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The epigenetic regulator PLZF represses L1 retrotransposition in germ and progenitor cells

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(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.)

Editor: Anke Sparman

1st Editorial Decision 19 December 2012

Thank you for submitting your research manuscript (EMBOJ-2012-83753) to our editorial office. It has now been seen by three referees and their comments are provided below. I apologize for the slightly protracted review process, which was caused in part by one delayed report.

All reviewers appreciate your study and are in general supportive of publication in The EMBO Journal. Nevertheless, they do raise a number of important concerns, and both reviewer #1 and #2 emphasize that a significant revision of the manuscript will be required. Referee #1 suggests that the study would be strengthened by data demonstrating that PLZF actually affects L1 retrotransposition. Referee #2 proposes to link your results to the germ cell phenotype of PLZF k.o. mice and referee #3 recommends to investigate if the deregulation of L1 sequences results in altered biological responses to stress. We encourage strengthening the data demonstrating the physiological significance of your observations.

Overall, I would like to invite you to submit a suitably revised manuscript to The EMBO Journal that addresses the concerns raised by the reviewers. I should add that it is our policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage. Please do not hesitate to contact me to further discuss the required revisions.

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, 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.

REFEREE COMMENTS

Referee #1

This is an interesting paper documenting a potential role for PLZF,a member of the POK family of transcription factors, in repression of L1s mainly in introns of genes. The authors have used PLZF-on and PLZF-off knock-in mutants along with mouse PLZF wildtype mice to show binding of PLZF mainly to a 7 bp region in the middle of ORF2 of mouse L1. They show that this binding leads to increased methylation of the L1 5'UTR by spreading of repressive chromatin factors. The PLZF-off mutant has increased L1 expression. The effect is also present at the L1 mRNA and translation levels.
One problem in the study is that the binding site in L1 is at about nt. 3000 (not ever explicitly stated) and that means that since the majority of 5' truncated L1s are less than 2kb in length from the 3' end and the PLZF site is at least 3 kb upstream of the 3' end that most intronic L1s will be unaffected by PLZF binding.
A second issue is that the effect is seen on L1 expression, but the authors have not checked for an effect on L1 retrotransposition. HEK293 cells that lack PLZF would be a great cell line for this experiment. They could assay for L1 retrotransposition using an L1 marked by a retrotransposition cassette plus/minus a PLZF construct. The assay could be done with an L1 containing the PLZF binding site and one with a mutant site. If the authors are correct, one would expect to see repressed L1 retrotransposition in the presence of PLZF and incresed retrotransposition with PLZF and a mutant (binding site) L1. This experiment could be done with transient transfection or a stably integrated L1. If retrotransposition was affected by PLZF, the paper would be much improved and convincing.
Minor comments on references: 1) bottom of page 1 reference Coufal, Muotri, Faulkner, Lee, Solyom on L1 activity in somatic cells. 2) Above that point reference Feng for endonuclease activity in L1. 3) For Alu retrotransposition driven by L1 reference Dewannieux with Heidmann. Other minor points- 1) page 5, first line under second heading-"equally present" is unclear. Delete equally.2) page 6 top- Rangwala et al. 2009 should be deleted in citing PLZF relocation.

Referee #2

Puszky et al.

Novel dual regulation of L1 retrotransposons by PLZF

The authors present a nice paper showing that PLZF is recuited to L1 elements where it triggers transcriptional repression and DNA methylation. They conclude their story as "... reveals a novel mechanism of action by which PLZF represses retrotransposons safeguarding normal progeneitor homeostasis". I think this is an interesting study and the L1 gene provides a useful system for investigating PLZF induced gene repression. However, it would be good to link the results presented here to the apparent phenotype in PLZF knockout mice where loss of PLZF triggers increased apoptosis in spermatogonia. Do the authors see apoptosis when they express the mutant PLZF in the knockin PLZF mouse lines? Can the authors better dissect the silencing mechanism of L1 elements? Is the gene silenced and then methylated or is DNA methylation the driver of silencing.

My biggest concern is that a number of experiments were done by transient transfection in 293T cells. In this situation the results maybe complicated by the plasmid will becoming chromatinised during the time course of these experiments (Fig 2). Is it possible to use an inducible expression system to investigate the effect of PLZF expression of L1 silencing?

Specific points

Please can the authors by more specific about what the DMRs correspond to in the main text? Why are there methylation differences between WT and PLZFoff. Does PLZFoff bind and recuit activator proteins?

Are there L1 DMRs that do not bind PLZF? How do the authors interpret this result?

Fig 2B/C. Would it be better to align reads with mouse L1 elements?

Fig 2E the labels for WT and Mut seem to have moved.

The authors show that PLZF interacts with 33 fragments, 19 of which are L1s. What else does it interact with? Known PLZF regulated proteins?

I might have missed this but what are the expression levels of L1 elements in 293T cells? Are they altered by WT and mutant PLZF expression.

Fig 3A. What result do you get if express PLZFon or PLZFoff instead?

The authors suggest DNMT1 is recruited and this can methylate the locus. I am surprised as DNMT1 is a maintenance methyltransferase not a de novo methyltransferase. Do they also see recruitment of DNMT3A/B?

The authors find that L1 sequences bound by PLZF are often located near or within the 3' UTR of coding genes and in intronic regions. What is the distribution of all L1s? Is it the same or different?

Fig 4C. The authors are separating the insulator function of L1 elements to the binding and silencing by PLZF. Can the authors provide statistics as it appears the expression goes down when they express PLZFwt (Fig 4C2). Is this significant? What results do the authors get if they express PLZWoff instead. Is it the same effect as mutating the L1 sequence?

Fig. 5B. The authors need to repeat with PLZFmut and L1 mutant to show result is specific.

The authors refer to Fig. 6C2. Is this data now in supplementary?

The title needs to be more specifc e.g. Novel regulation of L1 retrotransposons by the DNA and RNA binding activity of PLZF

The authors frequently refer to transcriptional repression as heterochromatinisation and that induction of L1 triggers heterochromatin formation. I think the authors need to be more specific as in my opinion this is not "heterochromatin" - it is transcriptionally repressed euchromatin. They also refer to genes being in a "closed" or "open" state. What do they mean by this? The authors have done no experiments that look at chromatin structure. It would be better to refer to the genes as active or inactive.

In general I found the figures a little confusing. Please can more labels be put on the figures e.g. Fig 3D, Fig 6B to show what each lane corresponds to without having to look at the legend. It might also be helpful to emphasise what cell types were used for experiments and that the 293T cell experiments are transient.

Referee #3

This is a very interesting paper that provides important advances in our understanding of the mechanism(s) of action of PLZF. The model proposes that PLZF is involved in the control of transcription of repeated DNA elements, and that this control is modulated by cellular stress. This fascinating model leads to the suggestion that in those tumors where PLZF activity is deregulated (such as PLZF-RAR leuekemias) will be very interesting to look at the regulation of L1 retrotransposons.
Major point:

• The Authors show that in KG1a cells, PLZF relocation following stress causes deregulation of L1 transcription. It would be of tremendous interest to look at the stress response in murine cells derived from the knock-in models, and check whether the hypothetical deregulation of L1 sequences could result in an altered biological response. I do not necessarily agree that the altered proliferative potential observed in the serial replating assays by the mutated PLZF constructs would mirror the response of the same cells upon X-rays treatment, or treatment with drugs.

Specific points:

• Missing reference: Martin et al., 2005;
• Legend to figure 1C: the authors should better explain the quantitation;
• Figure 1D: the analysis is done on whole bone marrow, is there any effect of the PLZF transgenes on the distribution of the hematopoietic subpopulations?
• Figure S1: I would prefer to see the absolute number of colonies.

1st Revision - authors' response 01 April 2013

Referee #1

- One problem in the study is that the binding site in L1 is at about nt. 3000 (not ever explicitly stated).

Answer:

In the first version of our manuscript, we reported the PLZF-BS to be located in the ORF2 region (page 3). In the revised manuscript, we have now added the specific nucleotides (nt 2634-2640) (page 3 and figure 2A).

As suggested by reviewer 2, since the majority of 5' truncated L1s are less than 2kb in length from the 3' end and that PLZF site is at least 3 kb upstream of the 3' end, PLZF will only interacts with a sub-fraction of truncated L1 sequences a nd that most intronic L1s will be unaffected by PLZF binding. A sentence was added to the manuscript underlying this specific point (page 5).

- …… and that means that since the majority of 5' truncated L1s are less than 2kb in length from the 3' end and the PLZF site is at least 3 kb upstream of the 3' end that most intronic L1s will be unaffected by PLZF binding

Answer:

Indeed, PLZF differentially methylated L1 DMRS are located in either intronic or 3'UTR sequences of genes. All the PLZF-interacting L1 sequences (containing PLZF-BS) are located in the one same region. This region of 460 pb corresponds to the L1 ORF2 sequence of 6kb (Figure 2B) and is situated 3kb upstream of the L1 3' end. No PLZF L1 DMRS were found in 5'UTR. These results are in line with a study reported by Faulkner *et al* 2009 showing that the most repressed L1 sequences were located in intronic/3'UTR sequences
We agree that not all L1s sequences will be affected by PLZF binding.

- A second issue is that the effect is seen on L1 expression, but the authors have not checked for an effect on L1 retrotransposition. HEK293 cells that lack PLZF would be a great cell line for this experiment. If retrotransposition was affected by PLZF, the paper would be much improved and convincing.

Answer:

As suggested a retrotransposition assay using the L1-EGFP reporter plasmid EF06R (described in Farkash *et al.* 2006) has been performed in the presence or absence of wild-type and mutant PLZFs. We are able to demonstrate that the expression of PLZF (whether from the $PLZF^{WT}$ or the $PLZF^{ON}$ expression plasmids) successfully protects from L1 retrotransposition. This result is now added in figure 6D1 and in the manuscript page 5. We thank the reviewer for this suggestion.

- Minor comments on references: 1) bottom of page 1 reference Coufal, Muotri, Faulkner, Lee, Solyom on L1 activity in somatic cells. 2) Above that point reference Feng for endonuclease activity in L1. 3) For Alu retrotransposition driven by L1 reference

Dewannieux with Heidmann. Other minor points- 1) page 5, first line under second heading-"equally present" is unclear. Delete equally.2) page 6 top- Rangwala et al. 2009 should be deleted in citing PLZF relocation.

Answer:
We have corrected and added the information required (in red in the revised manuscript).

Referee #2

Puszky et al. Novel dual regulation of L1 retrotransposons by PLZF.
The authors present a nice paper showing that PLZF is recruited to L1 elements where it triggers transcriptional repression and DNA methylation. They conclude their story as "... reveals a novel mechanism of action by which PLZF represses retrotransposons safeguarding normal progenitor homeostasis". I think this is an interesting study and the L1 gene provides a useful system for investigating PLZF induced gene repression.

- However, it would be good to link the results presented here to the apparent phenotype in PLZF knockout mice where loss of PLZF triggers increased apoptosis in spermatogonia. Do the authors see apoptosis when they express the mutant PLZF in the knockin PLZF mouse lines?

Answer:
This is an excellent point from the reviewer and these experiments had indeed been performed but not shown in the previous manuscript.
There is indeed as suspected by the reviewer an increased apoptosis in spermatogonia in the $PLZF^{OFF}$ mice. The study was performed on cells purified from testis. These results have been added to Supplemental Figure 1 and in the manuscript on page2.

- Can the authors better dissect the silencing mechanism of L1 elements? Is the gene silenced and then methylated or is DNA methylation the driver of silencing.

Answer:

- The silencing mechanism of L1 elements by $PLZF^{WT}$ and mutant $PLZF^{ON}$ were analyzed at different time points (0 to 24 hours) and at different molecular levels (histone acetylation, DNA methylation, recruitment of DNMT and HDAC and methyl-binding proteins)(Figure 3 and supplemental figures S3 and S4). In brief, these experiments allows to a) describe the decrease of L1 expression in the presence of PLZF to be slow (5 hours) and to give a precise scenario of how this silencing occurs: first, with the deacetylation of the L1 element at 2 hours at the PLZF-BS (PCR5) and at 18 and 24 hours in the L1 5'UTR region (PCR1) (figure 3). Then after 16-24 hours, the 5'UTR CpG island is methylated and the L1 element completely repressed. This monitoring is shown in Figures 2 and 3. Thus, in our view, first histone deacetylation, followed by DNA methylation drive L1 gene silencing (described in the manuscript page 3).

- I might have missed this but what are the expression levels of L1 elements in 293T cells? Are they altered by WT and mutant PLZF expression.

Answer:

- L1 elements are indeed expressed in 293T cells and they can easily be detected (qRT-PCR) in these cells that do not endogenously express PLZF. In the presence of $PLZF^{WT}$, a 3-fold decrease in the levels of L1 mRNA is noted. We have not shown this data. However, this information can now indirectly be inferred in the retrotransposition assay we have now performed in these cells using the L1-EGFP reporter plasmid. We show that expression of $PLZF^{WT}$ in these cells is correlated with a decreased of L1 retrotransposition frequency. These data have been added in Figure 6C1 and on page 5.

- My biggest concern is that a number of experiments were done by transient transfection in 293T cells. In this situation the results may be complicated by the plasmid will becoming chromatinised during the time course of these experiments (Fig 3). Is it possible to use an inducible expression system to investigate the effect of PLZF expression of L1 silencing?

Answer:
We were aware of this problem and took into account the possibility of the chromatinisation of the plasmid that could occur overtime. In the experiment shown in Figure 3 we had tested plasmid chromatinisation state in the absence of PLZF but had not shown the results. The plasmid is indeed

chromatinised (see Figure 3A anti-H3 ChIP) at 2 hours until the end of the time course (until 24 hours). However no spontaneous histone deacetylation (anti-AcH3 ChIP) occurs during the first 24 hours. Thus, the histone H3 deacetylation seen with PLZF ectopic expression at 2 hours (PCR4) and 24h hours (PCR1) in figure 3A, would not be related to chromatinisation of the plasmid in this time frame. Further experiments have been addressed this issue in another study of PLZF epigenetic function with the same results published (Guidez et al. 2007).

Specific points

1-Please can the authors by more specific about what the DMRs correspond to in the main text?
1-The DMRs correspond to DNA Methylated Regions identified in other published work (Meissner A, Cell Stem Cell 9, 338, 2011 and the associated work from Lienert *et al.* 2011). These DNA sequences have been characterized by their DNA methylation content (Highly GC rich), assessed by using the DNA methylated IP approach (immunoprecipitation of methylated cytosines using a specific antibody).
The DMRs referred to in the text page 2 are the DNA Methylated Regions present in the tissues of the PLZF mutants. These DMRs where identified after a bioinformatic analysis of the differentially methylated DNA sequences of the PLZF mutants' tissues. Validation by methylation analysis of known targeted PLZF sequences, allowed us to define a figure of 188 DMRs of interest (Figure 2A, supplemental figure S4 and table I).

2-Why are there methylation differences between WT and $PLZF^{OFF}$. Does $PLZF^{OFF}$ bind and recruit activator proteins?
The methylation differences observed are due to a lack of function of $PLZF^{OFF}$ (absence of DNA binding, HDAC and DNMT recruitment) and not to a direct positive effect of the $PLZF^{OFF}$ mutant. We have previously described this mechanism (Guidez *et al.* 2005 and shown also in Figure 1 B.2). The non acetylated PLZFmutant ($PLZF^{OFF}$) does not bind DNA. In the absence of DNA binding, there is no repressor activity of the PLZFmut protein ($PLZF^{OFF}$) thus no recruitment of HDAC and DNMT and DNA remains in an hypomethylated status. We have however taken the reviewer's concern into consideration and have added a more detailed sentence in the legend of figure 1B.

3-Are there L1 DMRs that do not bind PLZF? How do the authors interpret this result?
Answer:
We did not find L1 DMRs that do not interact with PLZF or contain a PLZF-BS. This is simply explained by the design of the analysis that was performed. As described above, the analysis retained the DMRs identified by the differential analysis of PLZF mutant DNA. Thus all the methylated L1 sequences that were identified by the analysis resulted for the methylation induced by PLZF and all these sequences were found to have a PLZF-BS.

4-Fig 2B/C. Would it be better to align reads with mouse L1 elements?
Answer:
The aim of Fig2B/C was to highlight the specific PLZF binding site. We thus aligned the L1 sequences identified with the anti-PLZF antibody used in the ChIP analysis and thus show the conserved ATGTAAA PLZF binding site throughout these sequences.
Nevertheless, zoo alignment of L1 sequences show that the PLZF-BS is conserved in mouse L1 elements (supplement figure S2). Thus, when mouse L1 elements were aligned in a selected L1 DMRS, the PLZF-BS is equally found. The figure is available if the reviewer and editor request its addition to the paper.

5-Fig 2E the labels for WT and Mut seem to have moved.
Answer:
We thank the reviewer and corrected this in Fig2E.

6-The authors show that PLZF interacts with 33 fragments, 19 of which are L1s. What else does it interact with? Known PLZF regulated proteins?
Answer:
The other (24) fragments contain non L1 repeat elements (mainly SINE/Alu sequences) correlating with the MeDIP-seq results (this was not mentioned in the previous manuscript because the study is focused on L1 elements). However we have now added this precision page 3.

7-Fig 3A. What result do you get if express $PLZF^{ON}$ or $PLZF^{OFF}$ instead?
Answer:
In Fig3A we have only looked at the effect of the PLZF mutants on local deacetylation. The $PLZF^{OFF}$ mutant does not induce direct epigenetic alteration because of its inability to interact with DNA. However, $PLZF^{ON}$ behaves like $PLZF^{WT}$. Furthermore, in Figure 4B, $PLZF^{OFF}$ was used as a negative control of repression, to assess its effect on L1 targets. The effects of the PLZF mutants been extensively described in Guidez *et al.* 2005.

8-The authors suggest DNMT1 is recruited and this can methylate the locus.

- I am surprised as DNMT1 is a maintenance methyltransferase not a de novo methyltransferase.

Answer:
ChIP analysis of DNMT1 was positive and confirmed (figure 2 C). PLZF appears to be a protein important for methylation maintenance, thus, recruitment of DNMT1 may be expected in this setting. As, L1 has been reported to be "constitutively" methylated in normal conditions they may expectedly be targets of DNMT1.

- Do they also see recruitment of DNMT3A/B?

Answer:
By ChIP analysis on the L1 sequence using a DNMT3A/B antibody, we were not able to observe a strong conclusive recruitment though we were able to see *in vitro an* interaction between PLZF and DNMT3A/B by GST-pull down. We have not shown these results but have added a sentence page 3 to this effect.

- The authors find that L1 sequences bound by PLZF are often located near or within the 3' UTR of coding genes and in intronic regions. What is the distribution of all L1s? Is it the same or different?

Answer:
As described in Faulkner et al. 2009, L1 can be found in various regions of genes: 5'UTR, coding and intronic regions and 3'UTR. L1 are overrepresented in introns (around 1/3 of L1 sequences). A sentence explaining this fact has been added page 6.
Furthermore, L1 levels of expression are associated with its genomic L1 localization as L1 elements found in introns and 3'UTR are not found to be expressed (Faulkner et al. 2009).

9-Fig 4C. The authors are separating the insulator function of L1 elements to the binding and silencing by PLZF. Can the authors provide statistics as it appears the expression goes down when they express PLZFwt (Fig 4C2). Is this significant? What results do the authors get if they express PLZWoff instead. Is it the same effect as mutating the L1 sequence?
Answer:
In fact as noted and detailed in figure 4B when $PLZF^{OFF}$ is expressed, there is no repression of these reporters. Absence of repression is also noted in the presence of $PLZF^{OFF}$ in the experiment described in figure 4C2 though this result was not added for clarity. $PLZF^{OFF}$ has indeed the same effect as mutating the PLZF-BS (as shown in figure 4B panels 1 and 2).

10-The title needs to be more specifc e.g. Novel regulation of L1 retrotransposons by the DNA and RNA binding activity of PLZF
Answer:
We have taken the reviewer's comment into consideration and have modified the title to reflect the more specific nature of the work.

11-The authors frequently refer to transcriptional repression as heterochromatinisation and that induction of L1 triggers heterochromatin formation. I think the authors need to be more specific as in my opinion this is not "heterochromatin" - it is transcriptionally repressed euchromatin. They also refer to genes being in a "closed" or "open" state. What do they mean by this? The authors have done no experiments that look at chromatin structure. It would be better to refer to the genes as active or inactive.
Answer:
To answer the reviewer's concern and to be more precise, we have changed these terms throughout the manuscript (pages 1, 3, 4 and 6)

In general I found the figures a little confusing. Please can more labels be put on the figures e.g. Fig 3D, Fig 6B to show what each lane corresponds to without having to look at the legend. It might also be helpful to emphasise what cell types were used for experiments and that the 293T cell experiments are transient.
Answer:
We have added more labels to facilitate the comprehension.

Referee #3

This is a very interesting paper that provides important advances in our understanding of the mechanism(s) of action of PLZF. The model proposes that PLZF is involved in the control of transcription of repeated DNA elements, and that this control is modulated by cellular stress. This fascinating model leads to the suggestion that in those tumors where PLZF activity is deregulated (such as PLZF-RAR leukemias) it will be very interesting to look at the regulation of L1 retrotransposons.

Major point:

• The Authors show that in KG1a cells, PLZF relocation following stress causes deregulation of L1 transcription. It would be of tremendous interest to look at the stress response in murine cells derived from the knock-in models, and check whether the hypothetical deregulation of L1 sequences could result in an altered biological response.
Answer:
We could not easily perform such an experiments in mouse cells. However, two novel experiments have been added which we believe answers this question:
First: We used HEK293 cells which do not express PLZF and studied the level of L1 retrotransposition in response to stress (Heat Shock) in the presence or absence of PLZF.
a) The retrotransposition assay was performed using the L1-EGFP reporter plasmid EF06R (described in Farkash *et al.* 2006). As shown in figure 6D1, the expression of $PLZF^{WT}$ and $PLZF^{ON}$ greatly decreased the spontaneous L1 retrotransposition in the HEK293 cells.
b) Retrotransposition was also tested under cellular stress conditions (heat shock). Under heat shock conditions, $PLZF^{WT}$ expression could no longer reduce L1 retrotransposition. (Fig6D2).

Second: We have also assessed the spontaneous apoptosis in spermatogonia to correlate with the phenotype found in the PLZF knock-out mouse characterized with increase apoptosis (Costoya *et al.* 2006). These experiments show that increased apoptosis is purified testis cells is only noted in the $PLZF^{OFF}$ model, recapitulating the result found in the PLZF knock-out model (see supplement figure 1 and manuscript page2).

- I do not necessarily agree that the altered proliferative potential observed in the serial replating assays by the mutated PLZF constructs would mirror the response of the same cells upon X-rays treatment, or treatment with drugs.

Answer:
We agree that this sentence was an overstatement as these conditions have not yet been tested. The sentence has been removed.

Specific points:
• Missing reference: Martin et al., 2005;
Answer:
This has been corrected and we apologize for the omission.
• Legend to figure 1C: the authors should better explain the quantitation;
Answer:
The quantitation has now been explained
• Figure 1D: the analysis is done on whole bone marrow, is there any effect of the PLZF transgenes on the distribution of the hematopoietic subpopulations?
Answer:
We have looked at different colony subpopulations obtained from the bone marrow of the mutant mice and found no effect of the expression of PLZF mutants on the frequency of the various

populations. However, these results need to be performed in detailed on different bone marrow and testis purified cell populations. New experiments will be performed in the laboratory and in collaboration, on newly bred mice to understand the full impact of PLZF on its target tissues (Bone marrow and Testis).

• Figure S1: I would prefer to see the absolute number of colonies.
Answer:
We have provided a figure with absolute number of colonies

2nd Editorial Decision 22 April 2013

Thank you for submitting your revised manuscript for our consideration. I am happy to inform you that in light of the comments from the original referees (provided below), we are ready to proceed with acceptance of the paper, pending modification of a few additional points.

- Both referees suggest several textual changes and clarifications that should be implicated.

- Please be sure to include information regarding the number of biological replicates and the statistical tests used to create error bars in all Figures.

- Please add an author contribution statement.

- Additionally, we encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Therefore, I would like to invite you to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.

- Finally, please complete and sign the linked license agreements (see below).

I will now return your manuscript to you for one additional round of minor revision. After that we should be able to swiftly proceed with formal acceptance and production of the manuscript!

If you have any questions, please do not hesitate to contact me directly.

REFEREE COMMENTS

Referee #1

This is an interesting paper that is improved in revision. The PLZF protein is shon to bind to a region of L1 ORF2 sequence and produce spreading of inhibitory chromatin marks such as methylation and deacetylation. My comments are relatively minor.

1) First, the references still need some work. On pg. 1 at the end of the first paragraph,Hancks eto al. Human Molecular Genetics 2011 is appropriat instead of Hancks 2012 and Raiz et al. NAR 2012 should be cited also for SVA retrotransposition in culture. On pag. 5, line 11, add Ostertag et al. NAR 2001 to Farkash et al. In the first paragraph of Discussion, Edwing is Ewing. Kroutter et al. is inappropriate. It should be Dewanieux et al. 2003, Hancks et al is the HMG 2011 ref. Raiz et al. should also be cited here. At the bottom of that page, Rangwala et al should be deleted. There is nothing in that paper on L1 expression reducing gene expression.
2) Check for errors in the text. Close chromatin in many places should be closed chromatin. In the middle of pg. 3 the mutations in the figure are T/A to C not G. The figure cited should be 2D and not 2E. A few lines above on pg. 3, PLZF binds to other non-LTR retrotransposons such as

SiNE/Alu. Is there binding to SVA also? Are the sites authentic PLZF binding sites? This is unclear. Bottom of pg. 2 the role of L1 in gene expression and oncogenesis-A role in oncogenesis has not been proven in any study. Please delete.
3) In the retrotransposition assay, please state the number of replicates. How many assays were carried out?
4) Realizing there are supplemental methods, it would be good to describe the qPCRs (primer sites, etc.) in the methods. Also the method for the retrotransposition assay should be in the standard methods.

The authors should state explicitly that the PLZF binding site is present in the ORF2 sequence of young, presently active mouse L1s (TF, GF, A families). I presume this is the case.

Referee #2

I am satisfied with teh changes in the MS and the additional experiments. My only outstanding concern is using the phrase "close chromatin" or "close chromatin state". The authors are not using any form of structure assay. I think the authors need to be explicit e.g.
Page 3, para 2. "A change in the acetylation and methylation of L1 chromatin by PLZF is correlated with the regulation of L1 expression"
start of discussion: "DNA binding activity of PLZF alters the local chromatin structure repressing the transcription of L1 retrotransposons"
discussion paragraph 2 "Thus, distal PLZF-induces deacetylation and DNA methylation at the L1 5' UTR and the specific recruitment of methyl binding proteins."

