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CHD1 remodelers regulate nucleosome spacing *in vitro* and align nucleosomal arrays over gene coding regions in *S. pombe*

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

1st Editorial Decision 17 August 2012

Thank you for submitting your research manuscript (EMBOJ-2012-82757) to our editorial office. It has now been seen by two referees and their comments are provided below.

All reviewers appreciate your study and are in general supportive of publication in The EMBO Journal. Nevertheless, they do raise a number of important concerns, especially regarding the absolute values measuring the increase in anti-sense transcription. Based on the comments provided, I would like to invite you to submit a suitably revised manuscript to The EMBO Journal that addresses this concern, including also the additional minor points suggested by the reviewers. I should add that it is our policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage. Nevertheless, given the very competitive nature of your research, it seems prudent to complete the revision at your very earliest convenience, ideally within 4 weeks. Please do not hesitate to contact me to discuss this time-line.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

This manuscript by Pointner et al reports that the ATP-dependent chromatin remodelers, Hrp1 and Hrp3, play a major role in the regular nucleosome positioning at 5' end of most genes in the fission yeast Schizosaccharomyces pombe. In total, the authors examined five different chromatin remodelers (Snf21, Swr1, Mit1, Hrp1 and Hrp3) on a genome-wide scale. They show that quite unexpectedly the RSC-type remodeler, Snf21, has no major role on the nucleosome organization downstream the transcription start site (TSS). Similarly, Swr1 and Mit1 remodelers have no major contribution at these same genomic sites. In contrast, the two CHD1-type remodelers, Hrp1 and Hrp3, are essential for the generation of nucleosomes arrays at these sites. In addition, the authors conducted in vitro experiments to show that Hrp1 and Hrp3 possess nucleosome-spacing activities. Moreover, they demonstrate that Hrp1 and Hrp3 critical function for the nucleosomal organization is limited to the 5' end of genes, rather than being general remodelers of chromatin. Finally, thanks to transcriptome analyses of hrp1Δ and hrp3Δ deficient strains, the authors show that the lack of Hrp1 and Hrp3 do not lead to a general change in mRNA expression but correlate with an increase of antisense transcription of some protein-coding genes.

The experiments and the data are globally of excellent quality. The conclusion are for the vast majority accurate and do not overstate the results. Furthermore, this work brings interesting new information on the identity of the remodelers that contribute to the ordered array of nucleosomes at the 5' end of genes in fission yeast. Finally, this work shall be of interest to both the expert and the more general reader in the field of chromatin biology. Thus, the manuscript shall deserve publication in The EMBO Journal provided the authors address the following points.

First, as part of the validation of their genome-wide approach of the nucleosomal organization next to the TSS, the authors should add a targeted analysis of 2 or 3 case study genes, especially for hrp1 and hrp3 mutants.

Second, the authors show that the nucleosome array located after the TSS is generally impaired in hrp1Δ and hrp3Δ, single and double mutants. However, from the available data it is not clear whether this compromised nucleosome organization is due to a complete eviction or a looser binding of histones. To address this important point it should be tested whether the lack of Hrp1, Hrp3 or both leads to an overall weaker binding of histones to chromatin. If the latter possibility appears to be true, this would also provide an explanation to the current discrepancy pointed out by the authors between results described in this manuscript and their previously published results (page 18).

Third, the in vitro nucleosome spacing experiment was done with purified Hrp1 and Hrp3. To control that the detected spacing activity indeed comes (mainly) from Hrp1 and Hrp3 (and not a contaminant present in the purification) the authors should at minimum test that a mock purification done with hrp1Δ and hrp3Δ extracts does not possess such activity.

Minor points:

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3. page 8, the conclusion mentioned in the sentence starting by "We now conclude that Mit1...", should also be limited to TSS regions, for the same reasons as point 1 and 2.

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5. page 12, the title section states that the "Bulk MNase ladders were also not much compromised...", which is indicative of a subtle effect. This is quite in contrast with the conclusion of this section which states that "the regular bulk nucleosome spacing was not entirely lost.", suggesting a more pronounced effect. These two descriptions of the same results appear quite different and should therefore be rephrased.

6. page 14, first sentence. It seems that "only" should be replaced by "do".

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Referee #2

The authors have made significant contributions to our understanding of remodeler function in S. pombe, which provides an informative comparison and contrast to budding yeast. Here, the authors take genomic, genetic and biochemical approaches to better understand the CHD family of remodelers in S. pombe, an issue of relevance due to the apparent absence of ISWI family remodelers. A comparison to the S. pombe RSC ortholog is also addressed. The central set of experiments is framed along the lines of the recent work on multiple ISWI and CHD knockouts in budding yeast from the Tom Owen-Hughes lab, who have demonstrated a semi-redundant function for ISWI complexes and CHD complexes in organizing chromatin on coding regions. This was inspired by earlier work from the Mellor and Tsukiyama groups on ISWI, and Arndt/Hartzog and others with CHD1, on coding region remodeling. Here, the authors extend these concepts to S. pombe, as well as providing initial biochemical experiments on its remodeling in vitro.

The work begins a little astride of the main issue with studies on H2A.Z, and show little evidence for its contribution (or SWR1) to nucleosome positioning. However, I found this interesting and think it should be kept in. Likewise, experiments with the S. pombe version of RSC are described, and essentially no affect is seen with their snf21 conditional mutant at the restrictive temperature, leading the authors to conclude that RSC from S. pombe has no role in nucleosome positioning. Here, the authors emphasize that they use the same criteria as previous studies from the Cairns lab and other labs for ATPase loss of function, where RSC was shown to have a clear affect in budding yeast. However, the criteria for the loss of function of the ATPase are actually very different, as the degron utilized in the budding yeast experiments eliminated the vast majority of the ATPase protein; this was the main and critical criterion, and this was not checked in the S. pombe snf21 conditional in this manuscript. Also, the RSC degron halted virtually all transcription in budding yeast, but not in S. pombe. Therefore, one can reasonably question whether this conditional mutant has completely lost function at the non-permissive temperature, or has simply lost one essential function. As the merit of the paper lies with Hrp/CHD results, and not on this negative result, I won't ask for additional experiments on snf21. Instead, I'll ask the authors to soften their interpretation considerably to acknowledge the issues above. The authors also discuss a notable correction of their previous work on Mit1, and the reasons for it, and this is well described and good to include here.

The authors then provide interesting and convincing data that Hrp1, and especially Hrp3, affect

positioning in the coding region. This is even more evident in the double mutant. This is the strongest and central data in the paper. I was intrigued by the correlation of Hrp3 occupancy with a large NFR, though Hrp3 is not needed for NFR creation. Here, I wonder if CHD remodelers might be attracted to the free DNA resident in the NFR, but work in the coding region.

The authors then claim increased antisense transcription, but I had a tough time understanding how this was calculated. Here, it appears that the authors simply used a fold change criteria, which has me quite concerned. Antisense transcription is low at most genes, so very slight/trivial increase over a background of 'near zero' can lead to high fold changes, but have a near-zero absolute value, and therefore very limited relevance. This must be addressed in a manner that conveys the absolute values. Also, the authors should examine baseline transcription outside genes - is the whole genome more permissive.

The authors then address bulk spacing, which I thought was an interesting control, and may speak to transcriptional promiscuity at coding regions versus intergenic regions. I also liked the set2 control, which lacked impact on spacing in coding regions occupied by H3K36me. Finally, the authors provide initial data on the nucleosome spacing activity of Hrp1 and Hrp3. The experiments are fine, though clearer in the Nap1 assembly/spacing assay. There are no mechanistic issues tested that might reveal interesting differences or properties, but they do support well the established view that these proteins are ATP-dependent nucleosome spacing remodelers.
Overall, this is a solid contribution to the CHD/ISWI remodeler literature that applies multiple lines of evidence to support the emerging view that both CHD and ISWI remodeler phase nucleosomes in coding regions, and helps the field understand the evolutionary roles of these proteins, as S. pombe only has CHD remodelers. I will support publication once the issues listed above regarding antisense and promiscuous transcription are addressed, as the impact on antisense is very important to resolve.

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Figure 4 could simply go to Supplemental, as the information content is modest and most of it repeats published work.

1st Revision - authors' response 17 September 2012

Referee #1

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rather than being general remodelers of chromatin. Finally, thanks to transcriptome analyses of hrp1 and hrp3 deficient strains, the authors show that the lack of Hrp1 and Hrp3 do not lead to a general change in mRNA expression but correlate with an increase of antisense transcription of some protein-coding genes.

The experiments and the data are globally of excellent quality. The conclusion are for the vast majority accurate and do not overstate the results. Furthermore, this work brings interesting new information on the identity of the remodelers that contribute to the ordered array of nucleosomes at the 5' end of genes in fission yeast. Finally, this work shall be of interest to both the expert and the more general reader in the field of chromatin biology. Thus, the manuscript shall deserve publication in The EMBO Journal provided the authors address the following points.

First, as part of the validation of their genome-wide approach of the nucleosomal organization next to the TSS, the authors should add a targeted analysis of 2 or 3 case study genes, especially for hrp1 and hrp3 mutants.

>We thank the Referee for this comment and agree that it is good to have single locus validation for genome-wide data. We analysed three loci in wt, *hrp1D*, *hrp3D* and *hrp1D hrp3D* cells by MNase indirect end labeling (new Suppl. Fig. 5). Also in this kind of analysis the chromatin patterns were different between wt and especially the *hrp3D* and *hrp1D hrp3D* mutants, which is consistent with our genome-wide results.

Second, the authors show that the nucleosome array located after the TSS is generally impaired in hrp1 and hrp3 , single and double mutants. However, from the available data it is not clear whether this compromised nucleosome organization is due to a complete eviction or a looser binding of histones. To address this important point it should be tested whether the lack of Hrp1, Hrp3 or both leads to an overall weaker binding of histones to chromatin. If the latter possibility appears to be true, this would also provide an explanation to the current discrepancy pointed out by the authors between results described in this manuscript and their previously published results (page 18).

>The Referee points towards the very important distinction between remodeling of nucleosomes such that histones leave the DNA or are still bound there, maybe in the form of a non-canonical nucleosome. Both cases would lead to increased MNase accessibility and can indeed not be distinguished in our assays. The most appropriate technique to distinguish both possibilities is anti-histone-ChIP. This experiment was already done for the *hrp1D* and *hrp3D* mutants by Walfridsson et al., 2007, EMBO J., Fig. 2 A-D. In both mutants histone H3 density went either up or down, depending on the locus, and Hrp1/3 targets tended to show increased H3 density, especially in promoter regions. We did not see these effects, and could not distinguish the targets as defined by Walfridsson et al. in our nucleosome occupancy data (see attached figure for the Referee's perusal). *[At end of this Review Process file.] We would like to underscore that our model (see Fig. 7) mainly invokes that regular arrays are not linked to the 5'end of genes in a uniform register anymore. It does not necessarily invoke that nucleosomes are gone. Nonetheless, if we see changed nucleosome occupancy it is rather decreased than increased. This is consistent with the lower H3 density seen by Walfridsson et al. at some loci, but remains at odds with the increased H3 density at other loci. We re-wrote the paragraph where we discuss this issue (p. 18/19: „We reported both increased and decreased histone H3 density at both coding (ORF) and promoter regions (IGR) in *hrp1D* or *hrp3D* cells (Walfridsson *et al*., 2007). Especially at Hrp1 and Hrp3 targets in promoter regions there was more H3 binding. We did not observe such an effect in promoter regions in terms of MNase resistant nucleosome occupancy (Figure 4A and Supplementary Figure 7A). If anything, we saw decreased nucleosome occupancy over promoter and coding regions at Hrp1 and Hrp3 binding targets. This discrepancy may be due to the different assays used. Here we employ MNase-chip methodology, i.e., score only MNase resistant DNA. The earlier study used anti-H3-ChIP-chip, i.e., scored all histone H3-DNA interactions regardless of formation of canonical nucleosomes. There is also a technical difference between the microarray platforms used possibly explaining the poor agreement between the results. We used spotted microarrays for anti-H3-ChIP-chip containing one 400 bp probe for each IGR and one 400 bp ORF probe with a 3' bias (Wiren et al., 2005), and here we used tiling arrays with 20 bp resolution. Another possible explanation is the formation of non-canonical nucleosome structures in the mutants. In *S. cerevisiae* it was shown that the absence of the histone chaperone Nap1 leads to increased formation of non-canonical nucleosomal particles (Andrews et al., 2010). We speculate that the absence of Hrp1 and/or Hrp3 has the same effect, which may translate into more DNA-bound H3 that can be detected by ChIP, but not by MNase digest.").

Third, the in vitro nucleosome spacing experiment was done with purified Hrp1 and Hrp3. To control that the detected spacing activity indeed comes (mainly) from Hrp1 and Hrp3 (and not a contaminant present in the purification) the authors should at minimum test that a mock purification done with hrp1 and hrp3 extracts does not possess such activity.

>This is a very valid point indeed. We conducted now in vitro spacing assays with mock purified material and clearly show that mock material is negative in this assay (new Suppl. Fig. 13). As shown already in the first version of our manuscript (old Suppl. Fig. 10A), Hrp1 binds

nonspecifically to anti-Flag M2 agarose beads. Therefore we used an *hrp1D hrp3D* mutant for the mock anti-Flag purification.

Minor points:

1. page 7. The title of the second section "The RSC remodeling complex... in global positioning." Appears to be too general. From the data presented, the effect of RSC was monitored at the 5' end of genes and not on the overall genome. Thus, a formulation such "at 5' end of genes" should be added at the end of this title.

>We changed the text accordingly (p. 7: „The RSC remodeling complex does not seem to be involved in nucleosome positioning around TSSs“).

2. page 8, 3rd sentence. Similarly to the previous point, according to the data presented the conclusion should be limited to the TSS regions

>We changed the text accordingly (p. 8: „...the RSC nucleosome remodeling complex does not seem to be much involved in nucleosome positioning around TSSs in *S. pombe*.“)

3. page 8, the conclusion mentioned in the sentence starting by "We now conclude that Mit1...", should also be limited to TSS regions, for the same reasons as point 1 and 2.

>We changed the text accordingly (p. 8: „We now conclude that Mit1 does not play a major role in nucleosome positioning in *S. pombe* euchromatin around TSSs.“)

4. page 9, the sentence starting by "This effect was not visible for Hrp1 binding genes in the hrp1 mutant, but also for this subgroup apparent..." is not clear. It maybe should be rephrased as "..., but was apparent for this subgroup...".

>We changed this sentence now (p. 9: „This increased effect was not really visible for Hrp1 binding targets (Supplementary Figure 7A and B), but also these targets were still clearly affected in the *hrp1D hrp3D* mutant.“).

5. page 12, the title section states that the "Bulk MNase ladders were also not much compromised...", which is indicative of a subtle effect. This is quite in contrast with the conclusion of this section which states that "the regular bulk nucleosome spacing was not entirely lost.", suggesting a more pronounced effect. These two descriptions of the same results appear quite different and should therefore be rephrased.

>We changed the title to „Bulk MNase ladders were also still detectable in an *isw1 isw2 chd1* triple mutant in *S. cerevisiae*.“ (p.12).

6. page 14, first sentence. It seems that "only" should be replaced by "do".

>We introduced „do so“ in front of „only“ (p.14, line 6).

7. page 16, "This notwithstanding, this linkage..."should be rephrased.

>We changed the sentence to „Nonetheless, this fixed linkage may on average lead to longer contiguous regular arrays, which may explain the more or less pronounced defects in bulk MNase ladders in some of these mutants.“ (p.17, line 3).

8. Figure 3 and Legend of Figure 3. The words "call" and "no call" should defined and explained in the figure legend.

>The words are defined. Note that former Figure 3 is now Figure 4.

9. Legend Figure 7 is missing a main title.

>We added now the title „Schematic illustrating the difference between bulk spacing and TSS-aligned genic arrays.".

10. Legend Supplementary Figure 1 and others following. It might preferable to replace "chromatin effects" (which is quite vague) by "defects in nucleosome positioning".

>We followed the Referee's suggestion.

11. Legend Supplementary Figure 2. "fluorescene" should be replaced by "fluorescence".

>Corrected.

12. Legend Supplementary Figure 11 is missing a main title.

>We introduced now the title „Drug sensitivity assays for *hrp* mutants.".

Referee #2

The authors have made significant contributions to our understanding of remodeler function in S. pombe, which provides an informative comparison and contrast to budding yeast. Here, the authors take genomic, genetic and biochemical approaches to better understand the CHD family of remodelers in S. pombe, an issue of relevance due to the apparent absence of ISWI family remodelers. A comparison to the S. pombe RSC ortholog is also addressed. The central set of experiments is framed along the lines of the recent work on multiple ISWI and CHD knockouts in budding yeast from the Tom Owen-Hughes lab, who have demonstrated a semi-redundant function for ISWI complexes and CHD complexes in organizing chromatin on coding regions. This was inspired by earlier work from the Mellor and Tsukiyama groups on ISWI, and Arndt/Hartzog and others with CHD1, on coding region remodeling. Here, the authors extend these concepts to S. pombe, as well as providing initial biochemical experiments on its remodeling in vitro.

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>We do not claim anymore that our criteria are the same as those from the Cairns lab. Instead, in the Discussion we explicitly point out now the controls for the stringency of RSC ablation that were employed by the Cairns lab but not by us and soften our conclusions (p. 15f: „We wonder if our restrictive conditions may have been too mild. They did induce a phenotype on cell growth and viability (Supplementary Figure 2) similar to the *sth1*^{td} phenotype in *S. cerevisiae* as used by (Parnell *et al*., 2008). However, in contrast to (Parnell *et al*., 2008), we did not test downregulation of new transcription or ablation of Snf21 on the protein level, which may reveal a different stringency of our *snf21ts* phenotype in *S. pombe* compared to the earlier *sth1*^{td} phenotype in *S. cerevisiae*. It remains to be clarified whether more complete ablation of Snf21 will reveal a role of RSC in nucleosome positioning in *S. pombe* after all.").

The authors also discuss a notable correction of their previous work on Mit1, and the reasons for it, and this is well described and good to include here.

The authors then provide interesting and convincing data that Hrp1, and especially Hrp3, affect positioning in the coding region. This is even more evident in the double mutant. This is the strongest and central data in the paper. I was intrigued by the correlation of Hrp3 occupancy with a large NFR, though Hrp3 is not needed for NFR creation. Here, I wonder if CHD remodelers might be attracted to the free DNA resident in the NFR, but work in the coding region.

>We thank the Referee for this point which is also valid for Hrp1 targets (Suppl. Fig. 7A,B) and we now mention this in the revised manuscript (p.10: „Interestingly, Hrp1 and Hrp3 targets both had a broader NFR in wt, which was not caused by these enzymes as it was still present in the *hrp1D hrp3D* mutant (Figure 4B and Supplementary Figure 7B). We wonder if such extended regions of free DNA serve as preferred recruitment sites for Hrp1 and Hrp3.“).

The authors then claim increased antisense transcription, but I had a tough time understanding how this was calculated. Here, it appears that the authors simply used a fold change criteria, which has me quite concerned. Antisense transcription is low at most genes, so very slight/trivial increase over a background of 'near zero' can lead to high fold changes, but have a near-zero absolute value, and therefore very limited relevance. This must be addressed in a manner that conveys the absolute values. Also, the authors should examine baseline transcription outside genes - is the whole genome more permissive.

>These points are well taken. We re-analyzed now all transcriptome data by using a double filter (see Materials and Methods). First, we require that the signal-to-noise-ratio (SNR) is larger than one, i.e., that the difference between wt and mutant is larger than between replicates of the same strain. Second, we took the average of the absolute wt expression levels of all intergenic regions (IGRs), i.e., all regions without annotated transcripts, as background expression level. For upregulated mutant expression levels we required that the final level was higher than 1.5fold this background. For downregulated mutant expression levels we required that the wt levels were not already lower than 1.5fold this background. This way we are on the safe side to count upregulated mutant transcripts only if they have substantial absolute levels, and downregulated mutant transcripts only if the wt level was not very low already to start with (see new Table I, Figure 2 and Materials and Methods). Please note that we use now the most recent *S. pombe* genome annotation (2008), which contains more annotated elements. Therefore the numbers in Table I are larger than in the first version of our manuscript, even though our double filter is more stringent now. Please also note that we define now „cryptic antisense transcripts“ as „transcripts that are antisense to an annotated element, but not annotated themselves“ (p. 10 and note to Table I and Materials and Methods). We also checked if transcription outside genes, i.e. at IGRs, was changed in the mutants. However, none of the IGRs made it through the first filter. We mention this now explicitly in the text (p. 10: „We also checked if intergenic regions lacking annotated transcripts became more transcribed in the *hrp* mutants. However, this was not the case.“).

The authors then address bulk spacing, which I thought was an interesting control, and may speak to transcriptional promiscuity at coding regions versus intergenic regions. I also liked the set2 control, which lacked impact on spacing in coding regions occupied by H3K36me. Finally, the authors provide initial data on the nucleosome spacing activity of Hrp1 and Hrp3. The experiments are fine, though clearer in the Nap1 assembly/spacing assay. There are no mechanistic issues tested that might reveal interesting differences or properties, but they do support well the established view that these proteins are ATP-dependent nucleosome spacing remodelers.
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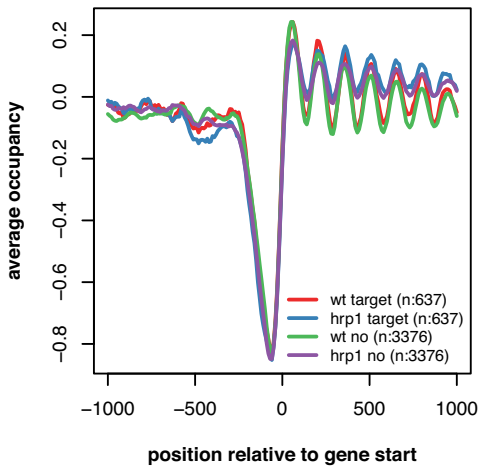
>The former Figure 4 is now Suppl. Figure 11.

Figure R1A

A



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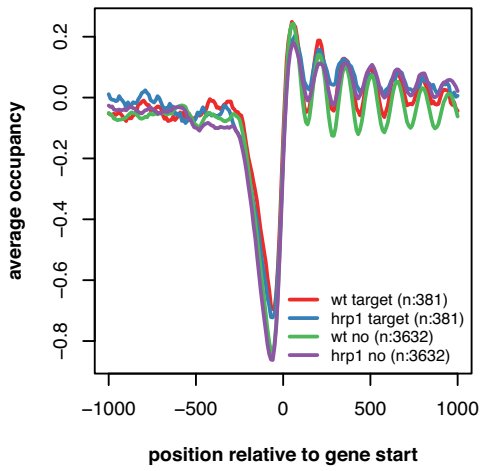

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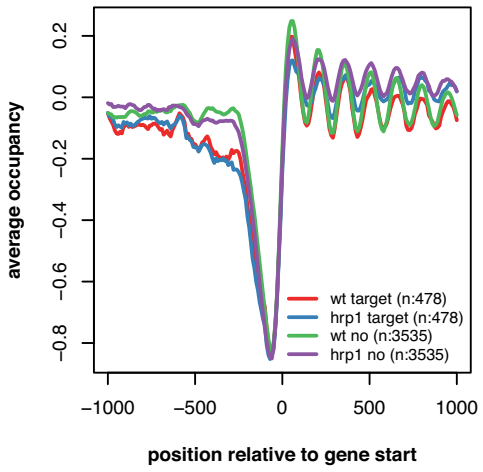

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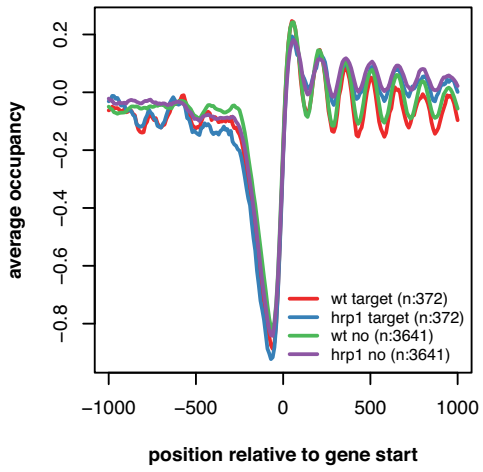

Figure R1A Poor correlation of changes in H3 density and nucleosome occupancy in *hrp1Δ*. Same data as in Figure 3A, but subgrouped according to Fig. 2A-D in Walfridsson et al. 2007 (**A**) low H3 density in *hrp1Δ* in IGRs (637 targets) or not (3376) (**B**) low H3 density in *hrp1Δ* in ORFs (381 targets) or not (3632), (**C**) high H3 density in *hrp1Δ* in IGRs (478 targets) or not (3535) and (**D**) high H3 density in *hrp1Δ* in ORFs (372 targets) or not (3641).

Figure R1B

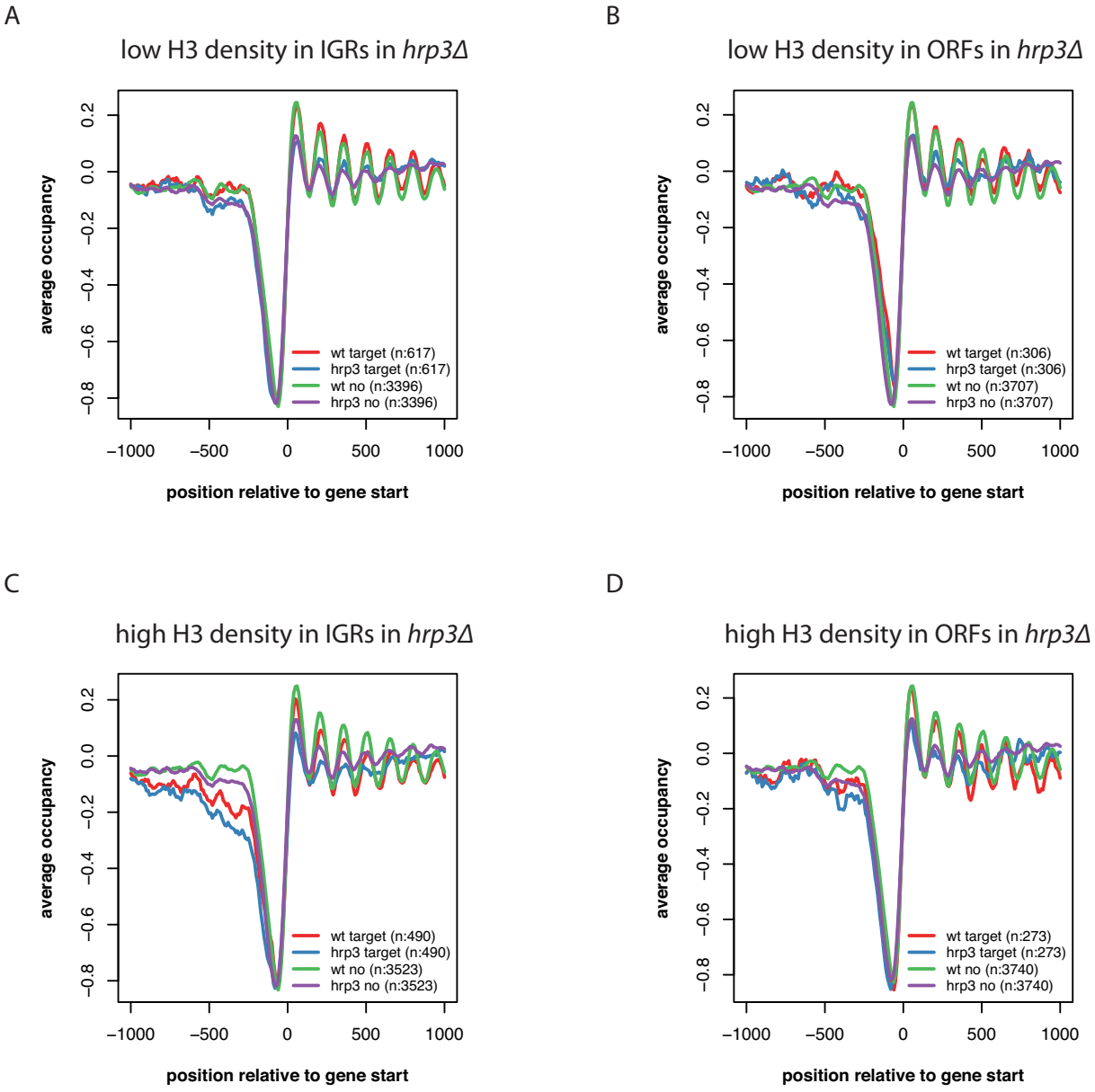

Figure R1B Poor correlation of changes in H3 density and nucleosome occupancy in *hrp3Δ*. Same data as in Figure 3B, but subgrouped according to Fig. 2A-D in Walfridsson et al. 2007(**A**) low H3 density in *hrp3Δ* in IGRs (617 targets) or not (3396) (**B**) low H3 density in *hrp3Δ* in ORFs (306 targets) or not (3707), (**C**) high H3 density in *hrp3Δ* in IGRs (490 targets) or not (3523) and (**D**) high H3 density in *hrp3Δ* in ORFs (273 targets) or not (3740).