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The histone demethylase Jmjd3 sequentially associates with the transcription factors Tbx3 and *Eomes* to drive endoderm differentiation

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Editor: Anke Sparmann

1st Editorial Decision 30 November 2012

Thank you for submitting your research manuscript (EMBOJ-2012-83638) to our editorial office. It has now been seen by three referees and their comments are provided below.

All reviewers appreciate your study and are in general supportive of publication in The EMBO Journal. Nevertheless, they do raise a number of important concerns, and emphasize that a significant revision of the manuscript will be required. I would like to especially highlight that following the suggestions of reviewer #2 (point 1), the interaction data needs to be considerably strengthened. Moreover, both referee #2 (point 2) and #3 (point 1&2) request further analysis of the significance of JmjD3-mediated H3K27-demethylation at the Eomes enhancer/promoter, which we strongly encourage. Of course, the additional constructive suggestions by all referees should be taken into careful consideration as well, particularly when concerning missing controls and further contextualization of your results.

Overall, I would like to invite you to submit a suitably revised manuscript to The EMBO Journal that addresses the concerns raised by the reviewers. I should add that it is our policy to allow only a

single major round of revision and that it is therefore important to address all criticism at this stage. Please do not hesitate to contact me to further discuss the required revisions.

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.

REFEREE COMMENTS

Referee #1

In this manuscript, the authors reported the chromatin dynamics of the regulatory elements of Eomesodermin (Eomes) in differentiation of embryonic stem (ES) cells to definitive endoderm. Eomes encodes a T-box transcription factor and its function is essential for generation of definitive endoderm in mouse development and human ES cells (Arnold SJ et al, Development, 2008; Teo Ak et al, Genes Dev, 2011). The authors focused on the transcriptional regulation of Eomes in differentiation of ES cells toward definitive endoderm. They found (1) The Eomes promoter possesses a bivalent histone mark characteristic for developmentally-regulated genes in mouse ES cells, (2) Eomes is rapidly induced in the culture for definitive endoderm differentiation before the addition of Activin A, a potent enhancer of definitive endoderm differentiation, with the recruitment of Tbx3 and Jmjd3, (3) Tbx3 and Jmjd3 facilitates the DNA looping for interaction of enhancer and promoter, (4) Activin signal promotes Smad2 recruitment to the Eomes enhancer-promoter loop to mediate the auto-regulation by replacing Tbx3, and (5) the Eomes-Smad2-Jmjd3 complex activates a set of genes involving in defivitive endoderm differentiation. They also demonstrated that the mechanism found in mouse ES cells is conserved in human ES cells to mediate definitive endoderm differentiation.
Mouse ES cells possess the character of the early stage of pluripotent stem cells in pre- and peri-implantation development and it was proposed that they are categorized in 'naïve' pluripotent stem cells (Nichols and Smith, Cell Stem Cell, 2009). To direct differentiation to embryonic cell lineages, mouse ES cells should acquire competency to respond the signals as the way found in gastrulation in post-implantation development. In the protocol to direct differentiation to definitive endoderm the authors applied in this manuscript, the first 2 days is the step to establish the competency to receive the Activin signal as a trigger of definitive endoderm differentiation. Here the authors succeeded to reveal what happens in the first step at Eomes locus. This is a very interest point of this manuscript and worth to be considered for publication in EMBO J. However, in parallel, this manuscript contains critical scientific flaw. The authors examined differentiation of human ES cells and claimed that the same molecular mechanism is conserved to direct differentiation toward definitive endoderm. It was proposed that human ES cells are categorized in 'primed' pluripotent stem cells and mimic the character of the late stage of pluripotent stem cells in post-implantation development. It was shown that the responses to the external signals of human ES cells are similar to that of mouse epiblast stem cells (EpiSCs) derived from post-implantation embryos and (Vallier L et al, PLoS One, 2009) and they directly respond to Activin without the step to establish competency as found in mouse ES cells. Therefore, the first step mediated by Tbx3 in the model proposed in this manuscript should be skipped in human ES cells. Indeed, mouse EpiSCs express low level of Tbx3 and high level of Eomes, which is opposite to mouse ES cells (Tesar PJ et al, Nature, 2007). Although I couldn't find an appropriate reference, I'm sure that human ES cells express Tbx3 and Eomes in similar manner as mouse EpiSCs. It will be good for the authors to examine the state of DNA loop in the Eomes regulatory region in mouse EpiSCs to confirm their two-step model. Alternatively, they should clearly argue the fundamental difference between mouse and human ES cells and extend it to the interpretation of their data. In addition, there are several points as listed below that should be revised for publication.

1. Recently Wolf XA et al reported that the conserved region located at ~20 kb pstream the Eomes coding region drives hsp68 promoter-GFP reporter at the sites of Eomes expression in gastrulation

(Gene Expr Patterns, 2012). This is different from the region the authors analyzed as the enhancer to direct expression in definitive endoderm. Cleat explanation will be required about this discrepancy.
2. Although the authors proposed the relay from Tbx3 to Eomes for stepwise activation of Eomes expression, there is no direct evidence to show that these two T-box factors bind to the same target sequence of the Eomes enhancer. T-box factors recognize the similar DNA sequence but they have distinct specificities as found in the cae of Brachyury, VegT and Eomes in xenopus (Conlon FL et al, Development, 2001).
3. The authors found that Tbx3 expression level is unchanged from mouse ES cells to differentiating cells at day 2 but the recruitment to the Eomes enhancer is promoted at day 1. What is the mechanism to prevent the binding in undifferentiated mouse ES cells? It was reported that Tbx3 protein expression is heterogeneous in mouse ES cells and it becomes homogeneous by inhibition of MAPK (Niwa H et al, Nature, 2009). Therefore, it will be worth to test protein distribution along the time course of differentiation.
4. Teo Ak et al reported that the pluripotency-associated transcription factors OCT4, SOX2 and NANOG bind to the EOMES enhancer and knock-down of NANOG impairs induction of EOMES whereas NANOG over-expression enhances it in human ES cells (Genes Dev, 2012). How about the case of Tbx3 in mouse ES cells? Do Oct4 and Sox2 co-bind to the enhancer with Tbx3? Does the Tbx3 overexpression enhance Eomes expression?
5. The knockout data indicated that Eomes and Smad2 are required for induction of definitive endoderm but Tbx3 is not. Are there any other T-box factors with overlapping function? Discussion about it will be required.

Referee #2

Kartikasari et al. address an interesting question: How are epigenetic machineries recruited to target promoters? They use an endoderm differentiation system which is based on embryoid body formation of ES cells followed by stimulation with Activin A to robustly initiate endoderm commitment. In this system they investigate how key regulators for endoderm development are activated during lineage commitment. In ES cells they detect a so-called bivalent chromatin state of H3K4me3 and H3K27me3 at key endoderm regulators. Upon differentiation, the repressive H3K27me3 is lost and lineage genes can be activated. The authors focus on activation of Eomes, an early-induced gene, and could identify a region upstream of the promoter which comprises a putative enhancer region. Upon differentiation, this enhancer switches from an inactive H3K4me1/H3K27me3 state to an active H3K4me1/H3K27ac state. This change coincides with binding of the Jmjd3 histone demethylase and of the transcription factor Tbx3 upon differentiation. The interesting point is that both Jmjd3 and Tbx3 are necessary to activate Eomes. The authors interpret their data by suggesting that Tbx3 is required for recruitment of Jmjd3, which, in turn, removes the repressive H3K27me3 from the Eomes enhancer. The authors further show that Eomes itself is important to mediate the subsequent activation of the endoderm transcription network by recruiting Jmjd3 and Smad2 to the promoter regions of a set of endoderm genes. Depletion of either Eomes, Jmjd3 or Smad2 prevents the full activation of these endoderm genes. The authors also investigate endoderm commitment of human ES cells and can in principal confirm association of Eomes, Smad2 and Jmjd3 at the human Eomes promoter.

Accumulating evidence suggest that epigenetic regulators are recruited to specific target genes by transcription factors. In this context the manuscript provides novel evidence for the specific recruitment of a histone demethylase through interactions with distinct transcription factors (Tbx3 and Eomes). Whereas the chromatin analyses are largely conclusive, the protein interaction tests are still premature. The following points need to be considered:

1. Interaction tests are essential to demonstrate that Jmjd3 is recruited to specific promoters through interaction with Tbx3 or Eomes. The quality of the experiments, however, is not sufficient to draw the conclusion that there is direct interaction or even complex formation between Jmjd3 and Tbx3/Eomes. For the IP experiments it is important to show all necessary controls. Input and IP results for all tested proteins need to be shown. Reverse IPs need to be done to demonstrate specificity of the interaction. Are the interactions mediated indirectly through DNA? Other, yet unknown factors might contribute to the recruitment of these proteins. Can recombinantly produced proteins interact or do they require specific post-translational modifications to interact?

As a clear demonstration of interactions between Eomes/Tbx3 and Jmjd3 is important for the line of argumentation, more solid datasets need to be provided. If this is not possible, the discussion needs to be toned down.
In particular the terms „complex" and „interactions" cannot be used.

2. Jmjd3 appears to be the major H3K27me3 demethylase at the Eomes promoter as binding of Utx could not be detected (page7). These data should be shown or the statement should be removed. The importance of Jmjd3 for removing H3K27me3 was not clearly demonstrated. Is H3K27me3 at the Eomes promoter altered in D2 Jmjd3 ko cells?

3. Many experiments were performed using knockdown cells (shJmjd3, shTbx3, shSmad4). Only single knockdown oligos were used, which poses a risk for off-target effects. The use of two independent oligos would reduce this risk.

4. For all ChIP experiments a „Neg" region is shown. What is the identity of this region and why is there always substantial enrichment with any antibody (always around 1% of Input, which is a lot)? Is there no mock control?

5. Figure legends should better describe the experiments and not just conclude the results.

Referee #3

In this study, Kartikasari et al. presented a novel epigenetic regulatory mechanism of gene activation during endoderm differentiation of ESCs. They described that the transcription factors, Tbx3, Eomes, and Smad2, cooperate with the H3K27me3 demethylase Jmjd3 to establish active chromatin structures for the sequential activation of endoderm genes. For instance, Jmjd3 demethylates H3K27me3 in the enhancer region of Eomes and help to form enhancer-promoter DNA loops at early differentiation stages. Later, Eomes recruits Jmjd3 as well as Smad2 to resolve the bivalent domains within the promoters of core definitive endoderm regulators, facilitating the expression of these target genes. Moreover, they showed that such a mechanism is conserved between mouse and human. These findings revealed crosstalk between transcription factors and Jmjd3-catalyzed H3K27me3 demethylaton in transcriptional regulation during definitive endoderm differentiation of ESCs.

Comments:

1. The authors showed that Eomes transcription is upregulated in D2 EBs as compared with ESCs (Fig 1A&B), which is accompanied by an increased RNAP-Ser2P occupation in the proximal promoter region of Eomes (Fig 1G). However, the bivalent domain structure in the proximal promoter region is unaffected at this stage (Fig 1E). Although authors found decline of the H3K27me3 level in the enhancer region of Eomes, it is unclear whether the Jmjd3-mediated H3K27me3 demethylation of the enhancer region is required for the transcriptional activation of Eomes at the early stages of endoderm differentiation. Chen et al (Genes & Dev. 2012) reported that Jmjd3-mediated H3K27me3 demthylation in gene bodies is involved in transcriptional elongation. Authors may want to investigate whether a similar mechanism is involved in the regulation of Ecomes.

2. It is unclear whether the demethylase activity of Jmjd3 is required for transcriptional activation of Eomes at D2 EBs. Previously, Miller et al. (Mol Cell. 2010) reported that Jmjd3 and UTX play a demethylase-independent role in T-box family member-dependent gene expression. Authors may want to investigate this issue in their system by performing genetic rescue using wildtype and catalytically inactive mutant of Jmjd3.

3. The authors revealed that Tbx3 and Jmjd3 are mutually required for their recruitment to the enhancer region of Eomes and that knockdown of either Tbx3 or Jmjd3 impairs transcriptional activation of Eomes in D2 EBs. In addition, Jmjd3 is co-immunoprecipitated with Tbx3 in D2 EBs. The underlying mechanism of this mutual dependence for recruitment is not clear. Is the active state of the enhancer, beginning with demethylation of H3K27me3 by Jmjd3 necessary for the

recruitment of Tbx3? If so, what's the signal that enables Jmjd3 to demethylate H3K27me3 at this region upon differentiation?

4. As the enhancer region of Eomes is anchored to the proximal promoter region through DNA looping, Jmjd3 bound at the enhancer region will have the opportunity to demethylate H3K27me3 in the proximal promoter region. However, H3K27me3 in the proximal promoter region is still present and "inactive" in D2 EBs but demethylated later in differentiation. The authors should discuss this issue.

Minor comments:
1. What are the expression (mRNA and protein) profiles of Tbx3 and Jmjd3 during definitive endoderm differentiation? Are their expression levels coordinately regulated during differentiation? Does Tbx3 affect Jmjd3 expression and/or vice versa?

2. Does Tbx3 interact with Jmjd3 directly or indirectly?

3. Many previous studies (Burgold et al. Plos One. 2008; Dai et al. J Cell Biochem. 2010; Miller et al. Mol Cell. 2010; Chen et al. Genes & Dev. 2012) have reported the functional role and epigenetic mechanism of Jmjd3 during neural differentiation or other cell differentiation systems. The authors should discuss the similarities and differences between their findings and the work from those other labs.

1st Revision - authors' response 21 December 2012

We would like to thank the reviewers for their constructive and diligent critiques of this manuscript.

Referee#1:

Mouse ES cells possess the character of the early stage of pluripotent stem cells in pre- and peri-implantation development and it was proposed that they are categorized in 'naïve' pluripotent stem cells (Nichols and Smith, Cell Stem Cell, 2009). Here the authors succeeded to reveal what happens in the first step at Eomes locus. However, this manuscript contains critical scientific flaw. The authors examined differentiation of human ES cells and claimed that the same molecular mechanism is conserved to direct differentiation toward definitive endoderm. It was proposed that human ES cells are categorized in 'primed' pluripotent stem cells and mimic the character of the late stage of pluripotent stem cells in post-implantation development. It was shown that the responses to the external signals of human ES cells are similar to that of mouse epiblast stem cells (EpiSCs) derived from post-implantation embryos and (Vallier L et al, PLoS One, 2009) and they directly respond to Activin without the step to establish competency as found in mouse ES cells. Therefore, the first step mediated by Tbx3 in the model proposed in this manuscript should be skipped in human ES cells. Indeed, mouse EpiSCs express low level of Tbx3 and high level of Eomes, which is opposite to mouse ES cells (Tesar PJ et al, Nature, 2007). Although I couldn't find an appropriate reference, I'm sure that human ES cells express Tbx3 and Eomes in similar manner as mouse EpiSCs. It will be good for the authors to examine the state of DNA loop in the Eomes regulatory region in mouse EpiSCs to confirm their two-step model. Alternatively, they should clearly argue the fundamental difference between mouse and human ES cells and extend it to the interpretation of their data.

The reviewer has an important point about conservation of the two-step model of differentiation to definitive endoderm in mouse and human ES cells given that hES cells are more similar to mEpiSCs than mES cells. hES cells are typically treated with activin (without EB formation) to differentiate into endoderm. The reviewer wondered whether hES cells (and mEpiSCs) have already undergone the initial step of genomic reorganization resulting in promoter-enhancer interaction of the Eomes locus. This competent state then allows hES cells to respond to activin and differentiate into endoderm. There are several reasons to suggest that the two-step model is conserved in both mouse and human ES cells. First, hES cells do not express Eomes and gene expression analysis shows that

expression of Eomes precedes Sox17 during differentiation supporting the two-step model of activation in hES cell differentiation (Fig 1A,B). To directly assess the chromatin structure around the Eomes locus in hES cells we carried out quantitative 3C analysis. The data shown in Fig 7A indicates that in hES cells, like mES cells (Fig 3F), do not show any evidence of long-range enhancer-promoter interactions within the *EOMES* locus, suggesting that the Eomes locus is not primed in both human and mouse ES cells. We do observe the formation of chromatin loops in activin treated hES cells supporting the view that the mechanism of definitive endoderm differentiation is similar in mouse and human ES cells.

1. Recently Wolf XA et al reported that the conserved region located at ~20 kb pstream the Eomes coding region drives hsp68 promoter-GFP reporter at the sites of Eomes expression in gastrulation (Gene Expr Patterns, 2012). This is different from the region the authors analyzed as the enhancer to direct expression in definitive endoderm. Cleat explanation will be required about this discrepancy.

The reviewer points to a recent study describing a conserved region located at ~20 kb upstream the Eomes coding region that drives hsp68 promoter-GFP reporter at the sites of Eomes expression in gastrulation. In our ChIP-seq analysis, we do not observe binding of ES cell factors such as Tbx3, Oct4 or Nanog at this region. In addition, the enrichment in H3K27Ac at this region was below the threshold used in our analysis. Having said that, our study does not preclude other important regulatory elements that could be involved in regulating Eomes expression. It is possible that genomic reorganization in the early steps of Eomes transcriptional activation involves a large portion of the upstream region that includes this element as well.

2. Although the authors proposed the relay from Tbx3 to Eomes for stepwise activation of Eomes expression, there is no direct evidence to show that these two T-box factors bind to the same target sequence of the Eomes enhancer. T-box factors recognize the similar DNA sequence but they have distinct specificities as found in the cae of Brachyury, VegT and Eomes in xenopus (Conlon FL et al, Development, 2001).

The reviewer has a question about whether the two T-box proteins, Tbx3 and Eomes share binding sites. We propose a relay model in which Tbx3 as part of a larger complex binds to the enhancer region of the Eomes locus. After genomic reorganization, Eomes replaces Tbx3 in the complex. In this model Eomes would be binding when the enhancer and promoter are in close physical proximity and sequence analysis identify an Eomes binding sites at the Eomes promoter region which is distinct from the Tbx3 binding site within the enhancer but these two sites are in close spatial proximity due to chromatin looping.

3. The authors found that Tbx3 expression level is unchanged from mouse ES cells to differentiating cells at day 2 but the recruitment to the Eomes enhancer is promoted at day 1. What is the mechanism to prevent the binding in undifferentiated mouse ES cells? It was reported that Tbx3 protein expression is heterogeneous in mouse ES cells and it becomes homogeneous by inhibition of MAPK (Niwa H et al, Nature, 2009). Therefore, it will be worth to test protein distribution along the time course of differentiation.

The reviewer wonders what prevents binding of Tbx3 to the Eomes locus in ES cells. We showed in the manuscript that Jmjd3 expression increase during endodermal differentiation and as Jmjd3 and Tbx3 are mutually required for binding to the Eomes promoter, we think the simplest explanation is that Tbx3 does not bind to the Eomes enhancer in ES cells due to the absence of Jmjd3.

4. Teo Ak et al reported that the pluripotency-associated transcription factors OCT4, SOX2 and NANOG bind to the EOMES enhancer and knock-down of NANOG impairs induction of EOMES whereas NANOG over-expression enhances it in human ES cells (Genes Dev, 2012). How about the case of Tbx3 in mouse ES cells? Do Oct4 and Sox2 co-bind to the enhancer with Tbx3? Does the Tbx3 overexpression enhance Eomes expression?

Below (figure not shown) is our Chip-seq data of Nanog, Tbx3, Oct4 and Sox2 on the *Eomes* locus in undifferentiated and D2-differentiated mES cells. ChIP-seq analysis show that Nanog, Oct4, Sox2 are bound to the Eomes enhancer in undifferentiated ES cells. Upon differentiation Tbx3 binds to the Eomes enhancer and Oct4 binding is also increased. However, Sox2 and Nanog binding are unchanged.

5. The knockout data indicated that Eomes and Smad2 are required for induction of definitive endoderm but Tbx3 is not. Are there any other T-box factors with overlapping function? Discussion about it will be required.

Our data shows that Tbx3 knockdown in ES cells prevents Eomes activation (Fig 3D) and Jmjd3 binding to the Eomes enhancer (Fig 3B). Thus Tbx3 is required for induction of definitive endoderm at the very early stages. Based on our microarray data, we do not find other T box factors expressed in the right place and time.

Referee#2:

1. Interaction tests are essential to demonstrate that Jmjd3 is recruited to specific promoters through interaction with Tbx3 or Eomes. The quality of the experiments, however, is not sufficient to draw the conclusion that there is direct interaction or even complex formation between Jmjd3 and Tbx3/Eomes. For the IP experiments it is important to show all necessary controls. Input and IP results for all tested proteins need to be shown. Reverse IPs need to be done to demonstrate specificity of the interaction. Are the interactions mediated indirectly through DNA? Other, yet unknown factors might contribute to the recruitment of these proteins. Can recombinantly produced proteins interact or do they require specific post-translational modifications to interact?As a clear demonstration of interactions between Eomes/Tbx3 and Jmjd3 is important for the line of argumentation, more solid datasets need to be provided. If this is not possible, the discussion needs to be toned down.In particular the terms „complex" and „interactions" cannot be used.

The interactions of Jmjd3, Smad2, Eomes are shown in Fig 5B. In this experiment we have used Jmjd3 and Smad2 antibodies for IP and blotted with Eomes antibody. We also done reverse IP by using Jmjd3 and Eomes antibody for IP and blotted with Smad2. Input and IgG controls are shown. To address whether the interactions require DNA we used DNAse 1 digestion to investigate whether the interaction is direct or dependent of DNA binding. Our data thus show that Tbx3 association with Jmjd3, independent of DNA.

2. Jmjd3 appears to be the major H3K27me3 demethylase at the Eomes promoter as binding of Utx could not be detected (page7). These data should be shown or the statement should be removed. The importance of Jmjd3 for removing H3K27me3 was not clearly demonstrated. Is H3K27me3 at the Eomes promoter altered in D2 Jmjd3ko cells?

We have removed the statement regarding Utx as this is not central to our study.

The enrichment of H3K27me3 at the Eomes enhancer was unaffected in D2 when Jmjd3-null cells were used in the differentiation (Supplementary Figure 3D). This indicated that the enzymatic activity of Jmjd3 was critical for the transcriptional activation of Eomes. To address this directly, we

overexpressed either JMJD3-wildtype or JMJD3-H1350A - a point mutation that renders deficiency in demethylase activity (Sen et al., 2008) in Jmjd3 null cells. Expression of the WT-Jmjd3 was sufficient to rescue transcriptional activation of Eomes. In contrast, expression of JMJD3-H1350A failed to activate Eomes transcription (Supplementary Figure 3E-F). These experiments indicate that the demethyase activity of Jmjd3 was essential for transcriptional activation of Eomes during differentiation.

3. Many experiments were performed using knockdown cells (shJmjd3, shTbx3, shSmad4). Only single knockdown oligos were used, which poses a risk for off-target effects. The use of two independent oligos would reduce this risk.

We have used an optimized algorithm to design these shRNA and assessed knockdown efficiency by QPCR. Furthermore, shJmjd3 and Jmjd3-null ES behaved similarly indicating that our shRNA knocdowns were efficient. Moreover, both Tbx3-shRNA and Smad4-shRNA have also been used in previously published studies (Han et al, Nature, 2010 and Deckers et al., CancerResearch, 2006)).

4. For all ChIP experiments a „Neg" region is shown. What is the identity of this region and why is there always substantial enrichment with any antibody (always around 1% of Input, which is a lot)? Is there no mock control?

The “neg” control region is described in Supplementary Information Table 2. We use the region in our ChIP-seq analysis and also used it in our ChIP experiments. We also have IgG controls and use the input in our calculation.

5. Figure legends should better describe the experiments and not just conclude the results.

We have edited the figure legend.

Referee#3:

1. The authors showed that Eomes transcription is upregulated in D2 EBs as compared with ESCs (Fig 1A&B), which is accompanied by an increased RNAP-Ser2P occupation in the proximal promoter region of Eomes (Fig 1G). However, the bivalent domain structure in the proximal promoter region is unaffected at this stage (Fig 1E). Although authors found decline of the H3K27me3 level in the enhancer region of Eomes, it is unclear whether the Jmjd3-mediated H3K27me3 demethylation of the enhancer region is required for the transcriptional activation of Eomes at the early stages of endoderm differentiation. Chen et al (Genes & Dev. 2012) reported that Jmjd3-mediated H3K27me3 demthylation in gene bodies is involved in transcriptional elongation. Authors may want to investigate whether a similar mechanism is involved in the regulation of Ecomes.

As suggested, we investigated whether enhancer activation resulted in transcriptional elongation. Towards this, we performed chromatin immunoprecipitation experiment of RNAP, RNAP-Ser5P (the initiation form of RNAP) amd RNAP-Ser2P (the elongation form of RNAP). We indeed observed high levels of RNAP-Ser2P within the gene body of *Eomes* in D2 EB suggesting transcriptional elongation, but not in ES cells (added as SupplFig1 B-C and explained in page 3).

2. It is unclear whether the demethylase activity of Jmjd3 is required for transcriptional activation of Eomes at D2 EBs. Previously, Miller et al. (Mol Cell. 2010) reported that Jmjd3 and UTX play a demethylase-independent role in T-box family member-dependent gene expression. Authors may want to investigate this issue in their system by performing genetic rescue using wildtype and catalytically inactive mutant of Jmjd3.

To address this directly, we overexpressed either JMJD3-wildtype or JMJD3-H1350A - a point mutation that renders deficiency in demethylase activity (Sen et al., 2008) in Jmjd3 null cells. Expression of the WT-Jmjd3 was sufficient to rescue transcriptional activation of Eomes. In contrast, expression of JMJD3-H1350A failed to activate Eomes transcription (Supplementary Figure 3E-F). These experiments indicate that the demethyase activity of Jmjd3 was essential for transcriptional activation of Eomes during differentiation.

3. The authors revealed that Tbx3 and Jmjd3 are mutually required for their recruitment to the enhancer region of Eomes and that knockdown of either Tbx3 or Jmjd3 impairs transcriptional activation of Eomes in D2 EBs. In addition, Jmjd3 is co-immunoprecipitated with Tbx3 in D2 EBs. The underlying mechanism of this mutual dependence for recruitment is not clear. Is the active state of the enhancer, beginning with demethylation of H3K27me3 by Jmjd3 necessary for the recruitment of Tbx3? If so, what's the signal that enables Jmjd3 to demethylate H3K27me3 at this region upon differentiation?

We show that Jmjd3 and Tbx3 associate independent of DNA binding. The signals that lead to recruitment of the complex is a great question that we are currently trying to address.

4. As the enhancer region of Eomes is anchored to the proximal promoter region through DNA looping, Jmjd3 bound at the enhancer region will have the opportunity to demethylate H3K27me3 in the proximal promoter region. However, H3K27me3 in the proximal promoter region is still present and "inactive" in D2 EBs but demethylated later in differentiation. The authors should discuss this issue.

We have discussed this issue in discussion page 15.

Minor comments:

1. What are the expression (mRNA and protein) profiles of Tbx3 and Jmjd3 during definitive endoderm differentiation? Are their expression levels coordinately regulated during differentiation? Does Tbx3 affect Jmjd3 expression and/or vice versa?

Based on our microarray and QPCR data, we saw increased Jmjd3 expression during differentiation, however Tbx3 expression did not change.

2. Does Tbx3 interact with Jmjd3 directly or indirectly?

Our analysis indicates that Jmjd3 and Tbx3 interacts directly and independent of DNA binding.

3. Many previous studies (Burgold et al. Plos One. 2008; Dai et al. J Cell Biochem. 2010; Miller et al. Mol Cell. 2010; Chen et al. Genes & Dev. 2012) have reported the functional role and epigenetic mechanism of Jmjd3 during neural differentiation or other cell differentiation systems. The authors should discuss the similarities and differences between their findings and the work from those other labs.

We have now discussed and added these studies into our discussion page 16.

2nd Editorial Decision 27 January 2013

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees and her/his comments are provided below.

Although the reviewer remains generally supportive of publication, s/he nevertheless stresses that a significant revision of your manuscript is still required, because crucial concerns raised in the first assessment have not been sufficiently addressed. As emphasized in my decision letter, the interaction assays need to be considerably strengthened and complete data sets need to be shown, i.e. westerns of all precipitated proteins, as well the reverse IPs (Reviewer #2, point 1). Additionally, providing evidence for direct interactions between the different partners using recombinant proteins should at least be considered.

Furthermore, given that establishing the importance of the demethylase activity of Jmjd3 in the activation of Eomes transcription was requested by two referees, the rescue experiments expressing either Jmjd3-wildtype or the demethylase-deficient mutant in the Jmjd3-null background should be integrated into the manuscript as part of the main set of figures.

In addition, due to the notorious off-target effects of shRNA knock-down constructs, we strongly encourage to conduct experiments with two independent oligos, at least in the case of Tbx3 and Smad4, even if the sequences used in your study have been published before.

Finally, I would like to stress once again that it is our policy at The EMBO Journal to allow only a single major round of revision. Given the potential we see in your study, and the overall positive assessment by all referees, I have decided to return your manuscript to you once more for the necessary amendments. However, let me emphasize that failure to address all criticism now will preclude publication at The EMBO Journal.

Please do not hesitate to contact me should any points require further clarification.

REFEREE COMMENTS

Referee #2

In their revised manuscript the authors have added new data demonstrating that enzymatic activity of jmjd3 is necessary for activation of the Eomes enhancer. They could further show that in their experimental setup interaction of Jmjd3 and Tbx3 appears DNA-independent. Unfortunately, the authors did not significantly improve the quality of their interaction assays. The strength of this manuscript is the analysis of chromatin changes during differentiation, not the interaction assays. I would recommend to either strengthen the interaction data with state-of-the-art controls (see below) or remove these data from the manuscript.
Detailed points:

>>1. Interaction tests are essential to demonstrate that Jmjd3 is recruited to specific promoters through interaction with Tbx3 or Eomes. The quality of the experiments, however, is not sufficient to draw the conclusion that there is direct interaction or even complex formation between Jmjd3 and Tbx3/Eomes. For the IP experiments it is important to show all necessary controls. Input and IP results for all tested proteins need to be shown. Reverse IPs need to be done to demonstrate specificity of the interaction. Are the interactions mediated indirectly through DNA? Other, yet unknown factors might contribute to the recruitment of these proteins. Can recombinantly produced proteins interact or do they require specific post-translational modifications to interact?As a clear demonstration of interactions between Eomes/Tbx3 and Jmjd3 is important for the line of argumentation, more solid datasets need to be provided. If this is not possible, the discussion needs to be toned down.In particular the terms „complex" and „interactions" cannot be used.

>The interactions of Jmjd3, Smad2, Eomes are shown in Fig 5B. In this experiment we have used Jmjd3 and Smad2 antibodies for IP and blotted with Eomes antibody. We also done reverse IP by using Jmjd3 and Eomes antibody for IP and blotted with Smad2. Input and IgG controls are shown. To address whether the interactions require DNA we used DNAse 1 digestion to investigate whether the interaction is direct or dependent of DNA binding. Our data thus show that Tbx3 association with Jmjd3, independent of DNA.

The authors claim to having identified many interactions between Jmjd3, Tbx3, Smad2 etc. To make these claims all co-immunoprecipitation experiments need to be properly controlled. For example, interaction between Jmjd3 and Tbx3 (Figure 2H) was only shown by precipitating Jmjd3 and testing for Tbx3 in the bound fraction. There is no reverse experiment. There is no control to demonstrate that Jmjd3 was efficiently precipitated.
Interaction between Jmjd3 and Smad2 (Figure 4G) was only shown in one direction. There is no control to demonstrate efficient precipitation of Jmjd3.
Interaction between Jmjd3, Smad2, Set7/9 with Eomes (Figure 5B, upper panel): no control for precipitation of Jmjd3, Smad2 and Set7/9. There is only a reverse experiment for the interaction between Eomes and Smad2 (Figure 5B, lower panel). Controls for precipitation of Jmjd3, Eomes and Set7/9 are missing.

The authors have performed an additional experiment to test whether interaction between Jmjd3 and Tbx3 is indirectly mediated through DNA. This experiment is not sufficiently controlled. There is no control for precipitation of Jmjd3. There is no reverse experiment. There is no control for efficient removal of DNA in the DNAse-treated sample.

The authors furthermore failed to show any data which would support direct interaction between the different proteins, e.g. using recombinant proteins.

>>2. Jmjd3 appears to be the major H3K27me3 demethylase at the Eomes promoter as binding of Utx could not be detected (page7). These data should be shown or the statement should be removed. The importance of Jmjd3 for removing H3K27me3was not clearly demonstrated. Is H3K27me3 at the Eomes promoter altered in D2 Jmjd3ko
cells?

>We have removed the statement regarding Utx as this is not central to our study. The enrichment of H3K27me3 at the Eomes enhancer was unaffected in D2 when Jmjd3-null cells were used in the differentiation (Supplementary Figure 3D). This indicated that the enzymatic activity of Jmjd3 was critical for the transcriptional activation of Eomes. To address this directly, we overexpressed either JMJD3- wildtype or JMJD3-H1350A - a point mutation that renders deficiency in demethylase activity (Sen et al., 2008) in Jmjd3 null cells. Expression of the WT-Jmjd3 was sufficient to rescue transcriptional activation of Eomes. In contrast, expression of JMJD3-H1350A failed to activate Eomes transcription (Supplementary Figure 3E-F).
These experiments indicate that the demethyase activity of Jmjd3 was essential for transcriptional activation of Eomes during differentiation.

The new experiment demonstrates that enzymatic activity of Jmjd3 is necessary to activate Eomes, supporting their argument that active removal of H3K27me3 at the Eomes locus is critical for activation.

>>3. Many experiments were performed using knockdown cells (shJmjd3, shTbx3,shSmad4). Only single knockdown oligos were used, which poses a risk for off-target effects. The use of two independent oligos would reduce this risk.

>We have used an optimized algorithm to design these shRNA and assessed knockdown efficiency by QPCR. Furthermore, shJmjd3 and Jmjd3-null ES behaved similarly indicating that our shRNA knocdowns were efficient. Moreover, both Tbx3-shRNA and Smad4-shRNA have also been used in previously published studies (Han
et al, Nature, 2010 and Deckers et al., CancerResearch, 2006)).

I agree that comparing Jmjd3 knock-down with Jmjd3 knock-out cells is a very valid approach to demonstrate specificity of the effects. This does not apply to shTbx3 and shSmad4 experiments, where additional controls are actually missing.

>>4. For all ChIP experiments a „Neg" region is shown. What is the identity of this region and why is there always substantial enrichment with any antibody (always around 1% of Input, which is a lot)? Is there no mock control?

>The "neg" control region is described in Supplementary Information Table 2. We use the region in our ChIP-seq analysis and also used it in our ChIP experiments. We also have IgG controls and use the input in our calculation.

The primers for the neg. control region were already shown in the first submission, however, it is still not clear why this region can be claimed to be negative for all the tested modifications and why there is still substantial enrichment. Furthermore it is not clearly described how the IgG control was used in the calculations.

>>5. Figure legends should better describe the experiments and not just conclude the results.

>We have edited the figure legend.

The figure legend have been improved.

Additional correspondence (author) 29 January 2013

Thank you for your continued support of our manuscript. I am writing to clarify the experiments we will carry out to address the points raised in by the reviews and in your decision letter.

Interaction tests are essential to demonstrate that Jmjd3 is recruited to specific promoters through interaction with Tbx3

1. We have IP and reverse IP data for Tbx3 and Jmjd3 along with the self IP control for these two proteins in Day 2 cultures.

As a clear demonstration of interactions between Eomes/Tbx3 and Jmjd3 is important for the line of argumentation, more solid datasets need to be provided. If this is not possible, the discussion needs to be toned down.In
particular the terms „complex" and „interactions" cannot be used.

We have been unable to carry out the reverse IP with Jmjd3 in sorted DE cells due to limited amount of cells to carry out Jmjd3 westerns.
Please advise whether we can revise the discussion.

2. We will move the rescue experiment of Jmjd3 WT and mutant to main figure.

3. We will use siRNA for Tbx3 and Smad4 and assess Eomes transcriptional activation as controls for the shRNA knock-down experiments.

Thanks for the time you have investing in improving this manuscript.

Additional correspondence (editor) 29 January 2013

Thank you for your comments in response to my decision. I appreciate the opportunity to clarify our demands.

Please include all relevant westerns in Figure 2H, i.e. IP, reverse IP and self-IP control. Since referee #2 asserts that the strength of your manuscript lies in the analysis of chromatin changes during differentiation, not in the interaction data, it will be acceptable to tone down your conclusions and discussion regarding these interactions. In this case, please be sure to remove all IPs that lacks the reverse IP and other pertinent controls and the textual references to these data from the manuscript.

Please do not hesitate to contact me if you have further questions.

2nd Revision - authors' response 15 February 2013

Referee #2

In their revised manuscript the authors have added new data demonstrating that enzymatic activity of jmjd3 is necessary for activation of the Eomes enhancer. They could further show that in their experimental setup interaction of Jmjd3 and Tbx3 appears DNA-independent. Unfortunately, the authors did not significantly improve the quality of their interaction assays. The strength of this manuscript is the analysis of chromatin changes during differentiation, not the interaction assays. I would recommend to either strengthen the interaction data with state-of-the-art controls (see below) or remove these data from the manuscript.

We have strengthened the interaction data between Jmjd3 and Tbx3 and included missing controls (Figure 2H). The control for an efficient removal of DNA using DNase1 treatment is now included in Supplementary Figure 2G.

Due to limited amount of DE, we have been unable to carry out the reverse IP experiment using Jmjd3. As suggested we have removed that data and all the textual references of these data from the manuscript.

In addition we have moved the rescue experiments expressing either Jmjd3-wildtype or the demethylase-deficient mutant in the Jmjd3-null background to the main figure (i.e. Figure 3E-G)

I agree that comparing Jmjd3 knock-down with Jmjd3 knock-out cells is a very valid approach to demonstrate specificity of the effects. This does not apply to shTbx3 and shSmad4 experiments, where additional controls are actually missing.

We used SMARTpool siTbx3 and SMARTpool siSmad4 and assessed Eomes transcriptional activation in differentiated cells to provide controls for the shRNA experiments. These data are presented as Supplementary Figure 3D-E and Supplementary Figure 5H.

The primers for the neg. control region were already shown in the first submission, however, it is still not clear why this region can be claimed to be negative for all the tested modifications and why there is still substantial enrichment. Furthermore it is not clearly described how the IgG control was used in the calculations.

The enrichment of ChIP is dependent on antibody specificity on genomic level and negative control area serves the best control for this. Enrichment in this region is therefore be regarded as background, thus any specific enrichment should be above the level of values from this negative region. This area is selected based on its nature in which it is known not to be bound by Smad proteins, T-box proteins, mediator complex and Pol2 complex, based on our own ChIP-seq data and others. This is also the region that is ORF-free and "promoter deserts" lacking any known or predicted structural genes.

We used Igg controls (usually <0.1%), in calculations to subtract/normalize the values of the ChIP enrichment.

3rd Editoral Decision 08 March 2013

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the reviewer. I am happy to inform you that we are ready to proceed with acceptance of the paper, pending modification of several important points.

- As emphasized in my previous communication, I have to insist that you include all relevant western blots in Figure 2H. Specifically, the top panel needs to be probed with the anti-Tbx3 antibody, whereas the second and third panels require probing with anti-Jmjd3. Inclusion of these data sets is an essential requirement for publication.

- Please amend the title for the second paragraph of the Results section ("Tbx3 recruits Jmjd3 to the enhancer region of Eomes locus") as the data shown here do not demonstrate a recruitment of Jmjd3 by Tbx3. Rather, you establish that both proteins are physically associated and co-occupy the Eomes enhancer during early differentiation steps. Indeed, data discussed in the next paragraph suggest that Tbx3 and Eomes are mutually required for their concurrent association with the Eomes enhancer.

- Please include a figure legend for Figure 8.

- In all figure legends, please indicate the number of biological replicates the data is based upon and add the information regarding the statistical tests used to create the error bars.

- Please add the data accession numbers for all high-throughput data sets.

- Please separate the 'author contribution' statement from the acknowledgements at the end of the manuscript text.

- I would like to suggest changing the current title to "The histone demethylase Jmjd3 sequentially associates with the transcription factors Tbx3 and Eomes to drive endoderm differentiation" as well as some minor alterations in the Abstract.

Stem cell differentiation depends on transcriptional activation driven by lineage-specific regulators as well as changes in chromatin organization. However, the coordination of these events is poorly understood. Here we show that T-box proteins team up with chromatin modifying enzymes to drive the expression of the key lineage regulator, Eomes during endodermal differentiation of embryonic stem (ES) cells. The Eomes locus is maintained in a transcriptionally poised configuration in ES cells. During early differentiation steps, the ES cell factor Tbx3 associates with the histone demethylase Jmjd3 at the enhancer element of the Eomes locus to allow enhancer-promoter interactions. This spatial reorganization of the chromatin primes the cells to respond to Activin signaling, which promotes the binding of Jmjd3 and Eomes to its own bivalent promoter region to further stimulate Eomes expression in a positive feedback loop. In addition, Eomes activates a transcriptional network of core regulators of endodermal differentiation. Our results demonstrate that Jmjd3 sequentially associates with two T-box factors, Tbx3 and Eomes to drive stem cell differentiation towards the definitive endoderm lineage.

- Finally, we encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. I am taking this opportunity to invite you to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.

I am now returning the manuscript to you for one last round of amendments. After the last concerns summarized above have been satisfactorily addressed, I hope to be able to proceed with formal acceptance of the manuscript!

If you have any questions in this regard, please do not hesitate to contact me directly.

