**Peer Review File**

**Manuscript Title:** Establishment and Function of Chromatin Organization at Replication Origins

## Reviewer Comments & Author Rebuttals

### Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1:

In this concise article the authors ask how the specific chromatin structure observed at S. cerevisiae replication origins (a nucleosome-free region (NFR) at the ARS consensus sequence flanked by phased nucleosome arrays) is established and what is its functional role.

Using chromatin reconstitution on a plasmid library of all 400 yeast origins, they show that: 1) chromatin assembly by salt gradient dialysis (SGD) reconstitutes the NFR but not the phased arrays; 2) when added to SGD chromatin, neither ORC alone, nor any of 17 tested chromatin factors, is able to reconstitute phased arrays; and 3) when ORC is assayed in combination with each of these 17 factors, only the chromatin remodellers INO80, ISW1a, ISW2 and Chd1 can generate origin nucleosome arrays in the presence of ORC.

In vivo analysis confirms a functional redundancy among the four remodellers and demonstrates a severe replication phenotype in a quadruple knock-out mutant compared to wild-type.

To explore the role of ORC, they analyse three different Orc1 mutants. The intrinsically disordered (IDR) domain of Orc1, of previously unknown function, turns out to be important for nucleosome positioning at origins and for chromosome replication, whereas the bromo-adjacent homology (BAH) domain, which can interact with nucleosomes, is not. The BAH deletion only results in minor replication defects. However, both domains together are required for viability, as is the ability of Orc1 to hydrolyze ATP.

In vitro experiments suggest that the death of both orc1-BAH-IDR and orc1-Walker B (deficient in ATP hydrolysis) mutants is caused by disrupted chromatin structure at origins of replication and not by problems in MCM loading.

In summary, this work demontrates that ORC collaborates with chromatin remodelers INO80, ISW1a, ISW2 or Chd1 to establish origin-adjacent chromatin architecture, and that this is an essential function for chromosome replication.

I found this article elegant, clear and convincing. I only have minor criticisms/questions.

1. When commenting on Fig. 2e, the authors state that "orc1-IDR mutants showed a mild but robust reduction of nucleosome occupancy". The data indeed show a weak effect for a single MNase seq

profile. However, I was unable to judge if this weak effect was robust. In the figure legend for most MNase-seq experiments, it is said that similar results were obtained with three independent replicates. Is the shown profile one of three replicates or is it an average of the three? Could they show as an Extended Data the three replicate experiments so the reader can be convinced that the said effect is robust?

2. When commenting on Fig. 3c, they state that "the Orc1-Walker B mutant showed reduced MCM loading (lane 4)". Looking at the gel, I was unconvinced of a weak MCM loading effect. Can they show replicate experiments and/or quantitate the gels to strengthen this statement?

3. The in vitro experiments with lethal orc1-BAH-IDR and orc1-Walker B mutants show convincingly that chromatin phasing is strongly affected. However, this does not demonstrate that this is responsible for cell death. Can they exclude that some other vital process than origin chromatin phasing is affected and is reponsible for the lethal phenotype?

4. What is the replication phenotype of single mutants in the four chromatin remodellers? Do these mutants replicate as well as wild-type or do they show some intermediary phenotype between wild-type and the quadruple knock-out?

Referee #2:

This study aims to identify the molecular basis for nucleosome organization at replication origins in yeast. Interestingly, each of the four major chromatin remodelers (INO80, ISW1a, ISW2 and Chd1) by themselves but necessarily in conjunction with the origin recognition complex (ORC) reconstitute nucleosome organization in a purified system. These are interesting preliminary findings. The authors conclude that ORC and remodelers organize nucleosomes at replication origins. However, much remains to be nailed down before the authors' conclusions become convincing and significant. In particular, the stated effects of certain key mutants are not convincing. The authors do not eliminate alternative interpretations of the data, which makes their findings preliminary. The authors’ results on the reconstitution and the effect of ORC and remodelers are dramatic, convincing, and exciting. The story remains under-developed.

Specific comments

1. Are there really 400 origins? I thought there were <300. This difference might actually diminish the magnitude of effects.

2. Plots need to be scaled from 0 not from 0.4, which exaggerates y-axis differences.

3. One does not get a sense of the variance among the 400 origins or among experiments. The data look highly smoothed.

4. One does not get a sense of what fraction of the 400 origins are actually responding to the remodelers, and whether they are bound by ORC, including in the ORC-only dataset.

5. Is it possible that the remodelers are “only” making chromatin more dynamic, thereby allowing ORC to bind? Then ORC is what organizes the chromatin rather than the remodelers. While I prefer the authors' model, alternative models are not excluded.

6. Is this statement “A-T-rich sequences, which are known to exclude nucleosomes” really true? They may have somewhat diminished affinity for nucleosomes, but clearly as this study and previous ones have shown, you need certain remodelers to really exclude nucleosomes.

7. The authors state “orc1-IDR mutants showed a mild but robust reduction of nucleosome occupancy”, and conclude “the IDR domain of Orc1 is important for nucleosome positioning”. This is simply not the case. The data show quite clearly that the IDR mutant is imperceptibly different from WT, requiring substantial smoothing and zooming-in to see a very minor effect that may or may not be due to experimental variation. Therefore, the conclusion about the IDR and nucleosome positioning is not supported by the data.

8. Much of the phenotypic studies in Suppl. Fig. 6 and Fig. 2 would be of interest to the replication community, but it is not evident that a broader readership would find interest here.

9. Fig. 3c lacks negative controls (mutant ARS1 DNA) and thus cannot be properly evaluated. Also it should be made clear that this is an in vitro experiment with a single ARS site.

Referee #3:

This study by Chacin and colleagues investigates the mechanism by which the yeast origin recognition complex, ORC, establishes phased nucleosome arrays near origins. They compare chromatin reconstitution in vitro using purified proteins with analysis of chromatin in mutant yeast strains. The work is technically very sound, and the data are of high quality; the text and figures are clear. The primary discoveries are that both a region of Orc1 containing both an intrinsically disordered region and the BAH domain and the ATPase activity of Orc1 are required to generate nucleosome arrays. Moreover, any one of four chromatin remodelers is sufficient with ORC to generate the arrays in vitro and are required in vivo. The inviability of cells with these Orc1 mutants or lacking all four remodelers suggests that the arrays near origins could be essential for normal replication. All in all, it’s a nice step forward, but unfortunately it isn’t the large leap I expect for studies published in Nature.

This evaluation of the magnitude of the advance is based on what is already known from prior studies, including the authors’ own work. A few relevant examples:

• The pattern of arrayed nucleosomes flanking yeast origins is known and ORC can establish a nucleosome-free region-but not the full pattern- in vitro, (Muller, 2010; Eaton, 2010).

• The Orc1 BAH domain can be essential for viability in the context of another orc mutation, and BAH contributes to establishing nucleosome positions at yeast origins in vivo (Muller, 2010).

• Chromatin remodeling enzymes (such as INO80 and ISW1) are required for replication using a combination of purified proteins and cell lysate (Azmi, 2017) and in a fully purified yeast replication reaction (Kurat, 2017).

• The requirement for these remodeling enzymes is to confer specificity to where ORC loads MCM complexes (Kurat 2017).

• ORC1 ATPase activity is important for yeast viability (Takehara, 2008), but not strictly for MCM loading itself (Evrin, 2013; Bowers, 2004).

What hasn't yet been done by the field is to directly connect the remodelers that are important for replication in vitro and in vivo to generating the nucleosome arrays. It is reasonable to hypothesize that the same remodelers required for controlling the site of MCM loading and stimulating DNA replication in vitro are also the ones that position nucleosomes near origins (such as INO80 and ISW1). The authors purified and tested eight chromatin remodelers as well as nine histone chaperones in addition to the set of licensing proteins and reconstituted the pattern of nucleosomes with a subset of remodelers and ORC; interestingly, the pattern did not require actual MCM loading.

While satisfying to connect ORC to the array assembly through these remodelers, it doesn't substantially change the way we think about chromatin at origins. Although the stated impetus is to determine the molecular basis by which ORC establishes nucleosome arrays, that mechanism isn't yet defined. The physical association of ORC with one or more remodelers, the differences among the four remodelers, and the function of the arrays that have been assembled are not addressed. Is chromatin assembled by one remodeler functionally different from another? We don't yet know how the IDR contributes to array assembly or if the BAH domain functions as a simple ORC recruiter, as has been suggested, or in some additional manner. Despite the title, one cannot definitively conclude that the arrays assembled are functional since the growth phenotype of the quadruple knockout strain could be attributed to other consequences of the remodeler mutations, as the authors acknowledge. The contribution of any histone post-translational modifications in the assembled chromatin (using Drosophila embryo histones) is not addressed. There's also no determination of how these results extrapolate to other species, and this point is particularly relevant because budding yeast origins and ORC are somewhat special for the importance of sequence specificity and DNA binding. For that reason, I'd expect to learn that metazoan ORC has the same function plus the full mechanism to be truly enthusiastic. Given what has already been learned by these authors and others, this study is very nicely done, but doesn't advance our understanding enough for this venue.

Referee #4:

This manuscript examines the factors that are involved in establishing spaced nucleosomes flanking the yeast origin of DNA replication, starting with in vitro reconstitution of ORCs on chromatinized templates with the addition of 17 different chromatin remodelers and histone chaperones. They found four chromatin remodelers already known to space nucleosomes (INO80, ISW2, ISW1a and

CHD1) that, when added with the ORC complex, can create a nucleosome spacing pattern flanking the ORC for 100s of ORCs, similar to that observed in vivo. The critical observation was any remodeler alone or ORC complex by itself was not sufficient to recapitulate this pattern of nucleosome spacing. In these assays the yeast genome was reconstituted in vitro into chromatin using purified histone octamers with a library of plasmid DNAs representative of the whole genome. Their assay is both powerful involving the purification of 17 different factors and yet direct and simple as well. Their observations suggest there is redundancy in factors that facilitate nucleosome spacing flanking the ARS of yeast, consistent with in vivo mapping of nucleosome positioning at the ORCs when any one of the remodelers is individually deleted. The authors did not comment on the observation that there appears to be an order of effectiveness for reconstituting the spacing in vitro, which appears to be in the order of INO80>ISW2>ISW1a>CHD1. They also find support for this redundancy by deleting critical subunits of each complex and examining the effect on the nucleosome spacing near ORCs in vivo and finding deletion of any one remodeler is not sufficient to disrupt the spacing. Although there is redundancy of function I wondered if all four are functionally redundant in this specific example or if maybe the two key factors that are redundant are ISW2 and INO80, given they have the more pronounced effects in their in vitro assay and have been indicated previously to be involved in DNA replication. Wouldn't it be important to test different combinations of remodeler subunit deletions to see that instead of having to mutate all four if merely deleting two, like Arp8 and Isw2, could block the spacing pattern?

To better understand the interplay between remodeler and the ORC complex the authors go on to delete the BAH or IDR regions of Orc1 and to make an ATPase catalytically-dead version of Orc1 to find which of these block nucleosome spacing and thereby imply possible interactions between remodeler and ORC complex or the functional interdependence. Based on no effect with deletion of the BAH domain and a barely detectable effect (not what I would call modest) with deletion of the IDR on the in vivo spacing it is surprising the combination of the two is lethal and blocks nucleosome spacing in vitro. The in vitro assays used for measuring ORC binding DNA and loading of the MCM helicase raises some questions given the size of the DNA target and potential nonspecific binding. How do they know for example if the ORC is binding properly to the ARS1 site and not elsewhere on the 2.8-kb fragment? The same is also true for the helicase. A simple end point experiment like that provided also does not address the question of potential affinity differences between wild-type and mutant Orc1 complexes, and is prone to gloss over any potential differences, and isn't clear if the amounts of complexes added are saturating or not. Given this is such a critical point I think there needs to be more done in this regard, especially in the mutant BAH-IDR ORC complex where spacing is so impacted. I think there remains a reasonable possibility that the change in nucleosome spacing observed with the BAH-IDR mutant is that ORC is not able to be properly positioned at the ARS and is more a reflection of barrier function of the ORC complex needed by the remodelers to establish nucleosome spacing.

Where this paper falls short is to provide insights into why ORC and remodeler together are needed to establish the nucleosome spacing pattern flanking the ARS. The explanation for why the AAA+-ATPase catalytically-dead Orc1 mutant could regulate nucleosome spacing doesn't make sense. Why would the ability of ORC to evict H2A-H2B dimers promote nucleosome spacing? I don't know of any example in the literature that would support this kind of model. The idea for the BAH domain interactions with nucleosomes promoting nucleosome spacing also doesn't provide a good rationale

for why the combination of ORC and remodeler are required together to promote spacing. Don't these remodelers intrinsically space nucleosomes as a general rule, and why would interactions of a BAH domain from the adjacent ORC complex assist in this process? The simplest explanation would be the barrier function of the ORC complex, which is dismissed in this paper and perhaps unfairly. In order for this to be a high impact paper I feel they need to better address this critical question rather than merely observing that the phenomena exists.

**Author Rebuttals to Initial Comments:**

We would like to thank all four reviewers for their time spent on reviewing our manuscript. We are delighted that the overall evaluations of our paper were very positive. We also take it as a good sign that often the same comments were brought up by several reviewers and that we could clarify and elaborate these obviously unclear or underdeveloped points and thereby strengthen our manuscript. Indeed, we addressed all the reviewers' points with new experiments as well as more detailed clarifications, which certainly improved the paper. In particular, reviewers' comments prompted us to establish unprecedented genome-scale *in vitro* replication experiments, both on naked DNA as well as on chromatinized templates. This way we could compare replication through chromatin for wild type and mutated ORC in a biochemically defined way. The results now really drive home our conclusion that the origin-flanking arrays of regularly spaced nucleosomes, as generated by the cooperation of ORC with remodelers, are functional and indeed crucial for efficient replication.

Compared to the previous 3 Figure version, we have now 4 main and also more Extended Data (ED) Figures. In particular, we added:

1. Heat map representations of chromatin structure at individual origins (new Fig. 2).
2. Point mutant *ars1* element controls (new Fig. 4c, f).
3. Genome-scale *in vitro* replication through chromatin (new Fig. 4i).
4. Chromatin reconstitutions with recombinant yeast histones (new ED Fig. 6).
5. *In vivo* effects on origin chromatin due to single and combined remodeler rapid ablations (new ED Fig. 7c, d and e).
6. *In vivo* effects on replication for single remodeler mutants (new ED Fig. 8a)
7. Genome-scale naked DNA and single *ARS1* locus chromatin *in vitro* replication (new ED Fig. 10a-d).
8. Genome-scale chromatin *in vitro* replication with various remodelers (new ED Fig. 10e,f).

In the following, please find a detailed response to all reviewer comments. To help to navigate through the revisions, we highlighted all direct citations from our revised text as well as new or altered Figures in green.

Referees' comments:

Referee #1:

In this concise article the authors ask how the specific chromatin structure observed at S. cerevisiae replication origins (a nucleosome-free region (NFR) at the ARS consensus sequence flanked by phased nucleosome arrays) is established and what is its functional role.

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In summary, this work demontrates that ORC collaborates with chromatin remodelers INO80, ISW1a, ISW2 or Chd1 to establish origin-adjacent chromatin architecture, and that this is an essential function for chromosome replication.

I found this article elegant, clear and convincing. I only have minor criticisms/questions.

We are very happy that the reviewer found our paper "*concise*" as well as "*elegant, clear and convincing*."

1. When commenting on Fig. 2e, the authors state that "orc1-IDR mutants showed a mild but robust reduction of nucleosome occupancy". The data indeed show a weak effect for a single MNase seq profile. However, I was unable to judge if this weak effect was robust. In the figure legend for most MNase-seq experiments, it is said that similar results were obtained with three independent replicates. Is the shown profile one of three replicates or is it an average of the three? Could they show as an Extended Data the three replicate experiments so the reader can be convinced that the said effect is robust?

We apologize that this was not presented clearly enough. Instead of showing one representative experiment, we now reformatted the figure so that the average of all four biological replicates is shown (new Fig. 3e). This way, the robustness of the difference in nucleosome positioning is more obvious. We thank the reviewer very much for pointing this out - we now re-analyzed all our data and always plot the average of replicates for all MNase-seq experiments in the manuscript.

Even though we are convinced that the effect of the *orc1*-IDR mutation was perceptible, robust and significant, we appreciate that this comment was raised by several reviewers (see also Referees #2 and #4) and now tone down our claim (page 8, line number 13: "..., while the *orc1*-IDR mutant showed a more pronounced, but still mild reduction of nucleosome occupancy (Fig. 3e) ..."), which does not affect our conclusions.

2. When commenting on Fig. 3c, they state that "the Orc1-Walker B mutant showed reduced MCM loading (lane 4)". Looking at the gel, I was unconvinced of a weak MCM loading effect. Can they show replicate experiments and/or quantitate the gels to strengthen this statement?

The reviewer is right that this perceived reduction in MCM loading was not a pronounced effect. To clarify, we repeated the ORC binding and MCM loading experiments now on chromatinised DNA templates, which are more relevant for our conclusions, and further probed origin specificity by comparing *ARS1* wild type to *ars1* mutant origins (new Fig. 4c). With this new experimental setup, we could not detect significant differences in MCM loading with the Orc1-Walker B mutant complex. In the light of the new experiments, we decided to omit our prior statement questioned by the reviewer from the main text. We point out that this strengthens our line of argument as it underscores that impaired replication with the Orc1-Walker B mutant is rather due to chromatin organization defects than impaired ORC binding or MCM loading.

3. The in vitro experiments with lethal orc1-BAH-IDR and orc1-Walker B mutants show convincingly that chromatin phasing is strongly affected. However, this does not demonstrate that this is responsible for cell death. Can they exclude that some other vital process than origin chromatin phasing is affected and is reponsible for the lethal phenotype?

We agree that this is an important concern and provide now more experimental evidence supporting our conclusion. As noted by the reviewer, our *in vitro* data strongly suggested that it was the most dramatic impairment of array generation with the Orc1-BAH-IDR or Orc1-Walker-B mutations that was responsible for the most dramatic lethal phenotype. Nonetheless, we agree that the link between chromatin defects and replication defects as cause for lethality was only inferred for these mutations. We had to demonstrate a direct causal link between impaired chromatin organization and impaired replication. This direct causality is difficult to establish in the convoluted *in vivo* setting and especially for lethal mutations. Instead, we used our biochemical fully defined chromatin replication system (Yeeles et al., 2015, *Nature*; Yeeles et al., 2017, *Mol Cell*; Kurat et al., 2017, *Mol Cell*). This enabled us now to directly show that these Orc1 mutations not only impaired origin chromatin organization but also specifically impaired replication of chromatinised templates. We observed these replication defects with a single origin (*ARS1*) chromatin template (new ED Fig. 10c,d), where MCM loading was similar for all Orc1 mutations (new Fig. 4c,f), as well as with the chromatinised genome-scale origin library (new Fig. 4i), but not with the origin library as naked DNA (new ED Fig. 10a,b), showing that replication defects were chromatin-dependent. In beautiful support for the causal link between origin chromatin organization and replication efficiency, the degree of replication defects *in vitro* correlated clearly with the degree of perturbed array structure generated by different ORC variants (Orc1-WT < Orc1-BAH < -IDR < -BAH-IDR/-Walker B; Fig. 4i vs. d,g). This order of defect severity in our *in vitro* experiments was also strikingly consistent with the respective order in our *in vivo* results, at least for the viable mutants where we could measure replication and if we take "dead" as "most compromised replication" for the Orc1-BAH-IDR/-Walker B mutants (Fig. 3d-f).

All this very strongly indicates that proper nucleosome organization at origins is crucial for replication through chromatin and depends on ORC. While such impaired replication by itself is sufficient to explain the lethal phenotype and most straight forward in the sense of Occam's razor, we agree that we cannot exclude that impairment of some other vital process might contribute to the lethal phenotype, too. We note that it is often difficult to pinpoint why exactly the absence of a factor (e.g., eliminating the RSC chromatin remodeler) is lethal and added now a respective note in the Discussion (page 12, line number 13: "Nonethless, we cannot rule out that impairment of some other vital process due to our Orc1 mutations may have contributed to the lethal phenotype.").

4. What is the replication phenotype of single mutants in the four chromatin remodellers? Do these mutants replicate as well as wild-type or do they show some intermediary phenotype between wild-type and the quadruple knock-out?

We performed FACS analyses with single chromatin remodeler deletion mutations and observed little replication defects with the exception of *arp8Δ*, which shows an appreciable delay in replication (new ED Fig. 8a), but much milder than the quadruple mutant (Fig. 3b). This is consistent with our model that INO80, ISW1a, ISW2 and Chd1 operate in a redundant manner.

Referee #2:

This study aims to identify the molecular basis for nucleosome organization at replication origins in yeast.

We realized that our previous version may have generated the impression that we mainly wished to "identify the molecular basis for nucleosome organization at replication origins in yeast." as perceived by the reviewer (and also by Referees #3 and #4). However, we wish to underscore that our study not only addresses the mechanism of nucleosome organization, but also the question if origin chromatin organization is functionally relevant for replication. As detailed below (point 8) and as also pointed out above, we added new experimental data (*in vitro* replication through chromatin with Orc1 mutant-ORC, new Fig. 4i) that provide now the previously missing link in our line of argumentation regarding the causal relationship between impaired nucleosome organization and impaired replication efficiency. We took great care to re-write the whole manuscript to make clear our two-pronged questions (nucleosome organization and functional importance) and conclusions.

Interestingly, each of the four major chromatin remodelers (INO80, ISW1a, ISW2 and Chd1) by themselves but necessarily in conjunction with the origin recognition complex (ORC) reconstitute nucleosome organization in a purified system. These are interesting preliminary findings. The authors conclude that ORC and remodelers organize nucleosomes at replication origins. However, much remains to be nailed down before the authors' conclusions become convincing and significant. In particular, the stated effects of certain key mutants are not convincing. The authors do not eliminate alternative interpretations of the data, which makes their findings preliminary. The authors' results on the reconstitution and the effect of ORC and remodelers are dramatic, convincing, and exciting. The story remains under-developed.

We appreciate that the reviewer finds our results "*dramatic, convincing, and exciting*", but also understand that the reviewer considers aspects of our study as "*preliminary*". As noted above and as detailed below, we took this as valuable incentive to develop our story further, and especially to "*nail down*" our conclusions.

Specific comments

1. Are there really 400 origins? I thought there were <300. This difference might actually diminish the magnitude of effects.

In our plasmid library we have ~400 origins, therefore we originally included them all in our analyses. Nonetheless, we agree with the reviewer that some of these origins are quite degenerate and differ significantly from the ACS consensus sequence. We now re-analyzed all our experiments with a smaller set of origins (~300, Soriano et al., 2014, *BMC Genomics*). Importantly, this did not diminish the magnitude of effects.

2. Plots need to be scaled from 0 not from 0.4, which exaggerates y-axis differences.

As suggested, we rescaled all our respective graphs in the paper, which did not invalidate y-axis differences.

3. One does not get a sense of the variance among the 400 origins or among experiments. The data look highly smoothed.

Yes, it is true that the ACS-aligned composite plots offer little information regarding the variance among individual origins. To address this point, we now added heat maps (new Fig. 2a-d), which show, row by row, the nucleosome organization at each origin. Data were smoothed using rolling means with a window size of 25 bp (Legend to Fig. 2; page 19, line number 3: "…smoothed using rolling means with a window size of 25 and square root normalised"), which is customary for these kind of analyses and does not obscure differences between origins. We thank the reviewer for raising this point as it led to this more informative and richer visualization of our data.

4. One does not get a sense of what fraction of the 400 origins are actually responding to the remodelers, and whether they are bound by ORC, including in the ORC-only dataset.

We agree that these are interesting aspects of our data that we should communicate to the reader. The first part of this comment (fraction of responding origins) is addressed now in the new heat map format (see also our reply to point 3 above) and taken up in the main text (page 5, line number 14: "No origin adopted the NFR-array nucleosome organization in either SGD chromatin alone (Fig. 2a) or plus ORC or remodeler alone (Fig. 2b, c), but most of them (> 80%) did if ORC was combined with remodeler (Fig. 2d)."). The second part of this comment (ORC binding to individual origins) we addressed both directly and indirectly. We directly monitored origin-specific ORC binding in our single locus assay for *ARS1* in a chromatin context (new Fig. 4c and f), but not across the 300 origins in our plasmid library SGD chromatin. Nonetheless, as not one single origin adopted the NFR-array nucleosome organization with remodeler alone but in most cases (> 80%) the combination of ORC + remodeler was required, we have a functional read-out that these origins bound ORC. We now note this also in the main text and also comment on the open question why some origins did not respond. (page 5, line number 16: "This functional read-out indirectly showed that these origins bound ORC. The few origins where no NFR-array pattern was reconstituted may have low-affinity ORC binding sites and/or may require additional factors, which needs future clarification.")

5. Is it possible that the remodelers are "only" making chromatin more dynamic, thereby allowing ORC to bind? Then ORC is what organizes the chromatin rather than the remodelers. While I prefer the authors' model, alternative models are not excluded.

This comment refers to a common misunderstanding regarding how such remodelers contribute to nucleosome organization. If it were true that remodelers just "lubricated" nucleosome dynamics while other factors determined the outcome of nucleosomal organization, then different remodelers, under otherwise identical conditions, should generate the same outcome. However, this is not the case as was observed earlier in mononucleosome assays (Brehm et al., 2000 *EMBO J*; Rippe et al., 2007, *PNAS*) and recently elaborated in genome-wide reconstitutions (Oberbeckmann et al., 2021, *Nat Comm*). Also in our present study we demonstrate this point: The combination of ORC with RSC did not generate the NFR-array organization (ED Fig. 5) even though RSC mobilizes nucleosomes under these conditions as seen by increasing the NFR depth and as known from previous observations under the same conditions (Krietenstein et al., 2016, *Cell*). We point out at several places in our manuscript, e.g., in the section title on page 4 ("ORC cooperates with chromatin remodelers in organizing origin chromatin") that it is the combination of ORC and remodelers that organizes origin chromatin. It is very likely, that RSC enables ORC to bind, but this was not sufficient for array generation. As it was shown before that RSC even inhibits *in vitro* replication through chromatin (Kurat et al., 2017, *Mol Cell*), we now point out that this is a further argument for the importance of arrays for replication efficiency (page 12, line number 21: "Indeed, our data suggested that just the generation of the NFR over origins, which allows ORC binding, was not sufficient for efficient replication as the RSC remodeler also generated the NFR (Extended Data Fig. 5), but no flanking nucleosomes nor arrays and was even inhibitory to replication *in vitro*[14]."). 

Regarding our model and alternative models, we now clarified our reasoning in the Discussion section (page 11, line number 3: "Conceptually, we see ORC as a "barrier" factor for nucleosome positioning. This concept is well established for GRFs like Reb1 or Abf1[21,22,26,40–42]. Barriers serve as alignment point so that remodelers with spacing activity, like INO80, ISW1a, ISW2 and Chd1, can phase arrays of regularly spaced nucleosomes to the barrier binding sites. In general, it is not known how barrier factors work. It was suggested[43,44] that special properties of the DNA binding domain are decisive for barrier factors, also in the context of the "pioneer factor" concept.

Our results here show that mere DNA binding was not sufficient for array generation. The Orc1-BAH-IDR and Orc1-Walker B mutations still allowed DNA binding (Fig. 4c, f) and NFR formation, but not array generation (Fig. 4d, g). It was suggested that GRFs allosterically regulate remodeler activity and that this enables remodelers to align arrays to such barriers[22]. We found that the BAH and IDR domains of Orc1 operated somewhat redundantly for the barrier function and speculate that Orc1 interacts, maybe also as an allosteric regulator, via these domains with the remodelers. We further speculate that the BAH domain may bind origin-flanking nucleosomes, which may be important for the accurate positioning of ORC at the ACS, consistent with previous results[30].

In comparison with established barriers like GRFs, our study suggests that ORC offers a wholly new aspect to barrier function as ATP hydrolysis by the $AAA^+$-ATPase domain was essential. A role for ATP hydrolysis by Orc1 is surprising, given that GRFs at gene promoters act as barriers without enzymatic activity[21,22]. We envision that ORC requires ATP hydrolysis for active manipulation of chromatin structure. Supporting this idea, recent work showed that INO80 remodels not only canonical nucleosomes containing histone octamers, but also hexasomes that lack one H2A-H2B dimer[45]. We speculate that such hexasomes may be generated by ATP hydrolysis by Orc1 and may be the actual substrate for INO80 at origins. In line with this idea, ORC can evict H2A-H2B dimers[46]. Such a mechanism involving ORC and INO80 may be especially relevant for efficient replication. We saw first hints for a maybe special role of INO80 as INO80 seemed particularly sensitive to the Orc1-BAH/IDR and Orc1-

Walker B mutations (Fig. 4d, g compared to Extended Data Fig. 9a,b) and particularly effective in our *in vitro* replications (Extended Data Fig. 10e, f).

We note that all three domains involved in ORC's barrier activity, BAH, IDR, ATPase, are highly conserved. Therefore it is very likely that ORC´s barrier function broadly applies across species[30,47–50].").

6. Is this statement "A-T-rich sequences, which are known to exclude nucleosomes" really true? They may have somewhat diminished affinity for nucleosomes, but clearly as this study and previous ones have shown, you need certain remodelers to really exclude nucleosomes.

We totally agree with the reviewer. One of our groups (Korber group) recently published a whole study where the argument for active remodeler-mediated nucleosome depletion over AT-rich sequences *in vivo* is comprehensively presented (Barnes and Korber 2021, *Int J Mol Sci*). However, in our case, we refer to the intrinsic nucleosome-exclusion properties of AT-rich sequences in salt gradient dialysis, not *in vivo*. We clarify this now (page 4, line number 13: "The latter was expected as origins are A-T-rich sequences[2,7], which intrinsically deplete nucleosomes in SGD chromatin[23,24].").

7. The authors state "orc1-IDR mutants showed a mild but robust reduction of nucleosome occupancy", and conclude "the IDR domain of Orc1 is important for nucleosome positioning". This is simply not the case. The data show quite clearly that the IDR mutant is imperceptibly different from WT, requiring substantial smoothing and zooming-in to see a very minor effect that may or may not be due to experimental variation. Therefore, the conclusion about the IDR and nucleosome positioning is not supported by the data.

This point was also raised by Referees #1 and #4 and we fully agree with its validity. Therefore, instead of showing one representative experiment, we now add a figure showing the averaged results of all four biological replicates, which underscores that the effect of the *orc1*-IDR mutation on nucleosome positioning *in vivo* is indeed significant (new Fig. 3e). Nonetheless, we also agree that the effect is not large and tone down our statement in the manuscript accordingly (page 8, line number 13: "..., while the *orc1*-IDR mutant showed a more pronounced, but still mild reduction of nucleosome occupancy (Fig. 3e) ..."), which does not compromise our conclusions. We thank the reviewer for bringing this up - we now applied this averaging over replicates throughout the manuscript for all graphs showing MNase-seq data of nucleosome positioning.

8. Much of the phenotypic studies in Suppl. Fig. 6 and Fig. 2 would be of interest to the replication community, but it is not evident that a broader readership would find interest here.

We agree that it is debatable what would be of interest to the broad versus the expert readership. Nonetheless, we feel strongly that the phenotypic results indeed contribute importantly to supporting our conclusions. There is a striking correlation between the order of severity of effects due to the Orc1 mutations on the one hand regarding chromatin organization and replication efficiency *in vitro* and on the other hand regarding chromatin and replication defects/growth defects/lethality *in vivo*: Orc1-BAH < Orc1-IDR < Orc1-BAH-IDR/-Walker B. We point this out explicitly in the main text (page 10, line number 6: "In contrast, replication of chromatinised templates showed decreasing efficiency with mutant ORC (Fig. 4i) and the severity of defects in replication efficiency scaled with the severity of defects in array formation (ORC WT < BAH < IDR < BAH-IDR/Walker B; Fig.

4d, g). Similar results were obtained with the chromatinised single *ARS1* locus (Extended Data Fig. 10c, d), where MCM loading was similar for all ORC (Fig. 4c, f).

Our *in vitro* experiments were strikingly consistent with our *in vivo* results (Fig. 3, ED Fig. 7a and 8) and supported our hypothesis that mutant ORC, especially the Orc1-BAH-IDR and Orc1-Walker B mutant-ORC, caused lethal replication problems due to their inability to generate nucleosomal arrays at the origins rather than compromised MCM loading.")

9. Fig. 3c lacks negative controls (mutant ARS1 DNA) and thus cannot be properly evaluated. Also it should be made clear that this is an in vitro experiment with a single ARS site.

We agree that this is an important point. We now added new experiments where we determined ORC binding and MCM loading on a chromatinised DNA templates bearing wild type *ARS1* or *ars1* mutant sequences. As can now be seen in new Fig. 4c and f, ORC binding and MCM loading is *ARS1*-specific. We also made clear that these are *in vitro* experiments on a single origin (page 9, line number 6: "They were also not compromised in ACS-dependent ORC-DNA binding or MCM loading with purified proteins *in vitro* on chromatin templates bearing the *ARS1* origin[14,38,39] (Fig. 4b, c). Specificity for the *ARS1* element was demonstrated by using a mutated *ars1* element that abolished ORC-DNA binding and MCM loading (Fig. 4c).").

Referee #3:

This study by Chacin and colleagues investigates the mechanism by which the yeast origin recognition complex, ORC, establishes phased nucleosome arrays near origins.

As also replied to Referees #2 and #4, we wish to underscore that our study not only addresses "*the mechanism by which the yeast origin recognition complex, ORC, establishes phased nucleosome arrays near origins*", but also the question if origin chromatin organization is functionally relevant for replication. We added new experimental data (*in vitro* replication through chromatin with WT and Orc1 mutant-ORC, new Fig. 4i) that provide now the previously missing link in our line of argumentation regarding the causal relationship between impaired nucleosome organization and impaired replication efficiency. We took great care to re-write the whole manuscript to make clear our two-pronged questions (nucleosome organization and functional importance) and conclusions.

They compare chromatin reconstitution in vitro using purified proteins with analysis of chromatin in mutant yeast strains. The work is technically very sound, and the data are of high quality; the text and figures are clear. The primary discoveries are that both a region of Orc1 containing both an intrinsically disordered region and the BAH domain and the ATPase activity of Orc1 are required to generate nucleosome arrays. Moreover, any one of four chromatin remodelers is sufficient with ORC to generate the arrays in vitro and are required in vivo. The inviability of cells with these Orc1 mutants or lacking all four remodelers suggests that the arrays near origins could be essential for normal replication. All in all, it's a nice step forward, but unfortunately it isn't the large leap I expect for studies published in Nature.

We appreciate the positive assessment and hope that the additional experiments and clarifications that we now provide (see below) are suited to convince the reviewer of the conceptual advance of our study.

This evaluation of the magnitude of the advance is based on what is already known from prior studies, including the authors' own work. A few relevant examples:

• The pattern of arrayed nucleosomes flanking yeast origins is known and ORC can establish a nucleosome-free region-but not the full pattern- in vitro, (Muller, 2010; Eaton, 2010).

Yes, as we write (page 3, line number 13: " The ACS element lies at the edge of an A-T-rich nucleosome-free region (NFR) and is flanked by arrays of regularly spaced nucleosomes[2–4] (Fig. 1a). ORC was implicated in the establishment of this organization as it was disrupted for ORC mutants defective in DNA binding[2,3]. However, it remains unknown how ORC achieves this origin chromatin structure and if it is functionally important for replication."), the NFR-array pattern around origins *in vivo* was described many years ago, but it was not clear how it was generated and if it was important for origin function. It is also true that studies suggested a role for ORC in NFR formation at origins. However, as also noted by the reviewer, the full picture was lacking - it was not at all clear how ORC is doing that and if it is involved in array formation. Therefore, we wish to point out that our results are in line with previous studies but now provide mechanistic explanation and the full picture (see also below).

• The Orc1 BAH domain can be essential for viability in the context of another orc mutation, and BAH contributes to establishing nucleosome positions at yeast origins in vivo (Muller, 2010).

Also this point is in line with our results but does not compromise the advance of our study.

• Chromatin remodeling enzymes (such as INO80 and ISW1) are required for replication using a combination of purified proteins and cell lysate (Azmi, 2017) and in a fully purified yeast replication reaction (Kurat, 2017).

Of course, we are aware of these findings, but wish to point out that it was never clear why these remodelers were important. Now we know that they are required for setting up the nucleosomal arrays (notwithstanding other roles, too) and that this is important for replication efficiency. We added now functional data using a fully reconstituted chromatin replication assay to support this conclusion (see below).

• The requirement for these remodeling enzymes is to confer specificity to where ORC loads MCM complexes (Kurat 2017).

We do not fully agree with this interpretation of our own published data. These assays were done with the ISW1a/Nap1 chromatin assembly system, never with other remodelers. The point was that highly assembled chromatin enforced origin specificity when ORC concentrations were high (Kurat et al., 2017, *Mol Cell*), but not that this was the main reason for, although related to, the remodeler requirement. Indeed, our new data show that just the NFR generation over origins, which should allow ORC binding, was not sufficient as the RSC remodeler also generated the NFR (ED Fig. 5), but was even inhibitory to *in vitro*

replication (Kurat et al., 2017, *Mol Cell*). Therefore, also this does not compromise the advance of our present study. We discuss this now (page 12, line number 21: "Indeed, our data suggested that just the generation of the NFR over origins, which allows ORC binding, was not sufficient for efficient replication as the RSC remodeler also generated the NFR (Extended Data Fig. 5), but no flanking nucleosomes nor arrays and was even inhibitory to replication *in vitro*[14]").

• ORC1 ATPase activity is important for yeast viability (Takehara, 2008), but not strictly for MCM loading itself (Evrin, 2013; Bowers, 2004).

We are aware of these points, cite them in our paper and they are perfectly in line with our results. However, a role for the ATPase activity of Orc1 in chromatin assembly at origins has not been proposed prior to our manuscript and is a wholly new aspect with regard to nucleosome barrier function as we explicitly point out now (page 11, line number 18: "In comparison with established barriers like GRFs, our study suggests that ORC offers a wholly new aspect to barrier function as ATP hydrolysis by the $AAA^+$-ATPase domain was essential. A role for ATP hydrolysis by Orc1 is surprising, given that GRFs at gene promoters act as barriers without enzymatic activity[21,22]. We envision that ORC requires ATP hydrolysis for active manipulation of chromatin structure. Supporting this idea, recent work showed that INO80 remodels not only canonical nucleosomes containing histone octamers, but also hexasomes that lack one H2A-H2B dimer[45].").

What hasn't yet been done by the field is to directly connect the remodelers that are important for replication in vitro and in vivo to generating the nucleosome arrays. It is reasonable to hypothesize that the same remodelers required for controlling the site of MCM loading and stimulating DNA replication in vitro are also the ones that position nucleosomes near origins (such as INO80 and ISW1).

As stated above, we now show that nucleosomal arrays have a crucial role in replication, beyond merely enforcing specific MCM loading.

The authors purified and tested eight chromatin remodelers as well as nine histone chaperones in addition to the set of licensing proteins and reconstituted the pattern of nucleosomes with a subset of remodelers and ORC; interestingly, the pattern did not require actual MCM loading.

We agree, the fact that MCM loading is not required for ORC´s chromatin organizing activity is interesting. We now point this out in the paper (page 10, line number 19: "ORC is also a master regulator of origin-adjacent chromatin organization, a mechanism that does not require MCM loading.").

While satisfying to connect ORC to the array assembly through these remodelers, it doesn't substantially change the way we think about chromatin at origins.

Although the stated impetus is to determine the molecular basis by which ORC establishes nucleosome arrays, that mechanism isn't yet defined. The physical association of ORC with one or more remodelers, the differences among the four remodelers, and the function of the arrays that have been assembled are not addressed.

Here we must disagree with the reviewer. As pointed out above, our study is not only about the mechanism of chromatin organization at origins but also about the functional importance of this organization for replication. This was not established before and does indeed change the way how "*we think about chromatin at origins*". We show that chromatin is not merely an obstacle that needs to be removed in order for the replication machinery to operate properly or for passively preventing unspecific MCM loading, but that the proper nucleosome array organization is a crucial prerequisite and gate keeper of physiological function.

We also disagree that the mechanism, by which ORC, together with the remodelers, generates the NFR-array organization, is not defined yet. Yes, it is not completely understood. However, we underscore that our finding that ORC works as a barrier in combination with spacing remodelers, similar to GRFs, is already pushing the mechanistic understanding as far as it gets in the context of the nucleosome positioning field. It would definitely be out of the scope of our study to also clarify how the cooperation between a barrier and remodelers work as this has been a long-standing question in the chromatin field that has not been resolved so far also in other contexts.

We now clarified our view of the mechanism in the Discussion section (page 11, line number 3: "Conceptually, we see ORC as a "barrier" factor for nucleosome positioning. This concept is well established for GRFs like Reb1 or Abf1[21,22,26,40–42]. Barriers serve as alignment point so that remodelers with spacing activity, like INO80, ISW1a, ISW2 and Chd1, can phase arrays of regularly spaced nucleosomes to the barrier binding sites. In general, it is not known how barrier factors work. It was suggested[43,44] that special properties of the DNA binding domain are decisive for barrier factors, also in the context of the "pioneer factor" concept.

Our results here show that mere DNA binding was not sufficient for array generation. The Orc1-BAH-IDR and Orc1-Walker B mutations still allowed DNA binding (Fig. 4c, f) and NFR formation, but not array generation (Fig. 4d, g). It was suggested that GRFs allosterically regulate remodeler activity and that this enables remodelers to align arrays to such barriers[22]. We found that the BAH and IDR domains of Orc1 operated somewhat redundantly for the barrier function and speculate that Orc1 interacts, maybe also as an allosteric regulator, via these domains with the remodelers. We further speculate that the BAH domain may bind origin-flanking nucleosomes, which may be important for the accurate positioning of ORC at the ACS, consistent with previous results[30].

In comparison with established barriers like GRFs, our study suggests that ORC offers a wholly new aspect to barrier function as ATP hydrolysis by the $AAA^+$-ATPase domain was essential. A role for ATP hydrolysis by Orc1 is surprising, given that GRFs at gene promoters act as barriers without enzymatic activity[21,22]. We envision that ORC requires ATP hydrolysis for active manipulation of chromatin structure. Supporting this idea, recent work showed that INO80 remodels not only canonical nucleosomes containing histone octamers, but also hexasomes that lack one H2A-H2B dimer[45]. We speculate that such hexasomes may be generated by ATP hydrolysis by Orc1 and may be the actual substrate for INO80 at origins. In line with this idea, ORC can evict H2A-H2B dimers[46]. Such a mechanism involving ORC and INO80 may be especially relevant for efficient replication. We saw first hints for a maybe special role of INO80 as INO80 seemed particularly sensitive to the Orc1-BAH/IDR and Orc1-Walker B mutations (Fig. 4d, g compared to Extended Data Fig. 9a,b) and particularly effective in our *in vitro* replications (Extended Data Fig. 10e, f).

We note that all three domains involved in ORC's barrier activity, BAH, IDR, ATPase, are highly conserved. Therefore it is very likely that ORC´s barrier function broadly applies across species[30,47–50].").

Is chromatin assembled by one remodeler functionally different from another?

This is a very interesting question that we would like to follow up in future work. For now, this is beyond the scope of our study, but we mention here some first hints in this regard.

Firstly, in contrast to fully symmetrical phasing around GRFs (Oberbeckmann et al., 2021, *Nature Commun*), reconstituted phasing around ACSs was a bit asymmetrical as also seen *in vivo* (Fig. 1a; Eaton et al., 2010, *Genes Dev.*; Berbenetz et al., 2010, *Plos Genet.*; Rossi et al., 2021, *Nature*). This asymmetry was different among the four remodelers. We speculate that this may differently influence replication efficiency, which may be reflected in the different DNA synthesis yields for the four remodelers (new ED Fig. 10f). This is very exciting, but preliminary and requires future clarification.

Secondly, regarding the proposed ORC/INO80/hexasome mechanism metioned above, we saw first hints for a maybe special role of INO80 compared to other remodelers, as we mention now in our Discussion (page 12, line number 4: "We saw first hints for a maybe special role of INO80 as INO80 seemed particularly sensitive to the Orc1-BAH/IDR and Orc1-Walker B mutations (Fig. 4d,g compared to ED Fig. 9a,b) and particularly effective in our *in vitro* replications (Extended Data Fig. 10e, f)."), but again, this is out of the scope of the manuscript.

Despite the title, one cannot definitively conclude that the arrays assembled are functional since the growth phenotype of the quadruple knockout strain could be attributed to other consequences of the remodeler mutations, as the authors acknowledge.

This is a central point. As pointed out at the very beginning of this rebuttal, we now provide *in vitro* replication data with wild type and mutant ORC that establish the functional importance of nucleosome organization at origins for replication efficiency. We write now in our manuscript: (page 10, line number 1: "So far our data strongly suggested that the inability of the Orc1-BAH-IDR or Orc1-Walker-B mutant-ORC to generate nucleosome arrays at origins was responsible for cell death and in particular due to impaired replication. To test this directly, we extended our purified and genome-scale *in vitro* reconstitution system to not only reconstitute chromatin organization but also replication. As control, replication on the naked DNA origin library was similar for WT and all mutant ORC (Extended Data Fig. 10a, b). In contrast, replication of chromatinised templates showed decreasing efficiency with mutant ORC (Fig. 4i) and the severity of defects in replication efficiency scaled with the severity of defects in array formation (ORC WT < BAH < IDR < BAH-IDR/Walker B; Fig. 4d, g). Similar results were obtained with the chromatinised single *ARS1* locus (Extended Data Fig. 10c, d), where MCM loading was similar for all ORC (Fig. 4c, f).

Our *in vitro* experiments were strikingly consistent with our *in vivo* results and supported our hypothesis that mutant ORC, especially the Orc1-BAH-IDR and Orc1-Walker B mutant-ORC, caused lethal replication problems due to their inability to generate nucleosomal arrays at the origins rather than compromised MCM loading.").

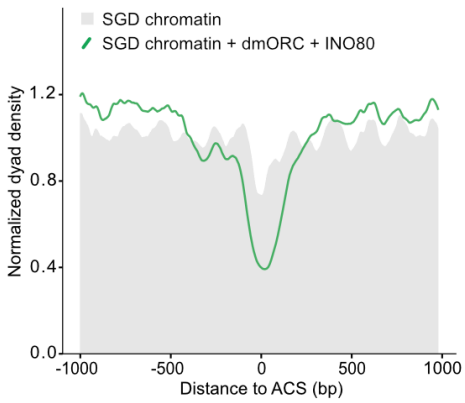
The contribution of any histone post-translational modifications in the assembled chromatin (using Drosophila embryo histones) is not addressed.

Yes, we wondered about such contributions, too, and added experiments with recombinant, i.e. modification-free, yeast histones and obtained the same results (new ED Fig. 6). We point this out explicitly in our main text (page 5, line number 5: "Recombinant yeast histones[25] gave similar results (Extended Data Fig. 6), showing that the mechanism did not depend on histone modifications."). Of course, this does not exclude a role for histone modifications in some other regard, but at least not for the core mechanism of phased array generation.

There's also no determination of how these results extrapolate to other species, and this point is particularly relevant because budding yeast origins and ORC are somewhat special for the importance of sequence specificity and DNA binding. For that reason, I'd expect to learn that metazoan ORC has the same function plus the full mechanism to be truly enthusiastic.

We agree that this comparison across species would be very interesting. At present, it is unclear how origin selection works in metazoans. As the reviewer points out, this is mainly due to a lack of conserved ORC binding sites. We do believe that the mechanism we describe here is conserved throughout species. (for example, page 12, line 8: "We note that all three domains involved in ORC's barrier activity, BAH, IDR, ATPase, are highly conserved. Therefore it is very likely that ORC´s barrier function broadly applies across species[30,47–50]."). However, determining how origins are defined in higher eukaryotes and to what extent our mechanism applies is an important goal for the future but clearly out of the scope of this study.

Nonetheless, we tried to address the reviewer's point by testing if purified *Drosophila* ORC could substitute for yeast ORC in our assay. Our expectations were not high mainly because of a) *Drosophila* origin libraries are not available and it is unlikely that *Drosophila* ORC would recognize yeast origins (Alessandro Costa, personal communication) and b) we used purified yeast remodelers in the assay and *Drosophila* ORC likely specifically interacts with *Drosophila* remodelers. We kindly obtained purified *Drosophila* ORC complex from the Bleichert lab and included it in our assay. As expected, *Drosophila* ORC could not substitute for the yeast complex (see Figure left; shown is the reaction with INO80. All four remodelers showed similar results.). Although a negative result, this does not change the way we think about homology of the mechanism. It rather shows that our assay is very specific.



Given what has already been learned by these authors and others, this study is very nicely done, but doesn't advance our understanding enough for this venue.

We always believed that our study represents a major advance. However, the review process helped to further develop our manuscript, especially the unprecedented and surprisingly pronounced functional link between origin chromatin organization and replication efficiency, and we are now even more confident that it represents a major breakthrough and should be made as visible as possible. We hope the reviewer agrees.

Referee #4:

This manuscript examines the factors that are involved in establishing spaced nucleosomes flanking the yeast origin of DNA replication, starting with in vitro reconstitution of ORCs on chromatinized templates with the addition of 17 different chromatin remodelers and histone chaperones.

We realized that our previous version may have generated the impression that we mainly wished to examine *„the factors that are involved in establishing spaced nucleosomes flanking the yeast origin of DNA replication*" as perceived by the reviewer (and also by Referees #2 and #3). However, we wish to underscore that our study not only addresses the mechanism of nucleosome organization, but also the question if origin chromatin organization is functionally relevant for replication. We added new experimental data (genome-scale *in vitro* replication through chromatin with WT and Orc1 mutant-ORC, new Fig. 4i) that provide now the previously missing link in our line of argumentation regarding the causal relationship between impaired nucleosome organization and impaired replication efficiency. We took great care to re-write the whole manuscript to make clear our two-pronged questions (nucleosome organization and functional importance) and conclusions.

They found four chromatin remodelers already known to space nucleosomes (INO80, ISW2, ISW1a and CHD1) that, when added with the ORC complex, can create a nucleosome spacing pattern flanking the ORC for 100s of ORCs, similar to that observed in vivo. The critical observation was any remodeler alone or ORC complex by itself was not sufficient to recapitulate this pattern of nucleosome spacing. In these assays the yeast genome was reconstituted in vitro into chromatin using purified histone octamers with a library of plasmid DNAs representative of the whole genome. Their assay is both powerful involving the purification of 17 different factors and yet direct and simple as well. Their observations suggest there is redundancy in factors that facilitate nucleosome spacing flanking the ARS of yeast, consistent with in vivo mapping of nucleosome positioning at the ORCs when any one of the remodelers is individually deleted. The authors did not comment on the observation that there appears to be an order of effectiveness for reconstituting the spacing in vitro, which appears to be in the order of INO80>ISW2>ISW1a>CHD1.

We agree that it is interesting to ask to what extent the four remodelers differ in their ability to generate nucleosome arrays at origins and if this matters for replication efficiency. As also replied to Referee #3, we would like to follow this up in future work. For now, this is beyond the scope of our study, but we mention here some first hints in this regard.

Firstly, in contrast to fully symmetrical phasing around GRFs (Oberbeckmann et al., 2021, *Nature Commun*), reconstituted phasing around ACSs was a bit asymmetrical as also seen *in vivo* (Fig. 1a; Eaton et al., 2010, *Genes Dev.*; Berbenetz et al., 2010, *Plos Genet.*;

Rossi et al., 2021, *Nature*). This asymmetry was different among the four remodelers. We speculate that this may differently influence replication efficiency, which may be reflected in the different DNA synthesis yields for the four remodelers (new ED Fig. 10f). This is very exciting, but preliminary and requires future clarification.

Secondly, we saw first hints for a maybe special role of INO80 compared to other remodelers, as we mention now in our Discussion (page 12, line number 4: "We saw first hints for a maybe special role of INO80 as INO80 seemed particularly sensitive to the Orc1-BAH/IDR and Orc1-Walker B mutations (Fig. 4d,g compared to ED Fig. 9a,b) and particularly effective in our *in vitro* replications (Extended Data Fig. 10e, f)."), but again, this is out of the scope of the manuscript.

They also find support for this redundancy by deleting critical subunits of each complex and examining the effect on the nucleosome spacing near ORCs in vivo and finding deletion of any one remodeler is not sufficient to disrupt the spacing. Although there is redundancy of function I wondered if all four are functionally redundant in this specific example or if maybe the two key factors that are redundant are ISW2 and INO80, given they have the more pronounced effects in their in vitro assay and have been indicated previously to be involved in DNA replication. Wouldn't it be important to test different combinations of remodeler subunit deletions to see that instead of having to mutate all four if merely deleting two, like Arp8 and Isw2, could block the spacing pattern?

This is an excellent suggestion that we incorporated now into our manuscript. We analysed published data of rapid remodeler depletion on nucleosome positioning (Kubik et al., 2019, *Nat. Struct. Mol. Biol.*) for effects on origin chromatin structure (new ED Fig. 7 c,d and e). We write now in the main text (page 6, line number 12 : "Similarly, re-analyzing rapid remodeler depletion experiments[18] showed that single depletions had little, double depletions intermediate, and depletion of all four remodelers had the strongest array defects (Extended Data Figs 7c,d, and e)."). As presumed by the reviewer, the combined ablation of INO80 and ISW2 had a stronger effect than that of ISW1 and Chd1. Nonetheless, as data for all double and triple combinations were not available, we abstain from drawing strong conclusions about more or less dedicated roles for individual remodelers. We merely speculate about a special role for INO80 in remodeling hexasomes, as noted above.

To better understand the interplay between remodeler and the ORC complex the authors go on to delete the BAH or IDR regions of Orc1 and to make an ATPase catalytically-dead version of Orc1 to find which of these block nucleosome spacing and thereby imply possible interactions between remodeler and ORC complex or the functional interdependence. Based on no effect with deletion of the BAH domain and a barely detectable effect (not what I would call modest) with deletion of the IDR on the in vivo spacing it is surprising the combination of the two is lethal and blocks nucleosome spacing in vitro.

This point was also raised by Referees #1 and #2 and we fully agree with its validity. Therefore, instead of showing one representative experiment, we now add a figure showing the averaged results of all four biological replicates, which underscores that the effect of the *orc1*-IDR mutation on nucleosome positioning *in vivo* is indeed significant (new Fig. 3e). Nonetheless, we also agree that the effect is not large and tone down our statement in the manuscript accordingly (page 8, line number 13: "..., while the *orc1*-IDR mutant showed a more pronounced, but still mild reduction of nucleosome occupancy (Fig. 3e) ..."), which does not compromise our conclusions.

The in vitro assays used for measuring ORC binding DNA and loading of the MCM helicase raises some questions given the size of the DNA target and potential nonspecific binding. How do they know for example if the ORC is binding properly to the ARS1 site and not elsewhere on the 2.8-kb fragment? The same is also true for the helicase. A simple end point experiment like that provided also does not address the question of potential affinity differences between wild-type and mutant Orc1 complexes, and is prone to gloss over any potential differences, and isn't clear if the amounts of complexes added are saturating or not. Given this is such a critical point I think there needs to be more done in this regard, especially in the mutant BAH-IDR ORC complex where spacing is so impacted. I think there remains a reasonable possibility that the change in nucleosome spacing observed with the BAH-IDR mutant is that ORC is not able to be properly positioned at the ARS and is more a reflection of barrier function of the ORC complex needed by the remodelers to establish nucleosome spacing.

This is a valid point that we address now with additional experiments. First, we demonstrate ACS-specific binding of ORC by using a chromatinized DNA template with a point-mutated *ars1* element as negative control (new Fig. 4c and f). Indeed, we did not detect ORC binding or MCM loading at the mutant *ars1* anymore and thus exclude non-specific ORC binding. Second, we provide now *in vitro* replication experiments using our assay as mentioned above on both the origin plasmid library as free DNA as well as on corresponding chromatinised templates where we compared wild type and mutant ORC complexes. We did not observe any difference on free DNA (new ED Fig. 10a,b), which makes it unlikely that there are affinity differences between binding these ORC complexes to DNA that would matter for replication and rather argues that the amounts of complex added were saturating. We consider such a functional readout more indicative of sufficient ORC binding than a mere binding assay. In addition, the binding assay on the chromatinized *ARS1* template (new Fig. 4c and f) confirmed equal binding of all WT and Orc1 mutant ORCs in the chromatin context. In contrast, the Orc1-mutant complexes showed decreased replication efficiencies with the chromatin templates (new Fig. 4i; new ED Fig. 10c,d), and the degree of impaired replication corresponded to the severity of their *in vivo* phenotype (Fig. 3f) and to the degree of impaired array generation *in vitro* (Fig. 4d and g) and *in vivo* (Fig. 3e). This strongly argues that this impairment in replication is not simply due to impaired DNA binding of Orc1-mutant complexes. Instead, we argue that it is the barrier function of the ORC complex that is compromised (see below).

Where this paper falls short is to provide insights into why ORC and remodeler together are needed to establish the nucleosome spacing pattern flanking the ARS. The explanation for why the AAA+-ATPase catalytically-dead Orc1 mutant could regulate nucleosome spacing doesn't make sense. Why would the ability of ORC to evict H2A-H2B dimers promote nucleosome spacing? I don't know of any example in the literature that would support this kind of model. The idea for the BAH domain interactions with nucleosomes promoting nucleosome spacing also doesn't provide a good rationale for why the combination of ORC and remodeler are required together to promote spacing. Don't these remodelers intrinsically space nucleosomes as a general rule, and why would interactions of a BAH domain from the adjacent ORC complex assist in this process? The simplest explanation would be the barrier function of the ORC complex, which is dismissed in this paper and perhaps unfairly. In order for this to be a high impact paper I feel they need to better address this critical question rather than merely observing that the phenomena exists.

We apologize that our thinking on all these important conceptual and mechanistic questions was not spelled out clearly enough. We are especially sorry that the reviewer got the impression that we dismiss a barrier function for ORC, while the contrary is true. We now take up all of the reviewer's questions and discuss them explicitly (page 11, line number 3: "Conceptually, we see ORC as a "barrier" factor for nucleosome positioning. This concept is well established for GRFs like Reb1 or Abf1[21,22,26,40–42]. Barriers serve as alignment point so that remodelers with spacing activity, like INO80, ISW1a, ISW2 and Chd1, can phase arrays of regularly spaced nucleosomes to the barrier binding sites. In general, it is not known how barrier factors work. It was suggested[43,44] that special properties of the DNA binding domain are decisive for barrier factors, also in the context of the "pioneer factor" concept.

Our results here show that mere DNA binding was not sufficient for array generation. The Orc1-BAH-IDR and Orc1-Walker B mutations still allowed DNA binding (Fig. 4c, f) and NFR formation, but not array generation (Fig. 4d, g). It was suggested that GRFs allosterically regulate remodeler activity and that this enables remodelers to align arrays to such barriers[22]. We found that the BAH and IDR domains of Orc1 operated somewhat redundantly for the barrier function and speculate that Orc1 interacts, maybe also as an allosteric regulator, via these domains with the remodelers. We further speculate that the BAH domain may bind origin-flanking nucleosomes, which may be important for the accurate positioning of ORC at the ACS, consistent with previous results[30].

In comparison with established barriers like GRFs, our study suggests that ORC offers a wholly new aspect to barrier function as ATP hydrolysis by the $AAA^+$-ATPase domain was essential. A role for ATP hydrolysis by Orc1 is surprising, given that GRFs at gene promoters act as barriers without enzymatic activity[21,22]. We envision that ORC requires ATP hydrolysis for active manipulation of chromatin structure. Supporting this idea, recent work showed that INO80 remodels not only canonical nucleosomes containing histone octamers, but also hexasomes that lack one H2A-H2B dimer[45]. We speculate that such hexasomes may be generated by ATP hydrolysis by Orc1 and may be the actual substrate for INO80 at origins. In line with this idea, ORC can evict H2A-H2B dimers[46]. Such a mechanism involving ORC and INO80 may be especially relevant for efficient replication. We saw first hints for a maybe special role of INO80 as INO80 seemed particularly sensitive to the Orc1-BAH/IDR and Orc1-Walker B mutations (Fig. 4d, g compared to Extended Data Fig. 9a,b) and particularly effective in our *in vitro* replications (Extended Data Fig. 10e, f).

We note that all three domains involved in ORC's barrier activity, BAH, IDR, ATPase, are highly conserved. Therefore it is very likely that ORC´s barrier function broadly applies across species[30,47–50]."). 

We strongly feel that our here provided data and mechanistic conclusions go beyond "*merely observing that the phenomena exists*" and hope that the reviewer can agree.

**Reviewer Reports on the First Revision:**

Referee #1:

I think the authors have done a nice and honest job to answer all the referees' comments resulting in a much improved manuscript that goes farther than the first version in the understanding of biological mechanisms. I have no further criticisms on this revised version.

Referee #2:

In this revised manuscript the author address issues raised by the reviewers. While I am still supportive of this work, there remain unresolved issues. What is most interesting is that ORC uses ATP hydrolysis to organize nucleosomes which differs from what is observed at promoters with GRFs, where only the ATPase of the remodelers is used. Also of interest is the redundancy of remodelers in nucleosome organization at ORCs, in which nucleosome positioning is the same regardless of the remodeler, again different from promoters.

Some unresolved issues persist from the first round, while others are new:

1. I must insist that all plots have their y-axis starting at zero. The authors claimed to have done this but have not.

2. The authors state that the reconstituted arrays are “reminiscent of promoters”. However, in Fig. 1h, arrays appear to start at the same distance from the ACS regardless of the tested remodeler, and inter-nucleosome distances also appear to be the same. How does this make sense given that these properties are normally different with different remodelers, when examined at promoters with GRFs? ED_Fig. 7b shows the same in vivo, which validates the authors in vitro findings, but needs to be explained in light of what is known about the spacing activity of these remodelers that is inherent in these enzymes. In the rebuttal the authors state “If it were true that remodelers just lubricated nucleosome dynamics while other factors determined the outcome of nucleosomal organization, then different remodelers, under otherwise identical conditions, should generate the same outcome”. But this is exactly what is observed. Different remodelers with different spacing activities do give the same result. Yes, RSC is the exception, but as the authors point out, RSC is not like the other remodelers since it lacks positioning activity.

3. That INO80 can produce arrays without a barrier at promoters is mentioned by the authors but not rationalized as to why promoters and origins differ in this regard.

4. The authors mention that arrays around ORCs are asymmetrical. This observation is not obvious from the referenced figure 1a. It looks symmetrical to me.

5. It is clear from the authors findings that ORC is required for nucleosome array generation. However, the authors try to walk a tight line about the BAH and IDR domains. Despite the in vivo effects on cell cycle, Fig. 3 is surprising by the relative lack of effect of BAH and IDR on nucleosome

organization. The authors were careful in this revised version to scale back their claims (although they still claim in the rebuttal that BAH+IDR mutants do not allow array generation). But now it becomes just an observation without a meaningful conclusion. My conclusion would be that BAH and IDR have a minor contribution to nucleosome organization at ACS (except in conjunction with INO80 alone, as shown in the much later Fig. 4g). Aspects of Fig. 3 adds little and may be better suited as a supplementary figure, unless the authors choose to whittle down Orc1 to find out what is the minimal region need for organization. Maybe only the DNA binding domain is needed, if it is solely a barrier function, but this is ruled out later with the orc1-Walker B mutation. The authors do assay for complex assembly and DNA binding for the mutants in Fig. 4, but without the lead-in for the orc1-Walker B mutation, there does not seem to be a strong basis for the proposed hypotheses that are to be tested (as written). The orc1-Walker B mutation has a substantial effect on nucleosome organization but this is not introduced until Fig. 4d. Also, the IDR mutation is more dramatic with INO80 than with the mix of the remodelers. This whole section could be consolidated and the limited effects of IDR to INO80 could be made clearer.

6. More on Fig. 3e: The authors' revised figure now reports an average of replicates in response to reviewer comments. However, the reviewers were seeking to understand the variance among replicates. Simply providing the average does provide a measure of variance. The authors should plot all four replicates.

Referee #3:

Kurat and colleagues have submitted a response and revision for their study of nucleosome phasing by ORC. In my opinion, the most substantial improvement in this revised submission is Fig. 4i (and ED10), which demonstrates strong replication defects in vitro from two Orc1 deletion mutants. These mutants load MCM but do not support nucleosome array assembly, are largely inactive for replicating chromatin templates in vitro, and do not support yeast cell viability. Thus, Orc1 has an essential function that can be separated from its essential role in MCM loading. This important addition goes beyond the original submission that partially dissected the ability of Orc1 to stimulate array assembly. Though not perfect proof of causation, the correlation between replication in vitro and array assembly plus the inviability of the ORC1 deletions despite no demonstrable effect on MCM loading in vitro is compelling. Why arrays are critical for chromatin replication independently of MCM loading is as yet unknown.

I'm somewhat surprised that the authors have not included any protein-protein interaction assays - either in solution or at origins- to determine if and how Orc1 interacts with the four chromatin remodelers. For example, Orc1 has been reported to bind INO80, but is that through the IDR-BAH or affected by the Walker B mutation? The lack of testing physical association of ORC with remodelers was mentioned in the initial review, but not addressed except in passing during the discussion as a possibility. Also does ORC bind all four, and are those interactions similarly affected (or not) in the Orc1 mutants? The barrier model discussion is fine but leaves quite a lot unanswered in terms of molecular mechanism.

Referee #4:

The authors clearly show the ATPase activity of Orc1 and the BAH and IDR regions of Orc1 are required for the nucleosome spacing activity around the ARS, but there is no experimental data to support the several speculations that are made about allosteric effects or even direct interactions between ORC and the remodelers. Besides the speculations there is no other data to provide supporting evidence for one model over another, so the report is more about cataloging the phenomena rather than providing insights as to why this might occur. ORC has been known before to be important for the nucleosome organization surrounding the ACS element, so the key question is how ORC achieves organizing chromatin at the origin and unfortunately remains uncertain in this study. I agree the authors have shown regions or aspects of ORC function are necessary for the "barrier function", but there is no data addressing why these domains might be important, thus leaving the reader to continue to wonder why ORC is important.

The addition of the heat maps for the various ARS to show how the positioning varies in the population of ARS sequences was a good addition to the paper. However, these analyses are most needed for the orc1 mutants in which the nucleosome spacing is the least affected, such as for the BAH and IDR mutants. It is not clear from the average plots if the difference is meaningful or not, and the heat maps would help in that regard.

The new in vitro replication assays are an essential addition to show the in vitro defects in nucleosome spacing caused by orc1 mutants are also defective in DNA replication and do further strengthen the paper.

I don't understand what the authors mean when referring to the "pioneer factor" in relationship to barriers of chromatin remodeling. Are we referring to as in pioneer transcription factors that can bind to nucleosomes and don't require the nucleosome to be moved off for the transcription factor to bind? Further clarification on this is needed.

The spot assays in Ext. Data Fig. 8 are missing some key time points. The 4 day is too long and the 1 day is too short. Why are there no views shown of the plates at day 2 or 3 which would have been more optimal?

**Author Rebuttals to First Revision:**

We would like to thank all four reviewers for their time and their thoughtful comments on our revised version of the paper. We are delighted that the overall impressions were very positive and that the reviewers felt that our study improved significantly. While Referee #1 is pleased with the revised version and has no further comments, Referees #2-4 have raised more points, which we all addressed in this new version of our manuscript. We added more experimental data and significantly reorganized text and figures.

In the following, please find a detailed response to all comments. To help to navigate through the revisions, we highlighted all direct citations from our revised text as well as new or altered figures in green.

**Referees' comments:**

**Referee #1:**

I think the authors have done a nice and honest job to answer all the referees' comments resulting in a much improved manuscript that goes farther than the first version in the understanding of biological mechanisms. I have no further criticisms on this revised version.

We are very happy that the reviewer feels that our efforts resulted in a "*much improved manuscript that goes farther than the first version in the understanding of biological mechanisms*".

**Referee #2:**

In this revised manuscript the author address issues raised by the reviewers. While I am still supportive of this work, there remain unresolved issues. What is most interesting is that ORC uses ATP hydrolysis to organize nucleosomes which differs from what is observed at promoters with GRFs, where only the ATPase of the remodelers is used. Also of interest is the redundancy of remodelers in nucleosome organization at ORCs, in which nucleosome positioning is the same regardless of the remodeler, again different from promoters.

Some unresolved issues persist from the first round, while others are new:

We are enthusiastic that the reviewer is supportive of our work and agree that there are remaining issues that need clarification.

1. I must insist that all plots have their y-axis starting at zero. The authors claimed to have done this but have not.

We apologize that we had not changed this for all plots of the *in vivo* experiments. We changed this now for all plots.

2. The authors state that the reconstituted arrays are "reminiscent of promoters". However, in Fig. 1h, arrays appear to start at the same distance from the ACS regardless of the tested

remodeler, and inter-nucleosome distances also appear to be the same. How does this make sense given that these properties are normally different with different remodelers, when examined at promoters with GRFs? ED_Fig. 7b shows the same in vivo, which validates the authors in vitro findings, but needs to be explained in light of what is known about the spacing activity of these remodelers that is inherent in these enzymes.

We agree that these points need clarification and have added now new experiments (new ED Fig. 4b) and a more detailed discussion in the text (see below). It is true that different remodelers with spacing activity have different "remodeler rulers" that set different spacing (Oberbeckmann, Niebauer et al., 2021, *Nat Comm*). However, these remodelers can only generate their remodeler-specific spacing if the nucleosome density is not too high. At high densities and if the remodelers cannot evict nucleosomes, which is the case for all remodelers with spacing activity, the spacing is dictated by the nucleosome density. Indeed, in our present study, we achieved a rather high assembly degree during salt gradient dialysis (SGD) so that all remodelers generated about the same spacing. Nonetheless, the distance between the ACS and the flanking nucleosomes (phasing distance) at the NFR, varied a bit, especially for ISW2 (new ED Fig. 4b).

To directly address the reviewer's point and to better link these results to our previous study, we assembled now SGD chromatin with the origin library also at lower (ca. halved) nucleosome density. In line with our previous results, the remodelers then also generated arrays with different spacing (new ED Fig. 4b). We note that the ranking of the remodeler-specific phasing differences were different at ORC versus at GRFs. This is yet another indication that ORC's mode of action as a nucleosome positioning barrier differs from that of GRFs.

We now write (page 5, line 8): "This mechanism was reminiscent of that at gene promoters, where the same set of chromatin remodelers cooperated with "barriers", like the general regulatory factors (GRFs) Reb1 or Abf1, in array phasing[18,20,25]. Nonetheless, there were some differences. In contrast to fully symmetrical phasing around GRFs[20], reconstituted phasing around ORC at ACSs was a bit asymmetrical as also seen *in vivo*[2–4] (Fig. 1a, h). Compared to previously reported array reconstitution at GRFs[20], the remodeler-specific ranking with regard to phasing (distance between ACS and -1/+1 nucleosomes) and spacing (internucleosomal linkers) distances were different, e.g., ISW2 generated shorter phasing distances than ISW1a instead of the other way around at GRFs, and linker lengths were similar among all four remodelers (Extended Data Fig. 4b). The latter indicated that the achieved nucleosome density was a bit higher than previously prepared, where linker lengths were remodeler-specific. At such *in vivo*-like density, the spacing was mainly determined by nucleosome density as these four remodelers cannot evict nucleosomes and did not have enough space along the DNA to generate their remodeler ruler-specified distances. In support of this interpretation, when we prepared SGD chromatin with lower density (histone : DNA ratio ca. halved), also spacing was remodeler-specific, similar to the previous report (Extended Data Fig. 4b)".

In the rebuttal the authors state "If it were true that remodelers just lubricated nucleosome dynamics while other factors determined the outcome of nucleosomal organization, then different remodelers, under otherwise identical conditions, should generate the same outcome". But this is exactly what is observed. Different remodelers with different spacing activities do give the same result. Yes, RSC is the exception, but as the authors point out, RSC is not like the other remodelers since it lacks positioning activity.

This is an interesting conceptual discussion. Our original argument as quoted by the reviewer was a response to point 5 of this reviewer's first comments ("5. *Is it possible that the remodelers are "only" making chromatin more dynamic, thereby allowing ORC to bind? Then ORC is what organizes the chromatin rather than the remodelers*.") and therefore referred just to the very general remodeling activity of "nucleosome dynamics", e.g., nucleosome sliding, which is catalyzed by RSC as well as by the remodelers with spacing activity. The mere fact that RSC can slide nucleosomes but not generate regular spacing validates our argument that there is something specific to remodeler mechanisms that co-determines the remodeling outcome in addition to the input by ORC and beyond just "making nucleosomes more dynamic".

In addition, and this is probably what the reviewer refers to now, there are remodeler-specific differences even among the remodelers with spacing activity. However, as pointed out in our response above, these differences cannot play out with regard to spacing differences at high nucleosome densities.

Given the conceptual importance, we wish to keep our original argument, but make it now more specific with regard to spacing activity (page 15, line 9): "This underscores that remodelers do not just lubricate nucleosome dynamics while the outcome is determined by other factors, but that there is remodeler-specific input, like spacing activity, that co-determines the nucleosome organization outcome".

3. That INO80 can produce arrays without a barrier at promoters is mentioned by the authors but not rationalized as to why promoters and origins differ in this regard.

The INO80-intrinsic nucleosome positioning at promoters relies on DNA shape/mechanics signals in the sequences underlying +1 nucleosomes (Krietenstein et. al, 2016, *Cell*; Oberbeckmann, Krietenstein et al., 2021, *Nat Comm*). Apparently, these have not evolved at origins. We speculate that origins always have one barrier protein (ORC), in contrast to gene promoters, which are more diverse in architecture and often do not have barrier proteins (the most abundant "UNB" promoter class in yeast; Rossi et al., 2020, *Nature*). Therefore, there may have been no need to evolve DNA sequences that are involved in positioning origin-flanking nucleosomes as ORC always takes care of it.

However, these are exciting speculations but we feel that they are too premature and would not contribute to our story at his point. We therefore decided to omit this statement and discussion from the text.

4. The authors mention that arrays around ORCs are asymmetrical. This observation is not obvious from the referenced figure 1a. It looks symmetrical to me.

This ACS/nucleosome asymmetry was also observed in previous *in vivo* studies (Eaton et al., 2010, *Genes Dev*.; Berbenetz et al., 2010, *PLoS Genet*.; Rossi et al., 2021, *Nature*). To facilitate the visualization of the asymmetry, we now added a vertical line at the alignment point of all composite plots.

5. It is clear from the authors findings that ORC is required for nucleosome array generation. However, the authors try to walk a tight line about the BAH and IDR domains. Despite the in vivo effects on cell cycle, Fig. 3 is surprising by the relative lack of effect of BAH and IDR on nucleosome organization. The authors were careful in this revised version to scale back their claims (although they still claim in the rebuttal that BAH+IDR mutants do not allow array generation). But now it becomes just an observation without a meaningful conclusion. My conclusion would be that BAH and IDR have a minor contribution to nucleosome

organization at ACS (except in conjunction with INO80 alone, as shown in the much later Fig. 4g).

Yes, as we agreed in our previous rebuttal, the effects due to the BAH and IDR single mutations were mostly mild and usually a bit more pronounced for the IDR mutation:

- The BAH mutation mildly affected cell growth under HU conditions, while the IDR mutation showed a mild effect already in rich media (Fig. 3b).
- The defects in nucleosome organization *in vivo* (now moved to the Supplemental Material, ED Fig. 6a, b, as suggested by the Reviewer) were slight for both single mutations but significant (see below regarding the significance).
- The defect in replication *in vivo* was mild for BAH and more pronounced for IDR (ED Fig. 6c).
- The defect in nucleosome organization *in vitro* (Fig. 4a, b) was very slight for BAH and again more pronounced for IDR.
- and the same was seen for the defects in replication *in vitro* (Fig. 4d)
- and also for the defects in remodeler interactions (new ED Fig. 8b).

We agree with the Reviewer that the conclusion is that both domains have minor contributions by themselves. However, we do strongly feel that it is important to show their mild effects in all our assays so that their redundancy can be appreciated in light of the strong effects of the BAH-IDR double mutation. We write now (page 9, line 10): "Because the individual *orc1*-BAH and -IDR mutations mildly influenced nucleosome organization and replication, suggesting redundancy between both domains, we speculated that lethality of the *orc1*-BAH-IDR mutation may result from synthetic effects due to lack of both domains together."

Please note that we took great care to point out the mild effects of the IDR and especially BAH mutations in all our assays (pages 8 - 11).

Aspects of Fig. 3 adds little and may be better suited as a supplementary figure, unless the authors choose to whittle down Orc1 to find out what is the minimal region need for organization.

As noted above, we follow the suggestion of the reviewer and moved now the *in vivo* chromatin data as well as the replication data for the *orc1*-BAH and -IDR single deletion mutants to the Supplementary material (new ED Fig. 6 a, b, and c).

We are certainly interested in finding minimal regions of BAH and IDR. However, this is out of the scope of the manuscript and would not add much to the story at this point.

Maybe only the DNA binding domain is needed, if it is solely a barrier function, but this is ruled out later with the orc1-Walker B mutation.

Yes. That not only the DNA binding domain is needed, is not just ruled out by the Walker B mutation. All Orc1-mutated ORCs can bind DNA (Fig. 3f).

The authors do assay for complex assembly and DNA binding for the mutants in Fig. 4, but without the lead-in for the orc1-Walker B mutation, there does not seem to be a strong basis for the proposed hypotheses that are to be tested (as written). The orc1-Walker B mutation has a substantial effect on nucleosome organization but this is not introduced until Fig. 4d.

Yes, we fully agree that it makes sense to introduce the Orc1-Walker B mutation earlier. We do introduce it now together with the other Orc1 mutations just before we raise our four hypotheses (page 9, line 10): "Because the individual *orc1*-BAH and -IDR mutations mildly influenced nucleosome organization and replication, suggesting redundancy between both domains, we speculated that lethality of the *orc1*-BAH-IDR mutation may result from synthetic effects due to lack of both domains together. We further hypothesized that lethality of the *orc1*-Walker B mutant might be related to a functon in nucleosome organization because ATP hydrolysis is dispensable for MCM loading, i.e., ATP hydrolysis may be required for an essential function that ORC has in addition to MCM loading.

We wished to determine the molecular basis for the lethal phenotypes of the *orc1*-BAH-IDR and the *orc1*-Walker B mutants and came up with four hypotheses."

Also, the IDR mutation is more dramatic with INO80 than with the mix of the remodelers. This whole section could be consolidated and the limited effects of IDR to INO80 could be made clearer.

We are not entirely sure what the reviewer refers to here. The effects of the IDR single mutation on nucleosome organization *in vitro* were similar for INO80 (Fig. 4a) and ISW1a (ED Fig. 7a), but weaker for ISW2 and Chd1. We never tested a "mix of remodelers" but always individual remodelers. Of course, effects *in vivo* reflect a "mix of remodelers", and here the IDR mutation showed only mild effects as discussed above, but this was not compared to INO80-specific effects.

Nonetheless, we agree that our data suggest that INO80 might be the major remodeler at origins of replication. We not only observed this with the IDR mutation, but also with the BAH-IDR double and Walker B mutations. We write (page 10, line 12): "Similar results as for reconstitutions with INO80 were obtained with ISW1a, ISW2 and Chd1 (Extended Data Fig. 7a, b), although in some cases to a lesser extent, suggesting that INO80 may be more sensitive to compromised ORC nucleosome organizer activity.", and (page 14, line 7): "We saw first hints for a maybe special role of INO80 as INO80 seemed particularly sensitive to the Orc1-BAH/IDR and Orc1-Walker B mutations in the chromatin context (Fig. 4a, b compared to Extended Data Fig. 7a,b) and was particularly effective in our *in vitro* replications (Extended Data Fig. 9e, f)." Nonetheless, we feel that our present data are not sufficient to firmly conclude a dedicated role for INO80 in nucleosome organization at origins.

6. More on Fig. 3e: The authors' revised figure now reports an average of replicates in response to reviewer comments. However, the reviewers were seeking to understand the variance among replicates. Simply providing the average does provide a measure of variance. The authors should plot all four replicates.

All agreed, we apologize for the misconception. We show now in new ED Fig. 6a composite plots of averages with SEM (standard error of the mean) bands as shaded areas around the trace. The SEM is so small that it can be hardly discerned from the average trace. This kind of plot underscores the significance of the small effects. For the reviewer, we show below the traces of the individual replicates. If preferred by reviewer and editor, we could also show the individual replicates as a supplementary figure. As MNase-seq peak heights depend on MNase digestion degree (Chereji et al., 2019, *Genome Biol*), we only used replicates with similar digestion degree. We took the median of the fragment length distribution (shown below as box plots for each replicate) as a proxy for digestion degree and used in the end only

replicates with a median between 150-154 bp. This is also stated in the Methods section (page 39, line 1): "Peak heights in MNase-seq data composite plots depend on MNase digestion degree and the underlying genomic sequence[80]. As we only compared same sequences, it was sufficient for meaningful comparison of peak heights to match digestion degrees between samples, which we did via similar medians of MNase fragment length distributions in the range of 150-154 bp for the data in Fig. 4a, ED Figs. 6a and 7a,b."

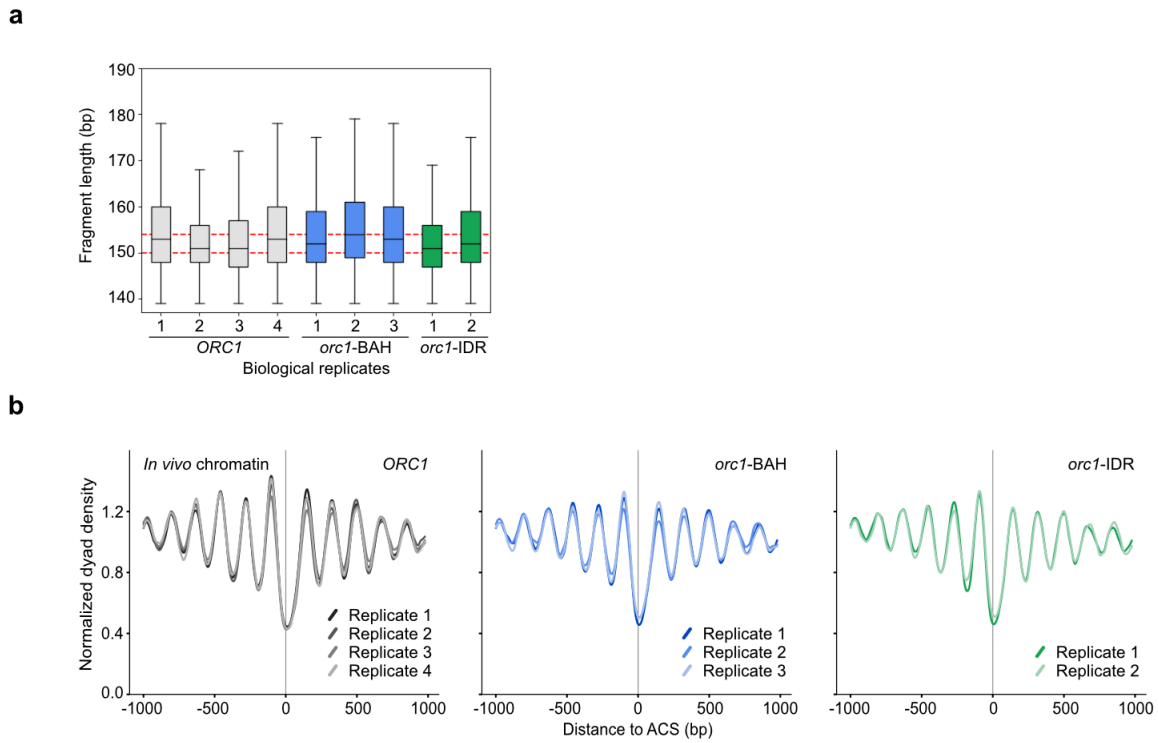



**Fig. 1: a)** Fragment length distribution of MNase digestions of replicates used in ED. Fig. 6a and b. **b)** Single replicates of the same experiment.

Taken together, we would like to thank the Reviewer again for all the very insightful and helpful comments on our paper and hope that the Reviewer can now agree that our paper matured and clearly conveys a well-supported message.

**Referee #3:**

Kurat and colleagues have submitted a response and revision for their study of nucleosome phasing by ORC. In my opinion, the most substantial improvement in this revised submission is Fig. 4i (and ED10), which demonstrates strong replication defects in vitro from two Orc1 deletion mutants. These mutants load MCM but do not support nucleosome array assembly, are largely inactive for replicating chromatin templates in vitro, and do not support yeast cell viability. Thus, Orc1 has an essential function that can be separated from its essential role in

MCM loading. This important addition goes beyond the original submission that partially dissected the ability of Orc1 to stimulate array assembly. Though not perfect proof of causation, the correlation between replication in vitro and array assembly plus the inviability of the ORC1 deletions despite no demonstrable effect on MCM loading in vitro is compelling.

We are very happy that the reviewer feels that our work improved and that the "*correlation between replication in vitro and array assembly plus the inviability of the ORC1 deletions despite no demonstrable effect on MCM loading in vitro is compelling*".

Why arrays are critical for chromatin replication independently of MCM loading is as yet unknown.

Yes, a lot remains unknown with regard to why arrays are important for DNA templated processes like transcription or replication and these are exciting questions. Nonetheless, our advance is that we demonstrate the importance of arrays for replication to start with. We also have some speculations that we now added to the Discussion and this will help the reader to envision why arrays might be important.

We now write (page 14, line 19): "Our conclusion that proper nucleosome organization at origins is crucial for replication and thereby for cell viability (Fig. 4e) may seem unexpected as chromatin *per se* is a major barrier to replication[12–14]. Analogously to the role of proper nucleosome positioning at promoters and in gene bodies for transcription[4,19,21,22], we propose that well-positioned and regularly spaced nucleosomes flanking origins may help activated helicases to escape origins in a bidirectional manner, which may be hindered by high levels of closely packed nucleosomes in irregular arrays as suggested for transcription[52]. Further, it was shown that ORC can, in principle, load the MCM complex regardless of the ACS sequence at any NFR[53,54]. Thus, arrays might stimulate specific bidirectional replication from origins rather than from other NFRs. Indeed, our data showed that just the generation of the NFR over origins, which allows ORC binding, was not sufficient for efficient replication as the RSC remodeler also generated the NFR (Extended Data Fig. 3b), but no flanking nucleosomes nor arrays. RSC even disrupts existing arrays and was shown to be inhibitory when added to replication reactions of chromatin templates with regularly spaced nucleosomes *in vitro*[14]. This underscores that remodelers do not just lubricate nucleosome dynamics while the outcome is determined by other factors, but that there is remodeler-specific input, like spacing activity, that co-determines the nucleosome organization outcome.

As Okazaki fragment length correlates with nucleosome positioning[12,55], it is tempting to speculate that regular arrays are instrumental for the coordination of leading and lagging strand synthesis and thereby for efficient replisome progression."

Further, stimulated by this Reviewer's comment in the first round of revision "…*interestingly, the pattern did not require actual MCM loading*", we now discuss in the text that our minimal reconstitution system also allows us to draw a similar conclusion for replication as was previously drawn for transcription. We write now (page 12, line 13): "The specialized nucleosome organization (NFR-arrays) at the starting point (origin or promoter) of the process (replication or transcription) is generated without setting up the process (MCM loading or formation of the preinitiation complex (PIC)). This prepones nucleosome organization one more step prior to the actual initiation of the process. *In vivo*, it is known that the promoter or origin nucleosome organization is set up prior to initiation of transcription[39] or replication[40], respectively. While *in vitro* reconstitutions showed in the context of promoters

that PIC formation is not required for nucleosome organization at promoters, it was unclear if, analogously, MCM loading is not required for nucleosome organization at origins, too."

I'm somewhat surprised that the authors have not included any protein-protein interaction assays -either in solution or at origins- to determine if and how Orc1 interacts with the four chromatin remodelers. For example, Orc1 has been reported to bind INO80, but is that through the IDR-BAH or affected by the Walker B mutation? The lack of testing physical association of ORC with remodelers was mentioned in the initial review, but not addressed except in passing during the discussion as a possibility. Also does ORC bind all four, and are those interactions similarly affected (or not) in the Orc1 mutants? The barrier model discussion is fine but leaves quite a lot unanswered in terms of molecular mechanism.

This point is well taken. As pointed out in the first rebuttal letter and also in our Discussion (page 13, line 4): "In general, it is not known how barrier factors work." It is a long-standing open question how barrier proteins work, not only in the ORC/origin context. We are currently working on a collaborative project addressing this in detail. This project is very much involved as it requires, for example, high resolution cryo-EM analyses. We feel that this is a logical follow-up on our current story and consider it out of the scope of the manuscript.

Nonetheless, we do agree with this reviewer, and also with reviewer #4, that a demonstration of a physical interaction between ORC and a remodeler and the investigation of the role of the BAH, IDR and Walker B domains would be a good addition and would help the reader to better understand how ORC achieves its barrier function. We would like to underscore that, although assumed, a physical interaction between other barriers like Abf1 and a remodeler was never clearly demonstrated.

We established an assay for testing this *in vitro* on SGD chromatin. We reasoned that it might be best to detect interactions between ORC and a remodeler on its natural substrate, origin chromatin, while generating arrays. We pulled on ORC and digested chromatin down to mono-nucleosomes. Indeed, using this assay we could detect interaction between ORC, nucleosomes and remodelers and could show that the BAH and IDR domains, together, are important, whereas binding did not depend on ATP hydrolysis by Orc1.

We now write (page 10, line 16): "We wondered if this compromised activity was due to compromised interactions between ORC mutants and the remodelers. Previous large-scale mass spectrometry results already indicated an interaction between ORC and INO80[31]. Consistent with this, pull-down experiments of WT and mutant ORCs incubated with remodelers and the SGD chromatin plasmid library, which was treated with limited amounts of MNase to obtain nucleosomal fragments, showed that WT ORC interacted with remodelers and nucleosomes (Extended Data Fig. 8a, b). The interactions with INO80, ISW1a and ISW2 were slightly decreased for the Orc1-BAH, more for the Orc1-IDR and mostly abolished for the Orc1-BAH/IDR mutant ORC, which corresponded again to the order of effect size among these mutations as observed in our other assays. Decreased interactions were not due to decreased pull-down of nucleosomes as monitored by histone H3 levels. Therefore, the ORC-remodeler interactions were likely direct and not only nucleosome-mediated. Pull-down of Chd1 was very weak for WT ORC, which may argue for a more transient interaction, but also this was abolished for all mutant ORCs. In contrast to the Orc1

deletion mutations, the Orc1 Walker B point mutation did not affect pull-down efficiencies, i.e., ATP hydrolysis was not important for ORC-remodeler interactions."

And further in the Discussion (page 13, line 21): "A role for ATP hydrolysis by Orc1 is surprising and offers a wholly new aspect to barrier function given that GRFs at gene promoters act as barriers without enzymatic activity[18,25]. We envision that ORC requires ATP hydrolysis for active manipulation of chromatin structure. Supporting this idea, recent work showed that INO80 remodels not only canonical nucleosomes containing histone octamers, but also hexasomes that lack one H2A-H2B dimer[44]. We cautiously speculate that such hexasomes may be generated by ATP hydrolysis by Orc1 and may be the actual substrate for INO80 at origins. In line with this idea, ORC interacts with the acidic patch in nucleosomes[30], which is a common feature of chromatin remodelers, and was reported to evict H2A-H2B dimers[45]."

We are grateful to the reviewer for suggesting these Co-IP experiments as we indeed feel that they now substantially strengthen the manuscript and provide more mechanistic detail for the role of the BAH and IDR domains.

**Referee #4:**

The authors clearly show the ATPase activity of Orc1 and the BAH and IDR regions of Orc1 are required for the nucleosome spacing activity around the ARS, but there is no experimental data to support the several speculations that are made about allosteric effects or even direct interactions between ORC and the remodelers. Besides the speculations there is no other data to provide supporting evidence for one model over another, so the report is more about cataloging the phenomena rather than providing insights as to why this might occur. ORC has been known before to be important for the nucleosome organization surrounding the ACS element, so the key question is how ORC achieves organizing chromatin at the origin and unfortunately remains uncertain in this study. I agree the authors have shown regions or aspects of ORC function are necessary for the "barrier function", but there is no data addressing why these domains might be important, thus leaving the reader to continue to wonder why ORC is important.

We agree that there remain open questions. Nonetheless, we would like to underscore that our study addresses two key questions, and not one as suggested by the Reviewer, and that we do provide major advances with regard to both our key questions.

The first key question, in agreement with the Reviewer, is how ORC achieves nucleosome organization at origins. We provide here several new mechanistic insights:

1) ORC works as a barrier together with certain remodelers to phase regularly spaced arrays at origins. This minimal system can already explain the generation of such nucleosome organization at origins and excludes the hypotheses that MCM loading or replication itself may be required for nucleosome organization (see below).
2) ORC's barrier function does not rely on DNA binding only but requires both the BAH+IDR domains.

3) As suggested by the Reviewer, we document now the physical interaction between ORC and the four remodelers. We reasoned it most relevant to detect interactions between ORC and remodelers on its origin chromatin substrate. We digested chromatin to mono-nucleosomes and pulled on ORC. Using this assay we detected interactions between ORC, nucleosomes and remodelers and could show that the BAH and IDR domains are important (new ED Fig. 8). We underscore that, although assumed, a physical interaction between other barriers like Abf1 and a remodeler was never clearly demonstrated. So we go substantially beyond current understanding of barrier mechanisms.
4) The ORC-remodeler interaction through the BAH-IDR domains is necessary but not sufficient as the Orc1-WalkerB mutation did not support array generation despite not compromised interaction (new ED Fig. 8). This is because ORC's barrier function requires ATP hydrolysis, which is a wholly new aspect of barrier function and provides a first answer to the question of why ATP hydrolysis by Orc1 is required for viability.

The exact role of ATP hydrolysis in Orc1 remains open, but we feel that this is an excellent starting point that will keep the reader excited about future work in this context. Similarly and as pointed out in our Discussion (page 13, line 4): "In general, it is not known how barrier factors work." It is a long-standing and still open question how phased arrays are generated, how remodelers and barrier proteins cooperate here, not only in the ORC/origin context. We agree that our understanding, despite major advances, is still incomplete and are currently working on a collaborative project addressing this in more detail. This project is very much involved as it requires, for example, high resolution cryo-EM analyses, and beyond the scope of this paper.

The second key question, in addition to the Reviewer's view, relates to the importance of NFR-array nucleosome organization for replication. While origin-flanking arrays were described over a decade ago, it was unclear if this NFR-array organization at origins was functionally important for replication. Here, we provide the first clear indication for functional importance of nucleosome organization by uncoupling ORC's nucleosome organization from MCM loading activity.

Related to this (see our point 1) above) and stimulated by the comment of Reviewer #3 during the first round of revision "…*interestingly, the pattern did not require actual MCM loading*", we now discuss in the text that our minimal reconstitution system also allows us to draw a similar conclusion for replication as was previously drawn for transcription. We write now (page 12, line number 13): "The specialized nucleosome organization (NFR-arrays) at the starting point (origin or promoter) of the process (replication or transcription) is generated without setting up the process (MCM loading or formation of the preinitiation complex (PIC)). This prepones nucleosome organization one more step prior to the actual initiation of the process. *In vivo*, it is known that the promoter or origin nucleosome organization is set up prior to initiation of transcription[39] or replication[40], respectively. While *in vitro* reconstitutions showed in the context of promoters that PIC formation is not required for nucleosome organization at promoters, it was unclear if, analogously, MCM loading is not required for nucleosome organization at origins, too."

The addition of the heat maps for the various ARS to show how the positioning varies in the population of ARS sequences was a good addition to the paper. However, these

analyses are most needed for the orc1 mutants in which the nucleosome spacing is the least affected, such as for the BAH and IDR mutants. It is not clear from the average plots if the difference is meaningful or not, and the heat maps would help in that regard.

We completely agree - this is an excellent suggestion. We now provide heat maps for the positioning experiments in ED Fig. 6a and Fig. 4a (new ED Fig. 6b and new Fig. 4b). This nicely complements the composite plots. For the latter, also in response to Reviewer #2, we now also provide SEM (standard error of the mean) data that support significance (see our reply to Reviewer #2, point 6).

The new in vitro replication assays are an essential addition to show the in vitro defects in nucleosome spacing caused by orc1 mutants are also defective in DNA replication and do further strengthen the paper.

Yes indeed, we agree that the addition of the *in vitro* replication reactions very much strengthen the paper.

I don't understand what the authors mean when referring to the "pioneer factor" in relationship to barriers of chromatin remodeling. Are we referring to as in pioneer transcription factors that can bind to nucleosomes and don't require the nucleosome to be moved off for the transcription factor to bind? Further clarification on this is needed.

We are sorry that this remark was confusing. The classical barrier factors, the general regulatory factors (GRFs) in yeast, have been compared to pioneer factors as at least some of them can also bind to nucleosomal DNA and may share with pioneer factors properties of the DNA binding domain that allow this binding mode. We just wished to refer to this discussion in the field and point out that just the DNA binding properties are not sufficient to explain how a barrier factor works. We now provide more clarification in the text (page 13, line 5): "It was suggested[43,44] that special properties of the DNA binding domain are decisive for barrier factors, as is also discussed in the context of pioneer factors[45], which is why the GRFs Reb1 and Cbf1 were likened to human pioneer factors[44].
Our results here show that mere DNA binding was not sufficient for array generation."

The spot assays in Ext. Data Fig. 8 are missing some key time points. The 4 day is too long and the 1 day is too short. Why are there no views shown of the plates at day 2 or 3 which would have been more optimal?

We agree that the time points were not ideal. We repeated the experiments and found that day 2 is appropriate to present the data (new Fig. 3b).

We would like to thank the reviewer again for a very fair and very thoughtful revision. We are delighted and truly believe that the review process helped strongly in the development of our story. We hope the reviewer can agree.

**Reviewer Reports on the Second Revision:**

Referees' comments:

Referee #2:

The manuscript is much improved and the responses to my concerns have been adequately addressed. This is a very nice body of work. Fig. 4b was nice to see. One final suggestion is to add in the in vivo distances (-1 to ACS, and +1 to ACS, and linker) to the table at the bottom of Fig. 4b. I don't think there is any explicit comparisons reported between the reconstituted distances and the in vivo distances.

Referee #3:

I am pleased the authors conducted the binding assays in Ext. Data Fig. 8, and that they support a role for those interactions. The manuscript and dataset have considerably improved during the revisions. I have no further criticisms.