THE
EMBO
JOURNAL

Manuscript EMBO-2010-76127

Eed/Sox2 regulatory loop controls ES cell self-renewal through histone methylation and acetylation

Hiroki Ura, Kazuhiro Murakami, Tadayuki Akagi, Keita Kinoshita, Shukuro Yamaguchi, Shinji Masui, Hitoshi Niwa, Hiroshi Koide and Takashi Yokota

Corresponding author: Hiroshi Koide, Graduate School of Medical Science, Kanazawa University

Review timeline:

Submission date:	30 September 2010
Editorial Decision:	03 December 2010
Revision received:	04 March 2011
Editorial Decision:	23 March 2011
Accepted:	24 March 2011

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 03 December 2010

Thank you for submitting your manuscript for consideration at The EMBO Journal. I apologise that it has taken longer than usual to have your manuscript reviewed, but we have been rather badly let down by one of the original referees. However I have now received the final report from the new referee, overall both find the study interesting and both support publication after some minor revisions and additional discussion have been included in the manuscript. Given this interest should you be able to address these issues we would be happy to consider a revised version of the manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor

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REFEREE COMMENTS

Referee #2:

In this manuscript, the authors investigate the functional relationship between Eed and Sox2 in embryonic stem cells. Using inducible KO ES cells for Eed or Sox2 they show mutual regulation of Eed and sox2 expression. Promoter reporter assays as well as CHIP analysis are used to demonstrate that Sox2 directly regulates Eed promoter and that Eed indirectly regulates Sox2 promoter through regulation of the expression of the repressor coup-TFII. The authors then demonstrate that Sox2 overexpression prevents change in gene expression triggered by Eed knock down. Sox2 overexpression prevents the loss of histone acetylation but not the loss of H3K27Me3 that follows Eed KO. Sox2 is shown to control histone acetylation level through regulation of the HATs TIP60 and ELP3.

The work presented in this paper is impressive and sheds new lights on the biology of ES cells. However, before publication the following questions need to be addressed to ensure the validity of the claim made in the manuscript:

1- Regarding the new binding sites identified in this work (Sox2 on eed promoter and COUP-TFII on SSR1) the CHIP analysis shown in the studies needs to be evaluated by qPCR (as are all the other CHIP data presented in the study) in order to be convincing.

2- The nature of the error bars as well as the number of experiments performed for each experiments need to be stated. The authors should include statistical analysis of the data (qPCR) in order to ensure that the observed differences between various experimental conditions are significant.

3- The authors should include more references to published literature concerning conventional KO of EED. Specifically, it would be of interest to comment on the differences that might exist in the early stage of Eed KO captured by the conditional KO approach as compared to traditional KO. In particular the propensity of these various cells to differentiate in the absence of differentiation cues should be commented. Along that line, some gene expression analyses in this paper are somehow different than in other reports (Chamberlain et al, 2008 for example report that oct4 is downregulated in ES eed -/- and that developmental genes are not so much upregulated after passaging). In the discussion section it would be interesting to have the authors' view on the effect of perturbing the eed/sox2 loop: Are the observed changes in gene expression following Eed KO happening in self-renewing ES cells or are they the result of perturbed self-renewal/differentiation decision (increased fraction of cell undergoing differentiation)?

4- The SRR1 and SRR2 luciferase reporter constructs in Fig2E shows very different levels of expression (6 and 60, respectively). In the natural context of the sox2 promoter both enhancers are presumably acting and one could conclude from the luciferase assay that the SRR2 enhancer activity is dominant. It would be therefore interesting to see to what extent a luciferase assay including both enhancers is affected by the KO of eed in ES cells.

5- On p13, bottom, the authors claim that, "these findings indicate that Eed positively regulates Sox2 expression through H3K27me mediated suppression of Coup-TFII." The data presented allow one to conclude that eed regulates coup-TFII expression but the need for H3K27me in this process can not be evaluated based on the evidence presented.

6- Scale bars should be included on confocal images.

Referee #3:

Comments on 'Eed/Sox2 regulatory loop controls ES cell self-renewal through histone methylation

and acetylation' by Ura et al.

In this manuscript the authors show a cross talk between Eed and Sox2. They find that their expression level is correlated in differentiating cells, that Eed is a Sox2 target, that Sox2 is significantly reduced in Eed deficient cells, and that Sox2 forced expression restores morphology and expression patterns in Eed deficient cells. They further show that Sox2 induces increased histone acetylation in the Eed depleted cells and that histone acetyltransferases (HATs) are downstream targets of Sox2. Finally, they show that selected HATs, namely Tip60 and Elp3 are sufficient to restore the Eed-deficient phenotype.

This is a very interesting and very thorough paper. The amount and strength of data is truly impressive.

The main caveat is the following:

In 2008, Chamberlain et al., (Stem Cells, 2008) showed that Eed is dispensable for pluripotency and the Eed-deficient cells seem to generate normal pluripotent colonies in their hands. If Eed is dispensable for pluripotency, it undermines the importance of these finding. There are also conflicting results with the Chamberlain et al. paper. Regardless, there may be a regulatory loop between Eed and Sox2 and the data that the authors present certainly support this view. However, they must discuss these previous finding by Chamberlain et al. (they only cite this paper to show that others have also seen increased pluripotency-related genes following Eed depletion and that Eed depletion results in loss of H3K7 methylation).

More minor issues:

Throughout the manuscript and inside the figures, the authors refer to 'H3K27me' but do not mention whether they are checking mono-, di- or tri-methylation. I imagine that they are referring to H3K27me3 but this should be always indicated.

Figure 1A. Representative image of the actual data (Western blots) should be provided.

Figure 1F. What is the control? In the figure legend it says: 'The reporter plasmid pGL2-Eed was transfected into HEK293 cells together with expression vectors for Sox2, Oct3/4, STAT3, or Nanog.' It doesn't say anything about the control. An empty vector or a control gene should be used together with the reporter plasmid.

Figure 1G. It seems that independently, all three mutations have, more or less, the same effect. However, double mutations do not have an additive effect but only a mild phenotype compared with the single mutations. Further, the triple mutation looks essentially similar to the double mutations. These are independent mutations and these results are therefore quite surprising. It may be that the control (no mutations) is problematic (it should also have an error bar). Perhaps the entire phenotype of these mutations is less severe?

Figure 3A. Scale bars should be shown.

Figure 3B and 6C. Is a bar missing in the control or did the authors always counted exactly zero colonies?

Figure 4A. Although the colonies are shown in similar sizes, they seem to be originally different. For example, the colony in the upper right corner seems to contain considerably less cells than the colony in the upper left corner, and therefore the cells look much bigger in the colony in the upper right corner. It seems that the colonies originally had different sizes and different cell numbers but were made to look similar. Scale bars should be provided.

It is also very difficult to discern the staining pattern of the different histone marks from such a bird's eye view. Higher magnification images should be shown to observe the nuclear morphology.

In the same figure, the differences in immunofluorescence (IF) seem dramatic while the differences shown by Western blots seems much milder. H3Ac and H4Ac, for example, seem to go down from

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very high to essentially no fluorescence intensity, but display less than 2-fold difference in Western blots. If this is a detection issue, perhaps the authors should comment / discuss.

Figure 6A. Add scale bars.

1st Revision - authors' response 04 March 2011

We are pleased to hear from both referees that our manuscript is impressive, interesting and thorough. We also appreciate the thoughtful and constructive comments from both referees. Below is our response to each of the comments made by the referees.

Referee #2

1- Regarding the new binding sites identified in this work (Sox2 on eed promoter and COUP-TFII on SSR1) the CHIP analysis shown in the studies needs to be evaluated by qPCR (as are all the other CHIP data presented in the study) in order to be convincing.

Response:
In response to this recommendation, we have performed qPCR analysis, and confirmed that Sox2 binds to eed promoter and that Coup-tfII binds to sox2 enhancer. We replaced the original Fig. 1I and Fig. 2I with these new data.

2- The nature of the error bars as well as the number of experiments performed for each experiments need to be stated. The authors should include statistical analysis of the data (qPCR) in order to ensure that the observed differences between various experimental conditions are significant.

Response:
In all experiments, the error bars represent standard deviation, and the number of experiments is three. We stated these informations in figure legends. Also, in response to the reviewer's recommendation, we performed statistical analysis of all qPCR experiments to show which differences are statistically significant (Fig. 1B, D and I; Fig. 2E, H, I, and J; Fig. 3C and D; Fig. 4C, E, F and G; Fig. 5C, D, E and F; Fig. 6D, E, F and G; and Fig. 7A and B).

3- The authors should include more references to published literature concerning conventional KO of EED. Specifically, it would be of interest to comment on the differences that might exist in the early stage of Eed KO captured by the conditional KO approach as compared to traditional KO. In particular the propensity of these various cells to differentiate in the absence of differentiation cues should be commented. Along that line, some gene expression analyses in this paper are somehow different than in other reports (Chamberlain et al, 2008 for example report that oct4 is downregulated in ES eed -/- and that developmental genes are not so much upregulated after passaging).

Response:
At first, we would like to mention the expression level of Oct4 in Eed KO ES cells of Chamberlain's group. In their paper in Stem Cells (2008), they performed immunostaining and claimed that their Eed KO cells express Oct4 at the similar level to the wild-type cells (Fig. 3C in their paper). Although they showed RT-PCR data where Oct4 is significantly downregulated (Fig. 3B in their paper), they explained in the text that this downregulation is due to "early differentiation resulting from feeder-free condition".
As the referee pointed out, several papers have described the phenotype of Eed KO ES cells. In our opinion, all Eed KO ES cells show similar phenotype, and we have not found any significant difference between conventional KO cells and our conditional KO cells. For example, Eed KO ES cells lose H3K27me activity. Eed deficiency results in leaky expression of some differentiation-associated genes and downregulation of some self-renewal genes. In spite of these changes in marker expression, Eed KO ES cells can still be maintained. Therefore, generally speaking, Eed KO ES cells can self-renew and do not undergo spontaneous differentiation without any differentiation

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cues. The observed induction of differentiation-associated genes seems to occur mainly because those genes are targets of PRC2, a protein complex containing Eed. However, downregulation of some self-renewal markers suggests that ES cell self-renewal is affected to a certain degree by induction of differentiation-associated markers. With these reasons, we now speculate that Eed is required for maintaining “complete” self-renewal and that Eed-deficient ES cells may stay in an intermediate state between undifferentiated and differentiated ES cells.
We think that the observed discrepancies in the level of some marker genes among several papers are probably due to difference in culture (e.g. with or without feeder cells), clonal variation and/or adaptation. In fact, Chamberlain et al. (2008) compared between low-passage and high-passage Eed KO cells and found that these two cell lines show few differences, indicating that the adaptation to low level of Eed may occur although the effect is not so big.

We described our opinions in the Discussion section (p24,line12- p25,line8) and added 2 more papers, Morin-Kensicki et al (2001) and Schoeftner et al(2006), as references concerning about Eed KO ES cells.

In the discussion section it would be interesting to have the authors' view on the effect of perturbing the eed/sox2 loop: Are the observed changes in gene expression following Eed KO happening in self-renewing ES cells or are they the result of perturbed self-renewal/differentiation decision (increased fraction of cell undergoing differentiation)?

Response:
We presume here that the referee recommends us to discuss whether the change in expression levels of several markers occur in all of the Eed KO cells or in a subpopulation of Eed KO cells.
It is difficult to answer this question for the moment, but Boyer et al. (2006) carried out immunostaining of Eed KO cells with antibody against Gata4, an endodermal marker. As a result, most of Eed KO ES cells look like to be positive for Gata4, while the wild-type cells are completely negative. This observation suggests that differentiation-associated genes are upregulated in most of Eed KO cells, although further detailed analyses, such as a single-cell PCR analysis, should be done to clarify these points. We described these opinions in the Discussion section (p25, line 9- 14).

4- The SRR1 and SRR2 luciferase reporter constructs in Fig2E shows very different levels of expression (6 and 60, respectively). In the natural context of the sox2 promoter both enhancers are presumably acting and one could conclude from the luciferase assay that the SRR2 enhancer activity is dominant. It would be therefore interesting to see to what extent a luciferase assay including both enhancers is affected by the KO of eed in ES cells.

Response: According to the paper by Tomioka *et al.*(2002), SRR2 shows higher enhancer activity than SRR1 in undifferentiated embryonal carcinoma cells. In support of this result, our present data (as well as our preliminary data) also suggest that it is true for self-renewing ES cells. In addition, SRR2 is regulated by two key transcription factors, Oct3/4 and Sox2. Therefore it is likely that SRR2 enhancer plays a dominant role in regulation of Sox2 expression in ES cells. As for the role of SRR1, we speculate that this region plays a regulatory role in Sox2 expression, because it contains Coup-tfII binding site that negatively regulates enhancer activity. Although, we tried to examine this possibility, we cannot arrive a solid conclusion so far. Therefore, in the revised manuscript, we described this possibility in the Discussion section (p21, line 15- p22, line 6) (as a result, Toyooka et al (2008) was added to the list of references), and will explore this possibility in a future study.

5- On p13, bottom, the authors claim that, "these findings indicate that Eed positively regulates Sox2 expression through H3K27me mediated suppression of Coup-TFII." The data presented allow one to conclude that eed regulates coup-TFII expression but the need for H3K27me in this process cannot be evaluated based on the evidence presented.

Response: We agree with the referee’s opinion that our data (shown in Fig.2J) only raise the possibility that Eed-mediated H3K27me may be involved in regulation of Coup-tfII expression. Therefore, we modified original text and legend for Fig. 2J as follows.

Original sentences in text:

•*Eed positively regulates Sox2 expression through H3K27-mediated suppression of COUP-TFII* (p13, line 18 –p14, line 1)
We removed the word " H3K27me-mediated".

•*We also found that Eed controls COUP-TFII expression through H3K27me.* (p21, line 12-13)
We replaced this sentence with "We also found that Eed negatively controls *COUP-TFII* expression."

Original sentence in legend of Fig. 2J:

•*Eed suppresses COUP-TFII expression through H3K27me.* (p47, line 13-14.)
We replaced this sentence with "Eed regulates H3K27me3 at the promoter region of the *coup-tfII* gene".

6- Scale bars should be included on confocal images.

Response:
We assume that the referee mentions about fluorescence micrographs (we did not use confocal microscopes in this study). Scale bars are inserted into bright-field images corresponding to the fluorescent images (Fig. 3A, 4A and 6A).

Referee #3

Major issue

In 2008, Chamberlain et al., (Stem Cells, 2008) showed that Eed is dispensable for pluripotency and the Eed-deficient cells seem to generate normal pluripotent colonies in their hands. If Eed is dispensable for pluripotency, it undermines the importance of these finding. There are also conflicting results with the Chamberlain et al. paper. Regardless, there may be a regulatory loop between Eed and Sox2 and the data that the authors present certainly support this view. However, they must discuss these previous finding by Chamberlain et al. (they only cite this paper to show that others have also seen increased pluripotency-related genes following Eed depletion and that Eed depletion results in loss of H3K7 methylation).

Response:
In their Stem Cells paper, Chamberlain et al concluded that Eed is dispensable for ES cell self-renewal, and proposed the "positive-only model" that expression of self-renewal markers may be sufficient to sustain self-renewal of ES cells, even when differentiation-associated factors are expressed. Although the referee pointed out that there are conflicting results, we think that, basically, we and Chamberlain's group have shown the similar data and reached the same conclusion that Eed is dispensable, because Eed-deficient ES cells express Oct3/4 at the similar level to the wild-type cells and can maintain their self-renewal, in spite of upregulation of several differentiation-associated genes (for example, Gata4 and Gata6). Nevertheless, the fact that several differentiation-associated genes are upregulated by Eed deficiency indicates that Eed is still important for the maintenance of "complete" self-renewal. The observed discrepancies in the level of some marker genes among our paper and Chamberlain's are probably due to difference in culture condition (e.g. with or without feeder cells), clonal variation and/or adaptation. In fact, Chamberlain et al compared between low-passage and high-passage Eed KO cells and found these two cell lines show few differences, indicating that the adaptation to low level of Eed may occur although the effect is not so big. We described these in the Discussion section (p24,line 12-p25,line 8).

More minor issues

Throughout the manuscript and inside the figures, the authors refer to 'H3K27me' but do not mention whether they are checking mono-, di- or tri-methylation. I imagine that they are referring to H3K27me3 but this should be always indicated.

Response:

We apologize for the lack of accuracy in our manuscript. As the referee imagined, antibody we used in this study recognizes H3K27me3. We indicated this in the text (for example, p8, line 6-7) and modified some figures (Figs. 2J, 4A, 4C, 6A, S2D, S3A, S3B, S4A and S4C).

Figure 1A. Representative image of the actual data (Western blots) should be provided.

Response:
Following the referee's recommendation, we added the representative image of Western blots in Fig. 1A.

Figure 1F. What is the control? In the figure legend it says: 'The reporter plasmid pGL2-Eed was transfected into HEK293 cells together with expression vectors for Sox2, Oct3/4, STAT3, or Nanog.' It doesn't say anything about the control. An empty vector or a control gene should be used together with the reporter plasmid.

Response:
We apologize again for inaccuracy of our manuscript. As a control, we used an empty vector. We described this in the legend for Fig. 1F.

Figure 1G. It seems that independently, all three mutations have, more or less, the same effect. However, double mutations do not have an additive effect but only a mild phenotype compared with the single mutations. Further, the triple mutation looks essentially similar to the double mutations. These are independent mutations and these results are therefore quite surprising. It may be that the control (no mutations) is problematic (it should also have an error bar). Perhaps the entire phenotype of these mutations is less severe?

Response:
We agree with the referee's comment that combination of mutations does not show significant additive effect. However, we do not think that the control plasmid (pGL2-Eed(-2600/-13)) is problematic by the following reasons. Firstly, we have confirmed that this plasmid contains intact binding sites for Oct4, Sox2 and STAT3 by sequencing. Secondly, we used this construct in the previous report (Ura et al. (2008) J. Biol. Chem.) and demonstrated that this construct responds well to downregulation of STAT3 and Oct3/4. It is therefore unlikely that the control plasmid has a problem. Instead, we think it is because the eed promoter is regulated not only by Oct3/4, Sox2 and STAT3. We now speculate that Oct3/4, Sox2 and STAT3 regulate about 60% of the eed promoter activity, and the rest of activity is regulated by other unknown factors. This speculation is based on our previous observation that, when ES cells undergo differentiation and expression of Oct3/4 and Sox2, as well as activity of STAT3, is downregulated, expression level of Eed mRNA is reduced but about 30-40 % of expression is still maintained. We think that because all of the three factors are likely to play a critical role in this promoter, mutation of either site might be enough to abolish most of the activity maintained by the three factors (i.e. 60% of the total activity). As a consequence, we could not detect the significant additive effect of combination, if any.
As for the error bar of the control, to minimize variation caused by cell conditions, we set the value of control to 1.0 and normalized the data in each experiment. As a result, the value of the control sample becomes 1.0±0. This is why there is no error bar. Apparently, we should have stated this in the manuscript. We added the following sentence in the legend for Fig. 1G.

In each experiment, the data was normalized by setting the value of the wild-type promoter as 1.0.

Figure 3A. Scale bars should be shown.

Response:
As suggested, we added scale bars.

Figure 3B and 6C. Is a bar missing in the control or did the authors always counted exactly zero colonies?

Response:

In the presence of tetracycline, none of Eed4 cKO ES cell colonies could form compact colonies in three independent experiments, unless we introduced Sox2 or histone acetyltransferase. As a consequence, the value of the control becomes zero.

Figure 4A. Although the colonies are shown in similar sizes, they seem to be originally different. For example, the colony in the upper right corner seems to contain considerably less cells than the colony in the upper left corner, and therefore the cells look much bigger in the colony in the upper right corner. It seems that the colonies originally had different sizes and different cell numbers but were made to look similar. Scale bars should be provided.

Response:
We thank for this comment. As the referee pointed out, the sizes of the colonies are slightly different from each other. We added scale bars to all the bright-field images.

It is also very difficult to discern the staining pattern of the different histone marks from such a bird's view. Higher magnification images should be shown to observe the nuclear morphology.

Response:
As pointed out by the referee, with this magnification, we cannot discern the different histone marks. However, unfortunately, we have only fluorescence microscope. Confocal laser microscope, which is required to observe nucleus at much higher magnification, is not available in our laboratory. Therefore, all we can do is to show fluorescence micrographs with higher magnification to demonstrate that antibodies actually stain the nucleus of ES cells. We presented these data as supplemental Fig. S3A (As a result, original supplemental Fig. S3 is now renamed as Fig. S4). Considering that the primary purpose of this experiment is to show that Sox2 overexpression leads to upregulation of histone acetylation without any change in H3K27me3, we feel that even the bird's views are still of help.

In the same figure, the differences in immunofluorescence (IF) seem dramatic while the differences shown by Western blots seems much milder. H3Ac and H4Ac, for example, seem to go down from very high to essentially no fluorescence intensity, but display less than 2-fold difference in Western blots. If this is a detection issue, perhaps the authors should comment / discuss.

Response:
We thank the referee to pointing this out. It is true that there are discrepancies between immunostaining and Western blotting. We have measured the intensity of fluorescence by NIH image J, and found that fluorescence intensity shows the similar tendency with signal intensity of Western blotting. Apparently, the original images have too strong contrast. So, we presented reprocessed images in revised Fig. 4A, and displayed the results of intensity measurement as supplemental Fig. S3B. With the same reason, we presented reprocessed images in revised Fig. 6A, and displayed the results of intensity measurement as supplemental Fig. S4A.

Figure 6A. Add scale bars.

Response:
As suggested, we added scale bars to Fig. 6A.

We thank again to both referees to improve the manuscript substantially. We hope that the above changes and responses are satisfactory and that our manuscript is now suitable for publication in *the EMBO Journal*.

2nd Editorial Decision 23 March 2011

Thank you for submitting your revised manuscript to The EMBO Journal. It has now been reviewed by the two original referees who find that you have satisfactorily

addressed the concerns. I am happy to accept the manuscript for publication in The EMBO Journal. I believe that it will make a great contribution to the Journal.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2
We have checked the revised version of the manuscript. All the changes we have requested have been implemented and were concerning only clarifications on minor points. The points raised by the other reviewer seems to have been equally well taken into account. Therefore we fully support the publication of this work in its present form.

I would like to apologize for our late reply,

Referee #3
The authors corrected the paper and answered my various queries and concerns.

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