**COOLAIR and PRC2 function in parallel to silence FLC during vernalization**

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Abstract
Non-coding transcription induces chromatin changes that can mediate environmental responsiveness, but the causes and consequences of these mechanisms are still unclear. Here, we investigate how antisense transcription (termed COOLAIR) interfaces with Polycomb Repressive Complex 2 silencing during winter-induced epigenetic regulation of Arabidopsis FLOWERING LOCUS C (FLC). We use genetic and chromatin analyses on lines ineffective or hyperactive for the antisense pathway in combination with computational modelling to define the mechanisms underlying FLC repression. Our results show that FLC is silenced through pathways that function with different dynamics: a COOLAIR transcription-mediated pathway capable of fast response; and in parallel a slow Polycomb Repressive Complex 2 (PRC2) switching mechanism that maintains each allele in an epigenetically silenced state. Components of both the COOLAIR and PRC2 pathways are regulated by a common transcriptional regulator (NTL8), which accumulates by reduced dilution due to slow growth at low temperature. The parallel activities of the regulatory steps, and their control by temperature-dependent growth dynamics, create a flexible system for registering widely fluctuating natural temperature conditions that change year on year, and yet ensure robust epigenetic silencing of FLC.

Significance statement
The role of non-coding transcription in chromatin regulation is still controversial. This controversy has extended to the role of transcription of antisense transcripts called COOLAIR in the Polycomb-mediated epigenetic silencing of Arabidopsis FLC, a key step in the process of vernalization. Here, we show that COOLAIR transcription and PRC2 silence FLC in parallel pathways; an antisense-mediated transcriptional repression capable of fast response, and a slow PRC2 epigenetic silencing, both of which are affected by growth dynamics and temperature fluctuations. These features explain the varied importance of COOLAIR transcription in cold-induced FLC epigenetic silencing seen in various studies using different conditions. The parallel repressive inputs and extensive feedbacks make the mechanism counter-intuitive but provide great flexibility to the plant.

Main text

Introduction
Non-coding transcription has emerged as an important mechanism in environmentally responsive gene regulation. In some cases non-coding transcription induces chromatin changes that are lost if the environmental signal is removed (1, 2). In other cases chromatin changes, particularly those involving the Polycomb mark H3K27me3, are epigenetically maintained providing a memory of the inductive signal. One well-characterised example of the latter is the winter-induced epigenetic silencing of the Arabidopsis
floral repressor gene, *FLC* (3, 4). This underpins the vernalization process, the acceleration of flowering by winter exposure. The process includes early induction of a series of antisense transcripts, called *COOLAIR* (5); a slow epigenetic switch from an active chromatin environment (marked by H3K36me3) to a silenced chromatin state (marked by H3K27me3) at an internal three nucleosome region (6); and spreading of the H3K27me3 Polycomb silencing over the whole locus (7-8). The switching mechanism involves canonical Polycomb Repressive Complex 2 (PRC2) and Arabidopsis PRC2 accessory proteins VIN3 and VRN5. VIN3 is slowly induced by cold exposure (9), interacts with PRC2 at the nucleation region downstream of the *FLC* transcription start site, and has a functionally important head-to-tail polymerization domain (10).

The timing of early antisense transcription and later VIN3 expression led to the view that antisense transcription was a prerequisite for PRC2 silencing. Consistent with this, single-molecule FISH experiments revealed that *COOLAIR* expression was mutually exclusive with *FLC* sense transcription at each allele (11). This sequence of events was initially tested through T-DNA insertions into the *COOLAIR* promoter. These had little effect on long-term vernalization (12). Similarly, *FLC* silencing was unaffected in studies using a CRISPR deletion of the *COOLAIR* promoter or mutation of *CBF* factors, known to facilitate cold-induction of *COOLAIR* (13). However, replacement of *COOLAIR* 5’ sequences (TEX1 line) attenuated *FLC* transcriptional silencing and disrupted the co-ordinated changes in H3K36me3 and H3K27me3 occurring at the *FLC* nucleation region (14).

*COOLAIR* had much stronger effects in experiments analysing *FLC* silencing in natural field conditions. *COOLAIR* expression was strongly induced on the first freezing night of autumn (15, 16), a result recreated in controlled environment cabinets (15). In these experiments, one freezing night was sufficient to induce *COOLAIR*, but several freezing nights were required to silence *FLC*, with silencing attenuated by disruption of antisense transcription. This data is reminiscent of many *S. cerevisiae* loci, where non-coding transcription plays an important role in environmental responsiveness (1, 17, 18). However, extensive feedback mechanisms between chromatin, transcription and co-transcriptional processes make functions of non-coding transcription difficult to elucidate. In particular, buffering between transcription and RNA stability leads to changed transcriptional dynamics with no change in steady state RNA (2).

To clarify the regulatory mechanism at Arabidopsis *FLC*, we have undertaken a series of genetic, molecular, and computational analyses to investigate the role of *COOLAIR* in cold-induced *FLC* silencing. Here, we show that *FLC* is silenced through parallel pathways. *COOLAIR* transcription can limit sense transcription, and this is associated with reduction in levels of the active histone mark
H3K36me3; this mechanism involves disruption of a 5'-3' FLC gene loop. In parallel, PRC2 silencing switches each allele from an epigenetically ON to an OFF state; this involves nucleation of H3K27me3 and subsequent spreading over the locus during subsequent growth, associated with further reduction in H3K36me3 (6). The nucleated and spread states differentially influence FLC transcription, which is still modulated by COOLAIR transcription. While FLC silencing by the PRC2 pathway operates on a slow timescale, the rapid induction capability of COOLAIR transcription, as seen in freezing conditions (15), enables this pathway in these conditions to silence FLC transcription on fast timescales. Components of both pathways are also regulated by their common transcriptional regulator NTL8 (19), which accumulates based on reduced dilution dependent on growth dynamics in the different cold phases. We integrate these parallel regulatory activities into a mathematical model that predicts FLC chromatin dynamics and transcription in different conditions. We argue that parallel activities converging onto a common target provides great flexibility in gene regulation, providing responsiveness to a wide variety of conditions. There are extensive similarities between how antisense transcription modulates FLC and how it alters sense transcription dynamics in yeast (2).

Results

COOLAIR rather than PRC2 nucleation is the major contributor to FLC repression in ntl8-D3. Two independent genetic screens in different genotypes had identified dominant mutations that revealed NTL8 regulates VIN3 and COOLAIR (15, 19). Ectopic COOLAIR expression leads to very low FLC levels in warm grown plants (15). We therefore confirmed that VIN3 and COOLAIR are both misregulated in the dominant mutant ntl8-D3 (Fig. 1A), and then used it to genetically activate both pathways simultaneously, independently of cold. FLC transcriptional output, histone modifications and chromatin topology were analysed. Paralleling cold effects on wild-type plants the ectopic COOLAIR expression in ntl8-D3 resulted in a clear decrease in H3K36me3, as compared to ColFRI, at the FLC transcription start site (TSS) and over the gene body (Fig. 1B). The high COOLAIR transcription in ntl8-D3 led to accumulation of H3K36me3 at the COOLAIR promoter (Fig. 1B), matching the cold-induced transient increase of H3K36me3 in ColFRI at the same position. The decrease in H3K36me3 was not accompanied by an increase in H3K27me3 observed during vernalization (Fig. 1C). Likewise, H2Aub, another histone modification that accumulates at FLC during early vernalization did not accumulate ectopically in ntl8-D3 (Fig. 1D). The lack of accumulation of H3K27me3 and H2Aub in ntl8-D3 compared to ColFRI in the absence of cold supports the view that VIN3 expression itself is not sufficient to cause Polycomb mediated silencing of FLC. These data indicate that antisense-mediated suppression rather than VIN3-mediated nucleation of H3K27me3 is the major factor causing FLC repression in ntl8-D3. Repression of
sense FLC transcription in ntl8-D3 is almost completely suppressed when COOLAIR transcription is blocked, giving further support to this conclusion (15).

Ectopically expressed VIN3 localises to FLC but fails to induce H3K27me3 nucleation. To understand what prevents the accumulation of H3K27me3 in ntl8-D3 despite ectopic VIN3 expression, we tested if other epigenetic factors are misexpressed in ntl8-D3. Only one of the tested genes changed slightly in expression (SI Appendix, Fig. S1). We then analysed association of VIN3 at the nucleation region in ntl8-D3. Despite the lack of H3K27me3 accumulation in ntl8-D3, we found VIN3-eGFP accumulated at the FLC nucleation region in warm conditions, mimicking the accumulation during vernalization (Fig. 1E). Thus, VIN3 accumulation at the nucleation region does not result in stable nucleation of H3K27me3. To distinguish VIN3 intrinsic binding to the FLC nucleation region, independently of COOLAIR transcriptional induction, we expressed VIN3-eGFP under the promoter of VRN5 (SI Appendix, Fig. S2A). This resulted in expression levels in non-vernalized plants that paralleled VIN3 induction after six weeks cold (6WT0) (SI Appendix, Fig. S2B). In this line VIN3-eGFP was enriched at the FLC locus in NV conditions (SI Appendix, Fig. S2C), showing VIN3 can remain associated with the nucleation region even when FLC is strongly expressed. We found that VIN3 association in ntl8-D3 led to H3K27me2 enrichment despite no accumulation of H3K27me3 (Fig. 1F). Thus, cold-induced features, possibly influencing residence time, are required to enable VIN3 functionality to deliver H3K27me3 to the nucleation region.

Ectopic induction of COOLAIR correlates with chromatin topology changes. A cold-induced feature at FLC is disruption of a gene loop conformation that links the transcription start site (TSS) and the transcription termination site (20). In ntl8-D3, we found that the gene loop was ectopically disrupted, mimicking vernalization (Fig. 1G). This suggests that gene loop disruption is linked with antisense-mediated reduction in FLC transcription. We also found that the TEX2.0 transgene, where a nos terminator promotes early COOLAIR termination, reduces gene loop formation (SI Appendix, Fig. S3), consistent with earlier reports using a similar, but not identical transgene (21). This result suggests a role for the activity of the antisense promoter/TSS, rather than antisense transcription per se, as being important for gene loop disruption.

Disrupting COOLAIR transcription perturbs H3K27me3 dynamics before and during cold, but not post-cold H3K27me3 levels. We further investigated the fact that ectopic expression of antisense transcription is enough to cause lower H3K36me3 around the FLC sense TSS and in the gene body, even
in the absence of cold. Antisense transcription could lower H3K36me3 levels, either through direct removal mediated by antisense transcription or indirectly by limiting sense transcription, thus preventing the co-transcriptional addition of H3K36 methylation. To dissect the interplay of H3K36me3 and H3K27me3, we studied the dynamic changes in these modifications using a vernalization time course.

Our previous analyses of TEX transgenes were in an flc-2 background, where part of the endogenous FLC genomic sequence remains (4). This limited the regions where the chromatin modifications on the transgene could be studied (14). To overcome this limitation, we generated a FRI +, FLC null (flclean) where the entire FLC genomic sequence had been deleted using CRISPR (SI Appendix, Fig. S4) and introduced the previously described TEX1.0 (replacement of the COOLAIR promoter) and TEX2.0 (insertion of a nos terminator to truncate COOLAIR transcription) transgenes. We also included a FRI FLCACOOLAIR CRISPR line, which deletes the COOLAIR promoter at the endogenous locus (22). Using these multiple defective COOLAIR lines and respective controls, we undertook a detailed time course of histone modifications during vernalization, including multiple time points post-cold (Fig. 2A, B).

The rate of accumulation of H3K27me3 during cold exposure was not reduced in the COOLAIR defective genotypes compared to the wild-type, and at some timepoints was even accelerated (Fig. 2C and SI Appendix, Fig. S5A,B), consistent with our previous data (14). By 6WT0, wild-type and COOLAIR defective genotypes show similar H3K27me3 levels in the nucleation region (Fig. 2A and SI Appendix, Fig. S5C). However, there were clear differences in the starting levels of H3K27me3, being significantly lower in the nucleation region in all defective COOLAIR genotypes (Fig. 2A and SI Appendix, Fig. S5D). Consistent with the differences in starting H3K27me3 levels, and supporting a role for antisense transcription in establishment of the initial FLC chromatin state (23), the defective COOLAIR genotypes showed a consistent trend of higher FLC RNA before cold exposure ( SI Appendix, Fig. S5E), although the differences were small. The similar trend in TEX1, TEX2 and FLCACOOLAIR argues against this being a specific TEX transgene effect. We interpret the H3K27me3 level in ColFRI before cold as representing a fraction of FLC alleles that have switched to a stable Polycomb silenced state. Thus, higher H3K27me3 levels in ColFRI compared to the COOLAIR defective genotypes may reflect the COOLAIR role in developmentally regulated PRC2 silencing of FLC (24, 25). After cold there was no significant difference in H3K27me3 levels in the nucleation region between ColFRI and any of the COOLAIR defective genotypes (Fig. 2A and SI Appendix, Fig. S5C). Spreading of H3K27me3 was also unaffected in the COOLAIR defective genotypes, as seen from the similar levels in the gene body at 6WT10 and 6WT20 (Fig. 2A). Overall, we find that H3K27me3 dynamics before and during cold are perturbed by COOLAIR, but that post-cold H3K27me3 levels are not.
Disrupting COOLAIR transcription attenuates H3K36me3 removal during vernalization.

H3K36me3 levels were similar in all genotypes before vernalization (Fig. 2B and SI Appendix, Fig. S5F) but decreased at different rates during cold exposure (Fig. 2D). This contrasts with the clear NV differences in H3K27me3 levels. However, this is consistent with the NV H3K27me3 levels coming from a small fraction of silenced alleles, while most alleles are transcriptionally active and contribute to the observed H3K36me3 levels, a scenario that generates bigger fold changes in H3K27me3 than in H3K36me3, as we observe (SI Appendix, Fig. S5G). H3K36me3 levels reduced more slowly in all defective COOLAIR genotypes at 6WT0 (Fig. 2B and SI Appendix, Fig. S5G), but after 2 weeks cold H3K36me3 levels increased in the gene body compared to NV (Fig. 2B). There were no differences in H3K36me3 levels between COOLAIR defective genotypes and wild-type ColFRI after transfer back to warm (Fig. 2B). In ntl8-D3, where VIN3 and COOLAIR are both overexpressed, faster reduction of H3K36me3 in the cold was observed, while H3K27me3 was less affected (SI Appendix, Fig. S6 A-D, SI Appendix, Fig. S9B-C). Together our results demonstrate that the Polycomb pathway is effective enough to completely silence the FLC locus, despite either an ineffective or hyperactive antisense pathway. The COOLAIR-mediated pathway mediates not only the removal of H3K36me3 but also H3K4me1 through the activity of the demethylase complex FLD-LD-SDG26 (23). H3K4me1, like H3K36me3, has been shown to be added co-transcriptionally in plants (26). Consistently, we found that in the COOLAIR defective lines, H3K4me1 reduction during vernalization was attenuated (SI Appendix, Fig. S7) showing the same trend as H3K36me3, including the increase at 2WT0. Overall, we find that COOLAIR defective genotypes have reduced rates of H3K36me3 removal, but after cold, any differences in H3K36me3 levels disappear. The relative changes of the unspliced FLC RNA levels did not match the corresponding H3K36me3 levels in the COOLAIR defective genotypes and effects on spliced FLC levels were different to unspliced (Fig. 2E-F). This suggests a similar interconnected mechanism linking chromatin modification to transcript stability as found in yeast, with unspliced and spliced transcripts affected in different ways (2).

H3K27me3 accumulation is not necessary for COOLAIR-mediated transcriptional downregulation.

A mutation in the core PRC2 component Su(z)12 (VRN2) only partially disrupted FLC repression (SI Appendix, Fig. S8A) (6), whilst H3K36me3 fold reduction at FLC during the cold was hardly changed (SI Appendix, Fig. S8B), despite accumulation of H3K27me3 being abolished (6). Thus, H3K36me3 reduction and FLC RNA downregulation do not rely on H3K27me3 nucleation. Analysis of a vrn5-TEX1.0 combination, defective in H3K27me3 accumulation and COOLAIR, had shown that the H3K36me3 reduction seen in an H3K27me3 nucleation mutant is mediated by COOLAIR (14). To
examine this aspect further, we analysed changes in the two modifications, H3K36me3 and H3K27me3, in fluctuating cold conditions, where we see the clearest indication of COOLAIR transcription regulating FLC expression (15). Under these conditions, COOLAIR was highly upregulated, causing significant downregulation of FLC sense transcript (15). Whilst we have previously shown that full-length COOLAIR transcription is essential for the FLC downregulation in these conditions (15), a role for H3K27me3 nucleation had not been investigated. Here we analysed H3K36me3 and H3K27me3 levels in ColFRI at 2WT0, under three different cold conditions (as in (15)), constant 5°C (CC, Constant Cold), mild 3°C - 9°C (FM, Fluctuating Mild), and strong fluctuating conditions -1°C - 12°C (FS, Fluctuating Strong). Zhao et al., 2021 showed that COOLAIR upregulation and FLC downregulation were greatest in the FS condition. Therefore, we would expect H3K36me3 to show the largest changes in this condition, and indeed this is seen in our data (Fig. 3A, SI Appendix, Fig. S9E). Both mild fluctuating and constant cold result in smaller changes (Fig. 3A, SI Appendix, Fig. S9E). In contrast, H3K27me3 accumulation showed little difference between the different conditions (Fig. 3B and SI Appendix, Fig. S9D), indicating that H3K27me3 is not the major contributor to the enhanced downregulation under strongly fluctuating conditions. The lack of difference in H3K27me3 accumulation, despite the relatively large change in antisense expression, further highlights the parallel and almost independent nature of these FLC repression pathways. To further confirm that COOLAIR transcription is necessary for the changes in H3K36me3 under fluctuating conditions, we subjected the TEX1.0 defective COOLAIR line to these conditions. As expected, the reduction in H3K36me3 was also attenuated relative to ColFRI (Fig. 3C, D). This is consistent with the lack of FLC sense transcriptional shutdown under FS conditions in a COOLAIR deletion line as recently described (27). Interestingly, for COOLAIR defective genotypes, the slight increase in H3K36me3 at 2WT0 observed in constant cold was also recapitulated in the FM conditions. The reduction under FS conditions was significantly attenuated in TEX1.0 (SI Appendix, Fig. S9F) and an analysis to test whether this is also the case in the COOLAIR deletion line is ongoing. Overall, we find that COOLAIR-mediated transcriptional repression does not strongly depend on H3K27me3 nucleation, supporting our earlier results in ntl8-D3. The contrasting dynamics of H3K36me3 and H3K27me3 under 2 weeks of fluctuating conditions further highlight the fast-response capability of the antisense-mediated repression. In ColFRI, the changes in nucleation region H3K36me3 after only 2 weeks of FS conditions (Fig. 3A,C) are comparable to H3K36me3 changes after 6 weeks in constant cold (Fig. 2B, SI Appendix, Fig. 5G). The fast response capability of FLC antisense transcription to temperature changes is also supported by field data for A. halleri FLC (28). They showed that H3K4me3 associated with COOLAIR transcription at the 3’ end of the FLC locus responds to temperature changes on a much faster timescale compared to the 5’ end (associated with FLC sense transcription), which responds mainly on a slow timescale.
Mathematical modelling of FLC regulation reconciles the different effects of antisense transcription on chromatin state. The dynamics of the two repression pathways are difficult to dissect quantitatively purely through molecular experiments. We therefore turned to mathematical modelling to see how the observed behaviour in COOLAIR defective mutants could be reconciled with our existing understanding of FLC repression in the cold. We have previously developed and experimentally validated a mathematical modelling framework describing dynamically changing fractions of active/silenced FLC alleles and their associated histone modifications (30, 31). Here we built on this framework to develop a new model, incorporating an antisense-mediated silencing component. A schematic of the model developed here is shown in Fig. 3E (details in Supplementary Information - the MATLAB code used to simulate the ODE model is available at https://doi.org/10.5281/zenodo.10257997). The model was built based on our main conclusion from the above data: namely that two pathways work in parallel to silence FLC, antisense transcription and PRC2 nucleation. We then interrogated the model to see whether it was capable of quantitatively reproducing histone modification dynamics in ColFRI and the various mutants.

The effect of the antisense-mediated pathway on sense transcription was modelled implicitly as a cold-dependent graded modulator of sense initiation/transition to productive elongation. This is consistent with high antisense transcription in ntl8-d mutants causing low levels of FLC transcription, independently of H3K27me3 nucleation. This is also consistent with previous data showing that sense and antisense transcription at FLC are anti-correlated in ColFRI, both in warm (32) and in cold conditions (11). This may be through a mutual exclusivity model for the FLC locus, similar to that reported for the CBF1-SVALKA locus, where full-length sense transcription is inhibited by antisense transcription (33). Another key aspect of the model is the co-transcriptional delivery of the H3K36me3 modifications. Changes in Pol II elongation behaviour can affect the H3K36me3 profile across mammalian genes (34), with slower Pol II speed allowing a larger window of opportunity for adding H3K36me3 at any given location. Any changes in transcription at FLC may be expected to produce corresponding changes in H3K36me3. However, to explain the increase in gene body H3K36me3 observed in the defective COOLAIR lines, specifically at 2WT0 compared to NV, despite the lack of any increase in transcriptional output over that time period (Fig. 2E), the model includes a cold-induced reduction in Pol II speed in this region, resulting in a longer dwell time. The SDG8 H3K36 methyltransferase, which we have shown co-transcriptionally associates with RNA PolII (29), is likely mediating these H3K36me3 changes. The model also allows for H3K36me3 removal on a timescale consistent with the experimentally observed lifetime of H3K36 methylation in other systems (35), so that its levels at the nucleation region would decay quickly in the
absence of sense transcription. The model also describes dynamic changes in \textit{FLC} mRNA levels as modelled in (30). However, due to the highly variable behaviour of spliced \textit{FLC} RNA observed in the different \textit{COOLAIR} defective mutants (Fig. 2F), which potentially reflects changes in RNA stability, we do not try to capture these levels using the model.

We also incorporate the PRC2 pathway and how it silences \textit{FLC} through H3K27me3 accumulation during vernalization. In cells which can have active or non-active cell cycles, we consider that \textit{FLC} alleles can be in three different states; non-nucleated (without H3K27me3 nucleation), nucleated, and spread, with the latter only attained in active cycling cells (31). To generate reasonable fits to our data, particularly the higher levels of H3K36me3 observed during cold in \textit{COOLAIR} defective mutants, we found that an extension to our previous models was needed, where we allow for different levels of \textit{FLC} transcription in the three states: highest in non-nucleated, much lower in nucleated and even lower in spread. Satisfactory fits also necessitated that the non-nucleated and nucleated states be capable of further downregulation by antisense transcription. Consistent with the possibility of some limited transcriptional activity in the nucleated state, we, therefore, allowed for potential co-existence of H3K27me3 and H3K36me3 on the same nucleosome. We then fitted the model to capture the qualitative changes in H3K36me3, H3K27me3, and transcription (sense and antisense) observed in the cold for Col\textit{FRI} and the defective \textit{COOLAIR} lines. We found that this model could capture all the qualitative features of the data observed in Col\textit{FRI} and the defective \textit{COOLAIR} lines (Fig. 4A-D), including both the increase of H3K36me3 at 2WT0 and the subsequent significantly slower reduction in H3K36me3 in the latter (Fig. 4A,B), as well as the reduction of H3K36me3 in the post-cold seen in all the lines (Fig.4A,B).

We then tested whether the model could capture our previous datasets by simulating other mutants that affect \textit{FLC} silencing in the cold (see previously published data (6)), including an H3K27me3 nucleation mutant (e.g., \textit{vrn2}, \textit{vin3}), a spreading mutant (e.g., \textit{lhp1,clf}). In all cases, the simulation outputs from the model are qualitatively consistent with data, including the post-cold behaviour of the two histone modifications (SI Appendix, Fig. S10A-D). Interestingly, in addition to recapitulating the behaviour captured by our previous models, the new model can capture the reduction of H3K36me3 in the post-cold seen in Col\textit{FRI} - a trend that could not be previously captured (Fig. 4B). This is because the new model allows for higher levels of transcription (and consequently higher H3K36me3) in a nucleated state relative to a spread state.

The model also incorporates a fast timescale response in the antisense mediated pathway, which can respond to temperature fluctuations (see Supplementary Information for details). Briefly, this consists of a
simple step increase in antisense mediated repression resulting from temperatures dropping below a threshold, which is incorporated into the slower timescale increase in antisense mediated repression. The fast timescale response allows the model to qualitatively capture the differences in H3K36me3 and FLC spliced RNA between the different cold conditions CC, FM, FS (SI Appendix, Fig. S11). The model predicts that H3K36me3 and FLC mRNA levels respond on a fast timescale, exhibiting oscillations in response to the daily repeated temperature profiles of FM and FS conditions. While the agreement between the model and experiments is overall good, the model did predict reduced H3K27me3 nucleation in FS conditions, which was not observed experimentally. This discrepancy may potentially arise from differences between the field conditions used to parameterise our model for H3K27me3 nucleation (30) and the FS experimental conditions used here. The model predicts that antisense transcription limits H3K36me3 through a graded, analog reduction in FLC transcription rather than by directly mediating H3K36me3 removal. The increased H3K36me3 at 2WT0 in COOLAIR defective lines, is predicted to arise from a combination of higher FLC sense transcription (since antisense mediated repression is disrupted) and cold-induced reduction in Pol II speed in the nucleation region. In a second slower response chromatin pathway involving Polycomb Repressive Complex 2 (PRC2), each allele progressively switches from a non-nucleated to H3K27me3 nucleated state during the cold and then to a spread H3K27me3 state during post-cold growth. The model indicates that intermediate levels of FLC transcription in the nucleated state, which can be further downregulated by antisense transcription, can explain how clear differences in H3K36me3 between defective COOLAIR lines and the wild-type can emerge in the cold yet subsequently disappear during growth after transfer to warm conditions. In these conditions, all the nucleated FLC alleles convert to the H3K27me3 spread state due to an active cell cycle (6), regardless of H3K36me3 levels and any residual expression. Hence, in the context of vernalization, the COOLAIR repressive pathway is most important during rather than after cold.

Discussion
Focused dissection of the mechanism underlying winter-induced FLC silencing has established a role for antisense transcription and PRC2 activity in registration of long-term exposure to noisy environmental signals (5, 15, 36, 37). However, the complexity of the mechanism, and its sensitivity to variable temperature and growth parameters, has led to different studies questioning the importance of the antisense transcription in cold-induced FLC silencing. Here, using a combination of experiments and mathematical modelling, we have elucidated the role of antisense transcription and PRC2 activity as parallel pathways, both leading to FLC silencing (Fig. 4B). The antisense-mediated pathway involves the FLC gene loop and represses FLC transcription (38). Two other lncRNAs have been described at FLC, COLDWRAP (39) and COLDAIR (40). We have detected cold-up-regulated FLC transcripts with
upstream transcription start sites including COLDWRAP that influence FLC expression levels but not cold-induced transcriptional silencing (41); we have not found COOLAIR equivalents.

The multiple effects of the COOLAIR-mediated transcriptional pathway on H3K36me3 in the 5’ region of FLC required modelling to deconvolve fully. In the wild-type, lower transcription leads to H3K36me3 reduction, but this is partly hidden in defective COOLAIR lines in the cold through a predicted increase in H3K36me3 from slower RNA PolII speed at the 5’ end of FLC. The slow PRC2 switch at each FLC allele from a non-nucleated to a H3K27me3 nucleated and then spread state is associated with decreasing frequencies of FLC transcription, consistent with previous findings of the relationship between H3K27me3 and FLC transcription (6). Both COOLAIR-mediated and PRC2 pathways are affected by the common transcriptional regulator NTL8, which accumulates slowly and variably dependent on reduced dilution by slower growth at low temperatures (19). Our observation that in ntl8-D3 both COOLAIR and VIN3 are ectopically expressed, yet the H3K27me2 modification but not H3K27me3 accumulates, implies a requirement for other cold-induced factors for vernalization (42, 43). These parallel repressive activities with multiple temperature inputs enables modulation of transcriptional silencing independently of robust epigenetic silencing. This gives the plants great flexibility to respond to autumnal conditions that vary in different geographical regions and from year to year yet ensure robust silencing. Indeed, variation in FLC transcriptional silencing has been shown to be an important adaptive determinant in A. thaliana accessions (16). It seems likely that similar parallel mechanisms may be involved in other seasonal responses, e.g., seed and bud dormancy and germination, for similar reasons.

The chromatin changes in COOLAIR-defective mutants are not directly reflected in steady-state unspliced and spliced FLC levels (Fig. 2E,F), similar to the situation in yeast (2). Which RNA stability mechanisms are involved remain to be determined, but m6A methylation has been shown to influence FLC regulation (38, 44, 45) and is enriched in the FLC 3’ UTR. This disconnect between chromatin dynamics and steady-state RNA levels is likely to have contributed to the controversy over the role of non-coding RNA in chromatin regulation generally. In addition, the effective combination of parallel pathways hides effects of mutations after saturating induction, e.g., mutations in CBF-binding factors (13). Another debate has been over the use of transgenes to modulate COOLAIR expression (13), but the use here of FLCΔCOOLAIR and ntl8-D3 for under/overexpression of COOLAIR argues against this. However, future studies need to generate a fully antisense null genotype, since all defective COOLAIR genotypes so far produced still contain cryptic antisense promoters, which become more active when the endogenous COOLAIR promoter is mutated/deleted (15). The difficulty of completely removing antisense transcription is also seen in other systems (46) and suggests transcription initiation from open chromatin regions rather than
specific promoter elements. Such a line would not only help elucidate the role of COOLAIR in the cold-induced silencing of FLC, but also in the starting FLC expression upon germination, a key determinant of natural variation underpinning adaptation (16).

The large number of plant chromatin regulators that interact with non-coding RNAs point to an important role of similar co-transcriptional mechanisms in environmental plasticity (47, 48). This is similarly true in yeast (2), where antisense expression has been associated with genes that are environmentally-silenced (1). Future work will address the evolutionary parallels and conservation of a mechanism enabling rapid transcriptional changes and switches to epigenetic silencing in response to noisy environmental cues.

Materials and Methods

Detailed descriptions of materials and methods are provided in the SI Appendix. A brief summery is provide here.

Plant materials

All mutant and transgenic lines were in the FRI°/° background. ntl8-D3 FRI was described previously (15). Generation of new mutant and transgenic lines are detailed in SI Appendix.

Expression analysis

RNA analysis was performed as previously described (49). Total RNA was extracted, DNAse treated, and cDNA was synthesised with SuperScript IV reverse transcriptase (Invitrogen). qPCR was performed using SYBR Green I Master (Roche) and analysed on a LightCycler 480 machine (Roche).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described in (49). ChIP was performed with antibodies: α-H3 (Abcam, ab1791), α-H3K36me3 (Abcam, ab9050), α-H3K27me3 (Abcam, ab192985), α-H3K27me2 (Upstate, 07-452), α-H2AK119ub (Cell Signaling Technology, #8240), α-H3K4me1 (Abcam, ab8895), and α-GFP (Abcam, ab290).

Chromatin Conformation Capture

Chromatin conformation capture was performed as described previously described (20) with minor modifications.
Mathematical modelling

The models used in this study are constructed within a framework we have previously developed and experimentally validated (29,30). In addition to what is captured by previous models, our new model also incorporates the dynamics of H3K36me3 and H3K27me3 at \(FLC\), as observed in Col\(FRI\) and the \(COOLAIR\) defective mutants.

Acknowledgements

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References


**Figures**

**Figure 1. ntl8-D3 mimics cold exposure, except for the accumulation of H3K27me3.**

(A) Expression of total COOLAIR and VIN3 in ntl8-D3 FRI and in ColFRI in non-vernalized conditions (NV). Data are presented as the mean ± s.e.m. Each open circle represents a biological replicate. (B-D) Enrichment of B) H3K36me3, C) H3K27me3, and D) H2A ubiquitin across FLC measured by ChIP in wild-type ColFRI and ntl8-D3 at NV conditions. H3K36me3 data are shown relative to H3, and actin. H3K27me3 data are shown relative to H3, and STM. H2Aub data are shown relative to H3. Error bars represent s.e.m (n ≥ 3 biological replicates). (E) VIN3-eGFP ChIP-qPCR enrichment at FLC at NV. Data are shown as the percentage input. Non-transgenic ColFRI plants were used as a negative control sample. Error bars represent s.e.m (n = 3 biological replicates). (F) Enrichment of H3K27me2 across FLC measured by ChIP in wild-type ColFRI and ntl8-D3 at NV conditions. Data are expressed relative to H3. Error bars represent s.e.m (n = 3 biological replicates). (G) Quantitative 3C-qPCR over the FLC locus in 10-day-old ColFRI and ntl8-D3 FRI seedlings. A schematic representation of the FLC locus is shown above. BamHI and BglIII restriction sites are indicated with dotted lines, and the respective regions are numbered with Roman numerals. Red arrows indicate the location of the primers used for 3C-qPCR. The region around the FLC transcription start site was used as the anchor region in the 3C analysis. The data below shows the relative interaction frequencies (RIF).

**Figure 2. Cold-induced chromatin and RNA dynamics in COOLAIR defective lines.**
(A-B) Enrichment of H3K27me3 (A) and H3K36me3 (B) across FLC measured by ChIP in wild-type ColFRI and the three defective COOLAIR lines, TEX1, TEX2, and FLC∆COOLAIR, before, during, and after vernalization. Data are expressed relative to H3 and STM. Error bars represent s.e.m. (n = 3 biological replicates). (C-D) Average levels of H3K27me3 (C) and H3K36me3 (D) in the nucleation region during vernalization. The averages were calculated by averaging the ChIP enrichment over three primers in the FLC nucleation region during vernalization in ColFRI and each of the defective COOLAIR lines. (E-F) FLC expression during a vernalization time course in ColFRI and the three defective COOLAIR lines, Unspliced (E) and spliced RNA (F), was measured and is shown relative to UBC and NV levels. Error bars represent s.e.m. (n = 3 biological replicates).

Figure 3. Fluctuating cold and mathematical modelling of the role of COOLAIR in histone modification dynamics. (A-B) Changes in H3K36me3 (A) and H3K27me3 (B) at FLC after two weeks of Constant Cold (CC), Fluctuating Mild (FM), or Fluctuating Strong (FS) conditions, measured by ChIP-qPCR. Data are expressed relative to H3. Error bars represent s.e.m. (n = 3 biological replicates). (C-D) Comparing changes in H3K36me3 at FLC between ColFRI (C) and TEX1 flclean (D) after two weeks of FM or FS conditions, measured by ChIP-qPCR. Data are expressed relative to H3 and STM. Error bars represent s.e.m. (n = 3 biological replicates). (E) Schematic of the mathematical model showing core components: PRC2 mediated silenced states (non-nucleated, nucleated and spread) at individual FLC alleles, antisense transcription mediated repression of FLC transcription, and the contribution of these components to the average population level H3K36me3 coverage at the FLC locus.

Figure 4. Model predictions of the impact of vernalization mutants on histone dynamics and schematic representation of parallel pathways that repress FLC expression. (A-D) Mathematical model predicted levels of H3K36me3 (A,B) and H3K27me3 (C,D) over a constant cold time course in a defective COOLAIR mutant (A,C) and the wild type, ColFRI (B,D). The predictions are compared to the ChIP-qPCR time course data for the different genotypes presented in Fig. 2(A,B). (E) Model for the parallel pathways that repress FLC. In the warm FLC forms a gene-loop conformation, which mediates a high expression state of FLC. The high expression state is marked by high levels of H3K36me3 around the FLC TSS. After cold exposure, the repressive pathways are activated: (1) The antisense-mediated pathway leading to disruption of the gene-loop and removal of H3K36me3 from the TSS of FLC. (2) The Polycomb pathway leading to deposition of H3K27me3 and repression of FLC transcription. The two pathways work in parallel rather than through a linear sequence of causation to give the final FLC expression outcome during vernalization.
Enrichment

H3K36me3

Col FRI (NV)
Col FRI (CC)
Col FRI (FM)
Col FRI (FS)

Enrichment

H3K27me3

Col FRI (NV)
Col FRI (CC)
Col FRI (FM)
Col FRI (FS)

Enrichment

H3K36me3

TEX1 FLClean
TEX1 FLClean
TEX1 FLClean

Distance from TSS (kb)

Non-nucleated

{7VS00ZWLLK (Lower in cold)}

Nucleated Spread

{TEX1 FLClean (NV)
TEX1 FLClean (FM)
TEX1 FLClean (FS)}

Model describes changes over time in the overall fraction of FLC alleles in each state.

Nucleation

Replication/division

Transcription frequency

AS mediated repression determines FLC transcription

Pol II speed

(Lower in cold)

H3K36me3 level
Supporting Information for
Antisense transcription and PRC2 repression function in parallel during vernalization

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SI Materials and Methods

Plant materials
All mutant and transgenic lines were in the \textit{FRI}^{02} background. Mutant alleles were described previously: \textit{ntl8-D3 FRI} (1), and \textit{FLC\textsubscript{ACOOLAIR}} (2). For this study, \textit{FLC\textsubscript{ACOOLAIR}} was introduced into Columbia with an active \textit{FRIGIDA} allele (3). The TEX1.0 and TEX2.0 constructs were described previously (1, 4), and for this study, the constructs were introduced into \textit{FLClean} by floral dipping. \textit{FLClean} was created by CRISPR-CAS9. The CRISPR guide-RNA sequences were: sgRNA\_1: attGAAAAGGGCAAGGAGGTGGTgttttagagctagaaatagcaag, sgRNA\_3: attGGCGGAGGAGCAGCGCAAGgttttagagctagaaatagcaag, sgRNA\_2: attGGGCGGACTCACGTTAGTCAgttttagagctagaaatagcaag, sgRNA\_4: attGTTGGAGCGCGTGAGGATCAgttttagagctagaaatagcaag.

Regions that map to the locus are in capital letters, mapping immediately upstream of the PAM motif (NGG). The long 3’-tail corresponds to the scaffold for Cas9 binding. Two \textit{FLClean} lines were created \textit{FLClean\_13}, with sgRNA 1 and 3, and \textit{FLClean\_24}, with sgRNA 2 and 4. The \textit{FLClean\_24} line was used as background for floral dipping with the \textit{TEX1.0} and \textit{TEX2.0} constructs. The \textit{pVRN5:VIN3-eGFP} line was generated by replacing the \textit{VIN3} promoter sequence from the \textit{pVIN3:VIN3-eGFP} construct described previously (5) with the promoter sequence of \textit{VRN5}. The construct was transformed into \textit{vin3-4 FRI} by floral dipping, and individual lines were selected. Two individual lines (#28 and #40) were used for ChIP analysis.

Growth conditions
Seeds were surface sterilized and sown on 1x Murashige and Skoog (MS) media without glucose. As \textit{FLC} shutdown in cold is sensitive to growth, seeds for expression analysis were sown at low density. Seeds were stratified for 2–3 days at 4°C and grown for 10 days under long-day conditions (16h light, 8h dark at 20°C). For vernalization treatment, seedlings were transferred to short-day conditions (8h light, 16h darkness at constant 5°C) after 10 days pre-growth. Plants harvested 10 days after vernalization were transferred back to long-day conditions and harvested from plates. For longer post-vernalization treatment (more than 10 days), the plants were transferred to soil and grown under long-day conditions. Fluctuating cold conditions used in Fig. 3 were as described previously (1).
Expression analysis

Total RNA was extracted using the hot phenol method as described previously (6). Genomic DNA contamination was removed with TURBO DNase (Invitrogen), following the manufacturer’s guidelines. cDNA was synthesized with SuperScript IV reverse transcriptase (Invitrogen). Gene-specific primers were used for reverse transcription (RT) of COOLAIR, FLC, and VIN3. For the RT reaction to analyse the expression of vernalization factors in SI Fig. 1, oligo(dT) primers were used. Quantitative PCR (qPCR) was performed using SYBR Green I Master (Roche) and analysed on a LightCyler 480 machine (Roche). Ct values were normalized to the geometric mean of UBIQUITIN CARRIER PROTEIN 1 (UBC) and SERINE/THRreonine PROTEIN PHOSPHATASE 2 A (PP2A). All primers are listed in SI Appendix, Table S1.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described in (7). ChIP was performed with antibodies: α-H3 (Abcam, ab1791), α-H3K36me3 (Abcam, ab9050), α-H3K27me3 (Abcam, ab192985), α-H3K27me2 (Upstate, 07-452), α-H2AK119ub (Cell Signaling Technology, #8240), α-H3K4me1 (Abcam, ab8895), and α-GFP (Abcam, ab290). ChIP enrichment was quantified by qPCR with primers listed in SI Appendix, Table S1. For histone, ChIP values were normalized to H3. For H3K36me3 and H3K27me3, values were further normalized to enrichment at the positive control loci ACTIN for H3K36me3 and STM for H3K27me3. Primers; FLC 0, FLC 250, and FLC 500 were used to calculate the average level in the nucleation region for the plots in Fig. 2 and SI Fig. 5.

Chromatin Conformation Capture

Chromatin conformation capture was performed as described previously (8, 9) with minor modifications. 1 g of 10 days old seedlings were crosslinked in 2 % formaldehyde for 20 mins. Crosslinking was stopped by the addition of 2M Glycin to a final concentration of 0.125 M and vacuum infiltrated for 7 mins. Nuclei were extracted with Honda buffer as for ChIP (7). After purification, chromatin was digested with 600 U of BamHI (NEB) and BglII (NEB) for 14-16 h, followed by 8h of ligation with T4 ligation (Promega) at 17°C. DNA was purified with Phenol:Chloroform:IAA (25:24:1) and precipitated with isopropanol. DNA was dissolved in water
and further purified using the ChIP DNA Clean & Concentration (Zymo Research) kit, following the manufacturer’s protocol. The 3C library was quantified as described previously (8).

The same primers were used to analyse TEX2.0, as the NOS sequence inserted in TEX2.0 contains no additional BglII or BamHI restriction sites.

Mathematical modelling

In this study we use mathematical modelling to dissect the complex interplay of Polycomb mediated silencing and antisense mediated repression involved in FLC regulation in the cold. The models used in this study are constructed within a framework we have previously developed and experimentally validated. This framework describes the dynamics of transcriptional shutdown and histone modification changes at the whole-plant level during cold-induced epigenetic silencing at FLC (10, 11). Therefore, the assumptions used in these previous models are carried through into the models developed here. Many of these assumptions are directly based on experimentally established details, while the validity of others has been established through experimental testing of model predictions in these previous studies (10, 11). The models developed in this study use these assumptions as a starting point before we add additional features.

H3K36me3 and H3K27me3 dynamics in COOLAIR defective mutants

Our previously developed (and experimentally validated) models successfully captured the behaviour of H3K27me3 at FLC in cold and post-cold conditions observed in ColFRI, nucleation mutants, spreading mutants, as well as reactivation in a natural variant of FLC. These models also captured some, although not all, aspects of H3K36me3 dynamics. However, these models did not capture the role of antisense mediated regulation. Here, we start with the existing modelling framework, and attempt to build a model that, in addition to what is captured by previous models, can also properly incorporate the dynamics of H3K36me3 and H3K27me3 at FLC as observed in ColFRI and the COOLAIR defective mutants. To do this we focus on only those trends which are consistent across the three different COOLAIR defective mutants. These features are as follows (Fig. 2):

1. Similar dynamics of H3K27me3 nucleation and spreading in ColFRI and COOLAIR defective mutants, with a significant difference in starting (NV) levels.
   - H3K27me3 nucleation is not slower in COOLAIR defective mutants.
   - H3K27me3 levels at 6WT0 are similar in ColFRI and COOLAIR defective mutants.
   - H3K27me3 spreading is similar in ColFRI and COOLAIR defective mutants.
2. Significant differences in H3K36me3 dynamics of the COOLAIR defective mutants during the cold.
   - H3K36me3 increases across the locus (relative to NV) in the COOLAIR defective mutants in the first two weeks of cold, while ColFRI does not show this trend.
   - The overall reduction in H3K36me3 levels over six weeks of cold is clearly weakened in the COOLAIR defective mutants, so that they exhibit significantly higher levels of this modification across the locus at 6WT0.
   - Consistent with H3K36me3 changes and the association of this modification with transcription, FLC unspliced also reduces more slowly in the COOLAIR defective mutants.

3. Similar behaviour of H3K36me3 in COOLAIR defective mutants and in ColFRI during post-cold growth.
   - A clear reduction of H3K36me3 levels is observed between 6WT0 and 6WT10/T20, in both COOLAIR defective mutants and ColFRI.
   - Consistent with H3K36me3 reduction, and the association of this modification with transcription, FLC transcriptional output also reduces further in the post-cold.

Building a model to capture the differences in H3K36me3 dynamics

The behaviour of H3K27me3 in the COOLAIR defective mutants (described above) can be captured by previous models, since the behaviour is similar to ColFRI, except for the difference in NV levels. However, these models cannot capture the H3K36me3 behaviour, which shows significant differences in the COOLAIR defective mutants. This is because: (i) these models do not explicitly include a regulatory role for antisense transcription, and (ii) these models treat H3K36me3 and H3K27me3 as being exclusively present in different states of the FLC locus (H3K36me3 only in a high transcriptional state, and H3K27me3 only in a nucleated or spread state).

Capturing FLC states at the whole plant level

The existing modelling framework describes FLC locus states in a population of cells representing the whole plant. This population is made up of dividing and non-dividing cells (10,11). One of the core assumptions of these models is that the FLC locus can undergo nucleation of H3K27me3 in both dividing (meristematic tissue) and non-dividing cells. The validity of this assumption is supported by both direct and indirect experimental evidence: (i) for dividing cells, measurements of FLC epigenetic silencing by Polycomb in root meristematic cells via fluorescent imaging of FLC-Venus in plants defective for spreading of H3K27me3 (12); (ii) for non-dividing cells, the direct measurement of H3K27me3 nucleation by ChIP in mature leaves during vernalization in ColFRI (13); (iii) for non-dividing cells, the indirect evidence from all of our own ChIP time course datasets for H3K27me3: If only copies in dividing cells were capable of
nucleation, the repeated division of these nucleated copies during rapid post-cold growth would cause a significant increase in population-level nucleation region H3K27me3. The fact that no such increase is observed by ChIP during post-cold growth indicates that FLC copies in both dividing and non-dividing cells can nucleate. This continues to be a core assumption in the models built here. As discussed in the main text, our data indicates that the antisense mediated repression and the PRC2/H3K27me3 mediated silencing function in parallel at FLC. This paradigm of two parallel pathways is therefore also central to the models developed here.

**Possible models with different behaviour in subpopulations of cells**

With the previously observed mutual exclusivity of H3K27me3 and H3K36me3 at FLC during vernalization (14), as well as other evidence for the mutual exclusivity of these modifications (15), it is tempting to consider models where apparent disruption of this mutual exclusivity in COOLAIR defective mutants arises from different subpopulations of FLC copies ending up in different states. While some tissue specific behaviour cannot be ruled out, here we examine two simple models with different behaviour between subpopulations and demonstrate that such subpopulation specific behaviour by itself is insufficient to capture the observed trends.

**Model 1:** A simple approach to explain the difference in H3K36me3 dynamics during the cold between ColFRI and the COOLAIR defective lines, using the existing models, would be to introduce a subpopulation of cells only in the COOLAIR defective mutants, in which H3K27me3 does not nucleate at FLC in the cold. These FLC copies would remain in an active transcriptional state, thus producing a higher level of H3K36me3 during the cold in the COOLAIR defective lines. However, the presence of such a non-nucleating subpopulation would be expected to produce a clear reduction of nucleation region H3K27me3 levels (compared to ColFRI) during the cold. Since this is not observed, particularly at 6WT0, we reject this model.

**Model 2:** A more sophisticated model to explain the difference in H3K36me3 dynamics in the cold is one where we again have a subpopulation of non-nucleating cells, but this subpopulation is common to both ColFRI and the COOLAIR defective mutants. This subpopulation has to include roughly the same proportion of dividing cells as the rest of the population (otherwise this model would produce a post-cold increase in nucleation region H3K27me3, which as discussed above, is inconsistent with all our data). The existence of such a subpopulation would allow H3K27me3 dynamics to be unchanged between these genotypes. The higher H3K36me3 in the COOLAIR defective mutants could then be explained by antisense mediated repression having a role specifically in the non-nucleating subpopulation. However, since the
non-nucleating population includes dividing cells, their active $FLC$ states (and hence high H3K36me3 levels) would be maintained and propagated during post cold growth. Therefore, without invoking additional, unknown mechanisms for H3K36me3 removal in this subpopulation of cells, even this model cannot explain the post cold reduction in H3K36me3 leading to essentially the same levels in Col$FRI$ and the COOLAIR defective mutants by 6WT10.

Thus, we are led to construct a model where H3K27me3 and H3K36me3 can co-exist at the same $FLC$ copy, and where antisense transcription mediated repression can modulate sense transcription associated H3K36me3 levels. We note that the coexistence of the two marks could potentially involve coexistence on the same H3 tail - H3K27me3 accumulation at $FLC$ during cold is mediated by a VRN2-PRC2 complex, whose activity has been shown to be insensitive to the presence of H3K36me3 on a substrate H3 tail (15). To capture the rapid response of this pathway observed under fluctuating temperatures (1), our model also includes a fast timescale response capability for the antisense mediated repression pathway. The mathematical model is detailed below.

**Model features**

**Chromatin states at $FLC$ copies:** The model allows each copy to be in one of three states: an active transcriptional state (no H3K27me3 nucleation), an H3K27me3 nucleated state, and an H3K27me3 spread state. Importantly, the model also allows H3K36me3 to be present at the locus in all of these states, at a level that is determined by the transcriptional activity possible in each state rather than direct mutual exclusivity with H3K27me3. This means that the $FLC$ copies in the active transcriptional state (no H3K27me3 nucleation) make the highest contribution to population level H3K36me3 levels; copies in the H3K27me3 nucleated state have lower transcriptional activity and hence make an intermediate contribution to population level H3K36me3; copies in the H3K27me3 spread state have the lowest transcriptional activity and hence make the lowest contribution to population level H3K36me3. We note that the above features replace the assumptions used in previous models (10,11):

1. Allowing H3K36me3 to be present in all states at a level determined by transcriptional activity replaces the previous assumption that this modification is only present in a high transcriptional state of $FLC$.

2. Having only three states of the locus – active, H3K27me3 nucleated, and H3K27me3 spread – replaces the assumption that there is a distinct, “inactive” state of the locus with neither H3K36me3 nor H3K27me3 accumulation, which is set up by a “VIN3 independent” pathway.
The new assumptions allow the model to capture transcriptional downregulation and H3K36me3 levels in parallel to H3K27me3 mediated changes of transcriptional state, and thus emphasises the paradigm that emerges from our data – that of parallel pathways (antisense transcription mediated and PRC2 mediated) converging to regulate $FLC$ expression.

**Dividing and Non-dividing loci:** The total number of dividing copies is fixed. We use a simplified division model (11), where each division produces one dividing copy and a fixed number of non-dividing copies.

**Nucleation and Spreading:**

- Transitions from an active to a nucleated state are allowed only in the cold, with the probability of nucleation dictated by the VIN3 protein concentration calculated from the LSCD model of VIN3 dynamics (10) – a predictive model of VIN3 expression that captures the effect of multiple thermosensory inputs operating at different timescales.
- The transition from a nucleated to a spread state occurs during replication/division, consistent with the dependence of spreading on an active cell cycle that we have previously shown (12).
- Replication/division causes a transition from a nucleated to a spread state: each division of a nucleated copy produces one dividing, spread copy and a fixed number of non-dividing, spread copies.
- The spread state is assumed to be stable – the model does not allow reactivation from the spread state.
- Except in simulations of the spreading mutant, we assume no loss of nucleation at nucleated, dividing copies.

**Division rate and pre-growth duration**

- The growth rate is assumed to undergo a step change in the cold (5°C) conditions - reduced by a factor of 40 relative to warm (22°C) conditions (11).
- The step change of growth rate is assumed to be the same in constant cold and fluctuating temperature conditions.
- The pre-growth duration is fixed at 10 days (11).

**Sense transcriptional activity (initiation rate):** Antisense mediated regulation of sense transcription (initiation rate) is assumed to be possible in the active and nucleated states. The highest level of transcriptional activity in the nucleated state is assumed to be 0.3 of the highest level in the active state. Based on our data, which shows that cold induced H3K27me3 nucleation and post-cold H3K27me3
spreading are not disrupted in the COOLAIR defective mutants, we assume that nucleation and spreading of H3K27me3 (i.e., the rates of transition to these states) are unaffected by the absence of the antisense mediated repression.

**Sense transcriptional activity (PolII speed):** The increase in H3K36me3 between NV and 2WT0 observed in the COOLAIR defective mutants cannot be captured by only having co-transcriptional addition of this modification – there is a general trend towards reduction rather than an increase in sense transcriptional activity between these timepoints (as measured by FLC unspliced and spliced transcript levels). Therefore, to capture this increase in H3K36me3, we introduce a reduction in the speed of RNA PolII in the nucleation region in the cold: it is assumed to undergo a step change drop to 0.6 of its NV value in the cold and also recovers post-cold. Such a reduction in Pol II speed in the nucleation region allows the Pol II dwell time to increase between the NV and 2WT0 timepoints, even with a reduction in transcriptional activity (initiation rate) between these timepoints. We assume that the same reduction in PolII speed in the nucleation region also occurs under the fluctuating temperature conditions.

**Antisense mediated (nucleation independent) repression pathway:** A nucleation independent repression pathway performs analogue control of the sense transcription level in active and nucleated states. The functioning of this pathway is assumed to rely on antisense (AS) transcription (i.e., this pathway is not functional in the COOLAIR defective mutants).

**Slow timescale component:** Under cold conditions, both constant and fluctuating cold, repression by the AS pathway is assumed to increase slowly in the cold and decrease quickly upon return to warm, consistent with NTL8 dynamics (16). The repression is modelled as a slow reduction in the sense transcription initiation rate in the cold, and a rapid recovery in the initiation rate in the post-cold. This is captured by a multiplicative factor set to vary between 1 and 0.5 with an exponential decay over time in cold and a faster exponential recovery over time in the warm (see model implementation below). This is consistent with our previous model-predicted NTL8 accumulation dynamics determined by slower growth in the cold (16), and rapid NTL8 reduction during post-cold growth, as well as the measured slow build-up of FLC antisense transcripts in constant cold measured by qPCR (17) This is also consistent with the analysis of the VIN3 independent pathway in (10) – the dynamics of this pathway was predicted to be temperature dependent, causing slow FLC reduction in the cold, but allowing rapid increase in the post cold in the absence of VIN3 dependent H3K27me3 nucleation. The reduction of the transcriptional initiation rate (caused by the AS pathway) is assumed to have the same dynamics at active (non-nucleated) and nucleated copies.
The transcriptional initiation recovery timescale in the post-cold is also assumed to be the same at non-nucleated and nucleated copies.

**Fast timescale component:** To capture the rapid response of the antisense mediated repression under fluctuating temperature conditions, we assume that low temperatures cause a large reduction in the sense transcription initiation rate (due to strong upregulation of antisense transcription). While freezing temperatures are seen to have the strongest effect on upregulating antisense transcription, we note that in our experimental data, both mild and strong fluctuating conditions cause significant downregulation of *FLC* sense transcription, with the reduction of both *FLC* mRNA and H3K36me3 being largest in FS conditions and intermediate in FM conditions. To capture these effects in both FM and FS conditions, we assume a simple step change in the antisense mediated repression. This is represented by the multiplicative factor defined above undergoing a step reduction to a value of 0.05 whenever the temperature falls below 4°C. Above this temperature, this factor takes a slow, exponentially decaying value (as a function of time) as described above (see Table S1 for definition).

Thus, under fluctuating temperature conditions we assume that, in addition to the large step changes to the multiplicative factor, a slow timescale reduction in this factor also occurs. Not including a slow timescale reduction under fluctuating conditions would cause the model to fail in capturing the observed changes to H3K36me3 and *FLC* mRNA under FM and FS conditions. Note that the reduction in Pol II speed in the nucleation region assumed for constant cold is also assumed to occur under FM and FS conditions. In the absence of this assumption – i.e., if the PolII speed is assumed to change only in constant cold conditions – the model would predict even larger changes in H3K36me3 under FM and FS conditions relative to NV.

We note that the above set of assumptions describing the antisense mediated pathway replaces population level H3K36me3 in the simpler description of a “VIN3 independent” pathway used in our previous models (10,11).

**H3K27me3 levels:** The contribution to H3K27me3 levels from an individual *FLC* locus depends on the state of the locus: low in the nucleation region (NR) and non-nucleation region (body) for active copies, high in the NR and low in the body for nucleated copies, high in the NR and body for spread copies.

**H3K36me3 levels in the NR depend on Pol II density:** For simplicity, the model describes the population level average H3K36me3 levels in the *FLC* nucleation region, but we note that the levels of this
modification across the gene body follow the trends in the nucleation region in all our data (Fig. 2B, (12,14)), with the only exception being the 3’ end of the locus, where H3K36me3 levels reflect the level of antisense transcription (increasing during the cold (Fig. 3A,C), (14) and high in ntl8-D3 (Fig. 1B)). H3K36me3 is represented as a dynamical variable in the model, and based on the above evidence, the rate of addition of H3K36me3 is assumed to be proportional to the Pol II density. This is consistent with co-transcriptional addition of this modification (18), as well as longer Pol II dwell time at a given location allowing a greater window of opportunity for adding this modification (19). The Pol II density is determined by the ratio of initiation rate to PolIII speed in the nucleation region. As described above, the initiation rate is determined by two factors - the H3K27me3 state and the antisense mediated repression pathway - while the PolIII speed undergoes a step change in cold conditions. We also assume a constant turnover rate of H3K36me3 - the value of the rate constant is assumed to be 1.21 day$^{-1}$, consistent with the half-life of H3K36me2 (0.571 day) estimated in (20), since no data was available for H3K36me3.

**FLC mRNA level:** The model describes the population level average *FLC* mRNA level as a dynamical variable, whose production is determined by the sense transcription initiation rates of the *FLC* copies in different states. As described above, these initiation rates are also determined by the antisense mediated repression. We fix a turnover rate for *FLC* mRNA consistent with a half-life of 6 hr as estimated in (21).

**Fluctuating temperature input to the model:** The temperature profiles used to simulate FM and FS conditions are shown in Table S2. We note that in the model, based on the above assumptions, this dynamic temperature input is affecting two components – the antisense mediated repression (as described above) and the LSCD model of VIN3 dynamics (thus determining the nucleation rate) (10). Other temperature dependent rates – Pol II elongation and growth – are assumed to undergo a step change in the cold, so they are not affected by other dynamics of the temperature input.

**Summary of changes from previous models**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Previous models</th>
<th>Current model</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>FLC</em> sense transcriptional downregulation</td>
<td>Captured by transition between an active (high) transcription state and an inactive state, controlled by a slow, ‘VIN3 independent’ pathway.</td>
<td>Captured by an antisense mediated repression that can modulate sense transcription in both an active transcription state and a nucleated state. This repression increases slowly in</td>
</tr>
<tr>
<td><strong>FLC states</strong></td>
<td>Five possible states: High transcription, Inactive, Nucleated, Spread, and Perpetuated.</td>
<td>Three possible states: Non-nucleated active state, Nucleated state, and spread state. The perpetuated state not included since this state is not expected to play a major role in the phases of silencing examined in this study. This state can be incorporated into the current model for studying aspects such as post-cold FLC reactivation.</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Nucleation</strong></td>
<td>Transition to nucleated state partly relies on transcriptional downregulation (i.e., prior transition to inactive state).</td>
<td>Transition to nucleated state does not rely on antisense mediated transcriptional repression.</td>
</tr>
<tr>
<td><strong>H3K36me3 contribution from nucleated and spread states</strong></td>
<td>Assumes no contribution to overall H3K36me3 level from either nucleated or spread/perpetuated states.</td>
<td>Assumes a contribution to overall H3K36me3 from all states, at a level determined by the amount of transcription (therefore determined by the antisense mediated repression).</td>
</tr>
<tr>
<td><strong>Link between H3K36me3 and RNA Pol II speed</strong></td>
<td>Not captured in previous models.</td>
<td>Rate of addition of H3K36me3 is assumed to be inversely proportional to the PolII speed in the nucleation region, consistent with a model of co-transcriptional H3K36 methylation, dependent on Pol II dwell time. The speed is constant cold but can respond to temperature fluctuations on a fast timescale.</td>
</tr>
</tbody>
</table>
Model implementation

Following the same approach as for our previous models (10, 11), an ODE (Ordinary Differential Equation) model is constructed using the above assumptions. The model equations are shown below. The model is simulated using the ode15s solver in Matlab version 2017a.

Model Variables:

- \( f_{a,d} \) (fraction of active, dividing copies)
- \( f_{n,d} \) (fraction of nucleated, dividing copies)
- \( f_{s,d} \) (fraction of spread, dividing copies)
- \( f_{a,nd} \) (ratio of active, non-dividing copies to total number of dividing copies)
- \( f_{n,nd} \) (ratio of nucleated, non-dividing copies to total number of dividing copies)
- \( f_{s,nd} \) (ratio of spread, non-dividing copies to total number of dividing copies)

The dynamics are such that \( f_{a,d} + f_{n,d} + f_{s,d} = 1 \) at all timepoints.

Basic Model (representing ColFRI):

\[
\begin{align*}
\frac{df_{a,d}}{dt} &= -k_sf_{a,d} \\
\frac{df_{n,d}}{dt} &= k_sf_{a,d} - g(T)f_{n,d} \\
\frac{df_{s,d}}{dt} &= g(T)f_{n,d} \\
\frac{df_{a,nd}}{dt} &= d_ng(T)f_{a,d} - k_sf_{a,nd} \\
\frac{df_{n,nd}}{dt} &= k_sf_{a,nd} \\
\frac{df_{s,nd}}{dt} &= d_ng(T)f_{n,d} + d_ng(T)f_{s,d}
\end{align*}
\]

\[
\frac{d[H3K36me3]}{dt} = k_{K36me3}^d \left( G_{K36} \left( F_a, F_n, F_s \right) - [H3K36me3] \right)
\]
Here, the total fractions of copies in each state (at any timepoint), i.e., $F_a, F_n, F_s$, can be computed as follows:

Total fraction of active copies: $F_a = \frac{f_{a,d} + f_{a,nd}}{1 + f_{a,nd} + f_{n,nd} + f_{s,nd}}$

Total fraction of nucleated copies: $F_n = \frac{f_{n,d} + f_{n,nd}}{1 + f_{a,nd} + f_{n,nd} + f_{s,nd}}$

Total fraction of spread copies: $F_s = \frac{f_{s,d} + f_{s,nd}}{1 + f_{a,nd} + f_{n,nd} + f_{s,nd}}$

Here, $g(T)$ represents the division rate, which undergoes a step change reduction in the cold. $g(T)$ is assumed to undergo a step reduction under FM and FS conditions, exactly as under constant cold conditions. The step change (factor of 40) is assumed to be the same under all three conditions. $k_r$ represents the nucleation rate, computed using the VIN3 level as in (11), where the VIN3 level is itself computed using the LSCD model (10). At all timepoints, the current temperature is an input to the LSCD model. $d_n$ represents the number of non-dividing copies produced at each division event (using the same simplified description as in (11)).

The rate of addition of H3K36me3 is assumed to be proportional to the function $G_{K36}(F_a, F_n, F_s)$:

$$G_{K36}(F_a, F_n, F_s) = \left( \frac{q(t)(r_a F_a + r_n F_n) + r_s F_s}{v(T)} \right).$$

$G_{K36}(F_a, F_n, F_s)$ represents the contribution to overall H3K36me3 addition from FLC sense transcriptional activity at FLC copies in different states, with the sense transcriptional activity dictated by both the H3K27me3 nucleation state (represented by $r_a$, $r_n$ and $r_s$) and the antisense mediated repression (represented by $q(t)$). The multiplicative factors $r_a$, $r_n$ and $r_s$ represent the maximum transcription initiation rate for active, nucleated, and spread copies respectively. $v(T)$ represents the Pol II speed in the nucleation region, which is assumed to undergo a step change (reduction) during the cold. $k_{K36me3}^d$ represents the turnover rate constant of H3K36me3. The H3K36me3 level is assumed to be normalised to its steady state level when $F_a = 1, v(T) = 1$, and $q(t) = 1$ (i.e., $G_{K36}(F_a, F_n, F_s) = 1$).
The time-dependent multiplicative factor $q(t)$ captures the repression by the antisense mediated pathway. This factor has a basal value of 1 and decays exponentially with time to 0.5 in the cold and recovers exponentially with time in the post-cold. $q(t)$ undergoes a step reduction to a value of 0.05 whenever the temperature falls below 4°C. When the temperature subsequently rises above 4°C, $q(t)$ returns to its slow decaying value, which decays continuously irrespective of the temperature fluctuations.

Note that having the parameter $r_n > 0$ above means that copies in an H3K27me3 nucleated state can also contribute to H3K36me3 addition. This reflects the model assumption that these two modifications can coexist in the nucleation region at a single $FLC$ copy during cold induced silencing, with the H3K36me3 levels being limited only by the level of sense transcription. This assumption is based on the ability of the VRN2-PRC2 complex (which mediates cold induced H3K27me3 accumulation at $FLC$) to methylate H3 histones even when they carry K36 methylation (15).

We describe $FLC$ mRNA as an additional dynamical variable, whose production rate is proportional to $G_{K36}(F_a, F_n, F_s) v(T)$ – i.e. the same function $G_{K36}(F_a, F_n, F_s)$ described above, which captures the contribution to $FLC$ sense initiation from antisense mediated repression and the different H3K27me3 states, but now multiplied by the Pol II speed in the nucleation region to cancel out the $v(T)$ factor in the denominator of $G_{K36}(F_a, F_n, F_s)$, so that the product represents a “transcriptional initiation rate”. $k_{F_{L C}}$ represents the turnover rate constant of $FLC$ mRNA. The $FLC$ mRNA level is also assumed to be normalised to its steady state level when $F_a = 1$, $v(T) = 1$, and $q(t) = 1$ (i.e., $G_{K36}(F_a, F_n, F_s) = 1$).

**Model for COOLAIR defective mutants:** For simulating the COOLAIR defective mutants, the same model is used, but with the antisense mediated pathway assumed to be non-functional. This is captured by setting $q(t) = 1$ throughout the simulation.

**Model for H3K27me3 Nucleation mutants:** The basic model is used with the nucleation rate $k_n$ set to zero throughout the simulation. The initial conditions in this case are still allowed to have a non-zero fraction of H3K27me3 spread $FLC$ copies, consistent with the NV level of H3K27me3 observed in cold-nucleation mutants including vrn2-1 and vin3-4 (12).

**Model for H3K27me3 Spreading mutant:** Here we modify the basic model to capture reactivation/loss of H3K27me3 nucleation. At each division event, a nucleated dividing copy is allowed to undergo three different scenarios (11):
• Reactivation, producing one active dividing copy and $d_n$ active non-dividing copies.
• Spreading, producing one spread dividing copy and $d_n$ spread non-dividing copies.
• Neither spreading nor reactivation, producing one nucleated dividing copy, $\beta_{nuc}d_n$ nucleated non-dividing copies, $\beta_{sprd}d_n$ spread non-dividing copies, and $\beta_{react}d_n$ active non-dividing copies.

\[
\frac{df_{a,d}}{dt} = -k_s f_{a,d} + \gamma g(T)f_{n,d}
\]
\[
\frac{df_{n,d}}{dt} = k_s f_{a,d} - \delta g(T)f_{n,d} - \gamma g(T)f_{n,d}
\]
\[
\frac{df_{s,d}}{dt} = \delta g(T)f_{n,d}
\]
\[
\frac{df_{a,nd}}{dt} = d_n g(t) f_{a,d} - k_s f_{a,nd} + d_n \gamma g(T) f_{n,d} + \beta_{react} d_n (1 - \delta - \gamma) g(T) f_{n,a}
\]
\[
\frac{df_{n,nd}}{dt} = k_s f_{a,nd} + \beta_{nuc} d_n (1 - \delta - \gamma) g(T) f_{n,d}
\]
\[
\frac{df_{s,nd}}{dt} = d_s \delta g(T) f_{n,d} + d_n g(T) f_{s,d} + \beta_{sprd} d_n (1 - \delta - \gamma) g(T) f_{n,d}
\]
\[
\frac{d |H3K36me3|}{dt} = k_{K36me3}^d \left( G_{K36} \left( F_{a}, F_{n}, F_{s} \right) - [H3K36me3] \right)
\]
\[
\frac{d |FLC|}{dt} = k_{FLC}^d \left( G_{K36} \left( F_{a}, F_{n}, F_{s} \right) v(T) - [FLC] \right)
\]

Here $\gamma$ represents the fraction of nucleated dividing copies undergoing reactivation and $\delta$ represents the fraction of nucleated dividing copies undergoing spreading, at each replication/division event. The parameters $\beta_{nuc}$, $\beta_{sprd}$, and $\beta_{react}$ were computed numerically (see SI Table 1 of parameter values), using a Monte-Carlo approach to carry out five successive replication/division events starting from one nucleated copy (five divisions is consistent with our assumption of $d_n = 32$ (11)).

Processing simulation output:
The total fractions of copies in each state (at any timepoint) can be computed as follows from the simulation output:

Total fraction of active copies: \( F_a = \frac{f_{a,d} + f_{a,nd}}{1 + f_{a,nd} + f_{n,nd} + f_{s,nd}} \)

Total fraction of nucleated copies: \( F_n = \frac{f_{n,d} + f_{n,nd}}{1 + f_{a,nd} + f_{n,nd} + f_{s,nd}} \)

Total fraction of spread copies: \( F_s = \frac{f_{s,d} + f_{s,nd}}{1 + f_{a,nd} + f_{n,nd} + f_{s,nd}} \)

The total fractions of copies in each state are then used to compute the H3K27me3 levels as follows:

NR H3K27me3 level: \( K27_{NR} = p_{K27} \left( (0)F_a + (1)F_n + (1)F_s \right) \)

Body H3K27me3 level: \( K27_{Body} = p_{K27} \left( (0)F_a + (0)F_n + (1)F_s \right) \)

The factor \( p_{K27} \) is used for scaling the model output for comparison to ChIP-qPCR data. Similarly, a factor \( p_{K36} \) is multiplied when comparing H3K36me3 levels to the ChIP-qPCR data.

**Comparing model predictions to experimental data under fluctuating temperature conditions:**

In Figure S11(B,C,D), we compare the model predicted H3K36me3, H3K27me3, and FLC mRNA levels for *ColFRI* at the 2WT0 timepoint under fluctuating conditions to experimental data.

The model assumes that the *FLC* transcriptional shutdown in fluctuating conditions is mediated mainly by an enhanced antisense pathway. Hence, in the case of a *COOLAIR* defective mutant, this model would predict that the *FLC* transcriptional shutdown is mostly disrupted even in FM and FS conditions. Therefore, this model would predict an increase in H3K36me3 after 2W FM and FS conditions, similar to the increase predicted under constant cold conditions at the same timepoint (as shown in Figure 4). The experimental data for the TEX1.0 *FLClean* mutant does show increased H3K36me3 across the locus under FM conditions (Fig. 3(D)), which is qualitatively consistent with the model prediction. This is similar to the increased H3K36me3 across the locus observed for all three *COOLAIR* defective mutants after 2W cold (Fig. 2(B)). However, under FS conditions, the data shows a reduction in H3K36me3 (although this reduction is significantly attenuated compared to *ColFRI* – Figure S9F). This indicates that there may be additional factors driving the transcriptional shutdown under FS conditions that are not captured by this simple model,
which require further experimental analysis of chromatin state changes under fluctuating temperatures to dissect.

**Note: Defining parameter values under fluctuating temperature conditions**

The instantaneous temperature input affects the parameters in the LSCD model of VIN3 dynamics and the rate of VIN3 mediated nucleation as described in (10). The parameter \( q(t) \), representing the antisense mediated repression undergoes step changes under the fluctuating temperature conditions as defined above. \( g(T) \), representing the division rate and \( v(T) \), representing the Pol II speed in the nucleation region, are set to the same, constant value under all three cold conditions (constant, FM, FS). See Table S1 for details.
**Fig. S1. Relative expression of other factors involved in vernalization.** Expression of vernalization factors in *nlt8-D3 FRI* compared to *ColFRI* in non-vernalized conditions. Data are presented as the mean ± s.e.m (n ≥ 3). Asterisks indicate significant difference (p ≤ 0.05, two-tailed t test). n.s, not significant. Each open circle represents a biological replicate.
Fig. S2. Constitutive expressed VIN3 binds the nucleation region at FLC. (A) Schematic of the construct used to generate transgenic lines that express VIN3 in the absence of cold. The VIN3 promoter was exchanged with the promoter sequence of VRN5 (pVRN5). (B) Expression of VIN3 in non-vernalized conditions (NV), VIN3 expression in ColFRI after six weeks of vernalization (6WT0) is shown for comparison. Data are presented as the mean ± s.e.m relative to the geometric mean of PP2A and ACTIN. Each open circle represents a biological replicate. The numbers under the bars refer to individual transgenic lines. (C) VIN3-eGFP ChIP-qPCR enrichment at FLC at NV. Data are shown as percentage input. Non-transgenic ColFRI plants were used as a negative control sample. Error bars represent mean ± s.e.m. (n = 3 biological replicates).
Fig. S3. Gene-loop is disrupted in TEX2.0. Quantitative 3C over the FLC locus in 10-day-old ColFRI and TEX2.0 flclean FRI with BamHI and BglII (similar to Fig. 1G). A schematic of the TEX2.0 transgene is shown above. BamHI and BglII restriction sites are indicated with dotted lines, and the respective regions are numbered with Roman numerals. The insertion of the NOS sequence does not result in additional 3C fragments when assayed with BglII and BamHI. Red arrows indicate the primers' location for 3C-qPCR. The region around the FLC transcription start site was used as the anchor region in the 3C analysis. The data shows the relative interaction frequencies (RIF) and are the average of at least seven biological replicates. Data are presented as the mean ± s.e.m. (n ≥ 7). The midpoint of the assayed 3C fragments is plotted.
**Fig. S4.** CRISPR/Cas9 mediated generation of *FLC* deletion lines (*FLClean*). (A) Schematic representation of the *FLC* locus with locations of sgRNA target sites (Red) and primer binding sites for genotyping (Blue). The size numbers refer to the region at 5’ and 3’ end of *FLC* removed in the *FLClean* lines. (B) Integrative Genomics Viewer (IGV) screenshot of *FLC* genomic region showing read coverage of whole genome DNA sequencing in *ColFRI* and two CRISPR *FLClean* lines. The *FLClean* lines were created through removal of the whole *FLC* locus with either sgRNA1 and 3 (*FLClean13*) or sgRNA 2 and 4 (*FLClean24*).
Fig. S5. Quantitative analysis of antisense role in histone modification dynamics. All comparisons shown consist of comparing the mean levels over three nucleation region primers between ColFRI to each of the defective COOLAIR lines (See supplementary information for details of primers). In cases where the qualitative trends were clear and consistent across the three defective COOLAIR lines, a one-tailed Student’s t-test was used for each comparison. Error bars represent s.e.m. (n = 3 biological replicates). In (A), where there was no clear trend, a two-tailed Student’s t-test was used. In all cases, the Bonferroni correction was used to adjust the significance level from $\alpha = 0.05$ to $\alpha = 0.0167$ (for three comparisons). (*) indicates $P < 0.0167$; ns indicates no significance ($P \geq 0.0167$). (A) Fold change (increase) of H3K27me3 in the nucleation region during first 2 weeks of cold treatment (2WT0/NV) is not significantly different between ColFRI and the defective COOLAIR lines. (B) Fold change (increase) of H3K27me3 in the nucleation region during the 2WT0 to 4WT0 period is significantly higher in the COOLAIR lines. (C) H3K27me3 levels at 6WT0 are not significantly lower in the defective COOLAIR lines. (D) NV level of H3K27me3 in the nucleation region is not significantly higher in ColFRI. (E) FLC expression in 10 days old seedlings before cold exposure in ColFRI and the three defective COOLAIR lines; TEX1, TEX2, and FLC∆COOLAIR. Unspliced RNA was measured and is shown relative to UBC and ColFRI. Error bars represent s.e.m. (n = 3 biological replicates). (F-G) Similar analysis as in (A-D). NV level of H3K36me3 is not significantly higher in the defective COOLAIR lines (F). The fold change in H3K36me3 over 6W of cold treatment indicates significantly smaller changes in H3K36me3 in the defective COOLAIR lines (G).
Fig. S6. Polycomb repression of *FLC* is not inhibited by hyperactive antisense pathway in *ntl8-D3*. (A) H3K27me3 ChIP in Col*FRI* and *ntl8-D3 FRI* across the *FLC* locus before, during, and after vernalization. H3K27me3 levels are expressed as relative to H3 and to the levels at the positive control gene *STM*. Data are presented as the mean ± s.e.m. (n ≥ 3). (B) H3K27me3 levels in the nucleation region in Col*FRI* and *ntl8-D3 FRI* during vernalization. The levels were calculated by averaging over three primers in the *FLC* nucleation region. (C) H3K36me3 levels in the nucleation region in Col*FRI* and *ntl8-D3 FRI* during vernalization. The levels were calculated as in B. (D) H3K36me3 ChIP in Col*FRI* and *ntl8-D3 FRI* across the *FLC* locus before, during, and after vernalization. H3K36me3 levels are expressed as relative to H3 and to the levels at the positive control gene *Actin*. Data are presented as the mean ± s.e.m. (n ≥ 3).
Fig. S7. H3K4me1 removal during the cold is attenuated in COOLAIR defective lines. H3K4me1 ChIP in ColFRI and the three COOLAIR mutant lines; TEX1, TEX2, and FLC<sub>ΔCOOLAIR</sub> across the FLC locus before, during, and after vernalization. Data are presented as the mean ± s.e.m. (n ≥ 3).
Fig. S8. *FLC* repression is only partially disrupted in *vrn2-1 FRI*. (A) *FLC* spliced levels and (B) H3K36me3 levels during vernalization in ColFRI and *vrn2-1 FRI*. (A) *FLC* spliced data shown relative to RNA levels in non-vernalyzed conditions (0 weeks of vernalization). (B) ChIP-qPCR data for H3K36me3 is normalized to H3 and shown relative to H3 normalized H3K36me3 levels at *ACTIN*. Data are presented as the mean ± s.e.m. (n ≥ 3). Reproduced from (12).
Fig. S9. Fold change comparisons for changes in nucleation region H3K36me3 and H3K27me3. This analysis is based on ChIP-qPCR time course data presented in Figs. 1,2. Error bars represent mean ± s.e.m. (n ≥ 3). All comparisons shown consist of comparing fold changes in the mean levels over three nucleation region primers in the indicated genotypes between different periods of cold and non-vernalized conditions (see supplementary information for details of primers). In all cases, the data showed clear trends, so a one-tailed Student’s t-test was used for each comparison. In (A-C) and (F), a significance level of alpha = 0.05 was used. (*) indicates P<0.05; ns indicates no significance (P≥0.05). In (D), the Bonferroni correction was used to adjust the significance level from alpha = 0.05 to alpha = 0.025 (for two comparisons). ns indicates no significance (P≥0.025). In (E), the Bonferroni correction was used to adjust the significance level from alpha = 0.01 to alpha = 0.005 (for two comparisons). (**) indicates P<0.005; ns indicates no significance (P≥0.005). (A) Comparison of NR H3K36me3 fold changes after 6 weeks cold (6WT0) in ColFRI and vrn2-1. (B) Comparison of NR H3K27me3 fold changes after 2 weeks cold
(2WT0) in *ColFRI* and *ntl8-D3*. (C) Comparison of NR H3K36me3 fold changes after 2 weeks cold (2WT0) in *ColFRI* and *ntl8-D3*. (D) Comparison of NR H3K27me3 fold changes after 2 weeks cold (2WT0) in *ColFRI* under different cold conditions – constant cold (CC), fluctuating mild (FM), and fluctuating strong (FS). (E) Comparison of NR H3K36me3 fold changes after 2 weeks cold (2WT0) in *ColFRI* under different cold conditions – constant cold (CC), fluctuating mild (FM), and fluctuating strong (FS). (F) Comparison of NR H3K36me3 fold changes after 2 weeks (2WT0) under FS conditions in *ColFRI* and *TEX1.0*. 
Figure S10. Model predictions of impact of vernalization mutants on histone dynamics. (A-D) Time course predictions from the mathematical model for other vernalization mutants: an H3K27me3 nucleation mutant, and a spreading mutant. The predictions are compared to previously published ChIP-qPCR time course data presented in (12).
Caption for Figure S11: Fast timescale response of the antisense mediated repression pathway to temperature fluctuations can capture experimentally observed changes under fluctuating temperature conditions. (A) Temperature profiles used in the fluctuating temperature experiments. (B) Comparison of experimentally measured (left) and model predicted (right) changes in *FLC* transcriptional output in *ColFRI* after 2W constant cold (CC), 2W fluctuating mild (FM), or 2W fluctuating strong (FS) conditions. The experimentally measured levels are normalised to the mean NV level. (C) Comparison of experimentally measured (left) and model predicted (right) changes in nucleation region H3K36me3 in *ColFRI* after 2W constant cold (CC), 2W fluctuating mild (FM), or 2W fluctuating strong (FS) conditions. (D) Comparison of experimentally measured (left) and model predicted (right) changes in nucleation region H3K27me3 in *ColFRI* after 2W constant cold (CC), 2W fluctuating mild (FM), or 2W fluctuating strong (FS) conditions.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_s$</td>
<td>Rate constant of H3K27me3 nucleation (day$^{-1}$)</td>
<td>Computed as in (11), with $p_{s2} = 0.007$ day$^{-1}$°C$^{-2}$</td>
<td>(10,11)</td>
</tr>
<tr>
<td>$g(T)$</td>
<td>Temperature dependent growth rate (day$^{-1}$)</td>
<td>0.4 for $T = 22$°C, 0.01 for $T = 5$°C Constant, FM, and FS conditions</td>
<td>(11)</td>
</tr>
<tr>
<td>$d_n$</td>
<td>Number of non-dividing copies produced per division of a dividing copy</td>
<td>32</td>
<td>(11)</td>
</tr>
<tr>
<td>$\delta$</td>
<td>Fraction of H3K27me3 nucleated dividing copies undergoing spreading</td>
<td>0.0025</td>
<td>This allows for a very small fraction undergoing H3K27me3 spreading, even in a spreading mutant. Consistent with very low levels of H3K27me3 spreading observed in (12).</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Fraction of H3K27me3 nucleated dividing copies undergoing reactivation during a replication/division event</td>
<td>0.03</td>
<td>Chosen to produce ~30% reactivation after 12 replication/division events starting from a single nucleated copy. This is based on the analysis of $FLC$ reactivation in a spreading mutant in (12).</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
<td>Value</td>
<td>Source</td>
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</tr>
<tr>
<td>$\beta_{sprd}$</td>
<td>Fraction of H3K27me3 spread non-dividing copies produced during replication of a nucleated copy in the spreading mutant model</td>
<td>0.0121</td>
<td>Numerically estimated from the same Monte Carlo simulation described above.</td>
</tr>
<tr>
<td>$\beta_{react}$</td>
<td>Fraction of non-dividing copies that have lost H3K27me3 nucleation, produced during replication of a nucleated copy in the spreading mutant model</td>
<td>0.1402</td>
<td>Numerically estimated from the same Monte Carlo simulation described above.</td>
</tr>
<tr>
<td>$q(t)$</td>
<td>Time-dependent multiplicative factor that captures repression by the antisense mediated pathway.</td>
<td>$q(t) = 1$ before cold (and during cold when simulating COOLAIR defective mutants) $q(t) = 0.5(1 + e^{-\alpha_1(t-10)})$ during cold (beginning at 10 days), for $T \geq 4{^\circ}C$ $q(t) = 1 - (1 - 0.5(1 + e^{-\alpha_1(t-10)}))e^{-\alpha_2(t-52)}$ during post-cold (beginning at 52 days) $q(t) = 0.05$ for $T &lt; 4{^\circ}C$</td>
<td>This study</td>
</tr>
<tr>
<td>$\alpha_1$</td>
<td>Rate constant of increasing antisense mediated repression during cold (day$^{-1}$)</td>
<td>0.08</td>
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<tr>
<td>$\alpha_2$</td>
<td>Rate constant of decreasing antisense mediated repression during post-cold (day$^{-1}$)</td>
<td>0.12</td>
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<tr>
<td>$v(T)$</td>
<td>Temperature dependent RNA Pol II speed in the nucleation region</td>
<td>1 for $T = 22{^\circ}C$ 0.6 for $T = 5{^\circ}C$</td>
<td>Constant, FM, and FS conditions</td>
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<tr>
<td>$r_a$</td>
<td>Maximum transcription initiation rate for active</td>
<td>1</td>
<td>This study</td>
</tr>
<tr>
<td>Parameter</td>
<td>Description</td>
<td>Value</td>
<td>Source</td>
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<tr>
<td>$r_n$</td>
<td>Maximum transcription initiation rate for nucleated copies (assumed normalised to rate at active copies)</td>
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<td>$r_s$</td>
<td>Maximum transcription initiation rate for spread copies (assumed normalised to rate at active copies)</td>
<td>0.025</td>
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<tr>
<td>$k_{36}\text{me}^3$</td>
<td>Turnover rate constant of H3K36me3</td>
<td>$1.21 \text{ day}^{-1}$</td>
<td>Consistent with the half-life of H3K36me2 (0.571 day) estimated in (20)</td>
</tr>
<tr>
<td>$k_{FLC}$</td>
<td>Turnover rate constant of FLC mRNA</td>
<td>$2.78 \text{ day}^{-1}$</td>
<td>Consistent with a half-life of 6 hr as estimated in (21).</td>
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<td>$p_{K36}$</td>
<td>Scaling parameter for comparing model output to ChIP-qPCR data</td>
<td>1.3 for <em>FRI clf</em>-2 and <em>FRI vrn2</em>-1 (Data from (12)) 1.3 for ColFRI, and COOLAIR defective mutants (Data from this study)</td>
<td>This study</td>
</tr>
<tr>
<td>$p_{K27}$</td>
<td>Scaling parameter for comparing model output to ChIP-qPCR data</td>
<td>1.1 for <em>FRI clf</em>-2 and <em>FRI vrn2</em>-1 (Data from (12)) 0.7 for ColFRI, and COOLAIR defective mutants (Data from this study)</td>
<td>This study</td>
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Table S2. Fluctuating temperature profiles input to model (matching conditions used in fluctuating temperature experiments). The temperature input is linearly interpolated between the hourly timepoints shown.

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<th>FS Conditions (°C)</th>
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Dataset S1. List of primers used in this study.
SI References


