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Multiple Cdt1 molecules act at each origin to load replication-competent Mcm2-7 helicases

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

1st Editorial Decision 15 June 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received the reports of three expert referees, which are copied below. As you will see, all referees acknowledge the interest and importance of your results for understanding the role of Cdt1 in Mcm double hexamer loading, and are thus in principle supportive of publication pending adequate revision of a number of specific points raised in their reports. I am thus happy to invite you to prepare a revised version of the manuscript in response to those comments.

Regarding requirements for such a revision, it will be essential to satisfactorily address all criticism that pertain directly to the presented experiments and their conclusiveness. On the other hand, some other referee points might be considered further-reaching and/or difficult to approach within the scope of the current submission - in those cases, it will nevertheless be important to at least diligently clarify these issues in your response letter. In any case, should you have any concerns regarding the experiments requested by the reviewers, I would encourage you to get back to me for further consultations.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will

form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The MCM helicase is loaded at origins of DNA replication as an inactive double hexamer, before subsequent activation during S-phase. ORC, Cdc6 and Cdt1 mediate the loading reaction, but the mechanism of double hexamer formation is not understood, as the free MCM complex before loading is a single hexamer. The authors provide evidence to indicate that two Cdt1-MCM complexes bind to a single ORC complex at an origin. Mutating one of the putative ORC-binding sites in Cdt1 still allows Cdt1-MCM formation but reduces recruitment to ORC, and blocks stable loading of MCM onto DNA. Intriguingly, the N-terminus of Cdt1 appears to be required for some late stage of the loading reaction, as Cdt1 lacking this region still loads MCM stably onto DNA, but this reaction does not allow subsequent recruitment of key helicase subunits Cdc45 and GINS, and so blocks replication. Overall these data represent a very interesting step forward in understanding a central question in the replication field. In my opinion, this paper would be appropriate for publication in EMBO Journal once several issues have been resolved.

Major points
1. One of the key arguments of the paper is that two Cdt1-MCM complexes are recruited to a single ORC at an origin, and that this is at the heart of the mechanism that leads to formation of a double-hexamer of MCM during the loading reaction. Mutating one of the presumed ORC-binding sites in Cdt1 apparently allows recruitment of just one Cdt1-MCM complex to the origin, and blocks loading (as monitored by a high-salt wash).
(a) Figure 5B/C show the data indicating that two Cdt1 molecules can bind to a single ORC complex at the origin. It's important to be clear that there is really just a single ORC complex bound to the origin. The authors have several plausible arguments in favour of this idea (the B2 element is not required for recruitment of multiple Cdt1-MCM complexes, and the delta N292 allele of Cdt1 does not pull down wild type Cdt1 in Figure 5C). In principle, they could test the idea more directly by doing a similar experiment to that in Figure 5, using tagged ORC instead of tagged Cdt1. Unless the efficiency of Cdt1-MCM is rather low, in which case most of the ORC-DNA complexes would lack Cdt1-MCM and the data would be harder to interpret. If possible, this experiment would serve as a useful control for the Cdt1 data.
(b) A strong prediction of the data in Figure 6A is that the delta N292 and delta N301 alleles of Cdt1 are only recruiting single hexamers of MCM, and do not support double hexamer formation. I realize this is not easy to prove, but doing so would add a lot to the argument. Have the authors tried releasing these complexes from DNA (as done by Evrin et al (2009) and Gambus et al (2011)), so that gel filtration could be used as a measure of single/double hexamer formation? Or are the MCM complexes recruited by the mutated Cdt1 molecules not sufficiently stable?

2. The other key point is that the N-terminal domain of Cdt1 has some role after MCM loading, which is needed for subsequent recruitment of Cdc45-GINS and so for replication. The authors suggest two possible explanations: formation of loaded single hexamers rather than loaded double hexamers, or formation of loaded double hexamers that are somehow not in the right conformation for the subsequent step.
(a) It would be very interesting to release the complexes loaded by full-length Cdt1 or delta N271

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from DNA and test their behavior during gel filtration, compared to unloaded MCM complex, in order to test the first idea.
(b) An alternative explanation for the observed effect could perhaps be that loading of the double MCM hexamer is normal, but release of Cdt1 from the loaded complex requires the N-terminus of Cdt1? The authors should show whether Cdt1 has been displaced from the beads in the experiment in Figure 6A. If there is a defect in release of Cdt1 without the N-terminus, maybe this could block subsequent steps, even though the MCM is loaded correctly? In that case, would removal of Cdt1 by the high-salt wash suppress the defect in Cdc45-GINS recruitment and replication, for the delta 271 Cdt1 mutant?
(b) A further possibility could be that the N-terminus of Cdt1 is needed for release of loaded MCM from ORC. As far as I am aware, the loading reactions in Figure 6 mostly lead to loading of a single double hexamer on the origin DNA, so we can't judge from the amount of loaded MCM whether release from ORC is occurring or not (failure to release would be expected to block subsequent rounds of MCM loading). Does mutant ORC without ATPase activity, published previously by Stephen Bell's group, also give the same phenotype in this assay (loaded MCM equivalent to control, but no subsequent recruitment of Cdc45-GINS or replication)? This idea also raises the question of whether the high-salt wash might suppress the subsequent defect in replication

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Recent evidence shows that yeast MCM2-7 complexes are loaded onto origins as head-to-head double hexamers (Evrin et al., PNAS 2009; Remus et al., Cell 2009), but how this occurs is mysterious, given that only a single ORC complex binds the origin. Takara and Bell use a structure-function analysis of yeast Cdt1 combined with in vitro pre-RC assembly assays to dissect the multi-faceted function of Cdt1 in pre-RC assembly. Several interesting points emerge from the analysis: (1) They map the MCM2-7 interaction domain in Cdt1 to the extreme C-terminus, which fits with previous results from metazoans. (2) They provide evidence that Cdt1 binding to pre-RCs is reduced by about half when they mutate a discrete region (IDR1) in the middle of the protein (which is interesting in light of results presented later in the paper), and it's completely eliminated when the MCM interaction domain is abolished. This suggests that Cdt1 must interact with MCM2-7 to load stably onto origins. This interpretation is confirmed in experiments showing that Cdt1 and MCM2-7 must both be present and in a complex for either to load stably onto DNA. (3) A Cdt1 mutant lacking the N-terminal 271 residues is completely normal for in vitro pre-RC assembly but does not support replication initiation, suggesting that Cdt1 not only recruits and loads MCM2-7, but also induces a conformation that is conducive for interaction with replication initiation factors. (4) The IDR1 mutant of Cdt1 with the 2-fold defect in Cdt1 loading and MCM2-7 recruitment has an absolute defect in MCM2-7 loading. (5) Based on co-IP of tagged and untagged Cdt1 molecules from assembled pre-RCs, and the absence of such co-IP in the IDR1 mutant, the authors conclude that two Cdt1 molecules bind to the origin in the process of MCM loading.

This paper makes a major step forward in solving the puzzle of how a single ORC can load two MCM2-7 complexes, and it contains many other useful and intriguing observations that will stimulate the field. I strongly support its publication in EMBO.

Specific Points
1. Although the authors have provided powerful evidence that pre-RCs contain multiple Cdt1 molecules, they have not formally proven that the number is two. Therefore, the sentence at the bottom of page 11 should be reworded to state "...recruitment of multiple Cdt1 molecules..."

2. The conclusions drawn from Figure 7 will be more convincing if MCM2-7 loading, Mcm4 and ORC phoshporylation, GINS and Cdc45 recruitment, and DNA replication are all quantified to show how much more severe the replication/initiation defect is than the pre-RC assembly defect for FL and deltaN271 Cdt1 proteins.

Referee #3 (Remarks to the Author):

Multiple Cdt1 molecules act at each origin to load replication-competent Mcm2-7 by Takara and Bell.

A double hexamer of Mcm2-7 replicative helicase is loaded to Orc-bound replication origins. This loading requires Cdc6 and Cdt1. How a double-hexamer of Mcm2-7 is loaded is currently unknown. The authors dissected the Cdt1 protein into three domains; the C-terminal domain of Cdt1 binds to Mcm2-7, the central domain functions for loading Mcm2-7 and the N-terminal for association of Mcm2-7 with Cdc45 and GINS to form active helicase. Lack of the inter domain region 1 (IDR1) between the N-terminal and central domains reduces association of Mcm2-7 with origin DNA and abolishes stable loading of Mcm2-7 to DNA. They showed further that multiple Cdt1s associate with origin DNA by coprecipitation of Cdt1 and tagged-Cdt1 and this coprecipitation is not observed for IDR1-deleted Cdt1. From these results, the authors argue that two Cdt1s complexed with Mcm2-7 bind to Orc6 and this is essential for stable loading of Mcm2-7 to origins. The N-terminal deletion of Cdt1 did not affect the loading of Mcm2-7 to origin DNA although it abolishes Cdt1 function in vivo. The authors revealed that this N-terminal deletion is defective in forming the Mcm2-7 complex competent for helicase-activation proteins, Cdc45 and GINS, using in vitro replication assay. This is a surprising new finding. Thus, the authors revealed important functions of Cdt1, which leads to further elucidation of molecular mechanism of helicase loading to replication origins. However, some more data are required to argue their idea.

(Major points)
1. Dr. Bell's group very recently reported that Orc6 has two binding sites for Cdt1 and CDK inhibits one of these sites (Chen and Bell, 2010). Although the authors showed coprecipitation of Cdt1 and tagged Cdt1, their argument for this point does not advance so much from the previous report. Moreover, coprecipitation may be undetected by reduced loading of Mcm2-7 (Fig. 5C). It might be circumvented by increasing the sample. DeltaN271 is a good control for this experiment, in addition to FL, because N271 efficiently loads Mcm2-7 and thus coprecipitate if the authors' argument is true. Furthermore, combination of deletion of Cdt1 and Orc6 mutant is recommended to define which binding site of Orc6 binds to IDR1-deleted Cdt1.
2. The N-terminal deletion of Cdt1 reduces the association of Cdc45 and GINS with origins. This is a very interesting and important finding. However, the authors did not show Cdt1 in Figures 6 and 7. It is important how much Cdt1 or mutant Cdt1 binds to the origin during in vitro replication assay; for example, how much Cdt1 associates with origins after DDK treatment? It is also necessary to describe the washing conditions between the steps in in vitro replication assay that affect the association of Cdt1 with origins.

(Minor points)
1. p.4, 5th line from the bottom, Muramatsu et al. (Genes Dev 24, 602-612, 2010) should be included as a reference.
2. p.7, 8th line from the bottom, 471-604 should read 472-604.
3. Fig.2, minor bands are seen in deltaN301, N433 and N471. Is Mcm2-7 easily degraded in these mutants? DeltaN271 and N292 migrated the same distance although deltaN301 migrated faster than N292. Comments on these points in the legend are appreciated.
4. Fig. 3, Mcm2-7 migrates as a single band in N292 and N301. The authors described that this is caused by the gradient acrylamide gel. They used the same gradient gel for Fig. 2 and appearance of the Mcm2-7 is so different from Fig.3. Is it caused by the difference of silver staining and western blotting or sub-assembly of Mcm coprecipitated with mutant Cdt1? Presentations of separate detection of Mcms clarify this problem.
5. The authors do not describe proteinase inhibitors in buffers. Is it true?

1st Revision - authors' response 12 September 2011

We thank all of the reviewers for their thoughtful comments on our manuscript.
We have addressed each of the comments below (our comments are in italics) and in many ways in the revised manuscript. We strongly believe that these additions have greatly strengthened the resulting manuscript.

Referee #1 (Remarks to the Author):
The MCM helicase is loaded at origins of DNA replication as an inactive double hexamer, before subsequent activation during S-phase. ORC, Cdc6 and Cdt1 mediate the loading reaction, but the mechanism of double hexamer formation is not understood, as the free MCM complex before loading is a single hexamer. The authors provide evidence to indicate that two Cdt1-MCM complexes bind to a single ORC complex at an origin. Mutating one of the putative ORC-binding sites in Cdt1 still allows Cdt1-MCM formation but reduces recruitment to ORC, and blocks stable loading of MCM onto DNA. Intriguingly, the N-terminus of Cdt1 appears to be required for some late stage of the loading reaction, as Cdt1 lacking this region still loads MCM stably onto DNA, but this reaction does not allow subsequent recruitment of key helicase subunits Cdc45 and GINS, and so blocks replication. Overall these data represent a very interesting step forward in understanding a central question in the replication field. In my opinion, this paper would be appropriate for publication in EMBO Journal once several issues have been resolved.

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(a) Figure 5B/C show the data indicating that two Cdt1 molecules can bind to a single ORC complex at the origin. It's important to be clear that there is really just a single ORC complex bound to the origin. The authors have several plausible arguments in favour of this idea (the B2 element is not required for recruitment of multiple Cdt1-MCM complexes, and the delta N292 allele of Cdt1 does not pull down wild type Cdt1 in Figure 5C). In principle, they could test the idea more directly by doing a similar experiment to that in Figure 5, using tagged ORC instead of tagged Cdt1. Unless the efficiency of Cdt1-MCM is rather low, in which case most of the ORC-DNA complexes would lack Cdt1-MCM and the data would be harder to interpret. If possible, this experiment would serve as a useful control for the Cdt1 data.

We have performed this requested experiment and our results are consistent with a single ORC molecule being associated with the DNA during the loading reaction. We have added this change to the text (starting at the bottom of page 10) and included the data in figure S4.

(b) A strong prediction of the data in Figure 6A is that the delta N292 and delta N301 alleles of Cdt1 are only recruiting single hexamers of MCM, and do not support double hexamer formation. I realize this is not easy to prove, but doing so would add a lot to the argument. Have the authors tried releasing these complexes from DNA (as done by Evrin et al (2009) and Gambus et al (2011)), so that gel filtration could be used as a measure of single/double hexamer formation? Or are the MCM complexes recruited by the mutated Cdt1 molecules not sufficiently stable?

We attempted to analyze the ATP S intermediate that retains Cdt1 using the gel filtration approach but even with wild-type Mcm2-7/Cdt1 we did not observe the formation of stable Mcm2-7 double hexamers at this stage. The analysis of the deltaN292 Cdt1 Myc IP experiment also argues that monitoring the associated Mcm2-7 complexes would be difficult, as we do not see Mcm2-7 co-precipitating with the tagged deltaN292 Cdt1-myc in these experiments (Fig. 5C). This is in comparison to the robust co-precipitation of Mcm2-7 with full length Cdt1-myc (Fig. 5B). We propose that the differential affinity is consistent with two Mcm2-7 being recruited in the presence of fulllength Cdt1 and the lack of both a second Cdt1 and Mcm2-7 results in a less stable association of the remaining Mcm2-7 (see top of page 18 and the paragraph starting on page 19) .

2. The other key point is that the N-terminal domain of Cdt1 has some role after MCM loading, which is needed for subsequent recruitment of Cdc45-GINS and so for

replication. The authors suggest two possible explanations: formation of loaded single hexamers rather than loaded double hexamers, or formation of loaded double hexamers that are somehow not in the right conformation for the subsequent step

a) It would be very interesting to release the complexes loaded by full-length Cdt1 or delta N271 from DNA and test their behavior during gel filtration, compared to unloaded MCM complex, in order to test the first idea.

We have performed this experiment and observe that both the wild-type and delta N271 Cdt1 form double hexamers on the DNA. We have included this data in figure 6E and describe the data starting on the bottom of page 13.

(*b) An alternative explanation for the observed effect could perhaps be that loading of the double MCM hexamer is normal, but release of Cdt1 from the loaded complex requires the N-terminus of Cdt1? The authors should show whether Cdt1 has been displaced from the beads in the experiment in Figure 6A. If there is a defect in release of Cdt1 without the N-terminus, maybe this could block subsequent steps, even though the MCM is loaded correctly? In that case, would removal of Cdt1 by the high-salt wash suppress the defect in Cdc45-GINS recruitment and replication, for the delta 271 Cdt1 mutant?*

We now include an analysis of the Cdt1 associated with the origin DNA prior to DDK treatment and Cdc45 and GINS association with the origin DNA (Fig. S7). We find that, as previously observed for wild-type Cdt1, deltaN271 Cdt1 is completely released from the origin DNA after Mcm2-7 loading. Thus, interference by deltaN271 Cdt1 after Mcm2-7 cannot explain the subsequent defects that we observe. We discuss this data in the final paragraph of the results (page 15).

(b) A further possibility could be that the N-terminus of Cdt1 is needed for release of loaded MCM from ORC. As far as I am aware, the loading reactions in Figure 6 mostly lead to loading of a single double hexamer on the origin DNA, so we can't judge from the amount of loaded MCM whether release from ORC is occurring or not (failure to release would be expected to block subsequent rounds of MCM loading). Does mutant ORC without ATPase activity, published previously by Stephen Bell's group, also give the same phenotype in this assay (loaded MCM equivalent to control, but no subsequent recruitment of Cdc45-GINS or replication)? This idea also raises the question of whether the high-salt wash might suppress the subsequent defect in replication.

This is an interesting possibility and we have added it to those discussed in the paper (first paragraph, page 22). One piece of data that argues against this possibility is that ORC is equally displaced from the DNA at the end of the replication reaction (see ORC blot in Fig. 7C). This suggests that release of ORC from the loaded Mcm2-7 is not reduced in the absence of the Cdt1 N-terminus. Although salt washing of the DNA templates is an interesting way to address this possibility, we have found that salt treatment leads to variable results in the subsequent steps of the replication assay and therefore makes the proposed experiment difficult to interpret. We are currently investigating why salt washing alters the results of the replication reaction but these studies are beyond the scope of the current manuscript.
As with the salt washing studies, experiments testing the effect of the ORC ATPase mutation on the replication events are interesting in their own right and we are pursuing these independent of this study. On the other hand, even if this mutant caused the same defect as N271 Cdt1 it would not say that the similar phenotype was due to the same defect in Mcm2-7 loading. Therefore, we have not added this analysis to the manuscript.

Referee #2 (Remarks to the Author):
Recent evidence shows that yeast MCM2-7 complexes are loaded onto origins as headto-head double hexamers (Evrin et al., PNAS 2009; Remus et al., Cell 2009), but how this occurs is mysterious, given that only a single ORC complex binds the origin. Takara

and Bell use a structure-function analysis of yeast Cdt1 combined with in vitro pre-RC assembly assays to dissect the multi-faceted function of Cdt1 in pre-RC assembly. Several interesting points emerge from the analysis: (1) They map the MCM2-7 interaction domain in Cdt1 to the extreme C-terminus, which fits with previous results from metazoans. (2) They provide evidence that Cdt1 binding to pre-RCs is reduced by about half when they mutate a discrete region (IDR1) in the middle of the protein (which is interesting in light of results presented later in the paper), and it's completely eliminated when the MCM interaction domain is abolished. This suggests that Cdt1 must interact with MCM2-7 to load stably onto origins.

This interpretation is confirmed in experiments showing that Cdt1 and MCM2-7 must both be present and in a complex for either to load stably onto DNA. (3) A Cdt1 mutant lacking the N-terminal 271 residues is completely normal for in vitro pre-RC assembly but does not support replication initiation, suggesting that Cdt1 not only recruits and loads MCM2-7, but also induces a conformation that is conducive for interaction with replication initiation factors. (4) The IDR1 mutant of Cdt1 with the 2-fold defect in Cdt1 loading and MCM2-7 recruitment has an absolute defect in MCM2-7 loading. (5) Based on co-IP of tagged and untagged Cdt1 molecules from assembled pre-RCs, and the absence of such co-IP in the IDR1 mutant, the authors conclude that two Cdt1 molecules bind to the origin in the process of MCM loading.
This paper makes a major step forward in solving the puzzle of how a single ORC can load two MCM2-7 complexes, and it contains many other useful and intriguing observations that will stimulate the field. I strongly support its publication in EMBO.

Specific Points
1. Although the authors have provided powerful evidence that pre-RCs contain multiple Cdt1 molecules, they have not formally proven that the number is two. Therefore, the sentence at the bottom of page 11 should be reworded to state "...recruitment of multiple Cdt1 molecules..."

Although it is most likely two Cdt1 molecules that associate with the origin for the reasons that we discuss, we agree with the reviewer that we have not definitively shown this is the case. Accordingly, we have changed the sentence as suggested by the reviewer.

2. The conclusions drawn from Figure 7 will be more convincing if MCM2-7 loading, Mcm4 and ORC phoshporylation, GINS and Cdc45 recruitment, and DNA replication are all quantified to show how much more severe the replication/initiation defect is than the pre-RC assembly defect for FL and deltaN271 Cdt1 proteins.

We have quantified the relative levels of Mcm2-7 loading, GINS and Cdc45 recruitment and DNA replication and added this to the text (see page 15) and they are consistent with the primary defect in the replication being due to the defect in Cdc45 and GINS recruitment. It is more difficult to quantify several of the phosphorylation events since they are measuring a shift in the band (Orc2, Orc6 and Mcm6). However, in each case this shift appears to be complete (Fig. S6). If we quantify the phosphospecific antibody signal (for DDK phosphorylation of Mcm4-S82-D83) we see that there is approximately equal (96%) phosphorylation in the wild-type and N271 Cdt1 lanes. We have included the quantification of the Mcm4-S82-D83 phosphorylation on page 15 but feel more comfortable leaving the reader to interpret the extent of shifted bands.

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Mcm2-7 and the N-terminal for association of Mcm2-7 with Cdc45 and GINS to form active helicase. Lack of the inter domain region 1 (IDR1) between the N-terminal and central domains reduces association of Mcm2-7 with origin DNA and abolishes stable loading of Mcm2-7 to DNA. They showed further that multiple Cdt1s associate with origin DNA by coprecipitation of Cdt1 and tagged-Cdt1 and this coprecipitation is not observed for IDR1-deleted Cdt1. From these results, the authors argue that two Cdt1s complexed with Mcm2-7 bind to Orc6 and this is essential for stable loading of Mcm2-7 to origins. The N-terminal deletion of Cdt1 did not affect the loading of Mcm2-7 to origin DNA although it abolishes Cdt1 function in vivo. The authors revealed that this N-terminal deletion is defective in forming the Mcm2-7 complex competent for helicase-activation proteins, Cdc45 and GINS, using in vitro replication assay. This is a surprising new finding. Thus, the authors revealed important functions of Cdt1, which leads to further elucidation of molecular mechanism of helicase loading to replication origins. However, some more data are required to argue their idea.

(Major points)
1. Dr. Bell's group very recently reported that Orc6 has two binding sites for Cdt1 and CDK inhibits one of these sites (Chen and Bell, 2010). Although the authors showed coprecipitation of Cdt1 and tagged Cdt1, their argument for this point does not advance so much from the previous report.

The previous study described above showed that one of two binding sites for Cdt1 on Orc6 is modified by CDK and this modification inhibited the association of Cdt1 with that site. These studies also strongly suggested that inhibition of this binding prevented subsequent loading.

In contrast, the current study is the first to show that there are multiple Cdt1 molecules associated with a helicase loading intermediate and provides strong evidence that the formation of a multi-Cdt1 intermediate is important for subsequent Mcm2-7 loading. In addition, the previous studies did not identify the parts of Cdt1 that are involved in these interactions, which is the focus of the current study. Although these studies are consistent with our previous studies of Orc6, they represent a substantial and important addition to our understanding of Cdt1 function rather than simple extensions of the previous observations.

Moreover, coprecipitation may be undetected by reduced loading of Mcm2-7 (Fig. 5C). It might be circumvented by increasing the sample. DeltaN271 is a good control for this experiment, in addition to FL, because N271 efficiently loads Mcm2-7 and thus coprecipitate if the authors' argument is true.

We disagree that the reduced Mcm2-7 and Cdt1 recruitment observed for deltaN292 Cdt1 can explain the difference in the extent of co-precipitated untagged molecules. As quantified in Fig. 3B, there is only a 50% reduction in the amount of recruited Cdt1 with this mutation. If there were still an equal amount of tagged and untagged deltaN292 present in the ATPgS-arrested complex, we would readily detect this. Instead, we observe a strong signal for the tagged deltaN292 and no detectable signal for the untagged deltaN292 Cdt1. Furthermore, we show in the control experiments in Fig. 5C that untagged deltaN292 is robustly associated with the DNA prior to the IP (Fig. 5C, lanes 2). Thus, the lack of detectable untagged deltaN292 Cdt1 is not due to insufficient protein to detect. We have revised the text to emphasize this point (last sentence on page 11 and top of page 12).

It is true that there is less Mcm2-7 recruited in these samples and, as we discuss (page 18, top), the reduced Mcm2-7 may reflect the formation of a less stable complex in the absence of both Orc6 binding sites on Cdt1. Nevertheless, we are asking about the association of Cdt1 in these assays and it is clear that the ability of untagged deltaN292 Cdt1 to associate with ORC has not been compromised. Thus, the simplest conclusion is that the mutant interferes with the binding of two Cdt1 molecules to ORC and this, in turn, interferes with Mcm2-7 recruitment and loading.

We do not agree that deltaN271 would be a better control than full-length Cdt1.
As we show in a number of ways, this mutant loads the same amount of Mcm2-7 in the form of a topologically-linked double-hexamer and also shows no defect in the initial recruitment of Cdt1 and Mcm2-7 relative to full-length Cdt1 (as would be expected if it had a defect in formation of the multi-Cdt1 helicase loading intermediate).

Furthermore, a combination of deletion of Cdt1 and Orc6 mutant is recommended to define which binding site of Orc6 binds to IDR1-deleted Cdt1.

We have not tested Orc6 mutants but we have done an experiment to address which of the two Orc6 binding sites for Cdt1 is bound to the IDR1-deleted Cdt1. We have measured association of IDR1-deleted Cdt1 (deltaN292 Cdt1) origin association in the presence of unmodified and CDK-phosphorylated ORC. Wild-type Cdt1 origin association is reduced 50% by CDK phosphorylation of ORC, consistent with one of two Cdt1 binding sites on Orc6 being eliminated by CDK phosphorylation. In contrast, when we perform the same experiment with N292 Cdt1, more than 90% of N292 Cdt1 origin association is lost. This strongly suggests that the IDR1-deleted Cdt1 binds to the Nterminal Cdt1 binding site on Orc6, since this is the site that is inhibited by CDK phosphorylation (Chen and Bell, 2011). These data add are discussed on page 12 (first full paragraph) and shown in Fig. S5 and add further depth to our understanding of the interactions between Orc6 and Cdt1.

2. The N-terminal deletion of Cdt1 reduces the association of Cdc45 and GINS with origins. This is a very interesting and important finding. However, the authors did not show Cdt1 in Figures 6 and 7. It is important how much Cdt1 or mutant Cdt1 binds to the origin during in vitro replication assay; for example, how much Cdt1 associates with origins after DDK treatment? It is also necessary to describe the washing conditions between the steps in in vitro replication assay that affect the association of Cdt1 with origins.

It is not possible to monitor Cdt1 in the replication reactions after the loading step because all of the Cdt1 has been released at that point. We have added a supplemental figure showing that both full-length and deltaN271 Cdt1 are undetectable after the removal of the G1 extract from the origin-DNA-associated beads (Fig. S7). There is no washing of the samples between the steps of the replication reaction (helicase loading, DDK phosphorylation and replisome assembly and initiation). Despite this, we observe no Cdt1 association with the beads after the removal of the G1 extract (Fig. S7).

(Minor points)

1. p.4, 5th line from the bottom, Muramatsu et al. (Genes Dev 24, 602-612, 2010) should be included as a reference.

We have added this reference.

2. p.7, 8th line from the bottom, 471-604 should read 472-604.

We have made this change.

3. Fig.2, minor bands are seen in deltaN301, N433 and N471. Is Mcm2-7 easily degraded in these mutants? DeltaN271 and deltaN292 migrated the same distance although deltaN301 migrated faster than N292. Comments on these points in the legend are appreciated.

We see approximately equivalent amounts of the full size Mcm2-7 bands in the N301, N433 and N471 Cdt1 preparations. Consistent with the extra bands not being due to Mcm2-7 breakdown products, western blotting with our Mcm2-7 antibodies indicates that these smaller products are not related to Mcm2-7. Instead, we believe that these are contaminants in these preparations due to lower overall yields. We have added a comment addressing this issue to the legend for Fig. 2.

4. Fig. 3, Mcm2-7 migrates as a single band in N292 and N301. The authors described that this is caused by the gradient acrylamide gel. They used the same gradient gel for Fig. 2 and appearance of the Mcm2-7 is so different from Fig.3. Is it caused by the difference of silver staining and western blotting or sub-assembly of Mcm coprecipitated with mutant Cdt1? Presentations of separate detection of Mcms clarify this problem.

The polyclonal antibody that is used to detect Mcm2-7 in our studies does not recognize all of the Mcm2-7 proteins equally. Thus, although two prominent bands are observed in the silver stained gel of Fig. 2, only one of these bands is strongly detected by the polyclonal antibody. We have indicated this in the legend to figure 3.

5. The authors do not describe proteinase inhibitors in buffers. Is it true?

Protease inhibitors are added during the preparation of extracts but not to subsequent buffers. We have indicated this in the materials and methods on page 23, last sentence of the "protein purification" section.

Acceptance letter 06 October 2011

Thank you for submitting your revised manuscript for our consideration. We have now heard back from the two original referees that have looked at it once more (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Before we will able to send you a formal letter of acceptance, I would kindly like to ask you to send us via email a modified text file of the paper, incorporating the minor text corrections requested by referee 3 (below). We would then upload and replace the version in our manuscript tracking systems, and after that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript.

Yours sincerely,
Editor
The EMBO Journal

Referee #1

(Remarks to the Author)
The authors have done a good job of revising their manuscript, which in my opinion is now appropriate for publication in The EMBO Journal.

Referee #3

(Remarks to the Author)
The revised version is well improved and satisfactory to me.
Now this is a good paper dealing with mechanistic aspect of formation of the pre-RC.

I have two minor comments.

1. p.18, 2nd line, "Similarly, we observed no Mcm2-7 co-precipitation with Cdt1 lacking IDR1"
Please refer a figure, probably Fig. 5C. and describe that it happened in the presence of ATPgS.

2. p.22, 2nd paragraph, 8th line,
Fig. 7C should read Fig. 6C.

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