EMBO reports

Manuscript EMBO-2015-40933

Yap1 is dispensable for self-renewal but required for proper differentiation of mouse embryonic stem (ES) cells

HaeWon Chung, Bum-Kyu Lee, Nadima Uprety and Jonghwan Kim

Corresponding author: Jonghwan Kim, University of Texas at Austin

Review timeline:

Submission date:	24 June 2015
Editorial Decision:	23 July 2015
Revision received:	23 October 2015
Editorial Decision:	16 November 2015
Revision received:	08 January 2016
Accepted:	22 January 2016

Editor: Barbara Pauly

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 23 July 2015

Thank you very much for the submission of your research manuscript to our editorial office and for your patience while we were waiting to hear back from the referees. We have just now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I would prefer not to repeat the details of them here. What becomes clear is that while all referees in principle agree on the interest of the study, they also point out instances in which they feel the data needs to be strengthened and they all provide feedback and suggestions on how to achieve this. For example, both referees 2 and 3 state that the contributions of TAZ and TEAD should be analyzed. In general, all reviewers point out instances in which additional controls, clarifications or details on experimental procedures should be provided and overstatements should be toned down. We would not insist on the request of referee 2 to provide further mechanistic insights into the function of Yap1 during differentiation, but you are, of course, welcome to add any data in this directions if you already have them at hand. The same goes for the request of this reviewer to provide a more detailed analysis of the different cell types that arise from the stem cells, but you should tone down the conclusions about the trophoblast/trophectoderm differentiation, as suggested by referee 1.

Given the potential interest of your findings, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees as listed above and in their reports should be addressed.

REFEREE REPORTS

Referee #1:

This MS is essentially claiming the opposite of a 5-years old paper, Lian et al., Genes and Development 2010. In that Genes and Development paper YAP was promoting ESC self-renewal and inhibiting differentiation. I was thus initially puzzled when I saw this MS, but after reconsideration of the literature and comparison with the present work, I have to say that the results of this MS are sound, indicating, admittedly quite convincingly, that YAP is in fact irrelevant for mouse ESC self renewal and instead instrumental for their differentiation. The key evidence of this MS are independent YAP knockout cell lines and other experiments in different ESC lines. It is thus important to offer the readers alternative views on the role of YAP in ES cells.

Specific comments.
1) In the way the MS is written, the authors are mainly centering their interpretations around the role of YAP in the mouse embryo. This is a bit old-fashioned, as ESC biology is now considered a field on its own; and, after all, ESCs are cell lines, with their own peculiarities, that only in part or only under specific conditions recapitulate what is going on in their tissue of origin, the ICM of the blastocyst. Moreover the role of YAP in the early embryogenesis (or those of YAP + TAZ), are poorly investigated and far to be clear. The authors should thus avoid overinterpretations.

To start, some statements from the abstract should be removed:
"whereas it is sequestered and inactive in the cytoplasm of the inner cell mass (ICM)"

this is an overstatement; I am not sure this is functionally proven.

"suggesting a functional discrepancy of Yap1 between in vivo and in vitro settings"; "which recapitulates Yap1 functions in early embryogenesis."

The ES cell field is full of these discrepancies with the blastocyst. This is not the point of this MS, and it would not be a problem.

There is an excessive emphasis on the supposed role of YAP in the trophoblast layer. Actually, YAP inactivation upregulates other differentiation markers, such as mesoderm and endoderm markers. Cdx2 is certainly a trophoblast marker but together with GATA6 it may well indicate endoderm regionalization/patterning. Again, please note that what matters here is YAP being required for differentiation not the fact that this recapitulates or not a corresponding role of YAP in the mouse gastrula, where, in fact, the role of YAP or YAP/TAZ is largely unknown as far as I can tell.

In essence, the author should stick to their data and tone down any blastocyst-centered interpretation of their results.

2) Strange enough, the authors are citing every bit literature (see below my comment on their Ref34, for example), but not a paper that is in fact consistent (not to say quite overlapping) with their own claim. Azzolin et al., in Cell 2014, figure 6, showed that double inactivation of YAP/TAZ has no effect of mouse ESC self-renewal (in line with what shown in this MS), at least in 2-inhibitors (2i) medium (Mek-i+GSK3-i). They also show that, in 1i medium (Mek-i alone), which is in fact a differentiation medium, loss of YAP and TAZ sustains pluripotency (again consistently with the claim of this MS). It appears to me that this 2014 Cell paper was centered on the role of YAP as Wnt inhibitor and did not emphasize this point sufficiently; moreover, Azzolen never showed what was actually going on with those cells nor what differentiation endogenous YAP was eventually promoting. In any case, the present MS does represent sufficient novelty and significantly extend over the Azzolen Cell paper.

In the present MS, the authors must find space (cutting short intro and other embryo-speculations) to discuss comprehensively the state of the art of YAP and mESCs, trying to help the reader in making sense of other reports, some consistent and other inconsistent. In sum:

a) they should discuss Azzolin et al., explain what is the overlap with the present MS and to what extend the new data extend those findings.

b) It is also puzzling to understand why Lian et al got remarkably opposite results. It should not be sufficient to state: "it is inconsistent". If one looks carefully at the Lian data, it appears only 1 out of 2 siRNAs (shRNA2, fig2) was really effective in their hands. In this MS at least there is YAP genetic inactivation and consistent YAP KD. Moreover, Lian et al used one ES cell type, the D3 ESC lines; here authors used the established J1 line for YAP knockout. Is there a cell line bias? What cells were used for shRNA KD? Are the KD or KO results similar to J1 when carried out in CJ7, E14?

c) There is another paper that the authors should try to mention and discuss if space allows it. Beyer et al., Cell reports 2013, suggested that YAP and TAZ operate as inhibitors of mesendoderm gene expression, at least in human ESCs, suggesting differences between human and mouse ESCs.

3) The MS is not very easy to read and in some part almost impossible to follow:
- dES, might be switched to differentiating- differentiated-ESCs

- Periods like these should be entirely rewritten for the benefit of the general reader:
We also investigated
the activity of previously defined functional modules in ES cells (Core and PRC) [26]. Previously, it has been shown that Core module activity is decreased while PRC module activity is increased upon differentiation of ES cells. While dES showed the expected Core and PRC module activities, ES cells with KD or KO of Yap1 showed reversed module activities compared to those of dES cells (Fig 2I).

Although the down-regulation of Pou5f1 and Nanog were not significantly affected, we found that some pluripotency factors, such as Sox2 and Esrrb were not down-regulated normally during differentiation of Yap1-depleted cells

As shown in Figure 3B, DEGs in Yap1-KD cells compared to the control ES cells under differentiation condition confirmed that the genes were not properly up or down-regulated upon differentiation (Table EV3). We further analyzed the activity of the Core and PRC modules, and confirmed that upon differentiation, the Core module activity was relatively higher while PRC module activity was lower in Yap1-KD cells than wild-type ES cells (Fig 3C), indicating that KD of Yap1 delayed or impaired proper differentiation of ES cells.

4) Figure 4. this whole figure is quite diluting the message and provides just correlative data. It is overall preliminary, requiring more experiments to complete the message they are trying to convey. Changing cell density, in turn correlated with changes in Yap1 localization, and then seeing some effects are three and not necessarily interconnected events; but there is no YAP functional link to demonstrate causality. Basically: as it is figure 4 might be deleted without any damage as the MS is on YAP (irrespectively of its regulation) and mESC differentiation and self renewal.

Other suggested/required changes:

5) Figure 1 (YAP upregulation during diff.): figure 1 seems to me a consequence of Figure 2. Please consider switching figure order. Moreover Fig 1 A-C are poorly informative, can be moved to Supplementary.
In general, I see only minor fluctuations in the initial panels; overall a weak start.

6) FIG 2A lacks of negative controls. Use Oct4 knockdown or Diff ESCs

7) FIG 2G ; Fig 3b and 3f: If they want to highlight specific genes, they must show the corresponding RT-PCR. Some of this (but only part) is in supplementary figures. They should show at least some of them in main figs (and the rest in supplementary info). It is impossible for the reader to know if an indicated gene in fig2g or 3b is black, red or green; thus, please delete gene names from RnaSeq panels, and eventually expand the RT PCR panels.

8) Fig2g is shown in a strange way. Now the comparison with differentiating ES cells is actually emphasizing the few genes that are different between YAP KD/KO and ES cells, rather than the (possibly much larger) overlap between dES cells and YAP KO cells (that is instead their claim and purpose of this panel). Please show a different graph. This can be done in several ways; as example, Fig 3B is of much more immediate comprehension. It should be possible to appreciate what happens after YAP knockdown: Is the gene set being up- downregulated in ESCs similarly up/down in the YAP KO? Now this conclusion can be only inferred from the black color!

9) Fig 3D is for Supplementary info.

10) Figure S1F GAPDH strip has severe problems, at least on my pdf.

11) It is imperative for all their cell line to show the effective knockout of YAP: by sequencing of the locus and western blot.

12) Fig1H is very hard to understand; and not even explained.

13) The following paragraph of the Conclusion on other reports supporting their own results, is incorrect (and a bit unfair) and should be deleted:
"This result is consistent with the role of Yap1 in human pluripotent stem cells where Yap1 does not affect the regulation of Pou5f1 or the generation of induced pluripotent stem cells [34,35]"
Ref34 is really on something else. It is on an Rnai screen and simply show YAP siRNAs as negative control, but by no mean this is meaningful (those ref34 oligos were not validated; for Ref34 YAP siRNAs are simply equivalent to GFP siRNAs or unrelated siRNA). By no mean this is a direct study of YAP.
Ref35 deals with reprogramming of human fibroblast to iPSCs; little is known, but this is a process perhaps only partially related to the preservation of self-renewal and differentiation of established mES cell lines here investigated.

Referee #2:

In this manuscript Chung et al. explore the roles of Yap1 in mouse ESC differentiation. Based on their observations the authors argue that Yap1 is not required for maintaining ESC pluripotency, but rather is required for proper differentiation of these cells. They show that ESCs depleted of Yap1 show no changes in markers of pluripotency, whereas overexpression of Yap1 induces the expression of genes associated with differentiation. The authors also reason that changes in cell fate markers that result from alterations in cell density, or disruption of cell adhesion, occur as a results of induced nuclear Yap1 activity.

Given that Yap1 been described as an ESC regulator, understanding what Yap1 is doing in these cells is of clear importance. This manuscript attempts to study the roles of Yap in ESCs, and while some interesting descriptive observations are made, no detailed insight is gained into Yap function. Additionally, several of the conclusions seem to be based on improperly controlled experiments (based on the presented methods). Therefore, in my opinion much more insight into the role of Yap in ESC fate specification is required before this manuscript can be considered for publication. Moreover, there are several issues that need to be addressed, some of which are outlined below:

- The manuscript fails to address or take into account several prior studies that address the roles of Yap1 in ESCs. Most importantly, prior work has shown that Yap1 functions redundantly with Taz in mouse pre-implantation development and in human ES cells. Therefore, depletion of Yap1 in mouse ESCs may not be sufficient to observe any clear phenotypes. The study would benefit from additional analyses of Taz and Yap1/Taz depletion in ESCs.

- No details are given for how the experiments were performed in Figure 2. The authors state that Yap1 is not required for the self-renewal of ES cells, but do not provide details regarding how the experiments being performed, how many passages were analyzed, or even at when the pluripotency markers were analyzed with respect to depletion.

- The authors argue that loss of Yap1 or overexpression of Yap1 affects the differentiation of ESCs, but no details are given into the specification of these cells. Rather, very broad statements and conclusion are made. A detailed analysis of the various cell fates that may be affected should be performed. Also, given that Yap1 is known to crosstalk with other signals in the context of ESCs (e.g. crosstalk with Lif, Wnt, BMP, TGF-beta, and Notch signaling) any analysis of cell fate should carefully consider these other signals, and if necessary, various culture and differentiation methods should be tested before drawing conclusions.

- Based on the data presented, ESCs appear to have significant changes in gene expression compared to the controls following the loss of Yap1. While this profile is more similar to ESCs than of differentiated EBs, these gene expression changes may be insightful into the roles of Yap1 in ESCs. Do the gene expression changes provide any clues into what Yap1 is doing in cell fate specification? Some analysis of this data, beyond simple correlation, should be included and discussed.

- The details for how Yap1 localization and activity was measured and compared in undifferentiated and differentiating ESCs is unclear. From the methods section it appears that the differentiating ESCs were continually being passaged during the analysis of Yap1, whereas the undifferentiated were not, and therefore dissociation of the cells may be the primary reason for observing increased nuclear Yap1 activity under these conditions. A more clear description of these experiments is required, and if culturing conditions were different, then conclusions with regard to Yap1 activity cannot be drawn from these experiments.

- How was Yap1 overexpression achieved? No details are given. For example, were these lines generated with viral transduction? Are they stably selected? Is the expression constitutive? Were the control cells generated in parallel, and similarly selected? These variables could have a huge influence on the cellular state and must be properly controlled. Additionally, the text states that overexpressed Yap1 mainly resides in the nucleus, but this does not appear to be different that the endogenous Yap shown in the control in Figure 3D. Rather, Yap just appears to be at higher levels.

- Based on the images in 1F, 1G and 3D and - it appears that Yap1 in abundantly localized in the nucleus of undifferentiated ES cells. The one field showing a possible increase in nuclear Yap1 levels in differentiated ES cells is unconvincing on its own - and all the conclusions regarding Yap1 localization would greatly benefit from quantitation and biochemical nuclear/cytoplasmic fractionation examined by immunoblotting.

- A control for Yap1 IF signal should be included in the study, particularly given that the authors have in hand a Yap1 deleted cell line.

- Differences in Yap1 localization in Figure 4A are not clear. To me, nuclear Yap1 levels look similar between the two conditions. Better images and quantitation would help make this conclusion more convincing.

- All the experiments related to cell density or E-cadherin depletion are vastly overstated, as the authors do not provide any evidence that the effects following changes in density or the loss of E-cadherin are mediated by Yap1. To draw any meaningful conclusions, the authors should perform similar experiments in their Yap1-deleted/depleted cell lines to show that fate changes are mediated by Yap (these experiment may also require Taz knockdown).

Referee #3:

This study describes the role of Yap1 in the self-renewal and differentiation of mouse embryonic stem (ES) cells. Authors of this study used 3 feeder-free ES cell lines and observed increased expression of Yap1, reduction in the phosphorylation of Yap1, and nuclear accumulation of Yap1 during the differentiation of these cells after LIF withdrawal. Knockdown of the gene encoding Yap1 did not significantly affect the self-renewal and gene expression profiles of ES cells. Cells lacking Yap1 showed defects in their differentiation while cells with Yap1 overexpression underwent differentiation. Alteration of subcellular localization of Yap1 by inhibiting E-cadherin-

mediated cell-cell adhesion and alteration of cell density also affected the differentiation of ES cells. Based on these results, the authors concluded that Yap1 was dispensable for the self-renewal but was required for the proper differentiation of ES cells. Interestingly, these observations are inconsistent with those of previous studies by Lian et al (2010) and Tamm et al (2011) that reported, Yap1-Tead activity was required for the self-renewal of ES cells. Although the results of the present study are of high quality and are convincing, the authors only examined Yap1. If the authors' conclusion is correct, one would expect that Tead activity is also dispensable for the self-renewal of ES cells. Addition of this information would further strengthen the authors' conclusion and would clarify whether functions of Yap were mediated by Tead in this context.

Major comment:
The data presented in this study showed that Yap1 was dispensable for the self-renewal but was required for the proper differentiation of ES cells. These results are inconsistent with those reported in studies by Lian et al (2010) and Tamm et al (2011), therefore, one should interpret these results with caution. Previous studies manipulated both Yap1 and Tead and obtained consistent results, thus, it is important to examine whether manipulation of Tead would also provide the same results as those obtained in the present study. I think that analyses of several key markers for the ES cells that are knocked down and/or overexpressed with active/inactive forms of Tead should be sufficient for this purpose.

Minor comments:
1. Throughout the manuscript, the authors considered that ICM and ES are equivalent, and therefore, that Yap performs similar roles in these cells. However, this is incorrect because ES cells are derived from the epiblast (a derivative of ICM) of hatched blastocysts and not from the ICM of early blastocysts. The role of Hippo signal/Yap1 during morula to early blastocyst stages is to specify the TE/ICM fates of the cells. However, roles of the Hippo signal/Yap1 in the epiblast are unknown. To date, no study has described the distribution of Yap in the epiblast of hatched blastocysts. Moreover, presence of Yap in the nuclei of epiblast cells cannot be ruled out.

2. Figure 2E-G. Please include the duration of culturing ES cells under differentiation conditions to obtain dES cells shown in these panels.

3. Figure 4E. The graph shows higher luciferase activity at high density. However, in the main manuscript, the result indicates reduced transcriptional activity at high density. Please recheck the accuracy of the data presented at both the instances.

1st Revision - authors' response 23 October 2015

RESPONSE TO REVIEWERS

Overall Response:

We appreciate all three reviewers for their critical and constructive comments on our manuscript. Common concerns addressed by the reviewers are inner cell mass (ICM) centered interpretation of our data as well as omission of investigating the possibility of involvement of other Hippo signaling pathway components such as Taz and Tead family proteins which may compensate Yap1 functions in Yap1 depleted embryonic stem (ES) cells. In order to address reviewers' comments, we performed additional experiments including generation of other Yap1 CRISPR-Cas9 mediated knock out (KO) cell lines with CJ7 and E14 ES cells, double knockdown (KD) of Yap1 and Taz, and triple KD of Tead 1/3/4 to examine whether deletions of these genes affect self-renewal of ES cells. We also substantially revised our main text to focus on ES cell biology and clarify methods to help the readers' understanding.

Referee #1:

This MS is essentially claiming the opposite of a 5-years old paper, Lian et al., Genes and Development 2010. In that Genes and Development paper YAP was promoting ESC self-renewal and inhibiting differentiation. I was thus initially puzzled when I saw this MS, but after reconsideration of the literature and comparison with the present work, I have to say that the results of this MS are sound, indicating, admittedly quite convincingly, that YAP is in fact irrelevant for mouse ESC self renewal and instead instrumental for their differentiation. The key evidence of this

MS are independent YAP knockout cell lines and other experiments in different ESC lines. It is thus important to offer the readers alternative views on the role of YAP in ES cells.
Specific comments. **1-1)** In the way the MS is written, the authors are mainly centering their interpretations around the role of YAP in the mouse embryo. This is a bit old-fashioned, as ESC biology is now considered a field on its own; and, after all, ESCs are cell lines, with their own peculiarities, that only in part or only under specific conditions recapitulate what is going on in their tissue of origin, the ICM of the blastocyst. Moreover the role of YAP in the early embryogenesis (or those of YAP + TAZ), are poorly investigated and far to be clear. The authors should thus avoid over interpretations.

Response: We appreciate the reviewer's constructive suggestion that the manuscript needs to focus on ES cell biology. Since our results are opposite to those of Lian et al., Genes and Development 2010 [1], we tried to support our data by comparing ES cells with their in vivo origin ICM. However, with the helpful comments and references suggested by the reviewer, we recognized that it is no longer necessary to compare our data with the data generated from ICM. So, in our revised manuscript we have focused on the roles of Yap1 in ES cells.

To start, some statements from the abstract should be removed:
1-1a) "whereas it is sequestered and inactive in the cytoplasm of the inner cell mass (ICM)" this is an overstatement; I am not sure this is functionally proven.
Response: Multiple papers suggested cytoplasmic localization of Yap1 in the ICM of pre-implanted embryos [2– 5]. However, as commented by the reviewer, the functions of Yap1 in ICM have not been well understood. We have modified the main text to address only well-established roles of Yap1 in early embryo development.

1-1b) "suggesting a functional discrepancy of Yap1 between in vivo and in vitro settings"; "which recapitulates Yap1 functions in early embryogenesis."
Response: Since we now mainly focus on ES cells rather than ICM, we have removed this sentence as suggested.

The ES cell field is full of these discrepancies with the blastocyst. This is not the point of this MS, and it would not be a problem.
There is an excessive emphasis on the supposed role of YAP in the trophoblast layer. Actually, YAP inactivation upregulates other differentiation markers, such as mesoderm and endoderm markers. Cdx2 is certainly a trophoblast marker but together with GATA6 it may well indicate endoderm regionalization/patterning. Again, please note that what matters here is YAP being required for differentiation not the fact that this recapitulates or not a corresponding role of YAP in the mouse gastrula, where, in fact, the role of YAP or YAP/TAZ is largely unknown as far as I can tell. In essence, the author should stick to their data and tone down any blastocyst-centered interpretation of their results.

Response: Again, we sincerely appreciate the reviewer's insightful comment to help us to improve our manuscript. We agree that comparing the roles of Yap1 in ES cell and ICM is not the main point of the manuscript. As indicated above, we now minimized blastocyst-centered interpretation of our data and focused on roles of Yap1 in ES cells throughout the revised manuscript.

1-2) Strange enough, the authors are citing every bit literature (see below my comment on their Ref34, for example), but not a paper that is in fact consistent (not to say quite overlapping) with their own claim. Azzolin et al., in Cell 2014, figure 6, showed that double inactivation of YAP/TAZ has no effect of mouse ESC self-renewal (in line with what shown in this MS), at least in 2-inhibitors (2i) medium (Mek-i+GSK3-i). They also show that, in 1i medium (Mek-i alone), which is in fact a differentiation medium, loss of YAP and TAZ sustains pluripotency (again consistently with the claim of this MS). It appears to me that this 2014 Cell paper was centered on the role of YAP as Wnt inhibitor and did not emphasize this point sufficiently; moreover, Azzolen never showed what was actually going on with those cells nor what differentiation endogenous YAP was eventually promoting. In any case, the present MS does represent sufficient novelty and significantly extend over the Azzolen Cell paper.

In the present MS, the authors must find space (cutting short intro and other embryo-speculations) to discuss comprehensively the state of the art of YAP and mESCs, trying to help the reader in making sense of other reports, some consistent and other inconsistent. In sum:

Response: We sincerely appreciate the reviewer's comments and suggested reference. As commented, Azzolin et al., claimed that Yap1 and Taz are not required for the self-renewal of ES cells in 2i medium [6], which is strongly supporting our observation. While we still do not clearly understand the causes of the discrepancy between our observation and Lian et al.'s, we systematically showed that Yap1 is not required for the self-renewal of ES cells using three different ES cell lines (J1, E14, and CJ7) with multiple shRNA-mediated KD experiments as well as CRISPR-Cas9 mediated KO followed by investigating morphology, AP activity and global gene expression profile, suggesting that dispensability of Yap1 in mouse ES cells is in fact general phenomenon. We also discussed genuine roles of Yap1 and a current controversy regarding functional roles of Yap1 in ES cells and other issues in the last paragraph of our revised manuscript.

1-2a) they should discuss Azzolin et al., explain what is the overlap with the present MS and to what extend the new data extend those findings.

Response: We again appreciate the suggested reference. As commented, Azzolin et al., showed that Yap1 and Taz are not required for the self-renewal of mouse ES cells in 2i medium [6], which is consistent with our data. We have cited this work and discuss about the results in multiple places in our revised manuscript.

1-2b) It is also puzzling to understand why Lian et al got remarkably opposite results. It should not be sufficient
to state: "it is inconsistent". If one looks carefully at the Lian data, it appears only 1 out of 2 siRNAs (shRNA2, fig2) was really effective in their hands. In this MS at least there is YAP genetic inactivation and consistent YAP KD. Moreover, Lian et al used one ES cell type, the D3 ESC lines; here authors used the established J1 line for YAP knockout. Is there a cell line bias? What cells were used for shRNA KD? Are the KD or KO results similar to J1 when carried out in CJ7, E14?

Response: We initially tried to reproduce the previously published data using the same shRNA sequences. However, instead of providing shRNA sequences, Lian et al., cited their previous paper providing shRNA sequences targeting human YAP gene. In the method section of Lian et.al.'s paper, they described "To generate YAP and TEAD1/3/4 knockdown cells, mES cells were infected with lentivirus containing shRNA targeting YAP and TEAD, respectively" [7]. The sequences of human YAP shRNAs used in their paper do not perfectly match with mouse Yap1 mRNA sequence. When we checked the cited sequences, shRNA sequence #1 shows two mismatches with mouse Yap1 mRNA. The sequence #2 did not match with mouse Yap1 transcript at all. Instead, the sequence partially matches with Mios, Zbtb40, and Tyw3 that are not implicated in self-renewal of ES cells. At this point, we assume that these discrepancies may be attributed to different shRNAs between Lian's and ours. Since Lian et al. did not provide sequence information in their manuscript, we could not directly address what was the cause of the discrepancy. We provide all shRNA sequences we used in the current study (Table EV1), all of which specifically target mouse Yap1 transcript.
In addition, we agree with the reviewer's comment on the possibility of cell line bias between D3 and other three ES cell lines that we tested. However, we were not able to find prior reports regarding the bias among those cell lines. In Fig EV2, we have shown that KD of Yap1 in CJ7 and E14 does not disrupt self-renewal of ES cells. Based on the reviewer's comment, we additionally performed CRISPR-Cas9 mediated KO of Yap1 in CJ7 and E14. Consistently, deletion of Yap1 does not disrupt self-renewal of both CJ7 and E14 ES cell lines (Fig EV2).

1-2c) There is another paper that the authors should try to mention and discuss if space allows it. Beyer et al., Cell reports 2013, suggested that YAP and TAZ operate as inhibitors of mesendoderm gene expression, at least in human ESCs, suggesting differences between human and mouse ESCs.
Response: We thank the reviewer for constructive comment. We cited and discussed the suggested paper in our revised manuscript.

1-3) The MS is not very easy to read and in some part almost impossible to follow:

Response: We changed our text as follows (comments 1-3a – 1-3d).

1-3a) dES, might be switched to differentiating-differentiated-ESCs

Response: As suggested, we changed dES to differentiating ES cells or ES cells upon differentiation throughout the manuscript.

1-3b) Periods like these should be entirely rewritten for the benefit of the general reader: We also investigated the activity of previously defined functional modules in ES cells (Core and PRC) [26]. Previously, it has been shown that Core module activity is decreased while PRC module activity is increased upon differentiation of ES cells. While dES showed the expected Core and PRC module activities, ES cells with KD or KO of Yap1 showed reversed module activities compared to those of dES cells (Fig 2I).

Response: We have modified the sentences and they now read as "We also investigated the activity of previously defined functional modules in ES cells (Core and PRC) [26]. Module activity is defined as an averaged expression of all genes in each module. Briefly, the Core module includes core pluripotency factors such as Pou5f1, Nanog, and Sox2, most of which are highly expressed in self-renewing ES cells. On the other hand, the PRC module includes many lineage-specific regulators, such as Fgf5, Bmp4, and Hand1, most of which are repressed in ES cells. Since differentiation of ES cells decreases the activity of Core module but increases the activity of PRC module, we sought to examine module activities upon KD or KO of Yap1 to test if cells undergo differentiation. As shown in Fig 1J, ES cells with depletion of Yap1 do not show down-regulation of Core module activity or up-regulation of PRC module activity, suggesting that Yap1-depleted ES cells largely maintain self-renewing and undifferentiated states."

1-3c) Although the down-regulation of Pou5f1 and Nanog were not significantly affected, we found that some pluripotency factors, such as Sox2 and Esrrb were not down-regulated normally during differentiation of Yap1depleted cells

Response: We have modified the sentence and it now reads as "We further found that expression levels of some pluripotency factors such as Sox2 and Esrrb were highly maintained in Yap1-depleted cells upon differentiation, although the expression of other core factors, Pou5f1 and Nanog, were decreased similar to their levels in control cells upon differentiation (Fig EV4A)."

1-3d) As shown in Figure 3B, DEGs in Yap1-KD cells compared to the control ES cells under differentiation condition confirmed that the genes were not properly up or down-regulated upon differentiation (Table EV3). We further analyzed the activity of the Core and PRC modules, and confirmed that upon differentiation, the Core module activity was relatively higher while PRC module activity was lower in Yap1-KD cells than wild-type ES cells (Fig 3C), indicating that KD of Yap1 delayed or impaired proper differentiation of ES cells.

Response: We have modified the sentences and they now read as "As shown in Fig 4B, gene expression patterns of DEGs (Yap1 KD ES cells/wild-type ES cells) upon differentiation showed an inverse correlation with the expression patterns of wild-type differentiating cells over self-renewing ES cells (Table EV3). The heatmap results clearly revealed that Yap1-depleted ES cells are not properly differentiated. Additional analyses of the Core and PRC module activity consistently indicate that Yap1 depletion causes stronger Core module activity with weaker PRC module activity during differentiation, indicating that KD of Yap1 delayed or impaired proper differentiation of ES cells (Fig 4C)."

1-4) Figure 4. this whole figure is quite diluting the message and provides just correlative data. It is overall preliminary, requiring more experiments to complete the message they are trying to convey. Changing cell density, in turn correlated with changes in Yap1 localization, and then seeing some effects are three and not necessarily interconnected events; but there is no YAP functional link to demonstrate causality. Basically: as it is figure 4 might be deleted without any damage as the MS is on YAP (irrespectively of its regulation) and mESC differentiation and self renewal.
Response: We agree to the reviewer's comment that our data related to the former Fig 4 may dilute the main message of our manuscript. Therefore, we deleted the data and description related to the former Fig 4 from our revised manuscript.

Other suggested/required changes:
1-5) Figure 1 (YAP upregulation during diff.): figure 1 seems to me a consequence of Figure 2. Please consider switching figure order. Moreover Fig 1 A-C are poorly informative, can be moved to Supplementary. In general, I see only minor fluctuations in the initial panels; overall a weak start.

Response: While our RT-qPCR results show up-regulation of Yap1 upon differentiation of ES cells, Lian et. al., claimed that mRNA expression levels of Yap1 decreased ~90% upon differentiation. Therefore, we wanted to clarify the level of Yap1 in self-renewing ES and differentiating ES cells using publicly available data and our own data (former Fig 1A-C). However, we also agree with the reviewer's comment that former Fig 1 (Fig 3 in the revised version) is not a strong start. As suggested, we have switched the order of figures.

1-6) FIG 2A lacks of negative controls. Use Oct4 knockdown or Diff ESCs

Response: We added data from Pou5f1 KD ES cells as suggested.

1-7) FIG 2G ; Fig 3b and 3f: If they want to highlight specific genes, they must show the corresponding RT-PCR. Some of this (but only part) is in supplementary figures. They should show at least some of them in main figs (and the rest in supplementary info). It is impossible for the reader to know if an indicated gene in fig2g or 3b is black, red or green; thus, please delete gene names from RnaSeq panels, and eventually expand the RT PCR panels.

Response: We have deleted gene names in the heatmap (Current Fig 1G) and tested expression levels of additional genes by RT-qPCR as suggested (Fig EV1C). We added Fig 1H to show expression levels of multiple lineage specific marker genes. As shown in Figs 1G, 1H, and EV1C most of the genes significantly up-regulated upon differentiation of ES cells do not show significant changes in gene expression upon KD or KO of Yap1, indicating that unlike previous study, Yap1 deletion does not promote differentiation of ES cells.

1-8) Fig2g is shown in a strange way. Now the comparison with differentiating ES cells is actually emphasizing the few genes that are different between YAP KD/KO and ES cells, rather than the (possibly much larger) overlap between dES cells and YAP KO cells (that is instead their claim and purpose of this panel). Please show a different graph. This can be done in several ways; as example, Fig 3B is of much more immediate comprehension. It should be possible to appreciate what happens after YAP knockdown: Is the gene set being up-downregulated in ESCs similarly up/down in the YAP KO? Now this conclusion can be only inferred from the black color!

Response: We apologize for the confusion. We used expression data obtained from differentiating ES cells as our experimental controls. Upon differentiation of ES cells, we see many differentially expressed genes (core pluripotency factors are down-regulated while lineage markers are up-regulated). However, we did not observe significant changes in these genes in Yap1-depleted cells (both KD and KO cells). The results suggest that KD or KO of Yap1 does not trigger differentiation of ES cells, which is different from the previous report claimed by Lian et. al. To make our results more clear, we have modified the figure as shown in Fig 1G.

1-9) Fig 3D is for Supplementary info.

Response: As suggested, we have moved Fig 3D to Fig EV4D

1-10) Figure S1F GAPDH strip has severe problems, at least on my pdf.

Response: We apologize for the quality of the blot. There was a technical glitch when we converted illustrator files to PDF files. We have fixed the problem.

1-11) It is imperative for all their cell line to show the effective knockout of YAP: by sequencing of the locus and western blot.

Response: We have generated CRISPR-Cas9 mediated Yap1 KO clones using mouse CJ7 and E14 ES cell lines as commented (please see our response to the comment 1-2b). We also added Western blot and sequencing results in Fig EV2G-L and Table EV1, respectively.

1-12) Fig1H is very hard to understand; and not even explained.

Response: We apologize for the lack of information. As commented, we have added additional explanation in the main text and now it reads as "The luciferase construct contains repeated Yap1-Tead binding motifs (8 times) in front of the minimal cTNT promoter followed by a luciferase reporter gene [15,32,33]."

1-13) The following paragraph of the Conclusion on other reports supporting their own results, is incorrect (and a bit unfair) and should be deleted: "This result is consistent with the role of Yap1 in human pluripotent stem cells where Yap1 does not affect the regulation of Pou5f1 or the generation of induced pluripotent stem cells [34,35]" Ref34 is really on something else. It is on an Rnai screen and simply show YAP siRNAs as negative control, but by no mean this is meaningful (those ref34 oligos were not validated; for Ref34 YAP siRNAs are simply equivalent to GFP siRNAs or unrelated siRNA). By no mean this is a direct study of YAP.Ref35 deals with reprogramming of human fibroblast to iPSCs; little is known, but this is a process perhaps only partially related to the preservation of self-renewal and differentiation of established mES cell lines here investigated.

Response: We thank the reviewer for the helpful comment. As suggested, we have deleted the sentence from the conclusion.

Referee #2:
In this manuscript Chung et al. explore the roles of Yap1 in mouse ESC differentiation. Based on their observations the authors argue that Yap1 is not required for maintaining ESC pluripotency, but rather is required for proper differentiation of these cells. They show that ESCs depleted of Yap1 show no changes in markers of pluripotency, whereas overexpression of Yap1 induces the expression of genes associated with differentiation. The authors also reason that changes in cell fate markers that result from alterations in cell density, or disruption of cell adhesion, occur as a results of induced nuclear Yap1 activity.

Given that Yap1 been described as an ESC regulator, understanding what Yap1 is doing in these cells is of clear importance. This manuscript attempts to study the roles of Yap in ESCs, and while some interesting descriptive observations are made, no detailed insight is gained into Yap function. Additionally, several of the conclusions seem to be based on improperly controlled experiments (based on the presented methods). Therefore, in my opinion much more insight into the role of Yap in ESC fate specification is required before this manuscript can be considered for publication. Moreover, there are several issues that need to be addressed, some of which are outlined below:

2-1) The manuscript fails to address or take into account several prior studies that address the roles of Yap1 in ESCs. Most importantly, prior work has shown that Yap1 functions redundantly with Taz in mouse preimplantation development and in human ES cells. Therefore, depletion of Yap1 in mouse ESCs may not be sufficient to observe any clear phenotypes. The study would benefit from additional analyses of Taz and Yap1/Taz depletion in ESCs.

Response: We appreciate the reviewer's helpful suggestion. As commented, Taz is homologous to Yap1 and shares similar domains. Since Taz may function redundantly with Yap1, we performed double KD of Yap1 and Taz using lentivirus-mediated shRNAs under drug selections (Blasticidin and Puromycin, respectively). Cells expressing both shRNAs survived and as shown in Fig 2A-D, ES cells with KD of Taz or both Yap1 and Taz still maintain undifferentiated state.

2-2) No details are given for how the experiments were performed in Figure 2. The authors state that Yap1 is not required for the self-renewal of ES cells, but do not provide details regarding how the experiments being performed, how many passages were analyzed, or even at when the pluripotency markers were analyzed with respect to depletion.

Response: We apologize for the lack of detailed information regarding cell culture condition. We added detailed experimental processes regarding virus infection and passaging of the cells in the Material and Methods section. In 'shRNA lentiviral production and infection' section, we added following sentences. "After one day of infection, cells are selected with appropriate antibiotics and

passaged every two days. Cell morphology, AP staining, protein and mRNA levels were examined two passages after the infection." We also examined the Yap1 KD and KO ES cells at earlier time points as well as later time points (more than a month of passaging), but we did not observe any significant differentiation morphology.

2-3) The authors argue that loss of Yap1 or overexpression of Yap1 affects the differentiation of ESCs, but no details are given into the specification of these cells. Rather, very broad statements and conclusion are made. A detailed analysis of the various cell fates that may be affected should be performed. Also, given that Yap1 is known to crosstalk with other signals in the context of ESCs (e.g. crosstalk with Lif, Wnt, BMP, TGF-beta, and Notch signaling) any analysis of cell fate should carefully consider these other signals, and if necessary, various culture and differentiation methods should be tested before drawing conclusions**.**

Response: We agree with the reviewer's comment that Yap1 crosstalks with other signaling pathways, such as LIF, Wnt, BMP, Tgf-beta, and Notch signaling. Combined with the fact that Yap1-KO embryos have defects in yolk sac vasculogenesis and placenta development [8] and our gene ontology (GO) term analysis shown in Fig EV4E, Yap1 seems to primarily affect mesoderm and trophectoderm differentiation. It would be great if we could further elucidate the roles of Yap1 in lineage specification or its connection with other signaling pathways. However, we think that these important questions are beyond the scope of our current manuscript. As another reviewer also suggested, we mainly claim that Yap1 is dispensable for self-renewal but required for proper differentiation of mouse ES cells.

2-4) Based on the data presented, ESCs appear to have significant changes in gene expression compared to the controls following the loss of Yap1. While this profile is more similar to ESCs than of differentiated EBs, these gene expression changes may be insightful into the roles of Yap1 in ESCs. Do the gene expression changes provide any clues into what Yap1 is doing in cell fate specification? Some analysis of this data, beyond simple correlation, should be included and discussed.

Response: We apologize to make the reviewer misunderstand the data. What we tried to convey in Fig 1G-K (formerly Fig 2G-J) was that loss of Yap1 in ES cells does not induce differentiation (please see our response to the comment 1-8). If Yap1 is required for the self-renewal of ES cells, Yap1-depleted ES cells would undergo differentiation and gene expression patterns must be similar to those of differentiated ES cells. However, as shown in Fig 1G-K, gene expression patterns of Yap1-depleted ES cells are more similar to normal ES cells than differentiating ES cells, supporting our idea that Yap1 is dispensable for self-renewal of ES cells.

2-5) The details for how Yap1 localization and activity was measured and compared in undifferentiated and differentiating ESCs is unclear. From the methods section it appears that the differentiating ESCs were continually being passaged during the analysis of Yap1, whereas the undifferentiated were not, and therefore dissociation of the cells may be the primary reason for observing increased nuclear Yap1 activity under these conditions. A more clear description of these experiments is required, and if culturing conditions were different, then conclusions with regard to Yap1 activity cannot be drawn from these experiments.

Response: We apologize for not providing detailed information in the previous manuscript. As we added in the figure legends 3E-G, both ES cells and differentiating ES cells were passaged every two days. Normal ES cells grow as colonies while ES cells under differentiation condition lose their ability to form colonies and become flattened. We think differences in cell-cell contact ability between ES and differentiating ES cells may contribute to the localization of Yap1. As shown in Figs 3E-G and EV3B-D, we observed that Yap1 translocates into the nucleus upon differentiation.

2-6) How was Yap1 overexpression achieved? No details are given. For example, were these lines generated with viral transduction? Are they stably selected? Is the expression constitutive? Were the control cells generated in parallel, and similarly selected? These variables could have a huge influence on the cellular state and must be properly controlled. Additionally, the text states that overexpressed Yap1 mainly resides in the nucleus, but this does not appear to be different that the endogenous Yap shown in the control in Figure 3D. Rather, Yap just appears to be at higher levels.

Response: We concur with the reviewer's comment that overexpression of Yap1 increases overall Yap1 levels. However, compared to control ES cells where we could observe Yap1 signals mainly in the peripheral of the nucleus, we could observe strong Yap1 signals overlapping with the nucleus of the cells upon Yap1 overexpression. To clearly show the result, we quantified nuclear Yap1 intensity in both control and Yap1overexpressing cells (Fig EV4E). In response to the reviewer's comment, we described the overexpression method in the supplemental experimental procedures section 'Generation of stable cell lines'. In short, we established stable cell lines constitutively expressing Yap1 under the control of EF1α promoter. To rule out the possibility of clonal variations, we also compared our results with the pool of Yap1-overexpressing clones. We also agree on the reviewer's comment that overexpression of Yap1 increases both the nucleus and the cytoplasmic portion. Based on the comment, we modified our text and it now reads "Yap1 mainly resides in the cytoplasm of self-renewing ES cells. While OE of Yap1 increases both nuclear and cytoplasmic Yap1 levels, we detected more nuclear Yap1 in Yap1 OE cells, indicating that exogenous Yap1 can translocate into the nucleus and act on its target genes (Fig EV4D and E)."

2-7) Based on the images in 1F, 1G and 3D and -it appears that Yap1 in abundantly localized in the nucleus of undifferentiated ES cells. The one field showing a possible increase in nuclear Yap1 levels in differentiated ES cells is unconvincing on its own -and all the conclusions regarding Yap1 localization would greatly benefit from quantitation and biochemical nuclear/cytoplasmic fractionation examined by immunoblotting.

Response: In response to the reviewer's comment, we quantified Yap1 localization with several different images and added new data in Fig EV3B-D.

2-8) A control for Yap1 IF signal should be included in the study, particularly given that the authors have in hand a Yap1 deleted cell line.

Response: We added IF data with Yap1-KO ES cells to confirm the specificity of the Yap1 antibody in Fig EV3A.

2-9) Differences in Yap1 localization in Figure 4A are not clear. To me, nuclear Yap1 levels look similar between the two conditions. Better images and quantitation would help make this conclusion more convincing.

Response: Please see our response to the point 2-10.

2-10) All the experiments related to cell density or E-cadherin depletion are vastly overstated, as the authors do not provide any evidence that the effects following changes in density or the loss of E-cadherin are mediated by Yap1. To draw any meaningful conclusions, the authors should perform similar experiments in their Yap1deleted/depleted cell lines to show that fate changes are mediated by Yap (these experiment may also require Taz knockdown).

Response to 2-9 and 10: Please also see our response to the comment 1-4. We agree with the reviewer's comment that our data related to the cell density or E-cadherin depletion may dilute the main claim of our
manuscript. Therefore, we deleted the data and description related to the former Fig 4 from our revised manuscript.

Referee #3:
This study describes the role of Yap1 in the self-renewal and differentiation of mouse embryonic stem (ES) cells. Authors of this study used 3 feeder-free ES cell lines and observed increased expression of Yap1, reduction in the phosphorylation of Yap1, and nuclear accumulation of Yap1 during the differentiation of these cells after LIF withdrawal. Knockdown of the gene encoding Yap1 did not significantly affect the self-renewal and gene expression profiles of ES cells. Cells lacking Yap1 showed defects in their differentiation while cells with Yap1 overexpression underwent differentiation. Alteration of subcellular localization of Yap1 by inhibiting E-cadherin-mediated cell-cell adhesion and alteration of cell density also affected the differentiation of ES cells. Based on these results, the authors concluded that Yap1 was dispensable for the self-renewal but was required for the proper differentiation of ES cells. Interestingly, these observations are

inconsistent with those ofprevious studies by Lian et al (2010) and Tamm et al (2011) that reported, Yap1-Tead activity was required for the self-renewal of ES cells. Although the results of the present study are of high quality and are convincing, the authors only examined Yap1. If the authors' conclusion is correct, one would expect that Tead activity is also dispensable for the self-renewal of ES cells. Addition of this information would further strengthen the authors' conclusion and would clarify whether functions of Yap were mediated by Tead in this context.

Response: As commented by the reviewer, Yap1 requires Tead family proteins to activate its downstream target genes [9], and confirming whether Tead proteins are dispensable for the self-renewal of ES cells is important. To address this issue, we sub-cloned each of Tead 1/3/4 shRNAs into three different versions of pLKO.1 vectors rr rcontaining three different antibiotics resistance genes (Neomycin, Puromycin, and Blasticidin). With drug selection, we obtained ES cells expressing all three shRNAs targeting Tead 1, 3, and 4. In short, ES cells with KD of all three Tead 1/3/4 maintain undifferentiated state of ES cells, supporting the idea that Tead family proteins are also dispensable for the self-renewal of ES cells (Fig 2E-I).

Major comment:
3-1) The data presented in this study showed that Yap1 was dispensable for the self-renewal but was required for the proper differentiation of ES cells. These results are inconsistent with those reported in studies by Lian et al (2010) and Tamm et al (2011), therefore, one should interpret these results with caution. Previous studies manipulated both Yap1 and Tead and obtained consistent results, thus, it is important to examine whether manipulation of Tead would also provide the same results as those obtained in the present study. I think that analyses of several key markers for the ES cells that are knocked down and/or overexpressed with active/inactive forms of Tead should be sufficient for this purpose.

Response: We appreciate the reviewer's constructive suggestions for the improvement of our manuscript. As suggested, we generated three different lentiviruses each of which contains shRNA targeting either Tead 1, 3, or 4. As shown in Fig 2E, ES cells infected with these lentiviruses showed normal ES cell morphology as well as high AP activity. We examined KD efficiency with RT-qPCR and Western blotting and found that all three Tead mRNAs/proteins are significantly down-regulated (Fig 2F and H). ES cells with triple KD express similar levels of pluripotency markers, such as Pou5f1, Nanog, Sox2, and Esrrb (Fig 2G and H), and we did not observe any significant up-regulation of lineage specific regulators (Fig 2I). These results support the idea that Tead family proteins are also dispensable for the self-renewal of ES cells.

Minor comments: **3-2)** Throughout the manuscript, the authors considered that ICM and ES are equivalent, and therefore, that Yap performs similar roles in these cells. However, this is incorrect because ES cells are derived from the epiblast (a derivative of ICM) of hatched blastocysts and not from the ICM of early blastocysts. The role of Hippo signal/Yap1 during morula to early blastocyst stages is to specify the TE/ICM fates of the cells. However, roles of the Hippo signal/Yap1 in the epiblast are unknown. To date, no study has described the distribution of Yap in the epiblast of hatched blastocysts. Moreover, presence of Yap in the nuclei of epiblast cells cannot be ruled out.

Response: We apologize for making the reviewer misunderstand the ES cell line that we used. Unlike human ES cells [10], which are derived from epiblasts as the reviewer commented, J1 mouse ES cells are derived from ICM of pre-implanted embryo [11]. However, we definitely agree with the reviewer's comment that the functions of Yap1 as well as its distribution have not been exploited in epiblast. As suggested by the reviewer 1, we minimized in vivo centered interpretation of our data, focusing mainly on the roles of Yap1 in ES cells and their differentiation.

3-3) Figure 2E-G. Please include the duration of culturing ES cells under differentiation conditions to obtain dES cells shown in these panels.

Response: We thank the reviewer for the comment. To clarify the experimental processes for current Fig 3E-G, we added a detailed description in the Material and Methods section (Cell culture). It reads "To differentiate ES cells, cells were washed three times with the media without LIF and then incubated for 4 days while passaging every 2 days."

3-4) Figure 4E. The graph shows higher luciferase activity at high density. However, in the main manuscript, the result indicates reduced transcriptional activity at high density. Please recheck the accuracy of the data presented at both the instances.

Response: We apologize for the mislabeling. We made a mistake while modifying the figures. The lower luciferase activity should be labeled as "High density" and higher as "Low density" as described in the main text.
However, after considering other comments from the reviewers, we decided to delete the data related to Fig 4 in our revise manuscript (Please see our response to the point 1-4).

REFERENCES

1 Lian I, Kim J, Okazawa H, Zhao J, Zhao B, Yu J, Chinnaiyan A, Israel M a, Goldstein LSB, Abujarour R, et al. (2010) The role of YAP transcription coactivator in regulating stem cell self-renewal and differentiation. *Genes Dev* **24**: 1106–1118.
2 Nishioka N, Yamamoto S, Kiyonari H, Sato H, Sawada A, Ota M, Nakao K, Sasaki H (2008) Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. *Mech Dev* **125**: 270–283.
3 Hirate Y, Hirahara S, Inoue K-I, Suzuki A, Alarcon VB, Akimoto K, Hirai T, Hara T, Adachi M, Chida K, et al. (2013) Polarity-dependent distribution of angiomotin localizes Hippo signaling in preimplantation embryos. *Curr Biol* **23**: 1181–1194.
4 Cockburn K, Biechele S, Garner J, Rossant J (2013) The Hippo pathway member Nf2 is required for inner cell mass specification. *Curr Biol* **23**: 1195–1201.
5 Wicklow E, Blij S, Frum T, Hirate Y, Lang R a., Sasaki H, Ralston A (2014) HIPPO Pathway Members Restrict SOX2 to the Inner Cell Mass Where It Promotes ICM Fates in the Mouse Blastocyst. *PLoS Genet* **10**: e1004618.
6 Azzolin L, Panciera T, Soligo S, Enzo E, Bicciato S, Dupont S, Bresolin S, Frasson C, Basso G, Guzzardo V, et al. (2014) YAP/TAZ incorporation in the β-catenin destruction complex orchestrates the Wnt response. *Cell* **158**: 157–170.
7 Zhao B, Ye X, Yu J, Li L, Li W, Li S, Yu J, Lin JD, Wang C-Y, Chinnaiyan AM, et al. (2008) TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* **22**: 1962–1971.
8 Morin-Kensicki EM, Boone BN, Howell M, Stonebraker JR, Teed J, Alb JG, Magnuson TR, O'Neal W,

Milgram SL (2006) Defects in yolk sac vasculogenesis, chorioallantoic fusion, and embryonic axis elongation in mice with targeted disruption of Yap65. *Mol Cell Biol* **26**: 77–87.

9. Zhao B, Ye X, Yu J, Li L, Li W, Li S, Yu J, Lin JD (2008) TEAD mediates YAP-dependent gene induction and growth control TEAD mediates YAP-dependent gene induction and growth control. 1962–1971.

10. Brons IGM, Smithers LE, Trotter MWB, Rugg-Gunn P, Sun B, Chuva de Sousa Lopes SM, Howlett SK, Clarkson A, Ahrlund-Richter L, Pedersen RA, et al. (2007) Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* **448**: 191–195.

11. Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69**: 915–926.

2nd Editorial Decision 16 November 2015

Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed reports from the referees that were asked to assess it. While referees 1 and 3 are, in principle, now supportive of its publication, referee 2 feels that some additional work is needed to make the study fully conclusive. Since all referees agree on the potential interest of this paper, I would thus like to give you the opportunity to revise the study a second time to address the remaining concerns of reviewer 2 and the minor issue raised by referee 3.

Referee #1:

This is an interesting and overall convincing paper and the authors have done a good job in revising the MS.

Referee #2:

The revised manuscript by Chung et al presents data arguing that Yap is not required for the maintenance of mESCs, but rather that Yap plays a critical role in mESC differentiation. The revised manuscript is stronger and written more clearly than the initial submission. However, there are a few additional comments I have that need to be addressed before potential publication.

Major comments:

- The data shown for how Tead knockdown affects mESCs is incomplete and needs to be better addressed. The primary Tead family member implicated in mESCs is Tead2 (see Tamm et al, 2011). In fact, Tamm et al state that loss of Tead2 function promotes mESCs differentiation. I think in order to make any conclusions about the Teads, Tead2 function has to be examined. Moreover, based on the image shown in Figure 2H, Tead4 knockdown is poor, making it hard for conclusions to be made about this family member.

- Figure 1b should show the reduction of Yap mRNA expression in the same samples by qPCR. Although it is not stated I am assuming the protein lysate shown in 1c is from a different sample.

Minor comments:

- It is unclear why the authors choose to only show the correlation between Yap-kd1 vs control in Figure 1K, but not the correlation with the Yap-KO data. Since the authors have the data in hand, I think that is important to also show the correlation of Yap-KO1 and Yap-KO2, at least as supplementary material.

- The rebuttal comments state: "We also examined the Yap1 KD and KO ES cells at earlier time points as well as later time points (more than a month of passaging), but we did not observe any significant differentiation morphology."
> This statement needs to be included in the manuscript. Moreover, analysis of the Yap-KO ES cells after one month for self-renewal markers should be included, as this would strengthen the conclusion that Yap is not required for mESC maintenance.

- The type of mESCs used for the Yap/Taz double knockdown and Tead knockdown experiments should be stated somewhere. Are these J1 cells? Also, details into how these cells were generated should be included in the methods section - selection conditions, passage number, etc.

- The Figure legend in Figure 1A (and other Figures) should mention the culture time in addition to the number of passages.

- Several statements throughout the manuscript should be modified. Specifically, the following:

* "Although importance of Yap1 in early cell fate decision has been well established, in particular in trophectoderm differentiation, its role in inner cell mass or embryonic stem (ES) cells remains elusive."
> The authors are not studying the ICM or trophectoderm, so this sentence should be simplified to only highlight the work in ES cells.

* "the roles of Yap1 in embryonic stem (ES) cells have been controversial"
> I would not refer to the prior studies as controversial, but rather that different observations have been made.

* "mouse ES cells grow as tightly packed colonies; considering the fact that high cell density or cell-cell contact activates Hippo signaling, and subsequent sequestration of Yap1 in the cytoplasm of various cell lines such as MDA-MB-231, HaCaT, and NIH-3T3"
> Referral to the MDA-MB-231 cells in this statement is inaccurate, as these cells do not exhibit robust density-mediated regulation of Yap1.

* "OE of Yap1 in ES cells enhances nuclear accumulation of Yap1 accompanied by induction of various lineage-specific marker genes."
> Based on the images shown it looks like there is no enhanced nuclear accumulation upon Yap expression, but rather that there is enhanced nuclear abundance. I would change this sentence accordingly.

Referee #3:

I am satisfied with the revision. This is a high quality paper with novel and important information. I think that the paper is suitable for publication in EMBO Reports after incorporating one minor comment described below.

Minor comment:

In the middle of page 10, authors discussed as "the Hippo pathway is at least dispensable for self-renewal of mouse ES cells". This sentence is misleading, because inactivation of the Hippo pathway causes nuclear localization of Yap, which should promote differentiation of ES cells. Therefore, the results suggest that active Hippo signaling is required for self-renewal of mouse ES cells. I think that it is more appropriate to rephrase this sentence, for example, as "Yap1/Taz/Tead are at least dispensable for self-renewal of mouse ES cells", or "the transcriptional effectors of the Hippo pathway are at least dispensable for self-renewal of mouse ES cells".

2nd Revision - authors' response 08 January 2016

RESPONSE TO REVIEWERS
We again are thankful for the reviewers' constructive comments. We mainly performed three additional experiments to address the points raised (mainly by the reviewer #2). First, we conducted KD and KO of Tead2 in ES cells. Second, we generated Tead4 KO ES cells and performed KD of Tead1/3 for the confirmation of the data presented in Figure 2H. We additionally maintained the Yap1 KO cells for a prolonged period of time (> a month) to test if the cells maintain self-renewal. Combined with these new data and analyses, we have substantially revised our manuscript in accordance with reviewers' suggestions. Below we provide a point-by-point response to each comment.

Referee #1:
This is an interesting and overall convincing paper and the authors have done a good job in revising the MS.

Referee #2:
The revised manuscript by Chung et al presents data arguing that Yap is not required for the maintenance of mESCs, but rather that Yap plays a critical role in mESC differentiation. The revised manuscript is stronger and written more clearly than the initial submission. However, there are a few additional comments I have that need to be addressed before potential publication.

Major comments:
2-1) The data shown for how Tead knockdown affects mESCs is incomplete and needs to be better addressed. The primary Tead family member implicated in mESCs is Tead2 (see Tamm et al, 2011).

In fact, Tamm et al state that loss of Tead2 function promotes mESCs differentiation. I think in order to make any conclusions about the Teads, Tead2 function has to be examined. Moreover, based on the image shown in Figure 2H, Tead4 knockdown is poor, making it hard for conclusions to be made about this family member.

Response: *We agree with the reviewer's comments that the roles of Tead2 in ES cells need to be addressed. Similar to the results of Tead1/3/4 KD ES cells, we found that KD of Tead2 does not disrupt self-renewal of ES cells (Fig EV4, newly added). Although significant KD of Tead2 in mRNA level was obtained (>90%, Fig EV4B), we could not detect specific Tead2 protein using multiple antibodies we tested (Abcam: ab123276, Proteintech: 21159-1-AP, and LSBio: LS-C119063). Therefore, we additionally generated CRISPR-Cas9 mediated Tead2 KO ES cells and, by sequencing, confirmed the frame shift mutation introducing premature stop codons on both alleles (Table EV1). In agreement with the Tead2 KD ES cells, three different Tead2 KO clones did not undergo differentiation and maintained comparable levels of pluripotency factors to wild-type ES cells (Fig EV4E-G).*

Although we believe that the KD of Tead4 in Figure 2H was significant, we generated CRISPR-Cas9 mediated Tead4 KO ES cells to clarify the roles of Tead1/3/4 in self-renewal of ES cells. Using these Tead4 KO ES cells, KD of both Tead1 and Tead3 was performed by shRNA-based approaches. Similar to the data shown in Figure 2H, Tead4 KO cells with KD of Tead1/3 self-renew and express comparable levels of pluripotency factors to the control cells (Fig EV4H-J, Table EV1).

2-2) Figure 1b should show the reduction of Yap mRNA expression in the same samples by qPCR. Although it is not stated I am assuming the protein lysate shown in 1c is from a different sample.

Response: *As commented, we included mRNA expression levels of Yap1 upon KD (Fig 1C) using the same samples used to generate Fig 1B.*

Minor comments:
2-3) It is unclear why the authors choose to only show the correlation between Yap-kd1 vs control in Figure 1K, but not the correlation with the Yap-KO data. Since the authors have the data in hand, I think that is important to also show the correlation of Yap-KO1 and Yap-KO2, at least as supplementary material.

Response: *As suggested, we have analyzed the gene expression correlation between Yap1 KO clones and wild-type ES cells, and confirmed that Yap1 KO cells and wild-type cells have similar gene expression profiles. The results were added to Fig 1L.*

2-4) The rebuttal comments state: "We also examined the Yap1 KD and KO ES cells at earlier time points as well as later time points (more than a month of passaging), but we did not observe any significant differentiation morphology." > This statement needs to be included in the manuscript. Moreover, analysis of the Yap-KO ES cells after one month for self-renewal markers should be included, as this would strengthen the conclusion that Yap is not required for mESC maintenance.

Response: *We appreciate the reviewer's comment. We have included new data obtained from the Yap1 KO clones cultured for more than a month (Fig EV3A-C).*

2-5) The type of mESCs used for the Yap/Taz double knockdown and Tead knockdown experiments should be stated somewhere. Are these J1 cells? Also, details into how these cells were generated should be included in the methods section -selection conditions, passage number, etc.

Response: *All experiments were done in J1 ES cells unless otherwise described. We have clearly indicated names of ES cell lines we used in the figure legends.*

2-6) The Figure legend in Figure 1A (and other Figures) should mention the culture time in addition to the number of passages.

Response: *All samples were collected 4 days after the infection unless otherwise mentioned. As suggested, we have added culture time in the figure legends.*

2-7) Several statements throughout the manuscript should be modified. Specifically, the following:
* "Although importance of Yap1 in early cell fate decision has been well established, in particular in trophectoderm differentiation, its role in inner cell mass or embryonic stem (ES) cells remains elusive." > The authors are not studying the ICM or trophectoderm, so this sentence should be simplified to only highlight the work in ES cells.

Response: *We appreciate the reviewer's suggestion to improve our manuscript. As commented, the main idea of the manuscript is to understand the roles of Yap1 in ES cells. Since we did not address the roles of Yap1 in trophectoderm or ICM, we have changed the text in the abstract and now it reads as "The importance of Yap1 in early cell fate decision during embryogenesis has been well established, though its role in embryonic stem (ES) cells remains elusive".*

2-8) "the roles of Yap1 in embryonic stem (ES) cells have been controversial" > I would not refer to the prior studies as controversial, but rather that different observations have been made.
Response: *As suggested, we have changed the text and now it reads as "Two different observations on the roles of Yap1 in embryonic stem (ES) cells are of note".*

2-9) "mouse ES cells grow as tightly packed colonies; considering the fact that high cell density or cell-cell contact activates Hippo signaling, and subsequent sequestration of Yap1 in the cytoplasm of various cell lines such as MDA-MB-231, HaCaT, and NIH-3T3" > Referral to the MDA-MB-231 cells in this statement is inaccurate, as these cells do not exhibit robust density-mediated regulation of Yap1.

Response: *We have removed "MDA-MB-231 cells" from the text.*

2-10) "OE of Yap1 in ES cells enhances nuclear accumulation of Yap1 accompanied by induction of various lineage-specific marker genes." > Based on the images shown it looks like there is no enhanced nuclear accumulation upon Yap expression, but rather that there is enhanced nuclear abundance. I would change this sentence accordingly.

Response: *As suggested, we have changed the text and now it reads as "OE of Yap1 in ES cells enhances nuclear abundance of Yap1 accompanied by induction of various lineage-specific marker genes".*

Referee #3:
I am satisfied with the revision. This is a high quality paper with novel and important information. I think that the paper is suitable for publication in EMBO Reports after incorporating one minor comment described below.

Minor comment: **3-1)** In the middle of page 10, authors discussed as "the Hippo pathway is at least dispensable for self-renewal of mouse ES cells". This sentence is misleading, because inactivation of the Hippo pathway causes nuclear localization of Yap, which should promote differentiation of ES cells. Therefore, the results suggest that active Hippo signaling is required for self-renewal of mouse ES cells. I think that it is more appropriate to rephrase this sentence, for example, as "Yap1/Taz/Tead are at least dispensable for self-renewal of mouse ES cells", or "the transcriptional effectors of the Hippo pathway are at least dispensable for self-renewal of mouse ES cells".

Response: *We appreciate the reviewer's constructive comment to improve our manuscript. As commented, we did not address the roles of the Hippo pathway in ES cells but rather focus on the transcriptional effector of the Hippo pathway in ES cells. To clarify this point, we have changed the text and now it reads as "implying that the transcriptional effectors of the Hippo pathway are at least dispensable for self-renewal of mouse ES cells".*

3rd Editorial Decision 22 January 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

EMBO REPORTS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

Corresponding Author Name: Dr. Jonghwan Kim
Manusript Number: EMBOR-2015-40933V3

Reporting Checklist For Life Sciences Articles

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 (see link list at top right).

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 only for independent experiments and sample sizes where the application of statistical tests is warranted (error bars should not be shown for technical replicates)
- when n is small (n < 5), the individual data points from each experiment should be plotted alongside an error bar.
- 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 (see link list at top right).

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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

USEFUL LINKS FOR COMPLETING THIS FORM

http://embor.embopress.org/authorguide
http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscie
http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanima
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-recomm
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/

B- Statistics and general methods

Please fill out these boxes ↓

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Appendix page 10.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Manuscript pages 18-21.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

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).	Appendix page 11.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Manuscript page 12.

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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 link list at top right). See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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' (see link list at top right).	NA
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' (see link list at top right).	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition' (see link list at top right). 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	Manuscript page 14.
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).	Manuscript page 14.
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).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section: Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Manuscript page 15-17.
22. 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.	NA

G- Dual use research of concern

23. 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.	NA