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Peer Review File

A pair of readers of histone H3K4 methylation recruit Polycomb repressive complex 2 to regulate photoperiodic flowering

Corresponding Author: Professor Yuehui He

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)
This study combines structural biology, biochemistry, and genetics to reveal the significant role of AtING1 and AtING2 in photoperiod-induced flowering in Arabidopsis and their epigenetic regulatory mechanisms. The research is comprehensive, with rigorous experimental design and abundant data, offering new insights into the molecular regulation of flowering time in plants.

Here are some minor suggestions:

In Figure 2F, the GUS staining shows a clear difference between the left and right leaves of the same plant, indicating potential variability in the staining process. To confirm the significance of the differences in FT-GUS expression between WT and ing1 ing2 backgrounds, statistical analysis should be performed.

Figure 3A should include 24-hour sampling (ZT0, 4,8,12,16,20,24) to compare FT expression in ing1 ing2 and WT, rather than just sampling at ZT16, as the proposed mechanism suggests that the function of ING1 and ING2 at ZT16 is relatively weak.

Reviewer #2

(Remarks to the Author)
In this study, Luo et al identified the PHD finger domains of AtING1 and AtING2 as specific readers of H3K4me2/3 via peptide binding assays. They further solved the crystal structures of the AtING1 PHD-H3K4me3 and AtING2 PHD-H3K4me3 complexes, providing a basis for the readout of H3K4me3 by AtING1 and AtING2. Analysis of the plants carrying ing1 and/or ing2 mutation indicated that under long-day conditions (LDs), ING1 and ING2 coordinately suppress the expression of florigen gene FT in a H3K4me2/3 interaction-dependent manner. Furthermore, they observed that ING1 enrichment at FT promoter undergoes an oscillation over a LD cycle and is antagonistic to CONSTANS (CO) protein, a transcription factor that activates the expression of FT. In addition, they found that AtING1 and AtING2 both interact with the PRC2 complex, and the CLF enrichment in ing1/ing2 mutant is reduced in the CCAAT and CORE regions. Based on these observations, they conclude that the PHD-H3K4me2/3 interaction-dependent association of AtING1 and AtING2 with FT locus functions to recruit PRC2 complex for suppression of FD expression in LDs. Overall, this study identified a new regulatory mechanism of flowering. The structures are of high quality. The conclusion that AtING1 and AtING2 bind to H3K4me2/3 to regulate expression of FT and flowering is well supported by structural and functional data. Nevertheless, there are some issues that need to be clarified.

1. The functional context of the ING1/2-PRC2 module in regulation of FT expression is not well articulated. Compared with clf81 mutation, the ing1/ing2 double mutation impacted the FT expression rather modestly (Fig. 7c). In the ing1/ing2 plant the FT expression and the CLF/H3K27me3 enrichment at CCAAT and CORE remain oscillated over an LD cycle (Fig. 7c,d and Extended Data Fig. 4d). These data suggest that other PRC2 regulatory pathways might play a dominant role in regulating the FT expression. Do ING1/2 cooperate with PRC1 in regulation of PRC2? The authors acknowledge that “the recruitment

of CLF-PRC2 to FT chromatin is partly dependent on ING1 and ING2" in line 390. But this is not reflected in Fig. 7e. Additional data or discussions are needed to clarify the relative role of ING1/2 in the PRC2 pathway.

2. In the method section, the authors state that the structure of AtING2-PHD-H3K4me3 complex was determined similarly as the AtING1-PHD-H3K4me3 complex structure. However, the data for the AtING2-PHD-H3K4me3 complex was collected on a different wavelength. Was ZN-SAD also used for the structure determination of AtING2-PHD-H3K4me3?

3. The authors need to include discussion to clarify the crystal packing effect on the structures. The H3K4me3 peptide is involved in crystal packing in both complexes. In the ING2-PHD-H3K4me3 complex, residue H3R2 interacts with residue E243 from a symmetry-related PHD domain, suggesting that crystal packing contributes to the structural difference between the two complexes.

4. The I/sigma I values for both complexes are much greater than 2. What is the rationale for such high cutoffs?

5. For the ITC experiments, please provide the N values to clarify the stoichiometry. Error estimates both Kd and N should also be provided.

6. Extended Data Fig. 2. A Fo-Fc or 2Fo-Fc omit map is more appropriate for analysis of the model-map fit.

7. Sequence information for the histone peptides for structure determination and ITC binding need to be provided.

8. English throughout the manuscript needs to be polished. To list a few:
Line 39. Remove word "that".
Line 180. Change "high-level methylation states" to "high-methylation states".
Line 189. Change "Generally" to "Overall".
Line 887. Change "attaches" to "is attached".

Reviewer #3

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The authors suggested that AtING1 and AtING2, the Arabidopsis homologues of the mammalian growth-inhibitory protein (ING), play the role of reader factors that read H3K4me2/me3 on chromatin of the FT gene and may also recruit PRC2 to the FT gene. The authors proposed the hypothesis that this complex suppresses FT expression during the night and early afternoon of the next day, after FT is activated at dusk in LD.

1. This paper can be appreciated as a report of new chromatin readers for the FT gene. However, most of the experiments were conducted concentrating only on the FT locus, and one cannot help but feel that the authors were forced to try to explain all flowering phenomena solely through FT regulation. In particular, we should investigate genome-wide histone modification ChIPs and changes in binding sites, such as how well the newly discovered AtING1 and AtING2 function genome-wide, including other loci.

2. The comparison of the protein structures of AtING1 and AtING2 is commendable. In the middle of page 11, the authors state "in contrast to the high levels of H3K4me2/me3 at ZT16, ING1 did not 296 bind to the FT chromatin, indicating that there is a factor to antagonize ING1 binding at this time point."
However, I do not understand why the antagonist only works at a specific time point.

Reviewer #4

(Remarks to the Author)
Overall, this is an interesting manuscript that merits publication pending resolution of minor concerns. The study elucidates how the H3K4me2/me3-ING1/2-PRC2 axis provides temporal repression of FT expression following its diurnal activation, revealing a critical mechanism for precise flowering time control. While the findings are compelling, several technical and presentational issues should be addressed:

1. The current figure layout requires restructuring to maintain logical flow. All figure panels should be cited sequentially according to their appearance (e.g., Fig. 2e should follow Fig. 2d; Figs. 4b-c should not appear after Fig. 5). Consistent panel ordering between figure presentation and text citation is essential for clarity.
2. The photoperiodic condition "(in LDs)" should be explicitly indicated in Fig. 2d to maintain consistency with other panels and prevent misinterpretation of growth conditions.
3. The differential emphasis on ING1/2 interactions with PRC2 components warrants clarification. Specifically: Does ING1 show equivalent interaction with core PRC2 structural subunits (EMF2, FIE) as ING2 in yeast two-hybrid assays? Conversely, does ING2 interact with EMF2 in Co-IP experiments similar to ING1? The current presentation (lines 365-367, 372-373) appears to overstate functional differences between ING1/2 without conclusive evidence. We recommend presenting complete interaction matrices for both proteins in Fig. 6.
4. The panels in Fig. 7 should be reorganized to follow the experimental chronology and the current text sequence. Showing Fig. 7a-b before7d disrupts logical progression from binding dynamics to functional outcomes.

5. The differential FT expression patterns between clf and ing1/2 mutants (Fig. 7c) require explanation. Given the proposed linear pathway (H3K4me2/me3-ING1/2-PRC2), one would expect similar arrhythmic FT profiles in both mutants.
These revisions would enhance the manuscript's clarity and reinforce the significance of the findings. The core conclusions about temporal coordination of FT repression through histone modification crosstalk are well-supported and advance our understanding of photoperiodic flowering control.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my concerns and I don't have further comments.

Reviewer #2

(Remarks to the Author)
The authors have addressed all of my concerns.

Reviewer #3

(Remarks to the Author)
I carefully checked the revised version of this manuscript.
I confirmed their revision according to reviewers' suggestion and comments.
I do not have additional comments on this manuscript.

Reviewer #4

(Remarks to the Author)
The authors have addressed all of my concerns, so I recommend for publication.

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Response to Referees

We are very grateful to the reviewers for positive comments and insightful suggestions, and have performed relevant experiments to address the raised concerns. The manuscript has been revised accordingly. Our point-to-point responses are presented below (reviewers' comments are in dark blue).

First, we would like to draw your attention on that Figures 2 to 6 have been reorganized to improve logic flow or revised with panel additions. Particularly, we have included the ChIP-qPCR data (Fig. 3c) showing that ING2, like ING1, dynamically binds to *FT* promoter chromatin over a long-day cycle to further solidify the main conclusion that these two proteins function in partial redundancy to regulate photoperiodic flowering.

Reviewer 1

This study combines structural biology, biochemistry, and genetics to reveal the significant role of *AtING1* and *AtING2* in photoperiod-induced flowering in Arabidopsis and their epigenetic regulatory mechanisms. The research is comprehensive, with rigorous experimental design and abundant data, offering new insights into the molecular regulation of flowering time in plants.

We truly appreciate the reviewer's positive comments.

Minor comments:

1. In Figure 2F, the GUS staining shows a clear difference between the left and right leaves of the same plant, indicating potential variability in the staining process. To confirm the significance of the differences in *FT-GUS* expression between WT and *ing1 ing2* backgrounds, statistical analysis should be performed.

Response:
We quantified the homozygous *FT-GUS* expression in WT and *ing1 ing2* backgrounds, and further conducted two-tailed *t*-test analysis on the means difference. Indeed, *FT-GUS* is expressed at a higher level in the double mutant compared to WT (see newly-added Fig. 2h).

2. Figure 3A should include 24-hour sampling (ZT0, 4,8,12,16,20,24) to compare *FT* expression in *ing1 ing2* and WT, rather than just sampling at ZT16, as the proposed mechanism suggests that the function of ING1 and ING2 at ZT16 is relatively weak.

Response:
We compared temporal *FT* expression in *ing1 ing2* and WT over a long-day cycle, and the results are presented in Fig. 6d.

Reviewer 2

In this study, Luo et al identified the PHD finger domains of AtING1 and AtING2 as specific readers of H3K4me2/3 via peptide binding assays. They further solved the crystal structures of the AtING1 PHD-H3K4me3 and AtING2 PHD-H3K4me3 complexes,

providing a basis for the readout of H3K4me3 by AtING1 and AtING2. Analysis of the plants carrying *ing1* and/or *ing2* mutation indicated that under long-day conditions (LDs), *ING1* and *ING2* coordinately suppress the expression of florigen gene *FT* in a H3K4me2/3 interaction-dependent manner. Furthermore, they observed that ING1 enrichment at *FT* promoter undergoes an oscillation over a LD cycle and is antagonistic to CONSTANS (CO) protein, a transcription factor that activates the expression of *FT*. In addition, they found that AtING1 and AtING2 both interact with the PRC2 complex, and the CLF enrichment in *ing1/ing2* mutant is reduced in the CCAAT and CORE regions. Based on these observations, they conclude that the PHD-H3K4me2/3 interaction-dependent association of AtING1 and AtING2 with *FT* locus functions to recruit PRC2 complex for suppression of *FT* expression in LDs. Overall, this study identified a new regulatory mechanism of flowering. The structures are of high quality. The conclusion that AtING1 and AtING2 bind to H3K4me2/3 to regulate expression of *FT* and flowering is well supported by structural and functional data. Nevertheless, there are some issues that need to be clarified.

We truly appreciate the reviewer's positive evaluation.

1. The functional context of the ING1/2-PRC2 module in regulation of *FT* expression is not well articulated. Compared with *clf81* mutation, the *ing1/ing2* double mutation impacted the *FT* expression rather modestly (Fig. 7c). In the *ing1/ing2* plant the *FT* expression and the CLF/H3K27me3 enrichment at CCAAT and CORE remain oscillated over an LD cycle (Fig. 7c,d and Extended Data Fig. 4d). These data suggest that other PRC2 regulatory pathways might play a dominant role in regulating the *FT* expression. Do ING1/2 cooperate with PRC1 in regulation of PRC2? The authors acknowledge that "the recruitment of CLF-PRC2 to *FT* chromatin is partly dependent on ING1 and ING2" in line 390. But this is not reflected in Fig. 7e. Additional data or discussions are needed to clarify the relative role of ING1/2 in the PRC2 pathway.

Response:

Polycomb-mediated repression plays an essential role to constitutively repress *FT* expression regardless of day-length conditions. Loss of function in Polycomb factors such as CLF-PRC2 and *EMBRYONIC FLOWER 1* (EMF1, a PRC1-like factor), results in strong constitutive *FT* de-repression and consequent early flowering in both LDs and SDs[1-3]. Even at dusk in LDs with CO (CO-NF)-mediated *FT* activation, PRC2 is only partly relieved from *FT* promoter (Fig. 6a); hence, in addition to ING1 and ING2, there are additional factors to mediate PRC2 enrichment at *FT*; for instance, it has been shown that the DNA-binding protein VIVIPAROUS1/ABI3-LIKE 1 (VAL1) recruits CLF-PRC2 to *FT* chromatin[4]. INGs function together with CO to mediate daily oscillation of CLF-PRC2 at *FT* in LDs. Loss of *ING1* and *ING2* functions leads to a great reduction in the CLF-PRC2 oscillation. Although the CLF enrichment on *FT* promoter chromatin still oscillates in *ing1 ing2*, the extent of oscillation is reduced compared to WT (Fig. 6a). These and other relevant discussions have been incorporated into the revision (Lines 516-530), to clarify the role of ING1/2 in PRC2-mediated *FT* regulation.

Regarding the functional cooperativity of ING1/2 with PRC1, it is likely that they may cooperate in PRC2 regulation of *FT* expression, but currently we do not have direct evidence.

Fig. 6e (the previous Fig. 7e) as well as the legends have been revised to reflect that the recruitment of CLF-PRC2 to *FT* chromatin is partly dependent on ING1 and ING2.

2. In the method section, the authors state that the structure of AtING2-PHD-H3K4me3 complex was determined similarly as the AtING1-PHD-H3K4me3 complex structure. However, the data for the AtING2-PHD-H3K4me3 complex was collected on a different wavelength. Was ZN-SAD also used for the structure determination of AtING2-PHD-H3K4me3?

Response:
We apologize for this mistake. The structure of AtING2-PHD-H3K4me3 was determined using molecular replacement method with the structure of AtING2-PHD-H3K4me3 complex search model. We have revised the relevant section in Methods.

3. The authors need to include discussion to clarify the crystal packing effect on the structures. The H3K4me3 peptide is involved in crystal packing in both complexes. In the ING2-PHD-H3K4me3 complex, residue H3R2 interacts with residue E243 from a symmetry-related PHD domain, suggesting that crystal packing contributes to the structural difference between the two complexes.

Response:
We thank the reviewer for bringing this to our attention, and have revised the relevant section accordingly.

4. The I/sigma I values for both complexes are much greater than 2. What is the rationale for such high cutoffs?

Response:
There is no rational design for this cutoff. These data are collected with a standard setting at the synchrotron facility. The 1.6-1.7 Å resolution is the edge of the CCD at that setting; thus, we obtained the data only at the resolution range.

5. For the ITC experiments, please provide the N values to clarify the stoichiometry. Error estimates both Kd and N should also be provided.

Response:
Since the figure panel space is limited to incorporate all these parameters, we kept the main figure unchanged. Instead, we have listed these parameters in the newly-added Extended Data Table 1.

6. Extended Data Fig. 2. A Fo-Fc or 2Fo-Fc omit map is more appropriate for analysis of the model-map fit.

Response:
As suggested, the maps have been replaced with Fo-Fc omit maps.

7. Sequence information for the histone peptides for structure determination and ITC binding need to be provided.

Response:
We have added the peptide sequence information to Extended Data Table 4.

8. English throughout the manuscript needs to be polished. To list a few:
Line 39. Remove word "that".
Line 180. Change "high-level methylation states" to "high-methylation states".

Line 189. Change "Generally" to "Overall".
Line 887. Change "attaches" to "is attached".

Response: All corrected.

Reviewer 3

The authors suggested that AtING1 and AtING2, the Arabidopsis homologues of the mammalian growth-inhibitory protein (ING), play the role of reader factors that read H3K4me2/me3 on chromatin of the *FT* gene and may also recruit PRC2 to the *FT* gene. The authors proposed the hypothesis that this complex suppresses *FT* expression during the night and early afternoon of the next day, after *FT* is activated at dusk in LD.

1. This paper can be appreciated as a report of new chromatin readers for the *FT* gene. However, most of the experiments were conducted concentrating only on the *FT* locus, and one cannot help but feel that the authors were forced to try to explain all flowering phenomena solely through *FT* regulation. In particular, we should investigate genome-wide histone modification ChIPs and changes in binding sites, such as how well the newly discovered AtING1 and AtING2 function genome-wide, including other loci.

Response:
We thank the reviewer for this constructive suggestion. A recent genome-wide study has revealed that ING1 occupies 12,989 loci and functions to repress the expression of over one thousand protein-coding genes at a seedling stage[5]. We extracted genome-wide mapping data of H3K4me3 and H3K27me3 distributions in Arabidopsis seedlings we generated previously (NCBI GEO accession nos.: GSE185121 and GSE185120), and observed that more than three quarter of ING1-bound loci overlapped with the list of H3K4me3-bearing genes (Extended Data Fig. 5a, newly added), consistent with ING1 reading H3K4me2/3. Moreover, over 600 ING1-bound loci may carry both H3K4me3 and H3K27me3, and more than a quarter of the ING1-repressed genes appear to be PRC2 targets (Extended Data Fig. 5b and Extended Data Table 3, newly added). These data together suggest that ING1 (and ING2) engage PRC2 to regulate the expression of a subset genes in the Arabidopsis genome. These results have been included into the revision (Lines 445-455).

Notably, the early flowering of *ing1 ing2* is caused by the de-repression of *FT*, because this phenotype is rescued by the *ft* mutation (Fig. 2e).

2. The comparison of the protein structures of AtING1 and AtING2 is commendable. In the middle of page 11, the authors state "in contrast to the high levels of H3K4me2/me3 at ZT16, ING1 did not bind to the *FT* chromatin, indicating that there is a factor to antagonize ING1 binding at this time point." However, I do not understand why the antagonist only works at a specific time point.

Response:
The antagonist, CO, starts to accumulate from the afternoon and reaches a high level only at ZT16 (dusk) in LDs. At night, this protein is degraded rapidly. Hence, the CO protein only functions around dusk.

Through the LD photoperiod pathway, *CO* mRNA expression is gradually upregulated towards the end of the day, and the coincidence of light-dependent CO protein stabilization

with the high level of *CO* mRNA in late afternoon results in a gradual accumulation of the CO protein from late afternoon towards dusk[6].

Reviewer 4

Overall, this is an interesting manuscript that merits publication pending resolution of minor concerns. The study elucidates how the H3K4me2/me3-ING1/2-PRC2 axis provides temporal repression of *FT* expression following its diurnal activation, revealing a critical mechanism for precise flowering time control. While the findings are compelling, several technical and presentational issues should be addressed:

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1. The current figure layout requires restructuring to maintain logical flow. All figure panels should be cited sequentially according to their appearance (e.g., Fig. 2e should follow Fig. 2d; Figs. 4b-c should not appear after Fig. 5). Consistent panel ordering between figure presentation and text citation is essential for clarity.

Response:
As suggested, we have re-structured Fig. 2 to Fig. 4, merging the previous Fig. 4 and Fig. 5 into the current Fig. 4, to improve logical flow and thus reading.

2. The photoperiodic condition "(in LDs)" should be explicitly indicated in Fig. 2d to maintain consistency with other panels and prevent misinterpretation of growth conditions.

Response: Indicated as suggested.

3. The differential emphasis on ING1/2 interactions with PRC2 components warrants clarification. Specifically: Does ING1 show equivalent interaction with core PRC2 structural subunits (EMF2, FIE) as ING2 in yeast two-hybrid assays? Conversely, does ING2 interact with EMF2 in Co-IP experiments similar to ING1? The current presentation (lines 365-367, 372-373) appears to overstate functional differences between ING1/2 without conclusive evidence. We recommend presenting complete interaction matrices for both proteins in Fig. 6.

Response:
Under stringent selection using the SD (-WLHA) dropout medium, we observed no interaction between ING1 and EMF2 or FIE in yeast cells. Subsequently, we conducted bimolecular fluorescence complementation (BiFC) assays, and found that ING1 was able to interact with both EMF2 and FIE in the nuclei of *Nicotiana benthamiana* epidermal cells (Extended Data Fig. 4b). In addition, we conducted the suggested co-IP experiments to confirm that ING2 indeed associates with EMF2 in Arabidopsis seedlings (Fig. 5f).

4. The panels in Fig. 7 should be reorganized to follow the experimental chronology and the current text sequence. Showing Fig. 7a-b before7d disrupts logical progression from binding dynamics to functional outcomes.

Response:
Fig. 6 (the previous Fig. 7) has been re-organized as suggested.

5. The differential *FT* expression patterns between *clf* and *ing1/2* mutants (Fig. 7c) require explanation. Given the proposed linear pathway (H3K4me2/me3-ING1/2-PRC2), one would expect similar arrhythmic *FT* profiles in both mutants.

Response:

Polycomb-mediated repression plays an essential role to constitutively repress *FT* expression regardless of day-length conditions. Loss of function in Polycomb factors such as CLF-PRC2 and *EMBRYONIC FLOWER 1* (EMF1, a PRC1-like factor), results in strong constitutive *FT* de-repression and consequent early flowering in both LDs and SDs[1-3]. Even at dusk in LDs with CO (CO-NF)-mediated *FT* activation, PRC2 is only partly relieved from *FT* promoter (Fig. 6a); hence, in addition to ING1 and ING2, there are additional factors to mediate PRC2 enrichment at *FT*; for instance, it has been shown that the DNA-binding protein VIVIPAROUS1/ABI3-LIKE 1 (VAL1) recruits CLF-PRC2 to *FT* chromatin[4]. INGs function together with CO to mediate daily oscillation of CLF-PRC2 at *FT* in LDs. Loss of *ING1* and *ING2* functions leads to a reduction in CLF-PRC2 enrichment on *FT* chromatin, but does not eliminate its enrichment (Fig. 6a). Hence, the *FT* expression profiles in *clf* and *ing1 ing2* are different. We have included a discussion on Polycomb-mediated *FT* repression (Lines 516-530).

References cited:

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2. Wang, Y., Gu, X., Yuan, W., Schmitz, R.J. & He, Y. Photoperiodic control of the floral transition through a distinct Polycomb repressive complex. *Dev Cell* **28**, 727-736 (2014).
3. Luo, X. *et al.* The NUCLEAR FACTOR-CONSTANS complex antagonizes Polycomb repression to de-repress *FLOWERING LOCUS T* expression in response to inductive long days in Arabidopsis. *Plant J* **95**, 17-29 (2018).
4. Jing, Y., Guo, Q. & Lin, R. The B3-domain transcription factor VAL1 regulates the floral transition by repressing *FLOWERING LOCUS T*. *Plant Physiol* **181**, 236-248 (2019).
5. Wu, C.J. *et al.* Conserved and plant-specific histone acetyltransferase complexes cooperate to regulate gene transcription and plant development. *Nat Plants* **9**, 442-459 (2023).
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