The reprogramming of epigenetic states in gametes and embryos is essential for correct development in plants and mammals\(^1\). In plants, the germ line arises from somatic tissues of the flower, necessitating the erasure of chromatin modifications that have accumulated at specific loci during development or in response to external stimuli. If this process occurs inefficiently, it can lead to epigenetic states being inherited from one generation to the next\(^2-4\). However, in most cases, accumulated epigenetic modifications are efficiently erased before the next generation. An important example of epigenetic reprogramming in plants is the resetting of the expression of the floral repressor locus *FLC* in *Arabidopsis thaliana*. *FLC* is epigenetically silenced by prolonged cold in a process called vernalization. However, the locus is reactivated before the completion of seed development, ensuring the requirement for vernalization in every generation. In contrast to our detailed understanding of the polycomb-mediated epigenetic silencing induced by vernalization, little is known about the mechanism involved in the reactivation of *FLC*. Here we show that a hypomorphic mutation in the jumonji-domain–containing protein ELF6 impaired the reactivation of *FLC* in reproductive tissues, leading to the inheritance of a partially vernalized state. ELF6 has H3K27me3 demethylase activity, and the mutation reduced this enzymatic activity *in planta*. Consistent with this, in the next generation of mutant plants, H3K27me3 levels at the *FLC* locus stayed higher, and *FLC* expression remained lower, than in the wild type. Our data reveal an ancient role for H3K27 demethylation in the reprogramming of epigenetic states in plant and mammalian embryos\(^5-7\).

Many *A. thaliana* accessions overwinter before flowering, as a result of FRIGIDA (FRI)-mediated high-level expression of a floral repressor called FLC\(^8\). Prolonged cold during the weeks of winter antagonizes this activation and progressively epigenetically silences *FLC*. This process enables other floral promotion signals, such as day length, to induce flowering in spring. The epigenetic silencing of *FLC* involves polycomb-mediated chromatin regulation\(^10-12\) and is maintained until embryogenesis, when *FLC* expression is reset to ensure a requirement for vernalization in every generation\(^13,14\). The resetting of *FLC* expression occurs in the early globular embryo\(^11,14\); then, *FLC* expression increases throughout embryo development until it reaches maximum levels when the seed has completely formed\(^15\). However, the molecular mechanisms underlying *FLC* resetting are unknown, and several factors that are required for the upregulation of *FLC* in vegetative tissues have been shown to be dispensable for *FLC* expression in the embryo\(^14\). One exception is the yeast SWR1 homologue PIE1 (ref. 14), although it is unclear whether *pie1* mutations are resetting-specific defects because these mutations strongly reduce *FLC* expression across the plant independently of vernalization status.

To dissect this resetting mechanism, we isolated mutants that are defective in the reactivation of *FLC* after vernalization (Extended Data Fig. 1a). The parental line was an *A. thaliana* Landsberg erecta (Ler) plant carrying an *FLC*:luciferase (FLC:LUC) transgenic fusion and an active FRI transgene\(^15\). We searched for plants in which *FLC* expression was silenced by vernalization but, unlike in the wild type, was not fully restored in the following generation (Fig. 1a), leading to inheritance of the vernalized state. The frequency of these mutations was low (with only 2 mutants identified from the progeny of 6,000 mutagenized parent lines), in contrast to the more common class of mutations, which involved early flowering before vernalization as a result of reduced *FLC* expression (Extended Data Fig. 1b). The first resetting mutant that was isolated was found to be recessive (Extended Data Fig. 2a) and flowered slightly earlier without vernalization than did the wild type (Fig. 1b). In the generation after vernalization, the mutant flowered even earlier and had significantly

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**Figure 1** | Isolation and characterization of the resetting mutant. a, The rationale for the genetic screen. The parental wild type (WT) is Ler (FRI) *FLC*:LUC, the mutant is a resetting mutant. b–d, The resetting mutant is early flowering (b), with fewer leaves when bolting (with flowering time assayed as total leaf number), and maintains low *FLC* expression, as shown by *FLC*:luciferase imaging of 8-day-old seedlings (c) and by quantitative reverse transcription PCR (qRT–PCR) analysis normalized to UBC levels (d). Pseudocolour bioluminescent images (c, top) from blue (least intense) to red (most intense) and normal images (c, bottom) of the same plants are presented. The data are presented as the mean ± s.e.m., *n* = 20 (b) and *n* = 3 (d); ***P < 0.001. NV, non-vernalized; NV(V), non-vernalized following vernalization in the previous generation; VER, vernalized.
reduced FLC expression (Fig. 1c, d), albeit about fourfold higher than in fully vernalized seedlings (Fig. 1d). The resetting mutant therefore causes transgenerational inheritance of a partially vernalized state. The early flowering phenotype was stable for at least three generations following vernalization (Extended Data Fig. 2b) but was not enhanced by a second vernalization treatment in the later generations. No other strong developmental phenotypes were observed.

The mutant phenotype was strongly affected by the segregation of modifiers in a traditional Ler × Columbia (Col) cross, which is normally used for genetic mapping, and the mutation was only narrowed to a ~500 kilobase region on chromosome 5. We therefore sequenced the whole genome of the mutant plant and analysed the linkage of candidate single nucleotide polymorphisms (SNPs) in an F2 population generated from a cross between the mutant and the isogenic progenitor line. This strategy identified a SNP in ELF6 (AT5G04240) that co-segregated with the resetting phenotype (Extended Data Fig. 3). To confirm that the resetting phenotype was caused by this mutant allele (named elf6-5), we complemented the mutation using the wild-type with the resetting phenotype (Extended Data Fig. 3). To confirm that the wild-type ELF6 gene under the control of its own regulatory sequences. Vernalized T2 transgenic elf6-5 lines carrying the wild-type ELF6 gene showed wild-type FLC expression levels in the siliques (Fig. 2a, b). Thus, we concluded that the single nucleotide mutation in ELF6 causes the mutant phenotype.

ELF6 is a jumonji-C-domain-containing protein that is closely related to the histone H3 trimethylated lysine 27 (H3K27me3) demethylase REF6 (ref. 16), and it is expressed at low levels in seedlings but at high levels in flowers and embryos (Fig. 2c–f). In the elf6-5 mutants, an alanine is replaced with a valine (amino acid 424) at the carboxy-terminal end of the elf6-5 mutant disrupts FLC expression, affecting an activity that is particularly important for resetting, the elf6-5 allele had a much larger effect on FLC expression in flowers and siliques than in seedlings (Fig. 3a, b). To define more precisely when the elf6-5 mutant disrupts FLC expression, we measured FLC messenger RNA levels at different stages of siliques development14, a proxy for FLC expression in the embryo13,14. Low FLCmRNA levels were detected in young siliques from the vernalized parental line (SQ16 and SQ17a) (Fig. 3c), and these levels increased as the siliques matured (SQ17b1 and SQ17b2), reaching a maximum when the siliques started to desiccate and the embryo became fully developed (SQ18). In the vernalized resetting mutant, FLC mRNA was detected in young, developing siliques, but it was not upregulated to wild-type levels at the later stages (Fig. 3c). Comparison of siblings differing only by an FLC::GUS (β-glucuronidase) reporter19 showed that FLC::GUS expression was lower in the early globular embryo of elf6-5 mutants than in the wild type (Fig. 3d). This finding suggests that ELF6 increases FLC expression as the embryo develops. There may be no clear mechanistic separation between reprogramming the epigenetic state and setting the FLC expression level.

The FLC locus has a complex transcriptional circuitry, including a set of antisense transcripts called COOLAIR that are induced during vernalization but are also expressed in the young plant20. We wondered whether the elf6-5 resetting mutant also affected COOLAIR expression. Surprisingly, no difference between the mutant and the wild type was found, and COOLAIR transcripts were upregulated normally in the mutant in developing siliques (Fig. 3e). Therefore, in contrast to mutants in which both FLC sense and total COOLAIR expression levels change coordinately (for example, fri mutants), the mutation in elf6-5 plants uncouples FLC sense and antisense regulation.

Many other loci are epigenetically modified during A. thaliana gamete formation and embryo development21,22. We tested whether the elf6-5 allele influences transposon expression, by analysing specific short interfering (si)RNA sequences using high-throughput sequencing (Fig. 3f). The elf6-5 allele caused the accumulation of transposon sequences, which may have implications for the early embryo.

**Figure 2** | Mapping of the resetting mutant. a, b, An ELF6 genomic construct complements the resetting mutant. FLC–luciferase imaging (a) and FLC qRT–PCR data (b) from mature siliques from the vernalized WT, the elf6-5 mutant and representative T2 elf6-5 (pELF6::ELF6) lines (Line 1 and Line 2). Pseudocolour bioluminescent image (a) from blue (least intense) to red (most intense). c–f, ELF6::GUS (blue) expression profile in a 7-day-old seedling (c), ovules (d), a globular embryo (e) and a mature embryo (f). Scale bars, 5 mm (c), 250 μm (d), 50 μm (e, f). g, The ELF6 amino acid residue that is mutated in elf6-5 mutants is conserved (red; A, alanine). A sequence alignment of the jumonji C domain of A. thaliana ELF6 and REF6 and human JMJD3 and UTX proteins is shown. Highly conserved residues are shaded in grey. The numbering refers to the ELF6 amino acid position.
The vernalization response in winter-annuals is known to occur preferentially in siliques. Using sensitive northern blot analyses, we compared with WT. In siliques (Fig. 3f), whereas regulation in

Figure 3 | Characterization of the elf6-5 resetting mutant. a, b, FLC-luciferase imaging (a) and FLC:qRT–PCR data (b) for tissues from the WT and the elf6-5 mutant in the generation following vernalization. The data are presented as the mean ± s.e.m., n = 6. Pseudocolour bioluminescent image (a) from blue (least intense) to red (most intense); c, qRT–PCR data for vernalized WT and elf6-5 siliques, immediately after fertilization, with petals still attached (SQ16); small and without petals (SQ17a); first (SQ17b1) and last (SQ17b2) mature green siliques; and yellow siliques (SQ18). The data are presented as the mean ± s.e.m., n = 4; *P < 0.05; **P < 0.01; ***P < 0.001 compared with WT. d, FLC:GUS (blue) expression in vernalized WT (top) and elf6-5 (bottom) early globular embryos. Scale bars, 100 μm. e, qRT–PCR data showing that COOLAIR levels are not affected in elf6-5 siliques.

Figure 4 | ELF6 shows H3K27 histone demethylase activity. a, b, Overexpression of a yellow fluorescent protein (YFP)-ELF6 fusion protein reduces H3K27me3 and H3K27me2 levels but not H3K27me1 levels (a, left). Overexpression of YFP-ELF6A424V has no effect on H3K27 methylation (b, left). Histone methylation was visualized by immunostaining (red). Nuclei (arrows) from transfected cells were visualized by the YFP signal (green) and stained with DAPI (4',6-diamidino-2-phenylindole) (blue). The histograms quantify the methylation levels in the nucleus of transfected (red) and non-transfected (blue) cells. The data are presented as the mean ± s.d. c, FLC regions analysed in ChIP, kb, kilobase. d, H3K27me3 levels in elf6-5 and WT siliques (stage SQ16–SQ17a) from vernalized plants. The data are presented as the mean ± s.e.m., n = 3. The H3K27me3 level in WT (V) plants is significantly lower than that in elf6-5 (V) in the FLC region +4,088 (*P < 0.05). e, The H3K27me3 levels in progeny derived from parents that had (V) or had not (NV) been vernalized. The H3K27me3 level in WT (V) plants is significantly lower than that in elf6-5 (V) in the FLC region +459 (*P < 0.05). The data are presented as the mean ± s.e.m., n = 3.
of this highly conserved residue (Fig. 2g) reduced H3K27 demethylease activity in our assay (Fig. 4b). To test whether this reduced activity influenced H3K27me3 levels at the FLC locus in vivo, we performed chromatin immunoprecipitation (ChIP) experiments. In wild-type Ler (FRI) plants, H3K27me3 levels increased by about twofold to fourfold in vernalized seedlings but were reduced to almost non-vernalized levels in vernalized siliques (Extended Data Fig. 7). When the resetting mutant was analysed, we found that the H3K27me3 levels were higher at FLC in vernalized young siliques of elf6-5 mutants than of the parental line (Fig. 4d). ChIP analysis on seedlings of the generation following vernalization also showed increased levels of H3K27me3 over various regions of FLC in elf6-5 mutants (Fig. 4e). These experiments were performed using whole seedlings or siliques; therefore, the data should be interpreted with caution because they are derived from a mixture of tissues. The vernalization-independent increase in H3K27me3 levels in elf6-5 mutants and the phenotype of the elf6 loss-of-function mutant make it likely that ELF6 has broader functions than simply resetting H3K27 methylation after vernalization. Nevertheless, all of these data are consistent with the reduced FLC expression during embryo development in elf6-5 mutants involving perturbed H3K27me3 dynamics that affect FLC resetting and result in the inheritance of a partially vernalized state.

This effective impairment of the reduction of H3K27me3 levels at FLC leads to transgenerational inheritance of a partially vernalized state (Fig. 1a–d). In nature, the consequences of this impairment would be to misalign the developmental program of the plant with respect to the environmental conditions. The sensitivity of FLC resetting to the reduced function elf6-5 allele may indicate that the requirement for H3K27me3 demethylase activity is highest at this post-vernalization stage of development, potentially explaining the differences in phenotype between elf6-5 and the null allele elf6-3. Functional redundancy between the three close homologues REF6, ELF6 and JM13 (AT5G46910) may also vary throughout development15. It will be interesting to see whether histone variants that are known to change in expression during embryogenesis16 are also involved in FLC resetting. In most eukaryotic genomes, a large proportion of chromatin is decorated with H3K27me3, probably explaining why the era of these methyl groups is a tightly controlled event during development and germ cell formation27. Further characterization of the FLC resetting process should provide greater insight into the molecular mechanism underlying genome reprogramming in eukaryotic organisms.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions P.C. and C.D. designed the research. P.C., H.Y. and C.G. performed experiments. M.T. conducted deep sequencing data analysis. X.Cao contributed new reagents and analytical tools. P.C., H.Y. and C.G. analysed the data and wrote the paper. All authors discussed the results and commented on the manuscript.

Author Information Genomic DNA deep sequencing data for the parental Ler-derived plant and the resetting mutant line have been deposited in the European Nucleotide Archive database under accession number PRJEB6498. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to C.D. (caroline.dean@jic.ac.uk).
METHODS

Plant material and growth conditions. All genotypes except for elf6-3 (ref. 17) were in a Ler background; an active FRI1U1223 allele and a genomic FLC::LUC construct were introduced by transformation to generate a vernalization-responsive line in which in vivo FLC expression could be monitored by luciferase imaging28. Genetic analyses and flowering time experiments were performed with plants sown on soil and grown in controlled environment chambers in long-day conditions (16 h light at 22 °C, 8 h darkness at 20 °C). For vernalization, seeds were pre-grown for 7 days in long-day, warm-growing conditions before being transferred to cold conditions (8 h light and 16 h darkness at 5 °C) for 6 weeks and then returned to warm conditions. 

Flowering time. Flowering time was scored as the total leaf number, including rosette and cauline leaves, before the first flower opened. Statistical evaluations were performed with Student’s t-test.

Reporter gene analysis. The parental Ler (FR1) FLC::LUC line has been described previously29,30, and luciferase bioluminescence imaging was detected using a NightOWL CCD photon counting camera (Berthold). Silique valves were opened longitudinally to detect the FLC::LUC signal from developing embryos. A complementing pELF6: ELF6::GUS; ELF6-3′UTR (3′ untranslated region) construct in an elf6-1 mutant background was used to monitor ELF6 expression. FLC::GUS was introduced into the resetting mutant, and β-glucuronidase activity was detected in A. thaliana thalli. Siliques were longitudinally cut, fixed for 2 h at 20 °C in 90% acetone and washed three times with 50 mM phosphate buffer, pH 7.0, before 24 h incubation at 37 °C in reaction buffer (0.19 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 10 mM EDTA, 0.1% Triton X-100, 1 mM potassium ferrocyanide and 1 mM potassium ferricyanide in 50 mM phosphate buffer, pH 7.2). Embryos were observed after clearing in Hoyer’s medium using a microscope under bright-field Nomarski optics.

RNA expression analysis. For seedlings, RNA analysis seeds were sown on GM media plates, stratified for 2 days and grown in long-day conditions for 11 days. RNA extraction from seedlings, DNase I treatment, cDNA synthesis and qPCR analyses (including the primers for FLC, total COOLAIR antisense and the UBC control gene) were as previously described31. The primers for the MAF-family genes were as previously described32-35. For the study of FLC expression in reproductive tissues, each biological replicate was obtained by extracting RNA31 from the main inflorescence or from three to five siliques from five plants. The expression data are presented as the average of several biological replicates, as indicated in the figure legends. Statistical evaluations were performed with Student’s t-test.

Illumina sequencing. To identify the resetting mutation, we performed genomic DNA deep sequencing on both the parental Ler-derived plant and the resetting mutant line (European Nucleotide Archive accession number, PRJEB6498). Total genomic DNA was isolated from 2 g inflorescence following a standard procedure. Briefly, inflorescences were ground in liquid nitrogen, and samples were homogenized in extraction buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 2 mM EDTA, 0.5% SDS and 100 mg ml−1 proteinase K) and incubated at 37 °C for 30 min. The proteins were removed by phenol–chloroform extraction, and the DNA was precipitated using 2.5 volumes of ethanol in the presence of 3 M sodium acetate, pH 5.

Illumina libraries were prepared using genomic DNA. The Illumina GAILX sequencing platform was used to generate 76-base paired-end reads for each sample. The alignment program MAQ v0.7.1 (ref. 32) was used, first to map reads from the parental line against the Col-0 (TAIR6) reference sequence. For this parental line, 10,867,014 reads from a total of 14,455,072 were mapped (with 10,265,540 of these mapped). As a control experiment, all T2 lines were also sown without antibiotic selection, and seedlings that did not carry the transgene were analysed: all lines without the transgenic ELF6 construct failed to complement the resetting phenotype.

Sequence alignment. The amino acid sequence alignment of the jumonji C domain of the A. thaliana ELF6 (UniProt ID, Q6BD8A0), A. thaliana REF6 (UniProt ID, Q9STM3), human (MJD3 (UniProt ID, AAH09994) and human UTX (UniProt ID, AAT68073) proteins was performed using the web-based software Multalin tool41.

Small RNA analysis. Total RNA extraction and northern blot analysis were performed as described previously36 using seed-specific probes37.

In vivo histone demethylation assay. The full length genomic coding sequences of wild-type ELF6 or mutant elf6-5 were cloned into the pEG104 vector35. The demethylation assay was carried out as previously described38. Briefly, N. benthamiana leaves were infiltrated with Agrobacterium tumefaciens EHA105 strains carrying a functional wild-type 35S::YFP; ELF6 or mutant 35S::YFP::ELF6-WT41. Nuclei from transgenic cells were isolated after 48 h. Immunoprecipitating of fixed nuclei was performed using histone-methylation-specific antibodies: H3K3me3 (Millipore 07-447, 1-100), H3K27me2 (Millipore 07-449, 1-100), H3K27me3 (Millipore 07-452, 1-200), H3K27me1 (Millipore 07-448, 1-200) and H3K36me3 (Abcam 9050, 1-100). The modified histones were revealed by Alexa-Fluor-550 conjugated goat anti-rabbit antibody (Invitrogen, 1-200). Transfected cells were revealed by monitoring the YFP signal. After staining, the slides were mounted in VECTAS HIELD Mounting Medium with 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and then photographed with an OLYMPUS BXSI fluorescence microscope. Histone methylation levels were quantified by comparing the staining density of a number of transfected 35S::YFP; ELF6 nuclei with that of the non-transfected nuclei in the same field. Image density was determined using ImageJ software. A negative result in this assay usually corresponded to 80% or less than the total wild-type histone demethylase activity.

ChiP experiments. ChiP experiments were performed using 11-day-old seedlings, using a previously described protocol32. For siliques, minor modifications were performed. About 0.5 g tissue was ground in liquid nitrogen, and then the powder was incubated for 10 min at room temperature in extraction buffer containing 1% formaldehyde to fix the tissue. We used anti-H3K27me3 (Millipore, 07-449) and anti-H3 core (Abcam, 1791) antibodies. All ChiP experiments were quantified by qPCR and analysed with previously described primers36. The ChiP data are presented as the ratio of H3K27me3 to total H3 normalized to non-vernalyzed wild-type levels. The ChiP seedling data were also normalized to STIM3 H3K27me3 levels36. Statistical evaluations were performed using Student’s t-test.

Statistical analysis. Statistical evaluations with Student’s t-test and graphical representation of the data were performed using the Prism software package (GraphPad). The means and s.e.m. are derived from independent biological samples.

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Extended Data Figure 1 | Screening for mutants with impaired epigenetic reprogramming of FLC. a, We started with a population of ethylmethane sulphonate (EMS)-mutagenized *A. thaliana* Ler plants carrying an FLC::LUC translational fusion and an active FRI transgene. We screened for mutants that were early flowering (as a result of low FLC expression) in the generation following vernalization but that did not flower early (and whose FLC expression was almost normal) without vernalization. b, To discriminate between early flowering and resetting mutants, early flowering M2 plants were backcrossed to the parental line, and the F2 phenotype was evaluated without vernalization. Those plants showing no early flowering segregants were considered to be resetting mutants. Superscript characters denote whether the plant was vernalized in the previous generation.
Extended Data Figure 2 | Characterization of the first resetting mutant.

a, The first resetting mutation was found to be recessive. F$_1$ plants were generated from a cross between the mutant in the generation following vernalization and the parental wild-type line. Flowering time was assayed as total leaf number under non-vernalized long-day conditions. The data are presented as the mean ± s.e.m., $n = 8$. b, The earlier flowering time of the mutant in the generation following vernalization was stable for at least three generations without vernalization. The data are presented as the mean ± s.e.m., $n = 10$. 
Extended Data Figure 3 | The elf6-5 SNP is linked to the resetting of FLC expression. Histogram showing the relationship between FLC::LUC levels in the reproductive organs of vernalized plants and the elf6-5 SNP (n = 154).
Extended Data Figure 4 | FLC expression levels in the null elf6-3 T-DNA insertion allele. a, The elf6-3 mutant expresses less FLC than the Col wild type. b, The null elf6-3 allele suppresses the high FLC expression induced by FRI before vernalization. This pre-vernalization phenotype of plants carrying the null allele precludes observation of the role of ELF6 during FLC resetting after vernalization. All graphs show 10-day-old non-vernalized seedlings. The data are presented as the mean ± s.e.m., n = 3.
Extended Data Figure 5 | siRNA production in elf6-5 mutants. The production of specific siRNAs associated with the epigenetic reactivation of transposable elements is not affected in elf6-5 mutants. Total RNA was extracted from vernalized mature siliques, and the detection of siRNAs was performed as described in Methods.
Extended Data Figure 6 | ELF6 has no H3K4me, H3K9me or H3K36me demethylase activity in an N. benthamiana transient assay. Overexpression of a yellow fluorescent protein (YFP)–ELF6 fusion protein, using the wild-type ELF6 sequence had no effect on H3K4me3, H3K9me2 or H3K36me3 methylation. Histone methylation was visualized by immunostaining with polyclonal rabbit modification-specific antibodies followed by Alexa-Fluor-555-conjugated goat anti-rabbit antibody (red; right). The nuclei of transfected cells were visualized by the YFP signal (green; centre). Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) (blue; left). Arrows indicate the nuclei of transfected cells.
Extended Data Figure 7 | H3K27me3 accumulation at the *FLC* locus.

a, Schematic representation of the *FLC* locus and the regions analysed in the ChIP assays. b, H3K27me3 levels at *FLC* in *Ler* (*FRI*) seedlings grown without vernalization (seedling NV), 7 days after vernalization (seedling VER) and in siliques from vernalized seedlings (siliques VER). The data are presented as the mean ± s.d., *n* = 2.