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CHROMATIN DEPENDENT AND INDEPENDENT REGULATION OF DNA REPLICATION ORIGIN ACTIVATION IN BUDDING YEAST

Marko Looke, Kersti Kristjuhan, Signe Varv and Arnold Kristjuhan

Corresponding author: Arnold Kristjuhan, University of Tartu

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1st Editorial Decision

10 July 2012

Thank you for the submission of your manuscript to EMBO reports. We have now received the referee reports on your manuscript, which are copied below.

As you will see, while all referees acknowledge the potential interest of the findings, they also all suggest additional experiments to further improve the study. All referees point out that the roles of the B3 element and the forkhead binding sites need to be clearly distinguished. Referee 1 and 3 further indicate that it should be investigated whether or not context-dependent origins bind Fkh1/2 and whether adding Fkh binding sites to such origins is sufficient to overcome the context-dependent timing of origin firing. Referee 1 adds that mutant origin analyses should be investigated in origin-containing contexts, and referee 2 points out that it needs to be excluded that Fkh binding site mutations affect origin efficiency. Referee 2 finally suggests to examine whether two Fkh binding sites are generally required for context-independent early firing.

Given these evaluations, we would like to invite you to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript

will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Also, the revised manuscript may not exceed 30,000 characters (including spaces and references) and 5 figures plus 5 supplementary figures, which should directly relate to the corresponding main figure. Shortening of the manuscript text may be made easier by combining the Results and Discussion section, which may eliminate some redundancy that is inevitable when discussing the same experiments twice. Parts of the materials and methods can also be moved to supplementary information, but materials and methods essential for the understanding of the experiments described in the main manuscript file must remain in the main materials and methods section. Please also add the number (n) of experiments performed in the figure legends.

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I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely,

Editor
EMBO Reports

REFeree REPORTS:

Referee #1:

In their manuscript, Kristjuhan and colleagues investigate factors that may be responsible for the different times of replication origin initiation within S phase. In particular they address whether an origin's proximal DNA sequence or its location in the genome are the primary determinants of its function. Previous studies (primarily from the Brewer and Fangman group) suggested that location in the genome rather than the proximal sequence had the most significant contribution. This has led to the hypothesis that the local chromatin environment is the major determinant controlling the time of origin firing within S phase in *S. cerevisiae* cells.

To address the possible contribution of local chromatin state to time of origin firing, the authors followed the recruitment of Cdc45 onto seven different early or late origins integrated into a single (adjacent to the VPS13 gene) locus. The VPS13 gene is located within a region of the genome that is normally late replicating but does not normally have a nearby origin. Interestingly, they find that origins that normally initiate later in S phase all acquired a similar late activation timing profile. From this they conclude that local chromatin contributes to this synchronization of origin firing.

On the other hand, when the authors integrated four origins that initiate early in S phase in their normal locus all but one (ARS605) retained their early time of initiation. This led the authors to propose that these early origins contain specific proximal DNA sequence elements that override the local chromatin effects on replication timing. To address the nature of these sequences, they analyze one of these origins in detail (ARS607). ARS607 contains two putative Fkh1/2 binding sites and a B3 element that binds Abf1. The authors analyze both truncations and site-specific mutants and follow origin activity either after origin integration at the VPS13 locus. They also address whether ARS607 replicates early or late when integrated into the sites of other origins that replicate early or

late. These experiments confirm the significance of the Fkh1/2 binding sites in early replication origin initiation (as was previously reported by Knott et al., 2012) and show that ARS607 can convert late replicating regions into early ones.

The strength of this manuscript is that it provides strong evidence that there are origins whose proximal sequence is sufficient to overcome the influence of the local chromatin environment and retain their replication timing profile. This is in stark contrast to previous data on a more limited number of origins suggesting the opposite was true. It is also of interest that all the origins that retain the timing of their original location normally initiate early in S phase. The authors provide strong evidence that (for one origin) this determinant involves the Fkh1/ transcription factors, consistent with previous studies indicating an important role for this factor in the control of replication timing.

There are two concerns with the authors conclusion that it is Fkh1/2 binding alone that is the determinants: (1) the authors do not address whether the early-firing origin that does not overcome the local chromatin structure (ARS605) binds Fkh1/2; (2) the authors should show that adding Fkh1/2 binding sites to an origin (e.g. to ARS501) is sufficient to isolate that origin from local chromatin effects. The latter experiment is particularly important given the previous data already implicating Fkh1/2 in this process. It would also improve the manuscript to make similar mutations in at least one of the other Fkh1/2 binding origins.

There are two additional experiments that would improve the author's conclusions. First, the authors also do not clearly distinguish between the role of the B3 element and the Fkh1/2 binding sites. The authors should assess the effects of a point mutation of ARS607 that eliminates B3 function specifically (currently the only B3 mutation also eliminates one of the two ARS607 Fkh1/2 binding sites). Second, all of the analysis of the mutant origins is done at the VPS13 locus that normally does not have an adjacent origin. The authors should show assay key mutations in ARS607 at the HXK1 or CLD1 locus that normally has a late-firing origin. It is possible that the local chromatin effects are more influential at sites that have an origin. Finally, the authors need to include controls to show that origin activity is lost upon deletion of HXK1- and CLD1-adjacent origins.

Overall, the data in this manuscript are interesting and provide additional evidence about the interactions between origins and their chromatin environment. Although previous data has implicated Fkh1/2 function in determining early replication timing if the authors can show that this is sufficient to isolate an origin from the influence of the local chromatin environment, in combination with the new data suggesting that only a subset of origins respond to their local chromatin environment, these data will be a significant addition to these previous studies.

Minor point:

1. The authors use Cdc45-ChIP to monitor origin firing. S phase replication timing profiles would be a nice addition to address how the authors genomic modifications influence the replication pattern of the local chromatin beyond the specific origins tested.

Referee #2:

The paper by L oake et al is an interesting analysis of timing determinants of replication origins in yeast. The approach is to relocate several minimal yeast origins (ARS elements ~100bp) and test their replication timing in a late-replicating genomic region that lacks origins. Thus, they show that there are two classes of ARS; one where the ARS timing changes to the timing characteristic of the inserted locus, and one that causes the inserted loci to become early firing like the origin. These early origins turn out to have Forkhead transcription factor binding sites. These results confirm recent results from the Aparicio lab (Knott et al 2012) that Fkh1/2 regulate origin timing, especially of the earliest origins. The current study extends this previous study by demonstrating that ARS607 inserted into late-replicating loci is early firing and this requires the Fkh1/2 binding sites.

An important issue that should be addressed is whether the Fkh1/2 binding site mutation that

overlaps with the ARS607 ACS affects the efficiency of this origin. I think plasmid stability analysis would be sufficient to show that the ACS hasn't been disrupted. Even better would be analysis of firing and/or ORC binding. This assurance should apply to all ARS mutant constructs.

Another interesting aspect where these results begin to extend the previous study is in the possibility that two Fkh1/2 sites are crucial. Here, they show that in the case of ARS607, both sites are required. In the abstract they refer to "several" ARS with double Fkh1/2 binding sites, are they referring only to ARS607 and ARS305? Are there two Fkh1/2 binding sites in ARS737? This is something that should be clarified. Analysis of ARS737 is warranted to determine whether the requirement for two sites extends beyond ARS607. ARS305 was analyzed by Knott et al., who reported that knocking out both sites affected timing, but they did not report the effect of knocking out a single site. I think this more thorough analysis here might add significantly to this paper.

A final suggestion is to be careful with continued use of B3 to describe the Fkh1/2 binding site in ARS607. It's unfortunate that Weinreich used this term (though I think he said B3-like) because it may become confusing, as the original B3 (in ARS1) is an Abf1 site and Kowalski used the term B4 to name a site in ARS305, which now seems to be revealed as a Fkh1/2 site by Knott et al. So, I would suggest using the term Fkh1/2 binding site rather than B3.

Overall, I think this is an interesting paper, nicely written, and timely.

Referee #3:

Looke et al. report on the existence of two classes of replication origins in *S. cerevisiae*: those whose time of activation during the S phase depends on their chromatin context and those that consistently replicate early regardless of their genomic localization. Sequence analysis and ChIP analyses lead the authors to propose that binding of the Fkh1 transcription factor to some origins is determinant for early activation.

These conclusions are well supported by origin translocation experiments to different locations and by monitoring their firing by ChIP analysis of Cdc45 protein binding at various times across the cell cycle in alpha-factor synchronized cells. The binding of Fkh1 to wild type or mutated origins was followed also by ChIP.

The manuscript is very concise and clearly written and the conclusions are interesting and well supported by the results. Previous contributions from many laboratories over the years have established that transcription factors contribute to replication in several systems but I find that an important contribution of this work is the involvement of specific transcription factors in the regulation of specific origins in their native chromosome context.

Specific points:

1. The sequence of the B3 element in ARS305, ARS737 and ARS607 (Figure 4A) is very degenerated. How clearly can be determined whether or not a specific origin has a B3 element? For example, ARS605 is not included in the sequence comparison to show that it lacks B3.
2. Not all early origins contain a B3 element (page 10). Is there a correlation between early origins (beyond the four studied here) and the presence of Fkh1 binding sites?
3. The consensus binding sequence for Fkh1 in ARS607 should be indicated in Figure 4A and also their localization in ARS305 and ARS737. Are these sequences also present in ARS605? Does Fkh1 bind to ARS605, which is activated early but is context-dependent (Figure 2D)?
4. Results show a clear correlation between early, context-independent origin activity and Fkh1 binding to ARS305, ARS607 and ARS737. Removal of Fkh1 binding by mutagenesis of its binding sites results in late activation of ARS607 (Figure 4). This indicates that Fkh1 binding is needed (but maybe is not sufficient) for early, context-independent activity. Could a late firing context-dependent origin (like ARS609) be engineered to incorporate binding sites for Fkh1 in its ACS and B3 (or equivalent) elements to test whether it would behave as an early, context-independent origin?

Response to referees' comments.**Referee #1:**

Q1. The authors do not address whether the early-firing origin that does not overcome the local chromatin structure (ARS605) binds Fkh1/2.

A: ARS605 doesn't bind Fkh1. This result is now shown on figure 5A.

Q2. The authors should show that adding Fkh1/2 binding sites to an origin (e.g. to ARS501) is sufficient to isolate that origin from local chromatin effects. The latter experiment is particularly important given the previous data already implicating Fkh1/2 in this process.

A: This is a great idea and we tried it twice: first before initial submission of the manuscript and second time during the revision. Apparently, things are not that simple, and the main problem is to get Forkhead factors bind the target site.

In the first attempt we made a fusion origin between ARS609 (which is a late one) and ARS607 (an early, chromatin-independent origin). We mutated a couple of nucleotides close to ACS of ARS609 to create a Fkh1/2 consensus sequence, and then we fused this origin to B3-box region from ARS607 (see supplementary figure 2A). The fused origin was inserted into VPS13 locus and Cdc45 binding to it was determined. The origin fired late (suppl. Fig. 2B). However, after careful analysis of localization of Fkh1/2 binding sites in all yeast origins, we realized that the distance between the sites might be important and we thought that it wasn't optimal in our ARS609/607 fusion origin (distance between Fkh1/2 sites in this origin was 62 bp).

In the second attempt we mutated ARS609 in its genuine locus and introduced Fkh1/2 binding sites in the way that they copied the orientation and distance of the sites in the ARS607. Also this origin fired late (suppl Fig. 2C).

Now we have realized that the binding of Forkhead factors to DNA is more complex than just recognizing its (very simple) consensus sequence: RTAAAYA. Those sequences are found frequently all over the genome, sometimes several motifs are reasonably close to each other - like in ARS305, ARS607, or ARS737, but not all of them are bound by Forkhead factors in vivo (Harbison et al., 2004; Nature 431:99). Therefore we also tested whether Fkh1 does actually bind our modified ARS609 sequences in vivo, and it doesn't, despite the presence of two Fkh1/2 consensus sites in these sequences (suppl Fig. 2D). Therefore, we propose that Forkhead factors have to bind the origin to make it fire early, however, the presence of Fkh1/2 consensus sequences doesn't guarantee the binding of Forkhead proteins.

Currently, we don't know what else is required for efficient binding of the Forkhead factors in vivo. However, we think this is not just replication origin binding issue, but affects Forkhead binding at any place in the genome: in vivo binding data (Harbison et al., 2004; Nature 431:99) does confirm only a fraction of all predicted Forkhead binding sites. We speculate that nucleosome positioning around the binding sites might be important for DNA accessibility to Forkhead factors. Currently, we are setting up the experimental system to investigate the possible mechanisms of DNA binding by Forkhead factors in vivo, but these results will be published in a separate study. In regard to regulation of early DNA replication origins, we can say that two Fkh1/2 binding sites are

required, but this is not sufficient (because it doesn't mean that Forkhead factors will bind the sequence).

Q3. It would also improve the manuscript to make similar mutations in at least one of the other Fkh1/2 binding origins.

A: Now we did it with ARS305 and ARS737 too. This data is presented on figures 4G and 4H.

Q4. There are two additional experiments that would improve the author's conclusions. First, the authors also do not clearly distinguish between the role of the B3 element and the Fkh1/2 binding sites. The authors should assess the effects of a point mutation of ARS607 that eliminates B3 function specifically (currently the only B3 mutation also eliminates one of the two ARS607 Fkh1/2 binding sites).

A: We made a revised version of figure 4A where we show the locations of Forkhead sites on ARS305, ARS607 and ARS737. We also show the locations of ARS607 B3-box (Chang et al. 2011; NAR 39:6523) and ARS305 B4-box (Huang & Kowalski 1996; NAR 24:816). Both boxes are almost entirely overlapping with Fkh1/2 consensus binding site.

Therefore, it's technically impossible to separate Fkh1/2 binding site from the B3 box. However, we took an alternative approach and replaced the original Fkh1/2 binding site in ARS607 with different one (some variability is allowed in Forkhead consensus sites - RTAAAYA) and we also mutated remaining two additional nucleotides of the B3-box that were not the part of Fkh1/2 site (we termed the mutant "ARS607-B3toFkh"; see figure 4A). In this origin the genuine B3/Fkh-box is replaced with different consensus site for Forkhead binding, and this origin behaves exactly as the wt ARS607 (shown on figure 4I). This suggests that the primary function of B3/B4 boxes is binding of Forkhead factors. Taken together, we suggest that B3 box of ARS607 is the same as B4 box of ARS305 and both are actually just Forkhead binding sites (please see also Q3 of Ref#2 on that topic).

The current terminology of these regulatory sequences is confusing; however, we hope this paper helps to clarify that issue.

Q5. All of the analysis of the mutant origins is done at the VPS13 locus that normally does not have an adjacent origin. The authors should show assay key mutations in ARS607 at the HXK1 or CLD1 locus that normally has a late-firing origin. It is possible that the local chromatin effects are more influential at sites that have an origin.

A: We analysed wt ARS607 in several loci and now we tested also ARS607 with mutated Fkh1/2 binding sites in HXK1 locus. As expected, this origin was activated in late S phase also in the HXK1 locus (the results are shown on Fig. 4J).

Q6. The authors need to include controls to show that origin activity is lost upon deletion of HXK1- and CLD1-adjacent origins.

A: Now we included these controls on figure 4J. These experiments are done in strains where the original ARS sequences were replaced with URA3 marker ("HXK1-URA3" and "CLD1-URA3" on figure 4J).

Minor point:

Q7. The authors use Cdc45-ChIP to monitor origin firing. S phase replication timing profiles would be a nice addition to address how the authors genomic modifications influence the replication pattern of the local chromatin beyond the specific origins tested.

A: We don't have resources to monitor genome-wide replication timing profiles. Most likely the introduction of an early origin into a late region, or disruption of an early origin in its genuine location causes some change of replication timing pattern in neighbouring areas, but those influences are likely restricted to

the size of the particular replicon - at some point two replication forks meet and there should be no influence beyond this point. Also in the study by Knott et al., 2012, where the genomic sequence of ARS305 was manipulated, the firing pattern of neighbouring origins was unchanged. From our own results we know that manipulations in the ARS609 locus have no influence on the firing of ARS607 (located approximately at 56 kb from each other and there is ARS608 between these two). Also our analysis of other origins (that are included in this study) indicates no changes of their firing patterns in response to manipulations in ARS-containing (HXK1, CLD1) or ARS-deficient (VPS13, DPB11) loci.

Referee #2:

Q1. An important issue that should be addressed is whether the Fkh1/2 binding site mutation that overlaps with the ARS607 ACS affects the efficiency of this origin. I think plasmid stability analysis would be sufficient to show that the ACS hasn't been disrupted. Even better would be analysis of firing and/or ORC binding. This assurance should apply to all ARS mutant constructs.

A: We confirmed that ORC does bind to all of these origins and now we show this result on supplementary figure 1. In addition, we'd like to point out that Cdc45 is recruited to all of these origins, which itself indicates that the origins are functional.

Mutation of Fkh1/2 site in the ARS607 ACS was really challenging, as these sequences are overlapping (see Fig. 4A). However, the ACS in ARS607 is not perfect: 10 out of 11 bases in ACS match the consensus sequence (T/A T T T A T/C A/G T T T T/A). In the ARS607 the first base is G (instead of T or A). Fortunately, this "G" is part of the Fkh1/2 site and when mutated to "T", the Forkhead binding consensus is disrupted, but the origin's ACS is actually improved, because now it has 11/11 match to the consensus sequence. This was the only nucleotide we could mutate in this context and we were very pleased to see that it actually disrupted Forkhead binding to the origin. During the revision of the manuscript we also disrupted ACS-proximal Fkh1/2 sites in the ARS305 and ARS737 (which are located very close to ACS, but don't overlap with it) and we saw the similar delay of origin activation (shown on figures 4G and 4H).

Q2. Another interesting aspect where these results begin to extend the previous study is in the possibility that two Fkh1/2 sites are crucial. Here, they show that in the case of ARS607, both sites are required. In the abstract they refer to "several" ARS with double Fkh1/2 binding sites, are they referring only to ARS607 and ARS305? Are there two Fkh1/2 binding sites in ARS737? This is something that should be clarified. Analysis of ARS737 is warranted to determine whether the requirement for two sites extends beyond ARS607. ARS305 was analyzed by Knott et al., who reported that knocking out both sites affected timing, but they did not report the effect of knocking out a single site. I think this more thorough analysis here might add significantly to this paper.

A: Yes, also ARS737 has two Fkh1/2 sites. Now we have made a revised version of figure 4A where we show the locations of Forkhead sites on ARS305, ARS607 and ARS737. In addition, we performed statistical analysis of replication initiation timing of origins vs. the presence of Forkhead binding sites, and found that the origins which contain two Fkh1/2 sites separated by 60 to 120 base pairs, are almost exclusively early-firing (shown on Fig. 5B). Please see also our response to Ref#3 Q2.

Q3. A final suggestion is to be careful with continued use of B3 to describe the Fkh1/2 binding site in ARS607. It's unfortunate that Weinreich used this term (though I think he said B3-like) because it may become confusing, as the original B3 (in ARS1) is an Abf1 site and Kowalski used the term B4 to name a site in ARS305, which now seems to be revealed as a Fkh1/2 site by Knott et al. So, I would suggest using the term Fkh1/2 binding site rather than B3.

A: We agree and now we avoid using "B3-box" in the revised manuscript. In addition, we did an experiment where we changed ARS607 B3-box to a different Forkhead binding site. This origin behaves like wt ARS607; strongly suggesting that Fkh1/2 site is functionally the same as the B3-box. Please see also our response to Ref#1 Q4.

Referee #3:

Q1. The sequence of the B3 element in ARS305, ARS737 and ARS607 (Figure 4A) is very degenerated. How clearly can be determined whether or not a specific origin has a B3 element? For example, ARS605 is not included in the sequence comparison to show that it lacks B3.

A: It seems that B3-box is functionally the Fkh1/2 binding site and ARS605 doesn't have it. Please see also our responses to Ref#1 Q4 and Ref#2 Q3 for more discussion on that topic. We also made a revised version of figure 4A, but we didn't include ARS605 on that, because the figure is already rather complex.

Q2. Not all early origins contain a B3 element (page 10). Is there a correlation between early origins (beyond the four studied here) and the presence of Fkh1 binding sites?

A: Yes and no: not all early origins contain Fkh1/2 binding sites (=B3 box), but those that contain two Fkh1/2 sites at the right distance, fire early. We performed statistical analysis of replication initiation timing of origins vs. the presence of Forkhead binding sites, and found that the origins, which contain two Fkh1/2 sites separated by 60 to 120 base pairs, are almost exclusively early-firing (shown on Fig. 5B).

Apparently, there are many ways for early activation of origins, for example, those that are located near centromeres, are usually early-firing (Pohl et al., 2012; PLOS Genetics 8:e1002677). Also, the origins might be located in an early-replicating region, but when relocated to different site, they might fire late. Therefore, early origins were found in all groups, regardless of the presence or number of Fkh1/2 sites in their sequence. However, late origins were missing from groups containing two Fkh1/2 sites separated by 60-120 base pairs.

Q3. The consensus binding sequence for Fkh1 in ARS607 should be indicated in Figure 4A and also their localization in ARS305 and ARS737. Are these sequences also present in ARS605? Does Fkh1 bind to ARS605, which is activated early but is context-dependent (Figure 2D)?

A: We made a revised version of figure 4A where we show the locations of Forkhead sites on ARS305, ARS607 and ARS737. There are no Fkh1/2 sites near ARS605 and we did not include this origin to figure 4A (because Fig 4A is very busy already and adding a non-related origin to the scheme would make it too confusing). However, now we do show that Fkh1 doesn't bind to ARS605 (Fig. 5A).

Q4. Results show a clear correlation between early, context-independent origin activity and Fkh1 binding to ARS305, ARS607 and ARS737. Removal of Fkh1 binding by mutagenesis of its binding sites results in late activation of ARS607 (Figure 4). This indicates that Fkh1 binding is needed (but maybe is not sufficient) for early, context-independent activity. Could a late firing context-dependent origin (like ARS609) be engineered to incorporate binding sites for Fkh1 in its ACS and B3 (or equivalent) elements to test whether it would behave as an early, context-independent origin?

A: This is an excellent idea and we tried it twice. Apparently, things are not that simple, and the main problem is to get Forkhead factors bind the target site. Please see our response to Ref#1 Q2 for detailed answer.

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees. Referees 1 and 3 still have minor suggestions that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

Referee 1 feels that ChIP data should be added to confirm that less or no Fkh1 binds to the mutated forkhead binding sites in ARS305 and ARS737. I assume that these ChIP assays are not difficult to do, and I therefore agree that they would be a useful addition. However, if you feel differently, then please do let me know and we can discuss this issue further. Referee 3 suggests to discuss the possible reasons for why Fkh1 does not bind to the inserted forkhead binding sites, and this should be done.

I also noticed that the legends for figure 4 and supplementary figure 2 say "Error bars indicate standard deviation of at least two experiments", which is not possible. If $n < 3$ then no error bars can be shown. Please remove the error bars from all figures when $n = 2$. It would certainly be better if n would equal or be greater than 3 so that error bars could be included for all experiments.

Lastly, I have a few minor suggestions for the title and abstract that I copy here. Please let me know if you agree with these or if you would like to change something.

CHROMATIN DEPENDENT AND INDEPENDENT REGULATION OF DNA REPLICATION ORIGIN ACTIVATION IN BUDDING YEAST

Regulation of DNA replication origin activation

To elucidate the role of the chromatin environment in the regulation of replication origin activation, autonomously replicating sequences are inserted into identical locations in the budding yeast genome and their activation times in S phase are determined. Chromatin dependent origins adapt to the firing time of the surrounding locus. In contrast, the origins containing two binding sites for Forkhead transcription factors are activated early in S phase regardless of their location in the genome. Our results also show that genuinely late-replicating parts of the genome can be converted into early-replicating loci by insertion of a chromatin independent early replication origin, ARS607, while insertion of two forkhead binding sites is not sufficient for conversion.

I look forward to seeing a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Reports

REFeree REPORTS:

Referee #1:

The revised and improved manuscript by Kristjuhan and colleagues addresses the requirements for an origin of replication to initiate early in a late replicating region. The authors suggest that a pair of forkhead binding sites proximal to the origin is required for an origin to overcome local chromatin effects and initiate early in S phase. The authors have significantly improved the manuscript by showing that this observation is true at three independent origins (rather than only one). This finding is consistent with recent data from Aparicio and colleagues showing that elimination of forkhead transcription factors dramatically changes replication timing across the genome. Also related to these authors findings, the Aparicio studies find that origins that fire later when forkhead genes are deleted tend to have proximal forkhead binding sites.

What is not demonstrated here or in the Aparicio paper is whether the forkhead transcription factors are sufficient to make an origin early firing. Indeed, these authors attempts to demonstrate such an effect have apparently been unsuccessful. Because they have not shown that the inserted sites bind forkhead it remains unclear whether this is due to a lack of binding or the (although it is not clear that the inserted forkhead binding sites were actually bound *in vivo*), suggesting that there is more to isolating an origin from local chromatin effects than simply having forkhead binding sites.

Overall, the authors provide new evidence that the time of replication origin firing is influenced by factors that act proximal to the origin. This changes the impression from previous studies arguing large regions of DNA must be co-translocated with an origin to maintain its time of initiation. Importantly, they show that this approach can be used to categorize origins as chromatin dependent and independent. In terms of the mechanism of this distinction, these studies do not extend significantly beyond those of the previous Aparicio studies. Although they suggest that forkhead proteins are responsible for these differences, they authors do not provide evidence that binding of these proteins is sufficient. Because there are only a few origins studied whether forkhead is the key protein distinguishing between these two classes remains unclear.

Specific points:

1. The authors should show Fkh1 ChIP data for the that the mutants proposed to eliminate forkhead binding at ARS305 and ARS737.

Referee #2:

I am satisfied with the revisions

Referee #3:

Looke et al. have addressed in the revised manuscript the points I made in my previous review:

The revised Figure 4 clarifies the link between the B3 element and the Fkh1/2 binding sites.

The new Figure 5B presents a statistical analysis of the correlation between the replication index and the presence of Fkh1/2 sites

The authors have done the important experiment of testing whether including Fkh1/2 sites into a late origin would advance its time of firing. Results in the new Supplementary Figure 2 shows that this is not the case, probably because Fkh1/2 fails to bind to the engineered sites in ARS609. This leaves my original question unanswered but this is not the fault of the authors. Instead, this result opens the interesting possibility that the architecture of Fkh1/2 sites in chromatin is important for binding. This is also suggested by the observation that a particular spacing between the 2 Fkh1/2 sites in ARS elements its important for their activation timing (Figure 5B). I know that space limitations in EMBO Reports are tight but I think it would be worth adding a couple of sentences in the text to discuss this interesting possibility. Previous studies have shown that the nucleosome organization of replication origins is essential for their activity (see, for example, Simpson (1990) *Nature* 343, 387; Lipford and Bell (2001) *Mol Cell* 7, 21; Eaton et al (2010) *Genes Dev* 24, 748)

As a further suggestion, Figures 2 and 3 could be combined into a single Figure since they describe 2 parts of the same set of experiments. The combined Figure would not be more complex than Figures 1 or 4.

Altogether, I think this is a very interesting work that will be of interest to many groups in the field of DNA replication.

Please find attached the revised manuscript entitled "Chromatin dependent and independent regulation of DNA replication origin activation in budding yeast"

We were able to generate the missing yeast strains a bit faster than expected and we did the experiments requested by Ref#1. The following modifications were made to the manuscript:

1. The title and abstract are modified according to your suggestion, but we did not compile the data from figures 2 and 3 onto a single figure as suggested by Ref#3, because we think the data is easier to follow in the current setup.

2. The following discussion is added under the section "Double binding sites for Forkhead factors are present in early origins":

"...We propose that the accessibility of Fkh1/2 sites might be hindered by nucleosomes in these loci. This possibility is supported by the genome-wide nucleosome localization data indicating that the ARS609 locus is covered by nucleosomes, while ARS305, ARS607 and ARS737 are located in nucleosome-free regions (NFRs) [25]. Therefore, at least one of the Fkh1/2 sites inserted into the ARS609 locus is likely covered by a nucleosome, which in turn might make the site inaccessible for Forkhead factors. Earlier studies have shown that the localization of ARS1 regulatory sequences in the NFR is crucial for the function of the origin [26], and the ORC is a key factor for precise nucleosome positioning at the borders of origins [27,28]. However, the NFRs are maintained in the ARS loci even in the absence of ORC [28], indicating that the establishment of NFR is directed by the origin itself. Therefore, we propose that the sequences of chromatin-independent origins are unfavourable for nucleosome formation, which ensures the accessibility of Fkh1/2 sites in these loci."

3. We made two new strains to see whether Fkh1 does bind to mutated ARS305 or ARS737. It doesn't, and these results are now added to figure 5A (and we refer to these results in text). We also rearranged the sequence of columns in this figure to make it easier to follow – wt and the corresponding mutated origins are shown next to each other and controls are grouped together.

4. Now the error bars on all main figures are based on three experiments. Previously, the graphs that were added during the revision of the manuscript were based on two experiments, because we were running out of time – revision deadline was approaching. Now we did the third experiment and we show the averages with standard deviations. However, we didn't do the third experiment for the results shown on supplementary figure 2B and 2C, and for those we show the average of two experiments without error bars.

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