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Chd1 chromatin remodelers maintain nucleosome organization and repress cryptic transcription

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Transaction Report:

(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 the submission of your manuscript to EMBO reports. We have now received the full set of referee reports, which is copied below.

As you will see, all referees in principle support publication of the study in EMBO reports. The main concern, raised by referee 1, is that it is unclear whether the genome-wide expression profiling of the Chd1-deficient strain was performed only once. The referee also suggests a MNase experiment that could further strengthen the role of Chd1 in nucleosome spacing. All other referee comments concern the presentation of the work, placing it into its scientific context, clarifications and statistics (that are important).

Given the number of very related studies in press and under consideration elsewhere, I think it is in both our interest that we proceed with this manuscript as quickly as possible. I therefore would like to know how often the genome-wide expression profiling was performed, and whether you think that the MNase experiment would be a valuable addition and could be performed quickly. Can you please comment on these two issues as soon as possible?
Assuming that the genome-wide assays were performed at least twice, I strongly suggest that you submit a revised version of your manuscript that addresses all the referee concerns within the next few weeks, and no later than the 14th of September.

You will need to rewrite and shorten the manuscript text in order to incorporate the referee suggestions and comply with our short format. We allow a maximum of 30.000 characters, including references, figure legends and spaces, and 5 main plus 5 supplementary figures. Shortening of the text may be made easier by combining the results and discussion section that may

help to eliminate redundancy that is inevitable when discussing the same experiments twice. Parts of the materials and methods can also be moved to the supplementary information, but the materials and methods essential for the understanding of the experiments described in the main body of the manuscript must remain in the main manuscript file.

Please let me know whether you agree with my suggestions of how to proceed and if you have any questions regarding manuscript shortening.

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

Yours sincerely,

Editor
EMBO Reports

Referee #1:

Hennig et al. present a very nice paper exploring the consequences of loss of the Chd1 remodelers Hrp1 and Hrp3 in S. pombe. In particular, they demonstrate that simultaneous loss of the these remodelers leads to:

1) a massive increase in cryptic, particularly anti-sense, transcripts;

2) that this increase in cryptic transcription shows striking similarity to that observed for alp13 and set2 mutations;

3) that the hrp1 hrp3 double mutant displays a significant disruption of nucleosome positioning with little, if any change in overall histone content;

4) that mutations that perturb chromatin, increase cryptic transcription and encode proteins that are thought to functionally interact with Chd1 do not show the chromatin disruption phenotype observed for Chd1.

In general, the data are high quality and the results presented are clear. The most difficult issue in evaluating this manuscript is that much of the data presented represent only an incremental advance in our knowledge. For example:

1) Several groups have shown that chd1 mutations in S. cerevisiae cause cryptic transcription (but have not shown the anti-sense transcription or genome-wide analysis presented here. (Cheung et al., PLoS Biol 6:e277; Quan and Hartzog, Genetics 2010)

2) Cryptic transcripts, including on the anti-sense strand have previously been observed in set2 mutations in S. cerevisiae. (e.g., Carozza et al., Cell 2005)

3) Disruption of nucleosome positioning similar to that reported here has previously been observed in a genome-wide study in S. cerevisiae. (Gkikopoulos et al., Science 2011).

Thus, the real value in this work seems to rest on the facts that: 1) important work on Chd1 has been extended from S. cerevisiae to S. pombe; 2) the observations of pervasive anti-sense cryptic transcription in the hrp1/3 double mutant, and 3) the observation that chromatin disruption (i.e. loss of nucleosome positioning or loss of significant loss of nucleosomes) is not required for cryptic transcription. I think that each of these observations potentially represents an important contributions to the field.

Specific comments:

1. The methods section is a bit thin on details. How many replicates were performed for the genome-wide analyses? I think that it is important that the key observations are based upon more than a single experiment. Also, how were the protein extracts used in the western blots prepared? Is it possible that apparent changes in histone content observed in the pob3 mutant reflect a change in chromatin solubility in the extract rather than a change in histone levels?

2. Did the authors examine the apparent differences in histone (total or acetylated) content in Figure 2 for statistical significance?

3. In addition to the work presented here and previously by Owen-Hughes and colleagues, a prior study used micrococcal nuclease digestion to show that nucleosome spacing is unaltered in chd1 mutants in S. cerevisiae. (Xella et al., Mol. Microbiol. 2006) It think that citation of this work would bolster the author's arguments. Furthermore, given the author's arguments here and the prior observations of Celona et al. (PLoS Biol., 2011), I would expect that careful analysis of MNase digests of hrp1 hrp3 chromatin would show a modest increase in sensitivity to MNase, but no change in apparent spacing. This simple independent test of the author's model could significantly strengthen the manuscript.

4. The significantly stronger effect of the alp13 mutation on histone H3 acetylation than that observed for the set2 mutation (Figure 2) is surprising and much different than what has been observed in S. cerevisiae by the Workman group (see Keogh et al., Cell 2005). This deserves more comment and a discussion of controls. For example, can the effect on bulk acetylation of histone H3 in the alp13 mutation be complemented by an ALP1 plasmid?

Minor comments:

1. The manuscript requires some light copy-editing for grammatical issues. The authors often inappropriately use or omit the definite and indefinite articles, the and a.

2. On page 6, in the results section, a citation should be provided for the statement "Both mutants are known to accumulate cryptic transcripts."

3. In the discussion of the interaction of Chd1 with the Paf and FACT complexes, page 15, the authors should cite Krogan et al., MCB 10(6):6979-92, 2002 and Kelly, Stokes and Perry, Chromosoma 108:10-25,1999, in addition to the Simic paper.

Referee #2:

This manuscript provides a timely description of various factors influencing the potential for non coding transcripts to be generated from within the coding regions of genes. A genetic screen is used to identify roles for two chromatin remodelling enzymes in this process. These enzymes related to the budding yeast Chd1 protein act to maintain nucleosome positioning over coding regions. Other mutations affecting non coding transcription include the alp13 histone deacetylates component and the histone methyltransferase set2. By investigating the effects of each of these mutants it is found that each affect chromatin organisation in different ways. It is of special interest to find that the nucleosome positioning is retained in the Se2 mutant, and that histone acetylation also is relatively unchanged in this mutant. These observations provide new insight in the process required to organise chromatin over coding regions that will be of interest to a general readership.

Minor points include:
In the introduction it is stated that K36 acts as a recruitment signal for the Cl6 HDAC. However, in budding yeast an alternative has been proposed in which K36 is required for HDAC activity but not recruitment. http://dx.doi.org/10.1016/j.molcel.2010.07.003

The manuscript is quite careful to point out the dangers in calculating overall occupancy for histone acetylation or nucleosomes from genome wide datasets. However, an estimation of the absolute

reduction in number of nucleosomes is still made using this approach in the results section describing the hrp1, hrp3 deletion (8%). This should presumably be qualified with the arguments made elsewhere in the manuscript.

Referee #3:

Hennig et al present a manuscript titled "Chd1 chromatin remodelers maintain nucleosome organization and repress cryptic transcription" examining the function of the conserved Chd1-class of remodeler in S. pombe in organizing transcribed chromatin and preventing widespread antisense transcription from cryptic promoters. Chd1 family members are more well studied in budding yeast where there is a single Chd1 homolog. This homolog has been implicated in transcribed chromatin and cryptic transcription, but has also been shown to collaborate with Isw1 in regulating chromatin and cryptic transcription. S. pombe lack ISWI family members so the function of Chd1 members and how these same types of activities are accomplished are not clear for S. pombe. Work on S. cerevisiae Chd1 has been relatively intensive very recently, but the results here are novel enough and extend analyses genome wide to a new system that has many important differences in chromatin control and transcription that make S. pombe a valuable model for higher eukaryotes that are distinct from S. cerevisiae. The experiments are clearly described and seem to be performed appropriately. The manuscript suffers much from lack of appropriate context for the reader in terms of recent and less recent Chd1 experiments in the S. cerevisiae system. These points should be addressed to facilitate readers' understanding of the system and the relative significance of the work. Points to consider are presented below in a numbered list. Some are minor questions of language, but many relate to appropriate citation and discussion of the literature. I cannot recommend where to cut sections of the paper to make room for essential context, but minimally a total of 2-3 paragraphs should be added to cover a range of uncited publications that relate highly to the current manuscript. Otherwise, with these caveats the work is a nice contribution to the literature.

Introduction

1. Page 4: "Since the passage of Pol II along the DNA during transcription elongation requires opening up the chromatin and disassembling the nucleosomes,"

Disassembly may correlate with transcription, but it is not necessarily clear if complete nucleosome disassembly is an absolute requirement for transcription. This is a subtle point but the sentence could be more clear.

2. Page 4: "Failure in this process leads to enhanced activity of cryptic promoters, and consequently to toxic accumulation of the resulting transcripts (Mason & Struhl, 2003; Kaplan et al, 2003; Jamai et al, 2009)."

It is not clear if any experiments have been performed to examine whether cryptic transcripts are toxic. This sentence should be rewritten. For example, the work here shows massive additional transcription with little or no effect on growth.

3. Page 4: "The Schizosaccharomyces pombe (S.pombe) homologue of this HDAC complex is the Clr6 complex-II, or Rpd3S complex in Saccharomyces cerevisiae (S.cerevisiae) (Carrozza et al, 2005; Nakayama et al, 2003)."

This sentence is awkwardly written.

4. Page 5: "Mutations in the FACT complex lead to decreased histone occupancy levels in transcribed genes and increased activity of cryptic promoters in these regions (Belotserkovskaya et al, 2003; Jamai et al, 2009; Mason & Struhl, 2003)."

Kaplan 2003 were the first to show spt16 mutant effects on cryptic transcription. Should be cited here and elsewhere for FACT roles in cryptic transcription.

Cheung et al 2008 (PLOS Biology, citation below) also did an extensive analysis of factors

implicated in cryptic transcription, and identified chd1 and isw1 has having this phenotype. This work should also be cited and discussed where appropriate.

5. Page 5: "In this study we attempted to identify other factors which might have a role in repressing cryptic transcription activity in S.pombe. We screened a deletion library for chromatin related factors and tested their effect on cryptic transcription. We found that deletion of the S.pombe Chd1-type chromatin remodelers, hrp1 and hrp3, results in a dramatic increase in cryptic transcription. To determine the underlying molecular mechanism, we mapped genome-wide nucleosome-position and histone acetylation patterns in Chd1-deficient strain."

Much very recent work and also not so recent work should be cited on this topic. The novelty of the results presented here are that they relate to the S. pombe system and anti-sense transcription is extensively monitored. In many previous analyses of S. cerevisiae, intragenic cryptic transcription of the sense strand has been monitored, many times for a small number of reporter loci and rarely if at all for antisense transcription. Because S. pombe contains the machinery for dsRNA-mediated silencing, anti-sense transcription has the possibility of being much more detrimental to gene expression and chromatin structure. However, there is no context given for what is actually known about Chd1 function in any system and this must be rectified in the introduction, and not the discussion. There are uncited papers that are less recent than Gkikopoulos et al (which is cited, but left to discussion) and they include Cheung et al 2008 mentioned above, Lee et al (2012, citation below) on Chd1 and transcribed chromatin, Radman-Livaja (2012) on Chd1 and H3 dynamics and transcribed chromatin, Quan and Hartzog (2010, citation below) showing synthetic effects between chd1 and isw1 on cryptic transcription in S. cerevisiae. These are major points of context that are missing from the manuscript.

Results

6. Page 6: "PCR reactions to quantify AS transcript levels. As positive controls, we used S.pombe strains lacking the histone methyl-transferase Set2 and the HDAC Clr6-complex II subunit Alp13. Both mutants are known to accumulate cryptic transcripts."

Citation missing. Presumably Nicolas 2007.

7. Page 6: "Interestingly, the changes observed in the sense transcripts were rather minor, with only 6% of the genes demonstrating significantly up- or down-regulated transcript levels."

The major cryptic transcripts that are generally studied in S. cerevisiae derived from intragenic cryptic promoters that are likely more obvious to study by northern blotting because they have defined 3' ends derived from the normal transcription unit's poly-A signals. Is there any evidence for altered 5'-3' ratios of sense mRNA that these transcripts might also be present for hrp3/hrp1 mutants. They might not be observed if if the total effect on average transcription for a gene was less than 2, but please see Figure S1 from Cheung et al, 2008. This seems like a missed opportunity to fully understand the magnitude of cryptic transcription hrp mutants.

8. Page 7: "Previous studies showed that genome-wide nucleosome depletion can result in activation of cryptic transcription in yeast (Keogh, 2012). Mutations in the FACT complex (Mason & Struhl, 2003; Jamai et al, 2009), Spt6 (Kaplan et al, 2003) or the histone chaperones Asf1/HIRA (Yamane et al, 2011; Keogh, 2012)"

As noted above, Cheung et al 2008 should probably be cited here and Kaplan 2003 should be cited for FACT. Again, results from 2008, 2010 (Quan) and 2011 (Owne-Hughes lab) need to be put into context here.

9. Page 13, top of page "exhibit" should be "exhibits"

10. "These results demonstrate that Chd1-remodelers repress cryptic transcription independently of Set2 and the Clr6-complex II."

It might be more precise to state that these results suggest that Chd1 regulates nucleosome positioning independently of Set2 and the Clr6-complex II, and it may be inferred that cryptic

transcription may also arise independently, because the experiment described is measuring.

Discussion

11. "During the course of our study, the Owen-Hughes lab reported that the chromatin remodelers Isw1 and Chd1 are responsible for the regular positioning of nucleosomes in coding regions in S.cerevisiae (Gkikopoulos et al, 2011)."

Because Quan and Hartzog 2010 had previously shown chd1 and isw1's relationship to cryptic transcription in yeast, the introduction should cover differences in the pombe system versus cerevisiae with regards to Iswi/Chd relationship)as noted above). See also Cheung 2008 (noting cryptic transcript activation for a cryptic transcription reporter for both chd1Δ and isw1Δ in S. cerevisiae, also noted above). Note that the link between chd1 and acetylation observed in Quan and Hartzog in S. cerevisiae is different than what is observed in pombe.

12. "Chd1 was also found to interact with transcription elongation complexes such as the Paf- and FACT-complexes (Simic et al, 2003)."

Quan and Hartzog (2010) also discuss the relationship between Paf-complex and Chd1. Also Warner et al (2007, MCB, citation below).

13. "The sequence requirements for a minimal promoter are not very specific, and such cryptic promoter sequences are present within many gene coding regions. These sequences are shielded by regularly placed nucleosome arrays, which repress their transcription initiation activity."

Are there relevant references that might be cited for these statements?

Methods

14. "Membranes were probed with either anti-H3- or anti-H3K9/K14ac-antibody and anti-actin antibody (dilution 1:2.000) following the manufacturer instruction. Actin membranes were additionally stained with Ponceau S to detect WCE loading."

The writing here makes it sound as if actin was probed separately on a different membrane. While this does not account for loading differences the presented quantitation perhaps is enough to rule out any systematic effects. However, if actin was probed on a blot from a separate gel it should be noted and not referred to as a loading control, but as an expression control. The authors are more diligent than most about their western blot quantitation and they are to be commended, but this point should be made clear if the wording is either in error or implies what it seems to imply.

15. "De-cross-linking and proteinase K (Ambion, AM2546) treatment were performed overnight at 65{degree sign}C. After a phenol-chloroform extraction and ethanol precipitation mono-nucleosomes were separated using a 1.7% agarose gel. Mono-nucleosomes were purified via NucleoSpin kit using NTC buffer (Macherey Nagel, 740609.250)."

At this point, mono-nucleosomes are not being purified, DNAs of a certain size range (should be noted) that are protected from MNAse digestion are (presumptive mono-nucleosomal DNA) are what is being purified. Same for next sentence as well. In the next sentence, it is stated "the pooled mono-nucleosomes were labeled"- I presume the pooling is the mononucleosomal DNA from multiple lanes (representing different concentrations of MNAse) were pooled? This should be made clear.

16. An additional point, the nature of the analysis was to focus on transcribed genes. Were there any non-repetitive heterochromatic regions present on the arrays that could be examined for hrp1/3 effects on nucleosome arrays of non-standard transcribed chromatin? Interestingly, much heterochromatin in pombe is transcribed but transcripts do not have traditional fates of coding genes, hence they appear to be silenced. It might be an interesting point to examine whether their cotranscriptional chromatin control were also distinct (similarly hrp1/3 dependent or not or on the other factors examined), and this might be simple to address with the data in hand.

Uncited references to be considered

Cheung V., Chua G., Batada N. N., Landry C. R., Michnick S. W., Hughes T. R., Winston F., 2008 Chromatin- and transcription-related factors repress transcription from within coding regions throughout the Saccharomyces cerevisiae genome. PLoS Biol 6: e277.

Lee J.-S., Garrett A. S., Yen K., Takahashi Y.-H., Hu D., Jackson J., Seidel C., Pugh B. F., Shilatifard A., 2012 Codependency of H2B monoubiquitination and nucleosome reassembly on Chd1. Gene Dev 26: 914-919.

Quan T. K., Hartzog G. A., 2010 Histone H3K4 and K36 methylation, Chd1 and Rpd3S oppose the functions of Saccharomyces cerevisiae Spt4-Spt5 in transcription. Genetics 184: 321-334.

Radman-Livaja M., Quan T. K., Valenzuela L., Armstrong J. A., van Welsem T., Kim T., Lee L. J., Buratowski S., van Leeuwen F., Rando O. J., Hartzog G. A., 2012 A key role for chd1 in histone h3 dynamics at the 3' ends of long genes in yeast. PLoS Genet 8: e1002811.

Warner M. H., Roinick K. L., Arndt K. M., 2007 Rtf1 is a multifunctional component of the Paf1 complex that regulates gene expression by directing cotranscriptional histone modification. Mol Cell Biol 27: 6103-6115.

Revision - authors' response 12 September 2012

Referee #1

Hennig et al. present a very nice paper exploring the consequences of loss of the Chd1 remodelers Hrp1 and Hrp3 in S. pombe. In particular, they demonstrate that simultaneous loss of the these remodelers leads to:

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2) that this increase in cryptic transcription shows striking similarity to that observed for alp13 and set2 mutations;

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Thus, the real value in this work seems to rest on the facts that: 1) important work on Chd1 has been extended from S. cerevisiae to S. pombe; 2) the observations of pervasive anti-sense cryptic transcription in the hrp1/3 double mutant, and 3) the observation that chromatin disruption (i.e. loss of nucleosome positioning or loss of significant loss of nucleosomes) is not required for cryptic transcription. I think that each of these observations potentially represents an important contributions to the field.

Specific comments:

1. The methods section is a bit thin on details. How many replicates were performed for the genome-wide analyses? I think that it is important that the key observations are based upon more than a single experiment. Also, how were the protein extracts used in the western blots prepared? Is it possible that apparent changes in histone content observed in the pob3 mutant reflect a change in chromatin solubility in the extract rather than a change in histone levels?

We corrected our methods section and included the information asked by the referee.
- Expression profiling experiments were repeated at least two times (biological replicates), some of the critical experiments more than two times. For example expression analysis of the *hrp1Δhrp3Δ* strain was repeated 4 times. ChIP-chip experiments were repeated two times. Nucleosome mapping experiments were carried out two times with the exception of the mutants which did not show any change compared to WT (*alp13Δ*, *set2Δ*, *mit1Δ*) and the *hrp1Δ* and *hrp3Δ* single deletion strains. We verified the deletions by checking the hybridization signals of the genomic DNA at the corresponding gene loci.

- Our protein extracts were done by TCA precipitation. We included this information into the Supplementary Material and Methods section. Some of the total cell extract preparation protocols use Laemmlie buffer or similar to extract proteins and get rid of the pellet fraction. These protocols risk losing proteins due to solubility issues as the referee pointed it out. In our TCA precipitation protocol we break the cells in the presence of TCA. TCA precipitates all proteins and we use the pellet fraction for further analysis. There is no additional centrifugation step in this protocol, which excludes the possibility of losing insoluble proteins. We can fully resolve the TCA pellet in Laemmlie-buffer.

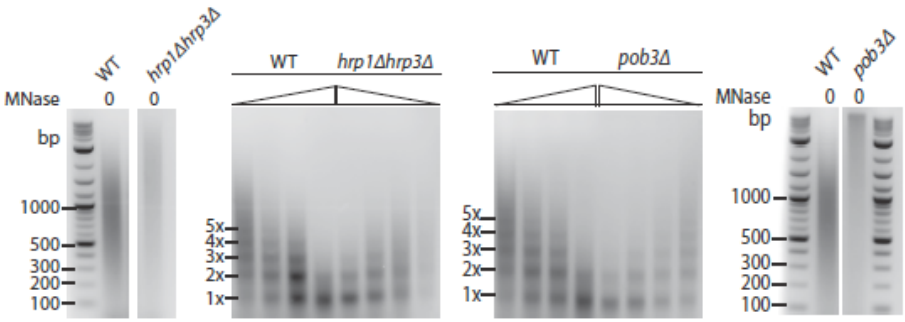
- Although we are confident to show the decreased nucleosome occupancy in the *pob3Δ* strain, we mainly used this mutant as a control, and concluded that *hrp1Δhrp3Δ* strain did not show comparable nucleosome loss. For example Celona et al. (PLoS Biol., 2011) did more extensive study with *nhp6Δ* in *S.cerevisiae,* normalized protein extracts to DNA content and demonstrated decreased H3 levels, comparable with our result.

2. Did the authors examine the apparent differences in histone (total or acetylated) content in Figure 2 for statistical significance?

Yes, we included this information in Figure 2. *pob3Δ* strain showed statistically significant reduction in its H3 content compared to WT (Standard T test). Other changes, including the slight decrease observed in *hrp1Δhrp3Δ* were not significant. In the H3K9/K14ac western-blot the only statistically significant change compared to WT is the increased H3K9/K14ac levels in the *alp13Δ* strain.

3. In addition to the work presented here and previously by Owen-Hughes and colleagues, a prior study used micrococcal nuclease digestion to show that nucleosome spacing is unaltered in chd1 mutants in S. cerevisiae. (Xella et al., Mol. Microbiol. 2006) It think that citation of this work would bolster the author's arguments. Furthermore, given the author's arguments here and the prior observations of Celona et al. (PLoS Biol., 2011), I would expect that careful analysis of MNase digests of hrp1 hrp3 chromatin would show a modest increase in sensitivity to MNase, but no change in apparent spacing. This simple independent test of the author's model could significantly strengthen the manuscript.

- Xella et al. showed that nucleosome spacing is altered in the *isw1Δ isw2Δ chd1Δ* triple deletion strain. Although it is a relevant study, due to space considerations we were unable to include all of the suggested references.
- We carried out the suggested experiment and the results are shown below. Although we find the suggestion useful, we decided not to include this experiment into the manuscript. The sensitivity and reproducibility of the MNase experiments is quite low. MNase sensitivity is also influenced by the zymolase sensitivity of the cell wall of the strains. Residual DNase activity also has a variable influence on the observed results. Furthermore, minimal variation in the handling of the samples can cause larger differences between the samples. We carried out this experiment 3 times, and we could detect a slightly enhanced MNase sensitivity in the *pob3Δ* strain (as it was shown in Celona et al.). Although *pob3Δ* is one of the strongest nucleosome depletion mutants, the observed differences in the MNase sensitivity is quite small. We can't see reproducible difference in MNase sensitivity of the *hrp1Δhrp3Δ* strain compared to WT. This supports our model which would predict a very small change in nucleosome occupancy in this mutant. We felt that the results of this experiment did not allow clear conclusions or contribute anything further to what we have already presented in the manuscript.



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Plasmids are relatively unstable in *S.pombe*, because *S.pombe* centromere is too big to incorporate into plasmids. Centromeric plasmids in *S.cerevisiae* stably propagate with the cells, while *S.pombe* cells quickly lose their plasmids. Because of this reason, plasmid complementation is not frequently used in the *S.pombe* system.

Several studies showed that targeting of the Rpd3S complex is independent of H3K36 methylation; Govind et al.(2010), Drouin et al. (2010).

The effect of the *alp13* deletion on histone H3 acetylation is not so surprising, since very similar effects were reported in independent studies; for example bulk H3K14ac level is increased 8 times and bulk H3K9ac level is increased more than two times in the *alp13Δ* strain in Nakayama et al. (2003). In contrast, H4 acetylation showed only a moderate increase in the *alp13Δ* strain. The surprising finding of our study is that the *set2Δ* strain does not show comparable increases in H3K9/K14 acetylation levels. We were able confirm the deletion of *set2* gene in our mutant strain by visualizing the hybridization signals at the *set2* locus.

Minor comments:

1. The manuscript requires some light copy-editing for grammatical issues. The authors often inappropriately use or omit the definite and indefinite articles, the and a.

We tried our best in the revised version to avoid these mistakes.

2. On page 6, in the results section, a citation should be provided for the statement "Both mutants are known to accumulate cryptic transcripts."

Done, (Nicolas et al, 2007) added.

3. In the discussion of the interaction of Chd1 with the Paf and FACT complexes, page 15, the authors should cite Krogan et al., MCB 10(6):6979-92, 2002 and Kelly, Stokes and Perry, Chromosoma 108:10-25,1999, in addition to the Simic paper.

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-We included this into the Introduction and cited Govind et al.(2010).

The manuscript is quite careful to point out the dangers in calculating overall occupancy for histone acetylation or nucleosomes from genome wide datasets. However, an estimation of the absolute reduction in number of nucleosomes is still made using this approach in the results section describing the hrp1, hrp3 deletion (8%). This should presumably be qualified with the arguments made elsewhere in the manuscript.

The referee is right and we decided to leave out the following speculation:

"The above-mentioned conclusion would explain the 10% decrease in histone H3 levels detected in the *hrp1Δhrp3Δ* strain. Although this change is not significant, it fits well with the results of the nucleosome mapping experiments. We found a moderate (8%) decrease in the number of nucleosomes detected in these cells, together with a 10bp increase in the median distance between nucleosomes."

The problematic part is to use the 8% decrease in the number of nucleosomes detected in the cells as an argument for nucleosome occupancy decrease. We agree that it is not correct to use in this context.

The following sentences are included in the revised manuscript:

"We also detected a 10bp increase in the median distance between nucleosomes. When nucleosome spacing is irregular, nucleosomes generally occupy a slightly longer DNA segment. A 10 bp increase in the average nucleosome distance would lead to the loss of roughly 1 nucleosome per gene for genes of average length. Extrapolating these numbers to the *S.pombe* genome would yield an approximately 6-8% loss in nucleosomes, which is compatible with our bulk H3 quantification results in this mutant."

In the revised version we also speak about 6-8% nucleosome loss, which might look similar to the previous version, but in this case the approximate calculation is based on the 10 bp increase in median nucleosome distance.

Referee #3:

Hennig et al present a manuscript titled "Chd1 chromatin remodelers maintain nucleosome organization and repress cryptic transcription" examining the function of the conserved Chd1-class of remodeler in S. pombe in organizing transcribed chromatin and preventing widespread antisense transcription from cryptic promoters. Chd1 family members are more well studied in budding yeast where there is a single Chd1 homolog. This homolog has been implicated in transcribed chromatin and cryptic transcription, but has also been shown to collaborate with Isw1 in regulating chromatin and cryptic transcription. S. pombe lack ISWI family members so the function of Chd1 members and how these same types of activities are accomplished are not clear for S. pombe. Work on S. cerevisiae Chd1 has been relatively intensive very recently, but the results here are novel enough and extend analyses genome wide to a new system that has many important differences in chromatin control and transcription that make S. pombe a valuable model for higher eukaryotes that are distinct from S. cerevisiae. The experiments are clearly described and seem to be performed appropriately. The manuscript suffers much from lack of appropriate context for the reader in terms of recent and less recent Chd1 experiments in the S. cerevisiae system. These points should be addressed to facilitate readers' understanding of the system and the relative significance of the work. Points to consider are presented below in a numbered list. Some are minor questions of language, but many relate to appropriate citation and discussion of the literature. I cannot recommend where to cut sections of the paper to make room for essential context, but minimally a total of 2-3 paragraphs should be added to cover a range of uncited publications that relate highly to the current manuscript.

Otherwise, with these caveats the work is a nice contribution to the literature.

Introduction

1. Page 4: "Since the passage of Pol II along the DNA during transcription elongation requires

opening up the chromatin and disassembling the nucleosomes,"
Disassembly may correlate with transcription, but it is not necessarily clear if complete nucleosome disassembly is an absolute requirement for transcription. This is a subtle point but the sentence could be more clear.

This section has been revised.

2. Page 4: "Failure in this process leads to enhanced activity of cryptic promoters, and consequently to toxic accumulation of the resulting transcripts (Mason & Struhl, 2003; Kaplan et al, 2003; Jamai et al, 2009)."
It is not clear if any experiments have been performed to examine whether cryptic transcripts are toxic. This sentence should be rewritten. For example, the work here shows massive additional transcription with little or no effect on growth.

This section has been removed in the revised version. However, several studies show that mutants with high levels of cryptic transcript accumulation show genomic instability, e.g., Nicolas et al., 2007.

3. Page 4: "The Schizosaccharomyces pombe (S.pombe) homologue of this HDAC complex is the Clr6 complex-II, or Rpd3S complex in Saccharomyces cerevisiae (S.cerevisiae) (Carrozza et al, 2005; Nakayama et al, 2003)."
This sentence is awkwardly written.

Yes. We re-worded this sentence: The evolutionarily conserved Rpd3S complex in *Saccharomyces cerevisiae* (*S.cerevisiae*) is responsible for histone deacetylation within gene coding regions{Keogh 2005}{Carrozza 2005}. Clr6 complex-II is the *Schizosaccharomyces pombe* (*S.pombe*) homolog of this complex{Nakayama 2003}.

4. Page 5: "Mutations in the FACT complex lead to decreased histone occupancy levels in transcribed genes and increased activity of cryptic promoters in these regions (Belotserkovskaya et al, 2003; Jamai et al, 2009; Mason & Struhl, 2003)."

Kaplan 2003 were the first to show spt16 mutant effects on cryptic transcription. Should be cited here and elsewhere for FACT roles in cryptic transcription
.
Cheung et al 2008 (PLOS Biology, citation below) also did an extensive analysis of factors implicated in cryptic transcription, and identified chd1 and isw1 has having this phenotype. This work should also be cited and discussed where appropriate.

Thanks to the referee for pointing out these highly relevant studies which we mistakenly omitted from our manuscript. We have now included these studies in the revised version.

5. Page 5: "In this study we attempted to identify other factors which might have a role in repressing cryptic transcription activity in S.pombe. We screened a deletion library for chromatin related factors and tested their effect on cryptic transcription. We found that deletion of the S.pombe Chd1-type chromatin remodelers, hrp1 and hrp3, results in a dramatic increase in cryptic transcription. To determine the underlying molecular mechanism, we mapped genome-wide nucleosome-position and histone acetylation patterns in Chd1-deficient strain."

Much very recent work and also not so recent work should be cited on this topic. The novelty of the results presented here are that they relate to the S. pombe system and anti-sense transcription is extensively monitored. In many previous analyses of S. cerevisiae, intragenic cryptic transcription of the sense strand has been monitored, many times for a small number of reporter loci and rarely if at all for antisense transcription. Because S. pombe contains the machinery for dsRNA-mediated silencing, anti-sense transcription has the possibility of being much more detrimental to gene expression and chromatin structure. However, there is no context given for what is actually known about Chd1 function in any system and this must be rectified in the introduction, and not the discussion. There are uncited papers that are less recent than Gkikopoulos et al (which is cited, but left to discussion) and they include Cheung et al 2008 mentioned above, Lee et al (2012, citation below) on Chd1 and transcribed chromatin, Radman-Livaja (2012) on Chd1 and H3 dynamics and transcribed chromatin, Quan and Hartzog (2010, citation below) showing synthetic effects between chd1 and isw1 on cryptic transcription in S. cerevisiae. These are major points of context that are missing from the manuscript.

We have included a brief section about Chd1 in the introduction, as suggested by the referee. Although all of these references are relevant, due to space considerations we were unable to include all of them. We have tried to include the earliest and most relevant studies. We also regret that we can not acknowledge all of the excellent studies on this topic.

Results

6. Page 6: "PCR reactions to quantify AS transcript levels. As positive controls, we used S.pombe strains lacking the histone methyl-transferase Set2 and the HDAC Clr6-complex II subunit Alp13. Both mutants are known to accumulate cryptic transcripts."

Citation missing. Presumably Nicolas 2007.

Yes, citation is inserted.

7. Page 6: "Interestingly, the changes observed in the sense transcripts were rather minor, with only 6% of the genes demonstrating significantly up- or down-regulated transcript levels."

The major cryptic transcripts that are generally studied in S. cerevisiae derived from intragenic cryptic promoters that are likely more obvious to study by northern blotting because they have defined 3' ends derived from the normal transcription unit's poly-A signals. Is there any evidence for altered 5'-3' ratios of sense mRNA that these transcripts might also be present for hrp3/hrp1 mutants. They might not be observed if if the total effect on average transcription for a gene was less than 2, but please see Figure S1 from Cheung et al, 2008. This seems like a missed opportunity to fully understand the magnitude of cryptic transcription hrp mutants.

The suggestion is valid, and indeed our analysis underestimates the magnitude of cryptic transcription in the mutant strains. However, sense transcription changes are moderate, and they are mostly below our threshold of minimum 2 times change compared to WT. Although further analysis of the data is possible with the suggested method, we decided to use a more conservative approach to estimate the changes in cryptic transcription.

8. Page 7: "Previous studies showed that genome-wide nucleosome depletion can result in activation of cryptic transcription in yeast (Keogh, 2012). Mutations in the FACT complex (Mason & Struhl, 2003; Jamai et al, 2009), Spt6 (Kaplan et al, 2003) or the histone chaperones Asf1/HIRA (Yamane et al, 2011; Keogh, 2012)"

As noted above, Cheung et al 2008 should probably be cited here and Kaplan 2003 should be cited for FACT. Again, results from 2008, 2010 (Quan) and 2011 (Owne-Hughes lab) need to be put into context here.

These studies have been included.

9. Page 13, top of page "exhibit" should be "exhibits"

The revised version of the manuscript has been checked for grammatical mistakes to the best of our ability.

10. "These results demonstrate that Chd1-remodelers repress cryptic transcription independently of Set2 and the Clr6-complex II."

It might be more precise to state that these results suggest that Chd1 regulates nucleosome positioning independently of Set2 and the Clr6-complex II, and it may be inferred that cryptic transcription may also arise independently, because the experiment described is measuring.

This suggestion has been implemented:

Discussion

11. "During the course of our study, the Owen-Hughes lab reported that the chromatin remodelers Isw1 and Chd1 are responsible for the regular positioning of nucleosomes in coding regions in S.cerevisiae (Gkikopoulos et al, 2011)."

Because Quan and Hartzog 2010 had previously shown chd1 and isw1's relationship to cryptic transcription in yeast, the introduction should cover differences in the pombe system versus cerevisiae with regards to Iswi/Chd relationship)as noted above). See also Cheung 2008 (noting cryptic transcript activation for a cryptic transcription reporter for both chd1Δ and isw1Δ in S. cerevisiae, also noted above). Note that the link between chd1 and acetylation observed in Quan and Hartzog in S. cerevisiae is different than what is observed in pombe.

The differences between *S.pombe* and *S.cerevisiae* are highlighted in the introduction.

12. "Chd1 was also found to interact with transcription elongation complexes such as the Paf- and FACT-complexes (Simic et al, 2003)."

Quan and Hartzog (2010) also discuss the relationship between Paf-complex and Chd1. Also Warner et al (2007, MCB, citation below).

We included Quan and Hartzog (2010) and other new references in this section.

13. "The sequence requirements for a minimal promoter are not very specific, and such cryptic promoter sequences are present within many gene coding regions. These sequences are shielded by regularly placed nucleosome arrays, which repress their transcription initiation activity."

Are there relevant references that might be cited for these statements?

There is only indirect evidence for this model, however this section has been removed from the revised version.

Methods

14. "Membranes were probed with either anti-H3- or anti-H3K9/K14ac-antibody and anti-actin antibody (dilution 1:2.000) following the manufacturer instruction. Actin membranes were additionally stained with Ponceau S to detect WCE loading."

The writing here makes it sound as if actin was probed separately on a different membrane. While this does not account for loading differences the presented quantitation perhaps is enough to rule out any systematic effects. However, if actin was probed on a blot from a separate gel it should be noted and not referred to as a loading control, but as an expression control. The authors are more diligent than most about their western blot quantitation and they are to be commended, but this point should be made clear if the wording is either in error or implies what it seems to imply.

We used actin as a loading control for the western blot experiments, but we noticed that to avoid saturation in the actin signal, we had to use higher sample dilutions for the quantitations. Technically, the referee is right, and we have modified the text and we no longer refer actin as a loading control.

15. "De-cross-linking and proteinase K (Ambion, AM2546) treatment were performed overnight at 65{degree sign}C. After a phenol-chloroform extraction and ethanol precipitation mononucleosomes were separated using a 1.7% agarose gel. Mono-nucleosomes were purified via NucleoSpin kit using NTC buffer (Macherey Nagel, 740609.250)."

At this point, mono-nucleosomes are not being purified, DNAs of a certain size range (should be noted) that are protected from MNAse digestion are (presumptive mono-nucleosomal DNA) are what is being purified. Same for next sentence as well. In the next sentence, it is stated "the pooled mono-nucleosomes were labeled"- I presume the pooling is the mononucleosomal DNA from multiple lanes (representing different concentrations of MNAse) were pooled? This should be made clear.

Yes, the referee is correct and we changed the wording.

16. An additional point, the nature of the analysis was to focus on transcribed genes. Were there any non-repetitive heterochromatic regions present on the arrays that could be examined for hrp1/3 effects on nucleosome arrays of non-standard transcribed chromatin? Interestingly, much heterochromatin in pombe is transcribed but transcripts do not have traditional fates of coding genes, hence they appear to be silenced. It might be an interesting point to examine whether their cotranscriptional chromatin control were also distinct (similarly hrp1/3 dependent or not or on the other factors examined), and this might be simple to address with the data in hand.

We did not analyze heterochromatic regions, because our nucleosome mapping data is not reliable at the repetitive probes.

Uncited references to be considered

Cheung V., Chua G., Batada N. N., Landry C. R., Michnick S. W., Hughes T. R., Winston F., 2008 Chromatin- and transcription-related factors repress transcription from within coding regions throughout the Saccharomyces cerevisiae genome. PLoS Biol 6: e277.

Lee J.-S., Garrett A. S., Yen K., Takahashi Y.-H., Hu D., Jackson J., Seidel C., Pugh B. F., Shilatifard A., 2012 Codependency of H2B monoubiquitination and nucleosome reassembly on Chd1. Gene Dev 26: 914-919.

Quan T. K., Hartzog G. A., 2010 Histone H3K4 and K36 methylation, Chd1 and Rpd3S oppose the

functions of Saccharomyces cerevisiae Spt4-Spt5 in transcription. Genetics 184: 321-334.

Radman-Livaja M., Quan T. K., Valenzuela L., Armstrong J. A., van Welsem T., Kim T., Lee L. J., Buratowski S., van Leeuwen F., Rando O. J., Hartzog G. A., 2012 A key role for chd1 in histone h3 dynamics at the 3' ends of long genes in yeast. PLoS Genet 8: e1002811.

Warner M. H., Roinick K. L., Arndt K. M., 2007 Rtf1 is a multifunctional component of the Paf1 complex that regulates gene expression by directing cotranscriptional histone modification. Mol Cell Biol 27: 6103-6115.

2nd Editorial Decision 12 September 2012

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Editor
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