

A Polycomb-based switch underlying quantitative epigenetic memory

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The conserved Polycomb repressive complex 2 (PRC2) generates trimethylation of histone 3 lysine 27 (H3K27me3)^{1,2}, a modification associated with stable epigenetic silencing^{3,4}. Much is known about PRC2-induced silencing but key questions remain concerning its nucleation and stability. Vernalization, the perception and memory of winter in plants, is a classic epigenetic process that, in *Arabidopsis*, involves PRC2-based silencing of the floral repressor *FLC*^{5,6}. The slow dynamics of vernalization, taking place over weeks in the cold, generate a level of stable silencing of *FLC* in the subsequent warm that depends quantitatively on the length of the prior cold. These features make vernalization an ideal experimental system to investigate both the maintenance of epigenetic states and the switching between them. Here, using mathematical modelling, chromatin immunoprecipitation and an *FLC:GUS* reporter assay, we show that the quantitative nature of vernalization is generated by H3K27me3-mediated *FLC* silencing in the warm in a subpopulation of cells whose number depends on the length of the prior cold. During the cold, H3K27me3 levels progressively increase at a tightly localized nucleation region within *FLC*. At the end of the cold, numerical simulations predict that such a nucleation region is capable of switching the bistable epigenetic state of an individual locus, with the probability of overall *FLC* coverage by silencing H3K27me3 marks depending on the length of cold exposure. Thus, the model predicts a bistable pattern of *FLC* gene expression in individual cells, a prediction we verify using the *FLC:GUS* reporter system. Our proposed switching mechanism, involving the local nucleation of an opposing histone modification, is likely to be widely relevant in epigenetic reprogramming.

In *Arabidopsis*, vernalization requires the plant-homeodomain-PRC2 complex (PHD-PRC2), whose components include SWINGER (an E(Z) histone methyltransferase homologue), together with VIN3, VRN5 and VEL1 (PHD proteins required to generate high H3K27me3 levels)^{7–10}. PRC2 complexes such as this are thought to initiate trimethylation of H3K27 and then spread this modification through a positive feedback loop where they both bind to H3K27me3 and trimethylate other nucleosomes^{7–10}. In principle, this process could generate stable maintenance of histone modifications even when perturbed by DNA replication, where on average half of the modifications could be lost by the insertion of unmodified nucleosomes. In general, however, how to establish stable epigenetic silencing at a level that depends quantitatively on the level of a transient stimulus is not understood.

In an effort to answer this question, we combined chromatin immunoprecipitation (ChIP) experiments with mathematical modelling to dissect the vernalization process. This required high-resolution analysis of H3K27me3 during vernalization as a function of space across the *FLC* locus and time. Previously, we had demonstrated that PRC2 was associated with the whole *FLC* locus before cold, and that this correlated with an epigenetically stable low level of silencing^{9,11}. After 8 weeks of cold, a PHD-PRC2 complex had formed at one particular site within the gene and only after plants were returned to the warm did the

PHD proteins associate more generally with PRC2 over the locus. This association resulted in high levels of H3K27me3, which epigenetically maintained the fully silent state. Therefore, we needed to explore the quantitative accumulation of silencing during the cold. Figure 1a (and Supplementary Fig. 1) shows the H3K27me3 profile across *FLC*, in non-vernalized plants and also after 2 and 4 weeks of cold (at 4 °C). A localized peak of H3K27me3 develops (close to exon 1, see Fig. 1), the level of which increases with increasing length of cold, in agreement with previous results¹². For longer periods of cold (6 and 8 weeks), this nucleation seems to saturate, with some limited general increase in H3K27me3 levels across the rest of the locus (Fig. 1b and Supplementary Fig. 2). During the cold, the rising levels of H3K27me3 in the nucleation region are correlated with an increase in expression of the PHD protein VIN3 (refs 7, 8), whose concentration levels may therefore constitute one of the primary read-outs of the length of cold. However, *VIN3* expression returns to low levels immediately (within one day) after return to the warm: the memory of the cold implicit in the *VIN3* concentration levels must therefore be converted into a

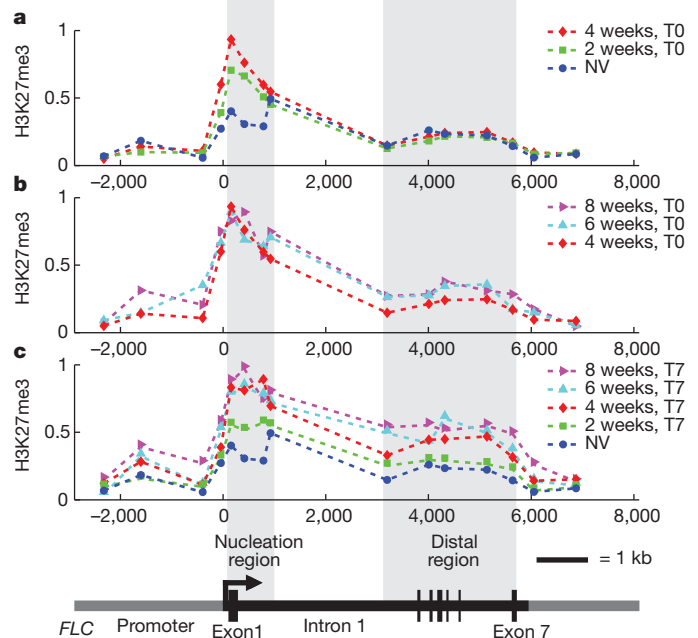


Figure 1 | H3K27me3 ChIP experiments. **a**, H3K27me3 profile across *FLC* for non-vernalized plants (NV) and after 2 or 4 weeks of cold, with 0 days of warm (T0). **b**, H3K27me3 profile across *FLC* after 4, 6 or 8 weeks of cold, with 0 days of warm (T0). **c**, H3K27me3 profile across *FLC* for non-vernalized plants and 7 days post-cold (T7) after 2, 4, 6 or 8 weeks of cold (top) and schematic layout of *FLC* gene (bottom), with definitions of nucleation and distal regions. Panels (a–c) show results from one complete experiment. Similar results were obtained with a biological replicate (Supplementary Figs 1–3).

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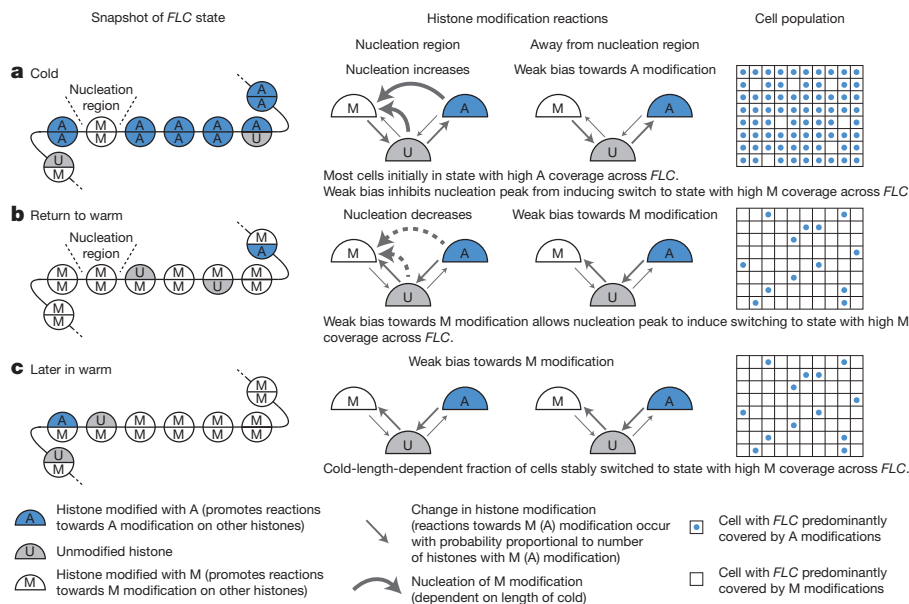
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second memory element in the warm, potentially H3K27me3 levels. We measured therefore the H3K27me3 profile across *FLC* 7 days after the plants had been transferred to the warm (Fig. 1c and Supplementary Fig. 3). The profile changed rather little in the nucleation region but rose quantitatively across the rest of the *FLC* locus according to the length of the cold period (Supplementary Fig. 4). This quantitative increase correlated with decreased *FLC* expression (Supplementary Fig. 5), and was consistent with H3K27me3 levels being a key element in epigenetic memory of the cold after return to the warm.

For shorter periods of cold, levels of ChIP-measured H3K27me3 at the nucleation region depended quantitatively on the length of the cold period, and could therefore potentially act to switch quantitatively the epigenetic state of the locus after return to the warm. Epigenetic states are, however, intrinsically stable (in the case of vernalization, over many weeks or months). It was therefore unclear whether the small size of the nucleation region (Fig. 1a) would be sufficient to cause a quantitative switch in the epigenetic state of the *FLC* locus. To answer this question, we turned to mathematical modelling^{13–19}. Previous modelling of epigenetic states had focused primarily on cell-autonomous dynamics in yeast^{13,15–18}. We wondered whether a similar cell-autonomous mechanism could also explain the quantitative response to the cold seen in vernalization. The above data provided an ideal opportunity to test such a model. In essence, our model is an implementation of a Polycomb-based switch, incorporating highly dynamic rewriting of histone modifications on a timescale of minutes (see Fig. 2 and Supplementary Information for details). The rapid dynamics of histone modifications are vital as they allow for the formation of bistable epigenetic states, despite the noisy turnover of nucleosomes, both continuously (on a timescale of tens of minutes²⁰) and discretely at DNA replication (on a timescale of days^{21,22}). The model utilizes a simplified set of possible histone modifications: M (H3K27me3), U (unmodified) and A (activating) (other possible assumptions are discussed in the Supplementary Information), where histones with the M or A modification antagonistically promote the transition of other histones towards the same modification, leading to positive feedback^{13,16}. At the high levels of noise potentially generated by noisy nucleosome turnover and subsequent random histone mark addition, the presence of spatially long-ranged interactions between histones is an important

factor in the formation of bistable states¹³. In our model, starting from a state with the *FLC* locus covered by A modifications, two additional processes contribute to the switch-like behaviour at the end of the cold into the opposing state, where the locus is predominantly covered by M modifications. The first process is a site-specific nucleation of the opposing M modification mediated by a cold-induced increase of *VIN3* expression (motivated by the localized increase of H3K27me3 levels, see Fig. 1a). The second process is a permanent PHD–PRC2-mediated bias in the histone dynamics towards the M modification on return to the warm, motivated by the observed post-cold spread of the PHD protein *VRN5* across the locus⁹. The combination of these two processes enhances the probability of switching between the two bistable epigenetic states, before nucleation is lost (due to clearance of *VIN3* on a timescale of days at the end of the cold) and the potential to switch is greatly diminished.

We simulated the above model in 10,000 individual *FLC* loci, and attempted to fit unconstrained model parameters to our experimental ChIP data (see Supplementary Table 2). In Fig. 3a–d we show the simulated population-averaged levels of the M modification averaged separately over the nucleation region (five-histones wide) and the rest of the locus. Also shown are the experimental H3K27me3 values for non-vernalized plants and 0 and 7 days after 2, 4, 6 or 8 weeks of cold, averaged separately over the nucleation and distal regions of *FLC* (as defined in Fig. 1). We find good overall agreement with the observed data. During the cold, some limited increase of H3K27me3 levels does occur in distal regions (Fig. 1b). However, by tracking the dynamics of individual simulated *FLC* loci, we predict that these low levels of H3K27me3 arise from a large subpopulation of cells with little or no H3K27me3 coverage away from the nucleation region and a small subpopulation where H3K27me3 substantially covers the locus (occupying ~90% of histones, limited by noisy dynamics such as nucleosome turnover). Switching into this latter subpopulation is limited by the lack of a (*VRN5*-dependent) bias towards the M modification. On return to the warm, however, this limitation is removed: our simulations then demonstrate that a transient narrow peak of H3K27me3 can indeed cause the epigenetic state of the locus to be efficiently switched. Again, this rise in H3K27me3 levels is predicted to be due to a subpopulation of cells with substantial coverage of H3K27me3 across the



histone modification reactions are biased towards M modification. This leads to a short window in which the system can switch from high A to high M modification coverage. **c**, After the cold, a subpopulation of cells, whose number depends quantitatively on the length of the cold, stably switched to a silenced state, with high levels of M modification coverage.

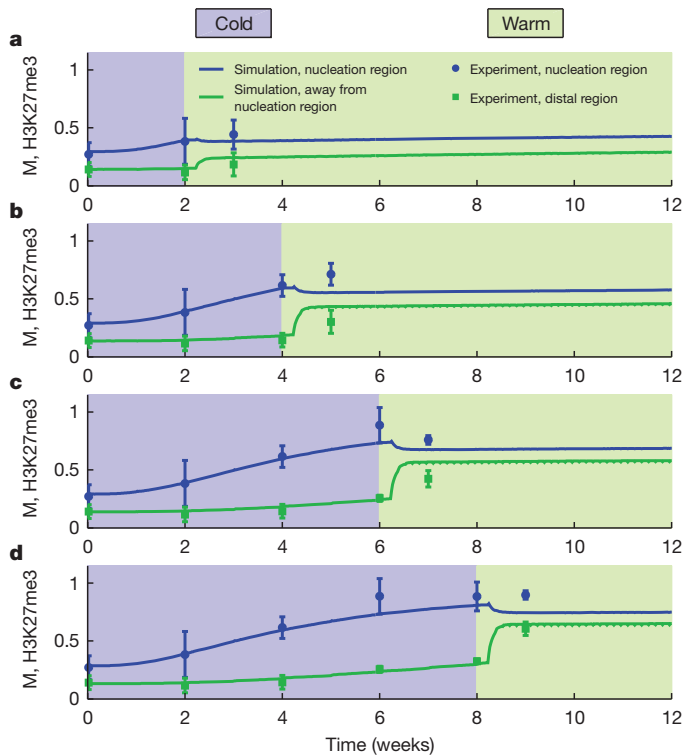


Figure 3 | Fitting model output to experimental ChIP data. a–d, Simulated population-averaged levels of M modification, averaged separately at and away from the nucleation region, as compared to experimental values of H3K27me₃, averaged separately over nucleation and distal regions, for non-vernalized plants and at 0 and 7 days post-cold after 2 (a), 4 (b), 6 (c) or 8 (d) weeks of cold. Error bars represent the range, $n = 2$.

FLC locus, with the remaining cells having low H3K27me₃ levels. Simulated population-averaged levels of the M modification from our fitted simulations are found to be approximately stable up to 30 days after the cold (Fig. 3), with a population-level change of less than about 10% from 7 days after the cold, in accordance with the stable long-term silencing observed experimentally. Simulated *FLC* expression levels 7 days after the cold also show a robust reduction from

non-vernalized levels, a reduction whose magnitude increases with increasing length of cold (Supplementary Fig. 5). However, with our fitted parameters, the simulated reduction is not as large as seen experimentally. The model therefore indicates that additional factors could reinforce repression of *FLC*, consistent with a role for the DNA-binding protein VRN1 (ref. 23).

According to the model, quantitative silencing on return to the warm is achieved through the fraction of cells that switch to the silenced state, a fraction that increases with increasing length of cold. Although the active or silenced epigenetic states are stable, a nucleating peak gives rise to a small probability per unit time of switching. This switching is generated by the noisy histone modification dynamics, which are able to amplify the signal from the nucleating peak of H3K27me₃ and use a rare fluctuation to flip the epigenetic state of the system. The timescale of a switch at an individual locus from predominant A to M coverage is predicted to be very rapid (about 45 min, see Supplementary Fig. 6).

To test the model prediction of bistable *FLC* expression, we assayed expression of an *FLC:GUS* translational fusion^{24,25} in tissue exposed to different cold treatments. For ease of imaging we analysed root tissue, in which cold-induced epigenetic silencing of *FLC* occurs, and which can regenerate into plants that have ‘remembered’ the cold exposure²⁶. The results are shown in Fig. 4a and Supplementary Fig. 8, and show that without cold the gene is evenly expressed in the dividing cells of the root tissue. This expression is reduced in all cells after 2 weeks of cold, supporting the fast reduction in *FLC* transcription seen previously²⁷. However, on return to the warm, the expression reactivates in a proportion of cells, in a cell-autonomous manner. These cells reflect the proportion of the population where the epigenetic switch to the silent state has not occurred. This heterogeneity in silencing would explain the flowering variation in regenerants from partially vernalized plants²⁸. The proportion of cells reactivating *FLC* expression reduces with increasing time in the prior cold (Fig. 4a), fully supporting the model predictions. The diversity of sector patterns reflects the stochastic nature of the silencing process: both single cells and clusters of *FLC:GUS*-expressing cells are observed, the latter probably reflecting clones from a single reactivating cell (Supplementary Fig. 8). The modelling also predicted that population-averaged levels of H3K27me₃ could rise rapidly (within 1–2 days) after return to the warm (Fig. 4b). Such increasing levels reflect individual *FLC* loci undergoing very rapid

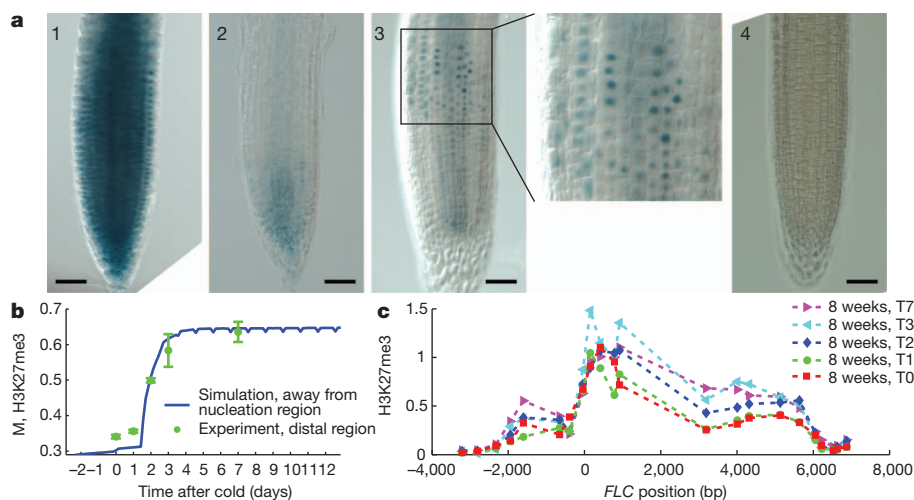


Figure 4 | Validating model predictions. a, *Arabidopsis* roots expressing *FLC:GUS* from plants with various cold treatments. Panels from left to right: (1) non-vernalized plants; (2) cold-treated for 2 weeks, harvested immediately on return to warm (T₀); (3) cold-treated for 2 weeks, then grown for a further 7 days in the warm (T₇); or (4) cold-treated for 4 weeks and then grown for a further 5 days in the warm (T₅). Scale bars, 50 μ m. b, Simulated population-averaged levels of M modification, averaged away from nucleation region, as

compared to experimental values of H3K27me₃ (from c and Supplementary Fig. 9), averaged over distal region 0, 1, 2, 3 or 7 days post-cold after 8 weeks of cold. Error bars represent the range, $n = 2$. c, Higher time resolution ChIP data for H3K27me₃ profile across *FLC* at 0, 1, 2, 3 or 7 days post-cold after 8 weeks of cold. Similar results were obtained with a biological replicate (Supplementary Fig. 9).

switching (taking only about 45 min) into the silenced epigenetic state, but at different starting times. To experimentally test the prediction of a rapid rise at population level, we performed additional higher time resolution (daily) ChIP experiments for the week immediately following the cold (Fig. 4c and Supplementary Fig. 9). In accordance with model predictions, we found a rapid rise of population-averaged H3K27me3 levels, with the H3K27me3 profile increasing mostly between 1 and 3 days after the cold.

Our combined experimental/modelling approach to vernalization has revealed that the quantitative level of epigenetic silencing in the warm is generated by an appropriate proportion of cells switching into a stable *FLC*-silenced state. Controlled switching of individual cells between bistable states, mediated by nucleation of an opposing histone mark may therefore be a common method of achieving a quantitative epigenetic response at a population level.

METHODS SUMMARY

The ChIP experiments and *FLC* expression analysis were carried out using *Col Fri-sf2* (ref. 29). H3K27me3 and H3 levels were assayed using anti-H3K27me3 (Millipore 07-449) and anti-H3 (Abcam 1791), respectively. *SHOOT MERISTEMLESS (STM)*³⁰ was used as the internal control for the ChIP experiments (primers are listed in Supplementary Table 1). Data are represented as the ratio of (H3K27me3 *FLC*/H3 *FLC*) to (H3K27me3 *STM*/H3 *STM*). *FLC:GUS* assay was performed using *FLC-Cok:GUS* in *Ler FR25*. Photoshop adjustment involved only image exposure using adjustment levels.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions C.D. and M.H. conceived the study, A.A., J.S., C.D. and M.H. designed the experiments, J.S. performed the experiments, A.A. and J.S. analysed the experimental data, A.A. and M.H. designed the numerical simulations, A.A. performed the simulations and analysed the simulation data. A.A., J.S., C.D. and M.H. wrote the manuscript.

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METHODS

Plant material and growth conditions. The *Columbia* line *FRI-Sf2* was described previously²⁹. Plant growth conditions were described previously⁹. Plants were vernalized for 2, 4, 6 or 8 weeks for the ChIP experiments, together with the non-vernalized control. T0 seedlings were harvested immediately after prolonged cold, whereas T1, T2, T3 and T7 seedlings were grown for 1, 2, 3 and 7 days, respectively, after transfer back to the warm.

ChIP and real-time quantitative PCR analysis. ChIP assays were performed as previously described⁹ with modifications. Trimethyl-histone H3 lysine 27 was assayed using anti-trimethyl-histone H3 lysine 27 from Millipore/Upstate (catalogue no. 07-449). Histone H3 levels were assayed using anti-H3 core antibody from Abcam (catalogue no. 1791). After immunoprecipitation, DNA was recovered using Chelex 100 resin (Bio-Rad, 10 g per 100 ml ddH₂O). All ChIP experiments were quantified by quantitative PCR (qPCR) in triplicates with appropriate primers (Supplementary Table 1). *SHOOT MERISTEMLESS (STM)*³⁰ was used as the internal control for the ChIP experiments. Data are represented as the ratio of (H3K27me3 *FLC*/H3 *FLC*) to (H3K27me3 *STM*/H3 *STM*).

Expression analysis. cDNA was synthesized using SuperScript III (Invitrogen) with a mixture of oligo d(T) and gene-specific primer (*FLC_unspliced_R* 5'-cttgtaatcaaagtg gagagc-3'), and analysed by qPCR on a LightCycler 480 II instrument (Roche), using LightCycler 480 SYBR Green mix (Roche). *FLC* sense unspliced (unspliced intron 2/3, *FLC_unspliced_F* 5'-cgcaatttcatagccttg-3', *FLC_unspliced_R*) quantification was normalized against the *Arabidopsis UBC* gene (*At5g25760*, *UBC_F* 5'-ctgcgactcagg gaatcttctaa-3', *UBC_R* 5'-ttgtgccattgaattgaacc-3').

***FLC:GUS* assay.** The *FLC-Col:GUS* in *Ler FRI* was described previously²⁵. The *FLC-Col:GUS* translational fusion contains the β -glucuronidase coding sequences cloned into the *NheI* site within *Col FLC* exon 6 (ref. 24). GUS staining was carried out by 4.5 h incubation at 37 °C in X-Gluc staining solution (0.5 mg ml⁻¹ X-Gluc, 2.0 mM K₃Fe(CN)₆, 2.0 mM K₄Fe(CN)₆, 100 mM phosphate buffer pH 7.0, 0.1% [v:v] Triton X-100, 10 mM EDTA). Seedlings were vacuum infiltrated for 5 min before incubation. After staining, seedlings were cleared in 75% (v:v) ethanol and then 8:1:3 (w:w:w) chloral hydrate:glycerol:water. Roots were imaged using DIC optics on a Leica DM6000 microscope with a Leica DFC420 camera controlled via Leica LAS AF7000 software. Photoshop adjustment involved only image exposure using adjustment levels.