nature portfolio

Peer Review File

Single amino acid mutations in histone H3.3 illuminate the functional significance of H3K4 methylation in plants

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The study addresses an important gap in understanding the functional significance of histone modifications, particularly H3K4 methylation, in plants. By using point mutations in H3 variants, the authors provide a direct approach to dissect the role of specific lysine residues, advancing the field beyond traditional histone methyltransferase disruption studies. This work highlights the differential roles of H3.1 and H3.3 in the deposition of H3K4 methylation. Additionally, the identification of key residues (H87, L90) in H3.3 as critical for its function and chromatin deposition provides new insights into the interplay between histone variants and post-translational modifications.

The authors employ a comprehensive array of techniques, including ChIP-seq, RNA-seq, ATAC-seq, and bisulfite sequencing, to thoroughly assess the impact of the H3.3 K4 mutation on chromatin modification and gene regulation. This multimodal approach strengthens the validity of their conclusions. The figures and supplemental data are well-organized, effectively illustrating the main findings. Additionally, the use of detailed control experiments, such as expressing HA-tagged H3 variants, enhances the mechanistic insights presented. Overall, this manuscript presents a well-executed and insightful study into the roles of H3.3 and H3K4 methylation in Arabidopsis. The work is generally strong; thus, I only have few points.

Some references in the text seem to emphasize animal studies where relevant plant studies exist, which may lessen the impact for plant-specific conclusions. For instance, at lines 51-54, the statement “non-histone substrates or enzymatic independent activities have been reported for these enzymes” could benefit from a plant-centered perspective, especially as only animal publications are cited here. Similarly, at lines 57-60, the exclusive citation of animal studies could be expanded to include plant-focused references, particularly those already cited later in the manuscript, such as refs. 66 and 67. Additionally, incorporating other relevant plant studies with similar approaches, like Iwakawa et al., 2017 (DOI: 10.1094/MPMI-12-16-0250-R); Lu et al., 2018 (DOI: 10.1038/s41467-018-02976-9); Yan et al., 2020 (DOI: 10.1242/dev.184895); and Fall et al., 2023 (DOI: 10.1111/nph.18666), would provide a more exhaustive background. Similarly, at lines 81-83, an animal study (ref. 31) is cited for the non-histone substrates of certain histone methyltransferases. There are also relevant plant studies here, such as those on ATXR7 (DOI: 10.1111/j.1365-313X.2006.02799.x), SDG7 (DOI: 10.1093/pcp/pcq171), and the cytosolic isoform of ATX1 (DOI: 10.1093/nar/gkq1300), that could strengthen the discussion.

Although the study employs a comprehensive set of assays, the in vitro methylation assay (Figure 5d) reveals that SDG2 does not preferentially methylate either H3.3 or H3.1, which appears inconsistent with the in vivo findings. The authors should consider offering potential explanations for this discrepancy, such as co-factor requirements or chromatin context dependencies that may be missing from the in vitro setup. It could also simply be that they only use the SET catalytic domain which may limit the assay's functional accuracy. Related to these assays and the usage of only the catalytic SET domain, I am wondering if it would be appropriate to use as a (negative) control other histone methylation marks such as H3K27 methylation ? Considering that “introducing K4A mutated H3.3 did not affect the endogenous H3K4 methylation levels “ (line 361-362), could it simply be that the mutated histone version is less or even not loaded on chromatin since the WT endogenous histone version is expressed ? A differential western blot comparing the soluble versus the non-soluble (i.e., containing chromatin) protein fractions could help to answer this issue.

The procedure used for western blot of histone methylation marks on exogenous versus endogenous H3.1 and H3.3 should be described in detail, as well as the procedure for histone/nuclear protein extraction. Indeed, the explanation provided in

the text (“so that the exogenous H3.1/H3.3 can be distinguished from endogenous H3 based on protein size differences” line 352-353) is definitely not sufficient for readers to understand the procedure, especially because the 3xHA tag brings only a +/- 3 kDa weight difference (without IP, exogenous and endogenous histones should be visible on the same H3K4 methylation blot). Also, the HA loading control is not clear to me. Did the authors perform an HA-IP before testing K4 methylation on exogenous histones ? Does the HA loading control really reflect an equivalent expression of exogenous histones between transgenic lines ? If an IP was done, are endogenous histone obtained from the IP flow-through ?

The reason for the low penetrance of the HTR5 T6A mutation described in Figure 7 is unclear. Could the authors clarify/discuss whether this is simply due to an insert location effect, or if it reflects quantitative differences in the loading of the mutated histone between plants? Finally, except for sterile ones, how the progeny of HTR5 T6A transgenic lines look like ? Did authors also observe such low penetrance with for example a range of weak to severe defects in progeny from weak parent plants ?

The manuscript also contains minor grammatical issues:
Line 44: “positive change” should be replaced with “positive charge.”
Line 576: "predominate" should be "predominant."

Reviewer #2

(Remarks to the Author)
In this manuscript, Xue et al. investigate the regulatory role of lysine 4 (K4) of Arabidopsis histone H3 by introducing point mutations at K4 in two major H3 variants, H3.1 and H3.3. As expected, the authors observed a drastic decrease in H3K4 methylation levels upon K4 mutation in H3.3, with associated transcriptome changes similar to those observed in an SDG2 mutant. Interestingly, the authors suggest that H3.3K4 and SDG2 are required for de novo gene activation and RNA Pol II elongation. They further propose that H3.3 may serve as a critical substrate for H3K4 methylation, which is important for gene expression regulation. Overall, this is an excellent study. The data and analyses are of high quality. However, I have one major concern:
The authors claim that H3.3K4 and SDG2 are required for de novo gene activation and transcriptional elongation. In my view, this conclusion is not fully supported by the presented data. To substantiate this claim, the authors should profile nascent RNA transcripts under both control and stress conditions in WT and mutant backgrounds. Since mature RNA sequencing (RNA-seq) primarily captures processed mRNA—the final product of transcription and RNA processing—it cannot distinguish between transcriptional regulation and post-transcriptional processing. A method that specifically tracks transcription dynamics is necessary to provide direct evidence that H3.3K4 and SDG2 are required for de novo gene activation.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have clearly addressed all my previous comments and questions. I appreciate their thorough responses and the improvements made to the manuscript. It was a pleasure to read such a well-prepared and insightful study.

Reviewer #2

(Remarks to the Author)
he authors have improved their manuscript and provided a convincing response to my comment.

Reviewer #1 (Remarks to the Author):

The study addresses an important gap in understanding the functional significance of histone modifications, particularly H3K4 methylation, in plants. By using point mutations in H3 variants, the authors provide a direct approach to dissect the role of specific lysine residues, advancing the field beyond traditional histone methyltransferase disruption studies. This work highlights the differential roles of H3.1 and H3.3 in the deposition of H3K4 methylation. Additionally, the identification of key residues (H87, L90) in H3.3 as critical for its function and chromatin deposition provides new insights into the interplay between histone variants and post-translational modifications.

The authors employ a comprehensive array of techniques, including ChIP-seq, RNA-seq, ATAC-seq, and bisulfite sequencing, to thoroughly assess the impact of the H3.3 K4 mutation on chromatin modification and gene regulation. This multimodal approach strengthens the validity of their conclusions. The figures and supplemental data are well-organized, effectively illustrating the main findings. Additionally, the use of detailed control experiments, such as expressing HA-tagged H3 variants, enhances the mechanistic insights presented. Overall, this manuscript presents a well-executed and insightful study into the roles of H3.3 and H3K4 methylation in Arabidopsis. The work is generally strong; thus, I only have few points.

Response: We sincerely appreciate the reviewer's positive and encouraging comments.

Some references in the text seem to emphasize animal studies where relevant plant studies exist, which may lessen the impact for plant-specific conclusions. For instance, at lines 51-54, the statement "non-histone substrates or enzymatic independent activities have been reported for these enzymes" could benefit from a plant-centered perspective, especially as only animal publications are cited here. Similarly, at lines 57-60, the exclusive citation of animal studies could be expanded to include plant-focused references, particularly those already cited later in the manuscript, such as refs. 66 and 67. Additionally, incorporating other relevant plant studies with similar approaches, like Iwakawa et al., 2017 (DOI: 10.1094/MPMI-12-16-0250-R); Lu et al., 2018 (DOI: 10.1038/s41467-018-02976-9); Yan et al., 2020 (DOI: 10.1242/dev.184895); and Fall et al., 2023 (DOI: 10.1111/nph.18666), would provide a more exhaustive background. Similarly, at lines 81-83, an animal study (ref. 31) is cited for the non-histone substrates of certain histone methyltransferases. There are also relevant plant studies here, such as those on ATXR7 (DOI: 10.1111/j.1365-313X.2006.02799.x), SDG7 (DOI: 10.1093/pcp/pcq171), and the cytosolic isoform of ATX1 (DOI: 10.1093/nar/gkq1300), that could strengthen the discussion.

Response: We thank the reviewer for this valuable suggestion. We have now incorporated the relevant plant references into the manuscript.

Although the study employs a comprehensive set of assays, the in vitro methylation assay (Figure 5d) reveals that SDG2 does not preferentially methylate either H3.3 or H3.1, which appears inconsistent with the in vivo findings. The authors should consider offering potential explanations for this discrepancy, such as co-factor requirements or chromatin context dependencies that may be missing from the in vitro setup. It could also simply be that they only use the SET catalytic domain which may limit the assay's functional accuracy. Related to these assays and the usage of only the catalytic SET domain, I am wondering if it would be appropriate to use as a (negative) control other histone methylation marks such as H3K27 methylation ? Considering that "introducing K4A mutated H3.3 did not affect the endogenous H3K4 methylation levels " (line 361-362), could it simply be that the mutated histone version is less or even not loaded on chromatin since the WT endogenous histone version is expressed ? A differential western blot comparing the soluble versus the non-soluble (i.e., containing chromatin) protein fractions could help to answer this issue.

Response: We thank the reviewer for raising these points.
1. Regarding the selective deposition of H3K4 methylation on H3.3 over H3.1, we have demonstrated that the COMPASS-like complex preferentially associates with H3.3 rather than H3.1 *in vivo* (Figure 6l), which is likely facilitated through its interaction with the H3.3 chaperone HIRA (Figures 6h-6j). This provides an explanation for the preferential deposition of H3K4 methylation on H3.3 *in vivo*.

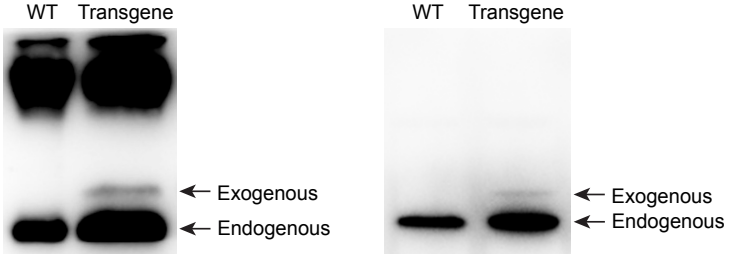
2. Regarding the specificity of the SDG2 SET domain, a previous study (Guo et al., 2010, https://doi.org/10.1073/pnas.1010478107) has already established that the SDG2 SET domain specifically methylates H3K4 but not H3K9, H3K27, and H3K36. In our experiment, we utilized the exact same sequences from this study for protein expression and methyltransferase assay.

3. For the loading of K4A mutated H3.3 on chromatin, we have shown through multiple approaches, including immunostaining and ChIP-seq, that K4A mutated H3.3 can be effectively loaded onto chromatin, and behaves similarly to non-mutated H3.3, despite the presence of wild-type endogenous histones (Line 184–207, Supplemental Figure 3).

The procedure used for western blot of histone methylation marks on exogenous versus endogenous H3.1 and H3.3 should be described in detail, as well as the procedure for histone/nuclear protein extraction. Indeed, the explanation provided in the text ("so that the exogenous H3.1/H3.3 can be distinguished from endogenous H3 based on protein size differences" line 352-353) is definitely not sufficient for readers to understand the procedure, especially because the 3xHA tag brings only a +/- 3 kDa weight difference (without IP, exogenous and endogenous histones should be visible on the same H3K4 methylation blot). Also, the HA loading control is not clear to me. Did the authors perform an HA-IP before testing K4 methylation on exogenous histones ? Does the HA loading control really reflect an equivalent expression of exogenous histones between transgenic lines ? If an IP was done, are endogenous histone obtained from the IP flow-through ?

Response: We thank the reviewer for raising this point.
1. We have now provided additional details on the detection of histone modifications for both exogenous and endogenous histones, as well as the procedure for nuclear protein extraction, in the methods section (Line 687-699).

2. As noted by the reviewer, due to the small size difference, the exogenous and endogenous H3 are in close proximity on the blot. This proximity poses challenges when detecting histone modifications on exogenous H3. Specifically, the exogenous H3 is expressed under the control of the *HTR5* or *HTR13* promoter, whereas the endogenous H3 is derived from a broader pool of genes, including three H3.3 coding genes (*HTR4*, *HTR5*, and *HTR8*), five H3.1 coding genes (*HTR1*, *HTR2*, *HTR3*, *HTR9*, and *HTR13*), and several other atypical H3 variants. This disparity in expression levels results in much stronger signals from endogenous H3 compared to exogenous H3. Consequently, when attempting to expose the blot for a long time to detect the weaker signals of exogenous H3, these signals are often obscured by the stronger signals of endogenous H3 due to their close proximity on the blot. This challenge is particularly evident when detecting some low abundant histone modifications, below are two examples.



To address this issue, we cut the membrane into two pieces based on the 15 kDa mark of the protein ladder, the exogenous H3 is above the 15 kDa mark, while the endogenous H3 is below the 15 kDa mark due to their size difference. This approach prevents interference from the endogenous H3 signals. Consequently, the exogenous and endogenous H3 are not visible on the same blot. The details of these experimental procedures have been included in the methods section (Line 687-699).

3. We did not perform HA-IP before detecting the H3K4 methylation levels on exogenous H3. Although the expression of exogenous H3 may vary between transgenic lines, we adjusted the protein loading (as indicated by the HA blot) to ensure that H3K4 methylation levels were detected on comparable amounts of exogenous H3.

The reason for the low penetrance of the HTR5 T6A mutation described in Figure 7 is unclear. Could the authors clarify/discuss whether this is simply due to an insert location effect, or if it reflects quantitative differences in the loading of the mutated histone between plants? Finally, except for sterile ones, how the progeny of HTR5 T6A transgenic lines look like ? Did authors also observe such low penetrance with for example a range of weak to severe defects in progeny from weak parent plants ?

Response: We appreciate the reviewer raising this question. As these are independent T1 transgenic plants with variations in transgene insertion sites and copy numbers, we believe the phenotypic differences among the lines are primarily due to variations in transgene expression levels. Supporting this, the progeny of T1 plants with weak phenotypes consistently exhibit weak phenotypes. We have incorporated this explanation into the manuscript (Line 510).

The manuscript also contains minor grammatical issues: Line 44: “positive change” should be replaced with “positive charge.” Line 576: "predominate" should be "predominant."

Response: We thank the reviewer for pointing out these errors. We have made the corrections accordingly.

Reviewer #2 (Remarks to the Author):

In this manuscript, Xue et al. investigate the regulatory role of lysine 4 (K4) of Arabidopsis histone H3 by introducing point mutations at K4 in two major H3 variants, H3.1 and H3.3. As expected, the authors observed a drastic decrease in H3K4 methylation levels upon K4 mutation in H3.3, with associated transcriptome changes similar to those observed in an SDG2 mutant. Interestingly, the authors suggest that H3.3K4 and SDG2 are required for de novo gene activation and RNA Pol II elongation. They further propose that H3.3 may serve as a critical substrate for H3K4 methylation, which is important for gene expression regulation. Overall, this is an excellent study. The data and analyses are of high quality. However, I have one major concern:
The authors claim that H3.3K4 and SDG2 are required for de novo gene activation and transcriptional elongation. In my view, this conclusion is not fully supported by the presented data. To substantiate this claim, the authors should profile nascent RNA transcripts under both control and stress conditions in WT and mutant backgrounds. Since mature RNA sequencing (RNA-seq) primarily captures processed mRNA—the final product of transcription and RNA processing—it cannot distinguish between transcriptional regulation and post-transcriptional processing. A method that specifically tracks transcription dynamics is necessary to provide direct evidence that H3.3K4 and SDG2 are required for de novo gene activation.

Response: We thank the reviewer's positive and encouraging comments.

Regarding the role of H3.3K4 and SDG2 in de novo gene activation, indeed traditional RNA-seq primarily captures processed RNA. However, our analysis revealed that disrupting H3.3K4 and SDG2 affects the intron splicing of only a small subset of genes (Supplemental Figure 5c). In addition, based on the reviewer's suggestions, we further investigated whether H3.3K4 and SDG2 are required for high temperature-induced de novo gene activation by analysing nascent RNA levels through extracting chromatin-associated RNA (Bhatt et al., 2012, DOI: 10.1016/j.cell.2012.05.043; Oesterreich et al., 2016, DOI: 10.1016/j.cell.2016.02.045) in both control and high temperature-treated WT and mutant plants. Our results show that the levels of nascent RNA for high temperature-induced genes are significantly reduced in *h3.3ko;HTR5 K4A* and *sdg2* mutants, providing further evidence that H3.3K4 and SDG2 are required for high temperature-induced de novo gene activation (Line 335-338, Figure 4a). The previous results from total RNA extracts have been relocated to the Supplementary Figure 6e.

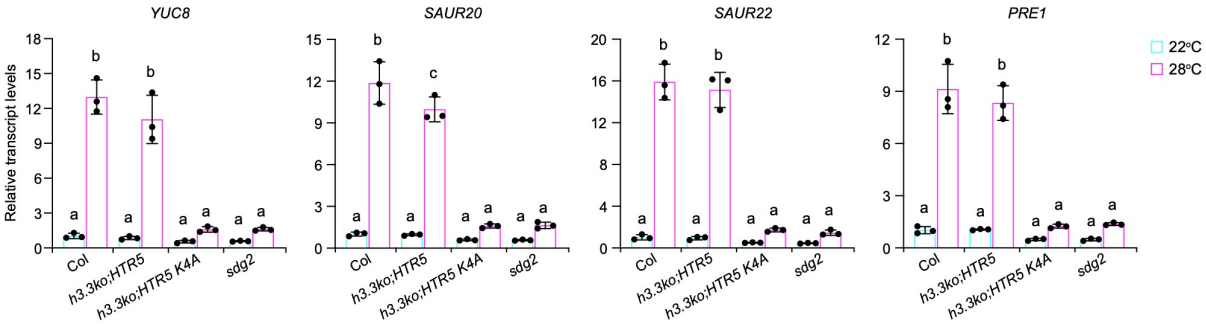


Fig. 4a. Relative chromatin-bound transcript levels of *YUC8*, *SAUR20*, *SAUR22* and *PRE1* determined by RT-qPCR at 22°C and 28°C.