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Epigenetic maintenance of the vernalized state in *Arabidopsis thaliana* requires LIKE HETEROCHROMATIN PROTEIN 1

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Vernalization is the process by which sensing a prolonged exposure to winter cold leads to competence to flower in the spring. In winter annual *Arabidopsis thaliana* accessions, flowering is suppressed in the fall by expression of the potent floral repressor FLOWERING LOCUS C (FLC)[1]. Vernalization promotes flowering via epigenetic repression of *FLC*[2]. Repression is accompanied by a series of histone modifications of *FLC* chromatin that include dimethylation of histone H3 at Lys9 (H3K9) and Lys27 (H3K27)[3,4]. Here, we report that *A. thaliana* LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) is necessary to maintain the epigenetically repressed state of *FLC* upon return to warm conditions typical of spring. LHP1 is enriched at *FLC* chromatin after prolonged exposure to cold, and LHP1 activity is needed to maintain the increased levels of H3K9 dimethylation at *FLC* chromatin that are characteristic of the vernalized state.

In many plant species, the timing of flowering is affected by seasonal cues. Plants that flower early in the spring often have a vernalization requirement; such plants require prolonged exposure to winter cold in order to achieve competence to flower. That vernalization typically requires prolonged cold permits plants to discriminate between transient warm periods in the fall and the actual arrival of spring[2].

In *A. thaliana*, vernalization promotes flowering primarily by repressing the flowering repressor FLOWERING LOCUS C (*FLC*)[5,6]. Vernalization-mediated *FLC* repression is epigenetic in the sense that it is maintained throughout subsequent mitotic cell divisions in the shoot apical meristem during spring growth. Expression of *FLC* resumes in the next generation, thus perpetuating the winter-annual growth habit[1].

Vernalization-mediated *FLC* repression is accompanied by deacetylation of H3K9 and H3K14 and methylation of H3K9 and H3K27 (refs. 3,4), which are likely to result in formation of a mitotically stable heterochromatin-like state at *FLC*. Maintenance of *FLC* repression requires VERNALIZATION2 (VRN2), the *A. thaliana* homolog of Suppressor of zeste 12 (Su(z)12)[7]. In animals, Su(z)12 is a component of Polycomb repression complex 2 (PRC2), a complex involved in maintaining Polycomb group (PcG) repression of target genes through methylation of H3K27 (ref. 8). VRN2 is likely to have a role similar to Su(z)12 in *FLC* repression, because in *vrn2* mutants the level of H3K27 methylation of *FLC* chromatin does not increase during cold exposure, and *FLC* repression is not maintained in subsequent cell division cycles[3,4]. In addition, *A. thaliana* has other PRC2 components; for example, there are three homologs of the H3K27 methyltransferase Enhancer of zeste (E(z))[9]. Thus, both animals and plants seem to use PRC2–type epigenetic repression involving H3K27 methylation.

In animals, maintenance of PRC2-mediated repression requires Polycomb repression complex 1 (PRC1)[8]. When H3K27 is methylated, PRC1 binds to histone H3 via the chromodomain of one of its components, Polycomb protein (Pc)[8]. Notably, no homologs of PRC1 components have been identified in plants[9]. Therefore, plants are likely to have a system different from that of animals for the maintenance of PcG-type silencing. Indeed, in animals, methylation of H3K27 seems to be a hallmark of genes that are stably repressed by PcG-mediated gene silencing[8], but the stable repression of *FLC* by vernalization requires methylation of not only H3K27 but also H3K9 (refs. 3,4).

One component of the system that might have a PRC1-like role in plants is the homolog of HETEROCHROMATIN PROTEIN 1 (HP1), LIKE HETEROCHROMATIN PROTEIN 1 (LHP1; also called TERMINAL FLOWER 2)[10,11]. In animals and yeast, HP1 preferentially localizes to, and is involved in maintenance of, constitutive heterochromatin, such as pericentric regions[12], although there are examples of a role for HP1 in the regulation of euchromatic genes in *Drosophila melanogaster* and mammals[13,14]. HP1 binds to chromatin by interacting with methylated H3K9 (ref. 12), a modification associated with maintenance of the vernalized state[3,4]. In plants, LHP1 localizes

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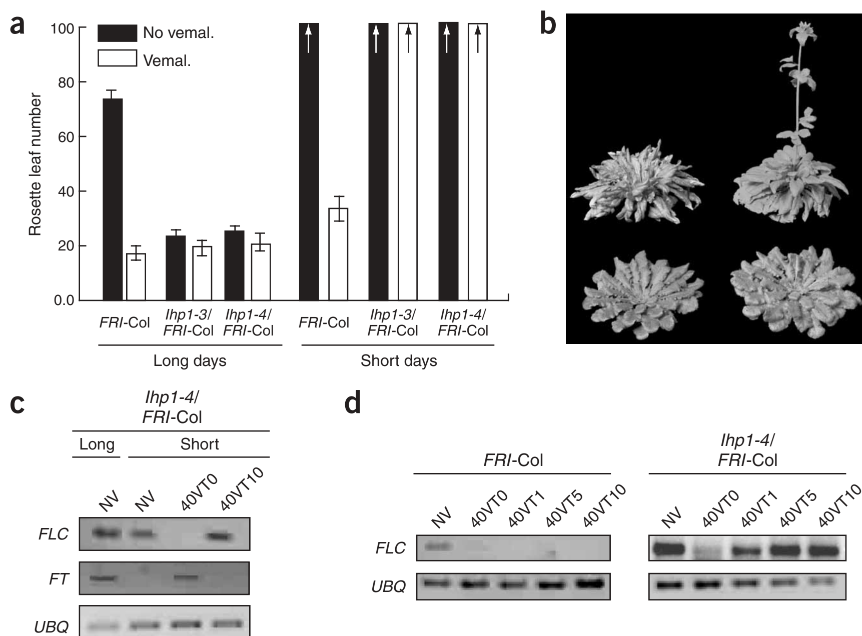

Figure 1 The vernalization response is impaired in *lhp1* mutants. (**a**) Flowering behavior of *lhp1* mutants with and without vernalization in long days and short days. Flowering was measured as the number of rosette leaves produced from primary meristems prior to flowering. Arrows at tops of bars indicate that plants had not flowered after producing more than 100 primary rosette leaves. (**b**) *lhp1* lesion blocks the vernalization response. *FRI*-containing winter annual lines without (upper left) and with (upper right) vernalization in short days. *lhp1-4*/*FRI*-Col lines without (lower left) and with (lower right) vernalization in short days. (**c**) mRNA levels of *FLC* and *FT* in *lhp1-4/FRI*-Col. *FT* levels are elevated in *lhp1-4/FRI*-Col, causing early flowering in long days despite higher levels of *FLC*. However, *FT* is only transiently expressed when *FLC* is transiently repressed during vernalization in short days. (**d**) Repression of *FLC* is not maintained in *lhp1-4/FRI*-Col.

primarily to euchromatic regions (**Supplementary Fig. 1** online)[15–17], similar to the distribution of PRC1 in animals[8]. Furthermore, LHP1 does not seem to be required for repression of loci that are silenced by DNA methylation, including pericentric regions, but it may have a direct role in the stable repression of euchromatic genes[15,18].

To evaluate whether *LHP1* has a role in vernalization, we introduced the *lhp1* mutation into the vernalization-responsive genetic background, *FRI*-Col (flowering of *FRI*-Col plants is extremely delayed in inductive photoperiods, unless the plants are vernalized[19] (**Fig. 1a**)). In long days without exposure to cold, however, the *lhp1* lesion in *FRI*-Col (*lhp1/FRI*-Col) causes rapid flowering (**Fig. 1a**); therefore, growth in long days precludes assessing the vernalization response. In contrast, without vernalization, *FRI*-Col plants with or without the *lhp1* lesion often fail to flower in noninductive photoperiods (that is, short days; **Fig. 1a**,**b**). Thus, growth in short days provides a means to assess the role of *LHP1* in vernalization. In short days, cold treatment strongly promotes flowering of the *FRI*-Col line but has no effect on the flowering of *lhp1/FRI*-Col (**Fig. 1a**,**b**), demonstrating that *LHP1* is required for the vernalization response.

To determine which phase of *FLC* silencing requires *LHP1*, we monitored *FLC* levels during and after cold exposure. *FLC* mRNA levels declined during cold exposure in *lhp1/FRI*-Col, similar to wild-type plants, but after we returned *lhp1/FRI*-Col plants to warm conditions, *FLC* mRNA increased to prevernalization levels (**Fig. 1c**,**d**)[20]. Thus, *LHP1* is required for the maintenance of *FLC* repression.

The ability of the *lhp1* mutation to suppress the *FLC*-mediated delay of flowering in long days but not in short days (**Fig. 1a**) is associated with differential expression of the floral promoter *FT* in the different photoperiods (**Fig. 1c**). In long days, *FT* expression is elevated in *lhp1/FRI*-Col plants despite high levels of *FLC*; in contrast, in short days, *FT* levels are low. In the absence of elevated *FLC* expression, *lhp1* lesions result in early flowering and elevated *FT* expression in short days[11,15]. Thus, *FLC* can repress *FT* in short days but not in long days in *lhp1* mutants. This photoperiod-dependent *FLC* repression of *FT* is likely to result from the differential activity of CONSTANS (CO), a promoter of *FT* expression[21], in long and short days. Specifically, the decreased CO activity in short days may shift the balance of promotive and repressive activities such that *FLC* is able to repress *FT* in short days in the absence of *LHP1*. Notably, in *lhp1/FRI*-Col, *FT* is only expressed while *FLC* is transiently repressed (**Fig. 1c**), indicating that the resumption of *FLC* expression may prevent the positive autoregulation of *FT* that was recently reported to occur in a rapid-cycling genetic background that does not express *FLC* to levels that repress flowering[22].

To test whether the association of LHP1 with *FLC* chromatin is enriched by vernalization, we performed chromatin immunoprecipitation (ChIP) assays using transgenic lines expressing a functional GFP-tagged LHP1. During vernalization, LHP1 association with *FLC* chromatin increases and the association is maintained in warm conditions (**Fig. 2**), supporting a direct *in vivo* role for LHP1 in the formation of repressive chromatin at *FLC*. Regions of *FLC* chromatin

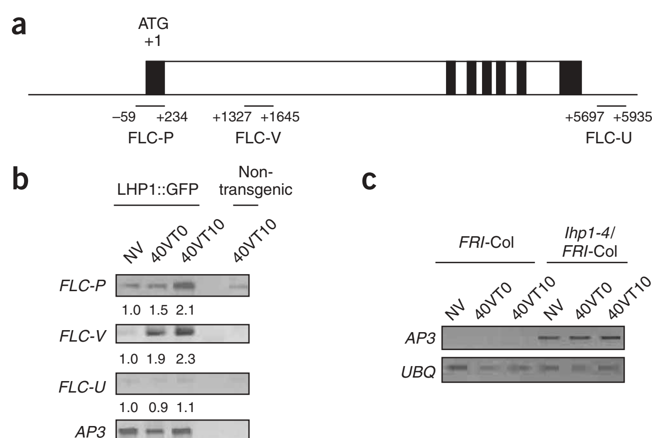

Figure 2 Role of LHP1 in *FLC* repression. (**a**) Relative positions of amplified *FLC* regions used in the ChIP assay. (**b**) ChIP assay using antibody against GFP and chromatin from transgenic plants carrying GFP-tagged LHP1 with and without vernalization. Three regions were examined for LHP1 enrichment in *FLC* chromatin (**a**). *AP3* was used for a loading control, because *AP3* is also repressed by LHP1 (ref. 15) and its repression is not affected by vernalization (**c**). (**c**) RT-PCR analysis of *AP3* expression in the *lhp1* mutant with and without vernalization.

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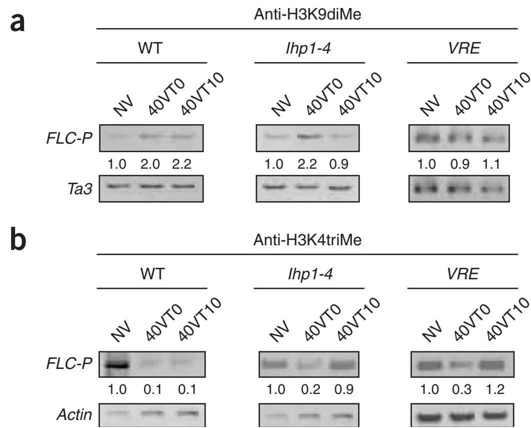

Figure 3 ChIP analyses of *FLC* chromatin. (**a**) H3K9 dimethylation levels of *FLC* chromatin before, during and after vernalization. (**b**) H3K4 trimethylation levels of *FLC* chromatin before, during and after vernalization. WT, wild-type.

enriched in LHP1 by vernalization (**Fig. 2b**) correspond to regions in which vernalization results in increased H3K9 dimethylation[3].

In animals, HP1 interacts with the Suvar3-9 class of histone methyltransferases to maintain the methylated state of H3K9 in newly assembled nucleosomes during cell division[12]. In *lhp1* mutants, the vernalization-mediated dimethylation of *FLC* chromatin on H3K9 is not maintained (**Fig. 3**). Notably, H3K9 dimethylation at *FLC* still occurs in the absence of *LHP1* during a vernalizing cold exposure (**Fig. 3a**). Thus, LHP1 is not required for the initiation of H3K9 dimethylation, but is essential for maintenance of H3K9 dimethylation and the silenced state of *FLC* after plants are returned to warm conditions.

In *D. melanogaster*, PcG and Trithorax group proteins act on target genes by association with specific DNA regulatory elements referred to as Polycomb and Trithorax response elements (PREs and TREs)[8]. A 2.8-kb region of the first intron of *FLC* is required for stable maintenance of *FLC* repression[23]. We find that a deletion of just 289 bp is sufficient to prevent maintenance of the silenced state of *FLC* (**Fig. 4a**; region labeled VRE for vernalization response element). Specifically, a deletion of this region in the first intron does not compromise *FLC* activation, nor does it prevent the repression of *FLC* expression during cold exposure (**Fig. 4b**); however, the VRE deletion prevents *FLC* repression from being maintained after a return to warm conditions (**Fig. 4b**) and it prevents vernalization from causing more rapid flowering (**Fig. 4c**).

Unlike the case in *lhp1* mutants in which H3K9 dimethylation increases only transiently during cold exposure, there is no increase in H3K9 dimethylation of *FLC* chromatin in the VRE deletion (**Fig. 3a**). Thus, the VRE region is required for the initial vernalization-mediated increase in H3K9 dimethylation. Whether or not the same H3K9 histone methyltransferase is involved in modifying *FLC* chromatin during and after cold exposure remains to be determined. Given that some of the proteins involved in the initiation versus maintenance phases of vernalization are different, and that there are 15 SET domain proteins that are predicted to methylate H3K9 in *A. thaliana*[24], it is possible that different histone methyltransferases are involved at different stages of vernalization.

The intronic DNA component defined by the VRE deletion may act in vernalization-mediated *FLC* repression similar to a PRE in PcG-mediated repression. The binding of the *D. melanogaster* Dorsal switch protein 1 (DSP1) to a minimal PRE can initiate recruitment of chromatin-remodeling complexes, including PRC2 and PRC1 (ref. 25). Similarly, the VRE region may serve as a site of recruitment of a vernalization-specific, chromatin-remodeling complex that is required for the initial increase in H3K9 dimethylation. Elevated H3K9 dimethylation alone is not sufficient to recruit LHP1, because not all chromatin with elevated levels of H3K9 dimethylation is associated with LHP1 (for instance, chromocenters; **Supplementary Figure 1**); therefore, additional factors, perhaps associated with the VRE, are required for LHP1 recruitment and stable silencing of euchromatic genes.

The failure to maintain *FLC* repression and the lack of a vernalization-mediated increase in H3K9 methylation at *FLC* chromatin in the VRE deletion are similar to the phenotypes of *vrn1* and *vrn2* mutants[3,4]. As in *vrn1* and *vrn2*, the initial repression of *FLC* during cold exposure occurs in the VRE deletion (**Fig. 4c**), indicating that this initial repression is mediated by other *cis* elements in *FLC* and does not involve increased H3K9 dimethylation.

The presence of *FRI*, which activates *FLC*, results in a higher level of H3K4 trimethylation at *FLC* chromatin than when *FRI* is absent[26]. During vernalization of *FRI*-Col, however, the level of H3K4 trimethylation in *FLC* chromatin is decreased, and this decrease is maintained after vernalization (**Fig. 3b**). Notably, in *lhp1* mutants the level of H3K4 trimethylation is also decreased during vernalization, but the reduced level is not maintained after plants are returned to warm conditions (**Fig. 3b**). Thus, *LHP1* is required to maintain the low level of H3K4 methylation associated with vernalization-mediated *FLC* silencing, but *LHP1* is not required for the initial decrease of H3K4 trimethylation during cold exposure. Similar to the *lhp1* mutant, trimethylated H3K4 levels at *FLC* chromatin are transiently reduced in the chromatin of the VRE deletion (**Fig. 3b**).

In animals, H3K4 trimethylation and active transcription of certain genes is mediated by a number of chromatin-remodeling proteins, including the PAF1 complex, which recruits the Trithorax group gene ASH1-like H3K4 methyltransferase[27]. PcG proteins act antagonistically to PAF1 and Trithorax group genes to maintain gene repression[8]. In *A. thaliana*, *FLC* regulation also seems to be mediated by antagonistic chromatin remodeling complexes. Active transcription and H3K4 methylation of *FLC* requires a PAF1 homolog[26] as well as an

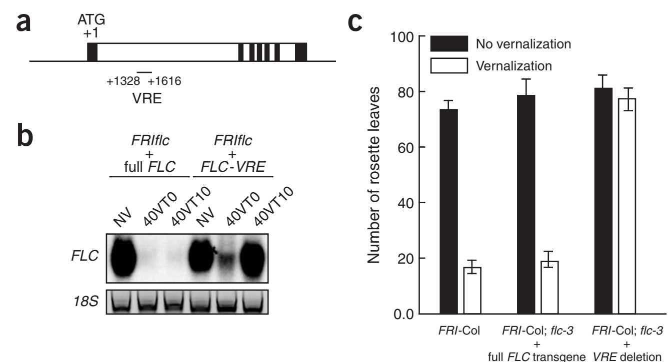

Figure 4 A region in the first intron of *FLC* is essential for maintenance of the vernalized state. (**a**) Location of an intronic region (VRE) required for maintenance. (**b**) RNA blot analyses of *FLC* expression before, during and after vernalization in lines described in **a** and **c**. (**c**) Flowering behavior of lines carrying either a full-length *FLC* construct or the construct with a VRE deletion in an *FRIflc-3* background. Vernalization response of representative homozygous T3 lines.

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ASH1-related H3K4 methyltransferase[28]. After a vernalizing cold exposure, the maintenance of the silenced state of *FLC* requires the association of LHP1 with *FLC* chromatin (**Fig. 2b**). This association might help to prevent complexes like PAF1 from activating *FLC* expression. Because *FLC* expression and H3K4 trimethylation transiently decrease during exposure to cold in *lhp1* mutants, the initial *FLC* repression system may involve other repressive complexes that compete with activating complexes.

In summary, vernalization-mediated epigenetic repression of *FLC* requires LHP1 as well as an intronic DNA component, and vernalization results in an increase in the localization of LHP1 at *FLC* chromatin. It is likely that LHP1 has a similar direct role in the PRC2-type repression of other genes in *A. thaliana* such as the floral homeotic gene AGAMOUS (*AG*)[15], which is repressed by the E(z) homolog *CLF*[9]. A common theme for epigenetically stable euchromatic gene repression in both plants and other organisms is the 'histone code hypothesis'—a requirement for specific histone modifications and binding of proteins that recognize such modifications[29]. However, in the absence of PRC1 in plants, LHP1 may have a central role in the creation of mitotically stable repression of euchromatic genes.

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METHODS

Plant materials. We used the terminal flower2 mutants *tfl2-1* and *tfl2-2* (hereafter referred to as *lhp1-3* and *lhp1-4*, respectively)[18]. *lhp1-3* and *lhp1-4* in Columbia were introgressed into *FRI*-Col[19].

Vernalization treatment. Seeds were germinated on agar plates for 5 d at 22 °C and vernalized for 40 d at 4 °C under 8 h of light and 16 h of dark. Post-vernalization samples continued to grow on agar plates under 8 h of light and 16 h of dark at 22 °C. All plants samples were prepared at 10 a.m. (2 h after light was turned on).

ChIP analysis. Constructs carrying LHP1::GFP were transformed into *lhp1-4* in *FRI*-Col, and homozygous lines were identified at T3 generation and used for ChIP analysis. Independently, *lhp1-4* in Col lines that carry LHP1::GFP were crossed to *FRI*-Col and F3 lines that are homozygous for both *FRI* and LHP1::GFP were used to confirm ChIP analysis. GFP antibodies were purchased from Molecular Probes. Antibodies against dimethylated H3K9 were purchased from Upstate. Both antibodies produced similar results. For biotin-conjugated antibodies, streptavidin agarose (Upstate) was used to precipitate immune complexes. ChIP assays were performed using a ChIP assay kit from Upstate following the manufacturer's suggested protocol, modified as previously described[30]. To detect the *FLC-P* region, we used a pair of primers that do not amplify a fragment from the *flc-3* deletion allele[5] (for primer sequences, see **Supplementary Table 1** online). FLC-V, FLC-U, and AP3 were amplified (for primer sequences, see **Supplementary Table 1**). The amount of chromatin was calculated as a multiple of the control using ImageQuant. All ChIP assays were performed at least three times from at least two biological replicates and produced similar results.

RNA analysis. Total RNA was isolated using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. First-strand cDNA synthesis was performed on 2 μg of RNA using the M-MLV System for RT-PCR (Promega) followed by PCR amplification with ExTaq DNA Polymerase (Takara Mirus) according to the manufacturer's recommendations. *FLC*, *UBIQUITIN*, *FT* and *AP3* were amplified (for primer sequences, see **Supplementary Table 1** online). Amplified fragments were separated on a 3% or 1.5% agarose gel. For RNA blot analysis, 15 μg of total RNA was separated on denaturing gel and transferred to a nylon membrane. The 3′-UTR of *FLC* was used as a probe. 18S RNA was visualized on the gel by ethidium bromide staining.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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