The Chromatin Remodelers PKL and PIE1 Act in an Epigenetic Pathway that Determines H3K27me3 Homeostasis in Arabidopsis

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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2017-00867-RA 1st Editorial decision – *revision requested* Dec. 18, 2017

We ask you to pay attention to the following points in preparing your revision. As you will see from the detailed comments of three reviewers, they agree on the general interest in the topic and the data, but they found several points that need clarification or complementation before the manuscript can be accepted for publication in Plant Cell. In several contexts, the reviewers noticed a lack of direct evidence beyond correlation, quite some missing controls, and they suggest a few experiments to better support the speculative model. They also recommend reducing some redundancy, to consider the different developmental stages over time between the mutants, and some improved data analysis and graphical representation.

I should stress that we are reluctant to see manuscripts undergoing multiple rounds of revision and would be unlikely to offer you more than one chance to satisfy the reviewers.

[Reviewer comments provided below with author responses]

TPC2017-00867-RAR1 1st Revision received Feb. 12, 2018

Reviewer comments and **author responses**:

Reviewer #1:

The manuscript by Carter et al. describes the interaction between PKL and PIE1, therefore a possible link among H3K27me3 and H2A.Z marks. They point out that H3K27me3 targeted genes are a subset of genes targeted by H2A.Z. The authors use a genome wide data approach carefully describing the bioinformatics data treatment and analysis and taking care of best practices for RNA and ChIP-seq. Importantly, the authors characterize a nucleosome maturation activity of PKL that contributes to bring light to the unknown mechanism by which PKL promotes

H3K27me3. Overall, this is a potentially strong manuscript, which addresses an interesting question and point new uncharacterized mechanisms of H3K27me3 homeostasis, useful for further studies.

Point 1. The authors propose that the incorporation of H2A.Z promotes H3K27me3 deposition and for that reason in pie1 mutant there is a reduction of H3K27me3 levels. The authors should explore the possibility of a reduced PRC2 activity in *pie1* mutant due to downregulation of PRC2 components.

RESPONSE: We have added a new Table S2 to address this point. The table makes clear that there are no significant differences in expression of genes encoding known PRC2 subunits in *pie1-5* relative to WT. We also added text to the Results section to explicitly address this point: "Importantly, transcript levels of known PRC2 subunits were not significantly altered in the pie1-5 mutant relative to WT (Supplemental Table 2), indicating that the effect of ablation of PIE1 on H3K27me3 levels is not due to decreased expression of genes encoding PRC2 machinery."

Point 2a. The reduced levels of H2A.Z in *pie1* and *clf* mutants are clearly shown in Figure 5. Although in the Figure 5C (and in the text, lane 213) the levels of H2A.Z in the *pie1* mutant are lower than in the *clf* mutant, the Figure 5D shows the opposite (although both are very low).

RESPONSE: The inclusion of genes that were not detectably enriched for H2A.Z made visual interpretation of Figure 5 difficult. Panels C and D of Figure 5 have been updated to include only genes containing at least one region of H2A.Z enrichment. As a result, the metagene plot in Figure 5D are now more representative of the heat map data in panel C.

Point 2b. Moreover, the metagene profile of *clf* is different than in *pie1*. The typical H2A.Z enrichment in the TSS is maintained in *clf* but not in *pie1* mutant. I suggest to provide a more complete description on that behavior in the text due to the importance given to the presence of H2A.Z in the gene body and in the TSS during the whole manuscript.

RESPONSE: We have revised the text describing Figure 5 in the Results section to address this point. It now reads: "In particular, we observed that the 5' peak of H2A.Z was dramatically reduced in the *pie1-5* line, revealing that preferential enrichment of H2A.Z observed at the TSS of genes is largely a *PIE1*-dependent phenomenon. Surprisingly, *clf-28* plants also exhibited reduced H2A.Z, although to a lesser degree than was observed in *pie1-5*. Further, a peak of H2A.Z enrichment at the TSS of genes was maintained in *clf-28* plants in contrast to its loss in *pie1-5* plants, indicating that *clf-28* affects enrichment of H2A.Z in a different manner than *pie1-5*."

Point 2c. The use of metagene plots is very informative and the manuscript would be strengthened by using them, complementing the distinction among TSS and Gene body. For example (a) would be interesting to see the H2A.Z metagene profile in the subset of genes with and without decreased H3K27me3 in the mutants; (b) the plots can be used instead of the visual inspection (lane 202) and (c) the association of loss and gain of H2A.Z with loss of H3K27me3 in *pie1* mutant (lane 232) can be more clearly addressed with metagene profiles.

RESPONSE: (a) We have added Figure S2, which makes use of the suggested metagene profile plots. We have also added the following text to the Results section that refers to this new supplementary figure: "A metagene profile of H2A.Z levels in *clf-28* does not reveal preferential loss of H2A.Z at H3K2me3-enriched genes (Supplemental Figure 2)." We also updated the relevant Discussion section text in the same manner: "…and loss of H2A.Z in *clf-28* does not occur preferentially at H3K27me3-enriched genes (Supplemental Figure 2)." (b) We have removed the "visual inspection" text and added references to figures 5 and 6. The altered text now reads: "Many genes exhibiting reduced H3K27me3 did so throughout the coding sequence in each of the lines (Figures 5A-B and 6D)." (c) In the new Supplemental Figure 2 mentioned above, we have included a panel examining this relationship in *pie1-5*. The following modified passage was added to the Discussion section referring to this panel: "However, not all loci exhibit significantly decreased levels of H2A.Z in *pie1-5*, indicating that other factors in addition to PIE1 can promote incorporation of this histone variant, at least at some fraction of the genome. In line with this proposition, we observed that H2A.Z levels were lower in *pie1-5* plants at H3K27me3-enriched genes compared to genes that were not enriched for H3K27me3 in WT (Supplemental Figure 2). Since H3K27me3 enrichment is correlated with very low transcript levels (Figure 4C), these data raise the possibility that PIE1-independent mechanisms exist that promote

incorporation of H2A.Z specifically at actively transcribed genes. These data thus also provide a possible rationale for the previous observation that *pie1* plants are not phenotypically equivalent to plants that are severely depleted for H2A.Z [33]."

Point 3. The authors should clarify in the text that genes classified as enriched in TSS and genes in enriched the Gene body have an important overlap. Having a look to the overlap of H2A.Z DEGs down in *clf* and *pie1* mutants, approx. 60% of them are marked in the gene body and the TSS in *clf*, and this number is even higher (80%) in *pie1.* Due to the strong overlap, the conclusion about the silencing role of H3K27me3 in the genes with gene body H2A.Z (lane 334) is difficult to be addressed.

RESPONSE: Additional text has been added to the Discussion section to address this point. It now reads: "Notably, previous studies have indicated that H2A.Z enrichment in the gene body contributes to transcriptional repression [18,54]. Our results strongly support a role for H2A.Z in transcriptional repression at these loci in part by contributing to enrichment of H3K27me3 (Figure 6). However, since 82% of genes with reduced H2A.Z in the gene body also exhibit reduced H2A.Z at the TSS in pie1-5 plants, it is difficult to determine whether enrichment specifically at one or both of these regions drives the observed changes in transcription and/or H3K27me3 levels."

Point 4. The detailed description of the bioinformatics analysis is highly appreciated. This information is important and necessary to fully understand the figures and very helpful for readers. However, sometimes it is unnecessary to place this information in the Results section. Moreover, in some cases the description is already in the Methods section and/or in the figure legend. I add few examples of this in the minor revisions section (from 1 to 4).

RESPONSE: We have substantially revised the Results section text on pages 5-7 in which we introduce the RNA-seq and ChIP-seq analyses to avoid repetition of the details included in the Methods section.

Reviewer #2:

Carter et al submitted a manuscript entitle "the chromatin remodelers PKL and PIE1 act in an epigenetic pathway that determine H2K27me3 homeostasis in Arabidopsis". The authors questioned the relationship between the H2A.Z histone variant deposition by PIE1 and the deposition and maintenance of H3K27me3 mark involving CLF and PKL proteins. By observing that the *pkl pie1* double mutant was severely impaired in organogenesis and development, the authors suggested that they are involved in the same epigenetic pathway and undertook the analysis of the transcriptomes of the two mutants, as well as *clf* mutant, as a control. They established H2A.Z and H3K27me3 chromatin profiling by ChIP-seq in the three mutant backgrounds. Furthermore, they performed in vitro assays showing that PKL has a function in prenucleosome maturation. Based on the comparisons of the transcriptomes and H2A.Z/H3K27me3 epigenomes and on this in vitro assay, the authors proposed that PIE1 first acts to established H2A.Z, then promoting H3K27me3 deposition and maintenance.

Point 1. This model is very attractive, the limitation being that it is essentially based on correlative analyses without taking into account the direct targets of these proteins in this developmental stage to solidify the conclusions. Direct comparisons of the epigenomes/transcriptomes give good directions but no strong conclusions on the mechanism especially due to the different developmental timing of the three mutants, analyzed after three weeks in soil (the floral transition probably occurred in clf, what about the others...).

RESPONSE: We have added notes to the Methods section clarifying that all plant material was collected prior to bolting, thus alleviating this concern.

Point 2a .The novelty and originality of the paper is based on the relevant question the authors addressed on the role of PKL in the conversion of prenucleosome particles into canonical nucleosomes, which is a recently developed method. This work is the first report with plant homologs. However, the experiments require samples to be run on the same gel, controls (see below comments Fig. 8) and complementary experiments showing that PKL acts in vitro at least, in an ATP-dependent manner and on mono-prenucleosomes, to be fully convincing and to be taken as a solid and established conclusion. The text relative to this assay and figure 8 need improvements and details to be understood from any reader, even not knowing what is a prenucleosome particle, how to obtained... For instance, the authors should mention at least the size of the DNA used to build the prenucleosomes to allow correct interpretation of the gel on figure 8.

RESPONSE: We have addressed these concerns by adding introductory text to the Results section. It now reads: "Based on in vitro and in vivo analyses, the histone octamer is first re- deposited by a histone chaperone in the form of a conformational isomer referred to as a prenucleosome. Prenucleosome particles comprise an octamer of core histones but occlude a much smaller length of DNA than do nucleosomes in the canonical conformation [46]." In addition, we have revised the Figure 8 legend and added additional details to the Methods section text for this assay.

Point 2b .They also need to precise in the manuscript the frame of validity of the results to avoid over interpretation : in this case that the PKL new function is proposed based on in vitro experiments and needs to be validated in vivo (line 380).

RESPONSE: We have changed the relevant text to make it clear that data were obtained in vitro and that these data are correspondingly only suggestive of activity in vivo. The passage now reads: "The discovery that PKL promotes maturation of prenucleosomes in vitro (Figure 8) provides a simple explanation for how it can contribute to global homeostasis of H3K27me3: it may act in vivo to promote retention of H3K27me3 after passage of a DNA and/or RNA polymerase."

Point 2c .A putative affinity for H3K27me3 enriched particles is suggested (Lines 308-311), which seems highly speculative for a result part in the absence of additional experimental proof.

RESPONSE: We believe this point is addressed by the text addition described in response to the comment below.

Point 2d .Line 322 " we undertook a biochemical analysis of PKL that supports a novel role in promoting retention of H3K27me3 (Figure 8) " is an over interpretation of the result showing that PKL is involved in prenucleosome maturation (Fig. 8). The authors should clarify the link between H3K27me3 and nucleosome maturation with experimental data if they wish to highlight the role of PKL in H3K27me3 retention by its maturation function (Line 32)."

RESPONSE: We have revised this text for clarity and to make it clear that the existing data only suggest an in vivo effect and do not establish it. It now reads: "Finally, we undertook a biochemical analysis of PKL and found that it possessed prenucleosome maturation activity in vitro, suggesting that potential maturation of H3K27me3-containing prenucleosomes by PKL in vivo helps promote their stability and retention in chromatin (Figure 8)."

Point 3. For the prenucleosome assay (Figure 8)

3a. all samples should be analyzed on the same gel with a ladder as control to be able to compare migration and determine band size.

RESPONSE: Ladder lanes are now displayed with size indicators for the gel images.

3b. missing controls: prenucleosome +ligase alone, prenucleosomes +ligase+ PKL +ATP.

RESPONSE: We have added a new panel to Figure 8 that demonstrates construction of the poly- prenucleosome template in the presence and absence of PKL. The addition of these data is such that Figure 8 now fully replicates the assays used to generate Figure S3C of Fei et al.

3c. missing controls: prenucleosomes + PKL +ATP & without ligase, prenucleosomes + ligase + PKL without ATP.

RESPONSE: While it would be of interest to determine whether the prenucleosome maturation activity of PKL is ATP-dependent, the ligation step that is performed prior to addition of PKL requires inclusion of ATP regeneration system to the sample. The published experimental conditions thus preclude analysis of ATP-dependence of the remodeler, which is likely why ATP-dependence was also not determined by Fei et al. for prenucleosome maturation by CHD1 and ACF. Although we strongly suspect that the maturation activity by these remodelers is ATP-dependent, we will note that the model depicted in Figure 9 does not require the maturation activity of PKL to be ATP-dependent.

3d. missing controls: if possible a comparison with the ACF protein used in Frei et al as a control.

RESPONSE: It was not feasible to generate the recombinant proteins for this control in the time frame given for manuscript revisions. Studies for a future manuscript include comparison of maturation of prenucleosomes using plant histones and plant remodelers, which is likely to be a more biologically meaningful comparison.

3e. Could the authors explain the 90 and 180 bp present in the lane III (prenuc/PKL+Mnase) compare to lanes I and II? Is the MNAse treatment sufficient? A gradient of concentration may be useful to check the nature of these bands and that the extra band is not due to degradation of the prenuc profile or just a difference in migration compare to lane I.

RESPONSE: The band at ~80 bp corresponds to the predicted size of the mono-prenucleosome and was also observed in Fei et al., Figure S7C. A description of the likely identify of this band is now included in the results. The band at ~250 bp may be the same band that was previously identified as "di-nucleosomes" in Figure 7B of the Lusser et al. reference, though the authors did not specify the apparent size of those bands. In the absence of confirmatory experiments, however, we have not ascribed an identity to this band in the text of the manuscript.

3f. In Materials & Methods, the authors refer to the protocol of Fei et al 2015. This paper describes mono-prenucleosome assays, which are not used in the present manuscript. The authors should provide precise information to be able to reproduce the experiments (DNA, protein quantity, conditions of reaction...).

RESPONSE: The mono-prenucleosome experiments in Fei et al. were performed using only ACF. Chd1 was shown to act on the prenucleosome array but not on mono-prenucleosomes. We faithfully followed the protocol of Fei at al 2015 with only the modifications that we now explicitly describe in the Materials and Methods.

Reviewer #3:

Carter and colleagues report on RNA-seq and ChIP-seq study of H3K27me3 and H2A.Z in Arabidopsis plants of wild-type and the mutants *pkl, pie1* and *clf*. They found that there were significant overlaps of misregulated genes in these mutants, suggesting a common regulatory pathway. A subset of genes enriched for H2A.Z was found also enriched for H3K27me3. They further showed that H2A.Z incorporation favors H3K27me3 deposition but impaired H3K27me3 deposition does not affect H2A.Z enrichment. Their genetic test indicated that the pkl pie1 double mutant exhibits more severe growth defects as compared to the pkl single mutant. Their in vitro test revealed that PKL has ATPase activity in converting prenucleosomes into canonical nucleosomes. They proposed a model in which H3K27me3 deposition by CLF locates downstream from H2A.Z incorporation by PIE1, and PKL acts in retention of H2A.Z/H3K27me3-mark nucleosomes after DNA replication and/or transcription.

The genome-wide profiling analyses are nicely detailed in this study, and the proposed model is attractive. Nevertheless, many interpretations remain largely hypothetical. It would have been more valuable to provide some mechanistic insights. I have several concerns.

Point 1. Dependence of H3K27me3 deposition on H2A.Z incorporation can be directly assessed by studying PRC2 (CLF) binding at chromatin in the *pie1* mutant compared to wild-type.

RESPONSE: Although we appreciate that testing this hypothesis is of interest, we feel that this experiment is more appropriate for a future manuscript.

Point 2. The genetic test can be more complete. For the *pkl pie1* double mutant phenotype, it is necessary to also include the *pie1* mutant. The double mutant has been made but had not been served in any follow up of the study.

RESPONSE: As described in the response to reviewer 2, we have added the image of the *pie1* single mutant to Figure 1 in panel C. With regards to more extensive characterization of the *pie1 pkl* plants, we are in the process of regenerating these lines as described in our response to Reviewer 2.

Point 3. Are there any data available on *clf pie1* and *clf pkl* mutants? If yes, do they provide support for the model?

RESPONSE: The short root phenotype associated with loss of PKL was shown to be epistatic to the (slightly) longer root phenotype associated with loss of CLF in the phenotypic characterization of the roots of *pkl clf* plants (The Plant Cell 23:1047). The shoot phenotype of *pkl clf* plants, however, was not described. The shoot phenotype of both *pkl clf* and *pkl swn* plants was characterized by Poethig and colleagues in The Plant Cell 28:28, where they

reported that *pkl clf* plants largely exhibited additive phenotypes whereas *pkl swn* plants exhibited synergistic phenotypes, particularly with regards to traits related to vegetative phase change. Similarly, characterization of *clf pie1* plants by Noh and Amasino in The Plant Cell 15:1671 revealed additive shoot phenotypes. The observation of additive shoot phenotypes is consistent with PKL, CLF, and PIE1 largely acting in a common pathway as proposed here as well as in independent pathways that are not functionally overlapping, whereas the observation of a synergistic phenotype for *swn pkl* plants raises the prospect that SWN plays other functionally related roles outside of this pathway. We have not raised these points in the manuscript, however, due to the fact that qualitatively different phenotypic outcomes have been emphasized (e.g. flowering time and phase change versus genome-wide levels of H3K27me3 and H2A.Z), and we felt that their inclusion as evidence in support of our model would be regarded as tenuous at best.

Point 4a. Can the 'Prenucleosome maturation assay' be performed using mono-nucleosomes derived from wild-type and a H2A.Z/H3K27me3-deficient mutant?

RESPONSE: We have not undertaken this experiment, in part due to the absence of biological material as mentioned above.

Point 4b. The current data shown in Figure 8 does not provide information about PKL function specifically in retention of H2A.Z/H3K27me3-mark nucleosomes as shown in the model.

RESPONSE: The reviewer is correct that these in vitro data do not address possibility that PKL preferentially acts on specific modification states in vivo. These types of studies would provide the bases for future publications. With regards to this manuscript, we feel that the modifications we have made to clarify interpretation of in vitro data versus in vivo implications as requested by Reviewer 2 address concerns that we are overinterpreting the significance of our in vitro data.

TPC2017-00867-RAR1 2nd Editorial decision – *revision requesed* Mar. 10, 2018

We have received reviews of your revised manuscript entitled "The Chromatin Remodelers PKL and PIE1 Act in an Epigenetic Pathway that Determines H3K27me3 Homeostasis in Arabidopsis." Thank you for submitting your best work to The Plant Cell and for considering the reviewers' input.

The editorial board agrees that the manuscript has improved in several aspects and that work you describe is substantive and falls within the scope of the journal. However, as you will see below, the reviewers are satisfied with some of your changes and answers to their previously expressed concern, but the comments of reviewer 2 and 3 indicate disappointment about some other points. This lack of enthusiasm was even more clearly stated in the comments to the editors, and we support the request for an optimal processing of constructive criticism. Therefore, we kindly ask you to discuss the comments from the first and second round of reviews among all authors to see where you could further improve the manuscript before its publication. We ask you specifically to improve the experiments about the prenucleosome maturation with recombinant PKL (Fig. 8) according to new and previous comments of reviewer 2, as this is a central element of your model, especially as long as the model is supported only by these in vitro data. We assume that this is feasible in the regular revision time, but let us know if you would need more time. There are also questions from reviewer 3 about references and the method to process the photos in Fig. 1.

As stated before, we are reluctant to see manuscripts undergoing multiple rounds of revision and would be unlikely to offer you more than this chance to satisfy the reviewers.

[Reviewer comments provided below with author responses]

TPC2017-00867-RAR2 2nd Revision received Apr. 25, 2018

Response to editor comments: [In an e-mail exchange], the specific request was made to demonstrate that prenucleosome maturation activity exhibited by PKL is ATP-dependent.

We have repeated the prenucleosome maturation assays with recombinant PKL in the presence and absence of additional ATP. Panel B in Figure 8 now clearly demonstrates that prenucleosome maturation activity exhibited by PKL is reduced in the absence of additional ATP, demonstrating that this activity is ATP-dependent as requested by

reviewers and the editor. The text in the results section and the material and methods has been modified accordingly.

Reviewer #1:

The authors have successfully answered and revised my previous comments and no major or minor revisions are required in the new version. The data presented in this work will be helpful for future studies addressing the link among H3K27me3 and H2A.Z.

RESPONSE: We are pleased to learn that our previous revisions have addressed the concerns of Reviewer 1.

Reviewer #2:

The authors improved the manuscript according to requests and added some additional data. In the letter, they answered to the questions addressed by reviewers, some were not fully addressed and left for another paper.

Point 1. A deeper investigation of the nucleosome maturation from prenucleosome with complementary experiments and techniques would have increased the value of the paper and comfort the similarity of action between CHD1 and PKL, not only at the sequence level. Whether the 147 bp band is resulting from prenucleosome maturation or a passive and partial protection of the 160 bp fragment in poly-prenucleosome by the addition of PKL remains open. Hopefully complementary paper will address the mechanism.

RESPONSE: We believe that the modification of Figure 8 panel B to include data demonstrating that prenucleosome maturation activity exhibited by recombinant PKL is ATP-dependent addresses the concerns expressed here.

Reviewer #3:

General paragraph.

Point 1. The revision clarified some points, but problematic issues remained on the mechanistic model. The H2A.Z incorporation in nucleosome occurs via exchange with H2A. A process largely associates with transcription, thus polymerase. Chaperones are known to chaperoning histone molecules but not 'prenucleosome'. Thus, the model drawn in Figure 9 is problematic.

RESPONSE: Our data demonstrate that H2A.Z and H3K27me3 co-localize (Figure 4B) and that genes that are enriched for H2A.Z and H3K27me3 exhibit very low levels of expression (Figure 4C. For this reason, we state that we favor the possibility that PKL acts after passage of a DNA polymerase through these regions of the genome rather than an RNA polymerase, but in no way do we rule out the possibility that PKL acts after passage of an RNA polymerase. The Kadonaga lab has demonstrated that histone chaperones are sufficient to promote formation of prenucleosomes in vitro when incubated in the presence of recombinant histones and DNA (Fei et al, Genes and Development 29:2563).

Point 2. The authors responded my question regarding knowledge on *clf pie1* and *clf pkl* mutants. I believe that the mentioned previous studies should be cited and discussed because these mutants are directly within interests of this current study.

RESPONSE: We have added the following paragraph to the Discussion:

"Previous characterization of double mutant plants that lack these or related genes is consistent with our observations. *clf pkl* plants largely exhibit additive shoot phenotypes whereas *swn pkl* plants exhibit synergistic shoot phenotypes, particularly with regards to traits related to vegetative phase change [Xu et al., 2016]. Similarly, characterization of *clf pie1* plants reveals additive shoot phenotypes [Noh and Amasino, 2003]. The observation of additive shoot phenotypes in *clf pie1* and *clf pkl* plants is consistent with *PIE1*, *CLF*, and *PKL* acting in a common pathway as proposed here. In contrast, the observation of a synergistic phenotype for *swn pkl* plants raises the prospect that *SWN* plays a functionally related role outside of the proposed pathway. Analysis of the molecular traits described here (genome-wide levels of H3K27me3 and H2A.Z) in these double mutants is likely to shed additional light into the respective roles of these factors with regards to these epigenetic phenotypes."

Point 3. I am not a specialist but intrigued by the photos shown in Figure 1: objective plant in green/yellow on top of background of black/white plants. How the photographs were taken?

RESPONSE: The images were collected using a standard light microscope camera. After collection, the background colors of the images were desaturated using image processing software. This can be done in Photoshop, GIMP, etc. by separating the region of interest (e.g. the seedling) into a separate layer in the image and then desaturating the color of the remaining background layer. Selecting the region of interest that will not be desaturated can either be done using an automatic "magic" selection too (if it works), or by manually tracing the region/seedling using the freeform selection tool on a high zoom level.

TPC2017-00867-RAR2 3rd Editorial decision – *accept with minor revision* May 2, 2018

We have received your revised manuscript entitled "The Chromatin Remodelers PKL and PIE1 Act in an Epigenetic Pathway that Determines H3K27me3 Homeostasis in Arabidopsis". The board of reviewing editors would like to accept your manuscript for publication in The Plant Cell. This acceptance is contingent on revision based on questions raised during the editorial consultation. In particular, please consider the following:

1) In your correspondence to the editors on March 22, 2018, it was suggested that it would not be possible (or would be very difficult) to examine the ATP-dependence of prenucleosome maturation, because "the assay includes an ATP regeneration system (3 mM phosphoenolpyruvate, 20 U/µL pyruvate kinase) during generation of the poly-prenucleosomal template that precludes inclusion of such a control". It was further stated that you did "not have any prenucleosomes or recombinant PKL on hand. The prenucleosomes in particular do not have a long shelf-life. It would take a significant investment of time and resources (particularly given our current funding status) to regenerate these reagents simply to have all of the lanes on one gel."

Nevertheless, a new Figure 8 was sent to editors on March 30, demonstrating ATP-dependence of prenucleosome maturation activity. It is noted in the figure legend and methods that the prenucleosome maturation assay performed as described by Fei et al. 2015 (Genes and Devel) was conducted in the presence of an ATP regeneration system. The editors are somewhat perplexed by the seeming discrepancy between the explanation given on March 22 and the new results shown in the revised Figure 8, received on March 30. We hope that you can provide a simple explanation, and, in particular, include an explanation in the figure legend or methods as appropriate, as to how the assay shown in Figure 8B, lane VI (without ATP) was performed (whether in presence or absence of ATP regeneration system). Please also indicate whether or not the experiment shown in Figure 8 was replicated.

TPC2017-00867-RAR3 3rd Revision received May 7, 2018

RESPONSE: All of the biochemical data that we have presented thus far were obtained in 2017. KK Ho, who carried out the prenucleosome maturation assays, was out of the country during the initial correspondence regarding the request for more biochemical data. Upon his return, he informed me that he had already undertaken the experiments requested. Ligation of the prenucleosomes takes place in the presence of an ATP regeneration system during a 16-hour incubation. An aliquot of this mixture is then diluted 1:1 for the subsequent maturation assay, at which point additional ATP is added as per the published protocol. Dr. Ho's data demonstrate that, in our hands, the maturation reaction does indeed require supplementation with additional ATP, indicating that the inclusion of the ATP-regeneration system as described at the initiation of the prenucleosomal ligation reaction is not sufficient to enable the PKL-dependent prenucleosomal maturation reaction after the subsequent treatment of the sample (an extended incubation followed by dilution). This observation, that PKL-dependent prenucleosomal maturation is dependent on concurrent addition of ATP, has been replicated. We have modified the relevant text in Methods (lines 572-3) to more clearly convey this information.

TPC2017-00867-RAR3 4th Editorial decision – *acceptance pending* May 11, 2018

Thank you for your comments and the requested revisions of your paper entitled "The Chromatin Remodelers PKL and PIE1 Act in an Epigenetic Pathway that Determines H3K27me3 Homeostasis in Arabidopsis". We are pleased to inform you that your manuscript has been accepted for publication in The Plant Cell, pending ...

Final acceptance from Science Editor May 24, 2018