anti-L-selectin monoclonal antibody Mel-14 (100 µg per mouse). At various time points during the following 48 h, mice were anaesthetized by an initial intraperitoneal injection of ketamine (50 mg kg^{-1}) and xylazine (10 mg kg^{-1}). The right popliteal LN was prepared microsurgically for intravital microscopy and positioned on a custom-built microscope stage. Care was taken to spare blood vessels and afferent lymph vessels. The prepared LN was submerged in normal saline and covered with a glass coverslip. A thermocouple was placed next to the LN to monitor local temperature, which was maintained at 36 °C. In some experiments the LN microcirculation was revealed by intravenous injection of 2% tetramethylrhodamine β-isothiocyanate–dextran or fluorescein isothiocyanate–dextran (500 or 2,000 kDa; Molecular Probes). Two-photon imaging was performed with an Olympus BX50WI fluorescence microscope equipped with a 20×, 0.95 numerical aperture objective (Olympus) and a Bio-Rad Radiance 2000MP Confocal/Multiphoton microscopy system, controlled by Lasersharp software (Bio-Rad). For two-photon excitation and second harmonic generation, a Tsunami Ti:sapphire laser with a 10-W MilleniaXs pump laser (Spectra-Physics) was tuned to 800 nm.

For four-dimensional analysis of cell migration, stacks of six squared x–y sections with 6 µm z spacing were acquired every 15 s with electronic zooming up to 4× to provide image volumes 30 µm in depth and 154–618 µm in width. Emitted light and second harmonic signals were detected through 400/40-nm, 525/50-nm and 620/100-nm band-pass filters with non-descanned detectors to generate three-colour images. Sequences of image stacks were transformed into volume-rendered four-dimensional movies using Volocity software (Improvision), which was also used for semi-automated tracking of cell motility in three dimensions. From x, y and z coordinates of cell centroids, parameters of cellular motility were calculated by using custom scripts in Matlab (MathWorks). As a measure of the cells' propensity to move away from an arbitrary point of origin, we calculated the motility coefficient by plotting each cell's absolute displacement against the square root of the time interval during which displacement occurred[2]. Interactions between T cells and DCs were defined as physical contacts lasting more than 1 min. Only contacts whose initiation and termination was observed or that lasted for the entire observation period (60 min) were included in the analysis.

Statistical analysis

Non-normally distributed data were presented as medians and compared with the Mann–Whitney U-test (two groups) or the Kruskal–Wallis test followed by Dunn's test to compare multiple samples. Otherwise, data are shown as means ± s.e.m. where appropriate.

1. Stoll, S., Delon, J., Brotz, T. M. & Germain, R. N. Dynamic imaging of T cell–dendritic cell interactions in lymph nodes. *Science* **296,** 1873–1876 (2002).
2. Miller, M. J., Wei, S. H., Parker, I. & Cahalan, M. D. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* **296,** 1869–1873 (2002).
3. Bousso, P. & Robey, E. Dynamics of CD8$^+$ T cell priming by dendritic cells in intact lymph nodes. *Nature Immunol.* **4,** 579–585 (2003).
4. Gowans, J. L. & Knight, E. J. The route of re-circulation of lymphocytes in the rat. *Proc. R. Soc. Lond. B* **159,** 257–282 (1964).
5. von Andrian, U. H. Immunology. T cell activation in six dimensions. *Science* **296,** 1815–1817 (2002).
6. Cahalan, M. D., Parker, I., Wei, S. H. & Miller, M. J. Two-photon tissue imaging: seeing the immune system in a fresh light. *Nature Rev. Immunol.* **2,** 872–880 (2002).
7. von Andrian, U. H. & Mempel, T. R. Homing and cellular traffic in lymph nodes. *Nature Rev. Immunol.* **3,** 867–878 (2003).
8. von Andrian, U. H. Intravital microscopy of the peripheral lymph node microcirculation in mice. *Microcirculation* **3,** 287–300 (1996).
9. Miller, M. J., Wei, S. H., Cahalan, M. D. & Parker, I. Autonomous T cell trafficking examined *in vivo* with intravital two-photon microscopy. *Proc. Natl Acad. Sci. USA* **100,** 2604–2609 (2003).
10. Gallatin, W. M., Weissman, I. L. & Butcher, E. C. A cell-surface molecule involved in organ-specific homing of lymphocytes. *Nature* **304,** 30–34 (1983).
11. Gunn, M. D. *et al.* Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J. Exp. Med.* **189,** 451–460 (1999).
12. Sallusto, F. *et al.* Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur. J. Immunol.* **28,** 2760–2769 (1998).
13. Bajenoff, M., Granjeaud, S. & Guerder, S. The strategy of T cell antigen-presenting cell encounter in antigen-draining lymph nodes revealed by imaging of initial T cell activation. *J. Exp. Med.* **198,** 715–724 (2003).
14. Langenkamp, A., Messi, M., Lanzavecchia, A. & Sallusto, F. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nature Immunol.* **1,** 311–316 (2000).
15. Saxton, M. J. & Jacobson, K. Single-particle tracking: applications to membrane dynamics. *Annu. Rev. Biophys. Biomol. Struct.* **26,** 373–399 (1997).
16. Tang, H. L. & Cyster, J. G. Chemokine up-regulation and activated T cell attraction by maturing dendritic cells. *Science* **284,** 819–822 (1999).
17. Dustin, M. L., Bromley, S. K., Zhengyan, K., Peterson, D. A. & Unanue, E. R. Antigen receptor engagement delivers a stop signal to migrating T lymphocytes. *Proc. Natl Acad. Sci. USA* **94,** 3909–3913 (1997).
18. Monks, C. R. F., Freiberg, B. A., Kupfer, H., Sciaky, N. & Kupfer, A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* **394,** 82–86 (1998).
19. Grakoui, A. *et al.* The immunological synapse: a molecular machine controlling T cell activation. *Science* **285,** 221–227 (1999).
20. Lee, K. H. *et al.* T cell receptor signaling precedes immunological synapse formation. *Science* **295,** 1539–1542 (2002).
21. Gunzer, M. *et al.* Antigen presentation in extracellular matrix: interactions of T cells with dendritic cells are dynamic, short lived, and sequential. *Immunity* **13,** 323–332 (2000).
22. Ingulli, E., Mondino, A., Khoruts, A. & Jenkins, M. K. *In vivo* detection of dendritic cell antigen presentation to CD4$^+$ T cells. *J. Exp. Med.* **185,** 2133–2141 (1997).
23. Norbury, C. C., Malide, D., Gibbs, J. S., Bennink, J. R. & Yewdell, J. W. Visualizing priming of virus-specific CD8$^+$ T cells by infected dendritic cells *in vivo*. *Nature Immunol.* **3,** 265–271 (2002).
24. Murphy, K. M., Heimberger, A. B. & Loh, D. Y. Induction by antigen of intrathymic apoptosis of CD4$^+$ CD8$^+$ TCR^{lo} thymocytes *in vivo*. *Science* **250,** 1720–1723 (1990).
25. Stefanova, I., Dorfman, J. R. & Germain, R. N. Self-recognition promotes the foreign antigen sensitivity of naive T lymphocytes. *Nature* **420,** 429–434 (2002).
26. Faroudi, M., Zaru, R., Paulet, P., Muller, S. & Valitutti, S. Cutting edge: T lymphocyte activation by repeated immunological synapse formation and intermittent signaling. *J. Immunol.* **171,** 1128–1132 (2003).
27. van Stipdonk, M. J. *et al.* Dynamic programming of CD8$^+$ T lymphocyte responses. *Nature Immunol.* **4,** 361–365 (2003).
28. Hurez, V. *et al.* Restricted clonal expression of IL-2 by naive T cells reflects differential dynamic interactions with dendritic cells. *J. Exp. Med.* **198,** 123–132 (2003).
29. Pircher, H., Burki, K., Lang, R., Hengartner, H. & Zinkernagel, R. M. Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. *Nature* **342,** 559–561 (1989).
30. Mora, J. R. *et al.* Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* **424,** 88–93 (2003).

Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3

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In biennials and winter annuals, flowering is typically blocked in the first growing season. Exposure to the prolonged cold of winter, through a process called vernalization, is required to alleviate this block and permit flowering in the second growing season[1]. In winter-annual types of *Arabidopsis thaliana*, a flowering repressor, *FLOWERING LOCUS C* (*FLC*), is expressed at levels that inhibit flowering in the first growing season[2]. Vernalization promotes flowering by causing a repression of *FLC* that is mitotically stable after return to warm growing conditions[2]. Here we identify a gene with a function in the measurement of the duration of cold exposure and in the establishment of the vernalized state. We show that this silencing involves changes in the modification of histones in *FLC* chromatin.

In winter-annual types of *Arabidopsis*, flowering is delayed unless plants are vernalized. The delayed flowering is due to dominant alleles of *FRIGIDA* (*FRI*) and *FLC*[3]. *FRI* elevates expression of the MADS-box transcriptional regulator *FLC* to levels that suppress flowering[4,5]. Vernalization promotes flowering primarily by repressing *FLC* expression[4–7]. The repressed state of *FLC* is maintained through mitotic cell divisions after a return to warm growing conditions[2]. Many summer-annual accessions of *Arabidopsis* flower rapidly without vernalization because such accessions lack an active *FRI* allele[8,9] or have a weak *FLC* allele[9,10] and thus have low levels of *FLC* expression.

To investigate the molecular mechanism of vernalization, we mutagenized a winter-annual *Arabidopsis* line (the Columbia accession into which an active *FRI* allele had been introgressed[3]) and

screened for mutants insensitive to vernalization. In this screen we found additional alleles of *vernalization 1* and *2* (*vrn1* and *vrn2*); these genes are necessary to maintain the repressed state of *FLC* after vernalization[11,12]. We also identified three independent mutants that defined a new complementation group, *vernalization-insensitive 3* (*vin3-1*, *vin3-2* and *vin3-3*). In the *vin3* mutants, the vernalization response was completely blocked (Fig. 1a, b).

In *Arabidopsis*, the autonomous pathway defines a group of genes that suppress *FLC* expression[13]. Autonomous-pathway mutants in a summer-annual background exhibit a vernalization-responsive late-flowering phenotype similar to winter annuals because both have a greater expression of *FLC* before vernalization[6,7,13]. The *vin3* lesion blocks the vernalization response in the autonomous-pathway mutant *luminidependens* (Fig. 1c). Vernalization also promotes flowering independently of *FLC* repression; specifically, vernalization can cause more rapid flowering in short days in both the wild type and a *flc*-null mutant[7]. The promotion of flowering by vernalization in short days is prevented in a *vin3 flc* double mutant (Fig. 1d). Thus, *VIN3* is required for all situations, both *FLC*-dependent and *FLC*-independent, in which vernalization can promote flowering. In both the parental winter-annual *FRI*-containing line and in the summer-annual Col background (*fri*), the *vin3* mutant displayed no pleiotropic phenotypes and had no effect on flowering other than the block to vernalization (Fig. 1a, b, and data not shown).

In *vin3* mutants, a vernalizing cold treatment is not able to repress *FLC* expression during and after cold exposure (Fig. 1e). This is in contrast to the behaviour of *vrn1* and *vrn2* mutants in which vernalization-mediated *FLC* repression is observed during the

period of cold exposure; in other words, *vrn1* and *vrn2* behave like the wild type with regard to *FLC* repression during a vernalizing cold treatment, but this repression is not maintained in warm growing conditions (Fig. 2b, c, and refs 11 and 12). Thus, unlike *VRN1* and *VRN2*, which are involved in the maintenance of *FLC* repression, *VIN3* has a function in the establishment of *FLC* repression during vernalization.

We cloned *VIN3* by a map-based approach (a rescued line is shown in Fig. 1a; *VIN3* is At5g57380; see www.arabidopsis.org). *VIN3* is composed of four exons encoding a 600-amino-acid protein that contains a putative nuclear localization signal and two domains found in other proteins (Fig. 2a): a PHD (plant homeodomain) domain, which is often found in proteins in chromatin remodelling complexes[14], and a FNIII (fibronectin type III) domain, which is often involved in protein–protein interactions[15].

A requirement for vernalization is an adaptation for ensuring that flowering does not occur before the onset of winter. To prevent flowering during short-term temperature fluctuations in the autumn, the vernalization process has evolved to distinguish a long exposure to cold from shorter cold exposures[2]. The pattern of *VIN3* induction is consistent with a role for *VIN3* in the process of distinguishing the duration of cold exposure (Fig. 2b). *VIN3* is expressed only after a duration of cold exposure that is effective for vernalization, and *FLC* repression does not occur until *VIN3* is induced (Fig. 2b). Moreover, the level of *VIN3* expression is correlated with both the duration of cold and the degree of *FLC* repression; after a long but not vernalization-saturating period of cold exposure, *VIN3* is partly induced and *FLC* is partly repressed (Fig. 2b; compare expression levels at 20 and 40 days of vernaliza-
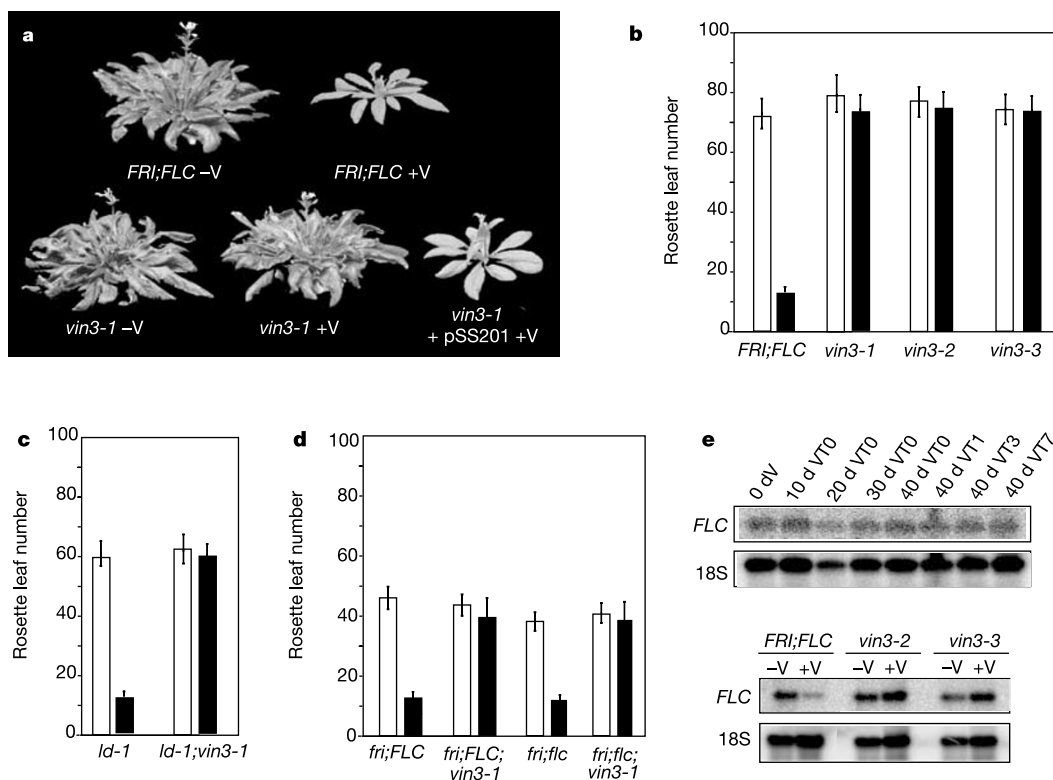
Figure 1 Characterization of *vernalization-insensitive 3* mutants. **a**, *vin3* blocks the vernalization response. Top, parental winter-annual line with and without vernalization. Bottom, *vin1-3* mutant with (+V) and without (−V) vernalization and rescue of *vin3-1* with a *VIN3* transgene. **b**, Block of the vernalization response in *vin3* mutants. Open bars, leaf number at flowering of non-vernalized plants; filled bars, values for plants vernalized for 40 days as described[6]. **c**, *vin3* causes vernalization insensitivity in the *luminidependens*

mutant. **d**, *vin3* blocks the vernalization response in a *flc* mutant in short days. **e**, *FLC* is not repressed by vernalization in *vin3* mutants. Top panel, length of exposure to 2–4 °C is shown as 'd V'. T0 samples were harvested directly from cold-grown plants. T1, T3 and T7 indicate the number of days for which the plants were grown at 22 °C after 40 days of cold. Bottom panel: +V samples were vernalized for 40 days, followed by 7 days at 22 °C.

tion). In addition, *VIN3* mRNA becomes undetectable within 3 days after return to a warm growth temperature (Fig. 2b, c). Thus, in contrast to *VRN1* and *VRN2*, which are constitutively expressed[11,12], *VIN3* is expressed only during cold exposure. Furthermore, the appearance of *VIN3* expression is a marker for cold exposures of a duration that are effective for vernalization. *VIN3* expression is induced by cold exposure in *vrn1* (Fig. 2c) and *vrn2* (data not shown), indicating that *VIN3* is upstream of *VRN1* and *VRN2*.

Vernalization causes an epigenetic change that provides competence to flower only in specific tissues such as shoot and root apices[2,16,17]. The pattern of *VIN3* expression, determined by a *VIN3* promoter fusion to *β-Glucuronidase* (*GUS*), matches the regions in which vernalization is effective (Fig. 2d). Furthermore, *VIN3* has the same pattern of expression as its target *FLC* (Fig. 2d). The *FLC–GUS* construct is subject to repression by vernalization in a wild-type background, whereas in a *vin3* mutant *FLC–GUS* repression does not occur during or after vernalization (Fig. 2e). In contrast, the *FLC–GUS* construct is repressed in a *vrn1* mutant during vernalization (Fig. 2e). The *VIN3–GUS* construct is expressed only when the plants are exposed to a long period of cold, and expression disappears after the plants are grown in warm conditions (Fig. 2d). Thus, the cold and tissue-specific expression of *VIN3* is likely to be controlled at the transcriptional level. These results, together with the RNA and phenotypic data described above, further support a role for *VIN3* in the vernalization-mediated

establishment of *FLC* repression in the shoot and root apex.

The epigenetic nature of *FLC* repression by vernalization, and the involvement of proteins related to chromatin-modelling proteins in this process, prompted us to examine whether histone modifications were involved in vernalization-mediated silencing of *FLC*. In humans and *Drosophila*, Su(z)12 polycomb group proteins, which are homologues of VRN2, mediate gene silencing by a series of histone modifications leading to methylation of histone H3 at Lys 9 (ref. 18). Methylation of Lys 9 is a marker for heterochromatin formation, and H3 Lys 9 methylation is thought to recruit heterochromatin protein 1 (and possibly to facilitate DNA methylation) to ensure a mitotically repressed state[19]. During the silencing process, a transiently acting repressor is often required to target specific genes for histone modifications[18]. One of the initial events that requires a transiently acting repressor can be histone deacetylation[20].

We therefore examined the acetylation of histone H3 at Lys 9 and Lys 14 in chromatin of *FLC*. We observed that a region of intron I (V1) and a region upstream of the transcriptional start site (P1) of *FLC* showed decreased acetylation during vernalization, and after transfer to warm growing conditions this decrease was maintained (Fig. 3a). However, *FLC* chromatin in the region (U1) encoding the 3′ untranslated region showed little change in acetylation levels. In *vin3* the vernalization-mediated changes in *FLC* acetylation do not occur (Fig. 4a; data shown for the V1 region). In *vrn1* and *vrn2* a decreased acetylation of *FLC* occurs during vernalization, but this
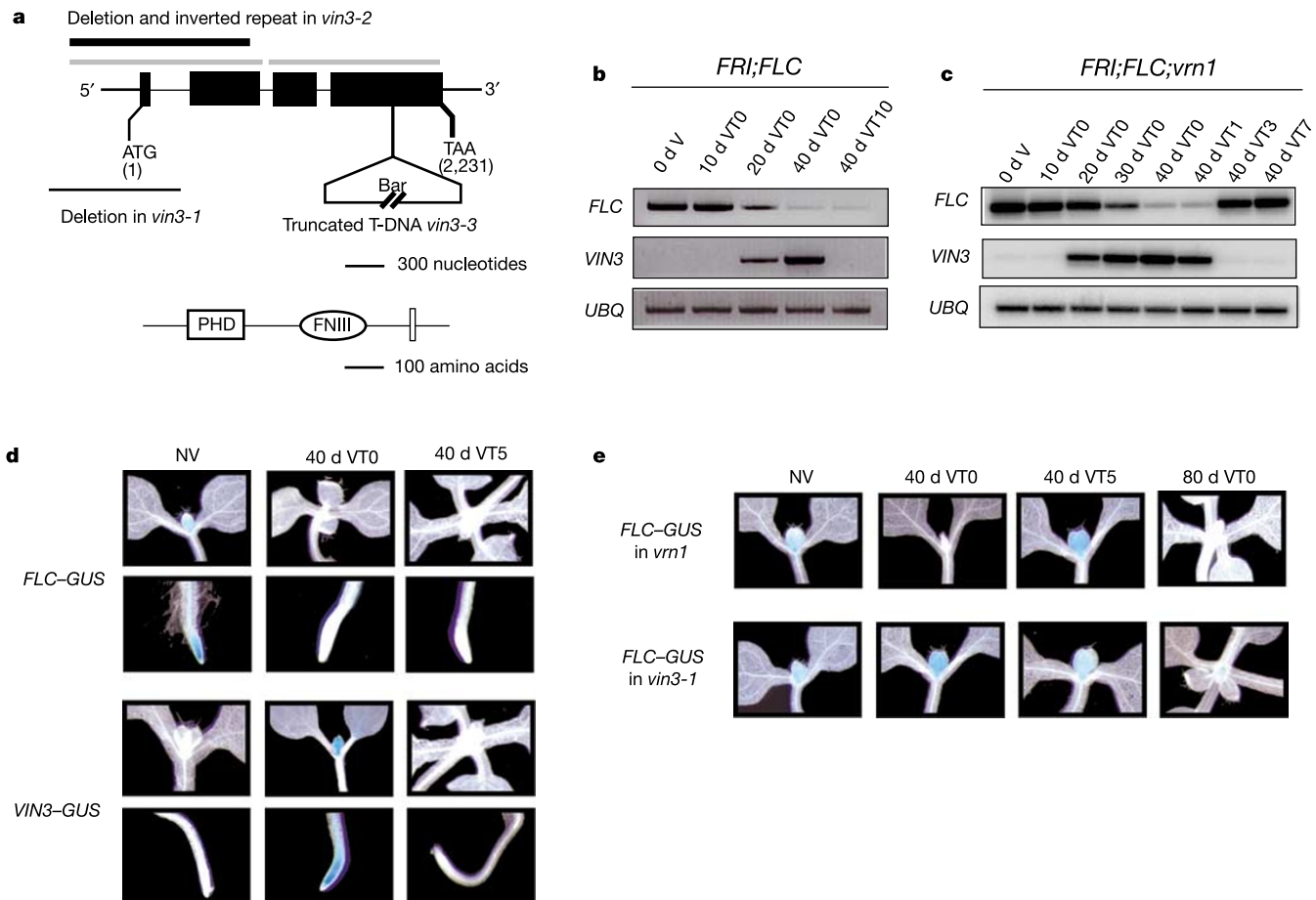
Figure 2 *VIN3* cloning and expression pattern. **a**, Positions of the domains in VIN3 (PHD finger, fibronectin type III, and (small rectangle) nuclear localization signal) and the lesions in *vin3* alleles. *vin3-1* is a fast-neutron allele, and *vin32* and *vin3-3* are T-DNA insertion alleles. **b**, RT–PCR analyses of the effect of vernalization on *VIN3* and *FLC* expression in the winter-annual wild type. Samples are as described in Fig. 1e. **c**, RT–PCR analyses of *VIN3* and *FLC* expression in *vrn1*. **d**, Spatial pattern of *VIN3* and *FLC* expression determined by reporter gene (*GUS*) fusions in the wild type without vernalization (NV), after 40 days of vernalization (40 d VT0), and after vernalization and 5 days at 22 °C (40 d VT5). **e**, *FLC* expression in *vrn1* and *vin3*. Designations are as in **d**.

decrease is not maintained in warm growing conditions (Fig. 4a, b). The lack of changes in *FLC* histone acetylation patterns in *vin3* mutants and the transient cold-specific expression of *VIN3* both in the wild type and in *vrn 1* and *vrn 2* during vernalization indicates that *VIN3* participates in a transient repression of *FLC* that involves histone deacetylation, and that this *VIN3*-mediated process is required for the establishment of *FLC* silencing.

A candidate modification for the maintenance of vernalization-mediated *FLC* repression is H3 dimethylation at Lys 9 (ref. 19). Indeed, we find that in the wild type, dimethylation levels at Lys 9 of H3 in chromatin of the *FLC* promoter and intron regions (P1 and V1) increase during vernalization and that this increase is main-

tained after vernalization (Fig. 3b). In *vin3*, as well as in *vrn1* and *vrn2*, the vernalization-mediated increase in H3 dimethylation at Lys 9 does not occur (Fig. 4). We also observe that in the wild type the level of dimethylation at Lys 27 of H3 increases during and after vernalization (Fig. 3b). Interestingly, the increase in H3 dimethylation at Lys 27 occurs in *vrn1* but not in *vrn2* (Fig. 4b, c) indicating that *VRN1* is not required for H3 dimethylation at Lys27 and that this dimethylation alone is not sufficient to maintain *FLC* repression. The lack of H3 dimethylation at both Lys 9 and Lys 27 in *vrn 2* indicates that VRN2 activity is required for both dimethylations, whereas VRN1 activity is necessary for H3 dimethylation at Lys 9. *VRN2* homologues in *Drosophila* and human (*SUPPRESSOR OF ZESTE; Su(z)12*) are involved in H3 methylation[18], indicating that the role of this type of polycomb group protein in gene silencing is evolutionarily conserved.

To address whether VIN3 can interact directly with the *FLC* locus, we evaluated whether VIN3 antiserum could precipitate *FLC* chromatin (Fig. 3c). Precipitation of chromatin from vernalized plants with VIN3 antiserum greatly enriched for *FLC* compared
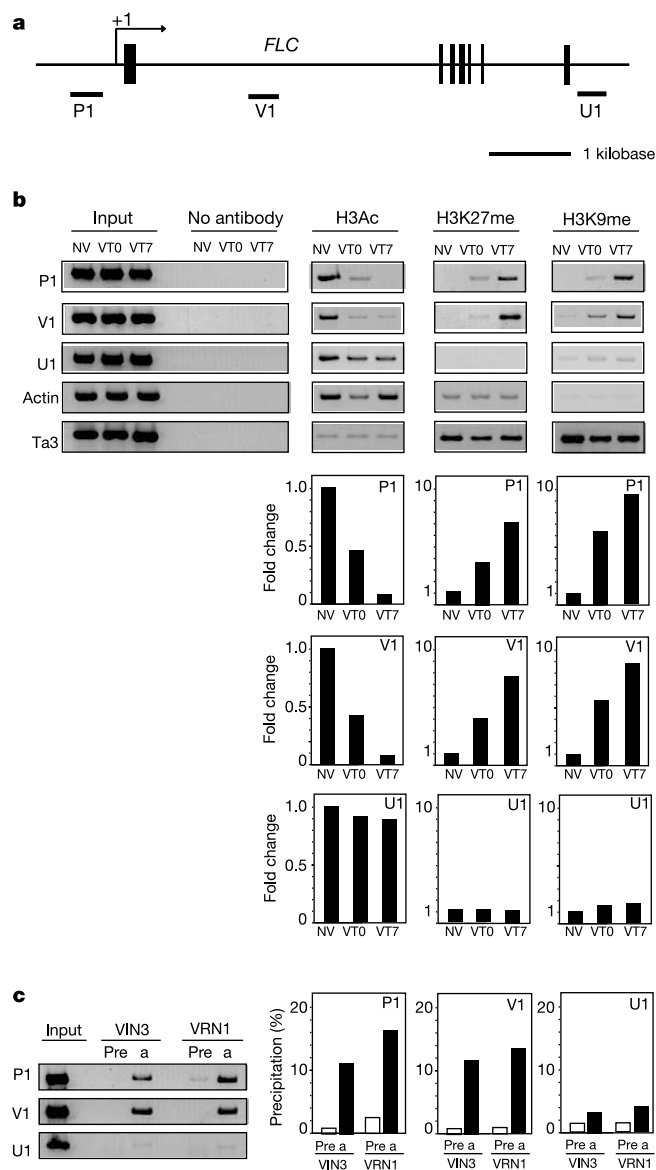
Figure 3 ChIP assays in wild-type winter-annual *Arabidopsis*. **a**, Regions of the *FLC* locus examined (see Methods for details). Coding regions are indicated by vertical lines. **b**, ChIP with antibodies against modified histones. Samples are identified as follows: NV, non-vernalized; VT0, vernalized for 40 days; VT7, vernalized for 40 days and subsequently grown for 7 days at 22 °C. Sample preparation was as described previously[30]. Signal intensities were normalized relative to actin and Ta3 control reactions with ImageQuant, and fold changes are presented in the bar graphs. **c**, ChIP with VIN3 and VRN1 antisera. Samples were from plants vernalized for 40 days without exposure to warm temperature. Signal intensities were normalized relative to input signal intensities. Pre, pre-immunized serum.
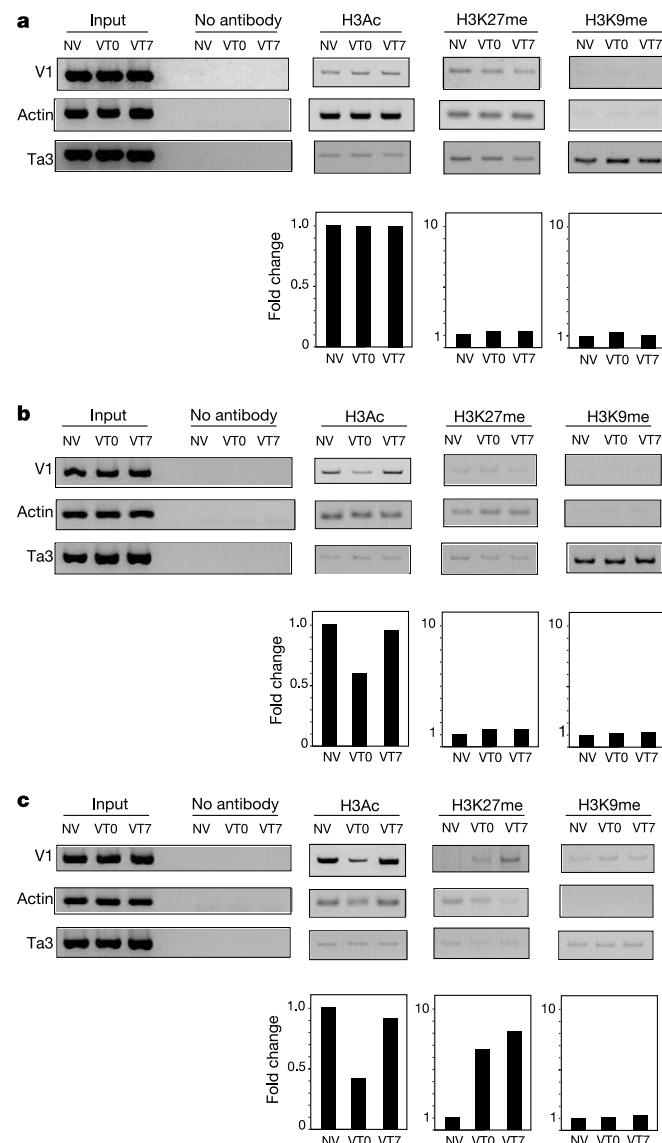
Figure 4 Chromatin immunoprecipitation (ChIP) assays in vernalization mutants. ChIP assays and designations are as described in Fig. 3. Genotypes were *vin3* (**a**), *vrn2* (**b**) and *vrn1* (**c**).

with the preimmune control. In addition, VRN1 antiserum greatly enriched for *FLC* chromatin compared with the preimmune control. The regions of the promoter (P1) and the intron (V1) were enriched in these immunoprecipitations to a much greater extent than the 3′ end (U1) of *FLC*. Thus, the regions of *FLC* chromatin with which VIN3 and VRN1 interact in immunoprecipitation experiments correspond to the regions of *FLC* chromatin in which changes in histone modification occur. The interaction of VIN3 and VRN1 with the 5′ promoter and the first intron regions and the vernalization-mediated histone modifications of these regions are consistent with the observation that the regions are required for a proper vernalization response[21] and that a DNase I-hypersensitive site in the intron region becomes insensitive after vernalization[11].

The epigenetic nature of vernalization was first recognized as a memory of winter in which the competence of an apical meristem to initiate flowering persists through mitotic cell divisions[22,23]. Our results provide a framework for the molecular basis of vernalization in *Arabidopsis*. The establishment of *FLC* silencing (and *FLC*-independent aspects of the vernalized state) requires the induction of *VIN3* expression by exposure to prolonged cold. *VIN3* expression is necessary for deacetylation of *FLC*, which in turn leads to histone methylation and the formation of mitotically stable heterochromatin at the *FLC* locus by a process involving *VRN1* and *VRN2*. *VIN3* is induced by cold only in tissues (root and shoot apices) in which vernalization occurs, and the spatial restriction of *VIN3* is likely to contribute to the restriction of the vernalization process to these tissues.

VRN1 and *VRN2* are expressed constitutively[11,12], and the recruitment of VRN1 and VRN2 to target genes such as *FLC* during vernalization is likely to require a factor to provide specificity. The spatial expression pattern and cold-specific induction of *VIN3* and the interaction of VIN3 with *FLC* chromatin indicates that VIN3 could be part of a transiently acting complex that leads to the initial establishment of target gene repression during vernalization. It is consistent with this model that *VIN3* is clearly the most upstream component of vernalization so far identified because *vin3* is the only mutant in which *FLC* repression is blocked during cold exposure, and in which no histone modifications are detected during vernalization. Moreover, *VIN3* is induced in *vrn1* and *vrn2*, and *VIN3*-mediated *FLC* silencing during cold exposure occurs in *vrn1* and *vrn2*.

The additional components that interact with VIN3, and VRN1 and VRN2, to repress *FLC* during and after vernalization are not known. One component might be a protein similar to ENHANCER OF ZESTE (E(z)). In human and *Drosophila*, E(z) acts as a histone methyltransferase in a complex with the VRN2 homologue Su(z)12, and this complex is involved in polycomb-complex-mediated gene repression[18]. MEDEA, CURLY LEAF and EZA1 are the closest relatives of E(z) in *Arabidopsis*[24]. Lesions in *CURLY LEAF* cause ectopic expression of the floral homeotic gene *AGAMOUS*[24] and do not affect the vernalization process (not shown). It is possible that MEDEA or EZA1 has a function in vernalization, or that there is redundancy between E(z) relatives in this process, or that other proteins are involved in vernalization-mediated histone methylation. It will be interesting to address nature of the complex that leads to establishment of *FLC* repression during vernalization.

One of the remarkable aspects of the vernalization process is that it has evolved to sense the prolonged cold of winter. Exposure to short periods of cold that are sufficient to initiate other cold responses such as cold acclimation[25] do not initiate a vernalization response[2]. Part of the mechanism for measuring the period of cold exposure during vernalization is the requirement for several weeks of cold treatment for *VIN3* induction. However, constitutive *VIN3* expression is not sufficient to substitute for cold treatment (data not shown); thus, VIN3 must interface with other cold-induced changes to cause vernalization. The recent demonstration that the PHD

finger domain can bind phosphoinositides[26] raises the possibility that VIN3 could be a nuclear phosphoinositide receptor, and that perhaps VIN3 is involved in perception of changes in the spectrum of nuclear phosphoinositides that might occur during cold exposure. It will be interesting to further explore the mechanism by which prolonged cold induces *VIN3* expression and achieves the vernalized state. □

Methods

Plant materials

Mapping

Chromatin immunoprecipitation (ChIP) assays

1. Chouard, P. Vernalization and its relations to dormancy. *Annu. Rev. Plant Physiol.* **11,** 191–238 (1960).
2. Michaels, S. & Amasino, R. Memories of winter: vernalization and the competence to flower. *Plant Cell Environ.* **23,** 1145–1154 (2000).
3. Lee, I., Michaels, S. D., Masshardt, A. S. & Amasino, R. M. The late-flowering phenotype of *FRIGIDA* and *LUMINIDEPENDENS* is suppressed in the Landsberg *erecta* strain of *Arabidopsis*. *Plant J.* **6,** 903–909 (1994).
4. Michaels, S. & Amasino, R. *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* **11,** 949–956 (1999).
5. Sheldon, C. C. *et al.* The *FLF* MADS box gene. A repressor of flowering in *Arabidopsis* regulated by vernalization and methylation. *Plant Cell* **11,** 445–458 (1999).
6. Sheldon, C. C., Rouse, D. T., Finnegan, E. J., Peacock, W. J. & Dennis, E. S. The molecular basis of vernalization: the central role of *FLOWERING LOCUS C (FLC)*. *Proc. Natl Acad. Sci. USA* **97,** 3753–3758 (2000).
7. Michaels, S. D. & Amasino, R. M. Loss of *FLOWERING LOCUS C* activity eliminates the late-flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. *Plant Cell* **13,** 935–942 (2001).
8. Johanson, U. *et al.* Molecular analysis of *FRIGIDA*, a major determinant of natural variation in *Arabidopsis* flowering time. *Science* **290,** 344–347 (2000).
9. Gazzani, S., Gendall, A. R., Lister, C. & Dean, C. Analysis of the molecular basis of flowering time variation in *Arabidopsis* accessions. *Plant Physiol.* **132,** 1107–1114 (2003).
10. Michaels, S. D., He, Y., Scortecci, K. C. & Amasino, R. M. Attenuation of FLOWERING LOCUS C activity as a mechanism for the evolution of summer-annual flowering behavior in *Arabidopsis*. *Proc. Natl Acad. Sci. USA* **100,** 10102–10107 (2003).
11. Gendall, A. R., Levy, Y. Y., Wilson, A. & Dean, C. The *VERNALIZATION 2* gene mediates the epigenetic regulation of vernalization in *Arabidopsis*. *Cell* **107,** 525–535 (2001).
12. Levy, Y. Y., Mesnage, S., Mylne, J. S., Gendall, A. R. & Dean, C. Multiple roles of *Arabidopsis VRN1* in vernalization and flowering time control. *Science* **297,** 243–246 (2002).
13. Simpson, G. G. & Dean, C. *Arabidopsis*, the Rosetta stone of flowering time? *Science* **296,** 285–289 (2002).
14. Fair, K. *et al.* Protein interactions of the *MLL* PHD fingers modulate *MLL* target gene regulation in human cells. *Mol. Cell. Biol.* **21,** 3589–3597 (2001).
15. Main, A. L., Harvey, T. S., Baron, M., Boyd, J. & Campbell, I. D. The three-dimensional structure of the tenth type III module of fibronectin: an insight into RGD-mediated interactions. *Cell* **71,** 671–678 (1992).
16. Wellensiek, S. J. Dividing cells as the locus for vernalization. *Nature* **195,** 307–308 (1962).
17. Burn, J. E., Bagnall, D. J., Metzger, J. D., Dennis, E. S. & Peacock, W. J. DNA methylation, vernalization, and the initiation of flowering. *Proc. Natl Acad. Sci. USA* **90,** 287–291 (1993).
18. Kuzmichev, A. R. D., Nishioka, K., Erdjument-Bromage, H., Tempst, P. & Reinberg, D. Histone methyltransferase activity associated with a human multiprotein complex containing the enhancer of Zeste protein. *Genes Dev.* **16,** 2893–2905 (2002).
19. Richards, E. J. & Elgin, S. C. Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. *Cell* **108,** 489–500 (2002).
20. Kehle, J. *et al.* dMi-2, a hunchback-interacting protein that functions in polycomb repression. *Science* **282,** 1897–1900 (1998).
21. Sheldon, C. C., Conn, A. B., Dennis, E. S. & Peacock, W. J. Different regulatory regions are required for

the vernalization-induced repression of *FLOWERING LOCUS C* and for the epigenetic maintenance of repression. *Plant Cell* **14**, 2527–2537 (2002).

22. Lang, A. & Melchers, G. Vernalisation und Devernalisation bei einer zweijährigen Pflanze. *Z. Naturforsch* **2b**, 444–449 (1947).

23. Lang, A. in *Encyclopedia of Plant Physiology* Vol. 15 Part 1 (ed. Ruhland, W.) 1371–1536 (Springer, Berlin, 1965).

24. Reyes, J. C., Hennig, L. & Gruissem, W. Chromatin-remodeling and memory factors. New regulators of plant development. *Plant Physiol.* **130**, 1090–1101 (2002).

25. Thomashow, M. F. So what's new in the field of plant cold acclimation? Lots! *Plant Physiol.* **125**, 89–93 (2001).

26. Gozani, O. *et al.* The PHD finger of the chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor. *Cell* **114**, 99–111 (2003).

27. Weigel, D. *et al.* Activation tagging in *Arabidopsis. Plant Physiol.* **122**, 1003–1013 (2000).

28. Michaels, S. D. & Amasino, R. M. The gibberellic acid biosynthesis mutant *ga1-3* of *Arabidopsis thaliana* is responsive to vernalization. *Dev. Genet.* **25**, 194–198 (1999).

29. Michaels, S. D. *et al. AGL24* acts as a promoter of flowering in *Arabidopsis* and is positively regulated by vernalization. *Plant J.* **33**, 867–874 (2003).

30. Johnson, L., Cao, X. & Jacobsen, S. Interplay between two epigenetic marks. DNA methylation and histone H3 lysine 9 methylation. *Curr. Biol.* **12**, 1360–1367 (2002).

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Vernalization requires epigenetic silencing of *FLC* by histone methylation

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To ensure flowering in favourable conditions, many plants flower only after an extended period of cold, namely winter. In *Arabidopsis*, the acceleration of flowering by prolonged cold, a process called vernalization, involves downregulation of the protein FLC, which would otherwise prevent flowering[1,2]. This lowered *FLC* expression is maintained through subsequent development by the activity of *VERNALIZATION* (*VRN*) genes[3,4]. *VRN1* encodes a DNA-binding protein[4] whereas *VRN2* encodes a homologue of one of the Polycomb group proteins, which maintain the silencing of genes during animal development[3]. Here we show that vernalization causes changes in histone methylation in discrete domains within the *FLC* locus, increasing dimethylation of lysines 9 and 27 on histone H3. Such modifications identify silenced chromatin states in *Drosophila* and human cells[5–7]. Dimethylation of H3 K27 was lost only in *vrn2* mutants, but dimethylation of H3 K9 was absent from both *vrn1* and *vrn2*, consistent with VRN1 functioning downstream of VRN2. The epigenetic memory of winter is thus mediated by a 'histone code' that specifies a silent chromatin state conserved between animals and plants.

The requirement for vernalization is an important trait in crop breeding and has resulted in the availability of winter- and spring-sown varieties, which has significantly extended the geographical range for the farming of many crops. An understanding of vernalization in the control of flowering has emerged from a molecular genetic analysis in *Arabidopsis*[8]. The pathways conferring a requirement for and ability to respond to vernalization converge on the regulation of *FLC*[1,2]. FLC is a MADS box transcriptional regulator that functions as a floral repressor by inhibiting the activation of a set of genes required for transition of the apical meristem to a reproductive fate[1,2,9–13]. *FRIGIDA* (*FRI*) confers a vernalization requirement by upregulating *FLC* expression, and this is antagonized by vernalization, which reduces *FLC* levels. Genes classified in the autonomous floral pathway, such as *FCA*, function in parallel with vernalization to downregulate *FLC*. *fca* mutants are late-flowering owing to increased *FLC*, and this phenotype is suppressed by vernalization[1,2]. Once *FLC* transcript levels are downregulated by prolonged cold exposure, they remain low during subsequent development, and this mitotic stability suggests that vernalization has an epigenetic basis. This led to the idea that DNA methylation might have a role in *FLC* regulation[14]. Despite the observation that *FLC* levels were reduced in plants expressing an antisense DNA methyltransferase[1], bisulfite sequencing has shown that there is no change in *FLC* DNA methylation caused by vernalization (J. Finnegan, personal communication).

To investigate the molecular basis of the cold-induced repression of *FLC*, a series of *Arabidopsis vrn* mutants, defective in the acceleration of flowering by vernalization, were identified[15]. VRN1 and VRN2 were shown to be required for the maintenance of *FLC* repression during subsequent development following prolonged cold exposure[3,4]. VRN2 is a zinc-finger protein homologous to SU(Z)12, a member of the ESC-E(Z) Polycomb group (Pc-G) complex, which maintains the silencing of *Drosophila HOX* genes[16]. Pc-G proteins act in multiprotein complexes to maintain a silenced chromatin state by modifying specific amino acids in the amino-terminal tails of histones through deacetylation or methylation[17]. The ESC-E(Z) Pc-G complex in mammals and flies has been shown to contain histone lysine methyltransferase activity for K27 and possibly K9 of histone H3[5,6,18,19]. Methylated K9 of histone H3 triggers the binding of HETEROCHROMATIN PROTEIN 1, leading to heterochromatin formation and silencing[7,20,21]. Phosphorylation, ubiquitination and sumoylation of specific amino acids of histone tails have also been described[17]. The varying marks placed on the histones combine to form a 'histone code' that specifies a chromatin state and subsequently determines whether a locus is transcriptionally active or silent[22].

To investigate whether histone modifications were involved in the vernalization-dependent regulation of *FLC*, we analysed the chromatin environment of the *FLC* locus by chromatin immunoprecipitation (ChIP). In this technique, chromatin is isolated from cells, sheared and immunoprecipitated with a range of antibodies specific to different histone modifications. The immunoprecipitates are then examined for specific DNA sequences by polymerase chain reaction (PCR) analysis. ChIP analysis on *fca-1* plants, undertaken in at least three separate experiments, using antibodies specific to H3 dimethyl K9, H3 dimethyl K27 and H3 dimethyl K4 (a modification associated with active loci[23]) revealed vernalization-specific modifications at *FLC* (Fig. 1). Sequences from the 5′ region of *FLC*, regions shown as A and B in Fig. 1, which cover the *FLC* promoter and the first exon, were found to show higher levels of H3 K9 dimethylation in vernalized tissue. H3 K27 dimethylation was higher in vernalized tissue, predominantly in region B, covering the 5′ end of the transcript, but was also found in some experiments in the promoter region (region A; data not shown). H3 K4 dimethylation was associated with all regions of *FLC* examined in non-vernalized tissue, but was reduced in region E, in the middle of intron 1, in vernalized samples. This could result from the targeted loss of H3 dimethyl K4 nucleosomes from this region or possibly