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Hrp3 controls nucleosome positioning to suppress non-coding transcription in eu- and heterochromatin

Young Sam Shim, Yoonjung Choi, Keunsoo Kang, Kun Cho, Seunghee Oh, Junwoo Lee, Shiv I. S. Grewal and Daeyoup Lee

Corresponding author: Daeyoup Lee, KAIST

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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 28 June 2012

Thank you for submitting your research manuscript (EMBOJ-2012-82049) to our editorial office. It has now been seen by three 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 confirmation that the Hrp3 point mutant used in the study indeed disrupts its ATPase activity, the strengthening of the data concerning the interaction between Hrp3 and Swi6 and the inclusion of a control regarding the size distributions of the sequenced nucleosomes. Given the comments provided, I would like to invite you to submit a suitably revised manuscript to The EMBO Journal that addresses the all raised concerns in full. 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.

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

The paper by Sam Shim and co-workers details the role of Hrp3 chromatin remodeler in the positioning of nucleosomes and the suppression of cryptic transcription on a genome-wide scale. More specifically, the authors show that the well-defined nucleosome arrangement at transcription start sites is disrupted in hrp3Δ cells. Moreover, transcripts resulting from cryptic transcription accumulate and histones are less tightly associated to genomic DNA in hrp3Δ cells. A point mutant in Hrp3 ATPase domain shows similar phenotype, suggesting that Hrp3 action on nucleosome position and cryptic transcription might go through ATP-dependent remodeling of nucleosomes. In addition, the authors show that Hrp3 contributes to transcriptional silencing at pericentric heterochromatin, where it is required for the cell cycle-dependent regulation of pericentric silencing. Chromatin immunoprecipitation and genetic studies provide further evidence of Hrp3 implication in pericentric silencing.

Overall, this is an interesting paper containing high quality data. The conclusions of the paper are globally justified and they shall be of interest for the general study of ATP-dependent chromatin remodeling's action on the regulation of DNA-linked processes such as transcription, both in the context of euchromatin and heterochromatin. I have however several concerns, most of them have to do with approximations and overstatements being made:

1. A point mutant of Hrp3 was made to examine how much of the defects observed in hrp3Δ cells are to be attributed to Hrp3 ATPase activity. Currently, no experiment shows that Hrp3 has an ATPase activity and that the point mutation made disrupts this activity. As this is an important element of the main conclusion of the paper, the authors should at least provide evidence that the point mutation does indeed destroy Hrp3 ATPase activity in vitro.

2. In the abstract the authors wrote "... Hrp3, is a global regulator that drives higher-order chromatin structure and genomic stability". In fact, there is no experiment in this paper that would allow such broad statement. A large-scale determination of nucleosome positioning and histone association to genomic DNA in wild-type and mutant strains is what was really addressed in the paper. Thus, "Higher-order" and "genomic stability" formulations should be avoided to not misinterpret the main message of the paper.

3. The interaction between Swi6 and Hrp3 has been described in a previous paper (Motamedi et al, Mol Cell, 2008). Although this previous paper did not further characterize the Swi6-Hrp3 interaction as it has been done here, the authors should mention the existence of this data in the main text and cite the paper.

4. The cell synchronization experiment using cdc25-22 temperature-sensitive mutant shows a dramatic loss of silencing in hrp3Δ cells after release of the cell cycle block. To exclude the possibility that this loss is linked to the way the cells were synchronized, the authors should redo the experiment with another mutant or another mean to synchronize the cell, and check whether the cell-cycle effect of the hrp3Δ mutation on the pericentric silencing is still observable.

Minor points:

1. Page 5, line 3 add an "s" to the word "region".
2. Page 6, last line the formulation "involvement between" is a bit confusing and should be replaced.
3. Page 8, title of the paragraph, the formulation "Set2/Clr6 HDAC complex II" should be avoided

as it makes it sound that Set2 and Clr6 a part of a same complex. This could be simply replaced by "Set2 and Clr6 HDAC complex II". The "Set2/Clr6 HDAC complex II" should also be replaced at other places in the text.
4. Page 10, title of the paragraph. It seems that the formulation "genomic stability" should be replaced by "nucleosome stability". This is also true throughout the text.
5. Page 13, last line. As it is written it sounds that Alp13 is and HDAC. The formulation should thus be changed to avoid such false interpretation.
6. Page14, lines 4 and 5. "... the PTGS (post-transcriptional gene silencing) factor, Clr4". This is misleading, as in fact Clr4 is a core component of the TGS (transcriptional gene silencing) machinery that is also required for PTGS at pericentric regions. Thus, Clr4 is not just a PTGS factor as it can be implied from reading the current formulation.
7. Page 15, line 7 "..., there were noticeable changes...". This formulation appears quite vague and should be replaced by a more explicit/descriptive formulation.
8. Page 16, Discussion section 1st paragraph line 7, the sentence starting by "In fact, pervasive..." is difficult to follow and should be rephrased.
9. Page 21, last line, "as described above" refers to what part in the "Materials and methods" section?
10. Page 27, legend of Figure 2A, C and D. State what the color code stands for.
11. Page 28, title of Figure 4. Unless shown "chromatin-remodeling activity" should be replaced by "ATPase activity" provided the authors show evidence of Hrp3 ATPase activity (as indicated in point #1 of this review).
12. Page 30, title Figure 7 "hrp3Δ perturbs nucleosome structure...". The experiments in this figure do not address the structure of the nucleosome but rather the position.
13. Figure 6 B and C. Add the pourcentage of inputs.
14. Legend Figure S4, the title "Hrp3 can exist independent of Hrp1" is too vague.

Referee #2

The manuscript by Shim et al. describes a comprehensive analysis of the role of the CHD-type chromatin remodeling factor Hrp3 on nucleosome positioning and transcription in fission yeast. MNase-seq studies in an Hrp3 mutant strain revealed strong perturbation of nucleosome positioning along gene bodies reflected by a loss in nucleosome periodicity and somewhat increased nucleosome occupancy towards the 3'region of genes (Fig. 1), an effect that is not seen in mutants of the other CHD-type remodelers Hrp1 and Mit1 (Suppl. Fig. S1). The authors claim that this effect is even more pronounced in highly transcribed genes although this is not really obvious from the graph shown in Fig. 1D. Interestingly, Hrp3 deletion caused a global increase in antisense transcription in a pattern that is similar to that of mutants of the Clr6 HDAC complex II and Set2 but not of heterochromatin proteins, such as Swi6, Clr4 or Pht1 (Fig. 2A-D). Moreover, antisense transcription of convergent genes was not more affected than that of non-convergent genes suggesting a role for Hrp3 in preventing transcription from cryptic promoters rather than in transcription termination (Fig. 2E). The authors also show that Hrp3 requires a functional ATPase domain for preventing increased antisense transcription (Fig. 4A). Analyses of double mutants of Hrp3 and Clr6 HDAC components and Set2, respectively, revealed synergistic effects in the regulation of antisense transcription (Fig. 3A, B) and histone H3 acetylation levels (Fig. 3C) suggesting that Hrp3 and Clr6/Set2 histone modifiers have non-redundant roles in the regulation of proper transcription elongation.

With respect to effects on bulk chromatin behaviour it is shown that Hrp3 deletion causes increased MNase sensitivity and salt-dependent loss of histones from chromatin preparations upon Hrp3 deletion, which confirms the MNase-seq data and in addition points to a destabilization of nucleosomes (Fig. 4 B-D).

The authors then went on to investigate the effects of Hrp3 deletion on pericentromeric transcription. They found that forward and reverse transcription of the dh/dg repeats was increased in Hrp3 mutants but that RNAi-mediated heterochromatin silencing appeared not to be affected (Fig. 5A-C). Conversely, H3K9 trimethylation levels were found to be reduced at the dh/dg repeats although Swi6 localization was not perturbed (Fig. 5D, E). In order to gain insight into the recruitment mechanisms of Hrp3 to pericentric chromatin, localization of Hrp3 was examined in swi6 mutants and found to be reduced (Fig. 6A). However, the levels of Hrp3 enrichment at dg

repeats seem to be very low even in wild type, therefore the interpretation that Swi6 is required for Hrp3 recruitment is not very soundly supported by the data. The results from in vitro and in vivo interaction experiments may or may not support an interaction between these two factors (Fig. 6 B, C; see comment below). Again, genetic interactions between Hrp3 and Clr6 complex, Clr3 and the Clr4 histone methyltransferase were examined and revealed synergistic defects in dh antisense transcription (Fig. 6D) and reporter gene silencing (Suppl. Figure S8), whereas the effects on sense transcription were slightly weaker and not obvious in the hrp3/clr3 double mutant (Fig. 6 D). With respect to chromatin structure in the centromeric region it is shown that nucleosome occupancy appeared to be increased at the central core region, while changes in the surrounding chromatin were not as pronounced (Fig. 7A). Finally, the authors show that the cell cycle dependent transcription of dh forward strand is disrupted in the absence of Hrp3 causing constitutive transcription instead of a transcriptional peak in S phase (Fig. 7B).

Collectively, this is a strong manuscript presenting an impressive amount of data that clearly contribute to our understanding of the cellular functions of CHD-type chromatin remodeling factors. In particular, the findings pertaining to genetic interactions with the Clr6 HDAC complex and Set2 or Clr4, respectively, contribute to the definition of Hrp3 action in the context of the network of chromatin modifiers that regulate transcription elongation. The technical quality is generally high and the manuscript is clearly written. Nevertheless, I should like to point out one weak part of the manuscript, i.e. the experiments designed to show interaction between Hrp3 and Swi6. As mentioned above, the ChIP data, at least for the dg repeat, show only very small changes in the enrichment of Hrp3 in the wild type compared to the delta hrp3 mutant. It is therefore not entirely clear, if Hrp3 is present at all at these loci. Second, the Co-IP experiments between Hrp3 and Swi6 are not very convincing, since Fig. 6C shows rather strong unspecific interaction of Swi6 with the Flag beads. Finally, it is not clear why whole cell (bacterial) extracts were used for GST-pull down assays instead of the purified proteins. Nucleic acids or other factors in these extracts might mediate an interaction between Hrp3 and Swi6. Unless the authors can provide more convincing evidence for this interaction and because this piece of data is not of great importance for the rest of the paper, I would recommend to delete this part of the manuscript.

Minor comments:
Title: It seems to me that the title should also reflect the fact that almost half of the presented data pertain to Hrp3's role in heterochromatin silencing.

Introduction: page 4, there is no CHD2 in Drosophila.

Fig. 1A: A legend should be provided in Fig. 1A to explain the color coding

Fig. 1D: The difference in nucleosome periodicity between highly and weakly expressed genes appears very small. What makes the authors think that this is significant?

Page 8, first paragraph: I assume the aim of the affinity purification strategy of tagged Hrp1 and Hrp3 was to determine, if the two factors interact? This should be stated more clearly. Also, it would be helpful to explain the CBP acronym as to avoid confusion with the mammalian CBP transcription factor. Along the same lines, for readers not familiar with the S. pombe names of conserved factors, it would be helpful to explain some of the ortholog names, e.g. H2A.Z and Pht1 on page 8, last line.

Fig. 3C: The difference in H3 acetylation levels between hrp3 and alp13 single and double mutants appears to be very small. Statistical significance should be determined.

Fig. 5A: Explain +/- in legend.

Fig. 6D: The increase in dh forward transcription in clr3/hrp3 double mutants is not synergistic compared to the clr3 single mutant as stated in the text on page 14. It might not even be additive.

Referee #3

This manuscript describes the role of the CHD remodeler Hrp3 in regulating nucleosome positioning

within the genome, and the consequences due to the loss of Hrp3. First the profiles of nucleosomes are strikingly less defined, in contrast to other Hrp1 or Mit mutants. Furthermore, the authors found an increase in antisense transcription, presumably due to the exposure of cryptic initiation sites within coding sequences. They also describe a role in maintenance of pericentric heterochromatin due to direct recruitment by an HP1 homologue, Swi6. Finally, they observe higher dg/dh repeat transcripts, and show an extension of transcription beyond the normal S phase.

Overall, the study is well designed and executed. The manuscript provides a nice illustration of the importance of chromatin remodelers in suppressing opportunistic, non-productive transcription. Although this has been implicated in previous reports, this paper directly tests this model with remodeler mutants and transcription measurements. Thus, the paper provides a solid advance in understanding remodeler function in S. pombe, and will be of interest to those in the transcription and chromatin fields. I recommend publication following their addressing the points below.

Major points:

The authors analyzed nucleosome occupancy and positioning by high-throughput, paired-end sequencing of MNase digested chromatin. This approach is best, as the exact size of each nucleosomal fragment can be determined. However, it is not clear whether the authors checked the size distributions of their sequenced nucleosomes, and whether they restricted to a particular size range. A library of degraded and/or sub-nucleosomal fragments can give a false interpretation of poor positioning. If the sequence libraries do have different mean lengths, is this due to interesting biological effects, or poor preparation?

The authors describe an interesting result where nucleosomes in the hrp3Δ mutant display increased sensitivity to salt extraction. The authors provide very little explanation of how the loss of a remodeler decreases general nucleosome stability. One possibility may be that an increased number of nucleosomes are no longer positioned onto energetically favorable DNA sequences (presumably by the Hrp3 remodeler), leading to increased sensitivity to salt disruption. An examination of the DNA sequence for canonical and alternate positions of the retained nucleosomes at moderate salt may address this. In a related issue, the statement "Hrp3 is important for the stability of chromatin structure during transcription" on page 11 is over-reaching for the data presented.

The authors examined sense and antisense transcription of both euchromatic and heterochromatic genes. They describe antisense transcription to their example gene, zer1 (the current name is prp1); I presume they are measuring the annotated ncRNA SPNCRNA1539? The authors also claim both forward and reverse transcription is increased for both heterochromatic dg and dh repeats; however, only dh readily shows a significant increase in Figure 5 without quantification. This is supported by the fact that Hrp3 is enriched by ChIP only over the dh repeat and very little over dg in Figure 6.

1st Revision - authors' response 17 August 2012

Referee #1

The paper by Sam Shim and co-workers details the role of Hrp3 chromatin remodeler in the positioning of nucleosomes and the suppression of cryptic transcription on a genome-wide scale. More specifically, the authors show that the well-defined nucleosome arrangement at transcription start sites is disrupted in hrp3Δ cells. Moreover, transcripts resulting from cryptic transcription accumulate and histones are less tightly associated to genomic DNA in hrp3Δ cells. A point mutant in Hrp3 ATPase domain shows similar phenotype, suggesting that Hrp3 action on nucleosome position and cryptic transcription might go through ATP-dependent remodeling of nucleosomes. In addition, the authors show that Hrp3 contributes to transcriptional silencing at pericentric heterochromatin, where it is required for the cell cycle-dependent regulation of pericentric silencing. Chromatin immunoprecipitation and genetic studies provide further evidence of Hrp3 implication in pericentric silencing.

Overall, this is an interesting paper containing high quality data. The conclusions of the paper are globally justified and they shall be of interest for the general study of ATP-dependent chromatin remodeling's action on the regulation of DNA-linked processes such as transcription, both in the context of euchromatin and heterochromatin. I have however several concerns, most of them have to do with approximations and overstatements being made:

Our comment: Before we respond to each point, we appreciate the enthusiasm of the reviewer. We have addressed the individual issues/concerns below.

1. A point mutant of Hrp3 was made to examine how much of the defects observed in hrp3Δ cells are to be attributed to Hrp3 ATPase activity. Currently, no experiment shows that Hrp3 has an ATPase activity and that the point mutation made disrupts this activity. As this is an important element of the main conclusion of the paper, the authors should at least provide evidence that the point mutation does indeed destroy Hrp3 ATPase activity in vitro.

Our comment: We thank the reviewer for this comment. To see the loss of ATPase activity of *hrp3*^{K406A}, we tested the ATPase activity of FLAG-tagged Hrp3 and Hrp3^{K406A} purified from yeast cells. We presented this data in Supplementary Figure S6. We also added the sentence on line 13-14, page 10 in the revised manuscript.

"The loss of ATPase activity in *hrp3*^{K406A} was observed in ATPase assay (Supplementary Figure 6)."

2. In the abstract the authors wrote "... Hrp3, is a global regulator that drives higher-order chromatin structure and genomic stability". In fact, there is no experiment in this paper that would allow such broad statement. A large-scale determination of nucleosome positioning and histone association to genomic DNA in wild-type and mutant strains is what was really addressed in the paper. Thus, "Higher-order" and "genomic stability" formulations should be avoided to not misinterpret the main message of the paper.

Our comment: We agree with this comment and toned down this sentence on page2, line5-6 in the revised manuscript.

"*Schizosaccharomyces pombe* CHD remodeler, Hrp3, is a global regulator that drives proper nucleosome positioning and nucleosome stability."

3. The interaction between Swi6 and Hrp3 has been described in a previous paper (Motamedi et al, Mol Cell, 2008). Although this previous paper did not further characterize the Swi6-Hrp3 interaction as it has been done here, the authors should mention the existence of this data in the main text and cite the paper.

Our comment: We are sorry for not including the previous data in the first manuscript. We added the sentence on page 13, line 21-22 in the revised manuscript.

"Consistent with these results, previous report has shown that Hrp3 copurified with Swi6 by tandem mass spectrometry (Motamedi et al., 2008)."

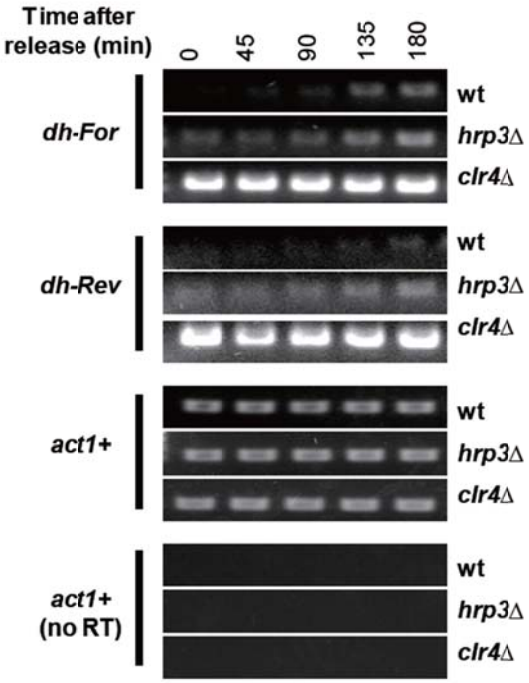
4. The cell synchronization experiment using cdc25-22 temperature-sensitive mutant shows a dramatic loss of silencing in hrp3Δ cells after release of the cell cycle block. To exclude the possibility that this loss is linked to the way the cells were synchronized, the authors should redo the experiment with another mutant or another mean to synchronize the cell, and check whether the cell-cycle effect of the hrp3Δ mutation on the pericentric silencing is still observable.

Our comment: We thank the reviewer for these comments. We carried out two different ways of experiments to exclude the background effect of *cdc25-22* mutant. Firstly, we used the *cdc25-22 hrp1*Δ mutant as a negative control. Unlike the *hrp3* mutant, the *cdc25-22 hrp1*Δ mutant did not affect cell-cycle dependent transcription of *dh* element. We presented this data in Supplementary Figure S12 and added an explanation for the data on page

16, line 12-13 in the revised manuscript.

"*cdc25-22 hrp1*Δ, however, did not cause apparent increase in transcript levels of *dh* repeats during cell cycle compare to wild-type cells (Supplementary Figure 12)."

Secondly, we performed the synchronized experiment using S-phase arrest drug hydroxyurea (HU). We present this data only in rebuttal.



Minor points:

1. Page 5, line 3 add an "s" to the word "region".

Our comment: We corrected this mistake.

2. Page 6, last line the formulation "involvement between" is a bit confusing and should be replaced.

Our comment: We replaced the formulation "involvement between" by "involvement of Hrp3 in RNAPII-

associated transcription" in the revised manuscript.

3. Page 8, title of the paragraph, the formulation "Set2/Clr6 HDAC complex II" should be avoided as it makes it sound that Set2 and Clr6 a part of a same complex. This could be simply replaced by "Set2 and Clr6 HDAC complex II". The "Set2/Clr6 HDAC complex II" should also be replaced at other places in the text.

Our comment: We corrected the formulation.

4. Page 10, title of the paragraph. It seems that the formulation "genomic stability" should be replaced by "nucleosome stability". This is also true throughout the text.

Our comment: We corrected the formulation.

5. Page 13, last line. As it is written it sounds that Alp13 is and HDAC. The formulation should thus be changed to avoid such false interpretation.

Our comment: We changed the formulation to "….*alp13*Δ, a component of Clr6 HDAC complex….".

6. Page14, lines 4 and 5. "... the PTGS (post-transcriptional gene silencing) factor, Clr4". This is misleading, as in fact Clr4 is a core component of the TGS (transcriptional gene silencing) machinery that is also required for PTGS at pericentric regions. Thus, Clr4 is not just a PTGS factor as it can be implied from reading the current formulation.

Our comment: Thank you. We corrected the formulation.

7. Page 15, line 7 "..., there were noticeable changes...". This formulation appears quite vague and should be replaced by a more explicit/descriptive formulation.

Our comment: We replaced the formulation to "….there were obvious changes…".

8. Page 16, Discussion section 1st paragraph line 7, the sentence starting by "In fact, pervasive..." is difficult to follow and should be rephrased.

Our comment: We rephrased the sentence in the revised manuscript.

> “Previous reports have shown that the histone chaperones such as Spt6 and Spt16 (Kaplan et al., 2003) and histone modifying enzymes including Set2, Clr6 HDAC complex II (Carrozza et al., 2005) are required to re-organize the nucleosome structure and prevent cryptic antisense transcription within intragenic regions.”

9. Page 21, last line, "as described above" refers to what part in the "Materials and methods" section?

Our comment: We referred to the ‘chromatin isolation and micrococcal nuclease digestion’ section

10. Page 27, legend of Figure 2A, C and D. State what the color code stands for.

Our comment: We corrected the legend of Figure 2A, C and D.

11. Page 28, title of Figure 4. Unless shown "chromatin-remodeling activity" should be replaced by "ATPase activity" provided the authors show evidence of Hrp3 ATPase activity (as indicated in point #1 of this review).

Our comment: We agree with this comment and changed the phrase to “ATPase activity of Hrp3”.

12. Page 30, title Figure 7 "hrp3Δ perturbs nucleosome structure...". The experiments in this figure do not address the structure of the nucleosome but rather the position.

Our comment: We modified the title of Figure 7.

13. Figure 6 B and C. Add the pourcentage of inputs.

Our comment: We corrected Figure 6B and C.

14. Legend Figure S4, the title "Hrp3 can exist independent of Hrp1" is too vague.

Our comment: We modified the title of Supplementary Figure S4.

"Hrp3 can be separated from Hrp1".

Referee #2

The manuscript by Shim et al. describes a comprehensive analysis of the role of the CHD-type chromatin remodeling factor Hrp3 on nucleosome positioning and transcription in fission yeast. MNase-seq studies in an Hrp3 mutant strain revealed strong perturbation of nucleosome positioning along gene bodies reflected by a loss in nucleosome periodicity and somewhat increased nucleosome occupancy towards the 3'region of genes (Fig. 1), an effect that is not seen in mutants of the other CHD-type remodelers Hrp1 and Mit1 (Suppl. Fig. S1). The authors claim that this effect is even more pronounced in highly transcribed genes although this is not really obvious from the graph shown in Fig. 1D. Interestingly, Hrp3 deletion caused a global increase in antisense transcription in a pattern that is similar to that of mutants of the Clr6 HDAC complex II and Set2 but not of heterochromatin proteins, such as Swi6, Clr4 or Pht1 (Fig. 2A-D). Moreover, antisense transcription of convergent genes was

not more affected than that of non-convergent genes suggesting a role for Hrp3 in preventing transcription from cryptic promoters rather than in transcription termination (Fig. 2E). The authors also show that Hrp3 requires a functional ATPase domain for preventing increased antisense transcription (Fig. 4A). Analyses of double mutants of Hrp3 and Clr6 HDAC components and Set2, respectively, revealed synergistic effects in the regulation of antisense transcription (Fig. 3A, B) and histone H3 acetylation levels (Fig. 3C) suggesting that Hrp3 and Clr6/Set2 histone modifiers have non-redundant roles in the regulation of proper transcription elongation.

With respect to effects on bulk chromatin behaviour it is shown that Hrp3 deletion causes increased MNase sensitivity and salt-dependent loss of histones from chromatin preparations upon Hrp3 deletion, which confirms the MNase-seq data and in addition points to a destabilization of nucleosomes (Fig. 4 B-D).

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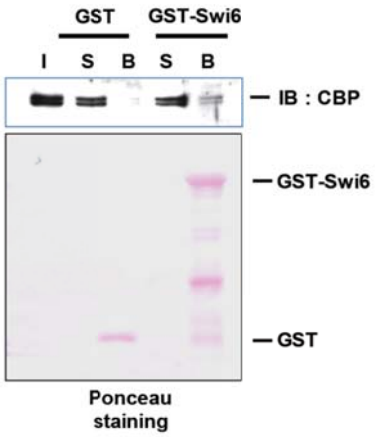
interactions between Hrp3 and Clr6 complex, Clr3 and the Clr4 histone methyltransferase were examined and revealed synergistic defects in dh antisense transcription (Fig. 6D) and reporter gene silencing (Suppl. Figure S8), whereas the effects on sense transcription were slightly weaker and not obvious in the hrp3/clr3 double mutant (Fig. 6 D). With respect to chromatin structure in the centromeric region it is shown that nucleosome occupancy appeared to be increased at the central core region, while changes in the surrounding chromatin were not as pronounced (Fig. 7A). Finally, the authors show that the cell cycle dependent transcription of dh

forward strand is disrupted in the absence of Hrp3 causing constitutive transcription instead of a transcriptional peak in S phase (Fig. 7B).

Collectively, this is a strong manuscript presenting an impressive amount of data that clearly contribute to our understanding of the cellular functions of CHD-type chromatin remodeling factors. In particular, the findings pertaining to genetic interactions with the Clr6 HDAC complex and Set2 or Clr4, respectively, contribute to the definition of Hrp3 action in the context of the network of chromatin modifiers that regulate transcription elongation. The technical quality is generally high and the manuscript is clearly written. Nevertheless, I should like to point out one weak part of the manuscript, i.e. the experiments designed to show interaction between Hrp3 and Swi6. As mentioned above, the ChIP data, at least for the dg repeat, show only very small changes in the enrichment of Hrp3 in the wild type compared to the delta hrp3 mutant. It is therefore not entirely clear, if Hrp3 is present at all at these loci. Second, the Co-IP experiments between Hrp3 and Swi6 are not very convincing, since Fig. 6C shows rather strong unspecific interaction of Swi6 with the Flag beads. Finally, it is not clear why whole cell (bacterial) extracts were used for GST-pull down assays instead of the purified proteins. Nucleic acids or other factors in these extracts might mediate an interaction between Hrp3 and Swi6. Unless the authors can provide more convincing evidence for this interaction and because this piece of data is not of great importance for the rest of the paper, I would recommend to delete this part of the manuscript.

Our comment: We warmly thank this reviewer for the critical comments. We completely agreed with the review's comments and replaced some data.

-To address the interaction between Hrp3 and Swi6, we referred the previous paper (Motamedi et al, Mol Cell, 2008) in main text. This reference revealed that Hrp3 copurified with Swi6 by tandem mass spectrometry. We also performed the GST pull-down experiment using TAP-purified Hrp3 instead of whole cell extracts. We present this data only in rebuttal.



We changed the co-IP data between Hrp3 and Swi6 in the revised manuscript (Figure 6C).

-We have difficulties in detecting the occupancy of Hrp3 using ChIP experiments as it is known that performing ChIP assay against chromatin remodelers including CHD family proteins is difficult. Although the enrichment of Hrp3 in the wild type is marginal, we could repeatedly detect Hrp3 at *dg* region. In addition, loss of Hrp3 caused the increase of transcripts at *dg* region in Figure 5A. Therefore, we interpreted that Hrp3 might be localized at *dg* repeat region as well as *dh* region. If we can make a high-quality antibody against Hrp3, we can probably solve this problem.

Minor comments:

Title: It seems to me that the title should also reflect the fact that almost half of the presented data pertain to Hrp3's role in heterochromatin silencing.

Our comment: Thank you. We modified the title to reflect the global role of Hrp3 in the revised manuscript.

"Hrp3 controls nucleosome positioning to suppress non-coding transcription in eu- and heterochromatin"

Introduction: page 4, there is no CHD2 in Drosophila.

Our comment: Thank you. We corrected this mistake.

Fig. 1A: A legend should be provided in Fig. 1A to explain the color coding

Our comment: Thank you. We added an explanation for the color coding in the revised manuscript.

Fig. 1D: The difference in nucleosome periodicity between highly and weakly expressed genes appears very small. What makes the authors think that this is significant?

Our comment: We focused on the defect of '-1' nucleosome and rapid disruption from '+3' nucleosome of *hrp3* mutant at highly expressed genes. Both '-1' nucleosome and nucleosome positioning in the transcribed regions play an important role in regulating transcription. Therefore, we thought that the changes of nucleosome positioning in *hrp3* mutant might be involved with RNAPII-associated transcription.

Page 8, first paragraph: I assume the aim of the affinity purification strategy of tagged Hrp1 and Hrp3 was to determine, if the two factors interact? This should be stated more clearly. Also, it would be helpful to explain the CBP acronym as to avoid confusion with the mammalian CBP transcription factor. Along the same lines, for readers not familiar with the S. pombe names of conserved factors, it would be helpful to explain some of the ortholog names, e.g. H2A.Z and Pht1 on page 8, last line.

Our comment: Thank you. We agree with these comments and have made the appropriate corrections.

- We added the sentence to clarify the aim of the purification strategy of tagged Hrp1 and Hrp3 on line 19-21, page 7 in the revised manuscript.

"Although Hrp1 and Hrp3 physically interact *in vivo*, they have distinct roles in chromosome segregation and heterochromatin silencing in central core region (Walfridsson et al., 2005)."

-We changed the words 'only-Hrp3 CBP' to 'only TAP-tagged Hrp3' on line 5, page 8 in the revised manuscript. And we modified supplementary Figure legend 4(A) describing α–CBP antibody.

-We added ortholog names of conserved factors.

Fig. 3C: The difference in H3 acetylation levels between hrp3 and alp13 single and double mutants appears to be very small. Statistical significance should be determined.

Our comment: Thank you. We agreed this reviewer for the critical comments. Statistical analysis was performed using *t*-test for *hrp3*, *alp13* single mutants and double mutants. As a result, we found that the increase of histone H3 acetylation levels in *hrp3Δ alp13Δ* double mutants was overanalyzed. Thus, we rephrased the sentence on page 10, line 1-3 in the revised manuscript.

"However, combining *hrp3Δ* with *set2Δ* resulted in cumulative increases in histone H3 acetylation, concomitant with elevations in the levels of antisense RNA, whereas *hrp3Δ alp13Δ* double mutants did not show any synergistic increase."

Fig. 5A: Explain +/- in legend.

Our comment: Thank you. We explained the meaning of '+/-' in the figure legend 5A.

Fig. 6D: The increase in dh forward transcription in clr3/hrp3 double mutants is not synergistic compared to the clr3 single mutant as stated in the text on page 14. It might not even be additive.

Our comment: Thank you. We agree with this comment and modified the sentence on 9-12, page 14 in the revised manuscript.

"In addition, the increase of *dh* reverse transcripts in *hrp3*Δ*clr3*Δ double mutant was also synergistic compared to those of the single mutants. However, we could not detect synergistic increase of *dh* forward transcripts indicating both redundant and non-redundant pathway between the two factors."

Referee #3

This manuscript describes the role of the CHD remodeler Hrp3 in regulating nucleosome positioning within the genome, and the consequences due to the loss of Hrp3. First the profiles of nucleosomes are strikingly less defined, in contrast to other Hrp1 or Mit mutants. Furthermore, the authors found an increase in antisense transcription, presumably due to the exposure of cryptic initiation sites within coding sequences. They also describe a role in maintenance of pericentric heterochromatin due to direct recruitment by an HP1 homologue, Swi6. Finally, they observe higher dg/dh repeat transcripts, and show an extension of transcription beyond the normal S phase.

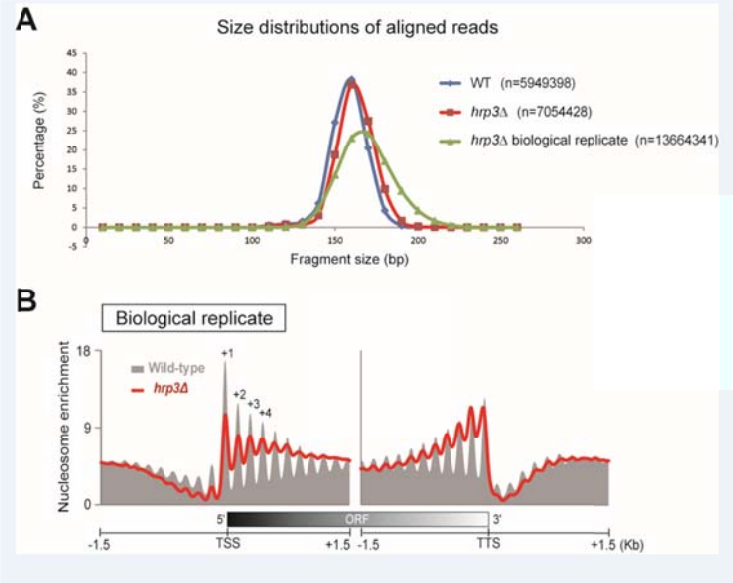
Overall, the study is well designed and executed. The manuscript provides a nice illustration of the importance of chromatin remodelers in suppressing opportunistic, non-productive transcription. Although this has been implicated in previous reports, this paper directly tests this model with remodeler mutants and transcription measurements. Thus, the paper provides a solid advance in understanding remodeler function in S. pombe, and will be of interest to those in the transcription and chromatin fields. I recommend publication following their addressing the points below.

Our comment: Before we respond to each point, we wish to express our appreciation for this reviewer's critical and constructive contributions for the improvement of our manuscript. We have taken advantage of the reviewer's invaluable input by revising the manuscript.

Major points:

The authors analyzed nucleosome occupancy and positioning by high-throughput, paired-end sequencing of MNase digested chromatin. This approach is best, as the exact size of each nucleosomal fragment can be determined. However, it is not clear whether the authors checked the size distributions of their sequenced nucleosomes, and whether they restricted to a particular size range. A library of degraded and/or sub-nucleosomal fragments can give a false interpretation of poor positioning. If the sequence libraries do have different mean lengths, is this due to interesting biological effects, or poor preparation?

Our comment: Thank you for your comment. We checked the size distributions of the samples before and after sequencing. We observed very similar size distributions between wild-type and *hrp3Δ*. To validate our findings in this study, we repeated the MNase-seq experiment with another *hrp3Δ* mutant (biological replicate) and sequenced it in a different sequencing facility. Even though the number of reads was different, we observed very similar nucleosome patterns in the *hrp3*Δ mutant. We present this data only in rebuttal.



The authors describe an interesting result where nucleosomes in the hrp3Δ mutant display increased sensitivity to salt extraction. The authors provide very little explanation of how the loss of a remodeler decreases general nucleosome stability. One possibility may be that an increased number of nucleosomes are no longer positioned onto energetically favorable DNA sequences (presumably by the Hrp3 remodeler), leading to increased sensitivity to salt disruption. An examination of the DNA sequence for canonical and alternate positions of the retained nucleosomes at moderate salt may address this. In a related issue, the statement "Hrp3 is important for the stability of chromatin structure during transcription" on page 11 is over-reaching for the data presented.

Our comment: Thank you for your comment. Until now, we did not have any data explaining the mechanism of nucleosome instability caused by loss of remodeler. We agree with your comment and are planning to do further dissect the mechanism. Thus we toned down this sentence on page 11, line 15-16 in the revised manuscript.

"Hrp3 is involved in the stability of chromatin architecture."

The authors examined sense and antisense transcription of both euchromatic and heterochromatic genes. They describe antisense transcription to their example gene, zer1 (the current name is prp1); I presume they are measuring the annotated ncRNA SPNCRNA1539? The authors also claim both forward and reverse transcription is increased for both heterochromatic dg and dh repeats; however, only dh readily shows a significant increase in Figure 5 without quantification. This is supported by the fact that Hrp3 is enriched by ChIP only over the dh repeat and very little over dg in Figure 6.

Our comment: We removed the annotated ncRNA *SPNCRNA1539* of Figure 1C in the revised manuscript to avoid confusion, because *SPNCRNA1539* was not found in the context of other genome annotations as a predicted region. In addition, *zer1*$^{+}$ has been often used to detect antisense transcripts in previous papers (Nicolas et al., 2007, Anderson et al., 2009). We also agree with your comments about the roles of Hrp3 at heterochromatic region, however, we repeatedly detected the increased transcript levels of *dg* repeat under our

condition. Thus, we interpreted that Hrp3 plays a role at *dg* repeat region. We mentioned the same point in major comments of the Referee #2. To clarify the role of Hrp3 at the heterochromatic region, we will perform further experiments.

2nd Editorial Decision 29 August 2012

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the original reviewers, and I am happy to inform you that we are ready to proceed with acceptance and production of the paper, pending modification of a few minor points.

- In response to reviewer #2's comment regarding the GST-pull-down experiments of Swi6 with Hrp3 using whole cell extracts, you have repeated the experiment with TAP-purified Hrp3 and included the data in your rebuttal. Given the raised concerns, it seems more appropriate to incorporate this experiment in the actual manuscript as a substitution to Fig. 6B.

- It is currently not obvious how many biological replicates the data in Fig. 1 and 2 are based upon - please clarify this in the figure legend. In case the data represent a single experiment, please add the biological replicate included in your rebuttal in the manuscript.

- The co-IP data in Fig. 6C is difficult to assess because of contrast/brightness settings that lead to loss of background signals. Please revise the respective figure panels by including less adjusted images.

- Please remember to add the data accession numbers for all high-throughput data sets.

- Finally, we encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. I am taking this opportunity to invite you to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.

I am now returning the manuscript to you for one last round of minor amendments, hoping that you will be able to upload and re-submit the final version at your earliest convenience. After that, we should be able to swiftly proceed with formal acceptance and production of the manuscript!

If you have any questions in this regard, please do not hesitate to contact me directly.

Yours sincerely,

Editor
The EMBO Journal

2nd Revision - authors' response 30 August 2012

In response to reviewer #2's comment regarding the GST-pull-down experiments of Swi6 with Hrp3 using whole cell extracts, you have repeated the experiment with TAP-purified Hrp3 and included the data in your rebuttal. Given the raised concerns, it seems more appropriate to incorporate this experiment in the actual manuscript as a substitution to Fig. 6B.

Our comment: We replaced GST-pull-down data in the revised manuscript. We also corrected some content in main text and legend of Figure 6B and described TAP purification protocol in the “materials and methods” section.

It is currently not obvious how many biological replicates the data in Fig. 1 and 2 are based upon - please clarify this in the figure legend. In case the data represent a single experiment, please add

the biological replicate included in your rebuttal in the manuscript.

Our comment: We added the biological replicate data in Figure 1B and additional explanations in legends of Figure 1 and 2. The data showing size distributions of the samples before and after sequencing was also added in Supplementary Figure S1.

The co-IP data in Fig. 6C is difficult to assess because of contrast/brightness settings that lead to loss of background signals. Please revise the respective figure panels by including less adjusted images.

Our comment: We replaced co-IP data in Figure 6C by less adjusted image.

Please remember to add the data accession numbers for all high-throughput data sets.

Our comment: We added GEO accession numbers of the data in the revised manuscript.

Finally, we encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. I am taking this opportunity to invite you to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.
Our comment: We have provided source data for electrophoretic gels and blots.

