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Regulation of the osteoblast-specific transcription factor Osterix by NO66, a Jumonji family histone demethylase

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1st Editorial Decision 06 February 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see the referees consider the manuscript as being an interesting one in principle. Still they raise a number of significant concerns that need to be addressed by further experimentation. More specifically, apart from a number of important additional controls and some additional data to strengthen the conclusions stronger evidence for the physiological significance of your findings will be needed (see referee 2). Furthermore referee 3 feels that at least some deeper analysis of how NO66 and Osx affect each other's occupancy of target gene promoters should be included. Taking together all issues raised I have thus come to the conclusion that we would be happy to consider a revised version of this manuscript, in which the referees' concerns need to be addressed in an adequate manner. 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.

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 (Remarks to the Author):

The authors have identified a novel Jumonji family histone demethylase (NO66) with activity against H3K4 and H3K36 and involved in regulating the transcriptional activation activity of the osteoblast-specific transcription factor Osterix. This finding is exciting not only because it adds to our understanding of how osteoblast differentiation is controlled and how transcription factors interact with chromatin modifying enzymes but also in the identification of a histone demethylase with novel substrate specificities. While the mechanism of NO66 inhibition of Osx transcriptional activity is not addressed in any detail, the work presented here lays a foundation for future work in understanding how histone demethylases regulate transcription factor activity. In reading the paper I had two major concerns:
1. The authors clearly show that NO66 HDM activity is required for Osx inhibition (Fig 5E). However, the claim (made from data presented in Fig 4C) that the interaction between NO66 and Osx is required for inhibition of Osx is not conclusively shown. While the delta-JmjC NO66 protein does not interact with Osx (Fig4B) and cannot fully inhibit Osx activation (Fig4C), this protein most likely does not have HDM activity. Therefore, because the authors later show that HDM activity is most certainly required for inhibition this mutant (delta-JmjC) cannot be used to conclude that NO66 and Osx interaction is required for inhibition. The conclusions drawn from the data presented in Fig4C and presented in the section titled "The JmjC domain of NO66 interacts with the transactivation domain of Osx to inhibit the transcriptional activity of Osx" need to be reconsidered. The authors could design additional experiments to distinquish binding and HDM activity by more finely mapping the Osx binding domain or, generating an Osx mutant unable to bind NO66 but still able to activate transcription.
2. The specificity of NO66 demethylating both H3K4 and H3K36 is (as far as I know) a unique combination of substrates. The authors do not address this and do not mention whether they tested/observed activity against any other substrates. I assume that since only K4 and K36 are mentioned that those were the only substrates NO66 was observed to act on, however it would be good for this to be mentioned specifically. On the same topic the above activity is shown convincingly in vitro however the in vivo data is presented in a way that does not clearly support the in vitro data. Only H3K4 in vivo demethylation is shown while the authors mention that H3K36me3 was observed. The authors should at least show the K36 data in a supplemental figure as it is important to clearly demonstrate such a unique combination of specificities in vivo to rule out artifact activity in an in vitro system. Concerns about substrate specificity under the extreme conditions of an in vitro assay and an over-expression in vivo system are somewhat allayed by the data presented in Fig 6D where the presence of NO66 on a promoter region is correlated with lower levels of H3K4me3 and H3K36me3, however no statistical analysis is shown to demonstrate a true difference (this is particularly noticeable for the H3K36 figure where the differences are much less pronounced). Again the case may also benefit from the mention that no other markers such as K9 or K27 were affected.
I also had several minor concerns while reading the article.
1. It might be informative to show a sequence alignment of NO66 and other Jumonji family proteins especially give that point mutants are made in conserved residues however we are give not information on this supposed conservation.
2. It is not clear from Figure 3B that knockdown of NO66 causes the cells to differentiate sooner as apposed to causing a greater percentage of the cells to differentiate. If it is possible to show a later time point for the WT where the same percentage of cells have differentiated just at a later time point. At the very least the authors might mention and site a typical time point when a similar percentage of the WT cells would be differentiated as that shown for day 24 of the knockdown.
3. In figure 4A labels for the two mutant proteins would be helpful. One protein is designated as a JmjC deletion mutant however both proteins shown in the schematic contain at least part of the JmjC domain. Calling a peptide delta-JmjC and concluding that the JmjC domain interacts with the transactivation domain of Osx (as stated in the heading of the fourth section of the results) is misleading since region deleted and shown to have an affect on binding includes only a portion of the JmjC domain but also a large part of NO66 outside the JmjC domain.

Referee #2 (Remarks to the Author):

Description

This study examines regulation of Osterix (Osx) an essential osteoblast-specific transcription factor for skeletal development. The focus of the paper is on the functional activity of Jumonji C (JmjC)-domain containing protein called N066 identified as an Osx-interacting protein using a proteomics approach. Convincing evidence is presented to show that N066 directly interacts with Osx, inhibits Osx-mediated promoter activation, and its cellular levels affect the program of expression of Osx target genes in knockdown study (Fig. 1, 2, 3). The mechanism for negative regulation was interrogated by characterizing N066 demethylase activity for both H3K4me and HeK36me (Fig. 4 and 5). The biological activity of N066 in regulating gene expression for bone formation was further shown by demonstrating a correlation between the decrease in N066 occupancy and the increase of Osx occupancy at Osx-target promoters (Fig. 6). Together these studies provide novel insights into requirements for regulation of a key transcription factor that establishes the osteoblast phenotype. In a broader context, the data set support a growing concept for transcriptional repression and activation of genes through very specific chromatin. However, some of the data needs clarification by added controls that are not found in the four supplementary figures to strengthen the conclusions.

Comments

1. Figure 1 shows Osx and N066 physically interact. What is missing is control Western for a known Osterix interacting protein that copurifies with Flag-HA Osx by tandem immunoaffinity column in figure 1B, for example ATF4 is a known OSX. Also this figure should show data for endogenous Osx and N066 proteins interacting in primary osteoblasts (mouse or rat). For figure 1D, do the authors have any coIP (both ways Osx→N066 or N066→Osx)? A coIP in preosteoblasts and differentiated cells should be compared to appreciate when N066 repressor activity of Osx predominates.

2. Figure 2 shows N066 expression represses Oxs transcription in promoter assay. This conclusion, Osx-270 mutant (-DNA binding but have Trans activation domain) would be strengthened if the Osx sites were mutated or use in an increasing fashion. Did the authors try mutated (Osx binding mutation in the proximal region) OCN or BSP promoter in this promoter activity studies? What is the justification of using HEK cells in this study? A comparison should be made, MC3T3 preosteoblasts, to identify if the N066 effects are specific to Osx in osteoblasts. In figure 2C, the N066 western is not of good quality. Please explain the duplex. Again, this study should be performed in mouse osteoblasts where BMP2 induction is not required. The greater effects on BSP could be due to an increased level in response to BMP2. Figure 2D, other osteoblast markers should be added, Runx2 and alkaline phosphatase expression.

3. Figure 3B control is not appropriate. One needs an sh knock-down to a scrambled sequence and sh stable clone of MC3T3, and not simply "WT" cells.

4. Fig. 4 maps the JmjC domain of N066 to the TA domain of OSX. Why in figure 4C does the 'bar graph' 8, show that in presence of ΔJmjC, which does not have functional demethylase domain, a 50% level of GAL-DBD- Osx activation? Please explain the contribution of the whole domain versus the partial deletion of JmjC. The conclusion needs to be modified. In the lower panel the western profile is different for each clone. How did you normalize your transfection experiment? In figure 4D, why is the basal transcriptional activity for 5xGAL-4 Luc two fold less in the treatment vs. nontreatment group (bar graph 2, 6 and 10)?

5. In figure 5B, did you try H3K9me3? If so add comment.

6. In the figure 6C as a control the authors need to use 3'UTR fragment of OC or BSP gene and show no binding. The N066 occupancy (fig. 6C Rt panel, fig 6D Lt panel) in OC and BSP promoter is very poor (0.02 to 0.04). Upon BMP2 treatment for 15 hrs the changes of occupancy for N066 are in the decimal range of 0.01% decrease. How the authors claim significant remodeling in H3K4me3 mark in both OC and BSP promoter? Could you please demonstrate these results in primary calvarial (mouse or rat) osteoblast? Also show that N066 binding will be reduced if you knock down Osterix (in Osterix het mouse model or siRNA knock down). In figure 6D please include the

actylation status, Osterix and PolII recruitment in {plus minus}BMP samples. With these added studies, the authors could suggest a molecular model for such epigenetic changes in bone specific promoters.

7. Other minor:

Abstract-line 3, not clear to readers to state "to understand transcriptional activation of Osx" and then characterize a negative regulator. Suggest rephrase to understand "regulation of Osx".

Introduction page 3, line 19 the authors wrote "Several others........osteoblast (....)". References for the homeodomain (Msx and Dlx) regulation of bone formation need to be included, e.g. Hassan et al; Cheng et al; add ref attached.

Referee #3 (Remarks to the Author):

Sinha et al. (de Crombrugghe), EMBO J.

The authors have identified the demethylase NO66 as a protein that interacts with the osteogenic transcription factor Osx. They define its histone demethylase activity and show that it represses Osx activity and, presumably as a consequence, osteoblast differentiation. Finally, they demonstrate that Osx and NO66 interact with the same regulatory DNA segment of an Osx target gene, and that the occupation of the chromatin by NO66 decreases as the occupation by Osx increases.

The identification of a physical and functional interaction of the histone demethylase NO66 and Osx is novel and interesting. The experiments are well done and the conclusions are justified.

My major problem with the manuscript is that the manuscript does not go far enough and, from my perspective, ends with a question: considering the interaction of Osx and NO66 (i.e. the first part of the manuscript), how come that NO66 is removed from the chromatin/DNA segment as Osx starts occupying it? The authors realize that this is a major issue and go through substantial speculation in the Discussion, but do not provide any experiments or data to address this issue. Even relatively simple experiments like evaluating whether this is competition, or requires the enzymatic activity of NO66 are not done. Maybe NO66 and Osx do/can not even associate with each other when bound to chromatin/DNA. So, my take on this manuscript is that the authors should extend the data with some mechanistic insight that demonstrates what is happening at the regulatory Osx target gene sequence.

Minor comment:
- It would be nice to show the coincident expression of Osx and NO66 in developing bones in the manuscript, rather than in supplementary data.
- page 9, last lines (i.e. the title) "interacts with the ..." should be "is required for the interaction with....".
- page 10, 1st line: This is the first time that I find out that the Osx used in Fig. 1E is in fact only it's transactivation domain. That information was not even in the figure legend. Please be clear about this when discussing Fig. 1E.

1st Revision - authors' response 11 July 2009

RESPONSE TO REVIEWERS

Reviewer 1

Major comments

1.The conclusions drawn from the data presented in the section titled "The JmjC domain of NO66 interacts with the transactivation domain of Osx to inhibit the

transcriptional activity of Osx" need to be reconsidered. The authors could design additional experiments to distinquish binding and HDM activity by more finely mapping the Osx binding domain or, generating an Osx mutant unable to bind NO66 but still able to activate transcription.

We fully agree with the reviewer that our previous data fell short of proving that interactions between NO66 and Osx are required for the inhibition of Osx activity. Although our results demonstrated that demethylase activity of NO66 was required for the inhibition of Osx, the NO66 mutants that we used in the interaction and reporter studies did not allow us to determine conclusively whether physical interactions between the two proteins were needed for this inhibition. For example, the NO66 mutant (deletion in JmjC domain, M2) was unable to inhibit Osx activation, but this could be either due to loss of demethylase activity in this mutant or due to loss of its interaction with Osx (Fig. 5C). Hence, as suggested by this reviewer to distinguish interaction and HDM activity, we mapped the NO66 interacting domain in Osx using Osx deletion mutants. These mutants were tested for their ability to interact with NO66 in co-IP experiments and reporter assays were used to determine whether NO66 could suppress activation of a target gene by the different Osx constructs. We have now added experiments that show an Osx mutant (89-428), that failed to interact with NO66, was not inhibited by wild type NO66 (Fig. 5D & E). Thus we have separated the effects of loss of HDM activity in NO66 from loss of interactions of Osx with NO66.

We think the reviewer will now agree that, with these experiments, we have adequately supported our conclusion.

2. The specificity of NO66 demethylating both H3K4 and H3K36 is (as far as I know) a unique combination of substrates. The authors do not address this and do not mention whether they tested/observed activity against any other substrates

A. We are thankful to this reviewer for his/her comments and have now added a text regarding the unique specificity of NO66 to the Discussion.

B. We have added a panel to Figure 5 (Fig. 5E) and a Supplemental Figure (Suppl. Fig. 4) to demonstrate that H3K9me3 and H3K27me3 are not substrates for NO66 demethylase activity in vitro.

C. As requested by this reviewer, we have added evidence that levels of H3K27me3 in the Osx-target Bsp gene were not changed in BMP-2 treated MC3T3 cells, whereas the levels of H3K4me3 and H3K36me3 increase in BMP-2 treated cells.

D. For ChIP experiment (Fig 6E), we used two sites, one in the promoter (A) and second in the coding region of the Bsp gene (B, +12kb). The levels of H3K36me3 occupancy (Fig 6E) is almost two fold higher in the coding region of the Bsp gene (at site B) after BMP-2 addition, which is consistent for H3K36me3 occupancy that is usually observed in the coding region of the active gene.

Minor comments

1. It might be informative to show a sequence alignment of NO66 and other Jumonji family proteins especially give that point mutants are made in conserved residues however we are give not information on this supposed conservation.

We originally referred to the paper by Klose et al. (2006), which provided sequence alignments for all jumonji proteins, highlighting putative conserved amino acids required for catalytic activity. In addition, in response to the reviewer, we have added a sequence alignment of conserved amino acids between NO66 and the previously studied JmjC-containing histone demethylase, JHDM1A (Takusuda et al, 2006) in Fig 4A.

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2. It is not clear from Figure 3B that knockdown of NO66 causes the cells to differentiate sooner as apposed to causing a greater percentage of the cells to differentiate. If it is possible to show a later time point for the WT where the same percentage of cells have differentiated just at a later time point. At the very least the authors might mention and site a typical time point when a similar percentage of the WT cells would be differentiated as that shown for day 24 of the knockdown.

To address this question and to address a criticism by reviewer 2 (see below) we have redone these experiments (Fig 3). Our results showed that at an early time point NO66 shRNA treatments resulted in more cells expressing high levels of alkaline phosphatase (ALP) and were positive for alizarin red relative to control shRNA-treated plates. In the control plates, a similar degree of ALP and mineralization as observed in NO66sh-treated cells was also seen at a much later time point. Thus, our data further confirm our initial conclusion that knockdown of NO66 caused an acceleration in differentiation.

3.In figure 4A labels for the two mutant proteins would be helpful.

We have added the labeling of these two proteins as M1 and M2 to the Fig 5.

Reviewer 2

Major comments
1A. Figure 1 shows Osx and N066 physically interact. What is missing is control Western for a known Osterix interacting protein that copurifies with Flag-HA Osx by tandem immunoaffinity column in figure 1B, for example ATF4 is a known Osx interacting protein.

We have responded to these comments, but in a slightly different way than the reviewer's example. We could not find evidence in the published literature that ATF4 is a known Osx interacting protein, so we used several other polypeptides which were copurified with Flag-HA-Osx and were identified in mass spectrometry. We confirmed that Yb-1 and Flightless-1 interacted with Flag-HA-Osx in co-immunoprecipitation experiments followed by western blotting (See Suppl. Figure 1).

B. Also this figure should show data for endogenous Osx and N066 proteins interacting in primary osteoblasts (mouse or rat).

We demonstrated that Osx and NO66 interacted in well-differentiated rat osteoblast UMR 106 cells and in BMP-2 induced C2-Osx osteoblast cells (Fig. 1). We also presented evidence for direct interaction between purified recombinant NO66 and Osx polypeptides. NO66 and Osx also colocalized in mouse skeletal tissues as shown by in situ RNA hybridization and antibody staining. Finally, expression of Osx is essentially restricted to the osteoblast lineage in vivo. Because expression of Osx decreases to undetectable levels during culture of primary osteoblasts unless induced by osteogenic media, as indicated by immunoblots, additional coimmunoprecipitation experiments in primary osteoblasts would be difficult and of limited value in further demonstrating interactions between NO66 and Osx..

C. For figure 1D, do the authors have any co-IP (both ways Osx→N066 or N066→Osx)?

We performed the experiments as the reviewer suggested. However we were not able to distinguish the Osx band from the immunoglobulin heavy chain, because they migrated very close to each other. As a result, we performed several co-IP and reverse co-IP in transfection experiments by expressing epitope tagged versions of Osx and NO66, and in

each case we observed clear evidence of the presence of the other polypeptide. We have now added one panel showing that Flag-HA-Osx was co-immunoprecipitated with myc-NO66 (Fig. 1C, bottom). We also showed reverse co-IPs in our experiments to map the domain in Osx required for interaction with NO66 (Fig 5D). In these new experiments Osx mutants were coimmunoprecipitated by NO66 M1 followed by western blots to detect interacting Osx fragments.

D. A co-IP in preosteoblasts and differentiated cells should be compared to appreciate when N066 repressor activity of Osx predominates.

We could not perform this experiment as described for several reasons. Osx was not expressed in pre-osteoblasts, but its expression was induced clearly after BMP-2 treatment (see Fig 6A). The cellular level of NO66 remained largely the same in MC3T3 pre-osteoblasts (before BMP-2 addition) and in BMP-2 induced MC3T3 osteoblasts. Hence a co-IP for interactions between Osx and NO66 is not possible in pre-osteoblasts. Figure 1B shows that in the Osx-expressing cell line (C2-Osx cells) interactions between NO66 and Osx occured only when both were present in the cells, but not when Osx expression was turned off by tetracycline (lane 2, 3 & 4).

2A. Figure 2 shows N066 expression represses Osx transcription in promoter assay. This conclusion, Osx-270 mutant (-DNA binding but have Trans activation domain) would be strengthened if the Osx sites were mutated or use in an increasing fashion.

The question seems to be whether the repression of Osx-mediated gene activation requires Osx binding to target promoters. However, we have shown in other experiments (Fig 5E, see below 2B) that if Osx does not bind to its targets sites, the activity of the reporters is strongly decreased, so it is difficult to examine NO66 repression in this situation. We do show in cotransfection experiments in Figure 2B, that full length Osx increases the activity of osteoblast-specific Bsp or Osteocalcin promoter reporters in a dose dependent fashion. Osx-270 is not able to activate Bsp-Luc reporter because it lacks a zinc finger DNA binding domain (See Fig 5D-E). Figure 2C and 6D both show dosedependent stimulation of a 2kb Bsp promoter activity by full length Osx.

In addition, the activity of GAL4-DBD-Osx (27-270) fusion polypeptide, consisting of a GAL4 DNA binding domain fused to the transactivation domain of Osx, was studied using a 5xGAL4-Luciferase reporter gene (See Fig. 4F, Fig. 5C and Suppl. Fig 3D). These studies again confirm that NO66 inhibits Osx-mediated activation post-recruitment of Osx to a target promoter.

B. Did the authors try mutated (Osx binding mutation in the proximal region) OCN or BSP promoter in this promoter activity studies?

In response to this question, we have performed several other experiments in which Osx binding sites in the proximal promoter of the Osteocalcin gene were mutated (data not shown). In transfection experiments, the response of the mutant promoter to Osx was markedly decreased compared to the wild-type promoter indicating that Osx was able to activate this promoter through its DNA recognition sites. The systematic mutation of the Osteocalcin promoter is a part of a separate project. We have added a sentence in the text of the result describing the loss of Osx-dependent promoter activity caused by an Osxbinding site mutation (See Page 16). We have also used this information to design ChIP primers flanking the Osx binding sites in the promoter of the Osteocalcin gene as shown in Fig 6C and Suppl Fig. 6).

C. What is the justification of using HEK cells in this study?

1. To study the effects of Osx on the Bsp and Osteocalcin promoter reporters, we decided to use HEK cells which do not express Osx. Thus, the promoter activation observed in

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our studies can be directly attributed to exogenously added Osx.
2. To address effects of NO66 on an endogenous Osx target, we also overexpressed NO66 in BMP-2 treated C2C12 cells, which resulted in a 50% decrease in the levels of endogenous Osteocalcin RNA. This result is now added to Fig 2 (Fig. 2C, Right)

D. A comparison should be made, MC3T3 preosteoblasts, to identify if the N066 effects are specific to Osx in osteoblasts.

As mentioned above Osx is not expressed in MC3T3 preosteoblasts, so we could not make this comparison. We believe our other data (Fig 2E-F, Suppl Fig. 3A-C, See Page 18, first paragraph discussing about unpublished observations) indicate that NO66 effects are specific to Osx targets in osteoblasts. However, effects of NO66 to the activity of other transcription factors in osteoblasts or other cell types could not be ruled out.

E. In figure 2C, the N066 western is not of good quality. Please explain the duplex.

We have redone this experiment to improve the quality of the blot. We do not usually observe duplex band, which may be due to degradation. It is not present in our repeat experiment and also not in Fig 3A.

F. The greater effects on BSP could be due to an increased level in response to BMP2. Figure 2D, other osteoblast markers should be added, Runx2 and alkaline phosphatase expression.

In preosteoblast MC3T3 cells, expression of the Osx, Bsp and Oc genes does not occur unless induced by BMP-2, but NO66 RNA and protein levels are largely unchanged before and after BMP-2 addition (Fig 6A). Mock and siRNAs transfected cells were all treated with BMP-2 at the same time after transfection. Hence the greater effect of the Bsp expression in siRNA (NO66 specific) treatment was specific due to knock-down of NO66. Osx is also a BMP-2 inducible gene; however, its RNA levels remained unchanged, thus suggesting that knock-down of NO66 specifically stimulates Osx-target genes. Levels of Runx2 RNA in this experiment were also not affected (data not shown), arguing against selective sensitivity to BMP stimulation

3.Figure 3B control is not appropriate. One needs an sh knock-down to a scrambled sequence and sh stable clone of MC3T3, and not simply "WT" cells.

As suggested, we have performed these experiments again using stably transfected nonspecific shRNA (Nsh) and NO66-specific shRNA MC3T3 cells (Fig 3). The new experiments did not affect our conclusions, because the ability of WT and the new control cells to produce alkaline phosphatase and to mineralize into extracellular matrix was essentially identical. Data are now presented for NO66sh and Nsh cells.

4A. Fig. 4 maps the JmjC domain of N066 to the TA domain of OSX. Why in figure 4C does the 'bar graph' 8, show that in presence of ΔJmjC, which does not have functional demethylase domain, a 50% level of GAL-DBD- Osx activation? Please explain the contribution of the whole domain versus the partial deletion of JmjC. The conclusion needs to be modified. In the lower panel the western profile is different for each clone. How did you normalize your transfection experiment? In figure 4D, why is the basal transcriptional activity for 5xGAL-4 Luc two fold less in the treatment vs. nontreatment group (bar graph 2, 6 and 10)?

1. We have redone this experiment (Fig. 5C) and observed reproducible data that mutant NO66 mutant lacking intact JmjC domain showed weak inhibition of Osx-dependent activation.

2. Based on in vitro binding assay using purified recombinant polypeptides (Fig 5), our results indicate that the JmjC domain of NO66 is necessary for interaction with Osx. NO66 mutant M1 (168-641), which contains an intact JmjC domain, was able to interact with Osx. But NO66 mutant M2 (a partial deletion in JmjC domain) failed to interact with Osx. Co-IP experiments using various NO66 deletion mutants revealed neither the N or C-terminal fragments of NO66 are needed for interaction with Osx (data not shown), strengthening our conclusion that the Osx interacting domain in NO66 lies within JmjC domain. It is already known that jmjC domain is required for protein-protein interactions in other contexts (Tronnersjo et al, 2007), consistent with our data.

Ref:

Tronnersjo S et al (2007) The jmjN and jmjC domains of the yeast zinc finger protein Gis1 interact with 19 proteins involved in transcription, sumoylation and DNA repair. Mol Genet Genomics 277(1): 57-70.

The weak inhibition of the reporter by our M2 NO66 mutant, which does not have functional demethylase domain, could be due to other inhibitory factors recruited by this protein

2. We normalized the transfection efficiency with -galactosidase activity. We indicated that in the original manuscript.

3. The higher activity of 5xGAL4-luc in the nontreatment group was most likely due to an overall drug toxicity effect. Importantly, inhibition by NO66 could not be overcome by these drugs.

5. In figure 5B, did you try H3K9me3? If so add comment.

NO66 did not demethylate H3K9me3. We added this result in the panel (now Fig.4C).

6A. In the figure 6C as a control the authors need to use 3'UTR fragment of OC or BSP gene and show no binding.

At the reviewer's suggestion, we performed ChIP experiment in BMP-2 treated MC3T3 cells and found that the occupancy of Osx at the promoter of the Osteocalcin gene was much higher than 3'UTR region of the gene. The result is now described in the supplemental figures (Suppl. Fig. 6).

B. The N066 occupancy (fig. 6C Rt panel, fig 6D Lt panel) in OC and BSP promoter is very poor (0.02 to 0.04). Upon BMP2 treatment for 15 hrs the changes of occupancy for N066 are in the decimal range of 0.01% decrease. How the authors claim significant remodeling in H3K4me3 mark in both OC and BSP promoter?

We are glad to reassure the reviewer about our claims. Two pieces of evidence show that the claim is reasonable.

1. The values of the NO66 ChIP at the Osx target promoters in Figure 6C were highly reproducible and much higher than the IgG values. The difference in NO66 occupancy at these promoters between untreated MC3T3 preosteoblasts and BMP-2 treated cells was also highly reproducible. The efficiency in precipitating NO66-containing chromatin fragments was clearly less than for other antibodies used in this study, despite the high degree of specificity of NO66 antibodies. A western blot of crude C2C12 cell extracts with NO66 antibody shows a single species of 66 kDa with a few minor bands only visible after long exposure. Furthermore, NO66 occupancy at the -actin gene appeared to be within background level thus showing the specificity of NO66 ChIP.

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2. Our data clearly showed that NO66 occupancy at the Bsp and Oc promoters gradually decreased during BMP-2 induced differentiation of MC3T3 cells. The levels of NO66 at the promoters of these genes were reduced by approximately 65% in BMP2-induced cells. Expression of these genes was seen only after 30 h of BMP-2 treatment, at which time there was a significant depletion of NO66 from these genes (Fig. 6C, Right). Furthermore, in another independent experiment (Fig 6E), NO66 was completely depleted from the promoter of the Bsp gene after 36 h of BMP-2 addition while there was a 3-fold increase in the H3K4me3 levels in the promoter of this gene, an indication of chromatin remodeling.

C. Could you please demonstrate these results in primary calvarial (mouse or rat) osteoblast?

1. Our study clearly indicates that binding of NO66 to the Bsp and Oc genes occurs in pre-osteoblasts, well before the activation of these genes; however its occupancy is decreased in osteoblasts when these genes are activated. Thus we used pre-osteoblast and BMP-2 induced osteoblast MC3T3 cells as cellular model systems to study whether any correlation is established between the interaction of NO66 with these genes and the activation of genes.
2. Primary osteoblasts are isolated from well formed calvarial bone of neonatal mice and thus are well differentiated cells in which all the matrix forming genes are highly expressed. Under such cellular conditions, we expect binding of Osx and other positive regulators to the Bsp and Oc genes because of the active state of these genes in primary osteoblasts. However, we expect the occupancy of NO66 to be low or undetectable. We would not be able to examine the inverse correlation between the occupancies of NO66 and Osx in target gene, because these genes are expressed in primary osteoblasts, unlike in the preosteoblast these genes are not expressed (inactive state) and but can be expressed only after BMP-2 (active state).
3. We did try to perform ChIP experiments using primary osteoblasts that were cultured for a week in the presence or absence of osteogenic media (β-glycerol phosphate and ascorbic acids) in order to induce expression of Osx and target genes, but the results were inconclusive because the target genes were expressed in both cases, though relatively more in the presence of osteogenic media. Occupancy of Osx at the Bsp promoter was observed only when cells were cultured in osteogenic media, however occupancy of NO66 was undetectable in both cases. One possibility is that during embryonic development in mice, NO66 was already depleted from the promoter of Bsp gene in vivo during osteoblast differentiation and formation of bone. The resulting primary osteoblasts from mice may not have NO66 occupied at the target gene.

Similarly, we also detected a high level of Osx occupancy in promoters of the Bsp and Oc genes in the rat UMR 106 cells, which are highly differentiated osteoblasts. NO66 was also detected on these promoters, but these results as in the case of primary cells do not allow us to compare the level of NO66 binding at repressed and activated genes.

D. Also show that N066 binding will be reduced if you knock down Osterix (in Osterix het mouse model or siRNA knock down).

1. Unfortunately, because Osx het mouse is normal and shows no defect in bone formation, there is no haploinsuffiency of Osx gene in bone formation Nakashima et al, 2002, Cell 108(1): 17-29.

2. As Fig. 2E-F and Fig. 3 show that in transiently or stably transfected NO66-specific siRNA, expression of Osx target genes Col1a1, Oc and Bsp was markedly stimulated.

3. Occupancy of NO66 was observed at the Osx-target promoters when Osx was not present in MC3T3 preosteoblasts (Fig 6A-C). However when Osx was induced in BMP2 treated MC3T3 cells and recruited to target promoters, occupancy of NO66 at these promoters decreased.

The above results suggest that in the absence of Osx, there would be an increase, not a decrease, in NO66 binding to the Osx target promoters.

E. In figure 6D please include the actylation status, Osterix and PolII recruitment in BMP samples.

We appreciate this suggestion and have now performed ChIP experiments for acetylated H3, RNA Polymerase II and Wdr5 (a BMP-2 induced histone methyl transferase subunit). Upon BMP-2 addition, the occupancy of these markers at the Bsp promoter was increased, consistent with the fact that this gene was being actively transcribed. The data now appear in Suppl. Fig. 8. This is further evidence that post-translational modifications of histone H3 by acetylation and methylation likely contribute to the active expression of the Bsp gene.

Minor comments

1. Abstract-line 3, not clear to readers to state "to understand transcriptional activation of Osx" and then characterize a negative regulator. Suggest rephrase to understand "regulation of Osx".

We rephrased the sentence as suggested by the reviewer

2. Introduction page 3, line 19 the authors wrote "Several others........osteoblast (....)". References for the homeodomain (Msx and Dlx) regulation of bone formation need to be included, e.g. Hassan et al; Cheng et al; add ref attached.

We apologize for not including these references and are thankful for the suggestion. We now have added them.

Response to Reviewer 3

The identification of a physical and functional interaction of the histone demethylase NO66 and Osx is novel and interesting. The experiments are well done and the conclusions are justified.

My major problem with the manuscript is that the manuscript does not go far enough and, from my perspective, ends with a question: considering the interaction of Osx and NO66 (i.e. the first part of the manuscript), how come that NO66 is removed from the chromatin/DNA segment as Osx starts occupying it? The authors realize that this is a major issue and go through substantial speculation in the Discussion, but do not provide any experiments or data to address this issue. Even relatively simple experiments like evaluating whether this is competition, or requires the enzymatic activity of NO66 are not done. Maybe NO66 and Osx do/can not even associate with each other when bound to chromatin/DNA. So, my take on this manuscript is that the authors should extend the data with some mechanistic insight that demonstrates what is happening at the regulatory Osx target gene sequence.

While we believe that the evidence we presented is at least consistent with an interpretation that interactions between Osx and NO66 are required in regulation of Osx target gene expression. We have, as the reviewer suggested, extended the data. These new data and the original results indicate that depletion of NO66 from Osx-target gene is mediated through Osx and/ or along with positive chromatin regulators.

In preosteoblasts before BMP2 addition, we initially observed high levels of NO66 occupancy and no or very low levels of Osx occupancy at the promoters of Osx target genes. After BMP-2 addition, NO66 occupancy decreased and Osx occupancy

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increased at these promoters. These target genes were then activated. We speculated that Osx had a role in chromatin remodeling of Osx target promoters including the displacement of NO66 from the chromatin of these target promoters.

To test this hypothesis (in response to the reviewer's suggestion), we performed two transient expression experiments. Note that the nature of chromatin of the transfected DNA in these experiments was obviously very different from what is happening in differentiating cells, but the experiments still yielded some mechanistic insights.

1. We first asked whether increasing intracellular levels of Osx could overcome the NO66-mediated inhibition of an Osx target promoter. Our new data showed that increasing concentrations of Osx indeed overcame NO66 inhibition, atleast in part (Fig. 6D).

2. Further, chromatin immunoprecipitation of transfected cells in parallel experiments indicated that NO66 was present at the promoter of reporter DNA in the absence of Osx. This occupancy decreased by almost 75% when Osx was cotransfected together with NO66 (Fig. 6D, Right). Our data suggest that there is competition between NO66 and Osx at the Bsp promoters in these experiments. Note that unlike Osx, which contains a zinc finger DNA-binding domain and binds DNA in a sequence-specific manner in the chromatin, NO66 does not contain a DNA-binding domain, however, it may well be recruited to chromatin through other factors.

3. In differentiating preosteoblasts, NO66 and Osx were occupied at the promoters of the Bsp and Oc genes at 15 h after BMP-2 treatment, when target genes were not yet activated (Fig. 6C). Similarly, sequential ChIP of Osx/NO66 in undifferentiated cells (Suppl.Fig. 10) colocalization of NO66 and Osx was also seen at the promoter of the Bsp gene. Thus we believe that both NO66 and Osx can interact with the chromatin of the target gene in preosteoblast or undifferentiated cells, but NO66 is removed later when cells undergo differentiation while Osx remains present at the gene for activation.

4. Our preliminary results, using an Osx-expressing stable cell line, suggest that interactions of NO66 with Osx negatively affect the binding of Osx to the target promoter. When NO66 was knocked-down by stably expressing shRNA in C2-Osx cells (this cell line was discussed in Fig 1A-B), the occupancy of Osx and levels of H3K36me3 in the target promoter markedly increased compared to that of control shRNA cells (part of separate project, unpublished observation).

These data suggest that interactions of NO66 with Osx inhibit recruitment of Osx to target genes thus compromising Osx activity and expression of Osx target genes. This conclusion is further supported by siRNA experiments in which expression of Osx-target genes was stimulated in NO66-deficient cells (Fig 2E & F, and Fig. 3). Hence we postulate that mechanism of NO66 inhibition requires both its demethylase activity, when present at the genes, and its interactions with Osx, that inhibit the recruitment of Osx to the target genes.

5. We also added ChIP experiments (Suppl. Fig. 8) in response to reviewer 2 and result of these experiments addressed the criticism of this reviewer.
During activation of Osx-target Bsp gene, not only occupancy of Osx at the promoter of the Bsp gene increased but also the levels of other chromatin regulators, Wdr5, H3 acetylation and methylation (H3K4me3 / K36me3), and RNA polymerase II increased. These data lead to the hypothesis that NO66 depletion from Osx-target gene during activation could be mediated through Osx, perhaps together with positive chromatin regulators such as factors that govern histone acetylation and methylation.

Minor comments:

1. It would be nice to show the coincident expression of Osx and NO66 in developing bones in the manuscript, rather than in supplementary data.

We have now shown these data in Fig. 2A.

2. page 9, last lines (i.e. the title) "interacts with the ..." should be "is required for the interaction with....".

We thank the reviewer for this correction, we modified it.

3. page 10, 1st line: This is the first time that I find out that the Osx used in Fig. 1E is in fact only it's transactivation domain. That information was not even in the figure legend. Please be clear about this when discussing Fig. 1E.

Please read Fig. 1F not E. We apologize for our mistake.

Reference cited

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108(1): 17-29

Tronnersjo S, Hanefalk C, Balciunas D, Hu GZ, Nordberg N, Muren E, Ronne H (2007) The jmjN and jmjC domains of the yeast zinc finger protein Gis1 interact with 19 proteins involved in transcription, sumoylation and DNA repair. Mol Genet Genomics 277(1): 57-70

2nd Editorial Decision 13 August 2009

Thank you for sending us your revised manuscript. Our original referees 2 and 3 have now seen it again. Referee 1 was not available to look at the manuscript at this time. Referee 2 is now positive about publication of the paper here and does not have any further comments. Referee 3 is not fully satisfied with the revision (see below). Apart from certain minor issues he/she expresses hesitations that the additional insight you provide into the functional interplay between NO66 and Osx remains on a level that he/she feels is rather limited (and I would like to add that we share his/her disappointment). Still, on balance and given the more positive vote by referee 2 as well as the support by referee 3 in principle we have come to the conclusion that the paper should be publishable here. Still, I would like to ask you to address the remaining minor issues raised by referee 3 in an amended manuscript.

Furthermore, there is one remaining editorial issue that needs further attention. Prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) to ask for the original scans (for our records).

In the case of the present submission there are a number of panels that appear to not fully meet these requirements: Figure 1B, figure 4C, figure 5B

I therefore like to kindly ask you to send us a new version of the manuscript that contains suitably amended versions of these figures. I feel that it would also be important to explain the assembly of these figures in the figure legends (i.e. that all lanes come from the same gel). Please be reminded

that according to our editorial policies we also need to see the original scans for the figures in question.

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final version of the paper.

Thank you very much for your cooperation.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #3 (Remarks to the Author):

Sinha et al. (de Crombrugghe), EMBO J. Revised

The authors have identified the demethylase NO66 as a protein that interacts with the osteogenic transcription factor Osx. They define its histone demethylase activity and show that it represses Osx activity and, presumably as a consequence, osteoblast differentiation. Finally, they demonstrate that Osx and NO66 interact with the same regulatory DNA segment of an Osx target gene, and that the occupation of the chromatin by NO66 decreases as the occupation by Osx increases.

As stated in my review of this manuscript before, the identification of a physical and functional interaction of the histone demethylase NO66 and Osx is novel and interesting. The experiments are well done and the conclusions are justified.

Previously, my major problem with the manuscript was that the manuscript did not go far enough and, from my perspective, ended with a question: considering the interaction of Osx and NO66 (i.e. the first part of the manuscript), how come that NO66 is removed from the chromatin/DNA segment as Osx starts occupying it? This question remains, and the authors realize that this is a major issue. While they speculate on this issue in the rebuttal, it remains essentially unclear how complex formation between NO66 and Osx can lead to displacement of NO66 by Osx upon BMP stimulation. In revising the manuscript, the authors evaluated whether there is competition of Osx with NO66 at the promoter (as requested), but did not go further in resolving this question. They did not address whether this competition requires the enzymatic activity of NO66, nor did they show whether NO66 and OSx do/can associate with each other when bound to chromatin/DNA (even though they clearly interact in solution) (as requested). So, essentially what it comes down to is the question how far the authors should go into gaining an answer to this mechanistic question, and that is to some extent an editorial judgment.

At times, the authors do not explain sufficiently well in the text of the Results or Figure Legends how exactly the experiments were done. For example, it is not clear what immunoprecipitates what, when I see e.g. a myc-precipitate followed by an HA western blot, if I don't know how the proteins are tagged. This and some other inaccuracies in the text (see below) give a sloppy impression. I urge the authors to meticulously go over the manuscript with this issue in mind.

Additional comments in order of appearance:
- page 6, line 8 from the bottom: The authors refer to Fig. 1B, but it looks to me that they should refer to Fig. 1A, and that Fig. 1B remains unexplained in the Results section.
- page 8, last paragraph going into page 9: In Fig. 2C (right panel), the authors only show the effect on endogenous osteocalcin expression. They should also show endogenous Bsp expression, considering that previous experiments use a Bsp reporter and that subsequent experiments will also deal with Bsp expression and the Bsp promoter.
- page 10 in reference to Fig. 3B, C: knocking down NO66 expression enhances and accelerates the expression of alkaline phosphatase. Alkaline phosphatase is an early marker of osteoblast expression

that is upregulated when osterix is not even expressed. So, how should we think now about the effect of the NO66 knockdown and the role of NO66? This certainly should not be explained through the NO66-Osx connection.
- page 11, 2nd paragraph halfway: This sentence is sloppy and inaccurate. For H339A, H404A, refer to Fig. 4A, since that information is not in Fig. 4C. Then move the reference to Fig. 4C, lanes 3 and 4 to the end of the sentence so that it makes more sense.
- page 11, related to Fig. 4D: The description of what should be seen in Fig. 4D does not make sense compared to what we see. Is there mislabeling? I just don't understand. Also, the minus sign next to NO66 in the top line of this panel has jumped away from where it should be.
- page 12, first few lines dealing with Fig. 4E: In that panel, does myc antibody detect NO66? This is not explained anywhere.
- page 12, first paragraph and Fig. 4E: why show K27me3 staining with wild-type NO66, if you don't show the effect of the catalytically inactive NO66 mutant. Also, this result is not even mentioned in the text.
- page 12, 2nd paragraph toward end: the text refers to "and in this Fig." Do you mean "Fig. 4F"?
- page 12, title of new section and first paragraph going into page 13: I do not agree with the conclusion that the JmjC domain is required for the interaction, based on the data shown. It could equally well be any sequence between aa 168 and the beginning of the JmjC domain.
- page 15, last sentence of first paragraph: the authors state that the levels of NO66 RNA and protein did not change. In contrast, the data indicate a decrease.
- page 16, lines 11-12: how can you conclude from the data shown that "inhibition by NO66 was seen only when its level was higher than that of Osx"? In fact, how can one even determine whether the level of one protein is higher than that of another protein?
- page 40, line 9 from the bottom: In this figure legend, the authors describe panel D as panel F (and there is no panel F).

2nd Revision - authors' response 08 October 2009

Changes in the manuscript in response to the reviewer 3

Editor: *In the case of the present submission there are a number of panels that appear to not fully meet these requirements: Figure 1B, figure 4C, figure 5B.*

1. We assure you that all lanes which are shown in Figure 1B came from a single SDS-gel. The blot as shown in Top panel was reprobed with anti-Flag antibody to show the immunoprecipitates of Flag-HA-Osx (Bottom panel). Input lane in bottom panel was exposed for longer time to detect the signal as the input was much diluted. We have indicated this in the legend.

2. In Figure 4C, two lanes were cropped out but all lanes that are shown came from the same gel. We have included original scanned image of the blots. We have now indicated this in the legend of Fig. 4C to read "All lanes in this panel came from the same gel".

3. In Figure 5B (Left panel), silver stained gel showing the preparation of recombinant proteins (lane 2 and 3) and coomassie stained gel showing GST-Osx (lane 4) were run on separate gel and hence separated in this panel. We have now indicated these two gels in legend (Page 36). We have also included original scan image of the blot for Figure 5B, right and left panels.

Reviewer 3*: It is not clear what immunoprecipitates what, when I see e.g. a myc-precipitate followed by an HA western blot, if I don't know how the proteins are tagged. This and some other inaccuracies in the text (see below) give a sloppy impression. I urge the authors to meticulously go over the manuscript with this issue in mind.*

1. We corrected Figure 1C and E following the suggestions of the reviewer.

2. We revised the legend of Fig 1C (Page 33) to further clarify the experiment that was performed. Please see below for the change in text as indicated by underline.

Top panel - immunoprecipitation of Flag-HA-Osx with HA-tag antibody followed by immunoblotting with myc-tag antibody for myc-NO66, Bottom panel- immunoprecipitation of myc-NO66 with myc antibody followed by immunoblotting with HA-tag antibody for Flag-HA-Osx.

3. We also corrected the text describing construction of NO66 and mutants (page 24).

Reviewer 3: *Page 6, line 8 from the bottom: The authors refer to Fig. 1B, but it looks to me that they should refer to Fig. 1A, and that Fig. 1B remains unexplained in the Results section.*

We indeed refer to Fig 1A on page 6, line 8. There was a typo error in the previous submission and now Fig. 1B is mentioned at the appropriate place (page 6, para 2, line 6).

Reviewer 3: *Page 8, last paragraph going into page 9: In Fig. 2C (right panel), the authors only show the effect on endogenous osteocalcin expression. They should also show endogenous Bsp expression, considering that previous experiments use a Bsp reporter and that subsequent experiments will also deal with Bsp expression and the Bsp promoter.*

1. We added the experiment in question (Fig. 2C, right panel) in the revised manuscript in response to reviewer 2 who questioned the rationale of using 293 cells for reporter assays (Fig 2C, left and middle panel) because the 293 cell line is not an osteoblastic line. Thus we did the experiment (Fig 2C, right panel) to show that ectopically expressed NO66 in BMP-2 induced C2C12 osteoblasts also down regulated the BMP-2-induced expression of the endogenous Osteocalcin gene.

2. Expression levels of osteoblast marker genes vary among osteoblast cell lines. In C2C12 cells after treatment with BMP-2, expression of Bsp is low compared to that of the Osteocalcin gene hence we have not tested the effect of overexpressed NO66 on the expression of the endogenous Bsp gene for this particular cell line.

Reviewer 3: *Page 10 in reference to Fig. 3B, C: knocking down NO66 expression enhances and accelerates the expression of alkaline phosphatase. Alkaline phosphatase is an early marker of osteoblast expression that is upregulated when osterix is not even expressed. So, how should we think now about the effect of the NO66 knockdown and the role of NO66? This certainly should not be explained through the NO66-Osx connection.*

We acknowledge the reviewer's concern why knocked-down of NO66 increased alkaline phosphatase activity (Fig. B & C). We do not know whether NO66 regulates the activity of other transcription factors which are needed for activation of early marker genes during osteoblast differentiation. Certainly this could be a possibility and needs to be tested in future studies.

We now added the following sentences in Discussion (Page 19, para 1, line 1-6) "One possible reason among others for the premature increase in alkaline phosphatase in MC3T3 cells containing a NO66-specific shRNA would be that NO66 regulates the activity of other factors needed for up regulation of early markers in osteoblasts, a hypothesis that needs to be tested. In this context, it is important to note that, ectopically expressed Osx in bone marrow stromal cells (BMSC) increased expression and activity of alkaline phosphatase (Tu et al, 2006) ."

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Ref: Tu et al (2006) Osterix enhances proliferation and osteogenic potential of bone marrow stromal cells. Biochem. Biophy. Res. Comm. 241í 1257-1265.

Reviewer 3: *Page 11, 2nd paragraph halfway: This sentence is sloppy and inaccurate. For H339A, H404A, refer to Fig. 4A, since that information is not in Fig. 4C. Then move the reference to Fig. 4C, lanes 3 and 4 to the end of the sentence so that it makes more sense.*

We agree with referee suggestions and modified the text accordingly (Page 11, para 2, line 5-6)

Reviewer 3: *Page 11, related to Fig. 4D: The description of what should be seen in Fig. 4D does not make sense compared to what we see. Is there mislabeling? I just don't understand. Also, the minus sign next to NO66 in the top line of this panel has jumped away from where it should be.*

The text is now modified so that the reader should understand more clearly the experiment that was performed (Page 11, para 2, line 7 till last sentence). The minus sign in figure 4D was also corrected and placed at the appropriate location.

Reviewer 3: *Page 12, first few lines dealing with Fig. 4E: In that panel, does myc antibody detect NO66? This is not explained anywhere.*

To clarify this issue we added a sentence in the Figure-legend of Fig 4E (Page 36) "Both wild-type and mutant NO66 were tagged with myc-epitope at their C-terminus and their expression was detected with anti-myc antibody".

Reviewer 3: *Page 12, first paragraph and Fig. 4E: why show K27me3 staining with wild-type NO66, if you don't show the effect of the catalytically inactive NO66 mutant. Also, this result is not even mentioned in the text.*

1. This experiment was shown in response to reviewer 1 who requested that we indicate specificity of NO66 for H3K4me3 / H3K36me3 by showing another histone methylated substrate which is not demethylated by NO66.

2. In Fig. 4C we showed that H3K27me3 was not demethylated by catalytically active or inactive NO66; hence it is not a substrate for demethylation by NO66. The result that H3K27me3 was not a substrate for NO66 demethylase activity was confirmed in transfected cells (Fig 4E). There is no need to add a mutant to support our conclusions.

3. This result was mentioned in the previous submission (see page 11, last line).

Reviewer 3: *Page 12, 2nd paragraph toward end: the text refers to "and in this Fig." Do you mean "Fig. 4F"?*

We corrected the sentence adding "Fig. 4F and Suppl. Fig. 3D".

Reviewer 3: *Page 12, title of new section and first paragraph going into page 13: I do not agree with the conclusion that the JmjC domain is required for the interaction, based on the data shown. It could equally well be any sequence between aa 168 and the beginning of the JmjC domain.*

1. We changed the title of this section from "the JmjC domain is required for the interaction" to "Domain of NO66 that interacts with the activation domain of Osx" (Page 12).

2. We agree with the reviewer's conclusion based on data shown for Fig 5A-B. Thus we modified the text as "that the segment between 168 and 386 containing the JmjC domain of NO66 was necessary for strong interactions with the activation domain of Osx" (Page 12-13, last line of Page 12 and first line of Page 13).

Reviewer 3: *Page 15, last sentence of first paragraph: the authors state that the levels of NO66 RNA and protein did not change. In contrast, the data indicate a decrease*

We corrected this sentence and modified the text (Page 14-15, last sentence of page 14 continuing next page) to read "In contrast, the cellular levels of NO66 protein levels showed little change during BMP-2-induced differentiation, although some decrease in NO66 RNA level was observed at 30 h after BMP-2 addition (Fig. 6A & B)".

We slightly modified the text by deleting one sentence which was repeated twice (page 14, para 2). The deleted sentence is "When BMP-2 was added, the genes for the transcription factors Runx2 and Osx were activated first in this process followed later by Bsp and Oc ".

Reviewer 3: *Page 16, lines 11-12: how can you conclude from the data shown that "inhibition by NO66 was seen only when its level was higher than that of Osx"? In fact, how can one even determine whether the level of one protein is higher than that of another protein?*

We acknowledge the reviewer's concern and the text was modified to read (page 16, line 5 and Suppl. Figure 7) "Our data showed that inhibition of the Osx-dependent activation by NO66 was overcome at least in part by increasing amount of Osx (Fig. 6D, left, bars 6-8)". In this context, we also somewhat modified the text of para 1 in page 16.

Reviewer 3: *Page 40, line 9 from the bottom: In this figure legend, the authors describe panel D as panel F (and there is no panel F.*

We corrected this typo (Page 37).

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