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Jarid1b targets genes regulating development and is involved in neural differentiation

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1st Editorial Decision 08 June 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by the same three referees that reviewed the Impey paper and I enclose their reports below. As you will see the referees find the study to be convincing but require further experimental analysis to strengthen the role of Jarid1b during neuronal differentiation and also to help resolve the discrepancies with the previous EMBO J paper. Firstly, referees #1 and #2 would like to see analysis of the methylation status of Jarid1b targets during neuronal differentiation and if these targets are direct and specific to NPCs. Referee #2 would like to see a more thorough analysis of the regulation of genes and regions containing intragenic Jarid1b peaks, which is also important. Referee #3 raises an important issue regarding the discrepancy with the Impey study, it would be very helpful to the field if you could remove all doubt that the knockout cells express a truncated form of Jarid1b. I realize that in part the data in the fig S7 suggest this may not be the case since there is a decrease in ChiP signal at TSS in the KO cells, but this is an important issue to be resolved. Upon reflection these concerns are important and central to the main conclusions of the study and should be satisfactorily addressed for the manuscript to be further considered for The EMBO Journal. Given the interest in the study, should you be able to address the concerns we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more

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http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

In this manuscript, Schmitz et al described the characterization of the roles of Kdm5b in regulating gene expression that are related to stem cell renewal and differentiation. Their major conclusions are in drastic contrast with those reported in a recent EMBO publication. The Soren group claimed that knock down of Kdm5b by shRNA disrupts ESC self-renewal; however, using a similar shRNA approach, Schmitz et al did not see such defects. To confirm this result, they constructed Kdm5b knock-out lines and convincingly demonstrated that Kdm5b is not essential for ESC maintenance. At the molecular level, this manuscript also disfavored the mechanism proposed by the Soren group that Kdm5b is enriched at coding regions to repress cryptic transcription. Instead, they found that Kdm5b is mostly functional around TSS. To reconcile this apparent difference, the authors provided some reasonable tests and showed that the antibodies used by the Soren group might be problematic. Although the Soren group showed a much cleaner western blot using the abcam antibody (Xie, et al 2011 Fig S4) comparing to the FigS8 shown here, Schmitz et al performed a critical control experiment for the ChIP, which is to measure the Kdm5b signal in Kdm5b knock out/down. Based on this control, they argue that the ChIP results from Soren group are likely misleading due to cross-reaction of the antibody.

Judging by the available data without seeing the respond from the Soren group, this reviewer feels that the conclusions from the previous publication are legitimately challenged. However, the new models presented in this manuscript also need to be scrutinized carefully before warranting publication.

Major concerns:

1. Using ChIP-seq, they showed that Kdm5b is occupied at H3K4me enriched regions in ES cells and knock down/out of Kdm5b leads to an increased level of H3K4 methylation on top of these H3K4 peaks. Their overall conclusion is that Kdm5b may be responsible for removal of K4me and down-regulating ESC specific-genes during ESC-NPC transition. However, they did not provide sufficient experimental supports for this model.
(1) Out of 2347 Kdm5b targets in ECS, how many of them undergo an active demethylation mediated by Kdm5b (or even just H3K4me reduction) upon neural differentiation? This can be tested by comparing the K4me3 pattern between ESC and NPC.
(2) Fig6A, as for those stem cell/germ cell specific genes indicated, do they represent the whole list of genes in this catalog based on genome-wide studies or they are hand-picked based on individual case studies in literature. Are K4me changed at those genes during differentiation and in Kdm5b knock-out cells? This experiment will address the most critical aspect of their model that is whether Kdm5b knock out/down fails to silence pluripotent genes and germ cell genes are due to the failure of demethylation

2. To provide some plausible explanations that Kdm5 colocalizes with its substrates, a potential recruitment mechanism should be tested. Since Kdm5b occupancy is not regulated by the Polycomb proteins, the question becomes whether the recruitment of Kdm5b even relies on H3K4 methylation, which will make their speculation about the contribution from PHD (on page16) reasonable. This can be tested by ChIPing Kdm5b in Set1/MLL histone methyltransferases knock down cells.

3. The experimental support for the claim that "Jarid1b is not required for neuronal differentiation of established NSCs" (on page 13) is not clear. Therefore it is inappropriate to include this statement in the result session. This is a major claim which should not be made merely based on "data not shown" (page 19)

Minor comments:

1. 2nd paragraph on Page6, it should be "Supplemental figure 1C-E", not 2C-E.
2. Kdm5b is recommended to be used throughout the manuscript to avoid confusing general audience.
3. The description (including text, figure legend, and material and methods) for Figure 6A is unclear. It is recommended to clearly indicate the labels at the top as different gene categories: ESC markers etc. They should also indicate the fold of changes as "Kdm5b vs control" to avoid being confused with "ESC vs NPC"

Referee #2

Schmitz et al.
Jarid1b targets genes regulating development and is involved in neural differentiation
EMBO J

In this manuscript, Schmitz and colleagues demonstrates that Jarid1b/KDM5b, a histone H3K4me3/me2 demethylase, is essential for neural differentiation of ES cells. By ChIP-seq analysis, they found that Jarid1b is predominantly enriched at transcription start sites (TSS) which are also marked by H3K4me3/me2. Moreover, they found that half of the Jarid1b targets are also bound by PcG proteins. Although a physical interaction between Jarid1b and PRC2 subunits was not observed, knockdown of Jarid1b affects the binding of PcG proteins to its target, and vise versa. Gene expression analysis showed that knockdown of Jarid1b results in up regulation of stem cells and germ cells genes in NPCs. It suggests that Jarid1b functions in regulation of neural development by silencing genes that are associated with pluripotency and germ cell related genes. Overall, their data are interesting and solid. However, a few points need to be further addressed.

Although the results in this manuscript are contradictory to the previously published paper (Xie et al. 2011), they demonstrated that the specificity of antibodies against Jarid1b used in ChIP-seq might be critical. Schmitz et al. showed that Jarid1b enrichment at intragenic regions of two genes tested can only be observed by using one of the commercially available antibodies (the one used in Xie's study), and the signal is not reduced when knocking down Jarid1b. However, Xie et al. showed that Jarid1b signal at intragenic peak region is reduced upon knocking down Jarid1b, although which genes they tested were not known from the paper (Fig 2C in Xie et al. 2011). In this manuscript, the result of Jarid1b ChIP-seq reveals that 34.8% of peaks are at TSS, but there is also a fairly high proportion (32.7%) of peaks present at intragenic regions (Fig3B). Although they claimed that the pattern of these peaks are individual peaks rather than spreading throughout the gene, it seems to be ignored in this study. Since it is almost as dominant as peaks present at TSS, the authors should also analyze these genes with intragenic peaks, e.g. GO terms, enrichment of Jarid1b and H3K4me3 and changes in gene expression in Jarid1b knockdown cells, etc. In figure 5C, they carried out genome-wide analysis of H3K4me3 levels in Jarid1b knockdown and control ESCs. They observed increased H3K4me3 levels at TSS as well as the gene body. It might be useful if they group genes with Jarid1b peaks at TSS or at intragenic region and analyze their H3K4me3 levels.

In figure 6, they showed that knockdown of Jarid1b in NPCs results in upregulation of stem cell and germ cell genes. Are these genes direct targets of Jarid1b in NPCs but not in ESCs? Are these genes also targeted by PcG proteins? In Jarid1b knocked down NPCs, do they observe increased H3K4me3 and decreased PcG binding and H3K27me3 levels?

In supplementary Figure 8B, in Eed knockout cells, enrichment of Jarid1b at promoters is reduced as seen in Rin1b KO cells. However, at Cebpa and Pax9 genes, the level of H3K4me3 is reduced instead of increased in Eed knockout cells. However, in page10, they stated that promoter H3K4me3 levels were increased in PcG knockout cells, which is not exactly what their data showed here. In

addition, the bar charts in sup Fig.8B lack error bars which should be added to the figure.

There are a few errors in figure legends, e.g. the figure legend of Fig5A should be Fig.5C; Fig. 1B and sup Fig.1D, they showed the level of Oct4/Nanog in the figure instead of Nanog/Oct4 described in the figure legends; the second paragraph in page 6, it should be sup Fig. 1C-E instead of sup Fig. 2C-E.

Referee #3 (Remarks to the Author):

Comments on Schmitz et al., Jarid1b targets genes regulating development and is involved in neural differentiation

This manuscript shows that Jarid1b, previously implicated in embryonic stem (ES) cell self-renewal and proliferation by other groups, is dispensable for ES cell maintenance but essential for ESC differentiation along the neuronal lineage. Using genome-wide localization analysis the authors show that Jarid1b occupies developmental regulators in ESCs and is responsible for modulating H3K4me3 levels. The authors also used Jarid1b conditional knockout cells to show the function of Jarid1b in early differentiation events towards the neural lineage. This article raises significant concerns about the specificity of the Jarid1b antibody used in a recent study and also contradicts the results of other studies regarding the establishment of Jarid1b knockout ES cell lines. Indeed, the authors reached very different conclusions from the previous studies regarding the function of Jarid1b in ES cells. Reading the other papers, they too, seem convincing (except the issue with the antibody which may have been unspecific), and should somehow be explained. Regardless, this is an interesting study, which dissects the role of Jarid1b in ES cell maintenance and differentiation very systematically and presents convincing data.

Major concerns:

Since this study contradict previous, convincing studies, it would be important to weigh all possibilities that cause this disagreement. Therefore, it would be important to answer, for example, if there's any possibility that the transcript resulting after the knockout can escape non-sense mediated decay (NMD) and produce a truncated protein? Have the authors checked for the complete absence of the Jarid1b protein, also as a truncated form after the knockout? If there is a chance that the first 5 exons still produce a ~240 aa protein product containing JmjN and ARID domains after the knockout, then it is possible that even though it may not be functional, it might still be capable of reacting to the antibody used by Xi et al. Since the antigen used to raise the antibody used by Xi et al includes the ARID domain. This could possibly explain some of the non-specificity issues arising regarding the antibody and even more importantly, may explain the partial rescue, perhaps, achieved by the truncated protein.

In this context it is also interesting that Catchpole et al were unable to establish Jarid1b knockout ES cells when they targeted exon 1 encoding JmjN domain and replaced it with the neomycin gene. But when they replaced exons 2-4 with the neomycin gene it resulted in a mutated protein that lacks the ARID domain and is expected to have no demethylase activity. Nevertheless, the ESCs with this mutated protein are viable and they were able to produce a mouse. Remarkably, the authors of this current manuscript succeeded in generating a Jarid1b knockout which conclusively shows the dispensability of the protein for ES cells. However, considering the Catchpole et al results, it is worthwhile to see if there are any novel splice forms of this protein, in addition to the existing isoforms, which lack one or other N-terminal domains possibly having different functions, rescuing, as indicated above, the lethal phenotype.

In any event, the authors should explain this apparent discrepancy, especially in regard to the KO study. For example, do differences between ES cell lines used in this study and other studies explain some of the observed discrepancies (E14 versus J1 in previous studies)?

Additional comments:

Do expression levels of other Jarid family members vary after Jarid1b knockdown or knockout of Jarid1b? Is there any compensating increase in their levels in ESCs?

LSD1 (KDM1A) was very recently shown to be essential for maintenance of hESCs pluripotency through the regulation of bivalent domains similar to Jarid1b as described in this manuscript. It appears that the modulation of H3Kme3 levels in ESCs is achieved by different regulators and the absence of one or two regulators doesn't have any perceptible effect in short term. However it is desirable to monitor the properties of Jarid1b knockdown ESCs in prolonged cultures for several passages.

1st Revision - authors' response 06 September 2011

Referee #1 (Remarks to the Author):

(1) Out of 2347 Kdm5b targets in ECS, how many of them undergo an active demethylation mediated by Kdm5b (or even just H3K4me reduction) upon neural differentiation? This can be tested by comparing the K4me3 pattern between ESC and NPC.

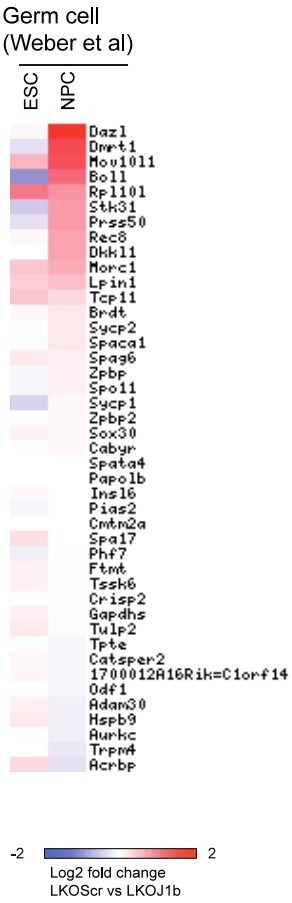
To address this question, we have compared Jarid1b targets genes with published H3K4 methylation profiles in ESCs and NPCs (Meissner, Nature 2008; Mohn, Mol Cell 2008). Of the Jarid1b target genes included in the respective datasets, 23.5% loose H3K4me3 from ESCs to NPCs while only 4.1% of Jarid1b target genes loose H3K4me2. This suggests that Jarid1b might contribute to demethylation of H3K4me3 during neural differentiation, while its contribution to removal of H3K4me2 is expected to be minor.

(2) Fig6A, as for those stem cell/germ cell specific genes indicated, do they represent the whole list of genes in this catalog based on genome-wide studies or they are hand-picked based on individual case studies in literature.

Stem cell genes included in Figure 6A comprise genes accepted in the stem cell field as markers of pluripotency. Specifically, the list was compiled based on two recent publications (Han, Cell 2011; Kamiya, Nature 2011). The references are now included in the manuscript. While, for example, gene ontology classification (GO:0019827) provides a longer list of genes annotated as stem cell maintenance (59 genes), many of these are not validated to be essential for ESCs, and therefore we have not used this list.

A list of germ cell genes was collected by annotating functions of genes overexpressed in NPCs based on published reports. In addition, we included genes annotated as germ cell markers in various stem cell studies (see references in the text). We are not aware of a complete list of germ cell genes relevant to our differentiation system. We have however transferred a list of human germ cell genes used by Weber et al (Nat Genetics 2007) to mouse and provide expression changes in the figure below (Reviewer Figure 1). While none of these germ cell genes is downregulated in NPCs, 9/43 are upregulated more than 2-fold, supporting our previous observations.

Finally, we have extended the lists of ecto-, endo- and mesoderm markers. References are provided in the manuscript.



Reviewer Figure 1. Microarray heatmap depicting expression of an unbiased list of germ cell genes in ESCs and NPCs. Coloring illustrates log2 fold changes between LKO Scramble and LKO *Jarid1b* ESCs (first column) and LKO Scramble versus LKO *Jarid1b* NPCs (second column) with blue and red colors representing down- and up-regulation in knockdown cells, respectively.

Are K4me changed at those genes during differentiation and in Kdm5b knock-out cells? This experiment will address the most critical aspect of their model that is whether Kdm5b knock out/down fails to silence pluripotent genes and germ cell genes are due to the failure of demethylation

As requested, we have analyzed H3K4me3 at stem and germ cell genes in both control and knockdown ESCs and NPCs (see new Figure 6b). During differentiation of Jarid1b knockdown cells, H3K4me3 is reduced to some extent at stem and germ cell genes but not to the levels of the control cells. Remaining H3K4me3 marks may therefore interfere with complete silencing of these genes. However, it cannot be ruled out that increased H3K4me3 levels are a mere consequence of persisting transcriptional activity.

2. To provide some plausible explanations that Kdm5 colocalizes with its substrates, a potential recruitment mechanism should be tested. Since Kdm5b occupancy is not regulated by the Polycomb proteins, the question becomes whether the recruitment of Kdm5b even relies on H3K4 methylation, which will make their speculation about the contribution from PHD (on page16) reasonable. This can be tested by ChIPing Kdm5b in Set1/MLL histone methyltransferases knock down cells.

We have addressed this question by knocking down Dpy-30, a core subunit of the SET1/MLL histone methyltransferase complexes, which recently was shown to regulate H3K4me3 levels at many loci in ESCs while not affecting ESC self-renewal (Jiang, Cell 2011). Lower H3K4me3 levels at Jarid1b target promoters result in reduced Jarid1b binding (new Supplementary Figure 14), suggesting that H3K4me3 is one of the mechanisms contributing to Jarid1b recruitment.

3. The experimental support for the claim that "Jarid1b is not required for neuronal differentiation of established NSCs" (on page 13) is not clear. Therefore it is inappropriate to include this statement in the result session. This is a major claim which should not be made merely based on "data not shown" (page 19)

The conclusion that Jarid1b is not required for neuronal differentiation of established NSCs is based on the data presented in Figure 2 using NSCs isolated from embryonic brain. The data referred to as "not shown" on page 19 relates to expression of stem/germ cell genes in these cells. We have rephrased the text to clearly state that these genes are not expressed upon Jarid1b deletion.

1. 2nd paragraph on Page6, it should be "Supplemental figure 1C-E", not 2C-E.

We apologize and have corrected the error.

2. Kdm5b is recommended to be used throughout the manuscript to avoid confusing general audience.

We would prefer to keep the traditional name "Jarid1b" which refers to the domain composition of the protein family it belongs to (JARID: Jumonji/ARID domain-containing). For the general audience, we have included all three common names (Jarid1b/Kdm5b/Plu1) in the abstract.

In addition to this, although we originally agreed on the content of the Cell paper in which the KDM nomenclature was suggested, we believe the nomenclature is not consistent and it appears used randomly. For instance:

- KDM6A and KDM6B correspond to UTX and JMJD3. These two demethylases are two very different genes without a co-linear structure. UTY, which is almost identical to UTX is not called KDM6C, because so far a catalytic activity was not assigned to UTY.
- KDM3A and KDM3B are closely related H3K9me2/me1 demethylases. PHF8 was named KDM7, even though it has H3K9me2/me1 demethylase activity. It should have been called KDM3C. But how about JMJD1C, when catalytic activity is assigned to this demethylase? How about KIAA1718, which is structurally related to PHF8, however, in addition to H3K9me2/me1 activity also has H3K27me2/me1 demethylase activity? Should KIAA1718 then be KDM7A or KDM8A, or ???

Without going into further inconsistencies in the KDM nomenclature, it is clear that it is not working and not precise. When CDKs were assigned a CDK name, they each got a unique number, i.e. CDK1, CDK2, CDK3 etc. A similar system might work for the KDMs and KMTs, however, currently the KDM/KMT nomenclature is not helpful.

3. The description (including text, figure legend, and material and methods) for Figure 6A is unclear. It is recommended to clearly indicate the labels at the top as different gene categories: ESC markers etc. They should also indicate the fold of changes as "Kdm5b vs control" to avoid being confused with "ESC vs NPC"

We thank the reviewer for this advice and have changed the figure, figure legend and text as recommended.

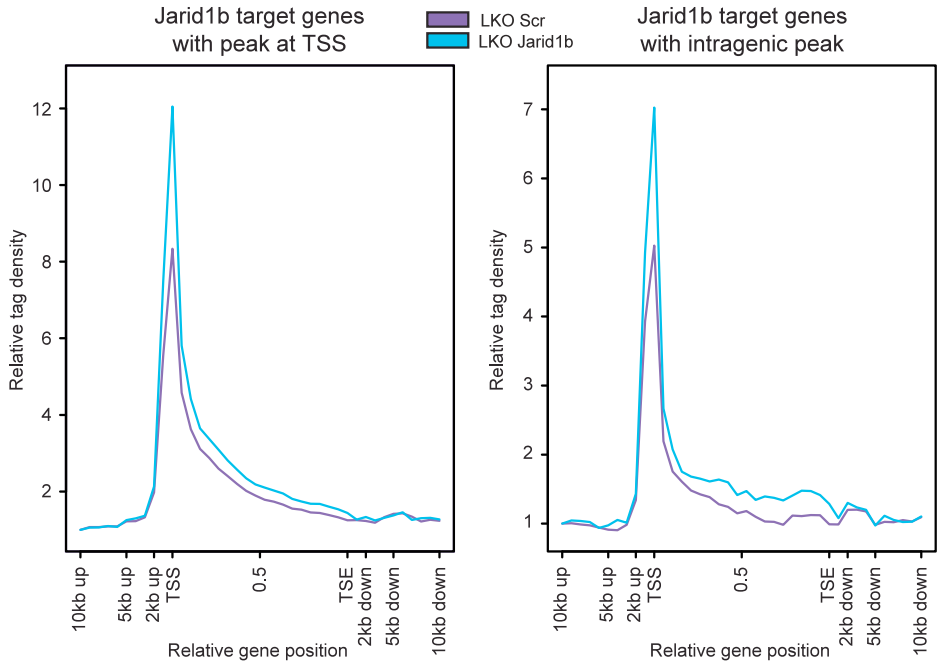
Referee #2 (Remarks to the Author):

In this manuscript, the result of Jarid1b ChIP-seq reveals that 34.8% of peaks are at TSS, but there is also a fairly high proportion (32.7%) of peaks present at intragenic regions (Fig3B). Although they claimed that the pattern of these peaks are individual peaks rather than spreading throughout the gene, it seems to be ignored in this study. Since it is almost as dominant as peaks present at TSS, the authors should also analyze these genes with intragenic peaks, e.g. GO terms, enrichment of Jarid1b and H3K4me3 and changes in gene expression in Jarid1b knockdown cells, etc. In figure

5C, they carried out genome-wide analysis of H3K4me3 levels in Jarid1b knockdown and control ESCs. They observed increased H3K4me3 levels at TSS as well as the gene body. It might be useful if they group genes with Jarid1b peaks at TSS or at intragenic region and analyze their H3K4me3 levels.

We agree that the intragenic Jarid1b peaks represent a major group and have analyzed them in more detail. The data is presented in new Supplementary Figure 7 and 8, and discussed in the text. We define several sequence features that account for intragenic Jarid1b binding, most abundantly CpG islands and GC-richness as well as a minor fraction of alternative transcription start sites. In addition, we compare intragenic Jarid1b binding to H3K4me3 and H3K36me3 and show that many intragenic peaks overlap H3K4me3 but are not marked by H3K36me3. We conclude that the intragenic Jarid1b binding sites observed in our study represent distinct sites, some of which may have regulatory functions, and which are different from the accumulation of Jarid1b in transcribed (intragenic) regions reported by Xie et al.

In addition, as suggested by this reviewer, we have compared H3K4me3 levels at Jarid1b target genes with peaks at the TSS versus target genes with intragenic peaks (Reviewer Figure 2). We find that H3K4me3 is increased at both groups of Jarid1b target genes. We believe that the analysis of gene expression changes is not meaningful here, since in general very few Jarid1b target genes change expression in Jarid1b knockdown ESC (3.4%).



Reviewer Figure 2. Mean distribution of tags across gene bodies for H3K4me3 ChIP-seq in control and Jarid1b knockdown cells at Jarid1b target genes with peaks at the TSS (left panel) and with intragenic peaks (right panel).

In figure 6, they showed that knockdown of Jarid1b in NPCs results in upregulation of stem cell and germ cell genes. Are these genes direct targets of Jarid1b in NPCs but not in ESCs? Are these genes also targeted by PcG proteins? In Jarid1b knocked down NPCs, do they observe increased H3K4me3 and decreased PcG binding and H3K27me3 levels?

To address these questions, we have performed ChIP-qPCR analysis of stem and germ cell genes for Jarid1b, H3K4me3 and H3K27me3 in knockdown and control ESCs and NPCs (see new Figure 6b). These genes were previously shown to loose H3K4me3 during neural differentiation (Meissner,

Nature 2008; Mohn, Mol Cell 2008), and we can confirm these results in control cells while Jarid1b knockdown cells maintain a substantial amount of H3K4me3 in NPCs. This is in agreement with incomplete silencing of these genes. Some of the stem/germ cells were reported in genome-wide studies to gain DNA methylation during differentiation and remain low for H3K27me3 (e.g. Rnf17), while others (e.g. Nanog) acquire H3K27me3 instead. Our ChIP data for H3K27me3 confirms these results, and in addition shows that H3K27me3 is lower in Jarid1b knockdown NPCs. However, we do not detect Jarid1b binding at the tested stem/germ cell genes, neither in ESCs nor in NPCs, indicating that silencing of stem/germ cell genes is not directly mediated by Jarid1b recruitment to their promoters. These findings are discussed in the manuscript and the proposed model has been revised.

In supplementary Figure 8B, in Eed knockout cells, enrichment of Jarid1b at promoters is reduced as seen in Rin1b KO cells. However, at Cebpa and Pax9 genes, the level of H3K4me3 is reduced instead of increased in Eed knockout cells. However, in page10, they stated that promoter H3K4me3 levels were increased in PcG knockout cells, which is not exactly what their data showed here. In addition, the bar charts in sup Fig.8B lack error bars which should be added to the figure.

We agree that H3K4me3 is more consistently increased in Ring1b KO cells, while an increase is only seen at a subset of genes in Eed KO cells. We have modified the text accordingly. In addition, error bars have been added to the Figure.

There are a few errors in figure legends, e.g. the figure legend of Fig5A should be Fig.5C; Fig. 1B and sup Fig.1D, they showed the level of Oct4/Nanog in the figure instead of Nanog/Oct4 described in the figure legends; the second paragraph in page 6, it should be sup Fig. 1C-E instead of sup Fig. 2C-E.

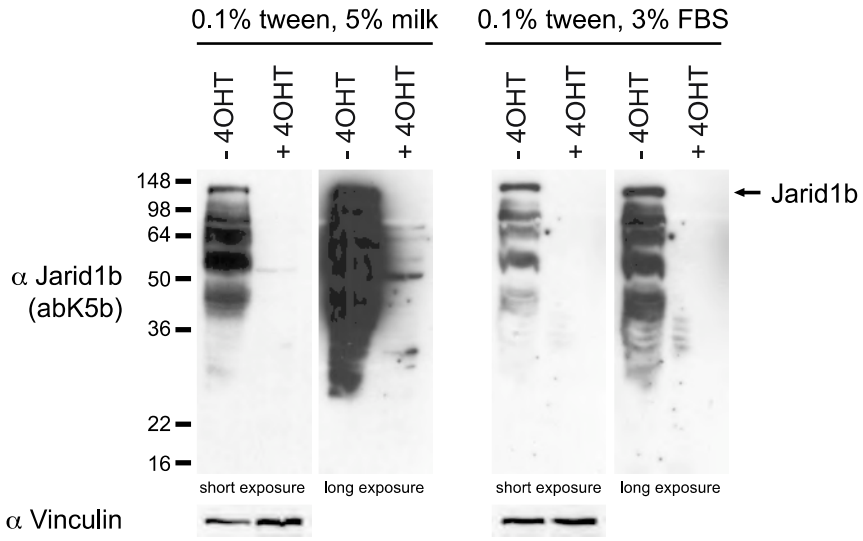
We apologize; these errors have been corrected.

Referee #3 (Remarks to the Author):

Therefore, it would be important to answer, for example, if there's any possibility that the transcript resulting after the knockout can escape non-sense mediated decay (NMD) and produce a truncated protein? Have the authors checked for the complete absence of the Jarid1b protein, also as a truncated form after the knockout? If there is a chance that the first 5 exons still produce a ~240 aa protein product containing JmjN and ARID domains after the knockout, then it is possible that even though it may not be functional, it might still be capable of reacting to the antibody used by Xi et al. Since the antigen used to raise the antibody used by Xi et al includes the ARID domain. This could possibly explain some of the non-specificity issues arising regarding the antibody and even more importantly, may explain the partial rescue, perhaps, achieved by the truncated protein.

We have analyzed expression of Jarid1b in knockout ESCs (see new Supplementary Figure 2D) and NSCs (data not shown) by qPCR using different primer sets covering all exons encoding functional domains, located both up- and downstream of the deleted exon. Transcription is very low along the entire locus, suggesting that there is no escape from NMD. If a truncated protein was produced, its levels should be very low and are unlikely to account for the ChIP signals obtained with the Ab-K5b antibody in intragenic regions.

To our knowledge there are no antibodies available that specifically target the N-terminal region of Jarid1b and would allow addressing whether a truncated protein is produced in knockout cells. However, as reviewer 3 points out, the epitope used to generate Ab-K5b antibody partially overlaps with the Jarid1b ARID domain and therefore might potentially recognize a truncated Jarid1b protein. As this antibody gives high background signal using our standard Western blot method, we have increased loading, optimized washing conditions and run high percentage gels to separate small proteins. We do not see any evidence for the appearance of a ~25 kDa protein in the knockout ESCs (Reviewer Figure 3).



Reviewer Figure 3. Western blot analysis of *Jarid1b* control (- 4OHT) and knockout (+ 4OHT) ESCs using the abK5b antibody (Xie et al). 50µg of cell lysates were resolved on a 13% polyacrylamid gel. Membranes were washed extensively to remove unspecific bands using the conditions indicated above each blot. Vinculin served as loading control.

However, considering the Catchpole et al results, it is worthwhile to see if there are any novel splice forms of this protein, in addition to the existing isoforms, which lack one or other N-terminal domains possibly having different functions, rescuing, as indicated above, the lethal phenotype.

Very low transcription along the entire Jarid1b locus (see above) suggests that there are no alternative splice variants present in the knockout ESCs and NSCs.

In any event, the authors should explain this apparent discrepancy, especially in regard to the KO study. For example, do differences between ES cell lines used in this study and other studies explain some of the observed discrepancies (E14 versus J1 in previous studies)?

The Jarid1b knockout mice generated by Catchpole et al were generated on a mixed C57BL/6/129/ola genetic background while our Jarid1b knockout mice are pure C57BL/6, which might account for phenotypic differences. The second Jarid1b strain generated by Catchpole et al, which expresses Jarid1b lacking the ARID domain at relatively low levels compared to wild-type, was backcrossed to C57BL/6, and therefore the genetic background is comparable to our knockout mouse. This mouse is viable and fertile (like our knockout), but displays a mammary phenotype. The Jarid1b ARID domain has previously been shown to be required for histone demethylase activity of Jarid1b (Yamane, Mol Cell 2007). However, since no further characterization of the ΔARID protein is provided by Catchpole et al, it remains speculative whether the ΔARID mouse is functionally comparable to a full knockout.

Regarding the ESCs, Catchpole et al used blastocysts of mixed C57BL/6/129/ola genetic background in which Jarid1b was already deleted for their attempt to establish ESC lines. Our Jarid1b knockout ESCs are of C57BL/6 background and Jarid1b deletion was induced in established ESC lines. Possibly, Jarid1b might be required for ESC establishment but is dispensable for ESC maintenance. The knockdown experiments conducted by Xie et al used J1 ESCs while our knockdown experiments were carried out in E14 cells. More likely, however, the different phenotypes are due to different specificities of the siRNA/shRNAs used. These possibilities are discussed in the manuscript.

Do expression levels of other Jarid family members vary after Jarid1b knockdown or knockout of Jarid1b? Is there any compensating increase in their levels in ESCs?

Expression of Jarid1a, Jarid1c and Jarid1d in ESCs remains unaffected by deletion of Jarid1b (new Supplementary Figure 3b).

LSD1 (KDM1A) was very recently shown to be essential for maintenance of hESCs pluripotency through the regulation of bivalent domains similar to Jarid1b as described in this manuscript. It appears that the modulation of H3Kme3 levels in ESCs is achieved by different regulators and the absence of one or two regulators doesn't have any perceptible effect in short term. However it is desirable to monitor the properties of Jarid1b knockdown ESCs in prolonged cultures for several passages.

Both Jarid1b knockdown and knockout ESCs were maintained in culture for several weeks without loosing their self-renewal properties. A comment was added to the manuscript.

2nd Editorial Decision 22 September 2011

Thank you for submitting your revised manuscript to The EMBO Journal, it has been re-evaluated by the three original referees who find that the study has been significantly strengthened. I am happy to accept it for publication in EMBO J. You will receive the official acceptance letter in the next day or so.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

Authors addressed my major concerns.

Referee #2

Schmitz et al.
Jarid1b targets genes regulating development and is involved in neural differentiation
EMBO J Revised manuscript

In this revised manuscript, Schmitz and colleagues answered most of the comments raised by the reviewers. They carried out new experiments including H3K4me3 ChIP at stem and germ cell genes in ESCs and NPCs, Jarid1b ChIP in Dpy-30 knockdown cells and more detailed data analysis of Jarid1b ChIP-seq results. Overall, this reviewer is satisfied with the revised manuscript.

Referee #3

This is an interesting study which shows the role of Jarid1b in the fine tuning of transcription and target gene expression through modulation of H3K4me3 levels. This study also clearly demonstrates the dispensability of Jarid1b for the maintenance of ES cells, revising earlier notions.

The authors satisfactorily addressed some of the concerns raised about the antibody specificities and alternative splicing events with additional experiments. The

possibility of a truncated protein at low levels still remains, but the qPCR experiments and the authors conclusion that the resulting protein levels, if at all present, should be very low are acceptable. They also cleared concerns about long term viability of Jarid1b knockout ES cells. However, their speculation that Jarid1b might be required for ES cell establishment but is not necessary for maintenance could have been easily tested during the generation of knockout mice. It would be interesting to see if Jarid1b is absolutely essential for the establishment of ES cells that would have pointed to alternative or yet unknown functions of Jarid1b in development.

