THE EMBO JOURNAL

Manuscript EMBO-2010-74427

Crucial function of histone deacetylase 1 for differentiation of teratomas in mice and humans

Sabine Lagger, Dominique Meunier, Mario Mikula, Reinhard Brunmeir, Michaela Schlederer, Matthias Artakar, Oliver Pusch, Gerda Egger, Astrid Hagelkruys, Wolfgang Mikulits, Georg Weitzer, Ernst W. Muellner, Martin Susani, Lukas Kenner and Christian Seiser

Corresponding author: Christian Seiser, Max F. Perutz Laboratories, Medical University of Vienna

Review timeline:

Submission date:	08 April 2010
Editorial Decision:	21 May 2010
Revision received:	20 September 2010
Additional Correspondence:	27 September 2010
Accepted:	28 September 2010

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 21 May 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first apologise for the delay in getting back to you with a decision: this was due to a delay in receiving the final referee's report. However, your manusript has now been seen by three referees whose comments are enclosed. As you will see, all three referees express interest in your finding that HDAC1 can regulate proliferation and differentiation in teratomas. However, the referees - #1 in particular - find that your study remains at a rather preliminary stage, and consequently state that the study needs to be significantly extended in order to be potentially for publication in the EMBO Journal. I would particularly draw your attention to point 1 of referee 1: while your data do suggest that HDAC1 represses Snail, the loss of HDAC1 would also be predicted to alleviate Snail's repression of E-cadherin; the downstream consequences here clearly need to be analysed in greater detail.

I do realise that a large amount of work will likely be required to address the concerns of the referees, but given the interest expressed, I would like to invite you to revise your manuscript according to their comments. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not

consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Apologies again for the delay, and thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Making use of teratoma formation in immunodeficient mice after the injection of ES cells, expressing or not HDAC1, Lagger and colleagues revealed new and unexpected properties of HDAC1. The approach is original and allowed the authors to make a number of unique observations. The most important observation is that in this particular context the phenotypes resulted from the absence of HDAC1 are unexpected and differ from all the described and molecularly dissected activity of HDAC1 under other contexts.

At this stage however this report presents only a descriptive study appealing for further molecular investigations. Moreover the absence of a precise analysis and quantifications of the data, makes it difficult to propose a clear hypothesis for the new activities of HDAC1 reported here.

Some examples are given below.

1 - The authors propose that the lack of HDAC1 leads to a loss of the negative autoregulatory feed back mechanism of Snail1 expression, leading to a dramatic accumulation of SNAIL1. This increased expression of Snail1 is suggested to be responsible for a "perturbed" E-cadherin expression.
It is not clear what does signify the term "perturbed". There is no precise quantification of Snail1 and E-cadherin expression. In fact, it seems that E-cadherin is expressed in HDAC1-/- teratomas but due to a disorganized epithelium, is found in cells present in diffuses areas.
Although the loss of HDACs could explain the abrogation of negative autoregulatory feed back mechanism of SNAIL1 on its own promoter, and its up-regulation, the authors have ignored the fact that SNAIL1 also uses HDACs to repress E-cadherin expression.
Therefore they provide no data explaining how and why the SNAIL1- dependent repression of E-cadherin is maintained. In fact the authors insinuate that the repression of E-cadherin is even more effective to support their proposed occurrence of EMT in the absence of HDAC1.

2 - The experiments performed in F9 cells are in general meaningless.
The authors have made use of HDAC inhibitors to show a derepression of Snail1 (due to its HDAC-dependent autoregulatory repressive activity). However, since SNAIL1 is known to use HDAC1/2/3 to ensure its repressive activities, this experiment does nor provide any new information relevant to the situations observed in the absence of HDAC1 in teratomas. The question is here the specific role of HDAC1.
Furthermore the demonstration of an increased H3 acetylation on Snail1 promoter after HDAC inhibitor treatment is of no surprise and only shows the expected increase in histone acetylation, as in the rest of the genome, after this treatment.
Finally the presence of HDAC1 on Snail1 promoter supports the previous findings that SNAIL1 uses HDACs to repress the transcription of its promoter.

As mentioned above the important question here is the role of HDAC1 in the repressive activity of SNAIL1 and also its specific contribution to the repressive activity of SNAIL1 on its own promoter

compared to the E-cadherin promoter.

3 - The Snail/E-cadherin situation is only a demonstration of the general lack of mechanistic consideration. This criticism also applies to the unexpected observations, in the absence of HDAC1, of enhanced cell proliferation and apoptosis. In fact no molecular mechanism is proposed to explain why in teratomas HDAC1 behaves drastically differently from ES, MEFs and tumour cells.

Referee #2 (Remarks to the Author):

This manuscript by Lagger and colleagues uses embryonic stem cells lacking Hdac1 to probe the contribution of this regulator of chromatin structure and gene expression to the formation of teratomas in a xenograft system. The tumors that are formed are generally smaller, but not significantly so. Examination of Ki67 and markers of apoptosis suggest that proliferation rates are somewhat higher with correspondingly higher rates of apoptosis. This is somewhat surprising given the links of Hdac1 to cell proliferation. The most remarkable difference in the tumors formed from Hdac1 deficient ES cells is that they are more immature, suggesting that Hdac1 contributes to differentiation. Mechanistically, this can be seen in continued expression of Oct3/4, a stem cell marker. In addition, these tumors are characterized by a lack of E-cadherin expression with the over expression of Snail. In fact, Snail appears to be a direct target for repression by Hdac1. These characteristics are conserved in immature human teratomas that express Oct3/4, Snail, and Hdac2, but not Hdac1.

Suggestions for improving the manuscript:

1) Because this is one of the first papers to describe Hdac1 loss as contributing to tumor formation, if enough sample is available the authors should consider addressing the mechanism underlying its loss of expression, which is an important consideration for the field. That is, is HDAC1 deleted, mutated, or methylated?

2) The authors should provide statistical rationale for the numbers of human samples examined to make a better argument that loss of Hdac1 is likely to be an important contributing event to teratoma formation (e.g., does the analysis of 4 immature tumors provide the statistical power needed to convince the reader).

3) It would help the reader to identify the more intense band that migrates slower than Oct3/4 in the left hand panel of Fig. 3B. Is this a phosphorylated form of Oct3/4 or a background band?

Referee #3 (Remarks to the Author):

HDACs are involved in tumor development, and several HDAC inhibitors are considered and tested as potential anti-cancer agents. HDAC inhibitors induce growth arrest, differentiation and/or apoptosis.
Studies from the same lab reveled the important role of HDAC1 during mouse development. HDAC1 knock out indeed is embryonic lethal. Seiser and collaborators also showed that in mESC HDAC1 is important for unrestricted proliferation, and is involved in epithelial cell differentiation. In this study, the authors investigate the specific role of HDAC1 in tumor formation.
Teratomas obtained with ES cells HDAC1-/- showed no difference in size when compared to those obtained from wild-type mESC. Surprisingly, loss of HDAC1 leads to increased proliferation and apoptosis and reduced differentiation of epithelial structures, together with an up-regulation of HDAC2. On the other hand, teratomas derived from wild-type ES cells were highly differentiated. These unexpected results are linked to a direct role of HDAC1 in the regulation of SNAIL1. ChIP analyses suggest a direct HDAC1 binding to Snail1 promoter. In HDAC1 knockout cells, SNAIL1 is constitutively expressed leading to disturbed E-cadherin expression and to a reduction of epithelial structures.
Interestingly, they found similar correlations of HDAC1/HDAC2 expressions and differentiation grades in human patient samples, and therefore the authors claim that HDAC1 and HDAC2 could be potential prognostic markers for carcinoma classification.

The manuscript is well written and experiments are well designed. The data presented in this manuscript are interesting but still preliminary. The authors should put a further effort into giving more strength to the result presented. To this purpose, I suggest the further experiments to address the following points:

1. In addition to HDAC inhibitor studies in embryonic carcinoma F9 cells, expression levels of SNAIL1 and E-Cadherin in cells interfered for HDAC1 or HDAC2 (or both) should be monitored.

2. It would be important to show increased HDAC2 recruitment to the E-Cadherin promoter in a HDAC1 deficient background.

3. Histone H3 acetylation within the Snail1 promoter is not highly increased upon HDAC inhibitor treatment. Is H4 acetylation more affected?

4. HDAC1 binding within the Snail1 promoter occurs together with SNAIL1 at E-box elements?

5. Are SNAIL and E-cadherin expression levels affected by overexpression and interference of HDAC1? Also the role of HDAC2 and histone acetylation must be addressed alone and together with HDAC1 interference and overexpression.

6. The authors suggest that HDAC1 and HDAC2 could represent valuable prognostic markers for carcinoma classification. The results in human teratoma samples are indeed interesting, and HDAC1 was mainly detected in differentiated teratomas, while HDAC2 in undifferentiated teratocarcinomas. Does this clear tendency mean that HDAC1 and HDAC2 do not act as heterodimers in such scenarios? Are HDAC1 and HDAC2 heterodimers predominantly found in not-transformed tissues?

7. How was the teratoma data analyzed? As far as the results might be field-dependent, statistics should be done to show results at least from five different teratoma fields.

Minor comments:
1) In Figure 3C, the low-right panel should be "HDAC1-/- ev" rather than "HDAC1-/- re".
2) In Figure 5C, Y axis must be indicated.
3) All Teratoma slides stained with an antibody should show quantification.
4) Data from Keratin 5 as an epithelial marker should be include together with the E-cadherin one.

1st Revision - authors' response 20 September 2010

Referee #1 (Remarks to the Author):

1 - The authors propose that the lack of HDAC1 leads to a loss of the negative autoregulatory feed back mechanism of Snail1 expression, leading to a dramatic accumulation of SNAIL1. This increased expression of Snail1 is suggested to be responsible for a "perturbed" E-cadherin expression. It is not clear what does signify the term "perturbed". There is no precise quantification of Snail1 and E-cadherin expression. In fact, it seems that E-cadherin is expressed in HDAC1-/- teratomas but due to a disorganized epithelium, is found in cells present in diffuses areas.
Although the loss of HDACs could explain the abrogation of negative autoregulatory feed back mechanism of SNAIL1 on its own promoter, and its up-regulation, the authors have ignored the fact that SNAIL1 also uses HDACs to repress E-cadherin expression.
Therefore they provide no data explaining how and why the SNAIL1-dependent repression of E-cadherin is maintained. In fact the authors insinuate that the repression of E-cadherin is even more effective to support their proposed occurrence of EMT in the absence of HDAC1.

Based on the criticisms of this reviewer, we have performed a whole set of new experiments in order to gain more insights into the mechanisms that might be responsible for the observed phenotype of HDAC1 deficient teratomas.
We have carefully quantified the expression of E-cadherin and the areas with cytosolic E-cadherin staining in wildtype and HDAC1 null tumors.
Originally, we interpreted the cytosolic E-cadherin staining as sign of downregulation of E-cadherin expression via internalisation and degradation of the protein. However, quantification of E-cadherin expression (mRNA and protein) in wildtype and HDAC1 deficient tumors indicated that in addition to the significantly increased areas with cytosolic E-cadherin also E-cadherin expression levels are enhanced in HDAC1 null teratomas. This is in perfect agreement with increased expression of E-cadherin in HDAC1 knockdown embryonal carcinoma cells.
Importantly, the areas with cytosolic E-cadherin also show high levels of its negative regulator SNAIL1. Due to the absence of the co-repressor HDAC1, both E-cadherin and SNAIL1 are induced. This finding is confirmed in F9 knockdown experiments discussed below. The absence of HDAC1 creates a very unusual scenario. On one hand SNAIL1 and its positively regulated targets such as MMP9 and ZEB1 are induced, on the other hand, the negatively regulated SNAIL1 target genes such as E-cadherin and Col2a1 are derepressed. This leads to the simultaneous presence of differentiationspecific factors and factors linked to tumor progression. SNAIL1 and (its positively regulated targets) are obviously dominant over the differentiation specific derepressed factors resulting in reduced differentiation and enhanced proliferation.
In order to examine in more detail a potential role of SNAIL1 for the phenotype of HDAC1 -/- teratomas we have reduced SNAIL1 expression in wildtype and HDAC1 null teratomas by lentiviral shRNA transduction. This led to two important findings. In agreement with previously published data on SNAIL1 knockdown in epidermal carcinomas we find a drastic reduction in tumor proliferation. In addition, we find that epithelial structures differentiate in HDAC1 deficient SNAIL1 knockdown teratomas as highlighted by reduced Ki67 staining and E-cadherin relocalization to the membranes.
Taken together we identify transcriptional regulator SNAIL1 and the downstream genes as important targets for the co-repressor HDAC1.

2 - The experiments performed in F9 cells are in general meaningless. The authors have made use of HDAC inhibitors to show a derepression of Snail1 (due to its HDAC-dependent autoregulatory repressive activity). However, since SNAIL1 is known to use HDAC1/2/3 to ensure its repressive activities, this experiment does nor provide any new information relevant to the situations observed in the absence of HDAC1 in teratomas. The question is here the specific role of HDAC1.
Furthermore the demonstration of an increased H3 acetylation on Snail1 promoter after HDAC inhibitor treatment is of no surprise and only shows the expected increase in histone acetylation, as in the rest of the genome, after this treatment.
Finally the presence of HDAC1 on Snail1 promoter supports the previous findings that SNAIL1 uses HDACs to repress the transcription of its promoter.
As mentioned above the important question here is the role of HDAC1 in the repressive activity of SNAIL1 and also its specific contribution to the repressive activity of SNAIL1 on its own promoter compared to the E-cadherin promoter.

In order to use the embryonal cell line F9 as useful tool to support the findings in the HDAC1 teratomas we silenced HDAC1 and HDAC2 in F9 cells by stable shRNA expression. Both knockdowns were highly efficient and showed the compensation (HDAC2 upregulation in HDAC1 knockdown and vice versa) also observed in HDAC1 null teratomas. Importantly, we found enhanced expression of SNAIL1 and E-cadherin in the absence of HDAC1. In addition we find SNAIL1 targets such as Col2a1 and MMP9 upregulated upon silencing of HDAC1. Thus, loss of HDAC1 deregulates the expression of the regulator SNAIL1, positively regulated SNAIL1 targets and negatively regulated SNAIL1

target genes.
Quantitative ChIP experiments demonstrate the presence of HDAC1 at the promoters of the Snail1 and Cdh1 genes. Loss of HDAC1 results in H3K9 and H3K56 hyperacetylation at these promoters strongly suggesting a direct regulatory role of HDAC1 in this context.

3 - The Snail/E-cadherin situation is only a demonstration of the general lack of mechanistic consideration. This criticism also applies to the unexpected observations, in the absence of HDAC1, of enhanced cell proliferation and apoptosis. In fact no molecular mechanism is proposed to explain why in teratomas HDAC1 behaves drastically differently from ES, MEFs and tumour cells.

Loss of HDAC1 affects proliferation in a cell type specific context. In ES cells and fibroblasts loss of HDAC1 leads to reduced proliferation due to upregulation of p21 and p57. This was shown by several groups including our lab. Very recently we could demonstrate that in T cells conditional ablation of HDAC1 results in enhanced proliferation of peripheral T cells most probably due to increased expression of specific cytokines. In teratomas our data indicate, as discussed above that the SNAIL1 regulatory network is a major target for HDAC1 and its deregulation contributes to the observed phenotype of HDAC1 deficient teratomas. We believe that our new data strongly support this model.

Referee #2 (Remarks to the Author):

Suggestions for improving the manuscript:

1) Because this is one of the first papers to describe Hdac1 loss as contributing to tumor formation, if enough sample is available the authors should consider addressing the mechanism underlying its loss of expression, which is an important consideration for the field. That is, is HDAC1 deleted, mutated, or methylated?

There was little material available from these tumors but our collaborator Gerda Egger a former postdoc of Peter Jones and specialist for DNA methylation performed DNA methylation assays for the HDAC1 and the HDAC2 genes. We did not detect DNA methylation in the HDAC1/2 promoter CpG islands in genomic DNA isolated from paraffin embedded teratomas of 18 patients using COBRA (combinded bisulfite restriction anaylysis) and bisulfite sequencing.

2) The authors should provide statistical rationale for the numbers of human samples examined to make a better argument that loss of Hdac1 is likely to be an important contributing event to teratoma formation (e.g., does the analysis of 4 immature tumors provide the statistical power needed to convince the reader).

We have statically analyzed all the data linked to IHC signals, mRNA expression, ChIP, areas with cytosolic localization and tumor size and indicated the statistic significance. The phenotype of HDAC1 deficient teratomas was consistently observed in tumors created with untransfected HDAC1 null ES cells, empty vector transfected HDAC1 null ES cells and NT shRNA control HDAC1 null ES cells as listed in the Supplementary Table.

3) It would help the reader to identify the more intense band that migrates slower than Oct3/4 in the left hand panel of Fig. 3B. Is this a phosphorylated form of Oct3/4 or a background band?

As documented in several publications this particular OCT3/4 antibody recognizes two bands on Western blots. To test whether one of the bands would be a phosphorylated form of OCT3/4 we performed phosphatase

experiments. As shown in the Supplementary Information for the reviewer treatment with calf intestinal phosphatase did abolish the signal for the control protein phosphoAKT but did not affect the ratio or intensity of the two OCT3/4 bands. According to the manufacturer the antibody recognizes two OCT3/4 isoforms.

Referee #3 (Remarks to the Author):

The manuscript is well written and experiments are well designed. The data presented in this manuscript are interesting but still preliminary. The authors should put a further effort into giving more strength to the result presented. To this purpose, I suggest the further experiments to address the following points:

1. In addition to HDAC inhibitor studies in embryonic carcinoma F9 cells, expression levels of SNAIL1 and E-Cadherin in cells interfered for HDAC1 or HDAC2 (or both) should be monitored.

As suggested by the reviewer and described above under Referee #1 Point 2 we have silenced HDAC1 and HDAC2 in F9 embryonal carcinoma cells. The simultaneous HDAC1/HDAC2 knockdown was much less efficient, but the cells that survived the selection might have escaped an efficient knockdown given the lethal phenotype of HDAC1/HDAC2 null fibroblasts (Haberland et al., 2009; Wilting et al., 2010; Yamaguchi et al., 2010).
Loss of HDAC1 but not HDAC2 results in up-regulation of both SNAIL1 and ECadherin.

2. It would be important to show increased HDAC2 recruitment to the E-Cadherin promoter in a HDAC1 deficient background.

Quantification of E-cadherin expression in HDAC1 null teratomas and the HDAC1 and HDAC2 knockdown experiments revealed that there is no efficient compensation by HDAC2 in the absence of HDAC1 with respect to E-cadherin expression. In ChIP assays we did not observe increased recruitment of HDAC2 in HDAC1 knockdown cells.

3. Histone H3 acetylation within the Snail1 promoter is not highly increased upon HDAC inhibitor treatment. Is H4 acetylation more affected?

We have performed now quantitative ChIP assays with specific histone acetyllysine antibodies in HDAC1 knockdown cells. Upon silencing of HDAC1 acetylation levels for histone H3K9 and K56 are increased at both promoters (Snail1 and Cdh1). The H3K56ac mark has been recently described as HDAC1 substrate.

4. HDAC1 binding within the Snail1 promoter occurs together with SNAIL1 at E-box elements?

We have tried hard but we could not get significant SNAIL1 signals higher than the IgG control at the Snail1 and Cdh1 promoters with commercially available ChIP antibodies.

5. Are SNAIL and E-cadherin expression levels affected by overexpression and interference of HDAC1? Also the role of HDAC2 and histone acetylation must be addressed alone and together with HDAC1 interference and overexpression.

As described above silencing of HDAC1 induces expression of both SNAIL1 and E-Cadherin. We have tried to overexpress HDAC1 in ES cells and teratomas. However we did not get significantly higher levels of HDAC1 upon retroviral infection. In fact, additional HDAC1 expression reduced the levels of endogenous HDAC1. this is most probably caused by the negative

autoregulatory feedback regulation of HDAC1 described in Schuettengruber et al., 2003.

6. The authors suggest that HDAC1 and HDAC2 could represent valuable prognostic markers for carcinoma classification. The results in human teratoma samples are indeed interesting, and HDAC1 was mainly detected in differentiated teratomas, while HDAC2 in undifferentiated teratocarcinomas. Does this clear tendency mean that HDAC1 and HDAC2 do not act as heterodimers in such scenarios? Are HDAC1 and HDAC2 heterodimers predominantly found in not-transformed tissues?

HDAC1 and HDAC2 can act as homo- and hetero-dimers and until now it is unclear if there is a difference in the function of HDAC1 only, HDAC2 only and HDAC1/HDAC2 co-repressor complexes. We have analyzed the presence of HDAC1 and HDAC2 in common complexes in untransformed cells and tumor cells and there is no clear difference. In some specific cell types such as neurons and glia cells only one of the two deacetylases is present but again both HDAC1 only and HDAC2 only complexes are enzymatically active.

7. How was the teratoma data analyzed? As far as the results might be field-dependent, statistics should be done to show results at least from five different teratoma fields.

With the HistoQuest software at least 10 High Power Fields were analyzed for each sample.

Minor comments:

1) In Figure 3C, the low-right panel should be "HDAC1-/- ev" rather than "HDAC1-/- re".

Corrected.

2) In Figure 5C, Y axis must be indicated.

We have labelled the Y axis in all graphs.

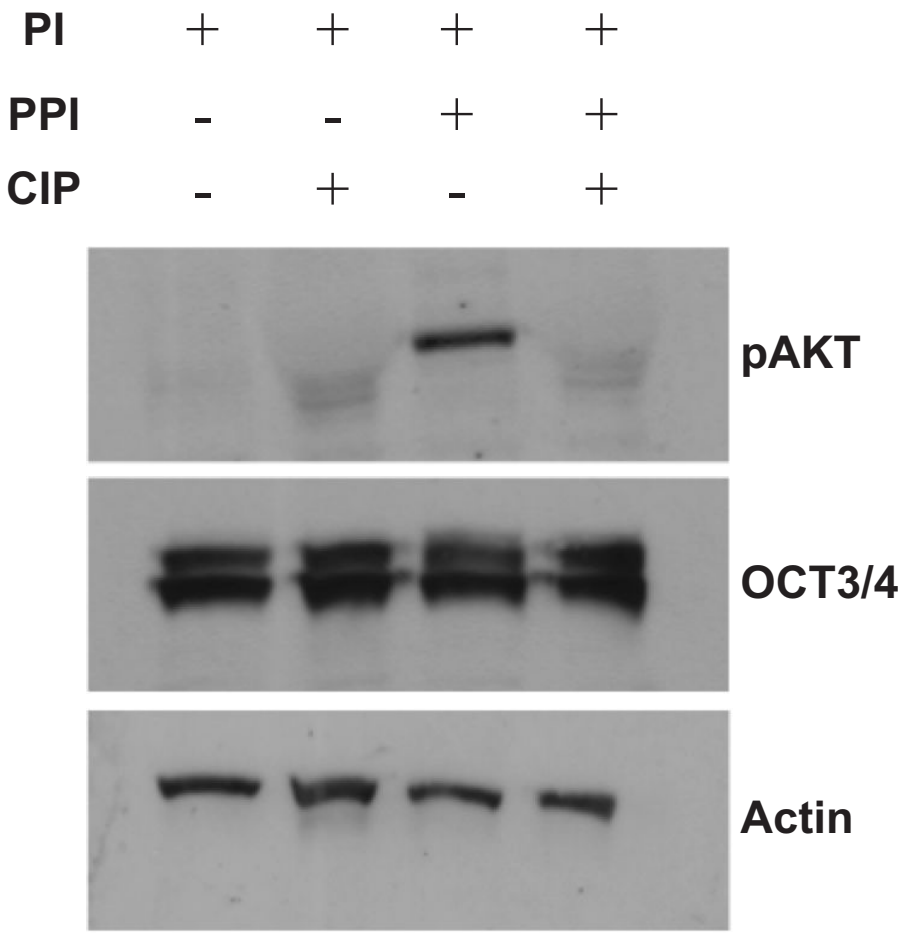
3) All Teratoma slides stained with an antibody should show quantification.

All stainings were quantified using by the HistoQuest Software. The evaluations are shown in separate graphs.

4) Data from Keratin 5 as an epithelial marker should be include together with the E-cadherin one.

We have included a Keratin staining of HDAC1+/+ and HDAC1-/- teratomas as Suppl. Figure 2.

Supplementary information for the reviewer



In order to test a possible phosphorylation of OCT 3/4, we incubated ES cell extracts harvested in the presence of protease inhibitors (PI) and in the absence or presence of phosphatase inhibitors (PPI).
We then incubated the extracts with CIP phosphatase (NEB) for 60 minutes at 37°C. We performed Western Blot analysis and incubated the membrane with a phospho AKT antibody to test protection of protein phosphorylation in the presence of phosphatase inhibitors. As a control, the phosphoAKT signal was lost upon CIP treatment. For OCT 3/4 signals, the upper band was persistent throughout all treatments indicating that this band is not a phosphorylated form of OCT3/4.

Additional Correspondence 27 September 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-74427R. It has now been seen again by referees 1 and 3, whose comments are enclosed below. As you will see, both referees find that you have responded well to the concerns raised in the previous round of review, and are now fully supportive of publication without further revision. I am therefore pleased to be able to tell you that we can accept your manuscript to be published in the EMBO Journal. However, before we do so, I have just one small request. We are currently implementing a policy of requesting an Author Contributions statement in all accepted manuscripts. Can I therefore ask you to send me a modified version of the manuscript text including such a statement (either in the Acknowledgements or as a separate section)? Once we have this, we can replace the previous version of the text, and will then be able to accept your study without further delay.

Thanks and best wishes,

REFEREE REPORTS

Referee 1 comments:

In responding to points No. 1 and 2, the authors have succeeded in unravelling a clear mechanism underlying the observed molecular events. These investigations also give a good explanation for the concern raised in point No. 3.
In conclusion the manuscript has been much improved and is now publishable in EMBO J.

Referee 3 comments:

The authors are commended for the work they have done to revise the manuscript based on the reviewer's suggesitons.

