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Regulating Genes Flanking Insulators involves dMes-4/Set2 as key players of Nucleosome dynamics

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

1st Editorial Decision 14 June 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. Three referees have now evaluated your study and I enclose their reports below. While the referees find that the current study is potentially interesting they unfortunately raise a number of important issues that preclude publication in the EMBO Journal.

I hope that you understand if I do not go though each individual criticism in this letter, but all the referees raise a number of important concerns. These include a lack of direct evidence that BEAF insulator activity is based on RNA Pol II pausing and insight into how BEAF-32 regulates pausing. There are also a number of important technical concerns especially regarding the flavopiridol experiments and the clarity of the data description and presentation. In its current form the referees do not strongly support publication in the EMBO Journal. Importantly, it is clear from the reports that addressing these concerns would take a extensive and significant amount of time and longer than the three months that the EMBO Journal allows for a period of revision. Therefore, given the amount of additional experimental work required and the fact that we can only afford to continue handling of papers that receive enthusiastic support from at least a majority of referees upon initial review, I am afraid, I see little choice but to come to the conclusion that we cannot offer to publish this study.

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Thank you in any case for the opportunity to consider your manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful. We also hope that this negative decision does not prevent you from considering our journal for publication of your future studies.

REFEREE COMMENTS

Referee #1

The manuscript by Hennion et al., entitled "Insulator-mediated regulation of gene expression through transcriptional profiling" is primarily a genomics study on the insulator binding protein BEAF-32. The authors map BEAF sites genome-wide in Drosophila-S2 cells and their results are similar to published data showing that only 10% are not near TSSs. They then show that knockdowns of BEAF lead to changes in expression of many genes. The majority of genes (2480) are down-regulated while 1464 genes were up regulated upon BEAF depletion. BEAF sites overlap NELF sites and are adjacent to the NFR at gene promoters containing paused polymerases. Depletion of BEAF leads to changes in the +1 nucleosome and their data suggest that BEAF and NELF function together to pause polymerases and maintain the NFR.
The data in the manuscript are interesting especially for laboratories interested in studying BEAF and RNA polymerase pausing. However the paper is very difficult to follow and needs clarity in writing. Thorough editing of the manuscript is absolutely essential.
While this manuscript focuses on BEAF, which was originally identified as an insulator binding protein, the results here clearly demonstrate that BEAF is a promoter bound transcription factor involved primarily in up-regulation of gene transcription. In chicken cells, USF1 is a promoter bound transcription factor that also binds insulators to mediate insulation. The same could be true of BEAF. However the conclusion/discussion described in this manuscript that BEAF insulator activity is based on pausing is not supported by the current data, as the BEAF sites here are not known to be insulators. If the authors wish to make this claim then they should specifically test this, in the context of paused and non-paused BEAF bound promoters. The authors need to demonstrate that BEAF bound promoters have insulator function that is dependent upon BEAF mediated polymerase pausing and NFR generation.
While most of the manuscript focuses on promoters that are downregulated upon BEAF depletion, the last section analyzes the promoters that are upregulated upon depletion. However the discussion focuses primarily on this latter class as a model of BEAF's role as an insulator protein. The discussion needs to be changed to sync with the preponderance of the results section. The discussion could be stronger and in sync with the results if the authors merely focused on how BEAFs role in pausing and nucleosome positioning differentiates it from other transcriptional activators. Also the title of the manuscript needs to be changed to better reflect the results.

The following additional points related to the results should be addressed.
1. In figure 1D where are the upregulated genes on the Venn diagram? The upregulated genes should be analyzed throughout the paper.
2. Figure 1E, and all the other figures where expression is binned arbitrarily and genes are given ranks based on expression or differential expression, it would be more informative if the data are presented as a scatterplot where each gene's expression is plotted against its BEAF binding at the proximal site. This would allow the authors to explicitly calculate the correlation between the two variables they are comparing.
3. Figure 2 is very difficult to follow. What does the heat map represent? Is each row depicting the differential nucleosome occupancy between wt and kd, or is it the number of reads in the wild type condition. The authors suggest there is a strong correlation between differential expression and nucleosome position, which is not obvious from the heatmap. It appears that for the top ~1000 genes this may be the case, but below that point little difference is seen. How different would this heatmap look if the rows were allowed to cluster on the basis of similarity of nucleosome profiles, rather than being manually ordered based on differential gene expression rank?
4. In Figure 2B/C my interpretation is that each node represents a specific nucleosome profile. Is each node in the 8x8 array the same in all the SOM maps (i.e. is 1,1 in panel B equal to 1,1 in panel C). The authors only show nucleosome profiles for two profiles - 3,8 and 4,8. Other (statistically significant?) differences are seen between BEAF-KD and WT and between pol II bound and

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unbound (see node 1,8 in WT vs BEAF-KD - which also is lost in the unbound pol II genes). What do these nucleosome profiles show?
5. In Figure 3 plots would be more informative if they were expressed as a scatterplot of expression of the gene vs its pausing index or its elongation index. The authors also state that all highly expressed genes have a shifted +1 profile, is this true for genes that are highly expressed with low pausing (i.e. those to the left of the plot in panel B).
6. Figure 4a is not very helpful. A venn diagram of BEAF32, NELF, and CTCF should be shown. This should be related to previous mapping studies, (Bushey 2009), which showed that only 18% of BEAF sites contained CTCF. The authors state >80% of BEAF sites colocalize with NELF, but there is no mention of the CTCF data.
7. Figure 5. I would like to see statistics to determine whether the BEAF+NELF+ and BEAF-NELF+ curves are significantly different. The data in figure 5A also suggest that NELF plays a bigger role than BEAF.
8. In Figure 5B the pause index for these genes should be shown, preferably this could be shown genome wide. Does pol II decrease at both the promoter and gene body, which would be expected for any transcription activator, or whether the change in expression is specifically tied to a change in the pausing at downregulated genes?
9. In Figure 5 the authors conclude that BEAF is reinforcing NELF-mediated pausing of pol II. A further prediction from figure 5B is that NELF RNAi should also similarly affect the expression and pausing at these genes. Are the same genes downregulated in both knockdowns? Either RNAseq should be done on these or microarray data from Gilchrist 2008 could be confirmed at a subset of genes by rt-qpcr to show this.
10. In figure 6B I am confused. The authors say some genes are BEAF bound while others are not, I do not see this labeled in the plot. Additionally the expression changes are weak in this subset of genes. Why were these chosen when the RNAseq data suggests that some genes were as much as 20 fold reduced? These changes are less than two fold at all WT vs BEAF-KD genes.
11. In Figure 6D what is the median values for CTCF at R30 vs WT, this fold difference looks very small. A significant wilcox pvalue is given, however if this data is normally distributed the pvalue will overestimate significance because of the small data set. What does a two-tailed t-test look like on this data? Is the fold change in expression normally distributed?
12. The authors suggest that CTCF also functions via pausing. The authors should either measure this following a CTCF kd or remove the claim. Is a +1 nucleosome shift seen in CTCF-KD? This would be critical for a model that CTCF like BEAF functions via a mechanism associated with pol II pausing regulation.
13. The authors finally talk about the upregulated genes in figure 7. I would prefer to see some of these analyses earlier. Specifically they identify 266 genes which contain a promiscuously activated gene adjacent to a neighboring gene containing a BEAF site. What is the pause index of these neighboring genes before and after BEAF-KD?
13a. Upregulation of some adjacent genes is what I expect of an enhancer-blocking protein, but the authors say these genes do not have BEAF in their vicinity. Can they explain this?
14. 89% of the above genes also bind NELF, which the authors say is highly significant, I'm curious if this is really the case, considering 80% of the 5000 BEAF bound sites bind NELF as well. If you randomly choose 266 of those 5000 sites, how many would also bind NELF.

Throughout the manuscript it is not clear what statistical analyses were performed especially how many times each experiment were repeated.

Referee #2

Hennion et al. examine the role of the insulator protein BEAF-32 near transcriptional start sites by performing BEAF-32 ChIP-seq and by analyzing the expression profile and nucleosome occupancy before and after BEAF-32 depletion. They find that genes downregulated by BEAF-32 depletion are normally bound by BEAF-32 at high levels suggesting that BEAF-32 promotes the expression of a large number of genes (at least 1800 genes). They then show that BEAF-32 depletion on average results in a shift of the +1 nucleosome further upstream. Since this has also been found for NELF depletion, the authors investigate whether BEAF-32 could be involved in the release of pol II pausing. They find that the +1 nucleosome shift is most prominent at genes that have both BEAF-32 and NELF binding. Furthermore, BEAF-32 depletion reduces the pol II occupancy at the TSS and this can be restored by treatment with flavopiridol, which blocks pTEFb-mediated release of pol II.

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Upon flavopiridol release, gene expression was restored to higher levels at 5 min but to lower levels at 30 min in BEAF-32 depleted cells versus wild-type cells. The authors conclude that BEAF-32 mediates the continuous release of pol II from the pause site. Finally, the authors show that BEAF-32 depletion also results in the upregulation of genes and that these genes are preferentially located near the second closest TSS of BEAF-32 binding sites, suggesting that BEAF-32 guides enhancer-promoter interactions.

While a connection between insulator function and pol II pausing is really interesting, the paper has not convinced me that there really is a direct one. I can see that the authors were serious with their genomics analyses but the presentation of the text and figures is often confusing and unnecessarily lengthy and complicated. Most importantly, even after closer inspection I have some concerns with the authors' interpretation of the data (see below). Thus, while I applaud the topic and the approach, the paper falls short for me in the execution. I hope this can be fixed.

First, I find it puzzling that BEAF-32 binding is enriched at DRE and E-box motifs, yet the authors claim that it overlaps well with NELF-bound sites (80%), since pol II pausing is known to be underrepresented at DREs and E-box motifs (e.g. see Gilchrist 2010). One concern is that BEAF, NELF and pol II are all most highly bound at actively transcribed genes and that this is why the overlap is so high but not necessarily specific. This idea is consistent with Fig. 4 and Fig. 5A, e.g. showing that pol II occupancy and expression is highest at genes with both BEAF-32 and NELF binding and they decrease if only one of them is present (it only made the cutoff in one dataset because of lower expression). More serious analysis on the motifs and the relationship between BEAF, NELF and pol II binding is required to establish a link between BEAF-32 binding and pol II pausing.

Second, I find the flavopiridol data hard to interpret and don't quite understand what the model is the authors propose (despite the many model figures). The authors suggest that "BEAF-32 functions to regulate NELF-mediated pol II pausing" but given the reduced occupancy of pol II upon BEAF-32 depletion, wouldn't the simplest explanation be that BEAF-32 mediates enhancer-promoter interactions and thus helps recruit pol II to paused promoters? BEAF-32 mostly binds upstream of the TSS and thus a more biochemical role in pol II pausing appears unlikely to me. If the authors did not imply such as direct role of BEAF-32 in pol II pausing, this should be more clearly explained. A more general connection between pol II pausing and insulator function has been established earlier (Chopra et al. 2009).

For a revised version, I propose to improve the language and logic of the text and shorten the figures. For example, Fig. 2A+B, Fig. 3. Fig, 4B+C, Fig. 5A, Fig. 8 could be moved to the Supplement or be removed.

Minor:

Fig S6 suggests that the +1 nucleosome shifts downstream upon BEAF-32 or NELF depletion. However, the published model suggests that NELF depletion shifts the +1 nucleosome upstream.

In Fig. 3B+D, the y axes are cut off at the bottom and the labels of both axes are misleading. What is plotted on the y axis is the gene rank, not the genes' RNA-seq data. The x axis should simply say "Genes binned by pausing indices".

Referee #3

This manuscript by Hennion et al. investigates the role of BEAF-32 binding near promoters. BEAF-32 is known to possess insulator function, but it has recently been shown to bind near promoters and its function in this context is unknown. The authors show that depletion of BEAF-32 leads to down-regulation of nearly 2000 genes that are also bound by BEAF-32 in their ChIP-seq data (suggesting that they are direct targets), indicating that BEAF-32 positively contributes to expression of these genes.
Since nucleosome occupancy is known to be low around insulator binding sites (GAF/BEAF-32/CTCF), the authors investigate nucleosome positions near TSS-associated BEAF-32 binding sites, and confirm that these too are nucleosome-deprived. Moreover, they use self-organizing maps

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to identify patterns in nucleosome profiles that are enriched at BEAF-32 associated genes, and find that these genes are characterized by a +1 nucleosome centered ~+145 with respect to the TSS. Depletion of BEAF-32 allows this nucleosome to shift back towards the promoter (to ~+135), to create a profile that is reminiscent of Pol II unbound genes, and indeed Pol II levels are shown to decrease at several BEAF target promoters upon depletion of BEAF. The authors then show that the nucleosome distribution at BEAF-32 regulated genes is similar to that recently reported at highly paused genes (including a shift of the +1 nucleosome), that BEAF-32 and NELF are often found together at promoters, and that BEAF-32 and NELF-bound promoters are highly paused. Consistent with the gene down-regulation observed, depletion of BEAF-32 is found to decrease Pol II levels near promoters and within BEAF-32-associated genes. Up to here, things look good, and convincingly indicate that BEAF-32 plays a role at paused genes. However, to get at how BEAF-32 might be working at these promoters, the authors perform experiments using FP to block pause release, but these assays are both hard to interpret, and don't appear to support the authors working model (see below), which represents a major problem with this manuscript in its current form. Finally, BEAF-32 knock-down led to the up-regulation of several hundred genes that are not bound by BEAF-32 nor paused Pol II, and appear to be neighbors of BEAF-32 down-regulated genes, leading to the sensible model that they are inadvertently up-regulated by nearby enhancers in the absence of BEAF-32 insulator activity.

Overall, there is some very nice, interesting data in this manuscript that I would like to see published. However, prior to publication, the authors should better flesh out how they think BEAF-32 is working to affect pausing, and thus gene expression at nearby promoters. The authors suggest that insulators may facilitate the release of paused Pol II, and say that "This interpretation is supported by the increase in Pol II levels at paused genes in the BEAF-KD", which would be true, if that is what they saw. However, my reading of Figure 5D is that BEAF-KD leads to less Pol II near target promoters, AND less Pol II within target genes, which is inconsistent with a role for BEAF-32 in facilitating pause release. In short, if BEAF-32 helped pause release, the its depletion should decrease pause release and lead to accumulation of Pol II near BEAF-32 -bound promoters, as is seen after FP treatment. But, BEAF-32 depletion and FP treatment are shown to have opposite effects on Pol II at these promoters. In addition a role for BEAF-32 in pause release is not supported by data showing that BEAF-32 depletion does not appear to reduce pause release immediately after FP wash-out (as they themselves state). I wonder why the authors don't favor a model wherein BEAF-32 acts like GAF near promoters- working to open chromatin so that Pol II and activators can bind, in a manner that both keeps Pol II occupancy high (favoring fast Pol II recruitment) as well as pause release through activator activity.

Additional comments and concerns:

1) The correlation between BEAF-32 and NELF binding is interesting, but since both factors seem to preferentially occupy highly active genes, how do the authors determine that this correlation is meaningful rather than being secondary/indirect?

2) The FP experiments are hard to interpret. I am not convinced that these experiments prove that recruitment is not impaired by depletion of BEAF-32. Since FP blocks Pol II release from the promoter, any Pol II recruited during the time course of this experiment would be held near the promoter as a trapped intermediate. As such, it is nearly impossible to say from this data whether the recruitment RATE is changed by BEAF-32 depletion, only that it is not blocked altogether. Given the data presented, it is still quite possible that BEAF-32 depletion impacts recruitment through its effects on nucleosome structure.

3) How many reads were obtained for MNase-seq in WT cells (Figure 2A). Although one can begin to discern a pattern in the heat map shown here, higher coverage data would be more compelling. Without knowing how many reads one has, it is impossible to determine how significant the absence of reads might be, or how significant the small shift in nucleosome position is (since the error in positioning is directly related to sequencing depth). This information must be provided for the reader to evaluate the confidence with which they can determine nucleosome positions.

4) The data are often not described in sufficient detail to permit a reader to understand what was done, nor to evaluate the analyses, and there are a number of places where useful information is omitted For example:

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Figure 1D legend reads "Venn diagram showing the intersection analysis between differentially expressed genes (2,480) whose expression is significantly higher (FDR<0.01) in WT cells compared to BEAF-KD cells as measured by RNA-Seq", and fails to mention that the intersection shown is between BEAF-32 down-regulated genes and BEAF-32 bound genes.
what is the number of genes in each group shown in figure 4C (e.g. BEAF+ NELF+, BEAF+ NELF-, etc.)?
The text reads that Pol II binding decreased at 10 out of 10 genes upon BEAF-32 depletion (Figure 5B), but Figure 5B only shows 7 genes where binding is decreased (+ 3 controls).

5) ChIP signal at up-regulated genes is too low to be interpreted, especially as graphed in Figure 7A. I recommend that the authors look at other up-regulated genes that have higher basal Pol II levels.

Minor comments:
1) For clarity, the authors should mention in the title or abstract that they are working in Drosophila.
2) The authors frequently use the word "juxtapose", which sounds awkward to a native English speaker. I think what they mean is that these BEAF-32 sites are near promoters, or "close to" them. I would recommend using these simpler words that are clearer and more accurate than juxtapose.
3) The authors point out in several places the fact that P-TEFb can be recruited by transcription activators, and cite the recent work by the Young lab as demonstrating this. In fairness, the recruitment of P-TEFb by c-myc was first shown by Peggy Farnham's group (Eberhardy, 2002), and other factors such as NF-kb, Brd4, CIITA can also serve this role. The more complete view of P-TEFb recruitment is described in a nice review by Peterlin and Price (mol cell, 2006), and I suggest modifying citations to better reflect the entire body of previous work on this topic.
4) Are the NELF ChIP-seq data shown in Figure 4A from another lab? If so, it should be made clear where these data come from. If not, much more information needs to be given about these data sets (antibodies used, westerns to show specificity, sequencing depth).

Resubmission 11 June 2013

2 years ago, our paper related to the role of Insulator proteins in nucleosome dynamics was rejected from the EMBO Journal (EMBOJ-2011-78176). We could show that Insulator proteins may impact nucleosome dynamics but the mechanism and/or co-factors involved were far from clear. At this time, the 3/3 referees gave a number of very positive feedback yet pointed out the need to provide further insights into how Insulators, maybe together with cofactors, regulate gene expression and nucleosome positioning.

We considered these criticisms and decided to biochemically purify Insulator complexes allowing to identify the H3K36 methyltransferase dMes-4/NSD as a novel co-factor of Insulator proteins. We now address the function of dMes-4 and show its pivotal role as a cofactor of Insulators favoring HAT recruitment to NFRs. Further, we show that Insulator and this HMT presets chromatin for H3K36me3 by Set2/Hypb thereby triggering nucleosome positioning along gene bodies. Of interest, defects in H3K36me3 deposition upon Insulator protein depletion further impact constitutive and alternative RNA splicing, depending on distinct (CTCF/Beaf32) insulator proteins.

We are now very pleased to submit our new manuscript entitled "**Regulating Genes Flanking Insulators involves dMes-4/Set2 as key players of Nucleosome dynamics**" to the EMBO Journal (including data from our previously submitted manuscript that are limited to Figure panels 1A-B and Supplementary Figures 1-2).

We feel that our findings will be of interest to the broad readership of EMBO Journal. In particular, our data highlight a pivotal role for H3K36me3 in triggering nucleosome positioning along gene bodies, in complete agreement with a recent Nature paper from the group of J. Workman showing that H3K36me3 regulates histone exchange/eviction. Further, recent papers have highlighted that chromosomes are partitioned into distinct physical domains that coincide with epigenetic domains bordered by Insulator protein sites. Given the implication of dMes-4 as an epigenetic regulator, our work may open new perspectives into how Insulator proteins together with specific HMTs may participate in setting up

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chromosomal borders epigenetically.

Thank you for your consideration.

2nd Editorial Decision 04 July 2013

Thank you for submitting your research manuscript entitled "Regulating Genes Flanking Insulators involves dMes-4/Set2 as key players of Nucleosome dynamics" (EMBOJ-2013-85965) to our editorial office. It has now been seen by three referees and their comments are provided below.

Referee 1 and 3 judge your findings as interesting and are in general supportive of publication in The EMBO Journal, provided that their concerns are addressed to significantly substantiate your conclusions. Reviewer 2 is more hesitant in this regard and finds the study more suitable for a less broadly oriented journal. Nevertheless, we would be willing to grant the opportunity to significantly extend and revise the current manuscript based on the constructive suggestions made by the reviewers. However, I would like to stress that your ability to extend the mechanistic depth of your manuscript is an important criterion in our editorial decision. As this will require challenging and time-consuming experiments, we would understand if you prefer to seek rapid publication elsewhere.

However, in case you do embark on revisions for our journal, please take the specified demands into careful consideration to avoid disappointments later in the process. I should add that it is our policy to allow only a single major round of revision and that it is therefore important to address the all raised concerns at this stage.
Thank you for the opportunity to consider your work for publication.

REFEREE COMMENTS

Referee #1

The manuscript titled, "Regulating genes flanking.....of Nucleosomal Dynamics", by Lhoumaud et al. uncover a mechanism by which Drosophila insulator binding proteins regulate gene expression of neighboring genes. Initially the authors show that loss of the insulator binding proteins results in altered nucleosomal occupancy over the adjacent genes. Interestingly, they find that the insulator binding protein Beaf-32 binds the histone modifying protein dMes-4, responsible for H3K36 di-methylation (H3K36me2). Loss of Beaf-32 affects H3K36 methylation, H4K16 acetylation and abrogates nucleosome positioning over promoters of genes. Interestingly, while loss of Beaf-32 does not affect dMes-4 expression or vice versa, a majority of genes regulated by Beaf-32 also depend on dMes-4. The authors suggest that H3K36me2 is necessary for NFR formation, while H3K36me3 is required for nucleosome positioning and gene splicing. Consistent with this observation, the authors show that loss of Beaf-32 results in loss of H3K36me3 over the gene body, altered nucleosome positioning and splicing defects. Additionally, the authors also find evidence of aberrant transcript production in the Beaf-32 mutant. The authors conclude that the insulator binding proteins recruit dMes-4, resulting in the promoter proximal H3K36me2, which leads to engagement of HATs and formation of the NFR. The subsequent recruitment of transcription factors such as DREF promotes elongation and the addition of the H3 K36me3 mark over the bodies of genes enhancing nucleosome positioning. The authors discuss the implications of Beaf-32 - dMes-4 interaction in regulating the functions of insulator binding proteins.
Overall, the paper is well organized and all the necessary controls are present. A few points detailed below need to be addressed before it can be accepted for publication in EMBO J.
1. Please quantify Fig. 2E and Supplementary Fig. S5 to validate the statements made in the manuscript.
2. It would be beneficial for readers if the authors included an explanation for why the ROC analysis was used in the supplementary methods section and include a reference to it in the main text.
3. On Pg 11, 2nd paragraph, the reference seems to be incorrect. Should it be Bell et. al 2007?
4. The authors conclude that Hypb mediated H3K36me3 is dependent on d-Mes4, which is why the levels of this mark are low in the Beaf-32 mutant. One interesting experiment would be to test the

levels of H3K36me2 over selected Beaf-32 dependent gene s upon Hypb KD by RTqPCR. This would conclusively show the directionality of the enzyme actions.
5. It would be conclusive if the authors take genes showing Beaf-32 binding AND change in gene expression upon loss of dMes-4 and check out the levels of a) K36me2 (promoter and gene), b) K36me3 (promoter and gene), c) nucleosome positioning (Mnase-Seq) in the Beaf-32 KD versus the WT as a control.
6. Please include the effect of H3K36 methylation in restricting the spread of silencing complexes in the discussion section (Cell Rep. 2012 Nov 29;2(5):1169-77 AND J. Biol. Chem., 286 (2011), pp. 7983-7989).

Referee #2

The manuscript by Lhoumaud et al entitled " Regulating genes flanking insulators involves dMes4/Set2 as key players of nucleosome dynamics" described a molecular dissection of how the BEAF32 protein functions at its binding sites in the Drosophila genome.
In this manuscript the authors use classic genome-wide expression analysis and ChIP analysis to dissect the mechanism by which BEAF32 recruits cofactors, modifies nucleosomes and alters chromatin structure.
The authors clearly show that BEAF32 functions by recruiting MES4 and possibly (but not shown) Set2, which then together methylate histone H3K36. They also show correlations of these histone modifications with histone acetylation but once again it is assumed that the methylation leads to acetylation but not definitively shown.

The main issue with this manuscript is it is not clear whether the authors are focused on understanding how BEAF32 functions at gene promoters or how it functions at insulators. While many IBPs were identified as proteins that bind insulators, recent studies have shown that many of these IBPs including dCTCF and BEAF32 also bind at or near promoters of genes. The authors in the introduction very succinctly and eloquently describe the fact that only some IBP bound sites function in enhancer-blocking and only a subset function as barriers. Do the remaining sites then function in gene activation (as traditional transcription activators)?
If the authors are suggesting that their results provide a basis for how insulators function then in the manuscript the authors make no attempt to determine if enhancer blocking or barrier function is compromised when BEAF32 or MES4 are down regulated.
If this manuscript is about insulators then they need to show that enhancer-promoter interaction/communication is altered upon reduction of these proteins while in the case of barrier activity they need to show changes in H3K27me3 domains upon reduction in BEAF and MES4.
If on the other hand this manuscript is describing the role of BEAF32 in transcription then the authors need to clarify how this protein is functioning given the observation that it binds the promoters of genes but affects H3K36me3 in the body of genes. Is it functioning in the release of paused polymerases and does it affect CTD phosphorylation or is it a different mechanism and if so what?
Finally the manuscript needs serious proof-reading and editing.

Referee #3

The authors addressed the question of co-factors mediating the function of Drosophila insulator factors dCTCF and Beaf32. They identified the histone methyltransferase NSD/dMes-4 interacting with Beaf32. dMes-4 co-regulates genes flanked by these insulators. Furthermore, the authors provide evidence that nucleosome positioning and RNA splicing are affected by Beaf32. These are interesting results on the function of chromatin insulation, provided that the points raised below can be addressed such that the above conclusions are still valid.

Major points:
1. Fig1A depicts all 4120 genes with a Beaf site within 500bp of the TSS. The illustrating scheme indicates that in all cases three features are together: a heterochromatic chromatin region, a Beaf32

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site and a promoter pointing away. This is very likely not the case for all 4120 Beaf sites. How often are these three features together?
2. The authors show 4120 promoters with Beaf32 binding. 2059 genes are differentially regulated after Beaf depletion with 1182 cases of promoter binding (Fig. S1). It is important to know the fraction of sites with the following features: 1. Beaf binding, 2. Beaf binding and responding to Beaf knockdown, but not control knockdown, 3. Beaf knockdown responding without Beaf binding. This class specific analysis should be done in experiments presented in Figures 1B, 1C, Fig.S3A, in order to see, how the data are supporting the proposed model in Fig.7.
3. The authors claim (page 7, bottom) "Beaf-KD significantly affected nucleosome positioning in thousands of genes (Figure 1C, see '+1') as evidenced by the changes in MNase-Seq reads along gene bodies compared to control cells."
There is an alternative explanation: Beaf32 -KD results in loss of gene activity which causes loss of nucleosomal positioning. Therefore, genes induced have to be separately compared in nucleosomal patterns with those reduced upon Beaf depletion.
4. Page 14: What are "constitutive splicing defects"? Are those seen in WT as well? And if so, can you exclude alternative splicing since exon/intron junctions are also found in alternative splice products.
5. Page 15 top: By which criteria were the genes scored as a gene with a nucleosome position defect? Change in the NFR or change in nucleosome +1?.
6. Materials and methods section explains the RNA knock down against Beaf32, dMes-4 and "control RNA". Which control RNA has been used? Throughout the manuscript and figures there is no mention of a control knock down, rather all the comparisons are against wildtype cells. If this is the case. none of the knock down experiments can be accepted knowing that unspecific knock down effects are usually observed.
7. Figure S9D: there are two out of five cases shown with an increase in exon/intron junctions. Explain why and how this is confirming splice defects as stated? On which basis have these five genes been chosen?

Minor points:
The title should read: Regulation of Genes...? In the current version it is not clear whether the genes regulate the insulator or vice versa.
Figure 3C: it is not clear what the percentages are. This requires a better explanation also by explaining the 100% level.
Figure 5C and page 14 top: Explain the meaning of 3% to 50% percentage. Which percentage? Also in Fig.5C the axis legend: "% of Genes with splicing defects in Beaf-KD / WT cells". What is 100%?
qPCR of which genes and how many have been analyzed?
Figure 5D: Again, the meaning of percentage is not evident. Explain in detail the percentage of what.
Figure 6A: Again the meaning of % is not evident. Are all genes with nucleosome position effects set to 100%?
Figure 6B: what are red and black arrows pointing at?
Page 14 center: Although dCTCF and Murine CTCF are similar, one cannot refer to Shukla et al. (Murine CTCF) when the role of dCTCF in alternative splicing is discussed.

1st Revision - authors' response 04 October 2013

We thank the three referees for their positive comments and very constructive remarks. Please see below for our detailed answer to each point (new Figure panels are indicated in bold characters).

Referee #1 (Remarks to the Author):
The manuscript titled, "Regulating genes flanking.....of Nucleosomal Dynamics", by Lhoumaud et al. uncover a mechanism by which Drosophila insulator binding proteins regulate gene expression of neighboring genes. Initially the authors show that loss of the insulator binding proteins results in altered nucleosomal occupancy over the adjacent genes. Interestingly, they find that the insulator binding protein Beaf32 binds

the histone modifying protein dMes-4, responsible for H3K36 di-methylation (H3K36me2). Loss of Beaf32 affects H3K36 methylation, H4K16 acetylation and abrogates nucleosome positioning over promsoters of genes. Interestingly, while loss of Beaf32 does not affect dMes-4 expression or vice versa, a majority of genes regulated by Beaf32 also depend on dMes-4. The authors suggest that H3K36me2 is necessary for NFR formation, while H3K36me3 is required for nucleosome positioning and gene splicing. Consistent with this observation, the authors show that loss of Beaf32 results in loss of H3K36me3 over the gene body, altered nucleosome positioning and splicing defects. Additionally, the authors also find evidence of aberrant transcript production in the Beaf32 mutant. The authors conclude that the insulator binding proteins recruit dMes-4, resulting in the promoter proximal H3K36me2, which leads to engagement of HATs and formation of the NFR. The subsequent recruitment of transcription factors such as DREF promotes elongation and the addition of the H3 K36me3 mark over the bodies of genes enhancing nucleosome positioning. The authors discuss the implications of Beaf32 - dMes-4 interaction in regulating the functions of insulator binding proteins.
Overall, the paper is well organized and all the necessary controls are present. A few points detailed below need to be addressed before it can be accepted for publication in EMBO J.
We thank this reviewer for such positive comments. Please see below for our detailed answer to each point.

1. Please quantify Fig. 2E and Supplementary Fig. S5 to validate the statements made in the manuscript.
The data have been quantified as suggested (**Supplem. Fig. S5B, S5G**) and similarly, quantification was performed to characterize the novel depletion of DREF as shown (**Supplem. Fig. S11D**).

2. It would be beneficial for readers if the authors included an explanation for why the ROC analysis was used in the supplementary methods section and include a reference to it in the main text.
A reference for ROC curve analysis has now been included as suggested, together with the rationale for using such approach.

3. On Pg 11, 2nd paragraph, the reference seems to be incorrect. Should it be Bell et. al 2007?
We have added this reference in the indicated section.

4. The authors conclude that Hypb mediated H3K36me3 is dependent on d-Mes4, which is why the levels of this mark are low in the Beaf32 mutant. One interesting experiment would be to test the levels of H3K36me2 over selected Beaf32 dependent genes upon Hypb KD by RTqPCR. This would conclusively show the directionality of the enzyme actions.
The article by Bell et al. (2007) has already reported that Hypb depletion increases H3K36me2 levels, supporting for the directionality in H3K36 methylation. We sought to test such directionality in the context of IBPs/dMes-4 and of subsequent H3K36me3 deposition. Our novel ChIP data now show that Beaf32 and dMes-4 recruit the transcriptional activator DREF to promoters (**Fig. 6A-B**). This extends recent report showing the high overlap between DREF sites and IBP sites including Beaf32 and dCTCF (Cell Cycle, 2013, Vol 12, pp 1605-1615), involving IBPs in recruiting DREF through unknown mechanism. Our novel results show that DREF depletion leads to a decrease in H3K36me3 levels (**Fig. 6E**) that is accompanied with increasing levels of H3K36me2 in promoters (**Fig. 6C**). In agreement, DREF depletion is not directly required to recruit dMes-4 (**Supplem**. **Fig. S11E**), in stark contrast to Beaf32 depletion. These results further support the directionality, i.e. from Beaf32/Mes-4 -mediated H3K36me2 to DREF/Hypb -mediated H3K36me3 upon transcriptional activation/elongation.
Please note that we could confirm the results by Bell et al. by performing ChIP of H3K36me2 in Hypb-depleted or control cells, as suggested. The results shows that Hypb-depletion leads to a moderate yet significant increase in the levels of H3K36me2

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over gene bodies as compared to control (**Figure R1**). Given that this has been published, and most important that our data already address this issue in the context of DREF recruitment through IBP/dMes-4 (**Figure 2F, 5A, 6A-F, S5D, S11E, S12A-B)**, we would rather prefer to provide such results as a separate figure panel for the referee –
(please see 'Figure R1' at the end of this file).

5. It would be conclusive if the authors take genes showing Beaf32 binding AND change in gene expression upon loss of dMes-4 and check out the levels of a) K36me2 (promoter and gene), b) K36me3 (promoter and gene), c) nucleosome positioning (Mnase-Seq)
We have performed such analyses and the results are shown in **Supplem. Fig. S7.**
The results show that Beaf32 bound genes harbor higher H3K36me2 and H3K36me3 levels compared to control genes (**Supplem. Fig. S7C-D**, compare 'Beaf32' and 'noBeaf32', respectively). Second, differentially expressed genes ('DE genes'; upon dMes-4-KD compared to control cells) are mostly enriched in high H3K36me2 levels even in the absence of Beaf32, supporting a strong linkage between such mark and the impact of dMes-4 on gene expression, in contrast to the lower H3K36me2 levels of control genes (whose expression does not vary upon dMes4-KD). Similarly, higher H3K36me3 levels are found in DE genes although in this case, a stronger dependence on Beaf32 binding is found (see below). In complete agreement, high nucleosome positioning is found for DE genes upon dMes-4KD, depending on Beaf32 binding (**Supplem. Fig. S7A-B**). In this case, we observe that changes in nucleosomepositioning upon Beaf32-KD are statistically linked to Beaf32 binding and to a lesser extent to DE genes upon dMes4-KD.
Our analyses have now been largely extended through ChIP analyses of H3K36me2 and H3K36me3 depending on presence/absence of Beaf32, dMes-4 and of the transcriptional activator DREF (**Figure 6**; please see our answer to point 4), which may better explain how Beaf32 and dMes-4 may impact H3K36me3 levels.

6. Please include the effect of H3K36 methylation in restricting the spread of silencing complexes in the discussion section (Cell Rep. 2012 Nov 29;2(5):1169-77 AND J. Biol. Chem., 286 (2011), pp. 7983-7989).
These highly relevant references have now been added and discussed with respect to insulator barriers.

Referee #2
The manuscript by Lhoumaud et al entitled " Regulating genes flanking insulators involves dMes4/Set2 as key players of nucleosome dynamics" described a molecular dissection of how the BEAF32 protein functions at its binding sites in the Drosophila genome.
In this manuscript the authors use classic genome-wide expression analysis and ChIP analysis to dissect the mechanism by which BEAF32 recruits cofactors, modifies nucleosomes and alters chromatin structure.
The authors clearly show that BEAF32 functions by recruiting MES4 and possibly (but not shown) Set2, which then together methylate histone H3K36.
We thank this reviewer for his/her positive comments. We now provide evidence that Beaf32 recruits dMes4 as shown by ChIP in Beaf32-KD compared to control cells (**Figure 2F**), in complete agreement with our novel ChIP analyses of H3K36me2 (**Supplem**. **Fig. S5D**). Furthermore, we have now clarified the function of Beaf32 and of dMes-4 with respect to H3K36me3 within the bodies of genes, which involves their requirement for the recruitment of a transcriptional activator, such as DREF (please see our answer below).

The main issue with this manuscript is it is not clear whether the authors are focused on understanding how BEAF32 functions at gene promoters or how it functions at insulators. While many IBPs were identified as proteins that bind insulators, recent studies have shown that many of these IBPs including dCTCF and BEAF32 also bind at or near promoters of genes. The authors in the introduction very succinctly and eloquently describe the fact that only some IBP bound sites function in enhancerblocking and only a subset function as barriers. Do the remaining sites then function in

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gene activation (as traditional transcription activators)?
If the authors are suggesting that their results provide a basis for how insulators function then in the manuscript the authors make no attempt to determine if enhancer blocking or barrier function is compromised when BEAF32 or MES4 are down regulated.
If this manuscript is about insulators then they need to show that enhancer-promoter interaction/communication is altered upon reduction of these proteins while in the case of barrier activity they need to show changes in H3K27me3 domains upon reduction in BEAF and MES4.
If on the other hand this manuscript is describing the role of BEAF32 in transcription then the authors need to clarify how this protein is functioning given the observation that it binds the promoters of genes but affects H3K36me3 in the body of genes.
We thank the reviewer for pointing out the need to clarify how Beaf32/dMes-4 recruitment to promoters may in turn affect H3K36me3 levels in the body of genes. Taken together with the above suggestions of the referee, it prompted us to consider how IBPs/dMes-4 might regulate transcription.
Our ChIP-Seq data previously pointed out the consensus of DREF in Beaf32 binding sites (Supplem. Fig. S7B). This transcriptional activator has been recently shown to share many binding sites with Beaf32 (Cell Cycle, 2013, Vol 12, pp 1605-1615) in agreement with previous report from our lab (Emberly et al., PloS Biol. 2008), prompting us to carefully consider the role of this factor with respect to H3K36me3 deposition.
Our novel analysis shows that the depletion of Beaf32 or dMes-4 specifically impairs the recruitment of DREF to promoters (**Fig. 6A-B**). The reverse is not true, i.e. depletion of DREF does not impair dMes-4 recruitment (**Supplem. Fig. S11**), contrasting with what we observed upon depletion of Beaf32 (**Fig. 2F**).
Additional ChIP analyses show that in contrast to Beaf32 depletion, depletion of DREF leads to increase the levels of H3K36me2 in the corresponding promoter regions (**Supplem. Fig. S5D** and **Fig. 6C,** respectively). Such increasing levels of H3K36me2 contrast with the decreasing levels of H3K36me3 upon DREF-KD as compared to controls (**Fig. 6E**). As such, the transcriptional activator DREF may be required for the transition from H3K36me2 to H3K36me3. Strongly supporting this interpretation, genome-wide analysis by ranking of genes according to H3K36me3/H3K36me2 levels shows a tight correlation between such ratio and the elongation rate of RNA Pol II (**Fig. 6E**). Moreover, the depletion of Hypb has been shown to similarly increase H3K36me2 levels (Bell et al., 2007). As such, our novel data highlight a role of Beaf32/dMes-4 in favoring the recruitment of transcriptional activators such as DREF, concomitantly with the presetting of chromatin through H3K36me2 that is required for subsequent H3K36me3 by Hypb and for RNA splicing.
Furthermore, it is interesting to note that H3K36 methylation has been involved in counteracting PRC2-mediated H3K27me3 in HeLa cells and in H3K27me3 spreading as shown in *C. elegans* further highlighting the relevance of functional interactions between IBPs and dMes-4.

Referee #3
The authors addressed the question of co-factors mediating the function of Drosophila insulator factors dCTCF and Beaf32. They identified the histone methyltransferase NSD/dMes-4 interacting with Beaf32. dMes-4 co-regulates genes flanked by these insulators. Furthermore, the authors provide evidence that nucleosome positioning and RNA splicing are affected by Beaf32. These are interesting results on the function of chromatin insulation, provided that the points raised below can be addressed such that the above conclusions are still valid.
We thank this reviewer for such positive comments. Please see below for our answer to each point.

Major points:
1. Fig1A depicts all 4120 genes with a Beaf site within 500bp of the TSS. The illustrating scheme indicates that in all cases three features are together: a heterochromatic chromatin region, a Beaf32 site and a promoter pointing away. This is very likely not the case for all 4120 Beaf sites. How often are these three features together?
Our scheme highlights the general linkage between Beaf32 binding and NFRs

preceding well positioned nucleosomes that are encountered at one side of the binding site (as shown in **Figure 1B**) unlike what is found the other side, heterochromatin or not. This is indicated in the Figure legend of panel A: " scheme illustrating the results obtained by MNase-Seq (panel B))."
To clarify this, we now indicate -in the Figure legend: ""+/- H3K27me3" indicates that although enriched within H3K27me3 domain borders, Beaf32 sites are not necessary flanking heterochromatin."
- in the Figure: "+/- H3K27me3" to clarify that the scheme does not solely apply to heterochromatin borders such as H3K27me3 borders. Furthermore, Beaf32 sites were already shown to be largely enriched at H3K27me3 domain borders (e.g. Sexton et al., Cell, 2012), an enrichment that was verified for our ChIP-Seq (930 Beaf32 sites are close to a TSS AND localize at a H3K27me3 border; p-value ~ 1e-216).

*2. The authors show 4120 promoters with Beaf32 binding. 2059 genes are differentially regulated after Beaf depletion with 1182 cases of promoter binding (Fig. S1). It is important to know the fraction of sites with the following features: **1**. Beaf binding, **2**. Beaf binding and responding to Beaf knockdown, but not control knockdown, **3**. Beaf knockdown responding without Beaf binding. This class specific analysis should be done in experiments presented in Figures 1B, 1C, Fig.S3A, in order to see, how the data are supporting the proposed model in Fig.7.*
The fraction of genes bound or not by Beaf32 and differentially expressed (DE), or the fraction of the DE genes that harbor a Beaf32 site within their promoter or not, are now indicated in **Supplem. Fig. S1D** and each percentage is clearly stated in the text (28.7%/7.9% and 57.4%/42.6%, respectively) together with the corresponding p-values (~ 1e-291 with Beaf32; p-value ~ 1 without).
We further conducted more systematic analyses of nucleosome-positioning according to Beaf32 binding (or not) and to differentially expressed (DE) genes or control genes (**Supplem. Fig. S3**). We also conducted similar analyses for DE genes upon dMes-4-KD (**Supplem. Fig. S7**). Our data show that the DE genes upon Beaf32-KD or dMes-4KD (as compared to control cells) harbor higher levels of nucleosomepositioning (panel S3A; S7A, respectively) compared to DE genes without a Beaf32 site.
In complete agreement, MNase-Seq upon Beaf32-KD shows that Beaf32 binding is a key determinant for the observed changes of nucleosome-positioning (S3B; S7B).
Furthermore, our novel data highlighting a key function of Beaf32/dMes-4 in recruiting the transcriptional activator DREF (**Fig. 6 ; Supplem. Fig. S11, S12)** further address the link between Beaf32/dMes-4 and H3K36me3 deposition/nucleosome positioning, thereby clarifying our previous model.
Please see also our response to point 3.

3. The authors claim (page 7, bottom) "Beaf32-KD significantly affected nucleosome positioning in thousands of genes (Figure 1C, see '+1') as evidenced by the changes in MNase-Seq reads along gene bodies compared to control cells."
There is an alternative explanation: Beaf32 -KD results in loss of gene activity which causes loss of nucleosomal positioning.
Our novel data support that nucleosome positioning is strongly linked to transcription indeed. This is illustrated through the role of Beaf32/dMes-4 in recruiting DREF that is required for H3K36me3 deposition upon transcriptional activation. Furthermore, the specificity for the defects in nucleosome positioning upon Beaf32-KD is best illustrated by the absence of enrichment for DE genes that are not bound by Beaf32 as shown in **Supplem. S3B-C**.

4. Page 14: What are "constitutive splicing defects"? Are those seen in WT as well? And if so, can you exclude alternative splicing since exon/intron junctions are also found in alternative splice products.
We replaced such confusing term ('constitutive') by "retained introns", i.e. the aberrant RNA product detected upon depletion of IBP and/or of dMes-4. We agree that such defects can be linked to alternative splicing products.

5. Page 15 top: By which criteria were the genes scored as a gene with a nucleosome

position defect? Change in the NFR or change in nucleosome +1?.
Nucleosome positioning in WT control or Beaf32-depleted cells was measured by adding the maximum peak intensity (in read counts) obtained from 3 separated widows (+100, +150), (+290, +320), (470, 500) and corresponding to +1, +2 and +3 nucleosome windows, as mentioned in the Supplementary Methods.
For genome-wide analyses, MNase-Seq reads within NFRs were also counted (250 to 0 bp from TSS, after normalization to the averaged read counts in neighboring regions (-1000 to -500 bp)). Note that lower levels of MNase-Seq reads were found in such regions as shown (e.g. **Fig. 2A**), thus limiting further analyses scoring variations in read counts.

6. Materials and methods section explains the RNA knock down against Beaf32, dMes-4 and "control RNA". Which control RNA has been used? Throughout the manuscript and figures there is no mention of a control knock down, rather all the comparisons are against wildtype cells. If this is the case. none of the knock down experiments can be accepted knowing that unspecific knock down effects are usually observed.
Control RNAs were synthesized against a gene encoding *luc* (*luciferase*) as a control of knock down, as now clarified in the Supplementary Methods section.

7. Figure S9D: there are two out of five cases shown with an increase in exon/intron junctions. Explain why and how this is confirming splice defects as stated? On which basis have these five genes been chosen?
We apologize for the confusing representation of the data. We now represent the levels in immature RNAs (using oligos that span exon-intron junctions) after normalization to the mRNA levels (using exon-specific oligos) for both WT control or Beaf32-KD cells as shown (**Supplem. Fig. S10**).
The list of genes was selected based upon the binding of Beaf32 or not (control genes), as in other Figure panels. This list has now been extended to those studied throughout the manuscript, as shown (**Supplem. Fig. S13)** with the exception of one gene whose levels of expression were not suitable for RTqPCR analysis (too low amounts of premRNA for accurate measures). The complete list is also provided in the Supplem.
Methods section.

Minor points:
The title should read: Regulation of Genes...? In the current version it is not clear whether the genes regulate the insulator or vice versa.
We made this change accordingly.

>Figure 3C: it is not clear what the percentages are. This requires a better explanation also by explaining the 100% level.
We now clarify this: “Percentages indicate differentially regulated genes over the total number of genes as measured for each category (harboring or not Beaf32 and/or dCTCF binding site in their promoters)”.

Figure 5C and page 14 top: Explain the meaning of 3% to 50% percentage. Which percentage? Also in Fig.5C the axis legend: "% of Genes with splicing defects in Beaf32-KD / WT cells". What is 100%?
We have now clarified this both:
- in Figure legend (now **Supplem. Fig. S10B**): “% of Genes with splicing defects in Beaf32-KD / WT cells / total number of genes in each quartile"
-in the text: “In addition, such defects tightly correlated with H3K36me3 levels over the corresponding gene bodies, the percentage of genes with splicing defects varying from <3% to ~50% over the total number of with low or high H3K36me3 levels, respectively (Supplementary Figure S10B, red bars)”

qPCR of which genes and how many have been analyzed?
All figure panels (box plots) were from analyses in 16 genes harboring a Beaf32 binding site (10) or not (6) as systematically used in all ChIP (qPCR) and expression (RTqPCR) analyses (the detailed lists are provided in the Supplem. methods section).

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Figure 5C: Again, the meaning of percentage is not evident. Explain in detail the percentage of what.
We now clarify this in the Figure legend ".../ total number of genes in each gene category"

Figure 6A: Again the meaning of % is not evident. Are all genes with nucleosome position effects set to 100%?
We now clarify this in the Figure legend ".../ total number of genes in each quartile"

Figure 6B (panel Fig. 7A for the revised manuscript): *what are the black and red arrows pointing at?*
The black and red arrows are pointing out at the linkage between NFR and H3K36me2 and nucleosome positioning and H3K36me3, respectively, as now indicated in the Figure legend.

Page 14 center: Although dCTCF and Murine CTCF are similar, one cannot refer to Shukla et al. (Murine CTCF) when the role of dCTCF in alternative splicing is discussed.
We clarified this by stating that the role of CTCF in alternative splicing (Shukla et al., 2011) was evidenced in mouse.

3rd Editorial Decision 18 November 2013

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by the same three referees; their comments are shown below.

Given the referees' recommendations, I would like to note that we are open to submission of second additionally revised version of the manuscript, if the comments of all three reviewers in the second round of review can we successfully addressed. While we normally avoid encouraging a second round of experimental revision, we note that one key experimental request made in the first round of review was not addressed in revision and we herewith reiterate the prominent previous referee request for a clear distinction between promoter and insulator function.
In summary, ref 1 raises numerous textual issues and requests more scholarly detail and rigour in the presentation - the lack of detail render it impossible to reproduce the current dataset, which is not acceptable. In further correspondence the referee also encouraged inclusion of the 'referee only figure' in supplementary information.
ref 2's key point remains that you have not clearly linked BEAF to H3K27me3 and an insulator role. While the referee does not outright ask for additional experimentation, it is in our view a key point of interest of the study and indeed reflects the statements made in title/abstract. As such, we request that this be addressed experimentally if a revision is pursued.
Notably, ref 3 agrees that the 'distinction between promoter and insulator function requested by ref 2 has not been addressed.'

If you are aiming to resubmit another revision, when preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. Please ensure that you accurately and specifically indicate where revision were made in the manuscript to address the points raised, be they textural or experimental in nature.
For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE COMMENTS

Referee #1:

The revised manuscript entitled, "Regulation of genes....Nucleosomal dynamics", by Lhoumaud et al. has satisfactorily addressed all of this reviewer's concerns. The current version of the manuscript needs to address few critical points as enumerated below before acceptance.
1. The authors should re-write the Results section to facilitate a better understanding of their manuscript by a wider audience. Since the authors have used a wide range of techniques and different pull down targets, it is absolutely essential to enunciate the exact experiment carried out. Please introduce which protein was pulled down, to carry out what experiment (e.g, Chip-Seq vs Chip q-PCR or Mnase Seq) and under what condition (e.g, WT or knock down of specific factors). By fragmenting this information among the results, methods and figure legends, the authors have missed out critical points leaving the reviewers and the readers confused. Please see specific examples below.
2. On Pg 7, last paragraph first line, was a subset of all genes used for this analysis or were the MNase profiles of all Drosophila genes averaged to generate the profile. Either way mentioning "thousands of genes" does not clarify this point.
3. Please label the y axis in Supplementary Figure 5 B correctly.
4. On Pg 10, Please refer to the correct sub figure in Supplementary Figure 5. I think it should be 5c.
5. Supplementary Figure 7 has been modified to include the previous comment of this reviewer. Although this point has been eloquently discussed in the rebuttal letter, it has been represented in a very confusing manner in the results section. The authors should include the details (as written in the rebuttal letter) in the results section and discuss the results in the Discussion. Please pay particular attention to the figure legends where the Supplementary figure 7A has either been mislabeled or misrepresented. Additionally have you represented the nucleosome profiles upon Beaf32 KD or the wild type? It is very unclear from the writeup in the results section and figure legends (an example of what I wrote in #1 above). Please include clearer explanations of the subsection C and D in this figure.
6. Page 12, last line: the heading and parts of the section "Beaf32 depletion....deposition" is confusing and misleading. H3K36me3 is not found over promoters, and the authors themselves show that. So the statement on page 13 line 7, "Genome wide....." needs to be altered along with the title. It is only the levels of H3 K36me2 that are affected near promoters.
7. Readers may be confused by the representation used by the authors in Figure 6 D and E where they show K36 methylation levels over the gene bodies, yet they mention Beaf 32 and control promoters under the graph to mean the genes where Beaf32 is bound over the promoter. Please choose an alternate representation to make the point clearer.
8. The discussion is well written, but for the first line of the 2nd paragraph on page 17. Did the authors mean that NSD/dMes4 is responsible for the active marks and DHS sites around the IBP site? The sentence certainly does not convey that meaning.

Referee #2:

The manuscript by Lhoumand et al describes the steps involved in gene regulation mediated by BEAF32/DREF, MES4 and SET2. The data delineate a pathway by which these factors cooperate to regulate a set of genes via the generation of a particular chromatin state. Their data in Drosophila are consistent with data in other organisms such as yeast and worms and are useful. The additional data in the revised manuscript support the conclusions and extend the previous results.
My main concern though is that given the focus of the introduction and discussion on the role of BEAF/DREF on insulation, the authors do not attempt to determine the changes in H3K27 me3 upon loss of these proteins. The current models in the field are that clustering of sets of promoters mediates the organization of chromatin domains. A prediction of this model is that alterations in a subset of promoters (changes in promoter architecture and activity via knockdown of BEAF or MES4) should lead to alterations in chromatin domain organization such as changes in H3K27me3.

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The authors of this manuscript are ideally placed to perform and directly test this model but unfortunately have not done so. While this does not in anyway reduce their results, at a minimum they should alter the title of the manuscript to be - Regulation of BEAF/DREF bound genes involves Mes4/Set2....

Referee #3:

This manuscript identifies dMes-4 as a co-factor for Beaf32. The authors provide support for a role of promoter bound Beaf32 in recruiting dMes-4, which methylates H3K36. Beaf32 depletion causes a reduction in dMes-4 binding, in H3K36 methylation, in H4K16 acetylation and in nucleosomal occupancy in gene bodies. These results are now further supported by additional experiments and by documenting the Beaf32 dependency of these functions. Overall the manuscript provides a substantial increase in understanding the molecular mechanisms of Beaf32. Nevertheless, the distinction between promoter and insulator function, as requested by referee 2, has not been addressed.

2nd Revision - authors' response 07 February 2014

Referee #1:
The revised manuscript entitled, "Regulation of genes....Nucleosomal dynamics", by Lhoumaud et al. has satisfactorily addressed all of this reviewer's concerns. The current version of the manuscript needs to address few critical points as enumerated below before acceptance.

1. The authors should ***re-write the Results section*** *to facilitate a better understanding of their manuscript by a wider audience. Since the authors have used a wide range of techniques and different pull down targets, it is absolutely essential to enunciate the exact experiment carried out. Please introduce which protein was pulled down, to carry out what experiment (e.g, Chip-Seq vs Chip q-PCR or Mnase Seq) and under what condition (e.g, WT or knock down of specific factors). By fragmenting this information among the results, methods and figure legends, the authors have missed out critical points leaving the reviewers and the readers confused. Please see specific examples below.*
We made every effort to provide a better explanation of the exact experiment carried out in the results section, when applicable, including the examples provided below.

2. On Pg 7, last paragraph first line, was a subset of all genes used for this analysis or were the MNase profiles of all Drosophila genes averaged to generate the profile. Either way mentioning "thousands of genes" does not clarify this point.
We have clarified this point.

3. Please label the y axis in Supplementary Figure 5 B correctly.
We have made this correction.

4. On Pg 10, Please refer to the correct sub figure in Supplementary Figure 5. I think it should be 5c.
We have made this correction.

5. Supplementary Figure 7 has been modified to include the previous comment of this reviewer. Although this point has been eloquently discussed in the rebuttal letter, it has been represented in a very confusing manner in the ***results section****. The authors should include the details (****as written in the rebuttal letter****) in the results section and discuss the* ***results in the Discussion****. Please pay particular attention to the figure legends where the Supplementary figure 7A has either been*

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mislabeled or misrepresented.
We now include such details in the results as written in the rebuttal letter. Furthermore, we made the correction in the legend of Supplementary Figure 7A.

Additionally have you represented the nucleosome profiles upon Beaf32 KD or the wild type? It is very unclear from the writeup in the results section and figure legends (an example of what I wrote in #1 above). Please include clearer explanations of the subsection C and D in this figure.
We now provide with a better description in the legends of figure panels Supplementary Figure S7A, S7C and S7D as well as in the results section.

6. Page 12, last line: the heading and parts of the section "Beaf32 depletion....deposition" is confusing and misleading. H3K36me3 is not found over promoters, and the authors themselves show that. So the statement on page 13 line 7, "Genome wide....." needs to be altered along with the title. It is only the levels of H3 K36me2 that are affected near promoters.
We have modified the title: "Beaf32 binding to promoters is indirectly required for subsequent H3K36me3 deposition over gene bodies" so it may be clearer to the reader that the link between Beaf32 depletion and such defects is not direct, as shown in the subsequent section of the results.

7. Readers may be confused by the representation used by the authors in Figure 6 D and E where they show K36 methylation levels over the gene bodies, yet they mention Beaf 32 and control promoters under the graph to mean the genes where Beaf32 is bound over the promoter. Please choose an alternate representation to make the point clearer.
We think that for clarity of the paper, it may be important to keep the same format for all box plots. However we clarify this point in the text.

8.The discussion is well written, but for the first line of the 2nd paragraph on page 17. Did the authors mean that NSD/dMes4 is responsible for the active marks and DHS sites around the IBP site? The sentence certainly does not convey that meaning.
We made this correction.

Referee #2:
The manuscript by Lhoumand et al describes the steps involved in gene regulation mediated by BEAF32/DREF, MES4 and SET2. The data delineate a pathway by which these factors cooperate to regulate a set of genes via the generation of a particular chromatin state. Their data in Drosophila are consistent with data in other organisms such as yeast and worms and are useful. The additional data in the revised manuscript support the conclusions and extend the previous results.
My main concern though is that given the focus of the introduction and discussion on the role of BEAF/DREF on insulation, the authors do not attempt to determine the changes in H3K27 me3 upon loss of these proteins. The current models in the field are that clustering of sets of promoters mediates the organization of chromatin domains. A prediction of this model is that alterations in a subset of promoters (changes in promoter architecture and activity via knockdown of BEAF or MES4) should lead to alterations in chromatin domain organization such as changes in H3K27me3. The authors of this manuscript are ideally placed to perform and directly test this model but unfortunately have not done so. While this does not in anyway reduce their results, at a minimum they should alter the title of the manuscript to be - Regulation of BEAF/DREF bound genes involves Mes4/Set2....

Referee #3:
This manuscript identifies dMes-4 as a co-factor for Beaf32. The authors provide support for a role of promoter bound Beaf32 in recruiting dMes-4, which methylates H3K36. Beaf32 depletion causes a reduction in dMes-4 binding, in H3K36 methylation, in H4K16 acetylation and in nucleosomal occupancy in gene bodies. These results are now further supported by additional experiments and by documenting the Beaf32

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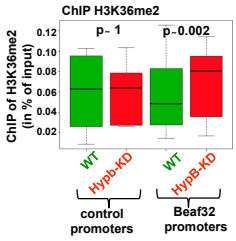
dependency of these functions. Overall the manuscript provides a substantial increase in understanding the molecular mechanisms of Beaf32. Nevertheless, the distinction between promoter and insulator function, as requested by referee 2, has not been addressed.

Lhoumaud et al., Figure R1



Legend of Figure R1:

Box plot showing the results of ChIP performed in Hypb-KD (red boxes) compared to WT-control (mock-depleted) cells (green boxes) in percent of input (y-axis) with anti- H3K36me2 antibodies or IgG control, for 16 promoters harboring a Beaf32 site or not (see Methods for a list of genes). ChIP data were analyzed by qPCR analyses in triplicates and for two independent measures normalized to three control loci.

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