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Sld7, an Sld3-associated protein required for efficient chromosomal DNA replication in budding yeast

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1st Editorial Decision 17 September 2010

Thank you for submitting your manuscript on the identification of Sld7 for consideration by The EMBO Journal. Three experts referees have now returned their evaluations, which you will find copied below. All of them acknowledge your findings as interesting and the conclusions on Sld7 functions in replication potentially important. However, there are also some major concerns whether the presented data are able to sufficiently strongly support these conclusions, which I feel still preclude publication in the present form. As you will see, most of these issues concern the conclusiveness of the genetic analyses and are brought up by referee 2 (although some of them are also mirrored in the comments of referee 3). Given the genetic entry point into the study, as well as the genetic-based nature of the key interpretations, I am afraid I have to agree with the critical reviewer that more definitive evidence will be required to make the study a suitable candidate for The EMBO Journal - including the proposed generation of some sort of conditional Sld7 allele to help exclude confounding suppressor mutations, and to be able to better understand whether the main role of Sld7 lies in the initiation or the elongation phase of replication.

I realize that extending the study along these lines (see also the detailed comments below) will likely entail a considerable amount of further work and effort, and would therefore understand if you were to decide to rapidly publish the study in its present form in a more genetics-oriented journal. Should you however be willing and able to improve the study as requested in the main criticisms, than we should be happy to consider a revised manuscript further for publication. Please note however that it is EMBO J policy to allow only one single round of major revision, and that it will thus be essential that you diligently answer to all the major and minor points raised at this stage if you wish for the paper ultimately to be accepted. Finally, when preparing a letter of response, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the

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community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding this decision .

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Review of EMBOJ-2010-75851 (Araki)

In this paper, Araki and colleagues report the discovery of a new protein called Sld7 from budding yeast that associates with Sld3 and promotes the efficient progression of DNA replication. Sld3, an essential target of CDK regulation, is an especially important replication protein. This protein and its relatives in other organisms are currently of great interest. Therefore, I feel that this paper would attract the attention of those working on DNA replication and related topics in both yeast and higher eukaryotes. From a technical standpoint, the experiments in the paper are for the most part impeccably done. I would therefore recommend publication in the EMBO Journal pending some minor revisions.

Points

1. Figure 2E and F. It is not explicitly stated whether these experiments were done with proteins expressed in bacteria or yeast.

2. Figure 5C. The results are not entirely clear-cut. There does seem to be some binding of Sld7 to origins in the absence of Sld3.

3. In view of the stated lack of homology between Sld7 and proteins outside of those found in yeast, it would be helpful for the authors to cite their methods for sequence analysis. For example, did they employ the methods used in the paper by Sanchez-Pulido and colleagues.

4. Page 20, line 13. The wording is not quite right. There is no direct evidence that Treslin/Ticcr has another subunit. Since lack of Sld7 does not abolish replication in yeast, the proposal that Treslin/Ticrr-depleted egg extracts containing recombinant Treslin/Ticcr do not replicate because of the lack of a putative Sld7 homologue is not entirely consistent with the yeast work.

Referee #2 (Remarks to the Author):

The genetic screens performed by Hiro Araki and colleagues in budding yeast have been instrumental in our understanding of the factors involved in replication initiation. These SLD screens have discovered the GINS complex and the two essential CDK substrates Sld3 and Sld2.
In this present study, Tanaka et al report the repetition of this SLD screen with a different allele of Dpb11 and they show the isolation of a novel factor, Sld7. It is encouraging to know that the genetic screens that have been done so far in budding yeast are still not saturated and there is enough circumstantial evidence in this work to suggest that one of the functions of Sld7 may be in DNA replication. Unfortunately there are some major limitations to this work and several of the findings in this paper need to be readdressed and possibly reinterpreted.

Major criticisms
1. It is a concern that 50% of sld7-delta spores are dead. This suggests that Sld7 is essential but may be suppressed at a high rate (perhaps there is a suppressor in the parental diploid which is why the viable number is close to 50%). If this is the case then the phenotypes of sld7-delta survivors may be misleading. It is worrying that in the growth assays 1D, 3B and 4A sld7-delta cells don't seem to be growth defective on YPD, yet the spores are very small and the S-phase is extremely slow. Ideally the authors need to generate a ts or a degron allele of Sld7 to verify the phenotypes they show in this paper. At the very least they should make a diploid with an episomal copy of Sld7 on a URA3 plasmid, and analyse the growth of resulting haploids as individual colonies on FOA plates.

2. The slow S-phase of sld7-delta cells is compelling (Figure 1B), but there is not a single assay to show whether these cells are defective in replication initiation or elongation. The inducible Sld7 mutants described in 1 would be useful in this regard. Since their model of Sld7 function is entirely based on a direct role in initiation, they need to show it.

3. The authors have based their model of Sld7 function in replication as being through Sld3. In figure 3A they isolate truncations of Sld3 that cannot bind Sld7, but are viable. If the authors are correct these alleles of Sld3 (such as 121-668) should phenocopy Sld7 delta mutants (e,g very slow S-phase). They must show this.

4. The authors state that the Sld7-5FLAG construct is synthetic lethal with sld3 mutant alleles. This means that the Sld7 tagged construct is not functional and therefore the results in Figure 2, with in vitro interactions and in Figure 5 with ChIP are open to mis-interpretation.

5. The interaction assays in Figure 2 are poorly controlled. There are no negative controls in C, D, E and G and there is no - Sld3/-Sld7 lane in 2B. They should be repeated, for example with another Flag-tagged protein in C, E and G.

6. It is not possible for the authors to conclude that Sld7 affects Sld3 protein levels from the experiment 4B. The asynchronous population of Sld7-delta cells has a different cell-cycle distribution to normal cells - see Figure 1b and 6a. in figure 2G there is a big difference in Sld3 levels from 15 mins versus 90 mins after G1 release. Therefore there may be a very simple explanation for changes in Sld3 levels in sld7-delta cells due to cell-cycle profile.

7. It is an interesting possibility that there must be a "handover" of Cdc45 from pre-IC to the replisome, but the data in Figure 7 is completely insufficient for the authors to claim a role for Sld7 in this event. First of all in 7a, the amount of Sld3 is lower in the sld3-sld7 input than in the sld3 alone input and this may be sufficient to explain the difference. In addition there is no Sld7 western, no negative control protein and both Sld3 and cdc45 are flag tagged, which together makes this experiment very difficult to interpret. The synthetic lethality between sld3-4, 5, 7 and 8 and gal-Sld7 also looks very unconvincing. The suppression by Jet1, or high levels of dpb11 in 7c is consistent with a role for Sld7 in replication initiation, not necessarily for disrupting the Sld3-Cdc45 interaction.

Other criticisms

8. Their Sld7 antibody recognizes a band of the same size as Sld7 that is present in sld7-delta cells (see Figure 2G and 4B). This makes interpreting figure 2G in particular, very difficult. I suggest affinity purification of this antibody and repetition of 2G inputs.

9. They need to show that Sld7 levels are unchanged in sld3-5 cells for figure 5c.

10. Figure 2a would look better if the silver stain and sld3 western were aligned.

Referee #3 (Remarks to the Author):

I found the manuscript by Tanaka et al to be interesting and well executed piece of work. The conclusions that a deletion of Sld7 leads to a slower S-phase and that these cells are sensitive to HU

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and MMS are quite clear. Furthermore I like the very careful biochemical and genetic interaction analysis of Sld3 and Sld7. Similar to Sld3, Sld7 also associates with replication origins in G1 phase and early S-phase, which makes sense knowing that Sld3 and Sld7 interact throughout the cell cycle. The authors show a reduced stability of Sld3 in a Sld7 deletion strain. This is an important finding to understand one functional role of Sld7; especially considering that protein levels of Sld3 are very low in yeast and therefore Sld3 is considered a rate limiting factor in initiation of DNA replication. In Figure 6 the authors show the influence of a Sld7 deletion on GINS association with an early and a late origin. It is interesting that specifically late origin firing is affected and GINS association is significantly delayed at ARS501. However it is unclear if the reason for this delay comes from the absence of Sld7 or the reduced Sld3 protein levels in a Sld7 deletion strain. To be conclusive this experiment should be repeated in a Sld7 deletion strain that has normal Sld3 levels. The authors showed that this experiment is possible, as an YCpSLD3 plasmid can be used to increase the Sld3 protein levels to near normal in a Sld7 deletion strain (Figure 4c).

The reduced Sld3 concentration in a Sld7 deletion strain can explain many of the findings in this publication, probably also the inefficient displacement of Sld3 from origins in Sld7 deletion strains. I agree with the authors that binding of Sld7 could stabilise a specific confirmation of Sld3. I am not sure if Sld7 could regulate in a specific way the dissociation of Sld3 from Cdc45, as the authors indicate as well, but the authors suggest that a protein interaction with Sld3 can in principle lead to a Cdc45 release.

In general this is technically very good work and it is important to understand the function of Sld7 in context of the replication field.

Minor comments:
The results section reads a bit funny, as the reader has to come to figure 4 to discover that the lack of Sld7 results in Sld3 instability. Probably this information can be incorporated in the title?

The manuscript needs some rewriting to correct mistakes in language.

1st Revision - authors' response 05 December 2010

In response to the suggestions of the referees, we have added four supplementary figures and modified the title, text and figures. We hope this version is satisfactory and is now suitable for publication in the EMBO Journal. Our responses to the comments of the referees are given below.

Referee #1

We thank the referee for recognizing our paper as an attractive work and for recommending it for publication. We have modified the text as suggested by the referee.

1. Figure 2E and F. It is not explicitly stated whether these experiments were done with proteins expressed in bacteria or yeast.

We apologize for the poor description of those experiments, in which we used yeast extracts. We have added sentences to the legend to Figure 2 to specify the organism used in the experiment.

2. Figure 5C. The results are not entirely clear-cut. There does seem to be some binding of Sld7 to origins in the absence of Sld3.

We would like to make two points. First, we used a temperature-sensitive allele of SLD3, sld3-5, at the nonpermissive temperature. Therefore, we cannot exclude the possibility that Sld3 still associates with origins very weakly, even at the nonpermissive temperature, and that this weak association was not detected with the ChIP assay. Second, the signal for the association between

Sld7 and ARS305 (+ antibody; closed bar) in sld3-5 cells was almost at background level (ñ antibody; open bar). Based on these points, we have modified the sentence in question to "association of the Sld7 protein with ARS305 was reduced in sld3-5 cells". We have also included an explanation of the closed and open bars in Figure 5C.

3. In view of the stated lack of homology between Sld7 and proteins outside of those found in yeast, it would be helpful for the authors to cite their methods for sequence analysis.

We used the common BLAST and FASTA programs. This is now described in the text.

4. Page 20, line 13. The wording is not quite right. There is no direct evidence that Treslin/Ticcr has another subunit. Since lack of Sld7 does not abolish replication in yeast, the proposal that Treslin/Ticrr-depleted egg extracts containing recombinant Treslin/Ticcr do not replicate because of the lack of a putative Sld7 homologue is not entirely consistent with the yeast work.

A protein that is important in yeast is also often essential in higher eukaryotes. However, we agree with the referee's comment. Therefore, we have modified the sentence to moderate our argument, "Treslin/Ticrr may have a subunit", and have introduced the argument of Kumagai et al (2010).

Referee #2

We thank this referee for his/her recognition of our work and the importance of the new factor Sld7. This referee argues that several of the findings in this paper should be readdressed and possibly reinterpreted. However, we disagree on several points, as described below. Our replies to each of the referee's comments are given below.

1. It is a concern that 50% of sld7-delta spores are dead. This suggests that Sld7 is essential but may be suppressed at a high rate (perhaps there is a suppressor in the parental diploid which is why the viable number is close to 50%). If this is the case then the phenotypes of sld7-delta survivors may be misleading. It is worrying that in the growth assays 1D, 3B and 4A sld7-delta cells don't seem to be growth defective on YPD, yet the spores are very small and the S-phase is extremely slow. Ideally the authors need to generate a ts or a degron allele of Sld7 to verify the phenotypes they show in this paper. At the very least they should make a diploid with an episomal copy of Sld7 on a URA3 plasmid, and analyse the growth of resulting haploids as individual colonies on FOA plates.

We understand this referee's concern regarding the viability of the spores. First, we apologize for the poor description of the results pertaining to tetrads. We do not think that the viability of the spores reflects the accumulation of suppressors, for the following reasons.

1) We analyzed one batch of spores with tetrad dissection. Other batches showed 80%ñ90% spore viability. The viability of spores depends on various factors, such as the duration of sporulation and the dryness of the agar plates. We have described this observation in the revised version of the manuscript.

2) As suggested by the referee, we sporulated the cells (SLD7/sld7) with plasmid encoding SLD7 and grew the sld7 cells on FOA plates, to select for the loss of SLD7. The cells that lost the SLD7 gene grew a bit slowly, as observed for the sld7 cells obtained from sporulation. We have described this observation in the revised version of the manuscript.

Therefore, we do not think that we need to construct a new ts allele and a degron of Sld7 to verify the phenotypes presented in this manuscript.

The referee was also concerned with the differences in the sizes of the colonies after tetrad dissection and vegetative growth. This point also worried us considerably, so we tested the cells that had lost the episomal copy of SLD7. We surmised that the sld7 spores germinated more slowly than those of the wild type. The sld7 cells showed an elongated S phase, but the doubling time of these cells was slightly longer than that of the wild-type cells (86.2 vs 77.5 min, respectively). This was probably because the G2 phase is reduced in sld7 cells.

2. The slow S-phase of sld7-delta cells is compelling (Figure 1B), but there is not a single assay to

show whether these cells are defective in replication initiation or elongation. The inducible Sld7 mutants described in 1 would be useful in this regard. Since their model of Sld7 function is entirely based on a direct role in initiation, they need to show it.

We do not think that another assay is required to show the defect in sld7 cells during the initiation step of DNA replication. We would like to explain our argument. The function of Sld3, mainly in the initiation step of DNA replication, is now well established. Our argument that Sld7 functions via Sld3 is based on several results:

1) Multicopy suppression of sld7 cells clearly indicates that Sld7 functions via Sld3. Flow-cytometric analysis of sld7 cells expressing high-copy SLD3 showed the complete restoration of DNA replication (this result is not described in the manuscript). The suppression of sld3-6 by Sld7 is also consistent with this idea. The referee suggests in comment 3 that the allele of Sld3 (121ñ668) should phenocopy Sld7 delta mutants. This was the case. The expression of the Sld3 (121ñ668) construct in sld7 cells induced their sensitivity to hydroxyurea (we have included this result in Supplementary Figure 4).

2) Most of the Sld7 protein associated with Sld3 throughout the cell cycle (Figure 2E, F, and G).

3) The ChIP assay demonstrated that Sld7 associates with the replication origins in an Sld3-dependent manner, but not with the replication forks, exactly as was observed for Sld3 (Figure 5A). Moreover, in the absence of Sld7, the association of GINS with origins and its dissociation from them were disturbed (Figure 6). These results clearly indicate that Sld7 functions in the initiation step.

4) We used the assays described above because these assays best illustrate our conclusion. Although there are two other ways to demonstrate the requirement for Sld7 in the initiation step, we think that those methods would provide ambiguous explanations.

i) The cells are arrested using hydroxyurea, released, and then examined for cell division or DNA synthesis. This is a classical method. However, it has been shown that this method alone cannot distinguish between the initiation and elongation steps. Moreover, it is hard to determine whether cell division or DNA synthesis is retarded in the case of sld7 because its defect is partial.

ii) The 2D agarose gel assay identifies the intermediates of replication. From the shape of the intermediates, we can distinguish initiation from elongation. This is also a good method for measuring the efficiency of initiation. However, it is insufficiently sensitive for cells with a partial defect in DNA replication, such as sld7 cells.

3. The authors have based their model of Sld7 function in replication as being through Sld3. In figure 3A they isolate truncations of Sld3 that cannot bind Sld7, but are viable. If the authors are correct these alleles of Sld3 (such as 121-668) should phenocopy Sld7 delta mutants (e.g. very slow S-phase). They must show this.

We agree with the referee's comment and have added the results of the test for HU sensitivity conferred by the N-terminally deleted Sld3 construct to Supplementary Figure 4. As expected, the expression of Sld3 lacking its N-terminal portion caused cellular sensitivity to HU.

4. The authors state that the Sld7-5FLAG construct is synthetic lethal with sld3 mutant alleles. This means that the Sld7 tagged construct is not functional and therefore the results in Figure 2, with in vitro interactions and in Figure 5 with ChIP are open to mis-interpretation.

We do not agree with this comment. The Sld7ñ5Flag construct was synthetically lethal with the sld3 mutant alleles, or the phenotype of the sld3 mutant was enhanced by Sld7ñ5Flag. This does not mean that the Sld7-tagged construct is not functional. Cells expressing Sld7ñ5Flag in the wild-type background behaved as wild-type cells, which suggests that Sld7ñ5Flag is functional. We often use tagged constructs and argue that cells expressing the construct behave like the wild-type cells. In these cases, we do not examine all the combinations between the tagged construct and the mutant alleles, as this is impossible. We also use various ts mutants and compare them under permissive

and nonpermissive conditions. These ts mutants show synthetic lethality with some other ts mutants or tagged constructs. Nonetheless, other researchers have trusted the results obtained with a single mutant and, so far, the results have been consistent with recent conclusions, because a single mutant under permissive conditions behaves like the wild-type cell.

5. The interaction assays in Figure 2 are poorly controlled. There are no negative controls in C, D, E and G and there is no - Sld3/-Sld7 lane in 2B. They should be repeated, for example with another Flag-tagged protein in C, E and G.

We have added the results of the control experiments to Supplementary Figure 3.

6. It is not possible for the authors to conclude that Sld7 affects Sld3 protein levels from the experiment 4B. The asynchronous population of Sld7-delta cells has a different cell-cycle distribution to normal cells - see Figure 1b and 6a. in figure 2G there is a big difference in Sld3 levels from 15 mins versus 90 mins after G1 release. Therefore there may be a very simple explanation for changes in Sld3 levels in sld7-delta cells due to cell-cycle profile.

This comment puzzled us. We do not think that there is a large difference in the levels of Sld3 in Figure 2G. Moreover, it has been shown repeatedly that the level of Sld3 is roughly constant throughout the cell cycle (Kamimura et al, EMBO J. 20, 2097, 2001; Kanemaki & Labib, EMBO J. 25, 1753, 2006; Tanaka et al, Nature 445, 328, 2007; Zegerman & Diffley, Nature 445, 281, 2007; Zegerman & Diffley, Nature 467, 474, 2010; Lopez-Mosqueda et al, Nature 467, 479, 2010).

7. It is an interesting possibility that there must be a "handover" of Cdc45 from pre-IC to the replisome, but the data in Figure 7 is completely insufficient for the authors to claim a role for Sld7 in this event. First of all in 7a, the amount of Sld3 is lower in the sld3-sld7 input than in the sld3 alone input and this may be sufficient to explain the difference. In addition there is no Sld7 western, no negative control protein and both Sld3 and cdc45 are FLAG tagged, which together makes this experiment very difficult to interpret. The synthetic lethality between sld3-4, 5, 7 and 8 and gal-Sld7 also looks very unconvincing. The suppression by Jet1, or high levels of dpb11 in 7c is consistent with a role for Sld7 in replication initiation, not necessarily for disrupting the Sld3-Cdc45 interaction.

This comment also puzzled us. We think that our argument is supported by the data presented here, for the following reasons.

1) We measured the protein levels carefully using the Odyssey Infrared Imaging System (LI-COR) and found that the levels of Sld3 protein alone and in the Sld3ñSld7 complex were almost the same.

2) The preferential binding of Sld3 to Cdc45 is also supported by the higher ratio of Sld3 to Sld7 in the Cdc45-associated fraction compared with that in the input sample (Figure 7A, Supplementary Figure 6A; as requested by the referee, we have shown the level of Sld7). This is probably because the preparation of Sld3ñSld7 contains complexes with various ratios of Sld3 to Sld7 and because the complex with the smallest amount of Sld7 binds preferentially to Cdc45.

3) The inhibition of sld3 ts by the overexpression of Sld7, although weak in some cases, was evident.

It is hard to infer the role of Sld7 unambiguously; therefore, we have discussed the role of Sld7 in the dissociation of Sld3 from Cdc45 in the Discussion section.

As requested by this referee, we have included an Sld7 western blotting experiment (we did not show this in the original version, although we had this result; Figure 7A) and control experiments (Supplementary Figure 6).

8. Their Sld7 antibody recognizes a band of the same size as Sld7 that is present in sld7-delta cells (see Figure 2G and 4B). This makes interpreting figure 2G in particular, very difficult. I suggest affinity purification of this antibody and repetition of 2G inputs.

We used purified antibodies directed against Sld7 in this study, which also react with a non-Sld7 protein. Therefore, we concluded that most of the Sld3 and Sld7 proteins in the cells were complexed throughout the cell cycle.

9. They need to show that Sld7 levels are unchanged in sld3-5 cells for figure 5c.

We have shown the levels of Sld7 in various sld3 mutant cells in the revised manuscript (Supplementary Figure 5). The level of Sld7 was identical to that observed in wild-type cells. We have also included the levels of Sld3 in these ts mutant cells.

10. Figure 2a would look better if the silver stain and sld3 western were aligned.

We ran these two experiments in separate gels, so the electrophoretic profiles differed slightly. Therefore, we have displayed them with the marker positions.

Referee #3

We thank this referee for recognizing our paper as an interesting and well-executed piece of work. Our replies to his/her comments are as follows.

1. In Figure 6 the authors show the influence of a Sld7 deletion on GINS association with an early and a late origin. It is interesting that specifically late origin firing is affected and GINS association is significantly delayed at ARS501. However it is unclear if the reason for this delay comes from the absence of Sld7 or the reduced Sld3 protein levels in a Sld7 deletion strain. To be conclusive this experiment should be repeated in a Sld7 deletion strain that has normal Sld3 levels. The authors showed that this experiment is possible, as an YCpSLD3 plasmid can be used to increase the Sld3 protein levels to near normal in a Sld7 deletion strain (Figure 4c).

We think that the reduced level of Sld3 mainly contributes to a delay in the association of GINS with the late-firing origins. However, as discussed in Figure 4, the reduced Sld3 level alone cannot account for the hypersensitivity of sld7 cells to HU. Therefore, based on the results reported in this paper, we discussed the possibility that Sld7 reduces the interaction between Cdc45 and Sld3 and that this reduction is important for efficient DNA replication. As suggested by the referee, the analysis of sld7 cells carrying YCpSLD3 should provide important theoretical insights. Here, we found that the level of Sld3 in sld7 cells fluctuated (e.g., see Figure 4C, which shows that a fluctuation of 20%ñ30% often occurred). This observation suggests that the data obtained from sld7 cells carrying YCpSLD3 will be vague and will not be easily explained. Therefore, we have moderated our argument.

2. I am not sure if Sld7 could regulate in a specific way the dissociation of Sld3 from Cdc45, as the authors indicate as well, but the authors suggest that a protein interaction with Sld3 can in principle lead to a Cdc45 release.

We did not argue that Sld7 regulated the dissociation of Sld3 from Cdc45 dynamically, but we stated that the Sld3ñSld7 complex dissociated from Cdc45 more easily than Sld3 alone. This observation of referee 3, together with comment 1, prompted us to tone down this point.

3. The results section reads a bit funny, as the reader has to come to figure 4 to discover that the lack of Sld7 results in Sld3 instability. Probably this information can be incorporated in the title?

We appreciate this referee's suggestion. However, we think that this is the best way to present and discuss our results.

4. The manuscript needs some rewriting to correct mistakes in language.

The revised manuscript has been edited to correct linguistic errors.

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2nd Editorial Decision 14 January 2011

Thank you for submitting your revised manuscript for our consideration, and also for your great patience awaiting our decision. I am very sorry about the unusual delay, but hope for your understanding in light of the complications associated with the re-review.
Your manuscript was sent back to the two referees with yeast replication expertise (2 & 3). While it appears that a number of the original concerns have been satisfactorily addressed, the most critical referee still retains a number of major concerns and reservations against publication in the present form. Referee 3 was kind enough to also comment directly on referee 2's original issues and your responses, which has clarified some but not all of these points. For this reason, I realized the necessity to myself carefully look at the problems in question, which unfortunately took a considerable amount of time in relation to the holiday season and the associated high submission rate we're experiencing at the moment.

The reports on your revised version are copied below. Upon my careful assessment of the points raised, I feel that some can be answered through a diligent response in the absence of adding further data. There are however also several points raised by referee 2 where I feel that at least some additional evidence needs to be provided:

- point 2: I am satisfied with the available evidence as discussed in your response; but please explain (in the response letter) why a 2D gel analysis as previously done in sld3-5 mutant cells would not be expected to be suitable here

- point 3: I think it is reasonable to at least directly compare HU sensitivity of the sld3(121-668) mutant strain with the sld7 deletion strain. To strongly support the evidence for Sld7 acting through Sld3, I hope you will also be able to conduct the originally requested test for slow S-phase of the sld3(121-668) mutant strain, and to test suppression by Sld7 overexpression

- point 4: please show the mentioned evidence for functionality of the Sld7-5FLAG protein in otherwise wild-type background. Am I correct in assuming that the Sld7-5FLAG version had been integrated at the endogenous SLD7 locus?

- point 6: since your data suggest that Sld3 level alterations are unlikely to underlie the sld7 phenotype, I do not think that clarifying whether sld7 deletion affects Sld3 levels on the transcriptional or post-translational level is of major importance for the conclusions of the paper - but the effects on Sld3 protein levels should be discussed with some more caution.

- point 7: this is the main point in need of further back-up, as also referee 3 agrees that some variability is apparent when comparing Figure 7A and Suppl. Figure 6A. Thus, to back up the conclusions on the 'Cdc45 handover' hypothesis, please revise these in vitro experiments in the spirit of the referees' comments, e.g. by adding the quantification asked for by referee 3. Please also further back up the synthetic lethality experiments in Figure 7B as asked for by referee 2.

As I stated before, given the genetic nature of the study and our aim to publish papers providing considerable functional insight, I feel it will be important to address at least the main issues that I have pointed out here. Should you be able to do so, I would be willing to ultimately consider the study for publication in The EMBO Journal, but please be aware that this will be the final round of revision we can allow for. In any case, I shall be happy to further discuss any questions or issues you may have in this regard.

Yours sincerely,
Editor
The EMBO Journal

REFEREE REPORTS:

Referee #2 (Remarks to the Author):

Using the same numbering as the original comments:

2) It is a shame that the authors do not think it is necessary to perform direct assays to show a role for Sld7 in replication initiation. In a previous EMBO paper (Kamimura et al 2001) these authors used 2D gels with the sld3-5 allele and even at the permissive temperature this assay showed a defect in initiation. It is not clear why this would not be effective for sld7 delta mutants.

3) The advantage of analyzing the 121-668 mutant of Sld3 is that it would give a read-out of the Sld3-Sld7 interaction without affecting the Sld7 gene. Supp. Figure 4 is a good start, although the HU sensitivity should be compared to the sld7 deletion. The 121-668 mutant should also have a slow S-phase when expressed at normal levels (compare to Sld7 mutant with wt sld3 expressed at normal levels) and should not be suppressed by high-doses of Sld7.

4) It is good to know that "cells expressing Sld7-5flag in the wild type background behaved as wild type cells". This should be shown in a supplementary figure with growth on YPD and HU. The reason for making sure that this allele is functional is because the authors extrapolate from negative data in Figure 5. If the tagged Sld7 does not bind to moving forks, but is not fully functional it is not possible to say whether the untagged protein binds to replisomes or not.

6) When the authors refer to figure 4b/c they say that it shows that Sld3 is unstable in an Sld7 deletion. However this figure does not say anything about protein stability; it could be Sld3 transcript levels that are affected. The authors need to do much more to show that Sld7 affects Sld3 stability.
As I have already stated, there is a difference in Sld3 levels in Figure 2G (compare 15 and 90 mins) therefore the authors cannot conclude about protein levels in an asynchronous population in their experiments. It is trivial to repeat this in a synchronized population, in the presence or absence of cycloheximide, together with an analysis of Sld3 mRNA.

7) By eye there appears to be less Sld3 in the Sld3-Sld7 input than in the Sld3 alone input. Supplementary figure 6 shows using the same experimental setup, there is now MORE Sld3 retained when Sld7 is present. This tells us that there is enough variability in this experiment to suggest that the small effects in Figure 7A should be disregarded.
The ratio of Sld3-Sld7 in this experiment is also a reflection of the off-rate of Sld7 from Sld3. The authors do not control for this and therefore they cannot draw any conclusions about a 'handover' between Sld7 and Cdc45.
For the synthetic lethality in Figure 7b it is important to know if the Sld3 alleles are equally expressed/stable when Sld7 is over-expressed akin to supp figure 5.

Referee #3 (Remarks to the Author):

I have been now through all Referee comments:

Referee 1:
All concerns addressed.

Referee 2:
Concern 1,3,8,9,10 - Addressed.
Concern 4 - I think that for the experiment in Figure 5C a Sld3 ts strain was used in combination with a tagged Sld7 allele, which is synthetical lethal/sick. This makes conclusions difficult.
Concern 5 - I would like to see information on the amounts of loaded protein in the input and IP lanes in %.
Concern 7 - Sup. Figure 6a does show a somewhat different result than Figure 7a. Could the authors add the quantification please?

Referee 3:
All concerns addressed.

I noticed in the Figure legend 5C a reference to the yeast strain YKK19, however this strain is not listed in supplementary table 1.

2nd Revision - authors' response 16 March 2011

Referee #2:

2) It is a shame that the authors do not think it is necessary to perform direct assays to show a role for Sld7 in replication initiation. In a previous EMBO paper (Kamimura et al 2001) these authors used 2D gels with the sld3-5 allele and even at the permissive temperature this assay showed a defect in initiation. It is not clear why this would not be effective for sld7 delta mutants.

As described previously, the 2D agarose gel assay is not sensitive enough for cells showing partial defect of DNA replication. This assay detects the intermediates of replication and thus provides efficiency of replication origins. Using this assay, we showed that origin firing efficiency reduces in sld3-5 cells at non-permissive condition; the initiation signal (bubble arc) was almost abolished. sld7 cells, however, reduces replication efficiency moderately. In this case, we cannot expect that the initiation signal is reduced significantly. Even if the signal is reduced partially (quantification itself is a bit difficult though), we cannot exclude possibilities, such that the bubble arc (the initiation signal) is destabilized by unknown reasons.

3) The advantage of analyzing the 121-668 mutant of Sld3 is that it would give a read-out of the Sld3-Sld7 interaction without affecting the Sld7 gene. Supp. Figure 4 is a good start, although the HU sensitivity should be compared to the sld7 deletion. The 121-668 mutant should also have a slow S-phase when expressed at normal levels (compare to Sld7 mutant with wt sld3 expressed at normal levels) and should not be suppressed by high-doses of Sld7.

Unfortunately, the strain harboring sld3 (121-668) is a shuffling strain for the plasmid bearing sld3, different from sld7 strain. Thus, we cannot compare sld7 and sld3 (121-668) precisely while rough comparison on the plate containing HU is possible. We therefore replaced the Supplementary Figure 4 by the new one containing sld7 (the rough comparison). The growth rates of wild-type and sld7 cells differ a little in the absence of HU and HU enhances this small difference. Hence, the difference shown in the presence of HU is enough for conclusion. Moreover, as this referee suggested, sld3(121-668) was not suppressed by high-doses of Sld7 (we included this result in the figure).

4) It is good to know that "cells expressing Sld7-5flag in the wild type background behaved as wild type cells". This should be shown in a supplementary figure with growth on YPD and HU. The reason for making sure that this allele is functional is because the authors extrapolate from negative data in Figure 5. If the tagged Sld7 does not bind to moving forks, but is not fully functional it is not possible to say whether the untagged protein binds to replisomes or not.

The comparison between WT and Sld7-5FLAG strain in the presence of HU (HU sensitivity) is a good idea. We did and confirmed that both strains are resistant to HU. The result is shown in Supplementary Figure 4.

6) When the authors refer to figure 4b/c they say that it shows that Sld3 is unstable in an Sld7 deletion. However this figure does not say anything about protein stability; it could be Sld3 transcript levels that are affected. The authors need to do much more to show that Sld7 affects Sld3 stability.

As I have already stated, there is a difference in Sld3 levels in Figure 2G (compare 15 and 90 mins) therefore the authors cannot conclude about protein levels in an asynchronous population in their experiments. It is trivial to repeat this in a synchronized population, in the presence or absence of cycloheximide, together with an analysis of Sld3 mRNA.

Since our main purpose of this manuscript is revealing the function of Sld7, we did not analyze expression of Sld7 further. Instead, according to this referee's concern, we removed the conclusive sentence in "Results section" and discussed it in "Discussion section".

7) By eye there appears to be less Sld3 in the Sld3-Sld7 input than in the Sld3 alone input. Supplementary figure 6 shows using the same experimental setup, there is now MORE Sld3 retained when Sld7 is present. This tells us that there is enough variability in this experiment to suggest that the small effects in Figure 7A should be disregarded.
The ratio of Sld3-Sld7 in this experiment is also a reflection of the off-rate of Sld7 from Sld3. The authors do not control for this and therefore they cannot draw any conclusions about a 'handover' between Sld7 and Cdc45.
For the synthetic lethality in Figure 7b it is important to know if the Sld3 alleles are equally expressed/stable when Sld7 is over-expressed akin to supp figure 5.

We are sorry. This is caused by poor explanation of Supplementary Figure 6A. We added this figure to show the specific interaction between Sld3 and Cdc45. So, we used larger amount of Cdc45, probably in this condition Sld3 and Sld3-Sld7 interaction with Cdc45 was saturated (although still Sld3 associates with Cdc45 slightly better than Sld3-Sld7). According to the comments of referees, we will present the results obtained exactly the same condition shown in Figure 7A.
This referee asked to present the protein level of the mutant Sld3 proteins in the presence of overexpressed Sld7 (synthetic dosage lethality). We present Figure 7B as a consistent result with the results shown in Figure 7A. We do not think that the presentation of Sld3 protein level improves the result. This referee probably expects that Sld3 level is constant as observed in Supplementary Figure 5 and would like to exclude the possibility if Sld3 protein level decreases. However, we do not know if Sld3 is stable when it dissociated from Cdc45 by overexpressed Sld7. Thus, we cannot exclude the possibility that Sld7-dependent dissociation of Sld3 from Cdc45 reduces the stability of Sld3. Genetic results are important to envisage the mechanism occurring in cells and confirm that the in vitro result is not artifact. However, as you wrote, these results alone cannot depict the mechanism but mostly support the idea; in other words they are consistent with idea but not sufficient to prove it.

Referee #3 (Remarks to the Author):

Concern 4 of Referee #2 - I think that for the experiment in Figure 5C a Sld3 ts strain was used in combination with a tagged Sld7 allele, which is synthetical lethal/sick. This makes conclusions difficult.

In Fig. 5C, we did not use the tagged version of Sld7. The referee #2 concerns the tagged-Sld7 is not functional. To exclude this concerns, we added the result that the strain bearing the tagged Sld7 behaves as the wild-type cells (Supplementary Figure 4).

Concern 5 of Referee #2 - I would like to see information on the amounts of loaded protein in the input and IP lanes in %.

We added information of the input and the IP lanes.

Concern 7 of Referee #2 - Sup. Figure 6a does show a somewhat different result than Figure 7a. Could the authors add the quantification please?

We replaced it by new data as described in the response to Referee #2. We described the measured amount of Sld3 and Sld7 in the legend.

I noticed in the Figure legend 5C a reference to the yeast strain YKK19, however this strain is not listed in supplementary table 1.

The strain name is YYK19. We thus corrected the yeast strain name in the legend to Figure 5C and added YYK19 to the supplementary table 1.

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Thank you for submitting your revised manuscript for our consideration. Following our editorial assessment of your re-revised manuscripts and your responses to the remaining concerns of the referees and editors, I am now happy to inform you that there are no further objections towards publication in The EMBO Journal.

You shall receive a formal letter of acceptance shortly.

Yours sincerely,
Editor
The EMBO Journal