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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], bisulphite 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 from the cross-linking of vernalization-specific proteins that block the epitope. A Mutator-like transposon has been found at the 3′ end of intron 1 of the Landsberg *erecta* (L*er*) *FLC* allele, and this seems to restrict high levels of *FLC* RNA accumulation[24,25]. Consistent with this, chromatin containing this transposon showed vernalization-independent H3 K9 dimethylation (data not shown). However, the transposon does not affect the vernalization response or the maintenance of *FLC* repression, so is unlikely to influence the vernalization-induced chromatin changes at *FLC*. It was interesting to note that H3 K4 methylation was still found at most regions within the *FLC* locus after vernalization. This could mean that K4 dimethylation is not reduced in silenced *FLC*[23], perhaps reflecting the reversibility of *FLC* repression later in development. Alternatively, it may reflect the fact that *FLC* expression is not silenced by vernalization in all cells. We have evidence for the latter from analysis of a *FLC–GUS* (β-glucuronidase) translational fusion in a transgenic *fca-1* line. Vernalization reduced FLC–GUS expression in most cells, but not in those of the vasculature, with strongest expression remaining in the vasculature of the cotyledons and hypocotyl (Fig. 2). In some of the ChIP experiments, an antibody to acetylated H3 was included (Figs 1 and 3); however, changes in *FLC* histone acetylation as significant as those seen for histone methylation were not observed, so this was not pursued further.

In summary, the ChIP analysis showed that regions within the promoter, the 5′ end of the transcript and intron 1 of *FLC* undergo vernalization-specific histone methylation changes that are normally associated with the maintenance of transcriptional repression. These regions can be defined only approximately (within ~750 base pairs (bp)) as sonication sheared the chromatin to between 500 and 1,500 bp. The non-uniform distribution of the histone modifications observed at *FLC* is unlike the situation at heterochromatic transposons from the knob on chromosome 4S, which are more uniformly associated with H3 dimethyl K9 (ref. 26, and Z.L. and R.A.M., unpublished data). In this respect, these modified domains within the *FLC* locus resemble the Polycomb response elements in *Drosophila*, *cis*-acting elements that recruit Pc-G complexes. Whether they function in this way remains to be established.

Analysis of deletion derivatives of a *FLC–GUS* fusion has defined *cis*-acting domains important for *FLC* regulation[27], and these coincide with the regions showing vernalization-induced histone methylation changes. Region E, which is less abundant in the H3 dimethyl K4 antibody immunoprecipitate after vernalization, is contained within a region defined as required for the maintenance of *FLC* repression. Intron 1 seems to contain multiple maintenance elements, as deletion of either end of the intron did not disrupt the maintenance of *FLC* repression. Region A, which shows H3 dimethyl K9 (and sometimes dimethyl K27) modification after vernalization, corresponds almost exactly to a potential negative regulatory promoter element whose deletion leads to higher *FLC* expression. Region B, which showed both H3 dimethyl K27 and H3 dimethyl K9 modification after vernalization, is part of a much larger region defined as being required for *FLC* expression in non-vernalized plants and for repression by vernalization, so a role in the maintenance of repression could not be directly tested[27]. Further definition of *FLC cis*-acting domains *in vivo* and further definition of the domains showing histone modifications will be important to

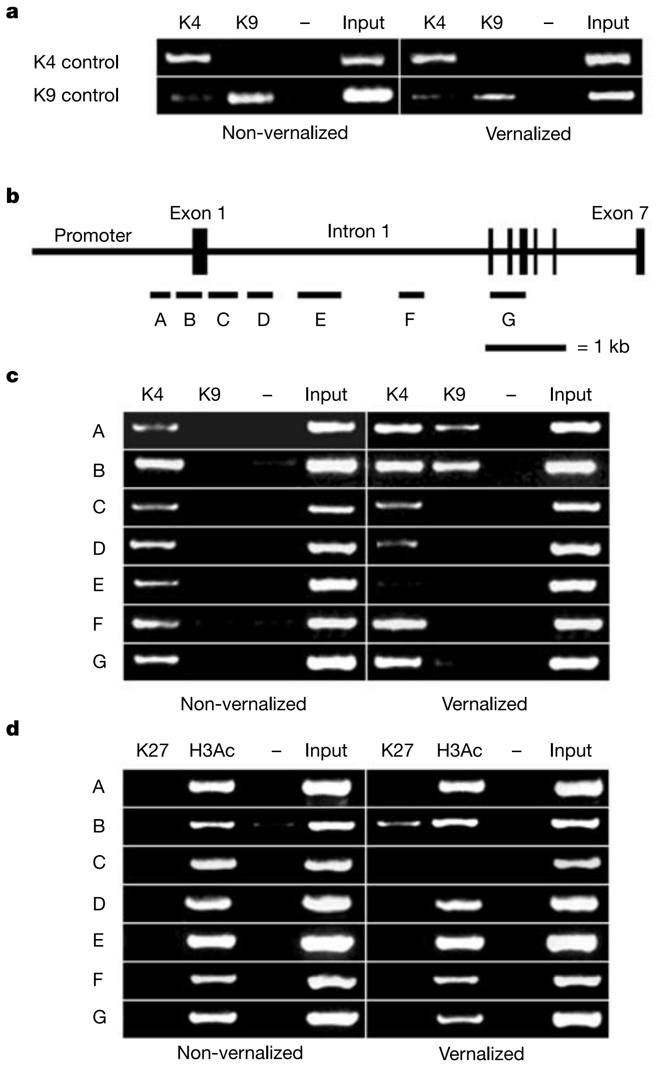

Figure 1 Histone modifications at *FLC* associated with vernalization. **a**, ChIP antibody controls: K4 primers amplify the transcribed phosphofructokinase gene on BAC T24H24.15, K9 control primers amplify the silenced Cinful-like retrotransposon on BAC T5L23.29. **b**, Genomic structure of *Arabidopsis FLC* (Columbia accession) and regions examined by ChIP. Black boxes represent exons; lines represent promoter and introns. Regions amplified by PCR are shown below as bars labelled A to G. For primer sequences, see Supplementary Fig. 3. **c**, ChIP analysis using H3 dimethyl K4 and H3 dimethyl K9 antibodies. *fca-1* plants were grown with and without prior vernalization. Vernalization resulted in increased precipitation of regions A and B with the H3 dimethyl K9 antibody, and reduced precipitation of region E with the H3 dimethyl K4 antibody. **d**, ChIP analysis using antibodies against H3 dimethyl K27 and acetylated H3 with *fca-1* plants. Region B was immunoprecipitated with the H3 dimethyl K27 antibody in vernalized seedlings. Identical results were obtained for L*er* and L*er* plants carrying an active *FRI* allele (Supplementary Fig. 2).

Figure 2 Vernalization-induced changes in expression of an *FLC–GUS* translational fusion. A translational fusion composed of the β-glucuronidase coding sequences cloned into the *Nhe*I restriction site (within *FLC* exon 6) of a complementing *FLC* genomic clone was introduced into *fca-1* seedlings. FLC–GUS expression is seen throughout the non-vernalized seedling but restricted to the vasculature (strongest in cotyledon and hypocotyl but also detectable in older leaves) in vernalized seedlings.

fully establish whether they correspond exactly.

We wanted to determine whether the genotype conferring a vernalization requirement affected the histone modifications. ChIP was repeated on non-vernalized and vernalized seedlings of L*er* and a L*er* line transformed with an active *FRI* gene (Fig. 3). The histone modifications mirrored those observed for *fca-1* plants, so the observed changes are specific to vernalization and not to the genotype conferring the vernalization requirement, and thus occur independently of the expression level of *FLC*. We also analysed, using ChIP, the role of the *VRN* genes in determining the vernalization-induced histone modifications. The vernalization-induced changes in H3 dimethyl K9 and H3 dimethyl K4 were lost in *vrn1*—regions A and B did not show increased H3 dimethyl K9 and region E was precipitated by anti-H3 dimethyl K4. However, the vernalization-induced H3 dimethyl K27 in region B was retained in *vrn1*. In vernalized *vrn2* seedlings, no increase in H3 dimethyl K9 in regions A and B or dimethyl K27 in region B was found; however, the vernalization-induced loss of H3 dimethyl K4 from region E was maintained or restored only weakly. Thus, both VRN1 and VRN2 must be required for the increase in H3 dimethyl K9 in regions A and B. VRN1 seems to be more important than VRN2 for the changes in region E, whereas VRN2 is sufficient for H3 dimethyl K27 of region B. Conclusive evidence that the VRN proteins function directly at the *FLC* locus has yet to be obtained, but preliminary ChIP experiments using a VRN2–tandem affinity purification (TAP)[28] complementing fusion showed enrichment of *FLC* 5′ sequences (region B) after immunoglobulin-γ precipitation (data not shown).

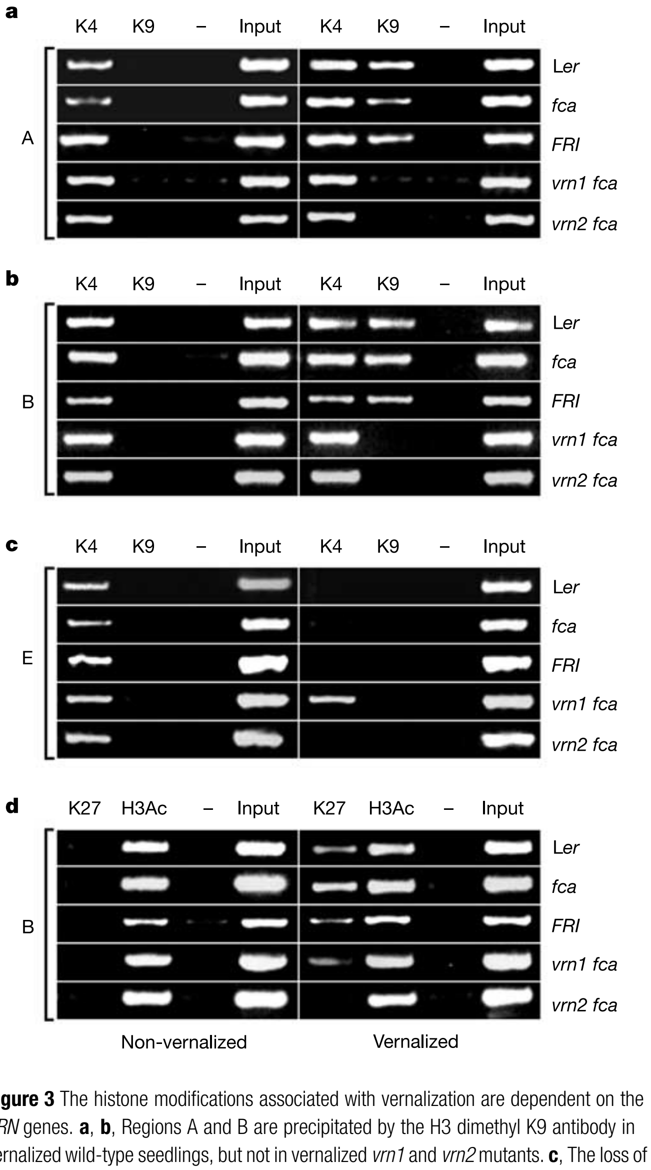

Figure 3 The histone modifications associated with vernalization are dependent on the *VRN* genes. **a**, **b**, Regions A and B are precipitated by the H3 dimethyl K9 antibody in vernalized wild-type seedlings, but not in vernalized *vrn1* and *vrn2* mutants. **c**, The loss of precipitation of region E with the H3 dimethyl K4 antibody is dependent on VRN1. Region E is precipitated from all samples of vernalized *vrn1* mutants, whereas it is only sometimes precipitated in *vrn2* mutants (in four biological replicates, low amounts of region E were precipitated in two sets; data not shown). **d**, The increased dimethylation of H3 K27 is dependent on VRN2. The H3 dimethyl K27 antibody precipitates region B after vernalization in wild-type and *vrn1* seedlings, but not in *vrn2*. Precipitation using the antibody to acetylated H3 is also shown.

A possible model suggested by these data is that the cold causes a reduction in *FLC* transcription and this process triggers recruitment of a VRN2 complex containing plant homologues of ESC-E(Z) to the 5′ region of *FLC* (including region B), causing H3 K27 dimethylation. How cold results in low *FLC* RNA and whether any post-transcriptional regulation occurs that feeds back to cause reduced transcription is unknown at present. Nuclear run-on experiments have been undertaken, but the levels of *FLC* transcription have proved too low to be detectable. The presence of the H3 dimethyl K27 may either stimulate the methylation of H3 K9 at the 5′ end of *FLC* by the ESC-E(Z) complex itself, or promote the recruitment of additional complexes that methylate H3 K9. If VRN2 functions in an ESC-E(Z) complex similar to that in *Drosophila* and mammals, it is likely to contain one of the three E(Z) homologues, CLF, CLK and MEDEA present in the *Arabidopsis* genome[29]. Analysis so far suggests that the vernalization response of *clf* mutant plants is not affected (R. Amasino, personal communication), so CLF is unlikely to function as the sole E(Z) component. The role of CLK and MEDEA in the VRN2 complex and any function in vernalization remains to be established. Biochemical and genetic data show the involvement of two complexes functioning in a cooperative manner to maintain long-term gene silencing in *Drosophila* and mammals. In mammalian cells, H3 dimethyl K27 acts as a signal for the binding of POLYCOMB (PC) and recruitment of PRC1, which restricts accessibility of nucleosome remodelling factors to the chromatin[5]. PC may also stimulate the H3 K9 histone methyltransferase activity of the ESC-E(Z) complex or promote methylation of H3 K9 through the recruitment of SU(VAR)3-9 (ref. 19). A similar mechanism for long-term silencing may exist in plants; however, homologues of PC and components of the PRC1 complex are not found in the *Arabidopsis* genome sequence[29]. The data presented here suggest that VRN1 could mediate this long-term repression. This would predict that the function of the VRN2 complex in methylating H3 K27 is the vernalization-specific factor shown to be required for *FLC* to be a target of VRN1 (ref. 4). ☐

Methods

Plant growth conditions and vernalization treatment

Landsberg *erecta* and *fca-1* lines were originally obtained from M. Koornneef. *vrn1-2 fca-1* and *vrn2-1 fca-1* have been described previously[4,15]. All seeds were surface sterilized and grown on MS media minus glucose. Vernalization was carried out by placing sterilized seeds at 4 °C under short-day conditions of 8 h white light (10 μmol photons $m^{-2} s^{-1}$), 16 h dark for 6 weeks. Seeds were then transferred to long-day conditions of 16 h white light (57 μmol photons $m^{-2} s^{-1}$), 8 h dark and grown for 20 days. By this stage, approximately 6 rosette leaves had been made and no flower buds were visible. Non-vernalized seeds were grown at 4 °C under short-day conditions of 8 h white light (10 μmol photons $m^{-2} s^{-1}$), 16 h dark for 2 days, then transferred to the long-day conditions for 20 days.

Chromatin immunoprecipitation

ChIP was carried out on 20-day-old seedlings that had or had not been vernalized using a previously described procedure[26]. Chromatin samples were immunoprecipitated with Upstate antibodies against histone H3 dimethyl Lys 4 (catalogue no. 07-030, lot no. 22672), histone H3 dimethyl Lys 9 (catalogue no. 07-212, lot no. 23424), histone H3 dimethyl Lys 27 (catalogue no. 07-322, lot no. 22264) and histone H3 acetylation (catalogue no. 06-599, lot no. 23793). The DNA isolated from these reactions was then amplified using primer pairs covering a region of *FLC* from the promoter to exon 5, a region of *FLC* previously characterized to contain *cis*-elements essential for *FLC* expression and function[27]. Primers, shown in Supplementary Fig. 3, were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). Default settings were used except for product size (250–500 bp), minimum GC content (40%) and maximum GC content (60%).

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Bcl10 activates the NF-κB pathway through ubiquitination of NEMO

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The NF-κB family of transcription factors is activated in response to many stimuli, including pro-inflammatory cytokines, environmental stresses and, in the case of B and T lymphocytes, by antigenic stimulation[1,2]. Bcl10 is essential for NF-κB activation by T- and B-cell receptors. T and B lymphocytes from Bcl10-deficient mice fail to activate NF-κB in response to antigen-receptor stimulation and, as a consequence, are unable to proliferate[3]. Bcl10 overexpression is sufficient to activate NF-κB, a process that requires the NF-κB essential modulator NEMO (also known as IKK-γ), which is the regulatory subunit of the IκB kinase complex[4]. However, the cellular mechanism by which Bcl10 activates the NF-κB pathway remains unclear. Here we show that Bcl10 targets NEMO for lysine-63-linked ubiquitination. Notably, a mutant form of NEMO that cannot be ubiquitinated inhibited Bcl10-induced NF-κB activation. Paracaspase and a ubiquitin-conjugating enzyme (UBC13) were both required for Bcl10-induced NEMO ubiquitination and subsequent NF-κB activation. Furthermore, short interfering RNAs that reduced the expression of paracaspase and UBC13 abrogated the effects of Bcl10. Thus, the adaptor protein Bcl10 promotes activation of NF-κB transcription factors through paracaspase- and UBC13-dependent ubiquitination of NEMO.

Activation of the 'classical' NF-κB pathway converges on the IκB kinase (IKK) signalosome, a protein complex composed of two kinase subunits (IKK-α and IKK-β) and a non-catalytic subunit NF-κB essential modulator (NEMO)[5]. Activated IKK phosphorylates IκB proteins that sequester NF-κB transcription factors in the cytoplasm. The SCF (Skp1–Cul1–F-box) ubiquitin ligase complex then modifies the phosphorylated IκBs by K48-linked multiubiquitination, and thereby targets them for proteolysis in the 26S proteasome[6]. NF-κB complexes are thus freed to translocate into the nucleus and engage κB enhancer elements of target genes[5,7]. Bcl10 is essential for NF-κB activation by T- and B-cell receptors, but it is dispensable for NF-κB activation in response to tumour-necrosis factor-α or interleukin-1 (ref. 3). Notably, Bcl10 expression is deregulated in B-cell lymphomas of mucosa-associated lymphoid tissue (MALT lymphoma) bearing the chromosomal translocation t(1; 14)(p22; q32), a rearrangement that places the *Bcl10* gene downstream of the strong immunoglobulin transcriptional enhancers. Enforced Bcl10 expression presumably contributes to transformation and antigen-independent lymphoma progression through constitutive activation of the pro-survival NF-κB pathway[8,9]. Bcl10 or its viral homologue E10 have been reported to interact with paracaspase/MALT1, cIAPs (inhibitor of apoptosis proteins) and TRAFs, but how these interactions affect Bcl10-induced NF-κB activation is unclear[10,11].

We used a biochemical purification method to identify components of the Bcl10 signalling complex. Cytosolic extracts (S100) that were prepared from Jurkat T cells either untreated or treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) plus ionomycin to mimic T-cell receptor activation were applied to a glutathione