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LncKdm2b controls self-renewal of embryonic stem cells via activating expression of transcription factor *Zbtb3*

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Transaction Report:

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

1st Editorial Decision 25 May 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by four referees whose comments are shown below.

As you will see, the referees think that a lncKdm2b-mediated control of ESC pluripotency and early embryogenesis is intriguing, and we agree with this view. However, the referees also think that your knock-out strategy is not suitable to substantiate such a role for lncKdm2b. Furthermore, they note that already published data do not convincingly support a reduction in lncKdm2b during differentiation. They also think that the SRCAP interaction data need further controls, that the pluripotency assays are not convincing, and, importantly, that the methods are not sufficiently described to be able to fully evaluate your study.

Given these numerous issues and the fact that the outcome of addressing these strong concerns is rather unclear, I am afraid we cannot offer to publish your study at this stage.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful. Should you be able to successfully address the concerns noted above, I would be prepared to take another look at your manuscript.

REFEREE REPORTS

Referee #1:

The manuscript by Ye et al., describes the function of the lncRNA lncKdm2b. The knock-out of lncKdm2b results in the reduction of ESC pluripotency and early embryonic lethality in mice. The authors show that lncKdm2b interacts with the SRCAP complex, which in turn activates the

expression of Zbtb3. Zbtb3 affects then ESC pluripotency in a Nanog-dependent manner.

Major concerns:
1) Intellectual:
I was very surprised that the authors did not reference their published study on lncKdm2b (Liu et al., 2017) until the last paragraph of the discussion. Indeed, several results presented in the current manuscript, including the generation of lncKdm2b KO and lncKdm2b RFP reporter mice and the embryonic lethal phenotype of lncKdm2b, were published in Liu et al., 2017 - "We identified an uncharacterized lncRNA required for the maintenance of embryonic stem cell pluripotency that we call lncKdm2b, because it is expressed divergently from the Kdm2b gene. LncKdm2b deficiency abrogates blastocyst development at embryonic day 3 (E3.0), leading to early embryonic lethality". As the manuscript is currently presented, the reader can easily have the impression that lncKdm2b is a novel, never before characterized lncRNA. To clearly distinguish the current work from the previous study, the results published in Liu et al 2017 should be clearly described in the introduction and only non-redundant new data should be presented in the current results. In line with this presentation, the section describing the generation of the mouse strains must be shorten and re-written to cite Liu et al., 2017, and other nearly identical phrases present in both manuscripts should be rewritten or removed.

Some parts of the current manuscript and the Liu et al., 2017 paper are almost identical (Discussion part):

Liu et al., 2017: "Divergent lncRNAs are called to be transcribed in the opposite direction of nearby protein-coding genes. In addition, lncRNAs are preferentially localized in the vicinity of gene promoters in the antisense orientation37. For example, the divergent lncRNA Evx1as promotes transcription of the nearby gene EVX1 to regulate mesendodermal differentiation25. We show that lncKdm2b deletion did not affect the expression of the nearby protein-coding gene Kdm2b or of other neighboring genes in mouse embryogenesis. We also show that lncKdm2b modulated the maintenance of ILC3s. LncKdm2b promoted the expression of Zfp292 in trans, without affecting its neighboring genes, in ILC3s."

Ye et al: "Divergent lncRNAs are transcribed in the opposite direction to nearby protein-coding genes. In addition, lncRNAs are preferentially localized at the vicinity of gene promoters in antisense orientation (Guttman et al., 2010; Sigova et al., 2013). For instance, the divergent lncRNA Evx1as promotes transcription of its nearby gene EVX1 to regulate mesendodermal differentiation (Luo et al., 2016). Interestingly, here we show that lncKdm2b deletion does not impact the expression of nearby protein-coding gene Kdm2b and other neighboring genes. LncKdm2b associates with the SRCAP subunit of the SRCAP remodeling complex to activate Zbtb3 expression for the regulation of ESC pluripotency in trans."

2) Technical:
My major concern about the study is the strategy to KO lncKdm2b - the authors delete an 800 bp region that is highly conserved at the DNA level, thus they are very likely removing cis regulatory motifs (such a disruption could explain the resulting mouse lethality, which is quite uncommon for lncRNA KOs). Indeed, the only evidence that could support that the mouse/ESC phenotype is triggered by the loss of lncRNA function rather than disruption of cis regulatory motifs are the shRNA KD experiments performed in ESCs. However, the authors provide no description of how the shRNA KD was performed, how many times the experiment was conducted or how many biological replicates were assayed, making it impossible to evaluate the outcome of these KD experiment. This information must be included. The authors show that upon KD of lncKdm2b the ESCs do not differentiate properly. However, ESC differentiation protocols (and particularly by LIF withdrawal used by the authors) are known to lead to aspecific differentiation defects e.g. this type of ESC phenotype is not robust and thus the shRNA experiments are a weak proof that the 800bp-region does not contain enhancers. As such, the authors should perform the following series of experiments to be able to conclude that this region does not contain functional enhancers:
a) The authors should map to this region chromatin modifications known to be associated with active enhancers (ex. H3K27ac; for example, using published, available data).
b) The authors should show in an enhancer assay (by expressing the 800pb region targeted for the deletion + minimal promoter + GFP) that the removed region does not act as an enhancer.

c) The most crucial experiment here would be to generate one of the two additional ESC lines (ideally both): (1) an inversion rather than a deletion of the 800pb region. Since this experiment might not be trivial to do, an alternative would be (2) to insert a premature polyA termination in the second exon, a minimally invasive technique that would maintain any potential cis regulatory elements while negatively impacting the lncRNA.

3) Material and methods:
Material and methods lack whole sections describing shRNA KD, lncRNA overexpression and rescue constructs, as well as how many biological replicates were used and how many times the experiments were performed. Thus, it was difficult to estimate the technical quality of the experiments throughout the manuscript. This whole section must be re-worked.

Other specific points:
1. Abstract:
- "Only a few divergent lncRNAs have been defined up to date" is incorrect. The authors should rephrase the statement and say that the biological and molecular functions of the divergent lncRNAs have been not characterized.
- "is conserved in different species..." - what are these species? This same vague terminology "highly conserved in different species" was used in the last paragraph of the introduction and in the first paragraph of discussion. The authors should be more precise and indicate whether the sequence conservation was detected among mammals or deeper in the evolution.
2. I could not find how the rescue/overexpression experiments were performed in ESCs. One needs a precise description of these experiments, as they are crucial for evaluating any potential enhancer activity of the removed region.
3. Fig 3D: Does the localization of SRCAP change in the absence of the lncRNA? The authors should add a SRCAP staining in the lncKdm2b-/- cells.
4. "We next performed transcriptome microarray analysis between lncKdm2b-/- ESCs and WT ESCs." How many biological replicates were used for these experiments? The authors should use at least 3 biological replicates (e.g. 3 independent lncKdm2b-/- clones).
5. "Therefore, these data suggest that lncKdm2b activates Zbtb3 transcription in a SRCAP-dependent fashion.". The data presented in the manuscript suggest that SRCAP activates Zbtb3 expression in a lncKdm2b-dependent manner, which is not what the authors claim. The sentence should be re-phrased.
6. The whole second paragraph in the discussion should be removed. Indeed, the authors' lncRNA KO strategy is not state of the art due to the deletion of a relatively big DNA fragment. As such, the reader is left with the question of whether the phenotype is due to the disruption of an enhancer effect or a lncRNA transcript effect (discussed in more detail in the following review: Bassett et al., 2014).
7. Fig 2A: Unclear how many clones were used for the Northern blot analyses. As the quality of the 18S rRNA loading control is fuzzy, the Northern blot should be repeated using 3 wildtype and 3 -/- samples. In addition, the authors do not explain what is the bottom panel. Finally, is the residual lncRNA transcript gone upon removal of the second exon?

Referee #2:

Buqing Ye and co-workers describe an lncRNA gene (that they recently discovered in a different publication), lncKdm2b, and which they now show to be involved in mouse pluripotency maintenance by regulating cascade of downstream targets, ultimately regulating Nanog levels and essential for embryogenesis. The story is compelling and of high interest, the data collected pointing to the effects observed following deletion of part of LncKdm2b sequence is convincing, however the evidence for the direct involvement of the lncRNA in the reported phenotype is currently lacking and needs strengthening.

Major comments
1. The authors suggest that LncKdm2B functions in trans, and to support that claim show that overexpression of LncKdm2b in the background of the deletion that engineered in one of its exons can rescue the loss of pluripotency in stem cells, assist embryoid body differentiation in mES LncKdm2b KO cells, and affect SRCAP complex activity. The evidence for rescue is currently images of the cells, but it is unclear what happens to downstream targets of LncKdm2b, such as

Zbtb3, which the author focuses on the majority of the paper, in the rescue setting. Does LncKdm2b restoration in LncKdm2b KO cells also restore Zbtb3 and Nanog expression to its WT levels? Same goes for the shRNA-mediated transcript knockdown that is shown to affect pluripotency. Does this knockdown affect Zbtb3 expression?

2. The author show that LncKdm2b is expressed in mESCs, and is downregulated upon differentiation both in-vitro and in-vivo (in an artificial system of PRF knock-in which may not faithfully recapitulate endogenous expression). Upon examination of multiple published RNA-seq datasets (e.g., PMIDs 25157815, 28285903, 25569111), which include ESCs, ESC-derived neurons, EBs and MEFs, it is difficult to see any down-regulation of this lncRNA during differentiation, and expression looks higher in MEFs and EBS than in mESCs - lncKdm2b appears to be up-regulated during differentiation, in what looks like a stark contradiction with Figure 1C. Could it be that the discrepancy is due to the very inefficient splicing of the 2nd intron? This point has to be explained and addressed.

3. The fact that the authors just published another paper on the same lncRNA is mentioned only in passing in the last paragraph of the discussion. It should be mentioned and explained in the introduction, to give better context to the results. It should also be mentioned that some of the constructs were already published previously (e.g., the RFP knock-in). It should be mentioned that the same fragment that interacts with SCRAP interacts with Satb1. Does the interaction with Satb1 take place also in mESCs? This has to be discussed.

Minor comments
1. Does the sequence deleted in exon2 overlap the SRCAP-interacting region?
2. Fig. S2f - No error bars are shown
3. Figure 3D- a zoom-in on a few cells should be shown to make the co-localizaton case more convincing.

Referee #3:

« LncKdm2b controls pluripotency of embryonic stem cells and early embryogenesis via activation of transcription factor Zbtb3 »

Ye et al.

The authors studied an uncharacterized lncRNA antisense to KDM2B whose expression decreased upon differentiation of ES cells. To investigate the role of this lncRNA, the authors generated a deletion of one of its exon. This deletion leads to early embryonic lethality. Deletion of this locus in ES cells is also interfering with pluripotency. The authors identified SRCAP as a specific interactor for LncKdm2b. The authors then analyzed gene regulation in response to LncKdm2b deletion and report that transcription factors, among which zbtb3, are mainly deferentially expressed. Finally they linked the phenotype of zbtb3 mis-regulation to LncKdm2b deletion.

To my knowledge, the phenotype resulting from the deletion of LncKdm2b is one of the most dramatic reported for lncRNA deletion. This astounding result prompts for many controls to validate it and would constitute a manuscript deserving publication by itself. Yet, the authors present a flurry of additional results, more and more surprising. Instead of supporting the initial observations, this in fine raises doubt about the overall study.

Specific comments.
1) Considering the proposed role for LncKdm2b, we assume that it should be highly expressed. The authors should provide a quantitative estimation of how many copies of LncKdm2b are expressed per cell.
2) The phenotype due to LncKdm2b deletion could either result from the lncRNA deletion or from mitigating important DNA sequences (e.g. enhancers) localized in the 0,8kb removed by the deletion of the exon 2. To circumvent this problem, the author should interfere with LncKdm2b expression by inserting a polyA for instance in its first exon. More details on the deleted region

should be included in the figure S1H.
3) To substantiate the ability of LncKdm2b to work in trans and rescue the phenotype of its deletion, the authors should define the regions required for this rescue. Precise quantification of the expression levels in the rescue should be provided.
4) RNA pull downs are notoriously dirty, yet this does not appear to be the case here. The authors should explain whether they used specific protocol. Controls with known lncRNA (e.g. Xist A repeats) should be included for comparison. Full mass spec result should be included. What happen to Satb1 and NURF? (see recent publication from the authors, Nat Immunology, 2017).
5) The authors conclude that LncKdm2b « modulate the assembly and activity of SRCAP-contained remodelling complex ». To confirm this result, the authors should show that RNA are required for the complex integrity. For instance, they could purify SRCAP, run it on sizing column and show that the complex is lost upon RNAse treatment.
6) If H2Az deposition is impaired genome wide upon LncKdm2b deletion, the authors should perform ChIP-seq to show the global consequences on chromatin regulation.
7) The authors should detail precisely how many genes are up and down regulated upon deletion of LncKdm2b.
8) It is unclear what the figure 4I means. More detailed on the protocol should be provided. Where the samples crosslink? When was the crosslink reversed? Chromatin modifiers whose elution pattern is not affected by LncKdm2b deletion should be analyzed.

Referee #4:

In this manuscript, the authors reported a novel role of divergent long noncoding RNA (lncRNA) in mouse embryonic stem cells (ESCs). They identified lncKdm2b for its specific expression in mouse ESCs. Using the Crispr/Cas9 system, they generated lncKdm2b-KO mice and found that the homozygous embryos die at early post-implantation stage. Then they generated KO ES cells with the same strategy and revealed that lncKdm2b-KO ES cells showed lower efficiency of stem cell colony formation, lower expression levels of pluripotency-associated genes and lower efficiency of teratoma formation, suggesting its function to maintain proper self-renewal. Then they performed co-Ip experiment and identify SRCAP for its binding to lncKdm2b RNA. They found that SRCAP requires lncKdm2b RNA as a scaffold to interact with its partners. They tested the impact of lncKdm2b KO on transcriptome in ESCs and found that Zbtb3 is down-regulated. They demonstrated that Zbtb3 promoter is occupied by SRCAP and the recruitment of SRCAP requires lncKdm2b. Finally, they revealed that Nanog is a functional target of Zbtb3 and its overexpression restore stable self-renewal of lncKdm2b, SRCAP and Zbtb3 KO ES cells.
The finding shown in this manuscript sounds interest and novel, so it looks suitable for publication in EMBO J. However, there are several points required revision as listed in below. Particularly, the authors' interpretation about the relation between the gene function and the maintenance of pluripotency is inappropriate. For example, the authors showed the results of colony formation assay followed by AP staining with the established KO ES cells and the poor AP-positive colony formation is interpreted as abolishment of pluripotency. This result just indicates the poor self-renewal capacity that could be due to the poor plating efficiency, slow proliferation, or high incidence of spontaneous differentiation or cell death. The lack of the detail information of the experiment is problematic for assessing these results. The ES cells could retain pluripotency with lower efficiency of self-renewal as found in the case of Nr0b1 KO (Fujii et al, Sci Rep, 2014). Pluripotency should be evaluated by the ability of differentiation to all germ layers, ideally by chimera assay for mouse ES cells.

Major points
1. Page 5 & Figure 2A: How about the expression of the truncated RNA in Crispr-KO ES cells after removal of exon 2? Are there any smaller bands in Northern blot? This is important point because the authors demonstrated that SRCAP binds to 450-700 of LncKdm2b and this region is just in exon 1. If the truncated form without exon 2 expresses in KO ES cells, it may retain the ability to bind to SRCAP.
2. Page 6: How were the mouse ES cell lines with deletion of exon 2 established? Were they clones after transient expression of the Crispr/Cas9? Indeed, there is no detail description in the materials and methods part about the establishment of KO ES cells for LncKdm2b, Scrap and Zbtb3 as well as their rescue experiments with the transgenes. These lacks made different to assess the results from these experiments.

3. How about the stability of self-renewal of LncKdm2b KO ES cells? Since Nanog expression is homogeneous in serum-free 2i culture condition, they may restore stable self-renewal in such culture condition. Moreover, this is essential to assess the function of LncKdm2b to maintain pluripotency definitively: whether it is absolutely essential or not.
4. Page 7: Since the fragment nt450-700 retains in the truncated transcript from the KO allele without exon 2, the ability of this truncated transcript to bind to SRCAP should be tested.
5. Page 8 & Figure S4D: What happened in Srcap KO ES cells? Do both alleles acquire the same deletion, or one frameshift allele and another large deletion?
6. Page 8 & Figure 5A: The authors showed that the KO ES cells for LncKdm2b, Srcap and Zbtb3 express lower levels of pluripotency-associated genes. However, if they were clonally expanded, they should retain self-renewing ability. What actually happens in these KO ES cells? Do they have lower efficiency of self-renewal, higher incidence of spontaneous differentiation, or something else? According to Fig 2H, the LncKdm2b KO ES cells showed just poor self-renewal capacity without the increased incidence of differentiation. Does it reflect slow cell cycle, high incidence of cell death or low plating efficiency?

Minor points
1. Page 3: Cellular reprogramming-what does it mean? Reprogramming of the gametic nuclei?
2. Page 4: differentiated ESCs removed LIF-ESCs differentiated by removal of LIF
3. Page 5: early embryonic establishment-embryonic development
4. Page 7: RNA electrical mobility shift assay-> electrophoretic mobility shift assay
5. Figure 2G and 2H were interchanged.

1st Revision - authors' response 7 November 2017

Thank you for your email of May 25 with the reviewers' comments on our manuscript (EMBOJ 2017-97174). We recognize that the four reviewers found our work to be novel and interesting, they also raised serious concerns that questioned the strengths of our conclusions from our previous studies. We have carried out many additional experiments based on their suggestions, carefully interpreted our data, and made extensive changes in the text and supplementary figures of the manuscript. We strongly believe that these changes, especially the new experimental data, have greatly strengthened our conclusions and our manuscript, and addressed all the concerns of these four reviewers. We sincerely hope that you will re-consider our manuscript for the EMBO Journal and forward it to the reviewers for their re-examination.

A detailed, point-by-point response to the reviewers' comments can be found in the following pages. I summarize here the major improvements in our manuscript based on the new experimental data:

1. We generated polyA insertion ESC lines with polyA insertion KO strategy to validate our previous results. We found that polyA insertion ESC lines displayed a similar phenotype to that of exon2-deletion ESCs (new Fig. S3D, E).
2. We analyzed published RNA-seq datasets. When ESCs were induced for neuron differentiation treated with RA (GSE85061) or activin (GSE36114), lncKdm2b was actually up-regulated during neuron differentiation. However, when ESCs were induced for spontaneous differentiation by LIF withdrawal (GSE48229), lncKdm2b was surely down-regulated during spontaneous differentiation. In parallel, for the Figure 1C, we showed that lncKdm2b was down-regulated during spontaneous differentiation by LIF withdrawal. As we showed that lncKdm2b was also expressed in the cranial/spinal accessory nerve at E10.5 (Fig. 1E), lnckdm2b may also play an important role in the regulation of neuron differentiation. We addressed this issue in the discussion section.
3. We purified the SRCAP complex with anti-SRCAP antibody from ESC lysates and treated with RNAse, followed by size fractionation assay. We found that RNAse treatment impaired the SRCAP complex integrity (new Fig. S4I). We identified that lncKdm2b deletion did not affect the NuRD complex integrity. We showed the elution pattern of NuRD complex as a negative control (new Fig. S4H).
4. We also provided the detailed protocols for main assays in the methods section.

Please let me know if you need any additional information. Thank you so much for your consideration and kind help.

Point-by-point response to the reviewers' comments

Referee #1:

The manuscript by Ye et al., describes the function of the lncRNA lncKdm2b. The knock-out of lncKdm2b results in the reduction of ESC pluripotency and early embryonic lethality in mice. The authors show that lncKdm2b interacts with the SRCAP complex, which in turn activates the expression of Zbtb3. Zbtb3 affects then ESC pluripotency in a Nanog-dependent manner.

Major concerns:

1) Intellectual:

I was very surprised that the authors did not reference their published study on lncKdm2b (Liu et al., 2017) until the last paragraph of the discussion. Indeed, several results presented in the current manuscript, including the generation of lncKdm2b KO and lncKdm2b RFP reporter mice and the embryonic lethal phenotype of lncKdm2b, were published in Liu et al., 2017 - "We identified an uncharacterized lncRNA required for the maintenance of embryonic stem cell pluripotency that we call lncKdm2b, because it is expressed divergently from the Kdm2b gene. LncKdm2b deficiency abrogates blastocyst development at embryonic day 3 (E3.0), leading to early embryonic lethality". As the manuscript is currently presented, the reader can easily have the impression that lncKdm2b is a novel, never before characterized lncRNA. To clearly distinguish the current work from the previous study, the results published in Liu et al 2017 should be clearly described in the introduction and only non-redundant new data should be presented in the current results. In line with this presentation, the section describing the generation of the mouse strains must be shorten and re-written to cite Liu et al., 2017, and other nearly identical phrases present in both manuscripts should be rewritten or removed. Some parts of the current manuscript and the Liu et al., 2017 paper are almost identical (Discussion part):

Liu et al., 2017: "Divergent lncRNAs are called to be transcribed in the opposite direction of nearby protein-coding genes. In addition, lncRNAs are preferentially localized in the vicinity of gene promoters in the antisense orientation37. For example, the divergent lncRNA Evx1as promotes transcription of the nearby gene EVX1 to regulate mesendodermal differentiation25. We show that lncKdm2b deletion did not affect the expression of the nearby protein-coding gene Kdm2b or of other neighboring genes in mouse embryogenesis. We also show that lncKdm2b modulated the maintenance of ILC3s. LncKdm2b promoted the expression of Zfp292 in trans, without affecting its neighboring genes, in ILC3s."

Ye et al: "Divergent lncRNAs are transcribed in the opposite direction to nearby protein-coding genes. In addition, lncRNAs are preferentially localized at the vicinity of gene promoters in antisense orientation (Guttman et al., 2010; Sigova et al., 2013). For instance, the divergent lncRNA Evx1as promotes transcription of its nearby gene EVX1 to regulate mesendodermal differentiation (Luo et al., 2016). Interestingly, here we show that lncKdm2b deletion does not impact the expression of nearby protein-coding gene Kdm2b and other neighboring genes. LncKdm2b associates with the SRCAP subunit of the SRCAP remodeling complex to activate Zbtb3 expression for the regulation of ESC pluripotency in trans."

Answer: Since this manuscript was completed and submitted earlier that Liu et al' one, we described more details such as lncKdm2b features and KO strategy. For our revision, we cited Liu et al' paper and rewrote the current manuscript.

2) Technical:

My major concern about the study is the strategy to KO lncKdm2b - the authors delete an 800 bp region that is highly conserved at the DNA level, thus they are very likely removing cis regulatory motifs (such a disruption could explain the resulting mouse lethality, which is quite uncommon for lncRNA KOs). Indeed, the only evidence that could support that the mouse/ESC phenotype is triggered by the loss of lncRNA function rather than disruption of cis regulatory motifs are the shRNA KD experiments performed in ESCs. However, the authors provide no description of how the shRNA KD was performed, how many times the experiment was conducted or how many biological replicates were assayed, making it impossible to evaluate the outcome of these KD experiment. This information must be included. The authors show that upon KD of lncKdm2b the ESCs do not differentiate properly. However, ESC differentiation protocols (and particularly by LIF withdrawal used by the authors) are known to lead to aspecific differentiation defects e.g. this type of ESC phenotype is not robust and thus the shRNA experiments are a weak proof that the 800bp-

region does not contain enhancers. As such, the authors should perform the following series of experiments to be able to conclude that this region does not contain functional enhancers:

Answer: We provided detailed information about shRNA KD experiments in Methods section and addressed all other concerns as follows.

a) The authors should map to this region chromatin modifications known to be associated with active enhancers (ex. H3K27ac; for example, using published, available data).

Answer: This is a good point. We used a published dataset GSE98063 to analyze modifications such as H3K27ac (marking transcriptional active region) and H3K4me1 (marking active enhancer) on the 800bp region. As shown in the new Fig. S3A, we noticed that H3K4me1 displayed no peaks, while H3K27ac had some peaks in this region, suggesting the 800bp region does not contain functional enhancers.

b) The authors should show in an enhancer assay (by expressing the 800pb region targeted for the deletion + minimal promoter + GFP) that the removed region does not act as an enhancer.

Answer: We performed this enhancer assay as suggested. As shown in the new Fig. S3B, we observed that the 800bp region did not promote GFP transcription, confirming the 800bp region does not act as an enhancer.

c) The most crucial experiment here would be to generate one of the two additional ESC lines (ideally both): (1) an inversion rather than a deletion of the 800pb region. Since this experiment might not be trivial to do, an alternative would be (2) to insert a premature polyA termination in the second exon, a minimally invasive technique that would maintain any potential cis regulatory elements while negatively impacting the lncRNA.

Answer: We generated polyA insertion ESC lines as suggested. We noticed that lncKdm2b was deleted in polyA insertion ESC lines (new Fig. S3C). PolyA insertion ESC lines displayed a similar phenotype to that of exon2-deletion ESCs (new Fig. S3D, E). We stated this result in the revised text.

3) Material and methods:
Material and methods lack whole sections describing shRNA KD, lncRNA overexpression and rescue constructs, as well as how many biological replicates were used and how many times the experiments were performed. Thus, it was difficult to estimate the technical quality of the experiments throughout the manuscript. This whole section must be re-worked.

Answer: We provided these detailed descriptions in the Methods sections.

Other specific points:

1. Abstract:
- "Only a few divergent lncRNAs have been defined up to date" is incorrect. The authors should rephrase the statement and say that the biological and molecular functions of the divergent lncRNAs have been not characterized.
- "is conserved in different species..." - what are these species? This same vague terminology "highly conserved in different species" was used in the last paragraph of the introduction and in the first paragraph of discussion. The authors should be more precise and indicate whether the sequence conservation was detected among mammals or deeper in the evolution.

Answer: We revised this wording in the abstract and provided detailed descriptions for the species accordingly.

2. I could not find how the rescue/overexpression experiments were performed in ESCs. One needs a precise description of these experiments, as they are crucial for evaluating any potential enhancer activity of the removed region.

Answer: We provided detailed descriptions in the methods section.

3. Fig 3D: Does the localization of SRCAP change in the absence of the lncRNA? The authors should add a SRCAP staining in the lncKdm2b-/- cells.

Answer: As shown in the new Fig. 3D, SRCAP was still localized in the nuclei of *lncKdm2b*-deficient ES cells and embryos.

4. "We next performed transcriptome microarray analysis between lncKdm2b-/- ESCs and WT

ESCs." How many biological replicates were used for these experiments? The authors should use at least 3 biological replicates (e.g. 3 independent lncKdm2b-/- clones).

Answer: We conducted transcriptome microarray between lncKdm2b$^{-/-}$ ESCs and WT ESCs with one biological replicate. However, the down-regulated TFs we selected were validated in at least three independent lncKdm2b$^{-/-}$ clones. Since the NimbleGen mouse microarray chips discontinued, we failed to perform three biological replicates for this assay.

5. "Therefore, these data suggest that lncKdm2b activates Zbtb3 transcription in a SRCAP-dependent fashion.". The data presented in the manuscript suggest that SRCAP activates Zbtb3 expression in a lncKdm2b-dependent manner, which is not what the authors claim. The sentence should be re-phrased.

Answer: We re-phrased this sentence in our revised manuscript.

6. The whole second paragraph in the discussion should be removed. Indeed, the authors' lncRNA KO strategy is not state of the art due to the deletion of a relatively big DNA fragment. As such, the reader is left with the question of whether the phenotype is due to the disruption of an enhancer effect or a lncRNA transcript effect (discussed in more detail in the following review: Bassett et al., 2014).

Answer: As we addressed above, the deleted 800 bp did not contain functional enhances (new Fig. S3C-E). In addition, polyA-insertion ESCs displayed a similar phenotype to that of exon2-deletion ESCs. These data suggest that the lncKdm2b KO strategy is fine to effectively delete whole lncKdm2b. We removed the whole second paragraph of the discussion section.

7. Fig 2A: Unclear how many clones were used for the Northern blot analyses. As the quality of the 18S rRNA loading control is fuzzy, the Northern blot should be repeated using 3 wildtype and 3 -/- samples. In addition, the authors do not explain what is the bottom panel. Finally, is the residual lncRNA transcript gone upon removal of the second exon?

Answer: We repeated Northern assay using 3 wildtype and 3 lncKdm2b KO samples and provided new data in new Fig. 2A. The bottom panel showed genotyping data by PCR. Indeed, the whole transcript of lncKdm2b was completely gone upon removal of the second exon.

Referee #2:

Buqing Ye and co-workers describe an lncRNA gene (that they recently discovered in a different publication), lncKdm2b, and which they now show to be involved in mouse pluripotency maintenance by regulating cascade of downstream targets, ultimately regulating Nanog levels and essential for embryogenesis. The story is compelling and of high interest, the data collected pointing to the effects observed following deletion of part of LncKdm2b sequence is convincing, however the evidence for the direct involvement of the lncRNA in the reported phenotype is currently lacking and needs strengthening.

Major comments:

1. The authors suggest that LncKdm2b functions in trans, and to support that claim show that overexpression of LncKdm2b in the background of the deletion that engineered in one of its exons can rescue the loss of pluripotency in stem cells, assist embryoid body differentiation in mES LncKdm2b KO cells, and affect SRCAP complex activity. The evidence for rescue is currently images of the cells, but it is unclear what happens to downstream targets of LncKdm2b, such as Zbtb3, which the author focuses on the majority of the paper, in the rescue setting. Does LncKdm2b restoration in LncKdm2b KO cells also restore Zbtb3 and Nanog expression to its WT levels? Same goes for the shRNA-mediated transcript knockdown that is shown to affect pluripotency. Does this knockdown affect Zbtb3 expression?

Answer: This is the case. As shown in the new Fig. S5H, we found that lncKdm2b restoration in lncKdm2b KO or knockdown ESC lines could rescue the expression levels of Zbtb3 and Nanog to their WT levels.

2. The author show that LncKdm2b is expressed in mESCs, and is downregulated upon differentiation both in-vitro and in-vivo (in an artificial system of PRF knock-in which may not faithfully recapitulate endogenous expression). Upon examination of multiple published RNA-seq datasets (e.g., PMIDs 25157815, 28285903, 25569111), which include ESCs, ESC-derived neurons, EBs and MEFs, it is difficult to see any down-regulation of this lncRNA during differentiation, and expression looks higher in MEFs and EBS than in mESCs - lncKdm2b appears to be up-regulated

during differentiation, in what looks like a stark contradiction with Figure 1C. Could it be that the discrepancy is due to the very inefficient splicing of the 2nd intron? This point has to be explained and addressed.

Answer: We checked these published RNA-seq datasets as this referee mentioned, the dataset 28285903 had no lncKdm2b reads. Dataset 25569111 conducted 3 replicates of WT ESCs and 2 replicates for EB by LIF withdrawal but showed bad repetitiveness for lnckdm2b (ENSMUSG00000056735) expression. We could not analyze the dataset 25157815 since we failed to convert the fastq file data. As such, we analyzed other published RNA-seq datasets. When ESCs were induced for neuron differentiation treated with RA (GSE85061) or activin (GSE36114), lncKdm2b was actually up-regulated during neuron differentiation. However, when ESCs were induced for spontaneous differentiation by LIF withdrawal (GSE48229), lncKdm2b was surely down-regulated during spontaneous differentiation. In parallel, for the Figure 1C, we showed that lncKdm2b was down-regulated during spontaneous differentiation by LIF withdrawal. As we showed that lncKdm2b was also expressed in the cranial/spinal accessory nerve at E10.5 (Fig. 1E), lnckdm2b may also play an important role in the regulation of neuron differentiation. We addressed this issue in the discussion section.

3. The fact that the authors just published another paper on the same lncRNA is mentioned only in passing in the last paragraph of the discussion. It should be mentioned and explained in the introduction, to give better context to the results. It should also be mentioned that some of the constructs were already published previously (e.g., the RFP knock-in). It should be mentioned that the same fragment that interacts with SCRAP interacts with Satb1. Does the interaction with Satb1 take place also in mESCs? This has to be discussed.

Answer: We introduced Liu et al' paper in the introduction section. As shown in the new Fig. S4G, Satb1 was not expressed in mESCs, suggesting that the interaction of lncKdm2b with Satb1 does not take place in mESCs. We discussed this issue in the discussion section.

Minor comments:

1. Does the sequence deleted in exon2 overlap the SRCAP-interacting region?

Answer: The SRCAP-interacting region (nt450-700) is located onto the exon1 and does not overlap with the sequence deleted in exon2.

2. Fig. S2f - No error bars are shown

Answer: We added error bars accordingly.

3. Figure 3D- a zoom-in on a few cells should be shown to make the co-localizaton case more convincing.

Answer: We provided zoom-in images in the new Figure 3D.

Referee #3:

«LncKdm2b controls pluripotency of embryonic stem cells and early embryogenesis via activation of transcription factor Zbtb3». The authors studied an uncharacterized lncRNA antisense to KDM2B whose expression decreased upon differentiation of ES cells. To investigate the role of this lncRNA, the authors generated a deletion of one of its exon. This deletion leads to early embryonic lethality. Deletion of this locus in ES cells is also interfering with pluripotency. The authors identified SRCAP as a specific interactor for LncKdm2b. The authors then analyzed gene regulation in response to LncKdm2b deletion and report that transcription factors, among which zbtb3, are mainly deferentially expressed. Finally they linked the phenotype of zbtb3 mis-regulation to LncKdm2b deletion.

To my knowledge, the phenotype resulting from the deletion of LncKdm2b is one of the most dramatic reported for lncRNA deletion. This astounding result prompts for many controls to validate it and would constitute a manuscript deserving publication by itself. Yet, the authors present a flurry of additional results, more and more surprising. Instead of supporting the initial observations, this in fine raises doubt about the overall study.

Specific comments:

1) Considering the proposed role for LncKdm2b, we assume that it should be highly expressed. The authors should provide a quantitative estimation of how many copies of LncKdm2b are expressed per cell.

Answer: This is the case. We conducted absolute quantitative PCR, we detected about 800 copies of lnckdm2b transcripts per cell. We showed these data in the new Fig. S1C.

2) The phenotype due to LncKdm2b deletion could either result from the lncRNA deletion or from mitigating important DNA sequences (e.g. enhancers) localized in the 0,8kb removed by the deletion of the exon 2. To circumvent this problem, the author should interfere with LncKdm2b expression by inserting a polyA for instance in its first exon. More details on the deleted region should be included in the figure S1H.

Answer: This is a very good suggestion. As we addressed the Qusestion#2 of Referee#1, we generated polyA insertion ESC lines and found that lncKdm2b was deleted in polyA insertion ESC lines (new Fig. S3C). PolyA insertion ESC lines displayed a similar phenotype to that of exon2-deletion ESCs (new Fig. S3D, E). In addition, we also performed an enhancer assay for the deleted 800 bp region and noticed that the deleted 800 bp did not contain functional enhances (new Fig. S3C-E). We addressed this issue in our revised manuscript.

3) To substantiate the ability of LncKdm2b to work in trans and rescue the phenotype of its deletion, the authors should define the regions required for this rescue. Precise quantification of the expression levels in the rescue should be provided.

Answer: We mapped the SRCAP-interacting region of lncKdm2b transcript (nt450-700) and rescue of this region could restore the lost stemness phenotype in lncKdm2b KO ESCs (new Fig. 5G). We provided the expression level of lncKdm2b fragment (nt450-700) in rescued lncKdm2b KO ESCs (new Fig. 5H).

4) RNA pull downs are notoriously dirty, yet this does not appear to be the case here. The authors should explain whether they used specific protocol. Controls with known lncRNA (e.g. Xist A repeats) should be included for comparison. Full mass spec result should be included. What happen to Satb1 and NURF? (see recent publication from the authors, Nat Immunology, 2017).

Answer: We repeated RNA pulldown experiment using Xist A repeats as control lncRNA and provided better data as shown in the new Fig. 3C. We described the conventional protocol that we used in the methods section. Mass spec results were provided in the Attached Table 1. However, Satb1, and the NURF complex such as Bptf and Snf2l components were not precipitated by lncKdm2b in mouse ESC lysates. Actually, Satb1 was not expressed in mouse ESCs (new Fig. S4G).

5) The authors conclude that LncKdm2b «modulate the assembly and activity of SRCAP-contained remodelling complex». To confirm this result, the authors should show that RNA are required for the complex integrity. For instance, they could purify SRCAP, run it on sizing column and show that the complex is lost upon RNAse treatment.

Answer: This is a good suggestion. We purified the SRCAP complex with anti-SRCAP antibody from ESC lysates and treated with RNAse, followed by size fractionation assay. We found that RNAse treatment impaired the SRCAP complex integrity (new Fig. S4I).

6) If H2Az deposition is impaired genome wide upon LncKdm2b deletion, the authors should perform ChIP-seq to show the global consequences on chromatin regulation.

Answer: We generated WT and lncKdm2b KO ESC lysates, and incubated with anti-H2A.Z antibody for ChIP-seq analysis. We noticed that lncKdm2b deletion surely decreased H2A.Z deposition genome wide (Attached Fig. 1A). In parallel, lncKdm2b deletion impaired H2A.Z deposition onto the promoter region of *Zbtb3* (Attached Fig. 1B). We addressed this point in our revised text.

7) The authors should detail precisely how many genes are up and down regulated upon deletion of LncKdm2b.

Answer: We analyzed differential genes upon deletion of lncKdm2b and stated this result in the revised manuscript accordingly.

8) It is unclear what the Figure 4I means. More detailed on the protocol should be provided. Where the samples crosslink? When was the crosslink reversed? Chromatin modifiers whose elution pattern is not affected by LncKdm2b deletion should be analyzed.

Answer: Mouse ESC cells were treated with 1% formaldehyde for crosslinking before lysis. Reverse crosslinking were preformed after ChIP for size fractionation assay. We identified that

lncKdm2b deletion did not affect the NuRD complex integrity. We showed the elution pattern of NuRD complex as a negative control (new Fig. S4H). We also provided the detailed protocol in the methods section.

Referee #4:

In this manuscript, the authors reported a novel role of divergent long noncoding RNA (lncRNA) in mouse embryonic stem cells (ESCs). They identified lncKdm2b for its specific expression in mouse ESCs. Using the Crispr/Cas9 system, they generated lncKdm2b-KO mice and found that the homozygous embryos die at early post-implantation stage. Then they generated KO ES cells with the same strategy and revealed that lncKdm2b-KO ES cells showed lower efficiency of stem cell colony formation, lower expression levels of pluripotency-associated genes and lower efficiency of teratoma formation, suggesting its function to maintain proper self-renewal. Then they performed co-Ip experiment and identify SRCAP for its binding to lncKdm2b RNA. They found that SRCAP requires lncKdm2b RNA as a scaffold to interact with its partners. They tested the impact of lncKdm2b KO on transcriptome in ESCs and found that Zbtb3 is down-regulated. They demonstrated that Zbtb3 promoter is occupied by SRCAP and the recruitment of SRCAP requires lncKdm2b. Finally, they revealed that Nanog is a functional target of Zbtb3 and its overexpression restore stable self-renewal of lncKdm2b, SRCAP and Zbtb3 KO ES cells.

The finding shown in this manuscript sounds interest and novel, so it looks suitable for publication in EMBO J. However, there are several points required revision as listed in below. Particularly, the authors' interpretation about the relation between the gene function and the maintenance of pluripotency is inappropriate. For example, the authors showed the results of colony formation assay followed by AP staining with the established KO ES cells and the poor AP-positive colony formation is interpreted as abolishment of pluripotency. This result just indicates the poor self-renewal capacity that could be due to the poor plating efficiency, slow proliferation, or high incidence of spontaneous differentiation or cell death. The lack of the detail information of the experiment is problematic for assessing these results. The ES cells could retain pluripotency with lower efficiency of self-renewal as found in the case of Nr0b1 KO (Fujii et al, Sci Rep, 2014). Pluripotency should be evaluated by the ability of differentiation to all germ layers, ideally by chimera assay for mouse ES cells.

Major points:

1. Page 5 & Figure 2A: How about the expression of the truncated RNA in Crispr-KO ES cells after removal of exon 2? Are there any smaller bands in Northern blot? This is important point because the authors demonstrated that SRCAP binds to 450-700 of LncKdm2b and this region is just in exon 1. If the truncated form without exon 2 expresses in KO ES cells, it may retain the ability to bind to SRCAP.

Answer: As shown in the new Fig. 2A with whole gel of Northern blot, there was no truncated RNA expression in lncKdm2b KO ES cells after removal of exon 2.

2. Page 6: How were the mouse ES cell lines with deletion of exon 2 established? Were they clones after transient expression of the Crispr/Cas9? Indeed, there is no detail description in the materials and methods part about the establishment of KO ES cells for LncKdm2b, Scrap and Zbtb3 as well as their rescue experiments with the transgenes. These lacks made different to assess the results from these experiments.

Answer: Yes, the mouse ES cell lines with deletion of exon 2 were cloned after transient expression of the CRISPR/Cas9. We provided detailed protocols for the establishment of KO ES cells and their rescue experiments in the methods section.

3. How about the stability of self-renewal of LncKdm2b KO ES cells? Since Nanog expression is homogeneous in serum-free 2i culture condition, they may restore stable self-renewal in such culture condition. Moreover, this is essential to assess the function of LncKdm2b to maintain pluripotency definitively: whether it is absolutely essential or not.

Answer: As shown in the new Fig. S2F, we used serum-free 2i culture condition and found that lncKdm2b KO surely impaired stable self-renewal of ESCs.

4. Page 7: Since the fragment nt450-700 retains in the truncated transcript from the KO allele without exon 2, the ability of this truncated transcript to bind to SRCAP should be tested.

Answer: We performed RNA immunoprecipitation (RIP) assay and found that anti-SRCAP antibody did not immunoprecipite any truncated transcripts of lncKdm2b in lncKdm2b KO ESC lysates (Attached Figure 2).

5. Page 8 & Figure S4D: What happened in Srcap KO ES cells? Do both alleles acquire the same deletion, or one frameshift allele and another large deletion?

Answer: We conducted genotyping for Srcap KO ES cells. We found that -5bp deletion took place in both alleles of the exon 5 of Srcap gene (new Figure S5D).

6. Page 8 & Figure 5A: The authors showed that the KO ES cells for LncKdm2b, Srcap and Zbtb3 express lower levels of pluripotency-associated genes. However, if they were clonally expanded, they should retain self-renewing ability. What actually happens in these KO ES cells? Do they have lower efficiency of self-renewal, higher incidence of spontaneous differentiation, or something else? According to Fig 2H, the LncKdm2b KO ES cells showed just poor self-renewal capacity without the increased incidence of differentiation. Does it reflect slow cell cycle, high incidence of cell death or low plating efficiency?

Answer: We found that lncKdm2b KO and Srcap KO ESC cells displayed slow cell cycle and high incidences of cell death (Attached Fig. 3).

Minor points:

1. Page 3: Cellular reprogramming-what does it mean? Reprogramming of the gametic nuclei?

Answer: The cellular reprogramming we used points to the embryonic development from one-cell zygote to formation of the blastocyst. We changed it to reprogramming.

2. Page 4: differentiated ESCs removed LIF-ESCs differentiated by removal of LIF

Answer: We changed it.

3. Page 5: early embryonic establishment-embryonic development

Answer: We changed it.

4. Page 7: RNA electrical mobility shift assay-> electrophoretic mobility shift assay

Answer: We corrected it.

5. Figure 2G and 2H were interchanged.

Answer: We corrected them accordingly.

(Table for referees not shown)

(Figures for referees not shown)

2nd Editorial Decision 12 December 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by the four original referees again whose comments are enclosed. As you will see, all referees express interest in your manuscript and are broadly in favour of publication, pending satisfactory minor revision.

I would thus like to ask you to address the remaining concerns and to provide a revised manuscript and a point-by-point response. Please note that the following needs to be addressed as well:

- please introduce the SRCAP complex better, and please also mention its alternative name SWR1
- please upload individual figure files and check whether all figure files are of adequate resolution and quality for production
- please rename your supplemental Information to Appendix and include a ToC at the beginning of the appendix. Appendix figures and tables should be renamed in the manuscript text from

'Supplemental Table S1' to 'Appendix Tables S1' and 'Supplemental Fig S1' to 'Appendix Fig S1'
- scale bars are missing in fig 2H, 6C and Appendix fig S2F, please add
- please change your reference list and manuscript section titles to EMBOJ format style
- please suggest (in a cover letter) a one-sentence summary 'blurb' of your paper, as well as 2-5 one-sentence 'bullet points', containing brief factual statements that summarize key aspects of the paper; this will form the basis for an editor-drafted 'synopsis' accompanying the online version of the article. Please see the latest research articles on our website (emboj.embopress.org) for examples - I am happy to offer further guidance on this if necessary.
- as you might know, we encourage our authors to provide original source data, particularly uncropped/-processed electrophoretic blots for the main figures of your manuscript. If you would like to add source data, we would welcome one PDF-file per figure for this information. These will be linked online as supplementary "Source Data" files.

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

REFEREE REPORTS

Referee #1:

In the revised manuscript by Ye et al., the authors have addressed the majority of my previous concerns. However, there are a few points that need to be clarified:

1. The authors claim that they do not remove any functional enhancers in their deletion mutants as shown they do not detect any H3K4me1 but H3K27ac accumulation in this region. However, multiple studies have associated H3K27ac with functional enhancers (Creyghton et al. 2010; Rada-Iglesias et al. 2011; Hnisz et al. 2013; Whyte et al. 2013 etc). The accumulation of H3K27ac in this region looks actually like an enhancer and the authors should change their statement about H3K27ac being associated with transcriptionally active regions only. However, I am pleased that the authors made a pre-mature poly(A) insertion mutant that resembles the deletion phenotype.
2. "Finally, we generated polyA insertion ESC lines and noticed that lncKdm2b was deleted in polyA insertion ESC lines (Fig. S3C)." The authors should re-phrase as insertion of a pre-mature polyA signal cannot "delete" a lncRNA.
3. The authors contradict themselves: they state in the abstract "The biological and molecular functions of the divergent lncRNAs have been not characterized", but have a long paragraph (highlighted in yellow) in the introduction about the known functions of divergent lncRNAs.

Referee #2:

The authors have addressed the concerns from the previous round of review in an overall satisfactory way.
Few minor comments:
1. Are the authors sure what they show in Fig. 2A is a Northern blot? It looks much more like a RT-PCR gel
2. Figure S5H also looks "too good to be true". The authors should show an RT-PCR quantification of the Zbtb3 levels in the shLncKdm2b cells. Its very surprising that the levels of Zbtb3 become undetectable in these conditions, where presumably not all lncKdb2b is eliminated.
3. In the abstract, its not accurate to say that "The biological and molecular functions of the divergent lncRNAs have been not characterized.", due to prior studies on Evx1-as and Uph. This sentence should be rephrased
4. Page 3: "initiate Zfp292 expression" should be "initiates Zfp292 expression"

Referee #3:

The authors have addressed all my major concerns, I recommend therefore publication.

Minor corrections :
1) P6: "In addition, (...) confirming the deleted 838 bp region
does not act as an enhancer" The authors should turn down this sentence as the presence of H3K27ac suggests that the deleted DNA sequence might be important. Furthermore, detecting the GFP by western blot is not very sensitive and might result in wrong conclusion.
2) "Material and methods" should be verified to insure that data can be reproduced by others. For example, no information is available on the way the microarray is processed. The number of genes reported to be differentially expressed is enormous (>11000) and suggest that the cutoff used for this analysis were rather low.

Referee #4:

In this revised manuscript, the authors addressed the points raised by the reviewers. Their plots look almost successful. Addition of the data of the poly-A knock-in ES cells is amazing progress. However, there are still several points required further revision for publication.

1. Page 5: Although the authors stated that LncKdm2b knockout abrogates ESC self-renewal, they indeed succeeded to establish LncKdm2b-null ESCs. Therefore, 'abrogates self-renewal' is too strong to interpret the phenotype of LncKdm2b-null ESCs since there are several genes reported for their absolutely essential function (complete lack of self-renewal/establishment of ESCs without the function of the gene such as Pou5f1).

2. Page 6: The authors added a new data of the enhancer assay on request by the other reviewer. However, the GFP reporter is not so sensitive and it will be better to use the luciferase reporter system to confirm the enhancer activity.

3. Page 8: Both Srcap- and Zbtb3-null ESCs can be established by the Crispr/Cas9 knockout, so they retain the ability of self-renewal, which is not abrogated.

4. Page 9: The authors claimed that the loss of self-renewal ability is expected for Nanog-null ESCs, but it is incorrect. Chambers et al demonstrated that Nanog-null ESCs can be propagated although they show higher incidence of spontaneous differentiation (Nature, 2007), and the self-renewal of Nanog-null ESCs is enhanced in 2i culture (Figure S4 in Silva et al, Cell, 2009).

5. The authors showed the reduction of Nanog expression in Figure 5A, but as mentioned above, the complete loss of Nanog still allow self-renewal of ESCs, so the reduction of expression would give very mild effect. How much degree did the Nanog expression decreased in protein level?

6. The authors analyzed the functions of LncKdm2b, Scrap and Zbtb3 in ESCs and show functional importance. However, according to Figure 4C and 6B, both LncKdm2b and Zbtb3 express in both ICM and trophectoderm, suggesting its general roles in cell proliferation/survival on embryonic lethality rather than the specific role in ICM/pluripotent stem cells. It should be mentioned and discussed.

2nd Revision - authors' response 5 February 2018

Point-by-point response to the reviewers' comments

Referee #1:
1. The authors claim that they do not remove any functional enhancers in their deletion mutants as shown they do not detect any H3K4me1 but H3K27ac accumulation in this region. However, multiple studies have associated H3K27ac with functional enhancers (Creyghton et al. 2010; Rada-Iglesias et al. 2011; Hnisz et al. 2013; Whyte et al. 2013 etc). The accumulation of H3K27ac in this region looks actually like an enhancer and the authors should change their statement about H3K27ac being associated with transcriptionally active regions only. However, I am pleased that the authors made a pre-mature poly(A) insertion mutant that resembles the deletion phenotype.
Answer: We changed this statement about H3K27ac.

2. "Finally, we generated polyA insertion ESC lines and noticed that lncKdm2b was deleted in polyA insertion ESC lines (Fig. S3C)." The authors should re-phrase as insertion of a pre-mature polyA signal cannot "delete" a lncRNA.
Answer: We changed this wording as suggested.

3. The authors contradict themselves: they state in the abstract "The biological and molecular functions of the divergent lncRNAs have been not characterized", but have a long paragraph (highlighted in yellow) in the introduction about the known functions of divergent lncRNAs.
Answer: We changed this wording accordingly.

Referee #2:
1. Are the authors sure what they show in Fig. 2A is a Northern blot? It looks much more like a RT-PCR gel
Answer: Yes, these are the Northern blot images. Since we loaded larger amounts of RNA sample, the images of lncKdm2b transcripts were much bigger with stronger contrast. We repeated this experiment and replaced the old images with better ones in the new Fig. 2A.

2. Figure S5H also looks "too good to be true". The authors should show an RT-PCR quantification of the Zbtb3 levels in the shLncKdm2b cells. Its very surprising that the levels of Zbtb3 become undetectable in these conditions, where presumably not all lncKdb2b is eliminated.
Answer: This is the case. We repeated these immunoblotting experiments for three times. We also conducted RT-PCR quantifications of the Zbtb3 mRNA levels in this experiment. LncKdm2b depleted ESC D3 cells displayed background levels of Zbtb3 mRNA, but undetectable for its protein levels. We included RT-PCR quantifications of the Zbtb3 mRNA levels in the new Fig. S5H.

3. In the abstract, its not accurate to say that "The biological and molecular functions of the divergent lncRNAs have been not characterized.", due to prior studies on Evx1-as and Uph. This sentence should be rephrased
Answer: We rephrased this sentence.

4. Page 3: "initiate Zfp292 expression" should be "initiates Zfp292 expression"
Answer: We revised it.

Referee #3:
1) P6: "In addition, (...) confirming the deleted 838 bp region does not act as an enhancer" The authors should turn down this sentence as the presence of H3K27ac suggests that the deleted DNA sequence might be important. Furthermore, detecting the GFP by western blot is not very sensitive and might result in wrong conclusion.
Answer: This is a good suggestion. In order to verify this result, we cloned the 838bp region to a pGL3-promoter vector and performed enhancer reporter luciferase assays. As shown in the new Fig. S3C, we observed that insertion of the 838bp upstream of the promoter-luc+ transcriptional unit did not induce luciferase gene transcription.

2) "Material and methods" should be verified to insure that data can be reproduced by others. For example, no information is available on the way the microarray is processed. The number of genes reported to be differentially expressed is enormous (>11000) and suggest that the cutoff used for this analysis were rather low.
Answer: We included the microarray processing information in the section of Material and methods. The cutoff used for this analysis is fold change >2.0, FDR<0.05.

Referee #4:
1. Page 5: Although the authors stated that LncKdm2b knockout abrogates ESC self-renewal, they indeed succeeded to establish LncKdm2b-null ESCs. Therefore, 'abrogates self-renewal' is too strong to interpret the phenotype of LncKdm2b-null ESCs since there are several genes reported for their absolutely essential function (complete lack of self-renewal/establishment of ESCs without the function of the gene such as Pou5f1).

Answer: We revised this wording accordingly.

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Answer: Chambers et al showed that Nanog-null ESCs displayed a reduced capacity to self-renew in LIF-contained culture (Figure 2f, Chambers I, et al. Nature, 2007). They deleted *Nanog* in ESC E14Tg2a cell line for their research. With the same LIF-contained culture condition, we found that *Nanog* knockout in ESC D3 cell line dramatically impaired the self-renewal capacity of ESCs (Fig. 5F). Moreover, rescue of *Nanog* in Nanog-null ESC D3 cells could recover the self-renewal capacity of ESCs (Fig. 5F). Additionally, rescue of *Nanog* in lncKdm2b-, Srcap- or Zbtb3-deleted ESC D3 cells could also restore the self-renewal capacity of ESCs (Fig. 5F). We repeated these experiments at least three times. In the ESC D3 cells, Nanog harbored much stronger potential to maintain self-renewal than that in E14Tg2a cells. By contrast, Silva et al used the Nanog-null E14Tg2a cell line and cultured them in 2i/LIF culture, which caused enhanced self-renewal capacity of ESCs (Figure S4, Silva, et al. Cell 2009), suggesting Nanog is not required for the established pluripotent cells in 2i/LIF culture. These data suggest that Nanog might play distinct roles in the regulation of self-renewal versus differentiation of different ESC cell lines. We addressed this issue in the revised text.

5. The authors showed the reduction of Nanog expression in Figure 5A, but as mentioned above, the complete loss of Nanog still allow self-renewal of ESCs, so the reduction of expression would give very mild effect. How much degree did the Nanog expression decreased in protein level?
Answer: We quantified these blots and showed ratios in the Fig. S5H.

6. The authors analyzed the functions of LncKdm2b, Scrap and Zbtb3 in ESCs and show functional importance. However, according to Figure 4C and 6B, both LncKdm2b and Zbtb3 express in both ICM and trophectoderm, suggesting its general roles in cell proliferation/survival on embryonic lethality rather than the specific role in ICM/pluripotent stem cells. It should be mentioned and discussed.
Answer: This is a good point. We addressed this issue in the discussion section.

3rd Editorial Decision 8 February 2018

Thank you for submitting your revised manuscript. I appreciate the introduced changes, and I am happy to accept your manuscript for publication in the EMBO Journal.
Congratulations!

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Zusen Fan
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2017-97174R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For western blotting experiments in this study, about 2e5 cells were analyzed in at least five separate experiments. For QPCR in this study, about 2e5 cells were analyzed in at least five separate experiments.For embryo analysis more than 100 typical embryos per stage were observed and calculated in at least five individual experiments, and the sample size is acceptable for the field. For the other statistic methods in the figures, we performed at least five independent experiments of each mouse from at least five mice for each group. The background of mice was C57BL/6, and mice were grouped by the same age and gender. We provided the statement about
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For animal studies, we used 5 mice with the same genetic background per group. We provided the statement about sample size in each figure legend.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We used littermates with the same age and gender for each group. We excluded the mice 5 g thinner than other littermates before any treatment or analysis. We provide the statement in Methods, "Generation of knockout mice" subsection.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	We did not use randomization in our animal studies.
For animal studies, include a statement about randomization even if no randomization was used.	We did not use randomization in our animal studies. We provide the statement in Methods, "Generation of knockout mice" subsection
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	We were not blinded to the group in our animal studies.
4.b. For animal studies, include a statement about blinding even if no blinding was done	We were not blinded to the group in our animal studies. We provide the statement in Methods, "Generation of knockout mice" subsection.
5. For every figure, are statistical tests justified as appropriate?	Yes

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes. Methods, 'Statistical analysis' subsection.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We showed these information in Methods, 'Antibodies and reagents' subsection.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	subsection. All the mice we used were C57BL/6 background, female, and 8-12 week old. We reported this in Method section " Generation of knockout mice" subsection.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	We provided the statement in page 15, Method section " Generation of knockout mice" subsection.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Transcriptome profiles for lncKdm2b-/- ESCs and WT ESCs ESCs were uploaded to GEO database. Microarray data were deposited in GEO with an accession number (GSE92868). RNA-seq analyses of mouse ESCs, EB, and differentiated ESCs by LIF withdrawal were performed by library construction and sequenced on Illumina Hiseq2000. RNA-seq data were deposited in GEO with an accession numbers (GSE93238).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We deposite these dataset to GEO
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A