

Manuscript EMBO-2014-88307

Dynamic loading and redistribution of the Mcm2-7 helicase complex through the cell cycle

Sara K. Powell, Heather K. MacAlpine, Joseph A. Prinz, Yulong Li, Jason A. Belsky and David M. MacAlpine

Corresponding author: David M. MacAlpine, Duke University Medical Center

Review timeline:

Submission date:	26 February 2014
Editorial Decision:	25 March 2014
Revision received:	25 September 2014
Editorial Decision:	21 October 2014
Revision received:	27 November 2014
Accepted:	02 December 2014

Editor: Hartmut Vodermaier

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.)

1st Editorial Decision

25 March 2014

Thank you again for submitting your manuscript on dynamic Mcm2-7 loading for our consideration. We have now received the reports from three expert referees, copied below for your information. As you will see, the referees acknowledge the potential interest of your findings on two-phase Mcm loading and the roles of active transcription and CyclinE-Cdk2 in the process. However, none of them is convinced that your present set of data has provided sufficiently compelling and decisive evidence to support such major conclusions at the current stage. A key point of criticisms in this respect is the reliance on two static arrest points, which may have artificially turned a normally continuous process into the observed two-stage process. Furthermore, there are potentially serious experimental caveats, as described mainly by referee 3. Finally, the referees point out the unclear mechanism of CyclinE-Cdk2-dependent recruitment in mitotic cells and the difficulties to reconcile the new models with previously published findings. In light of these combined and overlapping major concerns and their potential to confound the main interpretations and conclusions, I am afraid we cannot consider this manuscript at the present stage well suited for publication in The EMBO Journal.

In light of the importance of the topic and the apparent interest of your findings and conclusions, if validated, I would nevertheless like to give you an opportunity to address the referees' criticisms and suggestions through a major revision of this study. In this respect, we would not consider it required to establish the exact molecular mechanism by which CyclinE-Cdk2 achieves Mcm2-7 loading/redistribution, although experiments such as those suggested in referee 2's point 4 would clearly strengthen the study and their inclusion would certainly be encouraged. On the other hand, it would be essential to decisively strengthen the evidence for the proposed two-step model, by adding

the requested experiments in unperturbed/synchronized cell cycles and by comparing Mcm2-7/ORC binding datasets from cells arrested at the same stages - as we would only be able to undertake re-review if we felt that clear data to this effect are obtained, as asked by all three reviewers. Furthermore, it will also be important to address the various other experimental caveats, in particular the one about chromatin fractionation raised by referee 3.

Given the seriousness of the main concerns and their potential to undermine the current conclusions and significance of the study, I hope you understand that I am presently not able to make strong commitments regarding eventual publication of this work. Nevertheless, I notice that the referees' requests are in principle realistic and well-taken and that you may well be able to tackle them. Therefore, I would in this case be happy to discuss an extension of the three-month revision period we generally allow for, and I should also mention that any competing manuscripts published during such a revision period will have no negative impact on our final assessment of your revised study. Please be reminded however that it is our policy to allow a single round of major revision only, making it essential to carefully answer to all points raised by the reviewers at this stage.

Thank you again for the opportunity to consider this work for publication, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision.

REFeree REPORTS:

Referee #1:

Powell et al. describe experiments that are aimed at elucidating the mechanisms of replication origin licensing and replication origin specification in *Drosophila*, using mitotically growing *Drosophila* Kc tissue culture cells as a model. Upon RNAi depletion of cyclin E/cdk2 or conditional overexpression of CDK inhibitor, dacapo, the authors observe that only a limited amount (approx. 10%) of Mcm2-7 is loaded onto chromatin in G1 phase of mitotically growing *Drosophila* Kc cells, relative to the amount of Mcm2-7 associated with chromatin in cells arrested in early S phase with hydroxyurea (HU). This data is interpreted to suggest that Mcm2-7 loading during replication origin licensing in mitotically growing *Drosophila* Kc cells occurs in two stages and by two distinct mechanisms: 1) limited loading of Mcm2-7 early in early G1 phase independent of cyclin E-cdk2 activity and 2) more extensive cyclin E-cdk2-dependent Mcm2-7 loading in late G1. While differing in their dependency on cyclin E-cdk2 activity, both 'mechanisms' of Mcm2-7 loading are dependent on the canonical pre-RC components Cdc6 and Cdt1. Consistent with a bi-phasic model for Mcm2-7 loading in G1 phase the authors observe that Mcm2-7 initially continue to accumulate on chromatin upon synchronous release of Kc cells arrested in G1 phase by conditional dacapo overexpression, before being released from chromatin upon passage through S phase.

Using ChIP-chip the authors observe that the limited Mcm2-7 chromatin association in cyclin E-depleted Kc cells largely coincides along chromosome arm 2L with discrete ORC binding sites observed in asynchronous cells, consistent with these sites representing canonical pre-RC assembly sites. The Mcm2-7 chromatin association pattern along chromosome arm 2L appears drastically altered in cells arrested in early S phase by HU treatment: Mcm2-7 now appear to associate with broad domains of chromosome 2L that correlate with nontranscribed regions, rather than being localized to discrete peaks.

Based on the above observations this paper arrives at three major novel conclusions: 1) Mcm2-7 loading occurs by two mechanistically distinct mechanisms in early and late G1 phase; 2) Cyclin E-cdk2 is required for the chromatin loading of the full complement of Mcm2-7 in mitotically growing *Drosophila* cells; and 3) Mcm2-7 complexes are redistributed to non-transcribed regions of the genome upon entry into S phase. Although conceptually intriguing, the data presented in this paper fall significantly short of firmly supporting these notions.

Major concerns:

Figures 1-3: Cyclin E-cdk2 has been demonstrated by multiple labs in both *Drosophila* and mammalian cells to be important for origin licensing during the G0 to G1 transition, yet the existing data in the literature suggests that cyclin E-cdk2 does not play an essential role for replication origin licensing in a mitotic G1 phase. Powell et al. base a large part of their conclusions on the bi-phasic Mcm2-7 loading mechanism on the comparison of HU arrested cells with G1-arrested cells. However, the chromatin pelleting experiments inherently cannot actually distinguish between differences in Mcm2-7 chromatin stability versus loading efficiency. Alternatively, for example, it is possible that Mcm2-7 complexes in S phase, upon incorporation into replisomes (in HU or after release from dacapo-induced arrest), are simply more stably associated with chromatin than in G1. Additional experiments are, therefore, required to establish that cyclin E-cdk2 directly promotes Mcm2-7 loading, and not indirectly affects Mcm2-7 stability on chromatin. Moreover, given the fundamental novelty of a role for cyclin E-cdk2 in Mcm2-7 loading in a mitotic cell cycle, a basic mechanistic description of the reaction would seem essential to support this notion. For example, what could be the targets?

The authors cite the Anglana et al. paper (*Cell*, 2003) in support of their notion that 'more' Mcm2-7 is loaded in HU than in early G1. This is incorrect. The data in Anglana et al. merely demonstrate (as many other labs have) that impairment of replication fork progression by HU promotes dormant or otherwise inefficient origin firing.

Figure 4: Despite the good overlap of Mcm2-7 and ORC binding sites, this figure appears to compare data from asynchronous cells for ORC with those for cyclin E-depleted cells for Mcm2-7. Given the significance the authors ascribe to the cell cycle timing for origin licensing in this study the authors should compare datasets for the different proteins from cells arrested at the same cell cycle stage.

Figure 5: The authors claim that the Mcm2-7 signal here is derived from inactive Mcm2-7 helicase complexes at licensed origins, which were loaded at these sites by distal ORC complexes. Would it not seem more plausible to assume that the distributive Mcm2-7 chromatin association pattern is due to Mcm2-7 at replication forks that have moved away from the site of loading? In fact, I find the authors' suggestion that the data presented here are consistent with an Mcm2-7 loading mechanism at sites distal from ORC binding sites very farfetched, as such a Mcm2-7 loading mechanism is not actually supported by any other data here or elsewhere, and is mechanistically difficult to conceive. The Mcm2-7 distribution pattern here appears to have changed significantly compared to Figure 4. What about ORC? The authors should compare the ORC distribution in HU to that in cyclin E-depleted cells and to the Mcm2-7 distribution in HU in order to substantiate their claim that Mcm2-7 binding sites are distinct from the ORC binding sites.

Why is there no transcriptional effect on Mcm2-7 distribution in G1 phase (figure 4)? Or conversely, why would Mcm2-7 complexes only be affected in their distribution by transcription units in S phase (HU), but not in G1 phase? Would this not argue that the wide distribution of Mcm2-7 complexes in HU reflects that of replisomes, or at least a mixture of replisomes and inactive Mcm2-7 double hexamers at origins, which would complicate the mechanistic interpretation?

Can the effect of transcription on the Mcm2-7 distribution pattern be tested directly by altering transcription in G1 or S phase?

Referee #2:

This manuscript describes CyclinE/Cdk2-dependent, multiple rounds of Mcm loading and its re-distribution on a chromosome after early/mid G1 phase in *Drosophila* cells. The genome-wide mapping of Mcm binding indicates that chromatin-loaded Mcm co-localizes with ORC in early G1, but re-distributes to transcriptionally inactive or intergenic locus in the HU-treated cells (at the early S phase).

The results are interesting in two aspects. First, authors propose that Mcm is loaded onto chromatin in two steps and that the second step requires CyclinE/Cdk2. Second, they propose that transcription displaces Mcm from chromatin. These are interesting findings that may help resolve the mystery of MCM paradox, although it is not clear from the results presented what are the targets of CyclinE/Cdk2 to achieve redistribution of MCM during G1 phase.

The following issues need to be addressed before this manuscript is further considered for publication in EMBO J.

1 The difference of ChIP patterns between CycE-depleted cells and HU-treated cells appears to be striking when one compares Figure 4B and 5A. Authors propose two-step loading of Mcm. To demonstrate their model more convincingly, it is recommended that they synchronize the cells, for example, by nocodazol arrest/ release (or Dacapo release) and take time course, and show that the Mcm ChIP patterns change at lateM/ early G1 and late G1.

2 It is also not clear if the Mcm is redistributed over the entire genome before the onset of the S phase, which authors suggested but have not shown. It would be expected that Mcms bind to more randomly at late G1 before origins are fired and that those present in actively transcribed regions will be lost after cells enters S phase with no more reloading of Mcm, but is this actually the case? Treatment of HU for 24 hrs permits the firing of early origins and progression of replication forks to significant extent, while pre-RC formation is inhibited.

3 The ChIP analyses do indicate sequential binding of Mcm to chromatin that may be regulated by CyclinE/Cdk2. To further strengthen this conclusion and resolve the Mcm paradox in more convincing way, I would like authors to look at the Mcm localization on chromatin by immunofluorescence analyses. One would expect that Mcm and Orc colocalize in CycE-depleted cells as well as in late M/early G1 cells, but the signals are redistributed and increase as cells traverse into late G1.

4 It was reported that Cdc7-mediated phosphorylation of Mcm2-N-terminal region (and accumulation of active Dbf4/Cdc7 as well as Cdc6 dependent on CyclinE/Cdk2) promotes chromatin-loading of Mcm2 in cell cycle reentry from quiescence (Chuang, et al. Mol. Cell 2009). In light of this finding, it would be interesting to examine the CycE-dependent Mcm loading/redistribution during G1 can be achieved in the absence of CyclinE by ectopic expression of Cdc7 and Cdc6 or phosphomimetic forms of Mcm that mimic Cdk/Cdc7 mediated phosphorylation states.

5 In Figure 4, authors show that chromatin bindings of Mcm and Orc colocalize fairly well in CyclinE-depleted cells. I would like to know if these peaks also overlap with the actual initiation sites (active origins) observed during S phase. What fractions of these peaks are actually used for firing in S phase? Alternatively, are the active origins selected from the redistributed pre-RCs?

Referee #3:

Powell et al have identified 2 distinct phases during G1 where the licensing of replication origins by the Mcm2-7 proteins occurs: a Cyclin E/Cdk2 independent phase in which limiting amounts of Mcm2-7 are loaded and a Cyclin E/Cdk2 dependent phase that results in an increase and redistribution of chromatin-associated Mcm2-7. This is potentially an important new finding, as the current view is that origin licensing occurs from late anaphase and throughout G1, with no distinct substages. This new idea would be consistent with existing reports of an essential role for cyclin E in licensing origins specifically during the G0-G1 transition, and also with previous reports that licensing proteins (Cdc6 and Cdt1) are often degraded during early G1. The conclusions of the paper are in general well supported, and in principle are sufficiently novel and important for publication in EMBO journal; however, I have two major concerns with the manuscript in its current format.

The first potential problem is the lack of evidence to show that there actually are two stages that occur sequentially during G1. All the data presented in the paper are essentially static snapshots of Mcm2-7 on chromatin, using either cyclin E RNAi or HU to arrest cells at specific points. Although it seems highly plausible given what we know about the state cells should be in after these treatments, there is no evidence to show that the cells progress from the low Mcm2-7, low cyclin E/Cdk2 stage to a high Mcm2-7, high cyclin E/Cdk2. It is possible that in unperturbed cell cycles these two stages overlap with one another, or that Mcm2-7 are unloaded from DNA when cells arrest in Cyclin E RNAi. Hence, without some demonstration of the kinetics, it is unclear whether what the authors are describing actually refers to the sequence of events occurring in G1 or whether this occurs only at the specific arrest point of chemically-challenged cells. This issue could be easily

addressed experimentally using a mitotic inhibitor to synchronise cells in mitosis followed by release to allow visualization of the initial cyclin E/Cdk2 independent phase of Mcm2-7 loading followed by the cyclin E/Cdk2 dependent phase.

The second potential problem concerns the chromatin fractionation protocol that is used throughout the manuscript and on which all the interpretation depends. The technique and controls are shown in Supplementary Figure 5. In most standard protocols, the DNA-bound material is confirmed as such by release with DNase. This important control is missing, and other aspects of the fractionation are described in rather confusing terms. The non-structure-bound material is not a 'whole cell lysate' as it is called in text and figures, it is a soluble supernatant (even if it contains the bulk of the cellular Mcm2-7). It is unclear to me how the pellet could be washed with a '0 - 480 mM KCl gradient'. If this really was a gradient, it is unclear how it was applied, and it would be of value to see the elution profile of Mcm2-7. From the figure, instead I might guess that the 'salt washes supernatant' is the supernatant left after a low salt wash (presumably 0 KCl), the 'salt washed chromatin' is material that pellets in 480 mM KCl (not chromatin!!) and the 'chromatin bound fraction' is material eluted in 480 mM KCl (i.e. a high salt eluate). The protocol and the figure needs to be described more clearly. From the data as currently presented, there is no justification for any of the fractions being referred to as chromatin: in most cell extracts of this sort, chromatin represents a minor component of the pelletable material. It is important to demonstrate that the salt-washed material does in fact correspond to material that can be released from the pellet by DNase - that would provide justification for referring it to chromatin. This is an important point, because the majority of the cellular Mcm2-7 are not chromatin-bound, even in the HU sample, so even a minor contamination of the pellet with non-chromatin bound Mcm2-7 could have a big influence on the results. On this point, I note that the contrast of the blot in Supplementary Figure 5 seems rather high - no contrast enhancement should be made to the original data to obscure background bands.

Minor points:

1. There should be a blot to show the degree of knockdown that was achieved by Dup/Cdt1, Cyclin E and Cdk2 RNAi in Figure 1.
2. Quantification of the percentage number of cells in G1, S and G2/M would be useful for comparison in all the cell cycle Figures (Figures 1, 2 and 3 and Supplementary Figures 1 and 2).
3. The use of ORC2 as a loading control is not necessarily appropriate for normalizing samples in Figure 1 as it could change during the cell cycle. A better loading control for normalization would be histones.
4. The legend for Figure 1 describes a non-specific band detected by the Mcm2-7 antibody marked with an asterisk. Could the authors provide further details on which band this is referring to and why it only appears in Figure 1. The paper referenced to provide details on this antibody doesn't appear to have detected this non-specific band.
5. It would be useful if the authors could provide some clarification around the use of 3% DMSO to arrest cells in G2 in Supplementary Figure 1.
6. It would also be useful if the authors could provide some information on whether the cell cycle distribution shown for pUC RNAi in Supplementary Figure 2 is typical for *Drosophila* cells.
7. The data shown in Supplementary Figure 2 does not convincingly suggest the 'Cdk2 independent role in promoting pre-RC assembly' (page 6); the authors could provide some quantification of this 'reproducible' data.
8. The authors state that 'It is well documented that ORC, Cdc6, and Cdt1 are able to load multiple Mcm2-7 double hexamers onto DNA templates in vitro' (page 7); however, it is likely that this is more complex than was originally considered by the authors referenced, as ORC, Cdc6 and Cdt1 loading is likely to be highly dynamic (McNairn et al, 2005; Xouri et al, 2007; Sonnevile et al, 2012; Frigola et al, 2013).
9. Could the authors clarify why the ORC and Mcm2-7 ChIP experiments in Figure 4 were not

performed under the same experimental conditions i.e. both proteins analysed in both asynchronous and Cyclin E RNAi cells.

10. The recent work on Cdc6 degradation performed in the lab of Rob Wolthuis may be relevant to the second paragraph of page 11 (Clijsters et al, 2013; Clijsters & Wolthuis, 2014) - this may explain why Mcm2-7 loading is limited prior to cycE/Cdk2 activation. It may also be relevant that many of the pre-RC genes are E2F targets and are activated by cyclin E.

11. I was unconvinced by the explanation of how Cyclin E overexpression leads to reduced licensing in the discussion on page 12, as this seems to be counter-intuitive, as cyclin E overexpression ought to lead to increased Mcm2-7. The data presented in the manuscript doesn't seem to fit with the Ekholm-Reed et al observations.

1st Revision - authors' response

25 September 2014

(see next page)

Re: EMBOJ-2014-88307

Dynamic loading and redistribution of the Mcm2-7 helicase complex through the cell cycle

Dear Hartmut,

We are grateful for the reviewers' insightful comments on our manuscript. We have tried our best to address the concerns raised by the reviewers by additional analyses and new experiments. We believe that these revisions have resulted in a significantly improved manuscript. Below we summarize the findings from our new experiments, followed by a complete point-by-point response to each reviewer comment.

1. Genome-wide localization of ORC in HU. All of the reviewers expressed concern that we did not perform the genome-wide analysis of ORC and Mcm2-7 at the same cell cycle points. To allay these concerns, we have now analyzed the distribution of ORC in HU, and it remains the same as it did in the asynchronous sample (Supplemental Figure 4). Thus, as we concluded in our first experiments, there is a dramatic and specific genome-wide change in the Mcm2-7 distribution during HU arrest.
2. Another concern was the use of static arrest points (induced by cyclin E depletion or dacapo overexpression) to assess Mcm2-7 loading on chromatin. We've addressed this and similar concerns by visualizing Mcm2-7 loading in an asynchronous cell population by immunofluorescence using the cell cycle-regulated posttranslational histone modification, H4K20me1, as a marker for cells in late S and G2/M (Supplemental Figure 2). We find that cells in G1 and early S (lacking H4K20me1) exhibit a wide range of nuclear Mcm2-7 localization consistent with multiple phases of Mcm2-7 loading. Thus, we now provide an additional experimental type supporting our original conclusions.
3. Demonstrate that the chromatin associated proteins in the spin down assay are soluble by treatment with a nuclease. This is an important control suggested by one of the reviewers. We have clarified our original results and added an additional supplemental figure demonstrating that ORC, histone H3, and Mcm2-7 are released from the pellet by treatment with MNase (Supplemental Figure 8B).

We have also changed the author list to reflect contributions during the revision cycle.

Point by point response:

Referee #1:

Powell et al. describe experiments that are aimed at elucidating the mechanisms of replication origin licensing and replication origin specification in Drosophila, using mitotically growing Drosophila Kc tissue culture cells as a model. Upon RNAi depletion of cyclin E/cdk2 or conditional overexpression of CDK inhibitor, dacapo, the authors observe that only a limited amount (approx. 10%) of Mcm2-7 is loaded onto chromatin in G1 phase of mitotically growing Drosophila Kc cells, relative to the amount of Mcm2-7 associated with chromatin in cells arrested in early S phase with hydroxyurea (HU). This data is interpreted to suggest that Mcm2-7 loading during replication origin licensing in mitotically growing Drosophila Kc cells occurs in two stages and by two distinct mechanisms: 1) limited loading of Mcm2-7 early in early G1 phase independent of cyclin E-cdk2 activity and 2) more extensive cyclin E-cdk2-dependent Mcm2-7 loading in late G1. While differing in their dependency on cyclin E-cdk2

activity, both 'mechanisms' of Mcm2-7 loading are dependent on the canonical pre-RC components Cdc6 and Cdt1. Consistent with a bi-phasic model for Mcm2-7 loading in G1 phase the authors observe that Mcm2-7 initially continue to accumulate on chromatin upon synchronous release of Kc cells arrested in G1 phase by conditional dacapo overexpression, before being released from chromatin upon passage through S phase.

Using ChIP-chip the authors observe that the limited Mcm2-7 chromatin association in cyclin E-depleted Kc cells largely coincides along chromosome arm 2L with discrete ORC binding sites observed in asynchronous cells, consistent with these sites representing canonical pre-RC assembly sites. The Mcm2-7 chromatin association pattern along chromosome arm 2L appears drastically altered in cells arrested in early S phase by HU treatment: Mcm2-7 now appear to associate with broad domains of chromosome 2L that correlate with nontranscribed regions, rather than being localized to discrete peaks.

Based on the above observations this paper arrives at three major novel conclusions: 1) Mcm2-7 loading occurs by two mechanistically distinct mechanisms in early and late G1 phase; 2) Cyclin E-cdk2 is required for the chromatin loading of the full complement of Mcm2-7 in mitotically growing *Drosophila* cells; and 3) Mcm2-7 complexes are redistributed to non-transcribed regions of the genome upon entry into S phase. Although conceptually intriguing, the data presented in this paper fall significantly short of firmly supporting these notions.

We are thankful that the reviewer found our work novel and conceptually intriguing. We hope that the additional experiments we have performed will strengthen our conclusions and satisfy the reviewer.

Major concerns:

*Figures 1-3: Cyclin E-cdk2 has been demonstrated by multiple labs in both *Drosophila* and mammalian cells to be important for origin licensing during the G0 to G1 transition, yet the existing data in the literature suggests that cyclin E-cdk2 does not play an essential role for replication origin licensing in a mitotic G1 phase. Powell et al. base a large part of their conclusions on the bi-phasic Mcm2-7 loading mechanism on the comparison of HU arrested cells with G1-arrested cells. However, the chromatin pelleting experiments inherently cannot actually distinguish between differences in Mcm2-7 chromatin stability versus loading efficiency. Alternatively, for example, it is possible that Mcm2-7 complexes in S phase, upon incorporation into replisomes (in HU or after release from dacapo-induced arrest), are simply more stably associated with chromatin than in G1. Additional experiments are, therefore, required to establish that cyclin E-cdk2 directly promotes Mcm2-7 loading, and not indirectly affects Mcm2-7 stability on chromatin. Moreover, given the fundamental novelty of a role for cyclin E-cdk2 in Mcm2-7 loading in a mitotic cell cycle, a basic mechanistic description of the reaction would seem essential to support this notion. For example, what could be the targets?*

We appreciate the reviewer's concern and it is indeed difficult to discriminate between chromatin stability and loading efficiency. Using three different assays (chromatin fractionation, immunofluorescence - new to the revised manuscript, and chromatin immunoprecipitation), we have observed a dramatic increase in nuclear chromatin-associated Mcm2-7 and a marked difference in their genome-wide localization. This suggests that cyclin E/Cdk2 activity has profound effects on either Mcm2-7 loading and/or their chromatin stability. We do note that a limited amount of Mcm2-7 loading does appear to occur canonically at ORC binding sites in the absence of cyclin E/Cdk2 activity, and that cyclin E/Cdk2 activity is only needed for

loading the full complement of Mcm2-7. We suspect that Cdk2 activity is required for an intermediate factor (perhaps much like exit from quiescence, Cdk2 activity is needed for sufficient Cdc6 expression). These experiments are ongoing and will be the focus of a future manuscript.

The authors cite the Anglana et al. paper (Cell, 2003) in support of their notion that 'more' Mcm2-7 is loaded in HU than in early G1. This is incorrect. The data in Anglana et al. merely demonstrate (as many other labs have) that impairment of replication fork progression by HU promotes dormant or otherwise inefficient origin firing.

We apologize for the confusion. We intended to cite the Anglana paper for the fact that HU arrest induces dormant origins and that it remains possible that additional mechanisms may exist in response to HU or replicative stress that lead to additional Mcm2-7 loading. We have clarified this point in the revised text.

Figure 4: Despite the good overlap of Mcm2-7 and ORC binding sites, this figure appears to compare data from asynchronous cells for ORC with those for cyclin E-depleted cells for Mcm2-7. Given the significance the authors ascribe to the cell cycle timing for origin licensing in this study the authors should compare datasets for the different proteins from cells arrested at the same cell cycle stage.

We have found no differences in ORC chromatin association throughout the cell cycle. We have performed the requested experiment and have now also examined localization of ORC in HU-arrested cells. We observed no significant differences in ORC localization between asynchronous cells and HU arrested cells (Supplemental Figure 4). We did not examine ORC localization in cyclin E-depleted cells as ORC appears to be recalcitrant to ChIP in G1 due to issues with antibody accessibility during pre-RC formation (Aparicio et al, Cell, 1997).

Figure 5: The authors claim that the Mcm2-7 signal here is derived from inactive Mcm2-7 helicase complexes at licensed origins, which were loaded at these sites by distal ORC complexes. Would it not seem more plausible to assume that the distributive Mcm2-7 chromatin association pattern is due to Mcm2-7 at replication forks that have moved away from the site of loading? In fact, I find the authors' suggestion that the data presented here are consistent with an Mcm2-7 loading mechanism at sites distal from ORC binding sites very farfetched, as such a Mcm2-7 loading mechanism is not actually supported by any other data here or elsewhere, and is mechanistically difficult to conceive.

I would like to stress that we don't claim that Mcm2-7 are being loaded at sites distal to ORC; instead, we only present our observations regarding the distribution of ORC and Mcm2-7. In the discussion we speculate that there are at least two possible mechanisms - one of which is that Mcm2-7 loads distally and the other is that Mcm2-7 translocates away from ORC binding sites. We also agree that it would indeed be more plausible if the change in genome-wide Mcm2-7 distribution could be accounted for by early origin firing in the presence of HU. This was actually our first thought as well. However, we've mapped early origin firing in Drosophila by HU arrest (MacAlpine DM et al., Genes and Development, 2004; MacAlpine HK et al., Genome Research, 2010) and only 10% of the genome is replicated in the presence of HU. This is likely an overestimate because we are enriching for BrdU by immunoprecipitation and there may be cell to cell variability in the usage of early origins (e.g. a specific early origin may only be functional in a small fraction of cells). As we initially reported in the results, "We also considered that the bimodal distribution of Mcm2-7 enrichment between active and inactive genes might be due to the activation of early origins that are enriched for near actively transcribed genes. However, we found that

the bimodal distribution of Mcm2-7 was not dependent on early origin activity, but instead was a specific feature of annotated transcripts (Supplemental Figure 5)."

The Mcm2-7 distribution pattern here appears to have changed significantly compared to Figure 4. What about ORC? The authors should compare the ORC distribution in HU to that in cyclin E-depleted cells and to the Mcm2-7 distribution in HU in order to substantiate their claim that Mcm2-7 binding sites are distinct from the ORC binding sites.

We have performed the requested experiment and have also now examined the ORC localization in HU-arrested cells (Supplemental Figure 4). We observed no significant differences in ORC localization between asynchronous cells and HU arrested cells. We did not examine ORC localization in cyclin E arrested cells (see above).

Why is there no transcriptional effect on Mcm2-7 distribution in G1 phase (figure 4)? Or conversely, why would Mcm2-7 complexes only be affected in their distribution by transcription units in S phase (HU), but not in G1 phase? Would this not argue that the wide distribution of Mcm2-7 complexes in HU reflects that of replisomes, or at least a mixture of replisomes and inactive Mcm2-7 double hexamers at origins, which would complicate the mechanistic interpretation?

As we mention in the discussion, "We propose that Mcm2-7 residing in transcribed regions are displaced by the passage of RNA Pol II during transcription. The displacement of Mcm2-7 during transcription is analogous to the removal of Mcm2-7 from inactive origins during passage of the replication fork in S-phase (Madine et al., 1995; Krude et al., 1996; Romanowski et al., 1996b). The bimodal distribution of Mcm2-7 at intergenic and non-transcribed genes relative to active genes may only be established after the transition into S-phase. Prior to S-phase and the cessation of pre-RC assembly, Mcm2-7 are likely in a cycle of loading, translocation and subsequent eviction in transcribed regions; however, once pre-RC assembly is inhibited they are no longer able to re-occupy transcribed regions following eviction by active transcription."

Can the effect of transcription on the Mcm2-7 distribution pattern be tested directly by altering transcription in G1 or S phase?

This is an excellent suggestion. As we only see the difference in Mcm2-7 distribution during HU arrest when further pre-RC assembly is blocked, we would need to specifically alter transcription during the progression from G1 to S - which would likely interfere with the transcription of key cell cycle-regulated genes (e.g. E2F). In order to get around this conundrum, we examined the genome-wide localization of Mcm2-7 by ChIP-seq in two different *Drosophila* cell lines (Kc167 and S2) treated with HU (Supplemental Figure 7). Although much of the transcriptome is the same (Cherbas et al., Genome Research 2011), we identified approximately 100 genes that were significantly transcribed in one cell line and off in the other. As before, we found that the Mcm2-7 complex was excluded from actively transcribed genes in both cell lines. When we specifically examined those genes that were only transcribed in one cell line, we observed a significant decrease in Mcm2-7 occupancy that was specific for the transcribed cell line. Thus, we conclude that transcription and not some other feature like GC sequence content is responsible for the displaced Mcm2-7 complexes.

Referee #2:

This manuscript describes CyclinE/Cdk2-dependent, multiple rounds of Mcm loading and its re-distribution on a chromosome after early/mid G1 phase in Drosophila cells. The genome-wide mapping of Mcm binding indicates that chromatin-loaded Mcm co-localizes with ORC in early G1, but re-distributes to transcriptionally inactive or intergenic locus in the HU-treated cells (at the early S phase).

The results are interesting in two aspects. First, authors propose that Mcm is loaded onto chromatin in two steps and that the second step requires CyclinE/Cdk2. Second, they propose that transcription displaces Mcm from chromatin. These are interesting findings that may help resolve the mystery of MCM paradox, although it is not clear from the results presented what are the targets of CyclinE/Cdk2 to achieve redistribution of MCM during G1 phase.

We thank the reviewer for finding our work interesting.

The following issues need to be addressed before this manuscript is further considered for publication in EMBO J.

1 The difference of ChIP patterns between CycE-depleted cells and HU-treated cells appears to be striking when one compares Figure 4B and 5A. Authors propose two-step loading of Mcm. To demonstrate their model more convincingly, it is recommended that they synchronize the cells, for example, by nocodazol arrest/ release (or Dacapo release) and take time course, and show that the Mcm ChIP patterns change at lateM/ early G1 and late G1.

This is a great experiment that we would love to do. However, the Drosophila Kc167 cells do not exhibit sufficient synchrony coming out of a mitotic arrest. Having a mixed population of cells in early G1 and S significantly complicates the interpretation of the genome-wide localization data. We hope to address this question by flow sorting different populations of cells by DNA content and performing ChIP-seq. These experiments are currently a challenge because we have to sort live cells on DNA content using intercalating dyes which may disrupt chromatin structure. In the future we hope to use fluorescent cell cycle biosensors (eg. Fucci) to report and sort on specific cell cycle stages.

2 It is also not clear if the Mcm is redistributed over the entire genome before the onset of the S phase, which authors suggested but have not shown. It would be expected that Mcms bind to more randomly at late G1 before origins are fired and that those present in actively transcribed regions will be lost after cells enters S phase with no more reloading of Mcm, but is this actually the case? Treatment of HU for 24 hrs permits the firing of early origins and progression of replication forks to significant extent, while pre-RC formation is inhibited.

I hope we are interpreting this comment correctly. We have added an additional experiment to specifically address the question - Is transcription required to displace the Mcm2-7 complex from chromatin in HU arrested cells. We examined the genome-wide localization of Mcm2-7 by ChIP-Seq in two different Drosophila cell lines (Kc167 and S2) treated with HU (Supplemental Figure 7). Although much of the transcriptome is the same (Cherbas et al., Genome Research 2011), we identified approximately 100 genes that were significantly transcribed in one cell line and off in the other. As before, we found that the Mcm2-7 complex was excluded from actively transcribed genes in both cell lines. When we specifically examined those genes that were only transcribed in one cell line, we observed a significant decrease in

Mcm2-7 occupancy that was specific for the transcribed cell line. Thus, we conclude that transcription and not some other feature like GC sequence content is responsible for the displaced Mcm2-7 complexes. We also want to stress that the Mcm2-7 chromatin association we observe is dynamic in S-phase and that as S-phase progresses we see that the Mcm2-7 enrichment along the chromosome is specifically lost from early replicating domains (Figure 5D).

3 The ChIP analyses do indicate sequential binding of Mcm to chromatin that may be regulated by CyclinE/Cdk2. To further strengthen this conclusion and resolve the Mcm paradox in more convincing way, I would like authors to look at the Mcm localization on chromatin by immunofluorescence analyses. One would expect that Mcm and Orc colocalize in CycE-depleted cells as well as in late M/early G1 cells, but the signals are redistributed and increase as cells traverse into late G1.

We have performed the requested immunofluorescence experiments to the best of our ability. First, we were able to confirm and reproduce the chromatin association findings using immunofluorescence of Mcm2-7 localization. However, we were unable to convincingly resolve and co-localize ORC and Mcm2-7 in the cyclin E arrested cells (beyond the simple fact that both were localized to the nucleus). We believe that this is a limitation of our microscopy platform and that it will require FRET or super resolution microscopy to adequately address the co-localization of Mcm2-7 and ORC in early G1 - both of which are beyond our expertise. Another challenging factor is that the Drosophila nucleus is significantly smaller than a mammalian nucleus (~30-fold difference in genome size) which makes it difficult to resolve abundant factors.

We also examined Mcm2-7 loading in an asynchronous cell population by immunofluorescence (Supplemental Figure 2). Ideally, we would like to correlate Mcm2-7 levels with increasing cyclin E/Cdk2 activity during G1. However, to visualize chromatin-associated Mcm2-7 in the nucleus we need to permeabilize (PBS + 0.5% triton) and wash cells prior to fixation (Claycomb et al., Dev Cell, 2002). This step removes cytoplasmic and non-chromatin associated factors. As an alternative cell cycle reporter we used H4K20me1 staining as a marker of cells in late S phase and G2 (Supplemental Figure 2A). H4K20me1 is a well-characterized cell cycle-regulated chromatin mark that is generated by PR-Set7/Set8 in very late S-phase and persists on the chromatin up until mitosis (Oda et al., Mol Cell, 2010; Abbas et al, Mol Cell, 2010). Thus, cells in G1 and early to mid S-phase will be identifiable by very low levels of H4K20me1. Not surprisingly we found that H4K20me1 and Mcm2-7 staining cell populations were for the most part mutually exclusive with very little overlap consistent with Mcm2-7 being removed from chromatin by the end of S-phase (Supplemental Figure 2B). The remaining H4K20me1 negative cells displayed a range of Mcm2-7 staining from barely detectable (early G1) to robust signal (late G1 early S-phase) (Supplemental Figure 2C). Thus, even in an asynchronous population of G1 and S phase cells, we can clearly detect multiple stages of Mcm2-7 loading. Granted, we have not fully addressed that the different Mcm2-7 levels we observe by IF in G1 and early S-phase are correlated with cyclin E/Cdk activity, but the results are consistent with our chromatin fractionation and genome-wide approaches. Finally, these findings also parallel recent work from the Lygerou group which noted an increase in chromatin associated Mcms from late M phase into and peaking at the G1/S transition (Symeonidou et al, JBC, 2013).

4 It was reported that Cdc7-mediated phosphorylation of Mcm2-N-terminal region (and accumulation of active Dbf4/Cdc7 as well as Cdc6 dependent on CyclinE/Cdk2) promotes chromatin-loading of Mcm2 in cell cycle reentry from quiescence (Chuang, et al. Mol. Cell 2009). In light of this finding, it would be

interesting to examine the CycE-dependent Mcm loading/ redistribution during G1 can be achieved in the absence of CyclinE by ectopic expression of Cdc7 and Cdc6 or phosphomimetic forms of Mcm that mimic Cdk/Cdc7 mediated phosphorylation states.

These are all great suggestions that would provide key mechanistic insights. We are working to generate and test the specific constructs. However, we feel that these experiments are beyond the scope of the current manuscript.

5 In Figure 4, authors show that chromatin bindings of Mcm and Orc colocalize fairly well in CyclinE-depleted cells. I would like to know if these peaks also overlap with the actual initiation sites (active origins) observed during S phase. What fractions of these peaks are actually used for firing in S phase? Alternatively, are the active origins selected from the redistributed pre-RCs?

The Mcm2-7 complex co-localizes with ORC throughout the genome in cyclin E arrested cells. As we previously reported (MacAlpine HK et al., 2010, Genome Research), the ORC peaks are specifically enriched at the apex of BrdU incorporation of early origins of DNA replication. We have included a supplementary figure which also show the enrichment of Mcm2-7 in cyclin E depleted cells at sites that will function as HU resistant early origins of DNA replication (Supplemental Figure 3). We believe that the HU-resistant early origins are likely established in early G1 and may be defined by association with Cdc45 in early G1 (eg. in yeast Cdc45 associates with a subset of early origins in G1). As G1 progresses and Mcm2-7 loading increases and the distribution changes throughout the genome, we propose that the activation and location of activating origins will be more random as S-phase progresses.

Referee #3:

Powell et al have identified 2 distinct phases during G1 where the licensing of replication origins by the Mcm2-7 proteins occurs: a Cyclin E/Cdk2 independent phase in which limiting amounts of Mcm2-7 are loaded and a Cyclin E/Cdk2 dependent phase that results in an increase and re-distribution of chromatin-associated Mcm2-7. This is potentially an important new finding, as the current view is that origin licensing occurs from late anaphase and throughout G1, with no distinct substages. This new idea would be consistent with existing reports of an essential role for cyclin E in licensing origins specifically during the G0-G1 transition, and also with previous reports that licensing proteins (Cdc6 and Cdt1) are often degraded during early G1. The conclusions of the paper are in general well supported, and in principle are sufficiently novel and important for publication in EMBO journal;

We thank the reviewer for their enthusiasm in support of our findings.

however, I have two major concerns with the manuscript in its current format. The first potential problem is the lack of evidence to show that there actually are two stages that occur sequentially during G1. All the data presented in the paper are essentially static snapshots of Mcm2-7 on chromatin, using either cyclin E RNAi or HU to arrest cells at specific points. Although it seems highly plausible given what we know about the state cells should be in after these treatments, there is no evidence to show that the cells progress from the low Mcm2-7, low cyclin E/Cdk2 stage to a high Mcm2-7, high cyclin E/Cdk2.

We do show in Figure 4 that following a release from dacapo overexpression (low Cdk2 activity) there is a marked increase in Mcm2-7 chromatin association concomitant with entry into S-phase. The timing for

entry into S-phase not surprisingly coincides with the disappearance of dacapo - which is a known cyclin E/Cdk2 inhibitor. Thus, we conclude that dacapo overexpression blocks cyclin E/Cdk2 activity and upon the decrease in dacapo levels cyclin E/Cdk2 activity is gradually increased (resulting in more Mcm2-7 loading) until it passes some threshold that results in sufficient activity for entry into S-phase and inhibition of further pre-RC assembly. We have also analyzed the Mcm2-7 nuclear chromatin association by IF from an asynchronous cell population and observe a range of Mcm2-7 levels in G1 and early S phase cells (see below for a more detailed description).

It is possible that in unperturbed cell cycles these two stages overlap with one another, or that Mcm2-7 are unloaded from DNA when cells arrest in Cyclin E RNAi.

We considered the possibility that the Cyclin E RNAi may result in the dynamic loading and unloading of the Mcm2-7 complex in early G1. We do not believe this to be the case because we can deplete cells of cyclin E and then subsequently knock down Cdt1 and Cdc6, followed by cyclin E overexpression. During the Cdt1/Cdc6 knockdown, which blocks further pre-RC assembly, we don't see a further reduction in Mcm2-7 chromatin levels which would likely occur if they were unstable or being unloaded (Figure 3).

Hence, without some demonstration of the kinetics, it is unclear whether what the authors are describing actually refers to the sequence of events occurring in G1 or whether this occurs only at the specific arrest point of chemically-challenged cells. This issue could be easily addressed experimentally using a mitotic inhibitor to synchronise cells in mitosis followed by release to allow visualization of the initial cyclin E/Cdk2 independent phase of Mcm2-7 loading followed by the cyclin E/Cdk2 dependent phase.

As we noted in our response to reviewer #1 the Drosophila cells do not release synchronously from a mitotic arrest induced by either nocadazole, ecdysone or DMSO. The cells do eventually release, but not as a tight synchronous population. In Figure 4, we did perform a 'kinetic' experiment whereby cells were arrested by overexpression of the Cdk2 inhibitor dacapo (low Mcm2-7) and then released from dacapo overexpression. Following release from dacapo overexpression there was a build up of chromatin associated Mcm2-7 that peaked between 3 and 5 hours after release and which coincided with entry into S-phase.

We also attempted to partially address this question by immunofluorescence of Mcm2-7 loading in an asynchronous population (Supplemental Figure 2). Ideally, we would like to correlate Mcm2-7 levels with increasing cyclin E/Cdk2 activity during G1. However, to visualize chromatin associated Mcm2-7 in the nucleus, we need to permeabilize (PBS + 0.5% triton) and wash cells prior to fixation (Claycomb et al., Dev Cell, 2002). This step removes cytoplasmic and non-chromatin associated factors and thus we can't stain cyclin E or use cell cycle reporters like the Fucci system (Zielke et al., Cell Rep, 2014). As an alternative to a cell cycle reporter, we used H4K20me1 staining as a marker of cells in late S phase and G2. H4K20me1 is a well characterized cell cycle regulated chromatin mark that is generated by PR-Set7/Set 8 in very late S-phase and persists on the chromatin up until mitosis (Oda et al., Mol Cell, 2010; Abbas et al., Mol Cell, 2010). Thus, cells in G1 and early to mid S-phase will be identifiable by the absence of H4K20me1. Not surprisingly we found that H4K20me1 and Mcm2-7 staining were mutually exclusive with very little overlap consistent with Mcm2-7 being removed from chromatin by the end of S-phase. The remaining H4K20me1 negative cells displayed a range of Mcm2-7 staining from barely detectable (presumably early G1) to robust signal (presumably late G1 early S-phase). We could also detect a few cells in late S-phase with the Mcm2-7 complex localization limited to a tight region of late

replicating heterochromatin at the chromocenter. Thus, even in an asynchronous population of G1 and S phase cells we can clearly detect multiple stages of Mcm2-7 nuclear association. Although these results do not directly link the increased Mcm2-7 loading to increasing cyclin E/Cdk2 activity, we feel that they are consistent with and support our chromatin association studies and genome-wide localization. Finally, these findings are also consistent with recent work from the Lygerou group which noted an increase in chromatin associated Mcms from late M phase into and peaking at the G1/S transition.

The second potential problem concerns the chromatin fractionation protocol that is used throughout the manuscript and on which all the interpretation depends. The technique and controls are shown in Supplementary Figure 5. In most standard protocols, the DNA-bound material is confirmed as such by release with DNase. This important control is missing, and other aspects of the fractionation are described in rather confusing terms. The non-structure-bound material is not a 'whole cell lysate' as it is called in text and figures, it is a soluble supernatant (even if it contains the bulk of the cellular Mcm2-7). It is unclear to me how the pellet could be washed with a '0 - 480 mM KCl gradient'. If this really was a gradient, it is unclear how it was applied, and it would be of value to see the elution profile of Mcm2-7. From the figure, instead I might guess that the 'salt washes supernatant' is the supernatant left after a low salt wash (presumably 0 KCl), the 'salt washed chromatin' is material that pellets in 480 mM KCl (not chromatin!!) and the 'chromatin bound fraction' is material eluted in 480 mM KCl (i.e. a high salt eluate). The protocol and the figure needs to be described more clearly. From the data as currently presented, there is no justification for any of the fractions being referred to as chromatin: in most cell extracts of this sort, chromatin represents a minor component of the pelletable material. It is important to demonstrate that the salt-washed material does in fact correspond to material that can be released from the pellet by DNase - that would provide justification for referring it to chromatin. This is an important point, because the majority of the cellular Mcm2-7 are not chromatin-bound, even in the HU sample, so even a minor contamination of the pellet with non-chromatin bound Mcm2-7 could have a big influence on the results.

These are all excellent points raised by the reviewer and we apologize for the confusion.

In part, the problem is due to our poor description and the confusing order of lanes in supplemental figure 5 (now Supplemental Figure 8A).

Lane 1. The reviewer is correct, this is indeed a cytoplasmic fraction which contains the bulk of Mcm2-7. (We have now labeled this fraction as cytoplasmic throughout the manuscript).

Lane 2. This is the supernatant following a 480 mM KCl wash of the nuclear pellet (high salt eluate).

Lane 3. This is the pellet following the 480 mM KCl wash.

Lane 4. This is nuclear pellet following a 20 mM KCl wash.

Both ORC and Mcm2-7 are solubilized from the nuclear fraction with 480 mM KCl treatment.

However, the reviewer is correct that we have only demonstrated that there is a salt soluble nuclear fraction of ORC and Mcm2-7.

In the revised manuscript we have now digested the nuclear pellets with MNase to assess the chromatin solubility as suggested by the reviewer (Supplemental Figure 8B). We've found that there is significantly more nuclear ORC and histone H3 released into the supernatant following digestion with MNase in both the HU (lane 5) and DMSO (lane 11) treated samples. Similarly, we find that there is also more Mcm2-7 released from the nuclear pellet by treatment with MNase than without. However, we do note that there is an appreciable amount of Mcm2-7 released from the nuclear fraction in the mock MNase treatment and wash prior to the treatment. This is not carry-over from the cytoplasmic fraction because ORC is not detectable in this fraction. Together, these results suggest that although Mcm2-7 is highly enriched in the nuclear fraction, some of the Mcm2-7 complex may only be weakly associated or perhaps unstable in the low salt conditions used in the assay. Finally, as noted above we have also used IF to detect differences in Mcm2-7 nuclear chromatin association in the revised manuscript.

Thanks to the reviewer's insightful comments, we have now re-labeled all the chromatin fraction assays as either "Cytoplasmic fraction" or "Nuclear chromatin fraction" and also revised the methods and figure legends to be more accurate.

On this point, I note that the contrast of the blot in Supplementary Figure 5 seems rather high - no contrast enhancement should be made to the original data to obscure background bands.

We apologize for the poor quality of Supplementary Figure 5. It has been replaced with the original scan of the western blot.

Minor points:

1. There should be a blot to show the degree of knockdown that was achieved by Dup/Cdt1, Cyclin E and Cdk2 RNAi in Figure 1.

We are only able to readily detect endogenous Dup/Cdt1 and cyclin E by western blot. Representative examples of these knockdowns are shown in Figure 3. The efficacy of the Dup/Cdt1 knockdown is also shown in Supplemental Figure 4 from MacAlpine HK et al., Genome Research, 2010).

2. Quantification of the percentage number of cells in G1, S and G2/M would be useful for comparison in all the cell cycle Figures (Figures 1, 2 and 3 and Supplementary Figures 1 and 2).

Drosophila asynchronous cell cycle profiles are, unlike mammalian profiles, shifted towards G2. Also, given the much smaller genome size it is difficult to accurately assess the cells in S-phase relative to the G1 and G2/M populations. Our G1 arrests typically reflect approximately 70-80% of the cells exhibiting 2C DNA content. Of course, the remaining cells in G2 don't contribute to the Mcm2-7 nuclear signal.

3. The use of ORC2 as a loading control is not necessarily appropriate for normalizing samples in Figure 1 as it could change during the cell cycle. A better loading control for normalization would be histones.

We've found that ORC association on chromatin is relatively constant throughout the cell cycle. All of the experiments described were loaded based on starting cell counts. In supplemental figure 8B describing the MNase treated chromatin we've included histone H3 as an additional control.

4. The legend for Figure 1 describes a non-specific band detected by the Mcm2-7 antibody marked with an asterisk. Could the authors provide further details on which band this is referring to and why is only appears in Figure 1. The paper referenced to provide details on this antibody doesn't appear to have detected this non-specific band.

We are referring to the minor band that is present in all fractions including the Cdt1/Dup controls for no Mcm2-7 loading. We actually do not know what this band is or where it is coming from. We did not observe it in all samples or even from similar chromatin fractionations in our prior manuscripts (MacAlpine HK et al., Genome Research, 2010; Ding et al., Plos Genetics, 2010). We speculate that it may be due to a different prep of the monoclonal antibody but we have not formally tested this hypothesis.

5. It would be useful if the authors could provide some clarification around the use of 3% DMSO to arrest cells in G2 in Supplementary Figure 1.

We initially arrested cells in G2 by treatment with the hormone ecdysone which was solubilized in DMSO (Macalpine DM et al., Genes and Development, 2004). Several years ago we realized (by accident) that DMSO alone was sufficient for the arrest of cells in G2 (MacAlpine HK et al., Genome Research, 2010). Cells are able to re-enter the cell cycle following release from DMSO. We are not sure of the mechanism but it occurs before phospho-H3 staining and entry into M-phase.

6. It would also be useful if the authors could provide some information on whether the cell cycle distribution shown for pUC RNAi in Supplementary Figure 2 is typical for Drosophila cells.

The cell cycle distribution of asynchronous Drosophila cells is weighted towards cells in G2 or 4N content. This is unlike many mammalian cell lines which typically display a predominant G1 or 2N DNA content. We've added an additional panel to Supplemental Figure 1 depicting the cell cycle distribution of an asynchronous population treated with scrambled RNAi. A full profile of multiple Drosophila cycle phases is present in Figure 1 of our prior manuscript (MacAlpine et al, G&D, 2004).

7. The data shown in Supplementary Figure 2 does not convincingly suggest the 'Cdk2 independent role in promoting pre-RC assembly' (page 6); the authors could provide some quantification of this 'reproducible' data.

While we still stand by our data in the original Supplementary Figure 2 and do find the patterns reproducible across multiple experiments, we are cognizant that this is a very subtle effect and at best there would only be a minimal potential role for cyclin E to promote pre-RC assembly independent of Cdk2 activity. The data in quiescent mammalian cells regarding a CDK independent role for cyclin E in pre-RC assembly (Geng et al., Mol Cell, 2007) are relatively controversial (Chuang et al., Mol Cell 2009). Given that our experiments to directly address this issue are quite subtle, we've decided to remove this figure and discussion from the revised manuscript.

8. The authors state that 'It is well documented that ORC, Cdc6, and Cdt1 are able to load multiple Mcm2-7 double hexamers onto DNA templates in vitro' (page 7); however, it is likely that this is more complex than was originally considered by the authors referenced, as ORC, Cdc6 and Cdt1 loading is likely to be highly dynamic (McNairn et al, 2005; Xouri et al, 2007; Sonnevile et al, 2012; Frigola et al, 2013).

Indeed the dynamics of ORC binding and pre-RC formation in vivo are considerably more complex than in vitro. We have added some additional references to this section.

9. Could the authors clarify why the ORC and Mcm2-7 ChIP experiments in Figure 4 were not performed under the same experimental conditions i.e. both proteins analysed in both asynchronous and Cyclin E RNAi cells.

We have found no differences in ORC chromatin association throughout the cell cycle. We have performed the requested experiment and have now also examined localization of ORC in HU arrested cells. We observed no significant differences in ORC localization between asynchronous cells and HU arrested cells (Supplemental Figure 4). We did not examine ORC localization in cyclin E arrested cells as ORC appears to be recalcitrant to ChIP in G1 due to issues with antibody accessibility during pre-RC formation (Aparicio et al, Cell, 1997).

10. The recent work on Cdc6 degradation performed in the lab of Rob Wolthius may be relevant to the second paragraph of page 11 (Clijsters et al, 2013; Clijsters & Wolthius, 2014) - this may explain why Mcm2-7 loading is limited prior to cycE/Cdk2 activation. It may also be relevant that many of the pre-RC genes are E2F targets and are activated by cyclin E.

Thank you for the excellent suggestion -- we have expanded the discussion to include this work.

11. I was unconvinced by the explanation of how Cyclin E overexpression leads to reduced licensing in the discussion on page 12, as this seems to be counter-intuitive, as cyclin E overexpression ought to lead to increased Mcm2-7. The data presented in the manuscript doesn't seem to fit with the Ekholm-Reed et al observations.

We apologize for the confusion. Work from Sabrina Spencer has shown that there is a gradual increase in Cdk activity in G1 cells with cells entering S-phase exhibiting more Cdk2 activity than cells in G0/G1 (Spencer et al., Cell, 2013). We simply speculate that entry into G1 with deregulated levels of cyclin E/Cdk2 activity (very high) results in insufficient time to gradually build up pre-RC assembly prior to entry into S-phase. Thus, the levels of Cdk2 activity are critical for pre-RC assembly (low) and initiation of S-phase (high) and the gradual increase in Cdk2 activity provides a mechanism to ensure that sufficient pre-RCs are assembled prior to entry into S-phase.

Sincerely,

David MacAlpine
Associate Professor of Pharmacology and Cancer Biology
Duke University Medical Center.

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by the original three reviewers, who acknowledge the improvements and clarifications added to the revised manuscript. However, they all still retain considerable reservations, especially regarding the evidence supporting the key conclusion of Cdk2-Cyclin E-controlled two-step pre-RC loading. While we would be inclined to accept your responses and modifications on most aspects, and to discount the requests for further mechanistic/functional insights, I am afraid we feel the above-mentioned concern regarding the sequential loading steps continues to preclude publication in our journal.

As mentioned in my first decision letter, we generally only allow for one single round of experimental revision, in order to keep authors from getting caught up in a frustrating revision process extended over multiple rounds of unclear outcome and with editors thus remaining unable to commit to a given study. In the present case, I would nevertheless like to give you the opportunity for an exceptional second revision of the work, in light of the interest of the topic and given that the one key issue to be addressed is clearly defined. Thus, should you be able to decisively strengthen the evidence for two-step pre-RC loading (beyond confirming a previously reported general increase over the course of G1), we would be happy to consider a re-revised manuscript for one final round of review. I understand that this will not be trivial or even possible at all, given the difficulties in correlating chromatin-loaded Mcm2-7 with Cdk2-Cyclin E activity explained in your resubmission cover letter; however, maybe work along one of the alternative lines suggested by referee 2 in the current reports may be able to overcome these limitations.

Since this will have to be the final round of revision for this manuscript at our journal, I should make it clear that we would not insist on further experimental addressing any of the other remaining referee comments, but that we must insist on convincing additional evidence for the two-step loading conclusions given that this remains the major concern of all three referees. Should you have any questions in this regard, please do not hesitate to contact me and I'd be happy to discuss them. I look forward to hearing from you!

REFEREE REPORTS:

Referee #1:

This is a revised manuscript from the MacAlpine lab on the cell cycle-dependent distribution of Mcm2-7 proteins on chromatin in cultured *Drosophila* cells. Their main observations are: 1) Total chromatin-bound Mcm2-7 levels are high in early S phase (HU)-arrested cells, and significantly lower in Cyclin E / Cdk2-depleted cells; 2) Mcm2-7 proteins distinctly co-localize with ORC at early origins in Cyclin E / Cdk2-depleted cells, while being broadly distributed across transcriptionally inactive late replicating regions of the genome in HU-arrested and late S-phase cells. The authors suggest that lower levels of Mcm2-7 are loaded at ORC-containing origin sites early in G1 phase, while Cyclin E / Cdk2 activity later in G1 phase promotes the loading of the full complement of Mcm2-7 onto chromatin; upon entry into S phase the authors suggest that Mcm2-7, but not ORC, are redistributed by the transcription machinery to transcriptionally silent chromosomal regions.

This revised manuscript has improved in various minor ways from the original submission. For example ORC-chromatin localization has now been mapped in HU to allow direct comparison to the Mcm2-7 signal obtained at that arrest point, and an immune-fluorescence approach has been used to monitor the dynamics of Mcm2-7 proteins on chromatin in a normal cell cycle. Yet, this paper does not go far beyond the initial observations and some fundamental concerns remain:

Major concerns:

- 1) The Mcm2-7 chromatin association with transcriptionally inactive regions of the chromosome during HU arrest is only correlative. Although the authors try to address this shortcoming by comparing specific transcriptionally active/inactive regions in Kc versus S2 cells (supplementary Figure 7), a direct demonstration of elongating RNA Pol II causing the re-localization of Mcm2-7 is lacking.
- 2) It is not clear that Mcm2-7 loading occurs in two sequential steps (Cyc E/Cdk2 - independent and -dependent) in an undisturbed G1 phase. New supplementary Figure 2 tries to address this point, yet assignment of respective cell cycle stages in this assay is dependent on interpretation of the fluorescence images; a rigorous demonstration of the cell cycle stage at sufficient resolution that distinguishes early from late G1 phase is not possible here.
- 3) What is the biological significance of the CycE / Cdk2 -dependent MCM population on chromatin? For example, is this population required for normal replication, or does it become important under stress conditions? In Fig 3C the authors arrest cells in HU after Cdc6/Cdt1 shut-off during CDK depletion (lane 5). Therefore, the authors should be in position to characterize replication progression after HU release in this condition.

Referee #2:

The revised manuscript by Powell et al. is improved with additional data presented.

The increase of Mcm loading during G1 phase has been reported in Symeonidou et al, also cited in this manuscript. Thus, dynamic loading of Mcm during late M through G1/S is not particularly novel.

As I stated in my first comments, the novel aspects of this manuscript are two-fold. First is that Mcm is loaded onto chromatin in two steps and that the second step requires CyclinE/Cdk2. Second is that transcription displaces Mcm from chromatin.

With regard to the second issue, HU-treatment can drive the firing of early-firing origins and permits fork movement to a significant extent, thus raising a possibility that pre-RC present in the vicinity of early-firing origins may be removed by the moving forks not by transcription. Since early-replicating regions and high transcription activity is correlated, this possibility has been a concern. Although this possibility is not completely ruled out, and the direct evidence for transcription removing pre-RC has not been presented, the data in supplemental Figure 5 may argue against it and may provide support for authors' conclusion.

With regard to the first issue, the evidence for two-step loading, one independent on CyclinE/Cdk2 and the other dependent on CyclinE/Cdk2, is still weak. As commented by the other reviewers, a connection between Cdk activity and Mcm loading needs to be shown in unperturbed cell cycle progression from early G1 to S. If the synchronization is difficult, as the authors claim, correlation with CyclinE/Cdk2-dependent kinase activity (or at least CyclinE expression level -the use of fluorescence-tagged CyclinE is one possibility) and Mcm chromatin binding could be examined in asynchronous cell populations with cell staining.

I could think of using CyclinE-fluorescent protein (e.g. EGFP). CyclinE should be stabilized somewhere in early G1 and keeps accumulating until G1/S. If CyclinE-EGFP can be observed (hopefully in a stable line), one should know when CyclinE is stabilized (I am assuming that the level of CyclinE is indeed correlated with its activity). Then you can compare the signals with that of stained pre-RC. Although it takes some time to accomplish this, it is certainly worth testing. Another possibility is the use of human cell lines which can be easily synchronized. If this is an important finding, it should also be conserved in higher eukaryotes. Release from nocodazole block and sample cells with 15 min intervals. Measure CyclinE-Cdk2 activity at each time point. At the same time stain Mcm on Triton-washed cell at each timepoint. With these, one may be able to correlate pre-RC formation with CyclinE-dependent kinase activity.

Referee #3:

The authors have made some attempt to address the mainly technical issues I had with the work. It

appears that there is no way to look at accumulation of Mcm2-7 on chromatin plus cyclin E levels in cells progressing through G1. I accept the authors arguments around this point, but it still represents a weakness of the work. The second main issue I had was around the chromatin isolation procedure. The new supplementary data does the minimum to demonstrate that things are working as expected, and that the salt wash used throughout the manuscript is mirrored by the more rigorous nuclease release. However, it would have been nice to have seen a nuclease release on one or two of the key experiments.

Additional correspondence

29 October 2014

It was great talking to you earlier this week. We really appreciate the time and thoughtful consideration that you and the reviewers have given our manuscript. As we discussed, I would like to briefly respond to the reviewer's remaining concerns.

The reviewers all noted the following advances in our manuscript:

1.

Loading the full complement of Mcm2-7 during G1 requires cyclin E/Cdk2 activity. Although it has been recently shown in mammalian cells that Mcm2-7 loading gradually increases following exit from mitosis and peaks with entry into S-phase (Symeonidou et al., JBC 2013, Kuipers et al., JCB 2011), our data demonstrates that this increase in Mcm2-7 chromatin associated levels is dependent on cyclin E/Cdk2 activity. Specifically, we ablated cyclin E/Cdk2 activity by multiple approaches including RNAi of cyclin E or Cdk2 as well as overexpression of a Cdk2 inhibitor (dacapo) and observed only minimal amounts of Mcm2-7 loading.

2.

The genome-wide localization of Mcm2-7 throughout the Drosophila genome is dynamic (to our knowledge this is the first genome-wide survey of the Mcm2-7 helicase complex in a higher eukaryote).

- In the absence of cyclin E/Cdk2 activity Mcm2-7 tightly co-localizes with ORC.

- At the G1/S transition, not only is there an increase in Mcm2-7 chromatin associated levels, but there is also a marked and unexpected change in the distribution and localization of Mcm2-7 that is shaped by active transcription units. Importantly, the genome-wide changes we observe in Mcm2-7 localization at the G1/S transition are dynamic as we are also able to subsequently detect the replication-dependent displacement of the Mcm2-7 complex during S-phase progression.

However, a common concern shared between the first two reviewers was that we haven't adequately demonstrated that Mcm2-7 loading occurs in "two-distinct sequential steps" (cyclin E/Cdk2-independent and dependent) in an unperturbed cell cycle.

We believe this may be a semantic issue between how we and the reviewers think about the data and how we wrote the abstract and illustrated our model figure. We used RNAi against cyclin E and Cdk2 as well as overexpression of a specific Cdk2 inhibitor (dacapo) to conclude that maximal Mcm2-7 loading was 'dependent' on cyclin E/Cdk2 activity. However, even in the absence of cyclin E/Cdk2 activity (or very low levels), we

found that there were still minimal levels of chromatin associated Mcm2-7 (1/20th of those found at the G1/S transition) that specifically co-localized with ORC; thus, we concluded that there was also a cyclin E/Cdk2 'independent' phase of Mcm2-7 loading. However, we did not mean to imply that these were two distinct 'sequential' phases with a 'switch'-like state. Instead, we believe that in an unperturbed cell cycle the regulation of Mcm2-7 loading by cyclin E/Cdk2 activity is more akin to a 'rheostat' than a simple two-state 'switch'. As cells exit mitosis there is very low cyclin E/Cdk2 activity that is sufficient for minimal Mcm2-7 loading at ORC binding sites. As cyclin E/Cdk2 activity increases throughout G1 there is an increase in Mcm2-7 loading and a global redistribution in the genome-wide localization of the helicase. At some critical threshold, Cdk2 activity becomes sufficient to drive entry into S-phase and inhibit further Mcm2-7 loading. After further reflection on the reviewers comments and our description of the data in the original manuscript, we believe we can make minor edits to the manuscript to clarify these points and our model. Below we present the original abstract and our proposed changes in color (which we would also incorporate into the model figure and throughout the manuscript). We do not believe these changes would in anyway negatively affect the novelty and impact of our data.

We also thank reviewer #2 for their thoughtful experiment - namely that we try and further support the 'two-step sequential mechanism' by correlating increased cyclin E (GFP-reporter) or Cdk2 activity (biochemical) with Mcm2-7 loading. However, given that it is very well established, to the point of being dogma, that cyclin E/Cdk2 activity increases during G1 and that we (in this manuscript) and others (Symeonidou et al., JBC 2013, Kuipers et al., JCB 2011) have shown that Mcm2-7 chromatin association also increases during G1, a correlation (whether direct or indirect) is to be expected. Additionally, expression of simple cyclin E - GFP reporters is further complicated by the fact that subtle increases in cyclin E levels drive cells prematurely into S-phase - again underscoring the importance of cyclin E/Cdk2 regulation. Ideally, what will be needed in the future to further dissect the role of cyclin E/Cdk2 activity on G1 progression and Mcm2-7 loading are sophisticated single cell studies using biosensor reporters of cyclinE/Cdk2 activity (Spencer et al., Cell 2013) in conjunction with FRAP of Mcm2-7 chromatin association. However, even from the pioneering studies of Spencer et al., it is clear that we are just now beginning to understand the complex and stochastic nature of cell cycle progression at the level of individual cells.

We would be grateful if you would reconsider our manuscript for the EMBO Journal in light of these proposed changes.

Original Abstract

Eukaryotic replication origins are defined by the ORC-dependent loading of the Mcm2-7 helicase complex onto chromatin in G1. Paradoxically, there is a vast excess of Mcm2-7 relative to ORC assembled onto chromatin in G1. These excess Mcm2-7 are broadly distributed throughout the nucleus, exhibit little co-localization with ORC or replication foci, and can function as dormant origins. We used biochemical and genomic approaches to dissect the mechanisms regulating the assembly and distribution of the Mcm2-7 complex. We found that Mcm2-7 loading occurs in two phases during G1. In the first phase, limiting amounts of Mcm2-7 are loaded at ORC binding sites in a cyclin E/Cdk2 independent manner. Subsequently, there is a cyclin E/Cdk2 dependent phase of Mcm2-7 loading that results in a 20-fold increase in chromatin associated Mcm2-7 and a dramatic genome-wide reorganization of the distribution of Mcm2-7 that is shaped by active transcription. Thus, increasing cyclin E/Cdk2 activity over the course of G1 is not only critical for Mcm2-7 loading, but also for the distribution of the Mcm2-7 helicase prior to S-phase entry.

Revised Abstract

Eukaryotic replication origins are defined by the ORC-dependent loading of the Mcm2-7 helicase complex onto chromatin in G1. Paradoxically, there is a vast excess of Mcm2-7 relative to ORC assembled onto chromatin in G1. These excess Mcm2-7 are broadly distributed throughout the nucleus, exhibit little co-localization with ORC or replication foci, and can function as dormant origins. We used biochemical and genomic approaches to dissect the mechanisms regulating the assembly and distribution of the Mcm2-7 complex in the *Drosophila* genome. We found that in the absence of cyclin E/Cdk2 activity there was a 20-fold decrease in chromatin associated Mcm2-7 relative to the levels found at the G1/S transition. The minimal amounts of Mcm2-7 loaded in the absence of cyclin E/Cdk2 activity were strictly localized to ORC binding sites. In contrast, cyclin E/Cdk2 activity was required for maximal loading of chromatin associated Mcm2-7 and a dramatic genome-wide reorganization of the distribution of Mcm2-7 that is shaped by active transcription. Thus, increasing cyclin E/Cdk2 activity over the course of G1 is not only critical for Mcm2-7 loading, but also for the distribution of the Mcm2-7 helicase prior to S-phase entry.

Additional correspondence

07 November 2014

Thank you for your letter detailing your response to the remaining major concerns on your study of dynamic Mcm2-7 loading and redistribution. We have now carefully considered and discussed these points, and agree that these explanations clarify the lingering issues and confusion sufficiently to dispel the remaining reservations. I would therefore like to invite you to incorporate the proposed changes and clarification into a modified version of the text (in particular the abstract, but also at other relevant occasions such as the discussion), and to resubmit the study together with a brief response letter to the previous decision and referee reports.

In addition, there are at this stage a couple of editorial points to be taken care of:

- please double-check the completeness of the provided data accession numbers
- please complete and return the author checklist available in our Guide To Authors or at <http://emboj.emboPress.org/sites/default/files/additional-assets/EMBO%20Press%20Author%20Checklist%20-%20EMBO%20J.xlsx>
- please suggest (in your resubmission cover letter) 2-5 one-sentence 'bullet points' (complementary to the abstract) with factual statements summarizing key aspects of the paper - they will accompany the online version of the article as part of a 'synopsis'. Please see the latest research articles on our website (emboj.emboPress.org) for examples.
- In addition, I would encourage you to provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels - in this case I could imagine a simplified version of the final model figure from the manuscript.

Once we will have received your final version, we should in principle be in the position to proceed with swift acceptance and publication of your study.

2nd Revision - authors' response

27 November 2014

(see next page)

The reviewers all note the following advances in our manuscript:

1. Loading the full complement of Mcm2-7 during G1 requires cyclin E/Cdk2 activity. Although it has been recently shown in mammalian cells that Mcm2-7 loading gradually increases following exit from mitosis and peaks with entry into S-phase (Symeonidou et al., JBC 2013, Kuipers et al., JCB 2011), our data demonstrates that this increase in Mcm2-7 chromatin associated levels is dependent on cyclin E/Cdk2 activity. Specifically, we ablated cyclin E/Cdk2 activity by multiple approaches including RNAi of cyclin E or Cdk2 as well as overexpression of a Cdk2 inhibitor (dacapo) and observed only minimal amounts of Mcm2-7 loading.
2. The genome-wide localization of Mcm2-7 throughout the Drosophila genome is dynamic (to our knowledge this is the first genome-wide survey of the Mcm2-7 helicase complex in a higher eukaryote).
 - 2.1. In the absence of cyclin E/Cdk2 activity Mcm2-7 tightly co-localizes with ORC.
 - 2.2. At the G1/S transition, not only is there an increase in Mcm2-7 chromatin associated levels, but there is also a marked and unexpected change in the distribution and localization of Mcm2-7 that is shaped by active transcription units. Importantly, the genome-wide changes we observe in Mcm2-7 localization at the G1/S transition are dynamic as we are also able to subsequently detect the replication-dependent displacement of the Mcm2-7 complex during S-phase progression.

However, a common concern shared between the first two reviewers was that we haven't adequately demonstrated that Mcm2-7 loading occurs in "two-distinct sequential steps" (cyclin E/Cdk2-independent and dependent) in an unperturbed cell cycle.

We believe this may be a semantic issue between how we and the reviewers think about the data and how we wrote the abstract and illustrated our model figure. We used RNAi against cyclin E and Cdk2 as well as overexpression of a specific Cdk2 inhibitor (dacapo) to conclude that maximal Mcm2-7 loading was 'dependent' on cyclin E/Cdk2 activity. However, even in the absence of cyclin E/Cdk2 activity (or very low levels), we found that there were still minimal levels of chromatin associated Mcm2-7 (1/20 of those found at the G1/S transition) that specifically co-localized with ORC; thus, we concluded that there was also a cyclin E/Cdk2 'independent' phase of Mcm2-7 loading. *However, we did not mean to imply that these were two distinct 'sequential' phases with a 'switch'-like state.* Instead, we believe that in an unperturbed cell cycle the regulation of Mcm2-7 loading by cyclin E/Cdk2 activity is more akin to a 'rheostat' than a simple two-state 'switch'. As cells exit mitosis there is very low cyclin E/Cdk2 activity that is sufficient for minimal Mcm2-7 loading at ORC binding sites. As cyclin E/Cdk2 activity increases throughout G1 there is an increase in Mcm2-7 loading and a global redistribution in the genome-wide localization of the helicase. At some critical

threshold, Cdk2 activity becomes sufficient to drive entry into S-phase and inhibit further Mcm2-7 loading. After further reflection on the reviewers comments and our description of the data in the original manuscript, we have edited manuscript to clarify these points and our model. We do not believe these changes in anyway negatively affect the novelty and impact of our data.

Referee #1:

This is a revised manuscript from the MacAlpine lab on the cell cycle-dependent distribution of Mcm2-7 proteins on chromatin in cultured Drosophila cells. Their main observations are: 1) Total chromatin-bound Mcm2-7 levels are high in early S phase (HU) -arrested cells, and significantly lower in Cyclin E / Cdk2-depleted cells; 2) Mcm2-7 proteins distinctly co-localize with ORC at early origins in Cyclin E / Cdk2-depleted cells, while being broadly distributed across transcriptionally inactive late replicating regions of the genome in HU-arrested and late S-phase cells. The authors suggest that lower levels of Mcm2-7 are loaded at ORC-containing origin sites early in G1 phase, while Cyclin E / Cdk2 activity later in G1 phase promotes the loading of the full complement of Mcm2-7 onto chromatin; upon entry into S phase the authors suggest that Mcm2-7, but not ORC, are redistributed by the transcription machinery to transcriptionally silent chromosomal regions.

This revised manuscript has improved in various minor ways from the original submission. For example ORC-chromatin localization has now been mapped in HU to allow direct comparison to the Mcm2-7 signal obtained at that arrest point, and an immune-fluorescence approach has been used to monitor the dynamics of Mcm2-7 proteins on chromatin in a normal cell cycle. Yet, this paper does not go far beyond the initial observations and some fundamental concerns remain:

Major concerns:

1) *The Mcm2-7 chromatin association with transcriptionally inactive regions of the chromosome during HU arrest is only correlative. Although the authors try to address this shortcoming by comparing specific transcriptionally active/inactive regions in Kc versus S2 cells (supplementary Figure 7), a direct demonstration of elongating RNA Pol II causing the re-localization of Mcm2-7 is lacking.*

As the reviewer noted, we have indirectly linked to Mcm2-7 chromatin to active transcription by first showing that Mcm2-7 is depleted from actively transcribed regions in a single cell line, and second, demonstrating that cell line specific transcription patterns are predictive of Mcm2-7 levels. Importantly, by comparing multiple cell lines, we demonstrate that this phenomenon is conserved and not dependent on primary sequence. Future studies will directly assess the role of elongating RNA Pol II or transcription dependent chromatin modifications in excluding Mcm2-7 from actively transcribed genes.

2) *It is not clear that Mcm2-7 loading occurs in two sequential steps (Cyc E/Cdk2 - independent and -dependent) in an undisturbed G1 phase. New supplementary Figure 2 tries to address this point, yet assignment of respective cell cycle stages in this assay is dependent on interpretation of the fluorescence images; a rigorous demonstration of the cell cycle stage at sufficient resolution that distinguishes early from late G1 phase is not possible here.*

As noted in the introduction above, we apologize for the confusion in implying a sequential two-state 'switch' like model. Instead we believe that increasing cyclin E/Cdk2 activity modulates Mcm2-7 loading more akin to a 'rheostat' model.

3) *What is the biological significance of the CycE / Cdk2 -dependent MCM population on chromatin? For example, is this population required for normal replication, or does it become important under stress conditions? In Fig 3C the authors arrest cells in HU after Cdc6/Cdt1 shut-off during CDK depletion (lane 5). Therefore, the authors should be in position to characterize replication progression after HU release in this condition.*

This is an excellent suggestion and we are currently exploring the biological significance of cyclin E/Cdk2-dependent Mcm2-7 loading in our system.

Referee #2:

The revised manuscript by Powell et al. is improved with additional data presented.

The increase of Mcm loading during G1 phase has been reported in Symeonidou et al, also cited in this manuscript. Thus, dynamic loading of Mcm during late M through G1/S is not particularly novel.

As I stated in my first comments, the novel aspects of this manuscript are two-fold. First is that Mcm is loaded onto chromatin in two steps and that the second step requires CyclinE/Cdk2. Second is that transcription displaces Mcm from chromatin.

With regard to the second issue, HU-treatment can drive the firing of early-firing origins and permits fork movement to a significant extent, thus raising a possibility that pre-RC present in the vicinity of early-firing origins may be removed by the moving forks not by transcription. Since early-replicating regions and high transcription activity is correlated, this possibility has been a concern. Although this possibility is not completely ruled out, and the direct evidence for transcription removing pre-RC has not been presented, the data in supplemental Figure 5 may argue against it and may provide support for authors' conclusion.

With regard to the first issue, the evidence for two-step loading, one independent on CyclinE/Cdk2 and the other dependent on CyclinE/Cdk2, is still weak. As commented by the other reviewers, a connection between Cdk activity and Mcm loading needs to be shown in unperturbed cell cycle progression from early G1 to S. If the synchronization is difficult, as the authors claim, correlation with CyclinE/Cdk2-dependent kinase activity (or at least CyclinE expression level -the use of fluorescence-tagged CyclinE is one possibility) and Mcm chromatin binding could be examined in asynchronous cell populations with cell staining. I could think of using CyclinE-fluorescent protein (e.g. EGFP). CyclinE should be stabilized somewhere in early G1 and keeps accumulating until G1/S. If CyclinE-EGFP can be observed (hopefully in a stable line), one should know when CyclinE is stabilized (I am assuming that the level of CyclinE is indeed correlated with its activity). Then you can compare the signals with that of stained pre-RC. Although it takes some time to accomplish this, it is certainly worth testing. Another possibility is the use of human cell lines which can be easily synchronized. If this is an important finding, it should also be conserved in higher eukaryotes. Release from nocodazole block and sample cells with 15 min intervals. Measure CyclinE-Cdk2 activity at each time point. At the same time stain Mcm on Triton-washed cell at each timepoint. With these, one may be able to correlate pre-RC formation with CyclinE-dependent kinase activity.

As noted in the introduction above, we apologize for the confusion in implying a sequential two-state 'switch' like model. Instead we believe that increasing cyclin E/Cdk2 activity modulates Mcm2-7 loading more akin to a 'rheostat' model.

We also thank reviewer #2 for their thoughtful experiment - namely that we try and further support the 'two-step sequential mechanism' by correlating increased cyclin E (GFP-reporter) or Cdk2 activity (biochemical) with Mcm2-7 loading. However, given that it is very well established, to the point of being dogma, that cyclin E/Cdk2 activity increases during G1 and that we (in this manuscript) and others (Symeonidou et al., JBC 2013, Kuipers et al., JCB 2011) have shown that Mcm2-7 chromatin association also increases during G1, a correlation (whether direct or indirect) is to be expected. Additionally, expression of simple cyclin E - GFP reporters is further complicated by the fact that subtle increases in cyclin E levels drive cells prematurely into S-phase - again underscoring the importance of cyclin E/Cdk2 regulation. Ideally, what will be needed in the future to further dissect the role of cyclin E/Cdk2 activity on G1 progression and Mcm2-7 loading are sophisticated single cell studies using biosensor reporters of cyclinE/Cdk2 activity (Spencer et al., Cell 2013) in conjunction with FRAP of Mcm2-7 chromatin association. However, even from the pioneering studies of Spencer et al., it is clear that we are just now beginning to understand the complex and stochastic nature of cell cycle progression at the level of individual cells.

Referee #3:

The authors have made some attempt to address the mainly technical issues I had with the work. It appears that there is no way to look at accumulation of Mcm2-7 on chromatin plus cyclin E levels in cells progressing through G1. I accept the authors arguments around this point, but it still represents a weakness of the work. The second main issue I had was around the chromatin isolation procedure. The new supplementary data does the minimum to demonstrate that things are working as expected, and that the salt wash used throughout the manuscript is mirrored by the more rigorous nuclease release. However, it would have been nice to have seen a nuclease release on one or two of the key experiments.

We appreciate the reviewer's thoughtful concerns and comments.

Acceptance letter

02 December 2014

Thank you for submitting your final revised manuscript for our consideration. I have now looked through it and your responses, and I am pleased to inform you that we have decided to accept it for publication in *The EMBO Journal*.