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NuRD-mediated deacetylation of H3K27 facilitates recruitment of Polycomb Repressive Complex 2 to direct gene repression

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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 11 April 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below. As you will see the referees find the interdependence of the NuRD complex and PRC2 in ESC to be interesting. However, it is clear that further experimental analysis is required to make the manuscript suitable for The EMBO Journal. The main issues are the extent of this relationship in ESC by additional ChIP-seq of NuRD and PRC2 components in +/-Mbd3 cells, the recruitment and required for HDAC1/2 at these target genes and if JARID2 is required for the recruitment of NuRD and/or PRC2. Given the interest in the study, should you be able to address the concerns we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

This manuscript aims to address the functional and molecular relationship between two major and widely conserved repressors of gene expression: the Nucleosome Remodeling and histone Deacetylase complex (NuRD) and the Polycomb Repressive Complex 2 (PRC2). The mammalian NuRD complex possesses both an ATP-dependent chromatin remodeling activity, through its Mi-2 subunit, and a histone deacetylase activity, through the HDAC1 and HDAC2 subunits. Both activities are thought to work in concert to achieve transcriptional repression of target genes. The PRC2 is a distinct complex that possesses a histone methyl transferase activity through its catalytic subunit EZH2. The tri-methylation of the lysine 27 of the histone H3 (H3K27me3) is a hallmark of the Polycomb-mediated repression and is thought to mediate the PRC2-dependent repression. Both complexes are required for early mouse development and the maintenance of ES cell pluripotency. Studies in plant, worm, fly and mammalian cells suggested that NuRD and PRC2 may work together at some targets. However, the extent of such a relationship and the underlying mechanism is unknown.

The authors address these questions in mouse ES cells, using normal and knock-out cell lines combined with quantitative or high-throughput analysis of gene expression and chromatin immunoprecipitation (ChIP). They show that the NuRD complex is involved directly or indirectly in both repression and activation of several hundreds of genes in ES cells. Genes that are up-regulated in absence of NuRD are mainly associated with chromatin containing H3K4me3 alone (64%), whereas only 17.2% are associated with H3K27me3. This last association occurs almost exclusively in combination with H3K4me3. Among these 90 bivalent genes that require NuRD for their normal repression level, quantitative ChIP analysis on 5 promoters shows that 4 of them are significantly bound by NuRD in ES cells. In ES cells lacking Mbd3 (a NuRD component), binding of the PRC2 subunit Suz12 is reduced, H3K27me3 levels decreased, and H3K27Ac is increased. This suggests that the NuRD complex modulates PRC2 binding. Treatment with the class I and II mammalian HDAC inhibitor Trichostatin A (TSA) also leads to an increase of H3K27Ac levels and the decrease in Suz12 binding on these same 4 promoters, indicating that the histone deacetylase activity of NuRD might be involved in the full recruitment or the stable maintenance of PCR2 on these 4 target genes. PRC2 and NuRD are not binding to each others ruling out a direct and general PRC2 recruitment by NuRD as already suggested by the expression and ChIP-seq data. Based on these results, the authors propose a model in which the specific recruitment of the NuRD complex to a subset of bivalent genes and the consecutive deacetylation of H3K27 by its HDAC subunits, would specify and enable the stable recruitment of PRC2 and the deposition of the H3K27me3 repressive mark.

This work is in line with some previous studies showing that NuRD may modulate PRC2 function, however the extent of this relationship in mouse ESC should be addressed in a genome-wide manner. The proportion of NuRD-PRC2 common target genes where NuRD is actually able to modulate both PRC2 recruitment and activity is not analyzed and the actual degree of influence of NuRD on these PRC2 functions is not quantified. Moreover, experiments presented here do not allow a better understanding of the underlying mechanism. Indeed, observed effects on PRC2 recruitment in Mbd3-/- cells or in cells treated with TSA could be indirect effects due to the deregulation of hundreds of gene in both cases. In addition, appropriate experiments that would provide some evidence for a direct regulation of PRC2 function through NuRD deacetylase activity are lacking. In the absence of more direct data, we cannot recommend publication in EMBO journal.

Other comments:

1. ChIP-sequencing (ChIP-seq) data are lacking for NuRD despite authors have an antibody against the main NuRD subunit Mi-2beta that seems to work in ChIP (Fig.1B, 3B, 5B). Moreover, ChIP-seq for at least one PRC2 subunit (e.g. Suz12) binding should be performed in normal and Mbd3-/- cells. Such data would be critical for determining the actual extent of genes that are both targeted by NuRD and PRC2 and to properly analyze the effect of the absence of NuRD on PRC2 recruitment and activity at common target genes.

2. On page 15 the authors write "NuRD-dependent deacetylation of H3K27 makes NuRD target genes available for further repressive action by PRC2". That is a nice model, but the authors do not have evidence for this. It would be nice if the authors could support such a model by showing the sequential recruitment of NuRD and PRC2 to common target genes - for instance during the differentiation of ES cells.

3. The authors should support a role for NuRD in deacetylation of H3K27Ac on target genes by showing that HDAC1/2 bind to these target genes, and that their downregulation leads to increased H3K27Ac.

4. All quantitative ChIP analysis should be expressed in percent of input and not in relative binding to allow an estimation of actual degree of influence of NuRD on PRC2 recruitment and activity. Positive and negative control corresponding to known target and non-target should as well be included for all marks and chromatin factor tested. Finally, all ChIP or ChIP-seq data for a histone mark should be normalized by the corresponding histone (e.g. H3K27me3 should be normalized by H3).

Referee #2

The nucleosome remodelling and histone deacetylase (NuRD) complex and the Polycomb-repressive complex 1 and 2 (PRC1 and PRC2) have been implicated in mouse embryonic stem (ES) cell differentiation and lineage commitment. In this manuscript, Hendrich and colleagues describe an interesting interdependence between the two protein complexes in regulating a common set of genes (mainly bivalent genes).

The data presented support a role for the NuRD complex in deacetylating H3K27, which is a necessary step for subsequent methylation of the same residue by PRC2. Indeed, in ES cells, removal of the NuRD complex (by genetic deletion of the core component MBD3) led to a decreased occupancy of PRC2 and of the corresponding repressive marks (H3K27me3) at several NuRD target genes. Furthermore, ChIP-seq data for H3K27ac and for H3K27me3 in both wild-type and MBD3 -/- ES cells buttress this conclusion.
The data presented are convincing, and the manuscript is nicely written. The conclusion of this study, that NuRD and PRC2 act together to co-regulate transcriptional silencing in ES cells, is interesting and highly relevant for the field.

Major points:

1. NuRD complex lacking MBD3 has recently been found to be associated with Nanog in ES cells. Depletion of Mta1 or Mta2 results in different phenotypes that are distinct from ES cells with mutant MBD3, suggesting that different NuRD complexes co-exist in ES cells. This should be discussed in the text.

2. Jarid2 has been reported to be essential for PRC2 recruitment to target genes in ES cells. Did the authors check whether NuRD interacts with Jarid2? Is Jarid2 also necessary for NuRD recruitment? Is Jarid2 recruited to the common NuRD and PRC2 target genes in MBD3 wild type and mutant cells? Moreover, is the phenotype of Mi2beta-depleted ES cells similar to the MBD3 mutant ES cells or to the Eed mutant ES cells?

3. The authors should show that NuRD and PRC2 do not interact directly. How has the experiment been performed? Do NuRD and PRC2 interact in a DNA-dependent manner (e.g. in the absence of EtBr)?

4. In figure 1B, the authors should include ChIPs for MBD3, MTA2, and MTA1, to obtain better understanding of which of the NuRD "complex variants" binds to PRC2 target genes.

5. There is a significant reduction of H3K27me3 levels in the MBD3 mutant ES cells at the T promoter. The authors should comment on this.

6. A western blot of the histone deacetylases of the NuRD complex (HDAC1/2) should be included for TSA-treated cells, to verify that the observed effect is not due to a decrease in the protein levels of these enzymes.

7. What happend to the PRC1 complex in MBD3-/- cells?

8. It is not clear why the authors do not include the Chip-seq for Mi2beta in this manuscript.

9. Would TSA treatment cause a reduction of PRC2 complex occupancy in differentiated (non-proliferating) ES cells? In other words, is cell replication necessary for PRC2 displacement in the presence of H3K27ac?

Minor points:

- Abstract: the text "The Nucleosome Remodelling and histone Deactylase complex, NuRD" contains a typo (Deactylase);
- Several references do not comply with the EMBO Journal style;
- The authors should mention in the discussion section that PRC2 does not mono-methylate K27 on histone H3, and thus that an additional HMT is required to silence NuRD target genes.

Referee #3

In this manuscript, Reynolds et al showed that, in a subset of NuRD target promoters in ES cells, NuRD-mediated deacetylation of histone H3K27 facilitates PRC2 recruitment and subsequent H3K27 methylation. This study provides important mechanistic insights into the role of NuRD and PRC2 complexes in controlling the expression of genes required for early embryonic development. The experiments were performed carefully and the results are interpreted properly. There are several instances in which the conclusions could be strengthened by additional studies as described:

Several issues that need to be addressed:

1. In order to identify NuRD target genes in ES cells, the authors performed ChIP experiments using anti-Mi-2beta antibody (Fig. 1B). The enrichment of most of the sites was low likely due to the quality of the Mi-2beta antibody and/or the fact the Mi-2beta does not interact with chromatin directly. To be certain that the ChIP is working, it's necessary to perform ChIP in both wild-type and Mbd3-/- cells or in Mi-2beta knockout/knockdown ES cells. Alternatively, it will be more convincing to show the ChIP for HDAC1/HDAC2, which directly mediates deacetylation within the NuRD complex.

2. Similarly, in Fig. 1C, it is necessary to perform ChIP with Ezh2 besides Suz12 since Ezh2 is the enzymatic subunit of the PRC2 complex that directly mediates methylation of H3K27.

3. To confirm NuRD binding to its target genes, the authors only showed loci that are marked with H3K4me3 alone or H3K4me3/H3K27me3. It will also be interesting to show a few loci that only contain H3K27me3 mark.

4. To demonstrate reciprocal changes in H3K27 acetylation and methylation in the absence of NuRD (Mbd3), the authors examined two representative loci Htra1 and T (Fig. 3B). Since ChIP-seq was performed in WT and Mbd-/- ES cells (Fig. 3A), it will be more convincing to show the ChIP-seq data/plots rather than the ChIP-PCR data.

5. The authors may consider presenting the gene expression data (Fig. 6A) first before focusing on several representative genes (Fig. 1).

1st Revision - authors' response 07 October 2011

(Please see next page)

Referee #1 (Remarks to the Author):

This work is in line with some previous studies showing that NuRD may modulate PRC2 function, however the extent of this relationship in mouse ESC should be addressed in a genome-wide manner. The proportion of NuRD-PRC2 common target genes where NuRD is actually able to modulate both PRC2 recruitment and activity is not analyzed and the actual degree of influence of NuRD on these PRC2 functions is not quantified.

We now provide ChIP-seq for PRC2 component Suz12 in wild type and Mbd3-null ES cells to directly address this point. We find that approximately 25% of those peaks of Suz12 binding in wild type ES cells are not found in Mbd3-null ES cells. We further cross-reference this list with those found in our Mi2β ChIP-Seq data, and are left with a set of predominantly transcription factor genes, many of which are known to play important roles in ES cells and in early mouse development (Table 1).

Indeed, observed effects on PRC2 recruitment in Mbd3-/- cells or in cells treated with TSA could be indirect effects due to the deregulation of hundreds of gene in both cases. In addition, appropriate experiments that would provide some evidence for a direct regulation of PRC2 function through NuRD deacetylase activity are lacking.

We now include a time course experiment in which Mbd3 is restored to Mbd3-/- ES cells using a tamoxifen-inducible transgene. Within hours this restores NuRD-dependent transcriptional silencing of the Htra1 locus. We show that in the same time scale this silencing is associated with loss of H3K27Ac and gain of H3K27Me3 and PRC2 component association. This all occurs within 40 hours of induction, ruling out the possibility of artifacts of long term culture.

Other comments:

1. ChIP-sequencing (ChIP-seq) data are lacking for NuRD despite authors have an antibody against the main NuRD subunit Mi-2beta that seems to work in ChIP (Fig.1B, 3B, 5B). Moreover, ChIP-seq for at least one PRC2 subunit (e.g. Suz12) binding should be performed in normal and Mbd3-/- cells. Such data would be critical for determining the actual extent of genes that are both targeted by NuRD and PRC2 and to properly analyze the effect of the absence of NuRD on PRC2 recruitment and activity at common target genes.

Both Mi2β and Suz12 ChIP-seq datasets are now included in the manuscript.

2. On page 15 the authors write "NuRD-dependent deacetylation of H3K27 makes NuRD target genes available for further repressive action by PRC2". That is a nice model, but the authors do not have evidence for this. It would be nice if the authors could support such a model by showing the sequential recruitment of NuRD and PRC2 to common target genes - for instance during the differentiation of ES cells.

We now include a time course experiment in which Mbd3 is restored to the

nuclei of *Mbd3*$^{-/-}$ ES cells using a tamoxifen-inducible system. Mbd3 can quickly be detected at the promoter of a NuRD/PRC2 target gene, and Jarid2 association lags behind. Loss of H3K27Ac is paralleled by gain of H3K27Me3 (Figure 6).

3. The authors should support a role for NuRD in deacetylation of H3K27Ac on target genes by showing that HDAC1/2 bind to these target genes, and that their downregulation leads to increased H3K27Ac.

We now also provide Hdac1 ChIP data demonstrating its presence at many NuRD targets (Supplementary Figure 1). Our TSA experiments demonstrate that Class I and/or Class II HDAC activity is required for this deacetylation, and ChIP for histone modifications in our Mbd3-null cells demonstrate that NuRD is specifically required for deacetylation at NuRD targets only.

4. All quantitative ChIP analysis should be expressed in percent of input and not in relative binding to allow an estimation of actual degree of influence of NuRD on PRC2 recruitment and activity.

While the magnitude of binding of these complexes at individual loci may be of interest for those specific regions, the relative differences are actually the key point we are trying to make in this paper. For example, a region may have relatively low quantities of a particular histone mark or regulatory complex, but it is the change in levels which is important for any overall transcriptional changes. In all cases shown, we are comparing ChIP data for identical antibodies across different cell lines or drug treatment and feel that displaying relative amounts more aptly illustrates the effect we see.

Positive and negative control corresponding to known target and non-target should as well be included for all marks and chromatin factor tested.

Targets and non-targets are included throughout.

Finally, all ChIP or ChIP-seq data for a histone mark should be normalized by the corresponding histone (e.g. H3K27me3 should be normalized by H3).

In the literature there is no consensus on how to normalize ChIP-seq data of histone modification, and as a result numerous different normalization methods are being used, each has its own advantages and drawbacks. Methods currently used include background subtraction (Szulwach et al, 2011), fold change or percent of input (Bhandare et al, 2010), and normalization relative to nucleosome density (Dhami et al, 2010) A major disadvantage of the latter is that it is complicated to quantitatively compare signal levels obtained by two different antisera. Every antibody preparation has different epitope binding kinetics that are only linear in a specific range of input chromatin. The conclusions we draw from our ChIP-qPCR and ChIP-Seq data are based on relative quantities between cell types for the same antibody in every case. Rather than normalising to H3 signal, we have now normalised all ChIPSeq data using the background subtraction method. In addition, relative changes in H3 ChIP between wild type and Mbd3 null cells is

included in Figure 2, which is an important control demonstrating that the relative changes in histone marks that we see are not due to changes in nucleosome levels at the positions assayed.

Referee #2 (Remarks to the Author):

1. NuRD complex lacking MBD3 has recently been found to be associated with Nanog in ES cells. Depletion of Mta1 or Mta2 results in different phenotypes that are distinct from ES cells with mutant MBD3, suggesting that different NuRD complexes co-exist in ES cells. This should be discussed in the text.

We now cite papers showing Oct4-NuRD interactions in the text. Neither the van den Berg nor the Pardo studies found any evidence for the "NODE" complex reported by Liang et al., neither did a previous Nanog-interaction paper from the Orkin lab. Different Mta-containing subcomplexes will likely all contain Mbd3 or Mbd2, however Mbd2 is present a very low levels in ES cells (as compared to HeLa cells, for example) and our previous work showed that very little, if any functional NuRD can form in Mbd3-null ES cells (Kaji et al. 2006 Nature Cell Biology)

2. Jarid2 has been reported to be essential for PRC2 recruitment to target genes in ES cells. Did the authors check whether NuRD interacts with Jarid2? Is Jarid2 also necessary for NuRD recruitment? Is Jarid2 recruited to the common NuRD and PRC2 target genes in MBD3 wild type and mutant cells?

This is a very important control as Jarid2 is implicated in targeting PRC2 activity, and we thank the reviewer for pointing this out. We now include Jarid2 ChIP and use of Jarid2-null ES cells to address these points. We find that NuRD does not interact with Jarid2, but that Jarid2 recruitment is dependent upon NuRD activity.

Moreover, is the phenotype of Mi2beta-depleted ES cells similar to the MBD3 mutant ES cells or to the Eed mutant ES cells?

Knockdown of NuRD components in ES cells has proven unsatisfying in our hands as RNAi never completely removes gene activity. A manuscript describing the Mi2β-null phenotype is currently in preparation.

3. The authors should show that NuRD and PRC2 do not interact directly. How has the experiment been performed? Do NuRD and PRC2 interact in a DNA-dependent manner (e.g. in the absence of EtBr)?

We now include IPs for NuRD and PRC2 components, with and without nucleases (Supplementary Figure 5).

4. In figure 1B, the authors should include ChIPs for MBD3, MTA2, and MTA1, to obtain better understanding of which of the NuRD "complex variants" binds to PRC2 target genes.

ChIP for avi-Mbd3, Mta2 and Hdac1 are now included as Supplementary Figure 1

5. There is a significant reduction of H3K27me3 levels in the MBD3 mutant ES cells at the T promoter. The authors should comment on this.

There is a subtle change in H3K27methylation here, but is not of sufficient magnitude to be picked up in the ChIP-seq as a differentially methylated region. It is also not balanced by an increase in acetylation or significant change in expression.

6. A western blot of the histone deacetylases of the NuRD complex (HDAC1/2) should be included for TSA-treated cells, to verify that the observed effect is not due to a decrease in the protein levels of these enzymes.

This is indeed an important control and is now included as Figure 5D.

7. What happend to the PRC1 complex in MBD3-/- cells?

We have not addressed this, as we have focused on the methylation-deacetylation cycle, which only concerns NuRD and PRC2. However this could be an interesting point to follow-up later given the new questions regarding the relationship between PRC1 and PRC2 activities arising from the Wutz and Bickmore laboratories.

8. It is not clear why the authors do not include the Chip-seq for Mi2beta in this manuscript.

ChIP for Mi2beta does not produce the kind of enrichment normally seen for transcription factors or histones. While we have no problem generating significant enrichment levels by ChIP-PCR, this low enrichment proved a problem for ChIP-seq. We have subsequently repeated the ChIP-Seq including an extra cross-linking step, which did increase enrichment. The Mi2beta ChIP-seq data we now include produce very high confidence peaks, however we know that these data do not include all Mi2beta binding sites. Nevertheless it provides a large, conservative dataset with which to compare Mi2beta and PRC binding genome-wide.

9. Would TSA treatment cause a reduction of PRC2 complex occupancy in differentiated (non-proliferating) ES cells? In other words, is cell replication necessary for PRC2 displacement in the presence of H3K27ac?

This is an interesting question regarding the mechanism of action of PRC2, but as this study is primarily about NuRD function we have not addressed it here.

Minor points:
- Abstract: the text "The Nucleosome Remodelling and histone Deactylase

complex, NuRD" contains a typo (Deactylase);
- Several references do not comply with the EMBO Journal style;
- The authors should mention in the discussion section that PRC2 does not mono-methylate K27 on histone H3, and thus that an additional HMT is required to silence NuRD target genes.

We have addressed all of these points.

Referee #3 (Remarks to the Author):

1. In order to identify NuRD target genes in ES cells, the authors performed ChIP experiments using anti-Mi-2beta antibody (Fig. 1B). The enrichment of most of the sites was low likely due to the quality of the Mi-2beta antibody and/or the fact the Mi-2beta does not interact with chromatin directly. To be certain that the ChIP is working, it's necessary to perform ChIP in both wild-type and Mbd3-/- cells or in Mi-2beta knockout/knockdown ES cells. Alternatively, it will be more convincing to show the ChIP for HDAC1/HDAC2, which directly mediates deacetylation within the NuRD complex.

We now include ChIP for Hdac1 and Mta2 (Supplementary Figure 1). It is not straightforward to interpret ChIP for Mi2beta in Mbd3-null ES cells. In the absence of Mbd3, Mi2beta may still be recruited to some targets in the absence of the rest of the NuRD complex.

2. Similarly, in Fig. 1C, it is necessary to perform ChIP with Ezh2 besides Suz12 since Ezh2 is the enzymatic subunit of the PRC2 complex that directly mediates methylation of H3K27.

We show that both Suz12 and Jarid2 fail to be recruited in the absence of Mbd3. We feel that this is sufficient to report the presence or absence of PRC2, and are not aware of evidence that Ezh2 functions independently of PRC2. Indeed the lack of H3K27Me3 provides further evidence for the lack of Ezh2 activity at these targets.

3. To confirm NuRD binding to its target genes, the authors only showed loci that are marked with H3K4me3 alone or H3K4me3/H3K27me3. It will also be interesting to show a few loci that only contain H3K27me3 mark

This represents an extremely small proportion of genes (0.2%), so we decided not to consider these for this study.

4. To demonstrate reciprocal changes in H3K27 acetylation and methylation in the absence of NuRD (Mbd3), the authors examined two representative loci Htra1 and T (Fig. 3B). Since ChIP-seq was performed in WT and Mbd-/- ES cells (Fig. 3A), it will be more convincing to show the ChIP-seq data/plots rather than the ChIP-PCR data.

The ChIP-seq data are available and show the same trends, but our high

density q-PCR plots demonstrate this difference more clearly.

5. The authors may consider presenting the gene expression data (Fig. 6A) first before focusing on several representative genes (Fig. 1).

We have opted do use the gene expression data to address the question of overall significance at the end, while using our representative genes to explore mechanism from the outset.

Bhandare R, Schug J, Le Lay J, Fox A, Smirnova O, Liu C, Naji A, Kaestner KH (2010) Genome-wide analysis of histone modifications in human pancreatic islets. *Genome Res* **20**(4)**:** 428-433

Dhami P, Saffrey P, Bruce AW, Dillon SC, Chiang K, Bonhoure N, Koch CM, Bye J, James K, Foad NS, Ellis P, Watkins NA, Ouwehand WH, Langford C, Andrews RM, Dunham I, Vetrie D (2010) Complex exon-intron marking by histone modifications is not determined solely by nucleosome distribution. *PLoS One* **5**(8)**:** e12339

Szulwach KE, Li X, Li Y, Song CX, Han JW, Kim S, Namburi S, Hermetz K, Kim JJ, Rudd MK, Yoon YS, Ren B, He C, Jin P (2011) Integrating 5-hydroxymethylcytosine into the epigenomic landscape of human embryonic stem cells. *PLoS Genet* **7**(6)**:** e1002154

2nd Editorial Decision 26 October 2011

Thank you for submitting your revised manuscript for consideration by the EMBO Journal. It has now been seen once more by the three referees whose comments are enclosed. As you will see, all find that the study has been significantly strengthened, although there are a couple of issues from the first round that have not been satisfactorily addressed and should be taken care of before the manuscript is published in The EMBO Journal.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

This is an improved revised version of the manuscript, in which the authors have worked extensively to address the reviewers' comments and criticisms:

I have two related comments though:

1. We asked the authors to present the ChIP-qPCR data throughout the manuscript as "%bound/input". This is becoming a standard for ChIP data, because it gives an immediate idea of how robust the ChIP results are. I agree with the authors that the main point they would like to make is the relative differences. In fact, all papers would like to show relative differences, however, the question is whether "enrichment" over "background", i.e. "relative enrichment" is a good way to calculate a signal. "Background" will without doubt vary extensively from one experiment to another, and it does not give a good impression of how robust and reproducible the ChIP results are. In fact it is not clear, what the authors present in the ChIP figures. For instance in Figure 1b, do the authors show technical or biological replicates? On page 8 the authors state: " [they]... assigned an arbitrary threshold of 2-fold enrichment relative to the IgG control to designate genes as bona fide targets". How is 2-fold determined to be significant? Was it found to be statistically significant in biological replicates?

2. Related to the first comment (also raised by the two other reviewers). The authors need to use a proper control for the Mi2b ChIP, and ChIP-seq, i.e. Mi2b knockdown or knockout ES cells. In response to reviewer #2, the authors state that they have not been able to knock Mi2b down efficiently, however, they also state that they have generated Mi2b null cells. These cells should be used as controls for the specificity of the Mi2b ChIP experiments.

Referee #2

Reynolds et al. have now revised their work considerably by providing a number of additional experiments. Some of these expand on and lending further support of the proposed model. Moreover

a number of controls are now included. Together these additional experiments make the manuscript considerably stronger.

Referee #3 (Remarks to the Author):

the revision responds to the prior criticisms

2nd Revision - authors' response 27 October 2011

(Please see next page)

1. We asked the authors to present the ChIP-qPCR data throughout the manuscript as "%bound/input". This is becoming a standard for ChIP data, because it gives an immediate idea of how robust the ChIP results are. I agree with the authors that the main point they would like to make is the relative differences. In fact, all papers would like to show relative differences, however, the question is whether "enrichment" over "background", i.e. "relative enrichment" is a good way to calculate a signal. "Background" will without doubt vary extensively from one experiment to another, and it does not give a good impression of how robust and reproducible the ChIP results are.

I find ChIP data expressed as Relative Enrichment in papers currently in EMBO Journal's Advance Online Publications, e.g. Martin et al., Figures 5 and 6; Zwart et al., throughout, Yang et al. several figures. While it may be preferable to plot data as % input in some cases, this is not necessarily the most suitable way to show the data in all cases, and is clearly not EMBO Journal policy.

ChIP for Mi2beta does not produce the kind of enrichment normally seen for transcription factors or histones. This is likely to be due to the nature of Mi2beta, as overexpression of an Avi-tagged protein produced even lower ChIP enrichment (these data are in a paper under review elsewhere). With low amounts of template DNA variations in efficiency of PCR primers becomes more apparent, so we feel that displaying the ChIP data as relative enrichment would be far less likely to result in spurious positives and negatives. That the Mi2beta ChIP produces very low enrichment is evident from the ChIP-seq data and is mentioned in the text.

Nevertheless we have replaced Figures 1b and 1c with ChIP data expressed as % input. That we see significant changes in ChIP when the data are displayed using either method provides further confidence in the legitimacy of the results. This does not change any of our subsequent analyses or conclusions. The rest of the figures are designed to show relative enrichment in different conditions, so have been left unchanged.

In fact it is not clear, what the authors present in the ChIP figures. For instance in Figure 1b, do the authors show technical or biological replicates?

As stated in our Methods section under "Chromatin Immunoprecipitation": "All qPCR was carried out in triplicate for at least three biological replicates." Therefore it is clear what is presented in the figures. That we have displayed the data from at least three biological replicates demonstrates exactly how robust and reproducible the results are.

On page 8 the authors state: " [they]... assigned an arbitrary threshold of 2-fold enrichment relative to the IgG control to designate genes as bona fide targets". How is 2-fold determined to be significant? Was it found to be statistically significant in biological replicates?

2 fold was an arbitrary cut off since we find Mi2beta to ChIP broadly across many loci. Nevertheless we have removed this arbitrary cutoff and indicated all samples where Mi2beta and Suz12 chipping is significantly ($p<0.005$) enriched over IgG using a t-test.

2. Related to the first comment (also raised by the two other reviewers). The authors need to use a proper control for the Mi2b ChIP, and ChIP-seq, i.e. Mi2b knockdown or knockout ES cells. In response to reviewer #2, the authors state that they have not been able to knock Mi2b down efficiently, however, they also state that they

have generated Mi2b null cells. These cells should be used as controls for the specificity of the Mi2b ChIP experiments.

We stated that we are preparing a description of the Mi2beta null phenotype. This refers to embryos, not ES cells. ES cells die very quickly upon deletion of Mi2beta so cannot be used as a control for ChIP. Knockdown similarly depletes the population of cells lacking Mi2beta.