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Eaf5/7/3 form a functionally independent NuA4 submodule linked to RNA polymerase II-coupled nucleosome recycling

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Review timeline:

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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: Anke Sparmann/Hartmut Vodermaier

1st Editorial Decision 05 September 2013

Thank you for submitting your manuscript on a NuA4 Eaf5/7/3 submodule and its role for our consideration, and please excuse the delay in its evaluation during the summer vacation period. We have now received the comments of three expert referees, copied below for your information. As you will see, the reviewers find your observations and conclusions potentially interesting and important, and we should therefore in principle be happy to consider a revised manuscript further for publication in The EMBO Journal. Nevertheless, eventual acceptance will require satisfactory revision a number of points raised by referees 1 and 2; in particular, addressing the concerns of referee 2 will be required in order to strengthen the functional conclusions on the Eaf5/7/3 trimer.

I would therefore like to invite you to revise the manuscript in response to the referees' comments and criticisms. Please keep in mind that it is our policy to allow only a single round of major revision and that it is therefore important to carefully respond to all points at this stage. When preparing your letter of response to the referees' comments, please also remember that this will form part of the Review Process File, and will therefore be available online to the community.

Thank you for the opportunity to consider this work, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision.

REFEREE REPORTS:

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Referee #1:

The manuscript by Rosetto et al describes the characterization of the Eaf5/7/3 trimer within the NuA4 complex. Quite interestingly, they provide compelling evidence that the trimer also functions independently of the NuA4 complex. Notably, while the NuA4 complex targets both promoters and coding regions, the trimer seems to target coding regions specifically. The authors provide evidence that the trimer targets the elongating polymerase and acts as a histone chaperone in that it helps elongating through nucleosomes while also preserving nucleosome density. The data presented in that manuscript is solid and convincing. I have only two issues with this work. Addressing these two issues would greatly improve the manuscript.

1- Two pieces of evidence in this manuscript suggest that the trimer may target the ribosomal protein (RP) genes specifically. First, eaf6 mutants are much more sensitive to rapamycin than the eaf6 mutant. Second, the genes shown in Fig5E,F and the heat maps shown in FigS3 both point to RP genes rather than highly transcribed genes in general. Indeed, Eaf7 targets seem to be restricted to short highly transcribed genes, which are in essence composed of the RP genes in yeast. This possibility should be addressed by mapping the data on matching sets of genes with similar expression levels but containing no RP genes.

2- Fig6F suggests that Ctk1-dependent phosphorylation of the CTD on Ser2 triggers the recruitment of the trimer to genes. This model is quite interesting but the evidence for it fall a bit short. The authors should perform ChIP of Eaf5 or 7 (and also Epl1 as a control) in a ctk1 mutant strain. This would provide more direct evidence for the importance of phosphorylation of Ser2 by Ctk1 in the recruitment of the trimer (and eventually also NuA4) to genes.

Referee #2:

Rosetto et al. present a clearly written analysis of the structure and function of the NuA4 histone acetyltransferase complex from S. cerevisiae. This large, multi-subunit complex is the only histone acetyltransferase from yeast that is essential for life and is related to the histone acetyltransferase from humans. NuA4 is composed of at least 13 subunits, many of which appear to be arranged into subassemblies within the complex and several of which can also be found as participants in other complexes.

In this work, the authors focus on 3 particular subunits of NuA4, Eaf3, Eaf5 and Eaf7. In an extensive set of affinity purification experiments, the authors provide conclusive evidence that: Eaf5, Eaf7 and Eaf3 are bone-fide components of NuA4; that Eaf5 is required for association of Eaf7 and Eaf3 with NuA4; that Eaf7 is required for association of Eaf3 (but not Eaf5) with NuA4; that Eaf7 can interact with Eaf3 independently of Eaf5 or other NuA4 components; and that Eaf5 and Eaf7 can interact with NuA4 independently of Eaf3. They further show that, on a gel filtration column, a small amount of total cellular Eaf5, Eaf7 and Eaf3 co-fractionate in a peak that includes NuA4 and that a larger proportion of these proteins co-fractionate together in a distinct and apparently much smaller protein complex.

Genetic analysis of mutations in EAF3, EAF5, EAF7 and other members of the NuA4 complex are presented. The phenotypes and genetic interactions show good concordance between the phenotypes of eaf5 and eaf7 mutations, whereas the genetic behaviors of mutations in EAF3 and genes encoding other components of NuA4 are clearly distinct. Although they argue for shared phenotypes for mutations in these three genes, eaf5 and eaf7 are clearly distinct from ea3. The authors make the point that this may be due to the participation of Eaf3 in the Rpd3S complex, other reasonable interpretations of the data are also possible here.

ChIP and high resolution ChIP shows that NuA4 associates with both promoter regions and transcribed bodies of genes in a transcription-dependent manner. Furthermore, Eaf5 and Eaf7 associate with Pol II (including the elongating Ser2 phosphorylated form of Pol II) in an Eaf5-dependent manner. Interestingly, in an eaf5 mutant, the promoter association of Eaf7 is lost, whereas its association with the bodies of genes is largely preserved. Combined with the author's observation that an eaf5 mutation disrupts Eaf7's association with Pol II and the remainder of NuA4, this observation suggests that Eaf7/Eaf3 is capable of independent association with chromatin.
The authors next turned to the biological roles of Eaf3, Eaf5 and Eaf7. First, they examined the effect of eaf7 mutations on transcription elongation in vivo. Employing an assay developed in the Struhl lab to follow the last wave of polymerases as they move down a newly repressed gene, they observed what may be a modest decrease in Pol II density in an eaf7 mutant relative to wild type on their GAL1-FMP27 test gene, following glucose repression. Although the authors argue that this shows a Pol II processivity defect in the eaf7 mutant, I am not convinced. Looking at the 4 and 6 minute timepoints in Figs 6B and 6D, I am not persuaded that there are clear and significant differences between the wild type and mutant strains. Furthermore, if the eaf7 mutation did cause a processivity defect, shouldn't figure 6A show clear differences from wild type that increase in severity along the length of the gene?

In a second approach to investigating the functions of Eaf3, Eaf5 and Eaf7, the authors employed a genetic assay for cryptic transcription, which is an indicator of chromatin disruption. Here, they observed distinct sets of behaviors for an eaf3 mutation relative to the common behaviors of eaf5 and eaf7 mutations. This difference is likely due to the participation of Eaf3 in the Rpd3S complex. Nevertheless, the data presented are consistent with effects of all three mutations on transcribed chromatin. One deficiency here is that no northern blot data are presented and it is possible that the altered cryptic initiation observed in the eaf5 and eaf7 mutants actually reflects an underlying transcription initiation defect. The authors provide further evidence for a chromatin role of Eaf3, Eaf5 and Eaf7 by demonstrating genetic interactions with members of the yeast FACT complex. As above, an eaf3 mutation behaved differently than eaf5 and eaf7 mutations. Finally, using two different assays, the authors observed increased H3K56ac signals in the eaf7 mutant, suggesting increased de novo histone incorporation.

Overall, the authors have a convincing set of data for a Eaf3/Eaf5/Eaf7 submodule in the NuA4 complex and strong evidence that this set of proteins associates with both promoters and the transcribed bodies of genes. I am less convinced that they have a clear handle on other potential functions for Eaf3/Eaf5/Eaf7; their genetic data are open to multiple interpretations and their transcription elongation assay requires a more careful analysis and presentation of the data. In addition, although strong evidence is presented for increased histone exchange in the eaf7 mutation, a Northern blot or other RNA-based analysis is necessary for a definitive conclusion that eaf3, eaf5 or eaf7 effect cryptic initiation of transcription.

Other comments:

1. On page 13, the authors should more clearly acknowledge the fact that Ginsberg et al. (MCB, 2009) previously presented evidence that NuA4 associates with transcribed regions in addition to promoters.
2. On page 16, I am not sure why it should be unfortunate that no effect was observed on histone marks.
3. Doesn't figure 6E lane 5 show that Eaf1 is required for association of Eaf5 with Pol II (i.e. it does not merely enhance association of Eaf3/Eaf5/Eaf7 with Pol II as suggested on the bottom of page 17)?
4. In Figure 6F, it would be helpful if the blot was also probed for total Pol II.

Referee #3:

This is a thorough, high quality, accurately interpreted study that nicely combines biochemistry, genetics, and molecular analyses. They convincingly show that three of the subunits of the yeast HAT NuA4 exist as a separate subcomplex, and that these three proteins are enriched on coding regions of genes, where they interact with RNA polymerase II and promote its progression. They also provide genetic and physical evidence for a role for these three proteins in destabilization of nucleosomes ahead of the elongating polymerase. I find no minor or major problems with this very nice study.

1st Revision - authors' response 16 December 2013
Response to the referees’ comments

We wish to thank all three reviewers for their very positive and insightful comments, and for their excellent suggestions. We feel these changes and included new data greatly improve the manuscript, leading to a better molecular understanding.

Referee #1:

We really appreciate that this referee finds our data interesting, solid and providing compelling evidence. We addressed his two issues as requested. We thank the reviewer for suggesting the ChIP experiment with ctk1 mutant cells, which provided very strong support for the model of physical and functional interaction of the trimer with RNAPII CTD Ser2-P.

The manuscript by Rosetto et al describes the characterization of the Eaf5/7/3 trimer within the NuA4 complex. Quite interestingly, they provide compelling evidence that the trimer also functions independently of the NuA4 complex. Notably, while the NuA4 complex targets both promoters and coding regions, the trimer seems to target coding regions specifically. The authors provide evidence that the trimer targets the elongating polymerase and acts as a histone chaperone in that it helps elongating through nucleosomes while also preserving nucleosome density. The data presented in that manuscript is solid and convincing. I have only two issues with this work. Addressing these two issues would greatly improve the manuscript.

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We agree with the reviewers that the majority of RP genes being highly transcribed, this could have been misleading in relation to the trimer’s function. But as explained in the text with now some modifications to improve clarity, most mutations in NuA4 subunits lead to sensitivity to rapamycin, not only trimer mutants. This is certainly due to the previously reported role of NuA4 in RP gene transcription. The referee’s suggestion to distinguish in our ChIP-chip dataset RP genes and highly transcribed non-RP genes was done and results are presented in new supFig. 4. It seems that RP genes tend to have more equivalent binding of Eaf7 between promoter and coding regions, while highly transcribed non-RP genes show strong preference for the coding region. This analysis clearly shows that the trimer is also found associated to highly transcribed non-RP genes. The difference in profiles is certainly due to the
reported recruitment of NuA4 to the promoter of RP genes (Reid et al. Mol Cell 2000). We thank the reviewer for this excellent suggestion.

2- Fig6F suggests that Ctk1-dependent phosphorylation of the CTD on Ser2 triggers the recruitment of the trimer to genes. This model is quite interesting but the evidence for it fall a bit short. The authors should perform ChIP of Eaf5 or 7 (and also Epl1 as a control) in a ctk1 mutant strain. This would provide more direct evidence for the importance of phosphorylation of Ser2 by Ctk1 in the recruitment of the trimer (and eventually also NuA4) to genes.

We thank again the reviewer for this excellent suggestion and we performed the experiment as requested. The results are shown in new Fig. 6G and demonstrate that loss of Ctk1/CTD Ser2-P significantly decreases eaf7 binding to the coding region of several genes. Furthermore, we also included ctk1/eaf3 double mutants in the analysis and found an almost complete loss of Eaf7 on these regions. These data indicate that the trimer requires two distinct physical interactions to function on coding regions: RNAPII CTD Ser2-P and nucleosomes bearing H3K36me2/3 (Eaf3 chromodomain specificity). This is a nice parallel to what was reported for Rpd3S HDAC complex (Drouin et al. PLoS Genetics 2010).

Referee #2:

We thank this reviewer for his detailed comments and for finding our data convincing and clearly written. We answered all the suggestions/requests that were found in his text. We thank the reviewer for suggesting the RNA-based analysis of cryptic transcription initiation, which provided even stronger support for the model.

Rosetto et al. present a clearly written analysis of the structure and function of the NuA4 histone acetyltransferase complex from S. cerevisiae. This large, multi-subunit complex is the only histone acetyltransferase from yeast that is essential for life and is related to the histone acetyltransferase from humans. NuA4 is composed of at least 13 subunits, many of which appear to be arranged into subassemblies within the complex and several of which can also be found as participants in other complexes.

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small amount of total cellular Eaf5, Eaf7 and Eaf3 co-fractionates in a peak that includes NuA4 and that a larger proportion of these proteins co-fractionate together in a distinct and apparently much smaller protein complex.

Genetic analysis of mutations in EAF3, EAF5, EAF7 and other members of the NuA4 complex are presented. The phenotypes and genetic interactions show good concordance between the phenotypes of eaf5 and eaf7 mutations, whereas the genetic behaviors of mutations in EAF3 and genes encoding other components of NuA4 are clearly distinct. Although they argue for shared phenotypes for mutations in these three genes, eaf5 and eaf7 are clearly distinct from eaf3. The authors make the point that this may be due to the participation of Eaf3 in the Rpd3S complex, other reasonable interpretations of the data are also possible here.

As mentioned by the reviewer, we suggest in the text that the distinct phenotypes of eaf3 in comparison to eaf7/5 are caused by the presence of eaf3 in the Rpd3C HDAC complex. We believe that this is the most likely reason. We agree that there could be other interpretations (although no obvious ones came to mind beside maybe auto-regulatory function) and we modified the text to emphasize the fact that eaf3 mutants have distinct phenotypes than eaf5/7 mutants.

ChIP and high resolution ChIP shows that NuA4 associates with both promoter regions and transcribed bodies of genes in a transcription-dependent manner. Furthermore, Eaf5 and Eaf7 associate with Pol II (including the elongating Ser2 phosphorylated form of Pol II) in an Eaf5-dependent manner. Interestingly, in an eaf5 mutant, the promoter association of Eaf7 is lost, whereas its association with the bodies of genes is largely preserved. Combined with the author's observation that an eaf5 mutation disrupts Eaf7's association with Pol II and the remainder of NuA4, this observation suggests that Eaf7/Eaf3 is capable of independent association with chromatin.

The authors next turned to the biological roles of Eaf3, Eaf5 and Eaf7. First, they examined the effect of eaf7 mutations on transcription elongation in vivo. Employing an assay developed in the Struhl lab to follow the last wave of polymerases as they move down a newly repressed gene, they observed what may be a modest decrease in Pol II density in an eaf7 mutant relative to wild type on their GAL1-FMP27 test gene, following glucose repression. Although the authors argue that this shows a Pol II processivity defect in the eaf7 mutant, I am not convinced. Looking at the 4 and 6 minute timepoints in Figs 6B and 6D, I am not persuaded that there are clear and significant differences between the wild type and mutant strains.

We agree with the reviewer that the effect of the eaf7 mutant on PolII processivity could be seen as "modest", but we do provide the statistic showing the significant difference compared to wild type conditions. The effect is mostly seen at the 2 min time-point when PolII levels seem to remain the same at all loci over the coding region. This is in contrast to the wild type cells which show a progressive disappearance of PolII from the 5' region. Importantly, at the 4 min time-point, PolII signals in the eaf7 mutant cells suddenly decreased to the levels seen in the
WT or even below for the more downstream locus. This demonstrates a sudden strong loss of PolII throughout the gene compared to the previous 2 min time-point, supporting a dissociation of the polymerase instead of a defect in elongation rate. Thus, these results suggest that the trimer affects PolII processivity, most likely by regulating nucleosome dynamics. We have slightly modified the text to make our interpretation of the results clearer. We have also included another method to present these data in order to better visualize the differences between eaf7 and wild type cells at the different time-points (looking at the 4 different loci independently). This is now shown in new supFig. 5.

Furthermore, if the eaf7 mutation did cause a processivity defect, shouldn't figure 6A show clear differences from wild type that increase in severity along the length of the gene?

We do not think that this would be the case since PolII ChIP data presented in Fig. 6A represent steady state levels of PolII association throughout the coding region. Only kinetics of first or last waves of PolII can clearly detect relatively small effects on processivity.

In a second approach to investigating the functions of Eaf3, Eaf5 and Eaf7, the authors employed a genetic assay for cryptic transcription, which is an indicator of chromatin disruption. Here, they observed distinct sets of behaviors for an eaf3 mutation relative to the common behaviors of eaf5 and eaf7 mutations. This difference is likely due to the participation of Eaf3 in the Rpd3S complex. Nevertheless, the data presented are consistent with effects of all three mutations on transcribed chromatin. One deficiency here is that no northern blot data are presented and it is possible that the altered cryptic initiation observed in the eaf5 and eaf7 mutants actually reflects an underlying transcription initiation defect.

As suggested by the reviewer we have now included direct RNA expression analysis on different genes that are known to give rise to set2 mutant-dependent cryptic transcription. Using different sets of primers, RT-qPCRs clearly indicate that eaf7 mutant does not provoke an underlying transcription initiation defect. These data are presented in new Fig. 7C.

The authors provide further evidence for a chromatin role of Eaf3, Eaf5 and Eaf7 by demonstrating genetic interactions with members of the yeast FACT complex.

We now include data also showing physical interaction between the trimer and the FACT complex. This is in new Fig. 7F.

As above, an eaf3 mutation behaved differently than eaf5 and eaf7 mutations. Finally, using two different assays, the authors observed increased H3K56ac signals in the eaf7 mutant, suggesting increased de novo histone incorporation.
Overall, the authors have a convincing set of data for a Eaf3/Eaf5/Eaf7 submodule in the NuA4 complex and strong evidence that this set of proteins associates with both promoters and the transcribed bodies of genes. I am less convinced that they have a clear handle on other potential functions for Eaf3/Eaf5/Eaf7; their genetic data are open to multiple interpretations and their transcription elongation assay requires a more careful analysis and presentation of the data.

As stated above we have now included a more careful presentation of the transcription elongation assay (new supFig. 5). We have also modified the text and included data showing physical interaction with the FACT histone chaperone/elongation factor (new Fig. 6F). We feel that these data along the other new data about Eaf7 dependency on CTD Ser2-P (new Fig. 6G) and the RNA analysis of cryptic transcriptions (new Fig. 7C, see below) provide very strong additional information about Eaf5/7/3 function and mechanism of action.

In addition, although strong evidence is presented for increased histone exchange in the eaf7 mutation, a Northern blot or other RNA-based analysis is necessary for a definitive conclusion that eaf3, eaf5 or eaf7 effect cryptic initiation of transcription.

We closely followed the reviewer’s suggestion and performed RNA analysis to draw a definitive conclusion on eaf5/7/3 effect on cryptic initiation of transcription. We measured previously reported set2 mutant-dependent cryptic transcription on 4 genes and clearly demonstrate partial suppression of these cryptic initiation events, as suggested by our results with the reporter construct (old Fig. 7B). These RNA-based analysis are included as new Fig. 7C. We wish to thank again the reviewer for suggesting such an important experiment.

Other comments:

1. On page 13, the authors should more clearly acknowledge the fact that Ginsberg et al. (MCB, 2009) previously presented evidence that NuA4 associates with transcribed regions in addition to promoters.

   The text has been modified as suggested.

2. On page 16, I am not sure why it should be unfortunate that no effect was observed on histone marks.

   “Unfortunately” has been removed.

3. Doesn't figure 6E lane 5 show that Eaf1 is required for association of Eaf5 with Pol II (i.e. it does not merely enhance association of Eaf3/Eaf5/Eaf7 with Pol II as suggested on the bottom of page 17)?

   We agree that the blot presented could be interpreted as Eaf1 being a strong
requirement for Pol II association with Eaf5. On the other hand there is significantly less loading of Eaf5-TAP in the eaf1 mutant lane (compare lane 5 with lanes 4 and 6). Thus, we feel more comfortable being cautious and suggesting a facilitating action rather than a requirement. Furthermore, some Pol II signal is detected in the Epl1-TAP delat-eaf5 fraction presented in new Fig. 6F, suggesting secondary interaction surface on NuA4, beside the trimer module.

4. In Figure 6F, it would be helpful if the blot was also probed for total Pol II.

A blot for total Pol II has now been included (new Fig. 6F)

Referee #3:

Of course we were delighted by this reviewer’s comments about the quality, thoroughness of our study and the accuracy of our conclusions.

This is a thorough, high quality, accurately interpreted study that nicely combines biochemistry, genetics, and molecular analyses. They convincingly show that three of the subunits of the yeast HAT NuA4 exist as a separate subcomplex, and that these three proteins are enriched on coding regions of genes, where they interact with RNA polymerase II and promote its progression. They also provide genetic and physical evidence for a role for these three proteins in destabilization of nucleosomes ahead of the elongating polymerase. I find no minor or major problems with this very nice study.
Thank you for submitting your revised manuscript on the Eaf5/7/3 submodule to our editorial office. It has now been assessed once more by two of the original referees, and I am pleased to inform you that they both consider the original concerns well-addressed and the study now suitable for publication in The EMBO Journal. Upon incorporation of the following remaining editorial points, we shall happy to proceed with final acceptance and publication of this work:

Editorial points:

- Referees 2 brings up some specific (minor) comments, which you may want to incorporate into the final version.

- Please move all supplementary figure legends from the main manuscript into the single Supplementary Information PDF.

- In order to make the primary data more accessible and more directly represented, we encourage the inclusion of figure source data for gels, blots and autoradiographs, especially in cases where not a full gel but just assembled crops are shown (such as in Figs. 1B-E or 2A). We would ask for a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files can be uploaded upon resubmission selecting "Figure Source Data" as object type, and they would be linked as such to the respective figures in the online publication of your article.

- In addition to these presentational changes, I would like ask you to provide (in your resubmission cover letter) 2-5 one-sentence 'bullet points' (complementary to the abstract) that summarize key aspects of the paper - they will accompany the online version of the article as part of a 'synopsis'. Please see the latest research articles on our renewed website (emboj.embopress.org) for examples - I am happy to offer further guidance on this if necessary.

We should then be able to swiftly proceed with formal acceptance and production of the manuscript!

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REFEREE REPORTS:

Referee #1:

The authors have addressed all my concerns. The new manuscript is now widely improved.

Referee #2:

The authors have adequately responded to my prior comments. My only minor comment is that, on page 22 of the revised manuscript, the authors should check that their references to the panels in figure 7 are correct, that they should determine if they are satisfied with their labeling of panels E and F of figure 7 and that they should more clearly describe how the data is supplementary Figure 7C were corrected.
The requested minor editorial changes have been included. The source files (full scans) for the western blots in Fig. 1B, E, 2A and 6E-F have now been provided as jpeg files for each figure. It took us awhile to put them together as these data were dating back to work done in 2004. Supplemental figure legends have also now been transferred to the single PDF file of supplemental data. Finally, The 3 minor comments/corrections suggested by reviewer 2 about in-text references to Figures 7E-F, presentation of these panels and better description of the corrected data in supFigure 7C have been included as well.

Reviewer 2:
-Mistakes for in-text references to Figures 7E-F and supFig. 7 on pages 21 and 22 have been corrected.
-Labeling in panels in Figures 7E and 7F has been modified to be more consistent.
-A better description of the corrected data in supFig. 7C has been included in the figure legend.

Editorial points:
-Supplemental figure legends have been transferred to the single PDF file of supplemental data.
-The source files (full scans) for the western blots in Fig. 1B-E, 2A and 6E-F have now been provided as jpeg files for each figure. (supFig. 1F as well)

Accept letter

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.