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The PEAT protein complexes are required for histone deacetylation and heterochromatin silencing

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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.)

1st Editorial Decision 15th January 2018

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

As you will see from the reports, our referees all express interest in the findings reported in your manuscript, although they also raise a number of issues that would have to be clarified before they can support publication in The EMBO Journal. Most importantly, you will see that more data is needed on the nature and stoichiometry of the PEAT complex, its biochemical and biological activity and the directness of its interaction with chromatin. In addition, ref #1 and #2 both question the conclusiveness of several of the assays presented and ask for the inclusion of a number of additional controls as well as more biological replicas. Finally, the referees also point out that the manuscript text/structure will need extensive revision to better reflect the current literature, present a more unbiased analysis of the observed effects (especially regarding the phenotypes seen) and discuss the mechanistic/epistatic role for PEAT in the context of other known silencing complexes.

Should you be able to address these criticisms in full, we could consider a revised 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 in this revised version. I do realize that addressing all the referees' criticisms will require a lot of additional time and effort and be technically challenging. I would therefore understand if you wish to publish the manuscript rapidly and without any significant changes elsewhere, in which case please let us know so we can withdraw it from our system.

REFEREE REPORTS.

Referee #1:

Tan et al. describe the discovery of a new protein complex involved in transcriptional gene silencing. This is an important finding, given that forward genetic approaches seem to have been exhausted in studies of plant silencing mechanisms. The discovery of EPCR1 in an extensive reverse genetics study is followed by identification is its interacting partners, which together form the PEAT complex, which is required for histone deacethylation and proper development. The first half of the manuscript is not only interesting but also of high technical quality. The second half of the manuscript describes the importance of the PEAT complex for TE silencing, small RNA biogenesis and DNA methylation. It offers several interesting insights and somehow narrows down on the possible role of PEAT. However, it does not clearly place PEAT in context of known silencing pathways and is less developed and conclusive than the first half.

It is overall an interesting manuscript. While substantial shortcomings do exist, most of them should be possible to address.

Specific comments
1. The current version of the manuscript does not fully convince if the effects of mutations in PEAT subunits are direct. It is only partially addressed in the last paragraph of the results. The manuscript would be much more compelling if the presence of PEAT on its target loci could be shown by ChIP. Furthermore, it would be even better to show that binding of HDA6/9 and HAM1/2 requires the presence of PEAT. With multiple subunits to test, epitope-tagged lines available and the authors having the expertise, this may be doable.
2. Complementation shown on Fig. 1D seems to be only partial. A qPCR assay would be more quantitative and convincing.
3. Yeast two hybrid assay mentioned in line 193 is not shown. It should be included in the supplement.
4. In Fig 3C, soloLTR in arid234 is truncated. The plot should show all data points.
5. The paragraph showing knock down of TRB2 is less convincing than the rest of the manuscript and its relevance for the entire story is not well explained.
6. Stability of silencing in seedlings development does not mean much and is overinterpreted as evidence of PEAT being independent of development.
7. RNA-seq seems to have been performed in only one biological replicate. Given the well-established limitations of this method, three biological replicates are required.
8. All overlaps shown on Venn diagrams should be accompanied by statistical tests showing significance of the overlaps. Also, all Venn diagrams will be much more useful if their areas are proportional to the shown values.
9. Whole genome bisulfite sequencing is briefly mentioned on page 11 but is rather confusing and out of place there.
10. Results showing hyperacethylation in PEAT subunit mutants are important and may need to be expanded to be more convincing. An additional boxplot would make the profile in Fig. 6B easier to interpret. Also, presence of hyperacethylation is not discussed. It should be included in the analysis. Microscopy shown in Fig. 6C is not convincing and the categories appear arbitrary with the observation bias not excluded.
11. Effects of PEAT on sRNA shown in Fig. 6E would be more convincing if supported by a more quantitative analysis with appropriate statistics. As shown, they do not convince that the differences are significant and relevant.
12. Overlaps of the effects on PEAT mutations and hda6 are not interpreted in mechanistic categories. These are important results as HDA6 is proposed to be associated with PEAT. Even if the obtained insights are to some extent inconsistent, it should be better explained.
13. rDNA IGS transcription (Fig. 6H) should be studied directly by Pol II ChIP to claim effects on Pol II transcription. If not feasible, this result may be eliminated from the manuscript.
14. Effects of PEAT on DNA methylation shown in Fig 7A are small and possibly not significant. More quantitative analysis would be required to establish significance. Effects on pericentromeric regions shown in Fig 7B may be difficult to interpret given the highly repetitive nature of those regions.

15. Results shown in Fig 7C are very important as they justify one of the most important mechanistic insights into the function of PEAT - its role independent of DNA methylation. The number of tested genes and TEs is quite limited. This analysis may need to be significantly expanded to be more convincing.

Referee #2:

In this data-intensive, difficult-to-digest manuscript, Tan et al. begin with a reverse genetic screen that identified EPCR1 and EPCR2 (enhancer of polycomb-related proteins 1 and 2) as proteins that mediate transcriptional gene silencing of a solo-LTR element and several other well-known targets of RNA-directed DNA methylation (RdDM). One of these targets, SDC, is silenced by CHG DNA maintenance methylation and H3K9me2 histone methylation, in addition to RdDM, and the Jacobsen lab was the first to show that disruption of both pathways is needed for silencing to be lost. This fact is not discussed by the authors, but is an important clue, as it suggests that the EPCR proteins are either required for both the CHG maintenance and RdDM DNA methylation pathways or act in some way shared by both pathways, perhaps downstream of DNA methylation. Tests of DNA methylation patterns, shown later in the manuscript (Figure 7), show little effect of the mutations on DNA methylation, supporting the latter conclusion.
Based on mass spectroscopic assays of EPCR1/2 interacting proteins, the authors identified several paralogous PWWP-containing proteins (PWWP1, 2 and 3), paralogous AT-rich interaction domain-containing proteins (named ARID2, 3 and 4), and paralogous telomere repeat binding proteins (TRB1 and 2). None of these protein families are properly introduced in the (poorly written) Introduction section of the manuscript; one must wait until the Discussion to learn about these proteins and their history.
Genetic analyses indicate that the different EPCR, ARID and PWWP paralogs are functionally redundant, such that multiple paralogs must be knocked out to observe phenotypes. The phenotypes upon knocking out EPCR and ARID activities is severe, resulting in seeds that germinate but then arrest in development and never produce true leaves or elongate their roots, suggesting defects in stem cell maintenance. This is not a phenotype observed for any other known chromatin modifying activities, suggesting that gene-specific, rather than global effects, are responsible. The severe developmental phenotype is striking and interesting, and exploring the basis for the phenotype would have been a logical focus for the remainder of the paper. But the authors do not go this route. Instead, they ignore the developmental arrest phenotype to focus on the loss of silencing of heterochromatic elements in the dwarfed and developmentally arrested mutant seedlings. But one cannot help but question whether the loss of transposon/gene silencing is simply a genomic stress response due to a block in the normal developmental program. The authors are aware of this and do one (flawed) experiment to test it: they compare a time-course of wild-type seedlings to a single timepoint (10-day) for arrested mutant seedlings, assaying for loss of transposon silencing (Supplemental figure 5). But they do not show the same time course for the mutants, which would determine if the loss of silencing is apparent from day one of seedling germination, prior to the developmental arrest, or if it only occurs upon arrest. As such, little can be concluded from the experiment.
Based on pairwise co-IP and mass spec analyses of IPed target proteins, representing an impressive amount of work, the authors suggest that the PWWP, EPCR, ARID, and TRBs proteins associate to form a so-called PEAT complex, named for the firts letters of the component proteins. Proteins of the putative PEAT complex also interact with at least two histone deacetylases (HDACs, HDA6 and HDA9) and two histone acetyltransferases (HATs, HAM1 and HAM2). Finding HATs and HDAcs together in the same complex is surprising given that these chromatin modifiers tend to have opposite effects on gene expression. At one point in the manuscript, the authors propose that HAT activity may be promoted by the PEAT complex, as needed for gene activation, and this seems plausible, especially given that ROS1 and IBM expression are greatly reduced in arid and epcr mutants (Supp. fig 6), and without any significant effects on DNA methylation, which is needed for ROS1 expression. It is noteworthy that a recent paper showed that condensin smc4 mutants show increased ROS1 expression without any significant change in DNA methylation. This suggests that condensin is involved in repressing ROS1 expression levels, independent of DNA methylation. The epcr and arid mutants have the opposite effect, suggesting that they might be needed for ROS1 activation. However, throughout the paper, and in the model of Figure 7E, the PEAT complex is proposed to promote histone deacetylation and inhibit histone acetylation to bring about transcriptional silencing. This creates confusion, at least for this reviewer, and the sense that the

authors are not thinking objectively about their data.
Despite an impressive amount of molecular biology, genetic and genomic assays, a major shortcoming of the study is that there are no biochemical experiments to test for HAT or HDAC activity. There are also no tests for telomere shortening, as expected based on the finding of TRB proteins in the putative PEAT complex. And there are no biochemical experiments, such as gel filtration chromatography coupled with immunoblotting (or mass spec analysis of single fractions), to determine if there is a single PEAT complex or multiple sub-complexes that share some, but not all subunits. The fact that there are multiple paralogs of the PWWP, EPCR, ARID, and TRB proteins makes the existence of multiple complexes highly plausible, unless all of the paralogs are all together in one massive, multiply redundant complex. That seems unlikely. Moreover, a number of the indirect associations deduced in Figure 2G are not supported for all paralogs by the co-IP tests of Figures 2E (TRB1 is not co-IPed with ARID2) and 2F (EPCR1 is not co-IPed with either ARID2 or TRB1). Different sub-complexes may have distinct activities, and perhaps some mediate HAT activity. As the authors point out in their Discussion, PWWP domains are present in H3K4 methyltransferases, which is associated with transcriptional activation. Other sub-complexes may mediate HDAC activity. Inclusion of biochemical tests of partially purified complexes would likely sort this out to cut through the confusion, and would not be difficult to perform.

Specific points:
1. The introduction is currently a laundry list of facts concerning various proteins that do this or that, but without discussing what they have to do with one another. Also, the parts of the Discussion explaining the PWWP, EPCR, ARID, and TRB proteins, and how they get their names, belongs in the Introduction.
2. With respect to statements about the involvement of RdDM in chromatin condensation, Pontes and colleagues showed that the Pol V part of the pathway affects chromatin condensation, but not the Pol IV part of the pathway.
3. With respect to statements about yeast two-hybrid interactions, the authors should not state that interactions indicate that proteins homodimerize or heterodimerize- for all they know they could form hexamers or octamers.
4. For immunostaining, what is the specificity of the H3K27me antibody? Mono, di, tri-methylation?
5. In Figure 1D, the silencing of EPCR1/2 target loci was not fully restored by the EPCR1 transgene in the epcr1/2 mutant, which deserves some comment. Also, what are the two lanes shown for the rescue? Two sibs?
6. In Figure 2A and 2C, multiple protein bands of tagged EPCR1 were detected. Which band is the correct EPCR1? Does EPCR1 contain post-translational modifications?
7. Protein masses should be indicated in all of the figures.
8. There are no units on the Y axis of RNA-seq or DNA methylation profile figures.

Referee #3:

Tan et al. EMBOJ-2017-98770
«The PEAT 1 protein complex is required for histone deacetylation and heterochromatin silencing»
Tan et al report the identification of the PEAT protein complex, a multi-subunit protein complex required for heterochromatin silencing in Arabidopsis. This complex was initially revealed through the identification of its EPCR components, two proteins whose mutations affected transcriptional gene silencing at the soloLTR locus. Recently, several other mutations in TGS components have been reported by this team and others using similar screens, and the experiments presented in this manuscript follow the same experimental setup. In co-immunoprecipitation assays, ERPC were shown to interact with several other proteins proposed to be integral components or associated partners of the PEAT complex, including several PWWP and AT-rich interaction domain-containing (ARID) proteins, telomere repeat binding proteins (TRBs), histone acetyltransferases and deacetylases proteins. To test the role of PEAT, the authors characterize and further analyze several KO mutants in these genes and show that the PEAT complex mediates histone deacetylation, chromatin condensation and TGS. Genome-wide analysis of the epcr, arid and hda6 mutants revealed only a marginal impact on DNA methylation and small RNA accumulation at RdDM targets, while substantial transcriptional activation could be observed at regulated loci, suggesting that the PEAT complex represses transcription via histone deacetylation and heterochromatin

condensation.
The paper is interesting and addresses problems that are important to the fields of epigenetics, RdDM, and chromatin biology. The experimental work is well carried out, and the conclusions drawn by the authors are supported by the data. Although there are still many questions remaining about the exact mechanism of action of the PEAT complex in heterochromatin formation, this work improves our understanding of the role of several chromatin-related proteins in TGS and would be appreciated by the readers of the EMBO Journal. However, the specific minor points listed below have to be addressed by the authors.

1) Whereas co-IP and two-hybrid experiments are consistent with the idea that the ERPC, ARID and TRB proteins form a complex in vivo, the authors should provide gel filtration experiments to further support this conclusion.
2) To attest to the role of the PEAT complex in controlling the activity of HDA6 in vivo, the authors should provide an epistatic analysis comparing H4K5Ac levels of TEs in epcr1/2 versus epcr12/hda6 triple mutant.
3) What is the condensation status of nuclear bodies in the epcr1/2 double mutant (see figure 6C)?
4) It would be nice to provide WT and mutant plots on figure 6E instead of ratios.
5) The zoom in of the figure 7D indicates that the last two lanes have been sliced from another gel and therefore missed internal controls. The authors should provide the appropriate controls or repeat the experiment with all samples in the same gel.

1st Revision - authors' response 29th April 2018

Referee #1:
Tan et al. describe the discovery of a new protein complex involved in transcriptional gene silencing. This is an important finding, given that forward genetic approaches seem to have been exhausted in studies of plant silencing mechanisms. The discovery of EPCR1 in an extensive reverse genetics study is followed by identification is its interacting partners, which together form the PEAT complex, which is required for histone deacethylation and proper development. The first half of the manuscript is not only interesting but also of high technical quality. The second half of the manuscript describes the importance of the PEAT complex for TE silencing, small RNA biogenesis and DNA methylation. It offers several interesting insights and somehow narrows down on the possible role of PEAT. However, it does not clearly place PEAT in context of known silencing pathways and is less developed and conclusive than the first half.

It is overall an interesting manuscript. While substantial shortcomings do exist, most of them should be possible to address.

Response: We appreciate that the reviewer finds the importance of our work. We have improved our data accoding to the reviewer's suggestion point by point in the revised manuscript.

Specific comments
1. The current version of the manuscript does not fully convince if the effects of mutations in PEAT subunits are direct. It is only partially addressed in the last paragraph of the results. The manuscript would be much more compelling if the presence of PEAT on its target loci could be shown by ChIP. Furthermore, it would be even better to show that binding of HDA6/9 and HAM1/2 requires the presence of PEAT. With multiple subunits to test, epitope-tagged lines available and the authors having the expertise, this may be doable.

Response: We agree with the reviewer that it is important to determine whether the PEAT complexes directly mediate heterochromatin silencing. To address the concern, we used the well-expressed *ARID2-Flag*, *EPCR1-Flag*, and *TRB1-Flag* transgenic plants to determine whether the PEAT subunits are enriched on their target loci as determined by ChIP. Our result showed that all the three Flag-tagged proteins are enriched on *solo LTR*, *AtGP1*, *AtCOPIA28*, and *AT1TE42205* (Appendix Fig S5). The enrichment of the PEAT subunits on these loci supports the notion that the PEAT complexes directly mediate heterochromatin silencing. We did try to carry out ChIP assays for HDA6 and HAM1. However, although we were capable of detecting enrichment for several chromatin-related proteins, we failed to detect the enrichment of HDA6 and HAM1 on chromatin. HDA6 was primarily reported to be involved in heterochromatin silencing for more than a decade (Murfett et al., Plant Cell, 2001; Aufsatz et al., EMBO J., 2002). Thereafter, several excellent HDA6 related works have been published (Earley et al., Genes Dev., 2006, 2010; Blevins et al., Mol Cell, 2014). However, these studies also did not report the binding of HDA6 to heterochromatin regions even though the binding result is also important for their studies. Thus, due to technical limitation, we cannot provide data to show whether the PEAT complexes

affect the binding of HDA6 to chromatin at present. In this study, we have demonstrated the involvement of the newly characterized multi-subunit PEAT complexes in heterochromatin silencing and reported the function of the complex in histone deacetylation and heterochromatin condensation, which represent a significant progress in the epigenetic field.

2. Complementation shown on Fig. 1D seems to be only partial. A qPCR assay would be more quantitative and convincing.

Response: According to the reviewer's suggestion, we have evaluated the transcript levels of *solo LTR*, *SDC*, *AtSN1*, *AtGP1*, and *AtCOPIA28* by quantitative PCR (Fig 1C). The results showed that the transcript levels of these loci were weakly induced in the *epcr1* and *epcr2* single mutants; the induction was markedly enhanced in the *epcr1/2* double mutant. The *EPCR1* transgene in the *epcr1/2* double mutant can significantly restore the silencing of these loci even though the silencing is not completely restored to the wild type level. These results confirm that EPCR1 is involved in transcriptional silencing. There are two reasons that the *EPCR1* transgene partially complements transcriptional silencing in the *epcr1/2* double mutant. First, *EPCR2* is still defective in the *EPCR1* transgenic lines in the *epcr1/2* double mutant background. Second, the tag of the *EPCR1* transgene may partially affect its function in heterochromatin silencing.

3. Yeast two hybrid assay mentioned in line 193 is not shown. It should be included in the supplement.

Response: As suggested, the primary data of the yeast two-hybrid assay have now been shown in Appendix Fig S2. The data are summarized in Fig 2D.

4. In Fig 3C, soloLTR in arid234 is truncated. The plot should show all data points.

Response: We have shown all data points according to the suggestion.

5. The paragraph showing knock down of TRB2 is less convincing than the rest of the manuscript and its relevance for the entire story is not well explained.

Response: We agree with the reviewer that our genetic evidence for the function of TRB2 in transcriptional silencing is a little weak. This is due to the fact that, unlike the mutants of PWWPs, EPLs, and ARIDs, the *trb2* mutant is lethal. Thus, we have to generate *TRB2* knock down lines to evaluate the function of TRB2 in transcriptional silencing. Fortunately, we did identify *TRB2* knock down lines and demonstrate that the knock down of *TRB2* affects transcriptional silencing. Moreover, using mass spectrometry (Table 1), co-IP (Fig 2B and C), yeast two hybrid (Fig 2D), and pull down assays (Fig 2E and F), we infer that TRBs form complexes with PWWPs, ARIDs and EPCRs. We have explained the relevance of TRB1/2 for the entire story in the revised manuscript. Further, in the revised manuscript, we add our gel filtration data for these proteins and demonstrated that these proteins form a large molecular weight complex in Arabidopsis (Fig EV2).

6. Stability of silencing in seedlings development does not mean much and is overinterpreted as evidence of PEAT being independent of development.

Response: As suggested, we have revised the statement. Although previous studies reported that heterochromatin condensation and transcriptional silencing of rRNA genes go through transitions in early seedling development (Mathieu et al. 2003 Plant Cell, Earley et al. 2010 G&D), our result suggests that the release of silencing in the mutants of the PEAT complexes are not directly caused by the halted development of these mutants.

7. RNA-seq seems to have been performed in only one biological replicate. Given the well-established limitations of this method, three biological replicates are required.

Response: As suggested, we have performed three biological replicates for our RNA-seq experiment. The results from all the three biological replicates support the effect of *arid2/3/4* and *epcr1/2* on TE and gene expression as previously described in our study. We analyzed the results from the three biological replicates and showed the combined results in the revised Fig 4A-C and Supplemental Appendix Table S2.

8. All overlaps shown on Venn diagrams should be accompanied by statistical tests showing significance of the overlaps. Also, all Venn diagrams will be much more useful if their areas are proportional to the shown values.

Response: As suggested, we performed statistical analyses for the significance of the overlaps and showed statistical test for significance of the overlaps in the figure legends. Moreover, in the revised Venn diagrams, the areas have now been proportional to the shown values (Fig 4A, 6A, 6G).

9. Whole genome bisulfite sequencing is briefly mentioned on page 11 but is rather confusing and out of place there.

Response: We appreciate the comments raised by the reviewer. We have now moved the related text to the last section of the results part. In this section, the whole-genome DNA methylation data were described.

10. Results showing hyperacethylation in PEAT subunit mutants are important and may need to be expanded to be more convincing. An additional boxplot would make the profile in Fig. 6B easier to interpret. Also, presence of hyperacethylation is not discussed. It should be included in the analysis. Microscopy shown in Fig. 6C is not convincing and the categories appear arbitrary with the observation bias not excluded.

Response: As suggested, we have calculated the H4K5Ac levels of TEs in the mutants and the wild type and shown the results by boxplots (Fig 6C). The hyperacetylation is discussed in the revised manuscript. The hyperacetylation of TEs is consistent with the release of TE silencing in the *arid2/3/4* and *epcr1/2* mutants, suggesting that the PEAT components are required for histone deacetylation of TEs. Considering the interaction of the PEAT components with the deacetylases HDA6 and HDA9, we predict that the PEAT complexes may mediate histone deacetylation of TEs by facilitating the function of the histone deacetylases.

We carried out the experiment shown in Fig 6D （6C in the previous version） with the same method as previously reported (Moissiard et al. Science, 2012; Zhou et al. Plant Cell, 2013). In this study, the experiment was independently carried out by different researchers and similar results were obtained, confirming that the result shown in Fig 6D is reliable. To further confirm the result, we used a different assay to determine whether the condensation of heterochromatin is affect by the *arid2/3/4* mutation. In this assay, we counted the number of condensed foci in nuclei. We found that the number of condensed foci is significantly decreased in the *arid2/3/4* mutant relative to the wild type (Appendix Fig S8). This result was added in the revised manuscript.

11. Effects of PEAT on sRNA shown in Fig. 6E would be more convincing if supported by a more quantitative analysis with appropriate statistics. As shown, they do not convince that the differences are significant and relevant.

Response: As suggested, we analyzed the effect of the PEAT mutations on sRNA by a statistical analysis. The Fig 6F (6E in the previous version) indicated that siRNAs are significantly increased in pericentromeric heterochromatin regions but not in chromosome arms. To confirm the finding, we identified siRNA regions in which siRNAs are increased in the *arid2/3/4*, *epcr1/2*, and *hda6* mutants relative to the wild type (Fig 6G). Our boxplot analysis indicated that, in the siRNA regions that produced more Pol IV-dependent siRNAs in the *hda6* mutant, siRNAs are significantly up-regulated not only in the *hda6* mutant but also in the *arid2/3/4* and *epcr1/2* mutants (Fig 6H). Further, we analyzed the chromosome locations of the siRNA regions that produced more Pol IV-dependent siRNAs in the *arid2/3/4*, *epcr1/2*, and *hda6* mutants than in the wild type, indicating that most of these siRNA regions (>95%) are present in pericentromeric heterochromatin regions (Fig 6I; Fig EV4A). These results demonstrate that the PEAT complexes and HDA6 commonly repress the production of Pol IV-dependent siRNAs in pericentromeric heterochromatin regions.

12. Overlaps of the effects on PEAT mutations and hda6 are not interpreted in mechanistic categories. These are important results as HDA6 is proposed to be associated with PEAT. Even if the obtained insights are to some extent inconsistent, it should be better explained.

Response: Our study indicated that the PEAT complexes regulatePol IV-dependent siRNA production and RdDM in a similar manner with HDA6. We propose that, in the PEAT mutants, the increased DNA methylation in pericentromeric regions is mediated by the increased production of Pol IV-dependent siRNAs through the RdDM pathway. However, in the *hda6* mutant, DNA methylation was decreased in heterochromatin regions, while the production of Pol IV-dependent siRNAs from heterochromatin regions was increased (Fig 6F; Appendix Fig S9 and 12). HDA6 was known to interact with the DNA

methyltransferase MET1 and thereby facilitate maintenance of normal DNA methylation levels (Liu et al., 2012; To et al., 2011). We predict that, although the increased production of Pol IV-dependent siRNAs in the *hda6* mutant are also known to mediate DNA methylation through the RdDM pathway (Earley et al., 2010), it is not enough to compensate for the defect in the maintenance of DNA methylation.

13. rDNA IGS transcription (Fig. 6H) should be studied directly by Pol II ChIP to claim effects on Pol II transcription. If not feasible, this result may be eliminated from the manuscript.

Response: As suggested, we removed the figure from our revised manuscript.

14. Effects of PEAT on DNA methylation shown in Fig 7A are small and possibly not significant. More quantitative analysis would be required to establish significance. Effects on pericentromeric regions shown in Fig 7B may be difficult to interpret given the highly repetitive nature of those regions.

Response: In Fig 7A, the metaplot showed that DNA methylation at promoters of genes is slightly decreased in the PEAT mutants especially at CHH sites. DNA methylation at promoters of genes was known to be established by RdDM in Arabidopsis (Stroud et al., 2013). We therefore tested whether the *epcr1/2* and *arid2/3/4* mutations affect DNA methylation at RdDM target loci. The hypo DNA methylated regions (hypo-DMRs) identified in the Pol V mutant *nrpe1* was defined as RdDM target loci. Our boxplot analysis indicated that the DNA methylation levels were significantly decreased in both *epcr1/2* and *arid2/3/4* even though the decrease was much weaker in the *epcr1/2* and *arid2/3/4* than in *nrpe1* (Fig 7D). Further, heat maps showed that DNA methylation was decreased at CHH sites and to a lesser extent at CG and CHG sites at a subset of RdDM target loci (Fig 7E), suggesting that the PEAT complexes are involved in DNA methylation at a subset of RdDM target loci.

Although pericentromeric regions are highly repetitive, our whole-genome DNA methylation analysis assessed DNA methylation of regions only when those regions can be uniquely mapped on the genome. Thus, the effect of the PEAT mutants on DNA methylation at pericentromeric regions is convincing. To further confirm the result, we identified CG, CHG, and CHH hyper-DMRs in the *epcr1/2* and *arid2/3/4* mutants and determined whether these hyper-DMRs are enriched in pericentromeric regions. We found that CHG and CHH hyper-DMRs but not CG hyper-DMRs are enriched in pericentromeric heterochromatin regions (Fig 7C; Appendix Table S5), which is consistent with the observation that CHG and CHH sites are hypermethylated in pericentromeric regions in the *epcr1/2* and *arid2/3/4* mutants.

15. Results shown in Fig 7C are very important as they justify one of the most important mechanistic insights into the function of PEAT - its role independent of DNA methylation. The number of tested genes and TEs is quite limited. This analysis may need to be significantly expanded to be more convincing.

Response: We appreciate the reviewer's comments. Since our study identified both up- and down-regulated TEs and genes, we should test whether or not the effect of the PEAT mutations on transcription is independent of alteration of DNA methylation at both up- and down-regulated TEs and genes. The analysis indicated that, at both up- and down-regulated genes and TEs, DNA methylation is comparable between the PEAT mutants and the wild type except that CHH methylation in promoters of genes is decreased in the PEAT mutants (Fig 8A; Appendix Fig S13A). To confirm the results, we analyzed the DNA methylation levels of these loci by box plots and performed statistical analysis to determine whether the DNA methylation changes between the wild type and the mutants are significant. The result indicated that the DNA methylation levels of these loci are not significantly different between the wild type and the PEAT mutants except that CHH methylation in promoters of genes is decreased in the PEAT mutants (Fig 8B; Appendix Fig S13B).

Referee #2:

In this data-intensive, difficult-to-digest manuscript, Tan et al. begin with a reverse genetic screen that identified EPCR1 and EPCR2 (enhancer of polycomb-related proteins 1 and 2) as proteins that mediate

transcriptional gene silencing of a solo-LTR element and several other well-known targets of RNA-directed DNA methylation (RdDM). One of these targets, SDC, is silenced by CHG DNA maintenance methylation and H3K9me2 histone methylation, in addition to RdDM, and the Jacobsen lab was the first to show that disruption of both pathways is needed for silencing to be lost. This fact is not discussed by the authors, but is an important clue, as it suggests that the EPCR proteins are either required for both the CHG maintenance and RdDM DNA methylation pathways or act in some way shared by both pathways, perhaps downstream of DNA methylation. Tests of DNA methylation patterns, shown later in the manuscript (Figure 7), show little effect of the mutations on DNA methylation, supporting the latter conclusion.

Response: As suggested, we cited the *SDC*-related work from the Jacobsen lab and discussed the function of EPCR proteins in the regulation of *SDC* in the revised manuscript. Our results demonstrated that the mutants of the PEAT components identified in this study released the silencing of *SDC* and other loci that are silenced by DNA methylation and H3K9me2, thus identifying an previously uncharacterized complexes that are required for transcriptional silencing. This study started from a reverse genetic screening and identified a new silencing regulator. Subsequently, we demonstrated that this regulator functions redundantly with its paralogs and forms complexes with three other types of proteins, including PWWPs, ARIDs, and TRBs. We carried out genetic, molecular, and genomic methods to examine the function of the complexes, and demonstrated that the complexes regulate histone deacetylation and heterochromatin condensation. The large amount of data may make the manuscript a little difficult to digest. According to the reviewer's suggestion, we have point by point revised our manuscript. We believe that the results shown in this manuscript will significantly contribute to understanding the function of the newly identified complex.

Based on mass spectroscopic assays of EPCR1/2 interacting proteins, the authors identified several paralogous PWWP-containing proteins (PWWP1, 2 and 3), paralogous AT-rich interaction domain-containing proteins (named ARID2, 3 and 4), and paralogous telomere repeat binding proteins (TRB1 and 2). None of these protein families are properly introduced in the (poorly written) Introduction section of the manuscript; one must wait until the Discussion to learn about these proteins and their history.

Response: Our study initially identified EPCR1 and EPCR2 as silencing regulators by a reverse genetic screening. Further, by affinity purification in combination with mass spectrometric assays, we identified three types of paralogs: PWWP-containing proteins (PWWP1, 2, 3), AT-rich interaction domain-containing proteins (ARID2, 3 and 4), and telomere repeat binding proteins (TRB1 and 2). Except TRB1 and TRB2, PWWP-containing proteins and AT-rich domain-containing proteins were poorly studied in plants. This study aims to understand molecular mechanisms of DNA methylation and/or heterochromatin silencing. To help reader understand our study, we briefly introduced background knowledges in the field. However, none of the PEAT components identified in this study were previously reported to be involved in DNA methylation and heterochromatin silencing in plants. In the introduction part, we introduced the identification of the PEAT components and indicated how these proteins were named in this study. Moreover, we have rewritten the introduction according to the reviewer's suggestion.

Genetic analyses indicate that the different EPCR, ARID and PWWP paralogs are functionally redundant, such that multiple paralogs must be knocked out to observe phenotypes. The phenotypes upon knocking out EPCR and ARID activities is severe, resulting in seeds that germinate but then arrest in development and never produce true leaves or elongate their roots, suggesting defects in stem cell maintenance. This is not a phenotype observed for any other known chromatin modifying activities, suggesting that gene-specific, rather than global effects, are responsible. The severe developmental phenotype is striking and interesting, and exploring the basis for the phenotype would have been a logical focus for the remainder of the paper. But the authors do not go this route. Instead, they ignore the developmental arrest phenotype to focus on the loss of silencing of heterochromatic elements in the dwarfed and developmentally arrested mutant seedlings.

Response: We are pleased to know that the reviewer recognizes that the severe developmental phenotypes observed in *arid* and *epcr* mutants are striking and interesting. As stated by the reviewer, the phenotype was not reported for any other known chromatin modifying activities. Since the project initially aimed to identify and characterize new silencing regulators, this study focuses on the functions of these proteins in heterochromatin silencing. The results in this study indeed indicated that the PEAT complexes not only affect the development phenotype but also have significant effect on heterochromatin silencing. Although many regulators of development were identified in previous studies, few of those regulators were involved in heterochromatin silencing at the whole-genome level. Exploring the molecular mechanism of heterochromatin silencing is a significant topic in the epigenetic field. Thus, we focused our study on how the PEAT complexes are involved in heterochromatin silencing. Our study

does not deny the importance of the PEAT complexes in development. We agree with the reviewer that it is important to explore the basis for the development phenotype. Our RNA-seq data indicated that many genes involved in shoot apical meristem formation and maintenance, including *STM*, *CUC1*, *CUC2*, *KNAT1*, *KNAT2*, and *KNAT6*, are significantly increased in the *arid2/3/4* and *epcr1/2* mutants (Appendix Table S2). In the revised manuscript, the effect of *arid2/3/4* and *epcr1/2* on the expression of these genes was confirmed by qPCR at different developmental stages (Appendix Fig S3B and S4), suggesting that the developmental arrest of the *epcr1/2* and *arid2/3/4* mutants may be caused by the aberrant expression of these genes. These results provide valuable evidences for scientists who work on development to further study how these chromatin modifiers regulate shoot apical meristem formation and maintenance and thereby affect plant development.

But one cannot help but question whether the loss of transposon/gene silencing is simply a genomic stress response due to a block in the normal developmental program. The authors are aware of this and do one (flawed) experiment to test it: they compare a time-course of wild-type seedlings to a single timepoint (10-day) for arrested mutant seedlings, assaying for loss of transposon silencing (Supplemental figure 5). But they do not show the same time course for the mutants, which would determine if the loss of silencing is apparent from day one of seedling germination, prior to the developmental arrest, or if it only occurs upon arrest. As such, little can be concluded from the experiment.

Response: To address the concern raised by the reviewer, we carried our qPCR to examine the loss of transcriptional silencing in a time-course (2, 4, 7, 10 days after germination) not only for the wild type but also for the *epcr1/2* and *arid2/3/4* mutants. Our results demonstrated that the loss of silencing was observed in the *epcr1/2* and *arid2/3/4* mutants at all tested developmental stages (Appendix Fig S3B; Fig EV3), suggesting that the loss of silencing is not caused by the development arrest of the *epcr1/2* and *arid2/3/4* mutants.

Based on pairwise co-IP and mass spec analyses of IPed target proteins, representing an impressive amount of work, the authors suggest that the PWWP, EPCR, ARID, and TRBs proteins associate to form a so-called PEAT complex, named for the firts letters of the component proteins. Proteins of the putative PEAT complex also interact with at least two histone deacetylases (HDACs, HDA6 and HDA9) and two histone acetyltransferases (HATs, HAM1 and HAM2). Finding HATs and HDACs together in the same complex is surprising given that these chromatin modifiers tend to have opposite effects on gene expression. At one point in the manuscript, the authors propose that HAT activity may be promoted by the PEAT complex, as needed for gene activation, and this seems plausible, especially given that ROS1 and IBM expression are greatly reduced in arid and epcr mutants (Supp. fig 6), and without any significant effects on DNA methylation, which is needed for ROS1 expression. It is noteworthy that a recent paper showed that condensin smc4 mutants show increased ROS1 expression without any significant change in DNA methylation. This suggests that condensin is involved in repressing ROS1 expression levels, independent of DNA methylation. The epcr and arid mutants have the opposite effect, suggesting that they might be needed for ROS1 activation. However, throughout the paper, and in the model of Figure 7E, the PEAT complex is proposed to promote histone deacetylation and inhibit histone acetylation to bring about transcriptional silencing. This creates confusion, at least for this reviewer, and the sense that the authors are not thinking objectively about their data.

Response: As stated by the reviewer, our study indicated that the PEAT components interact not only with the histone deacetylases HDA6 and HDA9 but also with the histone acetyltransferases HAM1 and HAM2. The PEAT components are more likely to interact with the histone deacetylases and the histone acetyltransferases separately. Although the PEAT components interact with the histone acetyltransferases HAM1 and HAM2, our affinity purification experiment in combination with mass spectrometric analyses indicated that the PEAT components are not composition of the conserved HAM1/2-containing NuA4-type histone acetyltransferase complex in Arabidopsis (Table 2). However, as suggested by the reviewer, we cannot exclude the possibility that the PEAT components may facilitate histone acetylation by associating with the histone acetyltransferase HAM1 and HAM2 at euchromatin regions. Thus, we revised the related statement in the revised manuscript.

The expression of *ROS1* and *IBM1* was known to be reduced in the mutants defective in DNA methylation and/or heterochromatin (Mathieu et al., 2007; Lei et al., 2015; Williams et al., 2015; Rigal et al., 2012). In this study, we have demonstrated that heterochromatin is decondensed and silencing is released in the mutants of the PEAT components. Thus, the PEAT complexes may affect the expression of *ROS1* and *IBM1* through regulating their chromatin status even though the complex does not affect their DNA methylation. This finding is consistent with the results showing that the PEAT complexes are involved in heterochromatin condensation and silencing independently of alteration in DNA methylation at the whole-genome level.

To further clarify the issue, we examined the effect of *epcr1/2* and *arid2/3/4* on the histone H4K5 acetylation levels of *ROS1* and *IBM1*. If the PEAT components promote the expression of *ROS1* and *IBM1* through activating histone acetylation, the histone acetylation levels of *ROS1* and *IBM1* are

supposed to be reduced in the *epcr1/2* and *arid2/3/4* mutants. However, analysis of our whole-genome histone H4K5 acetylation data indicated that the H4K5 acetylation levels of *ROS1* and *IBM1* are not significantly affected in the *epcr1/2* and *arid2/3/4* mutants (Fig EV5). Therefore, our current results do not support the inference that the PEAT components promote the expression of *ROS1* and *IBM1* through activating histone acetylation. To avoid confusing, we have revised related statements in the discussion part.

Despite an impressive amount of molecular biology, genetic and genomic assays, a major shortcoming of the study is that there are no biochemical experiments to test for HAT or HDAC activity.

Response: We appreciate that the reviewer points out the impressive amount of works on molecular biology, genetic and genomic assays in our study. Based on these works, we have identified and characterized novel complexes (PEAT) which are composed of four types of chromatin-related proteins, among which PWWPs, EPCRs, and ARIDs were not previously reported in plants. Further, we demonstrated that heterochromatin condensation and histone deacetylation are required for the function of the PEAT complexes in heterochromatin silencing. We believe that these findings will significantly contribute to understanding the molecular mechanisms of heterochromatin silencing. As suggested, to understand how the PEAT complexes contributes to heterochromatin silencing, we carried out *in vitro* histone deacetylation assay to test whether the PEAT complexes affect the activity of the histone deacetylase HDA6. However, although we detected the histone deacetylation activity of HDA6, its activity is not affected by addition of components of the PEAT complexes as determined by the *in vitro* experiment (Appendix Fig S6), suggesting that the PEAT complexes may not directly affect the activities of the histone deacetylases. We predict that specific chromatin environments may be critical for the role of the PEAT complexes in regulating histone deacetylation and transcriptional silencing. We also tried to carry out histone acetylation assay for the PEAT components. However, since the histone acetyltransferases can be co-purified with the PEAT components, we cannot conclude how the PEAT complexes affect the histone acetylation activity of HAM1 and HAM2 from the *in vitro* assay.

There are also no tests for telomere shortening, as expected based on the finding of TRB proteins in the putative PEAT complex.

Response: As suggested, we measured the telomere length in the *arid2/3/4* and *epcr1/2* mutants and the wild type by Southern blotting. The result showed that the telomeres were clearly longer in *epcr1/2* and to a lesser extent in *arid2/3/4* than the wild type (Appendix Fig S7). Our result is consistent with the previous study showing that the telomere length was increased in the *trb2* mutant compared to the wild type (Lee et al., 2016 Nucleic Acids Research). The results suggest that, like TRB2, ARID2/3/4 and EPCR1/2 act as negative regulators of telomere elongation, confirming the molecular and functional connection between TRB proteins and AIRD2/3/4 or EPCR1/2 as identified in this study.

And there are no biochemical experiments, such as gel filtration chromatography coupled with immunoblotting (or mass spec analysis of single fractions), to determine if there is a single PEAT complex or multiple sub-complexes that share some, but not all subunits. The fact that there are multiple paralogs of the PWWP, EPCR, ARID, and TRB proteins makes the existence of multiple complexes highly plausible, unless all of the paralogs are all together in one massive, multiply redundant complex. That seems unlikely.

Response: As suggested, we performed gel filtration coupled with western blotting to determine whether PWWP, EPCR, ARID and TRB proteins form a large molecular weight complex (Fig EV2). For gel filtration, proteins were extracted from epitope-tagged transgenic plants and were separated on a Superose 6 increase (10/300 GL) column. The eluted fractions were examined by western blotting. The result indicated that PWWP2-Myc, EPCR1-Flag, ARID2-Flag and TRB1-Flag were predominantly eluted in large-size fractions (>443 kDa), supporting the notion that PWWP2, EPCR1, ARID2 and TRB1 form a large molecular weight complex *in vivo.* However, as mentioned by the reviewer, due to the fact that there are paralogs of the PWWP, EPCR, ARID, and TRB proteins, it is highly plausible that these proteins form multiple redundant complexes. Our affinity purification coupled with mass spectrometric analyses indicated that while ARID2-Flag could co-purify its paralogs ARID3 and ARID4, EPCR1-Flag and TRB1-Flag could not co-purify their paralogs (Table 1). The results suggest that while the paralogs of the ARID proteins can be present in one complex, the paralogs of the EPCR and TRB proteins are mutually exclusive in different complexes. Thus, these paralogs form multiply redundant complexes rather than one massive complex.

Moreover, a number of the indirect associations deduced in Figure 2G are not supported for all paralogs by the co-IP tests of Figures 2E (TRB1 is not co-IPed with ARID2) and 2F (EPCR1 is not co-IPed with either ARID2 or TRB1).

Response: In Fig 2G, we indicated that the EPCR, ARID, and TRB proteins cannot directly interact with each other. This is supported not only by the *in vitro* pull-down experiment shown in Fig 2E and F but also by the yeast two-hybrid assay (Fig 2D, Appendix Fig S2). For the *in vitro* pull-down assay, we

expressed EPCR1, ARID2, and TRB1 in *E. coli*. The pull-down experiment indicated that TRB1 does not directly interact with ARID2 and showed that EPCR1 does not directly interact with ARID2 and TRB1. Given the functional redundancy between paralogs of the EPCR, ARID, and TRB proteins, it is reasonable to deduce that all paralogs of the EPCR, ARID, and TRB proteins cannot interact with each other. Further, our yeast two-hybrid assays did not identify the interaction between all paralogs of the ARID proteins (ARID2/3/4) and TRB1 or TRB2 even though the interaction of the ARID and TRB proteins with the PWWP proteins were identified (Fig 2D), confirming the notion that all the three paralogs of the ARID proteins do not interact with the two paralogs of the TRB proteins.

Different sub-complexes may have distinct activities, and perhaps some mediate HAT activity. As the authors point out in their Discussion, PWWP domains are present in H3K4 methyltransferases, which is associated with transcriptional activation. Other sub-complexes may mediate HDAC activity. Inclusion of biochemical tests of partially purified complexes would likely sort this out to cut through the confusion, and would not be difficult to perform.

Response: We appreciate the reviewer's constructive comments. Since we have demonstrated that the paralogs of each group of proteins function redundantly in heterochromatin silencing, it is reasonable to deduce that the PEAT complexes function together in heterochromatin silencing but not function as different sub-complexes to mediate heterochromatin silencing. As suggested, we carried out histone deacetylation assays for purified complexes from Arabidopsis seedlings. The histone deacetylase HDA6 purified in this experiment was demonstrated to mediate histone deacetylation and was used as a control (Appendix Fig S6). In this experiment, we did not detect the effect of the PWWP, EPCR1, ARID2, and TRB1 co-purified proteins on the histone deacetylation activity. The results suggest that the PEAT complexes did not directly regulate the activity of the histone deacetylase HDA6 as determined by the *in vitro* assay. We predict that specific chromatin environments may be important for the regulation of the histone deacetylase by the PEAT complexes. Although PWWP domains were present in H3K4 methyltransferases, the PWWP domain proteins identified in our study were not shown to specifically associate with active histone marks as reported by a recent systematic profiling of histone readers in Arabidopsis (Zhao et al., Cell rep., 2018). In our study, all types of components of the PEAT complexes were demonstrated to function in heterochromatin silencing. Thus, we predict that the PEAT complexes function together to mediate heterochromatin silencing.

Specific points:
1. The introduction is currently a laundry list of facts concerning various proteins that do this or that, but without discussing what they have to do with one another. Also, the parts of the Discussion explaining the PWWP, EPCR, ARID, and TRB proteins, and how they get their names, belongs in the Introduction.

Response: As suggested, we revised the introduction and tried to connect different parts of the introduction. Our introduction provided background knowledges of DNA methylation and heterochromatin silencing, which help readers understand what we study and the significance of the study. Although our study focuses on PWWP, EPCR, ARID, and TRB proteins, none of these proteins were previously reported to be involved in DNA methylation and heterochromatin silencing. Thus, it is reasonable to discuss the function of PWWP, EPCR, ARID, and TRB proteins in the discussion part. Further, in the last paragraph of the introduction, we did show how these proteins were identified and how they got their names.

2. With respect to statements about the involvement of RdDM in chromatin condensation, Pontes and colleagues showed that the Pol V part of the pathway affects chromatin condensation, but not the Pol IV part of the pathway.

Response: Pontes and colleagues previously demonstrated that the Pol V part of the RdDM pathway but not the Pol IV part is involved in chromatin condensation (Pontes et al., Mol. Plant, 2009). As suggested, we cited the work from Pontes and colleagues in the revised introduction.

3. With respect to statements about yeast two-hybrid interactions, the authors should not state that interactions indicate that proteins homodimerize or heterodimerize- for all they know they could form hexamers or octamers.

Response: We revised the statement according the suggestion.

4. For immunostaining, what is the specificity of the H3K27me antibody? Mono, di, tri- methylation?

Response: H3K27me refers to H3K27 mono-methylation. We replaced H3K27me with H3K27me1 in the revised manuscript.

5. In Figure 1D, the silencing of EPCR1/2 target loci was not fully restored by the EPCR1 transgene in the epcr1/2 mutant, which deserves some comment. Also, what are the two lanes shown for the rescue? Two sibs?

Response: As we responded to the first reviewer, we performed quantitative PCR to examine the transcript levels of EPCR1/2 target loci and added the result in Fig 1C. The results indicated that the silencing of EPCR1/2 target loci is significantly restored by the *EPCR1* transgene in the *epcr1/2* double mutant even though the silencing is not restored to the wild-type level. In the *EPCR1* transgenic plants, *EPCR2* is still defect. Thus, the silencing was not completely restored. Two lanes shown for the rescue refer to two individual transgenic lines. As suggested, we added the explanation for the result.

6. In Figure 2A and 2C, multiple protein bands of tagged EPCR1 were detected. Which band is the correct EPCR1? Does EPCR1 contain post-translational modifications?

Response: The largest band corresponding to the expected molecular weight of the EPCR1-Flag fusion protein (~2.2 kDa) is the full-length *EPCR1-Flag* signal. The smaller bands are most likely to be degraded EPCR1 peptides. We did not find any post-translational modifications of *EPCR1*.

7. Protein masses should be indicated in all of the figures.

Response: As suggested, we added masses for all the protein figures.

8. There are no units on the Y axis of RNA-seq or DNA methylation profile figures.

Response: We indicated units on the Y axis of RNA-seq and DNA methylation profile figures.

Referee #3:

Tan et al. EMBOJ-2017-98770

«The PEAT 1 protein complex is required for histone deacetylation and heterochromatin silencing»
Tan et al report the identification of the PEAT protein complex, a multi-subunit protein complex required for heterochromatin silencing in Arabidopsis. This complex was initially revealed through the identification of its EPCR components, two proteins whose mutations affected transcriptional gene silencing at the soloLTR locus. Recently, several other mutations in TGS components have been reported by this team and others using similar screens, and the experiments presented in this manuscript follow the same experimental setup. In co-immunoprecipitation assays, ERPC were shown to interact with several other proteins proposed to be integral components or associated partners of the PEAT complex, including several PWWP and AT-rich interaction domain-containing (ARID) proteins, telomere repeat binding proteins (TRBs), histone acetyltransferases and deacetylases proteins. To test the role of PEAT, the authors characterize and further analyze several KO mutants in these genes and show that the PEAT complex mediates histone deacetylation, chromatin condensation and TGS. Genome-wide analysis of the epcr, arid and hda6 mutants revealed only a marginal impact on DNA methylation and small RNA accumulation at RdDM targets, while substantial transcriptional activation could be observed at regulated loci, suggesting that the PEAT complex represses transcription via histone deacetylation and heterochromatin condensation.
The paper is interesting and addresses problems that are important to the fields of epigenetics, RdDM, and chromatin biology. The experimental work is well carried out, and the conclusions drawn by the authors are supported by the data. Although there are still many questions remaining about the exact mechanism of action of the PEAT complex in heterochromatin formation, this work improves our understanding of the role of several chromatin-related proteins in TGS and would be appreciated by the readers of the EMBO Journal. However, the specific minor points listed below have to be addressed by the authors.

Response: We appreciate that the reviewer finds the novelty and importance of our study. In the revised manuscript, we have point by point addressed the issues raised by the reviewer.

1) Whereas co-IP and two-hybrid experiments are consistent with the idea that the ERPC, ARID and TRB proteins form a complex in vivo, the authors should provide gel filtration experiments to further support this conclusion.

Response: As suggested, we examined whether PWWP, EPCR, ARID and TRB proteins exist in high molecular weight complexes by gel filtration assays (Fig EV2). Proteins were extracted from epitope-tagged transgenic plants and were separated on a Superose 6 increase (10/300 GL) column. The eluted

fractions were examined by western blotting. The result indicated that PWWP2-Myc, EPCR1-Flag, ARID2-Flag and TRB1-Flag were predominantly eluted in large-size fractions (>443 kDa), supporting the notion that PWWP2, EPCR1, ARID2 and TRB1 form a large molecular weight complex *in vivo.* Given that there are paralogs of the PWWP, EPCR, ARID, and TRB proteins, it is highly plausible that these proteins form multiple redundant complexes.

2) To attest to the role of the PEAT complex in controlling the activity of HDA6 in vivo, the authors should provide an epistatic analysis comparing H4K5Ac levels of TEs in epcr1/2 versus epcr12/hda6 triple mutant.

Response: We appreciate the reviewer's constructive suggestion. Our results indicated that like HDA6, the PEAT complexes identified in this study are involved in histone deacetylation and heterochromatin condensation and silencing. However, unlike HDA6, the PEAT complexes are also required for plant development. Thus, the PEAT complexes are unlikely to only act as an upstream regulator of HDA6. We did try to introduce *hda6* into the *epcr1/2* double mutant by crossing. However, due to the time limitation and the competition of our study in the field, we feel sorry that we cannot provide the result at present.

3) What is the condensation status of nuclear bodies in the epcr1/2 double mutant (see figure 6C)?

Response: The nuclei used in this experiment were extracted from mature true leaves of Arabidopsis plants. Compared to the *arid2/3/4* mutant, the *epcr1/2* mutant has a more serious developmental defect and cannot grow true leaves. Thus, we cannot use the *epcr1/2* mutant in the experiment.

4) It would be nice to provide WT and mutant plots on figure 6E instead of ratios.

Response: As suggested, we provided siRNA distribution across chromosomes in wild type and mutants (Appendix Fig S9A). Further, we analyzed the effect of *arid2/3/4* and *epcr1/2* on siRNA accumulation by the box plots. The result indicating that, in the siRNA regions that produced more Pol IV-dependent siRNAs in the *hda6* mutant, siRNAs are significantly up-regulated not only in the *hda6* mutant but also in the *arid2/3/4* and *epcr1/2* mutants (Fig 6H). These results confirm that the PEAT complexes regulate the production of siRNAs in a similar manner with HDA6.

5) The zoom in the figure 7D indicates that the last two lanes have been sliced from another gel and therefore missed internal controls. The authors should provide the appropriate controls or repeat the experiment with all samples in the same gel.

Response: In this figure, the last two lanes of the *DRD1* signal were sliced from the same gel with the other lanes. Moreover, the experiment was repeated for two times and similar results were obtained. We will provide the origin figure when it is necessary.

2nd Editorial Decision 7th June 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by all three original referees and their comments are shown below.

As you will see the referees generally find that all major criticisms have been sufficiently addressed and recommend the manuscript for publication. However, ref #2 is still concerned that the molecular and functional consequences of PEAT complex binding remain unclear, given the simultaneous recruitment of HATs and HDACs. The referee therefore requests that you include a more detailed discussion of what this may mean for target gene regulation. In addition, I would also ask that you include a reference and discussion of the recent paper by Zhou et al that you mentioned to me by email.

At this point, I would therefore invite you to submit a final revised version of the manuscript in which you incorporate the text changes mentioned above as well as the following editorial points:

REFEREE REPORTS.

Referee #1:

The revised manuscript is significantly improved and my specific concerns have been generally addressed properly. One exception is ChIP with PEAT subunits, which shows weak enrichment supported by data from only two biological replicates. A third biological replicate would make this result much more solid.

Referee #2:

The authors did their best to address the reviewers' concerns and questions and the manuscript is improved relative to the first submission. Biologically, it is still not clear what the PEAT complexes are doing when associated with HATs or HDACs,due to the opposing functions of these chromatin modifiers, such that this paper remains confusing to this reviewer. But presumably, future manuscripts can build on the foundation laid by this study. For instance, the authors make the blanket statement that HDA6 suppresses Pol IV transcription. This may be true at many loci, but it is clearly not true at all loci. At a subset of loci, HDA6 has been shown to be required for Pol IV-dependent siRNAs to be produced (a paper by Blevins). This could be a result of HDA6's role in facilitating CG methylation, and its associated chromatin modifications, which are thought to recruit both Pol IV and Pol V. Maybe such heritable effects involving HDA6 are distinct from immediate effects, with PEAT complexes not involved in both? But clearly HDA6 does not always suppress Pol IV transcription, as the authors state. A more nuanced discussion would be useful.

Referee #3:

the authors have answered my comments in a satisfactory manner.

2nd Revision - authors' response 27th June 2018

Thank you for consideration of our manuscript for publication. As suggested by you and ref#2, we have a more detailed discussion of how the PEAT complexes are involved in gene regulation and Pol IV-dependent siRNA production. We have also cited and discussed the recent paper published by Zhou et al. (2018) and then raised future research directions in the field. In addition, all the editorial issues have been addressed in the revised manuscript.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Xinjian He
Journal Submitted to: the EMBO Journal
Manuscript Number: EMBOJ-2017-98770

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	In general, sample size was chosen based on the field's standards.
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.	Yes. When we analyzed the microscopy data(Fig 6D and E) ,the experiment was independently carried out by different researchers and similar results were obtained.
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?	Yes
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?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

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).	The information about the antibodies used are provided in the Materials and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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

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

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

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

* 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 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'. Please confirm you have submitted this list.	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'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	RNA-seq, BS-seq, ChIP-seq ,sRNA-seq data are deposited to Gene Expression Omnibus (GEO) database. Mass spectrometry data are provided in Appendix tables.
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).	NA
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. 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

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