Yeast X-Chromosome Associated Protein 5 (Xap5) Functions with H2A.Z to Suppress Aberrant Transcripts

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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.)

Editor: Esther Schnapp
1st Editorial Decision 15 July 2013

Thank you for the submission of your research manuscript to our journal. We have now received the enclosed reports on it.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also point out some technical concerns and indicate that a little more insight into how Xap5 inhibits antisense transcription would strengthen the paper. All three referees (after cross commenting on each others' reports) agree that it needs to be explained how the genes were chosen for the EMAP experiments, and that the genes with faulty termination need to be determined in the xap5 mutant. Referee 3 further agrees with referee 1 that ncRNA production should be assessed directly in xap5 and HDAC mutant strains and that Xap5 localization in pht1 mutants and vice versa should be investigated.

While referee 3 thinks that genome wide nucleosome mapping in xap5 mutants would go beyond the scope and length limits of EMBO reports, s/he suggests that nucleosome mapping in xap5 mutants could be investigated for selected genes. Both referees 2 and 3 remark that the data on Xap5 and Set1 antagonism should be kept and referee 3 further feels that deletion of Swr1 would not reveal substantial novel insight; this experiment is therefore not required.
Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and all the technical concerns) must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Can you please also specify the number "n" for how many experiments were performed and the error bars in the respective figure legends? This information is currently missing or incomplete.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have any further questions or comments regarding the revision.

REFEREE REPORTS:
Referee #1:

Review: "Yeast X-chromosome Associated Protein 5 (Xap5) Functions with H2A.Z to Suppress Aberrant Transcripts"

In this article Anver et al. investigate the function of Xap5. Xap5 is highly conserved across eukaryotes, yet little is known about its function in the cell. Based on a series of a genetic screen and a series of genome-wide gene expression analyses, the authors conclude that Xap5 likely functions in conjunction with H2A.Z to suppress the production of non-coding RNAs in wildtype fission yeast.

The experiments presented in this study were well executed and meticulously analyzed with regards to statistics. While this study has produced some interesting results, I feel that additional (biochemical) experiments are necessary in order to add weight to the paper's conclusions.

Major points:
The authors mention that Xap5 and Set1 antagonize each other. The only data presented for this include a correlation plot of the genetic interactions observed between set1 and xap5 (Fig. 1E) as well as a single growth experiment shown in Fig. S3E. Since nothing further is shown or discussed I think this part of the paper can be omitted completely (and may be more worthwhile being studied separately). Instead, it would make more sense to include similar data for Swr1 and Xap5. Since Swr1 is responsible for the incorporation of H2A.Z into nucleosomes, does deletion of the Swr1 complex lead to similar phenotypes as pht1 when compared to or combined with xap5?

Deletion of XAP5 leads to the production of ncRNA. Aberrant transcription is generally associated with changed nucleosome occupancy. Hence, it would be interesting to determine what happens to histone H3 and H2A.Z occupancy in a xap5 mutant compared to the wildtype. Is H2A.Z incorporation reduced in xap5 cells?

Does Xap5 itself associate with H2A.Z containing nucleosomes? A simple immunoprecipitation experiment should provide an answer. Alternatively, what happens to Xap5 localization in a pht1 mutant?

The authors compare the gene expression profiles of xap5 cells with certain histone deacetylase mutants. In my opinion this analysis by itself is completely insufficient. What happens to histone acetylation in xap5 cells? This can be checked on a global scale by Western blotting or locally by ChIP. In budding yeast both the Rpd3S and Set3C histone deacetylases have been implicated in the suppression of aberrant transcription. Did the authors also look at these HDACs? Rather than just looking at gene expression profiles between the HDAC and xap5 mutant strains, the authors should investigate ncRNA production directly, either by RT-PCR or Northern blotting.

I realize that the "choppy" appearance of the RNA-seq data (eg. Fig. S4) is probably due in part to using an rRNA removal kit. Has the data been processed by including a sliding window? If not, how have the authors determined which genes exhibit faulty termination in the mutant backgrounds since
it is unclear from the example genes shown whether a single or multiple transcripts are produced from individual genes?!

The RT-PCR experiment shown in Fig. S4G does not include a control amplicon, eg. ACT1.

Minor points:
The authors should mention in the introduction (not just in the conclusion) that nothing is known about Xap5 protein structure and function.

The authors performed an EMAP experiment with ca. half of the S.p. genome. Where the genes chosen at random or were they selected in some fashion?

When discussing misexpression of genes in various mutant backgrounds (p.5, paragraph 2), it would be helpful to mention the fold change in gene expression also in the manuscript text, not just the figure legend.

Referee #2:

Anver et al report a concise and interesting finding on the role of Xap5 proteins. Xap5 was not essential in S. pombe, but the xap5 deletion mutant had growth defects at colder and warmer temperatures. Strikingly, the Arabidopsis homolog could rescue this and other defects arguing for an extremely highly conserved function. Although the mechanistic details are not clear, the authors found functional interactions via E-MAP analysis and transcriptome correlations with chromatin associated factors, like the histone methyltransferases Set1/COMPASS or Clr4, the histone deacetylases Clr6 or Clr3, and especially the histone variant H2A.Z. Xap5 appears to suppress aberrant, especially antisense transcription, similar to and synergistically with the previously published role of H2A.Z. This role is likely direct as chromatin binding of Xap5 to loci with altered transcription in the xap5 mutant was demonstrated by ChIP-chip.

Overall I am in favour of this study. It offers a rather focussed but nonetheless significant insight, which will likely be the starting point of a more mechanistic analysis.

Nonetheless, I had some issues with data generation and analysis that have to be addressed.

Accurate detection of antisense transcripts is essential for this study. The Steinmetz group (PMID: 17897965) found false positive antisense signals due to artifactual second strand synthesis by reverse transcriptase. This is prevented by addition of actinomycin D. The protocol by Wang et al. (Suppl. reference no. 8) used by Anver et al. does not include actinomycin D. The authors should generate at least one data set plus and minus actinomycin D in order to check their false positive detection rate.

The authors should state the criteria for selecting the 2117 mutants for the E-MAP. In the present version this is unexplained and seems oddly arbitrary.

The authors overstate some of the effects. The S-score profiles in Fig. S2C do not look very similar. Is there no better way to quantify „likeliness“ of such profiles than eye balling? The correlations shown in Fig. 1D and E are not very impressive, too. Fig. 1F gives a clear correlation, but I would not call this „highly“ or „strongly“ correlated. In general, there is a deplorable tendency in the field to go after statistical significance rather than biological relevance. Comparisons of genome-wide data sets almost by default generate all kinds of significant effects, i.e. very low p-values, just due to the large numbers compared. However, the effects are often subtle and the biological relevance remains questionable. See for example the last sentence on p. 6. The high statistical significance is not a strong argument to make the weak correlation more convincing or relevant. (In addition, why gets the correlation worse for the xap5 phl1 double mutant?)

Even though I tend to believe the overall conclusions of this study, I recommend to report the actual results in a more balanced way with a focus on the magnitude of the effects rather than their statistical significance.
Given the antagonistic genetic interaction of xap5 with set1 (Fig. S3E), the authors should do the same kind of transcriptome correlations using published data as they did with clr6 etc mutants.

It seems that the labeling for „up“ and „down“ in Fig. 2D is erroneously reversed.

Issues with Venn diagrams: The overlap between misregulated genes in xap5 vs pht1 mutants in Fig. 2E-H is actually very small and even zero in Fig. S4 C-F, in contrast to the authors’ claim „many loci upregulated in...“ (p. 5, 16/15 from bottom). With view of the bar diagram in Fig. 3A I do not understand how the overlap between xap5 and pht1 mutants can be zero in Fig. 3B. I would like to see Venn diagrams for the overlap between transcript misregulation and ChIP enrichment, e.g. as additional panel for Fig. 4.

It should be stated more clearly that deltapht1 did not lead to upregulation of wtfs although it did bind there (Fig. 4A).

Referee #3:

Anver et al, EMBO Reports, July 2013

Anver et al. present a manuscript investigating the functions of the XAP5 gene of S. pombe. Previous work had shown that XAP5 is widely conserved across eukaryotes, that it maps near a locus of potential disease genes in humans and that it affects circadian rhythms and responses to external stimuli in Arabadopsis. However, the molecular function of XAP5 remained entirely unknown.

In this work, the authors remedy this gap in our knowledge by using the E-MAP genetic profiling technology to identify a potential function for the XAP5 protein family. In particular, the authors present E-MAP data suggesting a role for XAP5 in chromatin regulation and then use a combination of molecular techniques to show that loss of XAP5 function mirrors that of loss of histone H2A.Z and in particular, leads to transcription activation of sequences that are normally repressed (e.g. anti-sense and intergenic sequences, retroposons and other repeated elements). In addition, the authors demonstrate that the widely diverged XAP5 homolog from Arabadopsis, XCT, can complement a xap5 mutation in S. pombe, demonstrating conservation of function within this protein family.

In general, this paper is clearly written, brief and informative. In addition to providing new information on XAP5, a strength of the paper is that it provides a particularly clear example of a case in which results from E-MAP data alone were sufficient to determine the direction of a scientific investigation (rather than merely supporting or confirming a previously held point of view). Thus, the paper should be of general interest to the transcription community, and investigators interested in developing or extending genetic and genomic techniques.

Comments:

1. The description of the Xap5 ChIP data should be clarified. In particular, on page 6, the authors state that "...Xap5 protein is associated with chromatin throughout the genome, showing enrichment in both genic and intergenic regions..." If the protein is associated with the entire genome, how is it enriched with particular loci? How do the authors know that this does not simply reflect non-specific association with chromatin?

2. Is figure 2D correctly? The manuscript says that anti-sense transcripts are prominently up-regulated, not down-regulated as figure 2D seems to suggest.

3. Later, in the same paragraph, the authors talk about significant enrichment of Xap5 and H2A.Z in LTRs and wtfs. Significant relative to what? Enriched relative to what? Are these proteins associated with loci far from LTRs, wtfs or protein-encoding genes? I think it would be more accurate to just state that the ChIP data shows that these proteins are associated with the LTRs and wtfs.
4. In figure 2I, I assume that only the counts for the anti-sense strand are presented. Is this correct?

5. The description of the data presented in Figure 2K is opaque and should be clarified so that it is more obvious what data and data analysis is being presented in this figure panel.

6. The changes in gene expression as measured by RNA-seq that are presented in figure 4 are not immediately apparent. The paper would be more compelling if a quantitative measure of gene expression changes for the genes shown in the figure could also be presented.

7. I would have appreciated one or two sentences explaining or exploring the relationship between the changes in gene expression reported here and the phenomenon of cryptic transcription observed in S. cerevisiae.

8. The method for calculating doubling times should be described in the supplementary methods.

Additional correspondence (author) 02 December 2013

We have completed most of the experiments requested by reviewers (please see the attached response to editorial comments). Most notably, we have carried out a genome-wide ChIP-chip experiment with epitope tagged Xap5 in a wild type and delta-pht1/h2a.z background. We found that although genome-wide levels of Xap5 deposition are not significantly altered in yeast mutant for H2A.Z/Pht1, Xap5 abundance at repeated loci (LTRs, Tf2 transposons, and wtf repeats) is significantly enriched in the delta-pht1 mutant compared to wild type. These loci are enriched for both H2A.Z and Xap5 deposition in wild-type yeast (relative to genome-wide levels of these two proteins), are overexpressed in both single mutants, and are synergistically overexpressed in the delta-xap5/delta-pht1 double mutant. These new ChIP-chip data therefore reinforce our conclusion that Xap5 and H2A.Z play similar, partially redundant roles in the suppression of inappropriate gene expression.

We are planning on carrying out similar experiments to compare Pht1/H2A.Z deposition in wild-type and delta-xap5 yeast. However, unfortunately, we have run out of chips and will have to wait until more are manufactured, which could take some time. If we do these additional ChIP-chip experiments, we almost certainly will not be able to make the 6 month deadline that you mention below.

Under these circumstances, what would you recommend we do? I would hate to have the manuscript considered a new submission after all the work we have put in to address reviewers' comments.

Additional correspondence (editor) 03 December 2013

It seems a good idea to include the converse ChIP-chip experiment as well, as two referees agree that this should be done. I hope that all the data will fit into our short format. If you are unable to submit the revised version in December (or latest early January), you need to submit a new manuscript, that will, however be treated as a revised version (we will ask the same referees to review it as the revised version), except that the novelty of the findings will be re-assessed at the time of submission. So far, no similar paper has been published on the topic. If you are worried about re-assessing novelty, I suggest that you submit what you have, and we will see what the referees think about the study. But please keep in mind that EMBO reports allows a single round of revision only, and that extensive new experimental data, as the ChIP-chip data, cannot be added to the paper after this one round of revision.

Please let me know if you have further questions or comments.
We are pleased to re-submit the revised version of our manuscript “Yeast X-Chromosome Associated Protein 5 (Xap5) Functions with H2A.Z to Suppress Aberrant Transcripts” for possible publication in EMBO reports as a scientific report. We have performed a number of additional experiments and made changes to the manuscript to address most of the editorial and reviewer comments. In particular, we have now examined how loss of H2A.Z affects Xap5 association with chromatin and vice versa on a genome wide scale. Our new findings support our original conclusion that the novel protein Xap5 acts in a similar manner as H2A.Z to suppress expression of aberrant transcripts. Please see the attached “Response to the Editorial & Reviewer Comments” document for a point-by-point response to all editorial and reviewer comments.

In addition to the revised main manuscript, we also are submitting revised supplementary information. Three supplemental data files in .XLS format (Excel spreadsheets) with genetic interaction data (385 kb), expression data from the RNA-seq experiment (3.6 Mb, revised to include defective transcription termination analyses data in Δxap5, Δpht1 and Δxap5Δpht1 mutants) and ChIP-chip data (4.2 Mb, revised to include data from the Xap5 and H2A.Z/Pht1 ChIP-chip analyses of Δpht1 and Δxap5 mutants) have also been uploaded. The new ChIP-chip raw data files are available at the NCBI Gene Expression Omnibus (GEO) repository under the accession number GSE46506 which reviewers can access at via the following link:


Thank you very much for considering our manuscript; we very much hope that you now find it suitable for publication in EMBO reports.

Response to the Editor’s Comments

1. All three referees (after cross commenting on each others' reports) agree that it needs to be explained how the genes were chosen for the EMAP experiments.

We used all of the mutants in our library for our genetic analysis. A mutant library containing 2662 haploid deletions strains was originally constructed and supplied by the Bioneer Corporation and the Korea Research Institute of Biotechnology and Bioscience (http://pombe.bioneer.co.kr/). After quality control and removal of incorrect strains, the remaining 2117 mutants were crossed to the Δxap5 strain and growth of the double mutants was assessed. This is now described in the online methods section.

2. The genes with faulty termination need to be determined in the xap5 mutant.

We have more carefully analyzed our strand-specific RNA-seq data to identify genes with faulty termination of transcription. Of 4240 detectably expressed genes in our experiment, 178 have significantly higher levels of sequence reads in the 100 bp downstream of the transcriptional termination site in Δxap5 than in wild type. 113 genes have higher levels of transcript downstream of the transcriptional termination site in Δpht1 than in wild type, and 38 of these inappropriately terminated transcripts are shared between the two single mutants (significantly more overlap than expected by chance; p = 6.7 e-15, Fisher’s exact test). Please also see the manuscript and the online methods for a more thorough description of the analysis methods used.

3. Referee 3 further agrees with referee 1 that ncRNA production should be assessed directly in xap5 and HDAC mutant strains.

We carried out strand specific RT-PCR at selected ncRNA loci in strains mutant for xap5, pht1, clr3 (a histone deacetylase), set3, hos2/hda1 (two components of the Set3 / Rpd3L-expanded histone deacetylase complex), cph1 and clr6 (two components of the Rpd3S histone deacetylase complex) to determine whether ncRNAs are similarly upregulated in all of these mutants. We found that the assayed antisense and intergenic ncRNA loci were similarly upregulated in Δxap5, Δpht1, Δxap5Δpht1 double mutants and all of these HDAC mutants (new panel Fig. S5C). This further supports our conclusion that Xap5 cooperates with well-studied protein complexes to regulate chromatin.

4. Xap5 localization in pht1 mutants and vice versa should be investigated.
We constructed strains expressing epitope tagged Pht1 in a \( \Delta xap5 \) background and epitope tagged Xap5 in a \( \Delta pht1 \) background. We carried out chromatin IP experiments followed by a microarray analyses for these and comparable wild-type strains. We found that although genome-wide levels of Xap5 deposition are not significantly altered in yeast mutant for H2A.Z/Pht1, Xap5 abundance at repeated loci (Tf2 transposons and wtf repeats) is significantly enriched in the \( \Delta pht1 \) mutant compared to wild type (new panel Fig. 4D). These loci are enriched for both H2A.Z and Xap5 deposition in wild-type yeast (relative to genome-wide levels of these two proteins) (Fig. 4A), are overexpressed in both single mutants, and are synergistically overexpressed in the \( \Delta xap5/\Delta pht1 \) double mutant (Fig. 3). In contrast there were not significant changes in H2A.Z deposition in the \( \Delta xap5 \) mutant compared to wild type. These new ChIP-chip data reinforce our conclusion that Xap5 and H2A.Z play similar, partially redundant roles in the suppression of inappropriate gene expression, particularly at repeat loci.

5. While referee 3 thinks that genome wide nucleosome mapping in xap5 mutants would go beyond the scope and length limits of EMBO reports, s/he suggests that nucleosome mapping in xap5 mutants could be investigated for selected genes. Although nucleosome mapping of selected loci in xap5 mutants would be of interest, we feel that given the space constraints for manuscripts in EMBO Reports and the additional experiments we carried out in response reviewers’ comments, these experiments would be beyond the scope of the current manuscript.

6. Both referees 2 and 3 remark that the data on Xap5 and Set1 antagonism should be kept and referee 3 further feels that deletion of Swr1 would not reveal substantial novel insight; this experiment is therefore not required. We have retained the data on antagonism between Xap5 and Set1 and do not plan on examining phenotypes of Swr1 mutants.

7. Can you please also specify the number "n" for how many experiments were performed and the error bars in the respective figure legends? This information is currently missing or incomplete.

We have incorporated this information in the figure legends.

Response to Reviewer 1’s Comments

1. The authors mention that Xap5 and Set1 antagonize each other. The only data presented for this include a correlation plot of the genetic interactions observed between set1 and xap5 (Fig. 1E) as well as a single growth experiment shown in Fig. S3E. Since nothing further is shown or discussed I think this part of the paper can be omitted completely (and may be more worthwhile being studied separately). Instead, it would make more sense to include similar data for Swr1 and Xap5. Since Swr1 is responsible for the incorporation of H2A.Z into nucleosomes, does deletion of the Swr1 complex lead to similar phenotypes as pht1 when compared to or combined with xap5?

Please see response to editorial comment #6.

2. Deletion of XAP5 leads to the production of ncRNA. Aberrant transcription is generally associated with changed nucleosome occupancy. Hence, it would be interesting to determine what happens to histone H3 and H2A.Z occupancy in a xap5 mutant compared to the wildtype. Is H2A.Z incorporation reduced in xap5 cells?

Please see response to editorial comment #5.

3. Does Xap5 itself associate with H2A.Z containing nucleosomes? A simple immunoprecipitation experiment should provide an answer. Alternatively, what happens to Xap5 localization in a pht1 mutant?
We found that Xap5 chromatin localization is altered in Δpht1 mutants at selected loci (please see response to editorial comment #4).

4. The authors compare the gene expression profiles of xap5 cells with certain histone deacetylase mutants. In my opinion this analysis by itself is completely insufficient. What happens to histone acetylation in xap5 cells? This can be checked on a global scale by Western blotting or locally by ChIP. In budding yeast both the Rpd3S and Set3C histone deacetylases have been implicated in the suppression of aberrant transcription. Did the authors also look at these HDACs? Rather than just looking at gene expression profiles between the HDAC and xap5 mutant strains, the authors should investigate ncRNA production directly, either by RT-PCR or Northern blotting.

We performed RT-PCR directly in the various HDAC mutants and Δxap5 to assess ncRNA production at selected loci (previously shown to be upregulated in clr6-1). As predicted by the reviewer, we found these loci to be regulated in a range of HDAC mutants. Please see response to editorial comment #3.

5. I realize that the "choppy" appearance of the RNA-seq data (eg. Fig. S4) is probably due in part to using an rRNA removal kit. Has the data been processed by including a sliding window? If not, how have the authors determined which genes exhibit faulty termination in the mutant backgrounds since it is unclear from the example genes shown whether a single or multiple transcripts are produced from individual genes?

Please see response to editorial comment #2.

6. The RT-PCR experiment shown in Fig. S4G does not include a control amplicon, eg. ACT1. act1 expression was measured in all of our experiments but these data were not included in the figure to reduce the size of the panel. However, we have now added this data at the reviewer’s request (new gel added to Figure S4G).

7. The authors should mention in the introduction (not just in the conclusion) that nothing is known about Xap5 protein structure and function.

We do mention that “The cellular function of Xap5 proteins has not been described in any organism” in the introduction. Due to space constraint we feel it best limit further discussion of Xap5 protein structure to the discussion section.

8. The authors performed an EMAP experiment with ca. half of the S.p. genome. Where the genes chosen at random or were they selected in some fashion?

Please see response to editorial comment #1

9. When discussing misexpression of genes in various mutant backgrounds (p.5, paragraph 2), it would be helpful to mention the fold change in gene expression also in the manuscript text, not just the figure legend.

Since these refer to gene groups/categories, members of which have different fold changes, we included cutoff fold changes as “(p≤0.01, fold change≥2)” in the manuscript text to address this point.

Response to Reviewer2’s Comments

1. Accurate detection of antisense transcripts is essential for this study. The Steinmetz group (PMID: 17897965) found false positive antisense signals due to artifactual second strand synthesis by reverse transcriptase. This is prevented by addition of actinomycin D. The protocol...
by Wang et al. (Suppl. reference no. 8) used by Anver et al. does not include actinomycin D. The authors should generate at least one data set plus and minus actinomycin D in order to check their false positive detection rate.

While the comment is very well taken, our methodology is generally accepted in the transcriptomics world and many published work in peer-reviewed journals have used a similar approach [1-6].

2. The authors should state the criteria for selecting the 2117 mutants for the E-MAP. In the present version this is unexplained and seems oddly arbitrary.

Please see response to editorial comment #1.

3. The authors overstate some of the effects. The S-score profiles in Fig. S2C do not look very similar. Is there no better way to quantify „likeliness” of such profiles than eye balling? The correlations shown in Fig. 1D and E are not very impressive, too. Fig. 1F gives a clear correlation, but I would not call this „highly” or „strongly” correlated. In general, there is a deplorable tendency in the field to go after statistical significance rather than biological relevance. Comparisons of genome-wide data sets almost by default generate all kinds of significant effects, i.e. very low p-values, just due to the large numbers compared. However, the effects are often subtle and the biological relevance remains questionable. See for example the last sentence on p. 6. The high statistical significance is not a strong argument to make the weak correlation more convincing or relevant. (In addition, why gets the correlation worse for the xap5 pht1 double mutant?) Even though I tend to believe the overall conclusions of this study, I recommend to report the actual results in a more balanced way with a focus on the magnitude of the effects rather than their statistical significance.

We completely agree with the reviewer that statistical significance does not prove biological relevance. We also agree that genomics-scale data must be analyzed using objective and consistent approaches, and for that reason have avoided drawing any conclusions based solely on datasets ‘looking’ similar to each other. However, as the reviewer points out, it can be useful to provide both the results of statistical tests and graphical representations of the data to allow the reader to assess how biologically important a statistically significant similarity (or difference) is likely to be. For example, we have taken this approach in Fig. S2. Panel B provides a statistical analysis of overlapping gene lists while panels A and C provide a more graphical representation of these data.

We have tried to present our data in a balanced way. We described the genetic interaction profiles reported in Figure 1 as “similar to Δxap5”, which we think is a reasonable description given both the correlation coefficients and the high statistical support. In response to the reviewer’s concerns we have changed our description of the relationship between gene expression changes in xap5 and pht1 mutants from “highly correlated” to “well-correlated”. We think that our description of the similarity between the gene expression profiles in the xap5 mutant and known chromatin modifiers as “moderate but statistically significant” is both accurate and informative. The biological significance of this statistical analysis is further bolstered by the new experiments analyzing gene expression in HDAC mutants described in our response to editorial comment #3.

4. Given the antagonistic genetic interaction of xap5 with set1 (Fig. S3E), the authors should do the same kind of transcriptome correlations using published data as they did with clr6 etc. mutants.

Although this analysis would be of interest, we feel that given the space constraints for manuscripts in EMBO Reports and the additional experiments we carried out in response to other reviewers’ comments that this analysis would be beyond the scope of the current manuscript.

5. It seems that the labeling for „up” and „down” in Fig. 2D is erroneously reversed.

Thank you for your careful reading of the figures; we have corrected this error.

6. Issues with Venn diagrams: The overlap between misregulated genes in xap5 vs pht1 mutants in Fig. 2E-H is actually very small and even zero in Fig. S4 C-F, in contrast to the authors' claim.
many loci upregulated in ... (p. 5, 16/15 from bottom). With view of the bar diagram in Fig. 3A I do not understand how the overlap between xap5 and pht1 mutants can be zero in Fig. 3B.

In fact the overlap is high when you note that the loci shared between all the three genotypes are also shared between Δxap5 and Δpht1. For instance in Fig 2E the overlap between Δxap5 and Δpht1 is 85 (84+1) and not 1. In figure 3B it is 7 and not zero.

7. I would like to see Venn diagrams for the overlap between transcript misregulation and ChIP enrichment, e.g. as additional panel for Fig. 4.

We agree that this would be an interesting addition. Unfortunately, we are already at the limit for the number of figures and panels permitted by the EMBO Reports format.

8. It should be stated more clearly that deltapht1 did not lead to upregulation of wtfs although it did bind there (Fig. 4A).

In fact there is an upregulation of wtf transcription in Δpht1 (albeit not as great an upregulation as seen in Δxap5). This is described on page 6, 1st paragraph after the subheading: “Relative to all genes, these different types of repeat loci are preferentially upregulated in both Δxap5 and Δpht1 mutants (Fisher’s exact test, p-values for LTRs =1.203e-05 and wtfs <2.2e-16 in Δxap5 and LTRs <2.2e-16 and wtfs = 0.006443 in Δpht1).”

Response to Reviewer 3’s Comments

1. The description of the Xap5 ChIP data should be clarified. In particular, on page 6, the authors state that “...Xap5 protein is associated with chromatin throughout the genome, showing enrichment in both genic and intergenic regions...” If the protein is associated with the entire genome, how is it enriched with particular loci? How do the authors know that this does not simply reflect non-specific association with chromatin?

We mentioned that Xap5 protein is associated throughout the genome to indicate that it is not exclusively associated with a particular region of chromosomes such as heterochromatic regions (centromeres, telomeres etc.) as generally observed for Clr proteins. Please also see our response to comment #2 below.

2. Later, in the same paragraph, the authors talk about significant enrichment of Xap5 and H2A.Z in LTRs and wtfs. Significant relative to what? Enriched relative to what? Are these proteins associated with loci far from LTRs, wtfs or protein-encoding genes? I think it would be more accurate to just state that the ChIP data shows that these proteins are associated with the LTRs and wtfs.

The enrichment is relative to overall protein binding in the genome. In other words, the protein association in the repeat element loci is significantly higher than the association in other loci. We also observed a higher association of these proteins with many protein coding genic loci and intergenic loci that don’t belong to any single (functional or structural) category. We have added more details about how enrichment was calculated in the on-line methods.

3. Is figure 2D correctly? The manuscript says that anti-sense transcripts are prominently up-regulated, not down-regulated as figure 2D seems to suggest.

Thank you for your close reading of the figure! The labels are now corrected.

4. In figure 2I, I assume that only the counts for the anti-sense strand are presented. Is this correct?

rps2402 and cnt5 are convergent genes and transcribed from opposite strands. The figure shows the transcript in one direction (rps2402 to cnt5) or from one strand. Therefore it depicts the sense counts for rps2402 and antisense counts for cnt5.
5. The description of the data presented in Figure 2K is opaque and should be clarified so that it is more obvious what data and data analysis is being presented in this figure panel.

We agree and have added a section to the supplementary text under online methods “Antisense Read Comparison: TSS vs TTS (Figure 2K)”.

6. The changes in gene expression as measured by RNA-seq that are presented in figure 4 are not immediately apparent. The paper would be more compelling if a quantitative measure of gene expression changes for the genes shown in the figure could also be presented.

We agree and have now added a supplementary table (Supplementary table S5) with these data.

7. I would have appreciated one or two sentences explaining or exploring the relationship between the changes in gene expression reported here and the phenomenon of cryptic transcription observed in S. cerevisiae.

We have added a short discussion of to the results and discussion section (page 7 paragraphs two).

8. The method for calculating doubling times should be described in the supplementary methods.

We have now added this to the supplementary text under online methods.

References


2nd Editorial Decision

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the reports from the referees that were asked to assess it, and as you will see, both support publication of the study in our journal now.

However, both referees also point out that important information regarding the methods is missing from the manuscript text and needs to be included. It would be good if the manuscript text could be a little more shortened to make room for the missing information. Referee 2 also told us that you do not have to perform new experiments but should discuss explicitly that the applied methodology is
"not up to the state of the art regarding detection of antisense transcripts and that the genome-wide data may be technically flawed and should be taken with a grain of salt". S/he suggests that you can resort to the points mentioned in his/her report that support the conclusions independently of the potentially flawed genome-wide data. Please also see below the cross-comments from referee 1.

Regarding the statistics, can you please specify "n" and the error bars for figure 4 and S4H in the legends? I also noticed that in figures 2 and 3 n=2, in which case no error bars can be shown. Please remove the error bars and instead show the actual single data points along with the mean.

Since January this year, EMBO press papers are accompanied online by 1) a short 1-2 sentence summary of the findings and their significance, 2) 2-3 bullet points highlighting key results and 3) a synopsis image. Can you please send us this information, and may be draw a model for the synopsis image, which needs to be exactly 211x157 pixels large? Otherwise, a key result/figure panel could also be depicted in the synopsis image.

I look forward to seeing a revised form of your manuscript as soon as possible. Please let me know if you have any questions.

REFEREE REPORTS:

Referee #1

The revised manuscript, "Yeast X-chromosome Associated.....suppress aberrant transcripts", by Anver et al. has satisfactorily addressed the points raised by this reviewer. The current version of the manuscript can be accepted for publication after the authors include the response #2 to the Editor's comment in the text. While they have discussed the methods used to analyze the data, they have not discussed this piece of analysis provided to the editors and reviewers.

Referee #2

I have strong feelings regarding the authors' response to my previous comment #1: It is not true that the RNA-seq protocol used by Anver et al. is "generally accepted in the transcriptomics world and many published work in peer-reviewed journals have used a similar approach [1-6]". Whenever cDNA is generated by reverse transcription using more or less random hexamer primers and if accurate detection of antisense transcripts is important for a study, it is strongly advised to use actinomycinD. A bench mark comparison of different RNA-seq protocols from the Broad Institute (LevinJZ et al. 2010 Nat Methods) explicitly explored this issue in the context of a NNSR library. They also included a dUTP protocol, which would be the equivalent to the dUTP protocol used by Anver et al. However, this approach only allows keeping the strand information starting from the cDNA but it cannot distinguish if erroneous second strand synthesis already happened during cDNA synthesis. This is why Levin et al. included actinomycinD for first strand cDNA preparation prior to generation of their dUTP library (online methods) and this is why Anver et al. should do so, too. Anver et al. attempt to quote six publications using their protocol. Ironically, three of them do not. DeGennaro et al., 2012 and Marguerat et al., 2012 employ an RNA ligation strategy where the strand information is kept due to ligation of asymmetric adapters directly to the RNA. Shim et al., 2012 do not use RNA-seq but hybridize labeled cRNA (generated from cDNA by in vitro transcription) to microarrays. Here the strand information is preserved due to the use of a polyA specific primer for cDNA synthesis that includes a T7 polymerase promoter that is orientation specific during the in vitro transcription step (Agilent kit). The remaining three references use exactly the same protocol as Anver et al. but in two of these studies, Bao et al., 2013 and Yang et al., 2013, the detection of antisense RNA is of no importance. The word "antisense" does not come up in a whole-manuscript search.

Collectively, the authors' attempt to justify their protocol is not valid upon closer inspection and does not concur with my own expertise. Therefore I seriously question their genome-wide conclusions with regard to increased antisense transcription and ask for proper methodology. Nonetheless, I do realize that there are several single locus strand specific RT-PCR experiments supporting their conclusion. In addition, the inverse correlation between changes in sense and antisense transcripts (Fig. S4) argues for true effects apart from possible artefacts due to the omission of actinomycinD. So I tend to believe the overall conclusion, as already stated in my first
review, but I strongly feel that there should not be another article added to the literature where improper methodology is used as if it were "generally accepted in the transcriptomics world".

Upon the Editor's request I also went through the authors' response to the comments by Referee #3 and found the response satisfactory.

Cross-comments from referee 1:

I have gone through the manuscript and I agree with the 2nd reviewer that they need to include a statement to the effect that they have not included actinomycin D in their experiments. While they do not have to repeat the experiments, it is imperative that they define the method they have used to identify the antisense transcripts. While the omission of Actinomycin D may produce false positives, the authors have included strand specific RT-PCR of individual genes to justify their conclusion. Therefore, the authors need to spell out clearly that they have not included Act D in their methods and discussion sections.

1st Revision 15 May 2014

We are very pleased that you and the reviewers feel that our manuscript is in principle appropriate for publication in *EMBO reports*. We have made the requested changes as detailed below.

1. To address the issue of potential spurious antisense transcripts in our RNA-seq libraries, we have added a short discussion of the pros and cons of using actinomycin D during library preparation and detailed the independent evidence supporting the upregulation of antisense transcripts in the mutants on page 6. We also noted that actinomycin D was not used during library preparation in the online methods.

2. We have added a brief discussion of the number of transcripts with faulty transcriptional termination in the different mutants on page 6 to fulfill the request of Referee #1.

3. We have added information on the number of replicate experiments presented in figures 4 and S4 in the respective legends. The ChIP data depicted in Fig. 4 are derived from a single experiment, although the same trends were observed in two independent biological replicates (NCBI Gene Expression Omnibus accession number GSE46506). Directly combining the data from the two experiments was not feasible due to large differences in background signal. However, we were able to determine statistical significance for our findings from the single depicted ChIP experiment because we averaged protein enrichment at many different loci in the three classes of genes examined. For example, in panel 4D, n for LTRs = 238, n for TF2s = 13, n for wtfs = 25.

4. The data presented in figures 2 and 3 are based on at least three independent biological replicates, as noted in the figure legends.

We suggest the following brief summary for the manuscript:

Xap5 is a protein highly conserved across eukaryotes but with no known molecular function. We now show that it is associated with chromatin and acts in a similar manner as the variant histone H2A.Z to suppress expression of antisense and repeat element transcripts.

And we suggest the following points be listed as our key results:

- Genome-wide genetic interaction analysis implicates Xap5 in chromatin regulation
- Xap5 function is conserved between widely diverged eukaryotes, suggesting a fundamental role in cellular processes
- Gene expression, chromatin immunoprecipitation, and epistasis experiments together suggest that Xap5 and the histone variant H2A.Z work in a similar manner to suppress expression of aberrant transcripts
Finally, we have included an image that we think provides an appropriate visual synopsis of the manuscript.

Thank you very much for considering our manuscript; we very much hope that you now find it suitable for publication in *EMBO reports*.

3rd Editorial Decision 21 May 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.