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Dual histone H3 methylation marks at lysines 9 and 27 required for interaction with *CHROMOMETHYLASE3*

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Both DNA methylation and post-translational histone modifications contribute to gene silencing, but the mechanistic relationship between these epigenetic marks is unclear. Mutations in two *Arabidopsis* genes, the *KRYPTONITE* (*KYP*) histone H3 lysine 9 (H3K9) methyltransferase and the *CHROMOMETHYLASE3* (*CMT3*) DNA methyltransferase, cause a reduction of CNG DNA methylation, suggesting that H3K9 methylation controls CNG DNA methylation. Here we show that the chromodomain of CMT3 can directly interact with the N-terminal tail of histone H3, but only when it is simultaneously methylated at both the H3K9 and H3K27 positions. Furthermore, using chromatin immunoprecipitation analysis and immunohistolocalization experiments, we found that H3K27 methylation colocalizes with H3K9 methylation at CMT3-controlled loci. The H3K27 methylation present at heterochromatin was not affected by mutations in *KYP* or in several *Arabidopsis* PcG related genes including the Enhancer of Zeste homologs, suggesting that a novel pathway controls heterochromatic H3K27 methylation. Our results suggest a model in which H3K9 methylation by KYP, and H3K27 methylation by an unknown enzyme provide a combinatorial histone code for the recruitment of CMT3 to silent loci.

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Introduction

Gene silencing in eukaryotes is associated with several epigenetic mechanisms including post-translational histone modifications, DNA methylation, and small RNAs, and accumulating evidence suggests extensive crosstalk between these mechanisms (Elgin and Grewal, 2003; Fischle *et al*, 2003a). Transcriptionally active or silenced genes are associated with distinct combinations of post-translational histone modifications important for regulating gene expression (Strahl and Allis, 2000; Turner, 2000). Methylation of the histone H3 tail at lysine 9 (H3K9) is associated with heterochromatic gene silencing (Lachner *et al*, 2001; Nakayama *et al*, 2001; Schotta *et al*, 2002). H3K9 methylation creates a binding site for the chromodomain of Heterochromatin-associated Protein 1 (HP1), an important silencing protein found in many different organisms (Bannister *et al*, 2001; Jacobs *et al*, 2001; Lachner *et al*, 2001; Nakayama *et al*, 2001). Lysines can accept three methyl groups, and can therefore be monomethylated, dimethylated, or trimethylated (hereafter denoted m, m^2, and m^3), and recent evidence suggests that there may be functional differences between these methylation states (Dutnall, 2003). For instance, in mammalian cells, H3K9m^3 is preferentially localized to pericentromeric heterochromatin, while H3K9m and H3K9m^2 are localized to euchromatin (Peters *et al*, 2003; Rice *et al*, 2003). In *Neurospora*, silent loci are associated with H3K9m^3 (Tamaru *et al*, 2003). However, in *Arabidopsis*, both H3K9m and H3K9m^2 are found at silent loci, with H3K9m^3 virtually absent (Jackson *et al*, 2004).

Methylation of histone H3 lysine 27 (H3K27) is also associated with gene silencing in several systems. For instance, mammals show an enrichment of H3K27m at heterochromatin and H3K27m^2 in euchromatin (Peters *et al*, 2003), and methyl H3K27 also marks the inactive X chromosome (Plath *et al*, 2003; Okamoto *et al*, 2004; Rougeulle *et al*, 2004). Furthermore, homeotic gene silencing in animals is associated with H3K27 methylation controlled by polycomb group (PcG) proteins (reviewed in Cao and Zhang, 2004). In *Arabidopsis*, silencing of the *FLC* locus is associated with H3K27 methylation, which depends on VRN2, a homolog of the PcG member Su(z)12 (Bastow *et al*, 2004; Sung and Amasino, 2004).

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The eukaryotic enzymes shown thus far to methylate H3K27 are the mammalian SET domain-containing protein G9a and the PcG Enhancer of zeste (E(Z)) proteins. G9a can methylate both H3K9 and K27 *in vitro* (Tachibana *et al*, 2001). However, while its role in methylating H3K9 *in vivo* has been characterized, its ability to methylate H3K27 *in vivo* has yet to be demonstrated (Tachibana *et al*, 2002; Rice *et al*, 2003). E(Z) proteins exist in conserved protein complexes responsible for H3K27 methylation of homeotic genes and of the inactive X chromosome (Cao *et al*, 2002; Czermin *et al*, 2002; Kuzmichev *et al*, 2002; Muller *et al*, 2002). One component of these complexes is the Extra Sex Combs (ESC) WD-40 protein, which is required for H3K27 methylation activity *in vivo*, and which is found in both animal and plant PcG complexes (Czermin *et al*, 2002; Kuzmichev *et al*, 2002; Muller *et al*, 2002; Hsieh *et al*, 2003). The polycomb protein (Pc) binds to methyl H3K27 via its chromodomain suggesting a possible mechanism for its action in the silencing of homeotic genes (reviewed in Cao and Zhang, 2004).

The *Arabidopsis* genome encodes three E(Z) homologs, *CURLY LEAF* (*CLF*), *SWINGER* (*SWN*) (formerly named *EZA1*), and *MEDEA* (*MEA*) (reviewed in Hsieh *et al*, 2003). *CLF* encodes a repressor of the floral homeotic gene *AGAMOUS* (Goodrich *et al*, 1997). *SWN* is a very close homolog of *CLF* and appears to act in a partially redundant fashion with *CLF* (Chanvivattana *et al*, 2004). *MEA* is an imprinted gene encoding a repressor of gene expression and endosperm development, and is expressed in the developing female gametophyte and endosperm (reviewed in Hsieh *et al*, 2003). The *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*) gene encodes the sole *Arabidopsis* ESC homolog. *FIE* mutations were initially identified by their endosperm development phenotypes, which are similar to those of *mea* mutants. Subsequently, using a *fie* partial loss-of-function allele, *fie TK114*, *FIE* was shown to be involved in floral homeotic gene repression (Kinoshita *et al*, 2001). Thus, the plant polycomb complexes likely act in the same fashion as in animals in the long-term repression of developmental regulatory genes (Hsieh *et al*, 2003).

Recent reports suggest a close relationship between histone H3K9 methylation and DNA methylation in several organisms (Tamaru and Selker, 2001; Jackson *et al*, 2002; Lehnertz *et al*, 2003). For instance, *Neurospora* mutants lacking histone H3K9 methylation show a complete loss of DNA methylation in all sequence contexts (Tamaru and Selker, 2001). In addition, in a screen for mutations that derepress the silencing of the heavily CNG (where N = A, T, C, or G) methylated and silenced *Arabidopsis SUPERMAN* locus, we previously isolated two genes with similar loss-of-function phenotypes, encoding the DNA methyltransferase CMT3 (Lindroth *et al*, 2001) and the H3K9-specific histone methyltransferase KYP (Jackson *et al*, 2002). These same two genes were cloned from an independent mutant screen for suppressors of silencing at the *PAI* loci (Bartee *et al*, 2001; Malagnac *et al*, 2002). The *cmt3* and *kyp* mutants show a loss of methylation, primarily at CNG sites, and cause reactivation of the expression of a subset of retrotransposons. We found that *kyp* mutants, but not *cmt3* mutants, show major losses of H3K9 methylation at affected loci, suggesting that H3K9 methylation acts upstream to control CNG DNA methylation (Johnson *et al*, 2002).

Here we address possible mechanisms by which H3K9 methylation targets CMT3 to methylate CNG sites. We previously found that, unlike the chromodomain of HP1, the chromodomain of CMT3 did not bind to K9 methylated histone H3 peptides. We therefore proposed a model in which CMT3 was indirectly tethered to methylated histones by the plant HP1 homolog, LHP1 (Gaudin *et al*, 2001; Jackson *et al*, 2002). However, our genetic analyses of *lhp1* mutants, as well as that previously published (Malagnac *et al*, 2002), do not support a role for LHP1 in the control of DNA methylation. Instead, we find that the CMT3 chromodomain can indeed interact with methylated histone H3 tails, but only if they are simultaneously methylated at H3K9 and H3K27. Furthermore, we show that H3K9 and H3K27 methylations are both enriched at silent loci where CMT3 acts. This suggests that a dual methylation mark is required for CMT3-dependent CNG methylation. Interestingly, the H3K27 methylation present at heterochromatin is not affected by mutations in *KYP* or in several *Arabidopsis* PcG related genes including the E(Z) homologs, suggesting that a novel pathway controls heterochromatic H3K27 methylation.

Results

LHP1 is not required for CMT3 activity in vivo

We previously showed that the *Arabidopsis* HP1 homolog, LHP1 (Gaudin *et al*, 2001), binds methylated H3K9 (Jackson *et al*, 2002). Furthermore, we showed that LHP1 binds to CMT3 *in vitro*. We therefore proposed a model in which KYP methylates H3K9, which results in binding of LHP1 and the subsequent recruitment of CMT3 to methylated chromatin (Jackson *et al*, 2002). This model predicts that loss-of-function alleles of *LHP1* should resemble loss-of-function alleles of *kyp* and *cmt3*. To test this, we obtained two recessive alleles of *lhp1* including a complete deletion (*LHP1* is the only HP1 homolog in *Arabidopsis*). These alleles were previously described as the *terminal flower2* mutants, and show a number of interesting developmental abnormalities including early flowering and inflorescences that prematurely terminate in floral structures (Larsson *et al*, 1998; Kotake *et al*, 2003). Both *tfl2* alleles have the same phenotype, and are here referred to as *lhp1-3* and *lhp1-4*.

Using Southern blot analysis with methylation-sensitive endonucleases, we tested several methylated DNA sequences known to be affected by *kyp* and *cmt3* for a loss of methylation in the *lhp1* mutants. We have previously shown that CMT3 methylates heterochromatic regions such as the pericentromeric *Ta3* retrotransposon and centromeric 180 bp (CEN) repeats, as well as the silent *FWA* and *SUP* loci, which are present in otherwise euchromatic regions. The *lhp1* alleles did not affect DNA methylation at either CG or CNG at any of the loci tested (Figure 1A–C). We also found that two retrotransposon related sequences (*Ta3* and *TSI*) that show reactivation of expression in the *cmt3* and *kyp* mutants are not reactivated in the *lhp1* mutants (Figure 1D) (Jackson *et al*, 2002).

In fission yeast, it has been shown that spreading and distribution of methyl H3K9 are dependent on *Swi6* (the yeast HP1 homolog) (Hall *et al*, 2002). We therefore tested if *lhp1* mutants altered dimethyl H3K9 distribution in *Arabidopsis*. The *lhp1* mutant did not show a change in the distribution of dimethyl H3K9, since staining was still clearly localized to heterochromatic chromocenters (Figure 1E).

CMT3 and KYP are important in maintaining non-CG methylation and gene silencing of the *SUP* locus in a stably

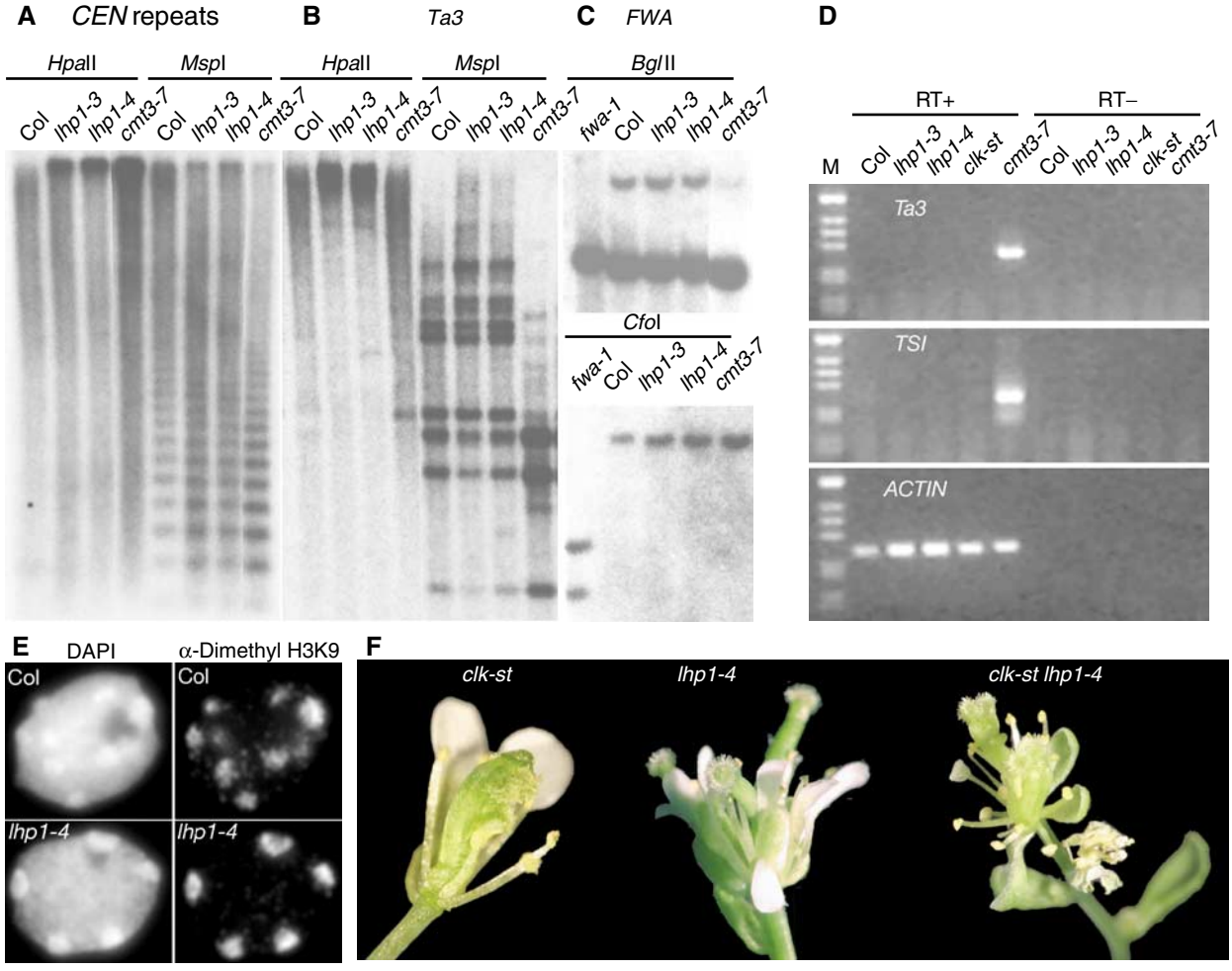

Figure 1 *lhp1* mutants do not affect DNA methylation or gene silencing in heterochromatin. (**A–C**) Southern blot analysis with the DNA methylation-sensitive endonuclease *Cfo*I and *Hpa*II for CG sites, and *Bgl*II and *Msp*I for CNG sites, probed with centromeric 180 bp repeat (*CEN*), *Ta3*, and *FWA*. *fwa*-1 is a mutant lacking methylation of the *FWA* gene. *cmt3-7* (included as a control) reduces CNG methylation, and therefore shows a downshifted banding pattern. (**D**) Semiquantitative RT–PCR showing reactivation of retroelement sequences in *cmt3* but not in *lhp1* mutants. (**E**) Immunohistochemical staining of wild-type and *lhp1* interphase nuclei with dimethyl H3K9 antibodies preferentially labeling heterochromatic chromocenters. The *lhp1-4* mutant does not affect distribution of methyl H3K9. (**F**) Floral phenotype of the *clark kent-st* (*clk-st*) *lhp1-4* double mutant. The left flower, a *clk-st* mutant, shows supernumerary stamens and a defective gynoecium. The middle flower, an *lhp1-4* mutant, shows a terminal flower with three normal gynoecia. The right flower, a *clk-st lhp1-4* double mutant, shows a compound terminal flower containing two *clk-st*-like defective gynoecia.

hypermethylated *superman* allele, *clk-st*. To analyze if *LHP1* is involved in this process, we introduced the *lhp1-4* mutant into the *clk-st* strain and looked for suppression of the *superman* floral phenotype. We found that, unlike the *cmt3* and *kyp* mutants, the *lhp1* mutants do not suppress silencing of *clk-st* (Figure 1F). Thus our genetic analysis does not support a role for LHP1 in recruitment of CMT3. These results also agree with data showing that a weak *lhp1* allele (Gaudin *et al*, 2001) does not reduce DNA methylation of the *PAI* loci or of the *CEN* repeats (Malagnac *et al*, 2002). We therefore conclude that LHP1 is unlikely to play an important role in the recruitment of CMT3 to methylated chromatin.

Binding of CMT3 to the doubly methylated N-terminus of histone H3

We revisited the hypothesis that CMT3 might bind directly to methylated histones through its chromodomain. The CMT3 chromodomain sequence is closely related to those of HP1 and Pc that bind to methylated H3K9 and H3K27, respectively (Figure 2A and B). Analyses of the structures and thermodynamics of the HP1 (Jacobs and Khorasanizadeh, 2002; Nielsen *et al*, 2002) and Pc chromodomains (Fischle *et al*, 2003b; Min *et al*, 2003) in complex with methylated H3 tails have shown that each of these chromodomains makes a 1:1 complex with its target histone H3 tail. Further, both HP1 and Pc use an aromatic cage involving three conserved aromatic residues to recognize the methyllysine side chain in the H3 tail. The CMT3 chromodomain also contains aromatic residues located at the same sequence positions (F382, W409, and Y412; Figure 2B).

Using fluorescence anisotropy binding assays, we measured the affinity of the CMT3 chromodomain for various histone H3 tail peptides methylated at K9, K27, or both. We found no appreciable binding to peptides with either methylation mark alone. However, when both methylations were present in the same histone H3 peptide, we found a strong interaction ($K_D = 0.9 \pm 0.1\,\mu M$) (Figure 2C). This mode of binding suggests that the CMT3 chromodomain may form

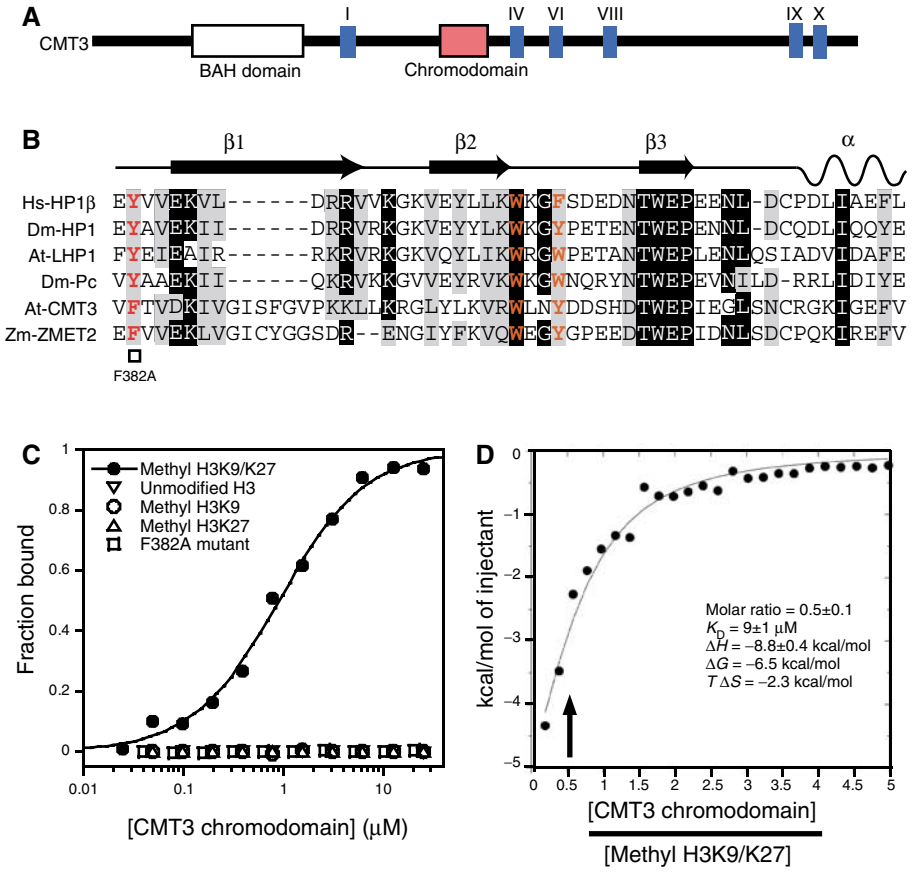

Figure 2 Interaction of the CMT3 chromodomain with the N-terminal tail of histone H3. (**A**) Domain composition of CMT3. Roman numerals denote the conserved motifs within the catalytic DNA methyltransferase region. (**B**) Alignment of the chromodomains of CMT3 and ZMET2 (a functional CMT gene from *Zea mays*; Papa *et al*, 2001) proteins with those of the HP1, LHP1, and Pc proteins. Secondary structure elements drawn above the sequence correspond to the structure of the HP1 chromodomain. Positions F382, W409, and Y412, highlighted in red, show the sites of conserved aromatic residues that form an aromatic cage. (**C**) Fluorescence polarization-based binding results for the interaction of the CMT3 chromodomain with H3 peptides. The CMT3 chromodomain binds to a doubly methylated H3K9/K27 peptide, but not to either the unmodified or singly methylated peptides. Binding was eliminated by mutation of the conserved aromatic residue F382 to alanine. (**D**) Binding of CMT3 chromodomain to methyl H3K9/K27 as measured by ITC. The solid line corresponds to the fit of the integrated heats of injections to an interaction stoichiometry of 0.5; see the inset for the thermodynamic parameters of the best fit of the data using the MicroCal software.

two independent binding sites for each methyllysine, or alternatively form a homodimer for binding to the doubly methylated H3 tail in a 2:1 complex.

To establish more thoroughly the thermodynamics and stoichiometry of this interaction, we carried out isothermal titration calorimetry (ITC) and found that the molar ratio of the CMT3 chromodomain bound to the doubly methylated peptide is 0.5 ± 0.1, with the remaining parameters measured as follows: $K_D = 9 \pm 1\,\mu M$, $\Delta H = -8.8$ kcal/mol, $\Delta G = -6.5$ kcal/mol, and $T\Delta S = -2.3$ kcal/mol. This indicates that the association of two CMT3 chromodomains as a homodimer allows the high-affinity interaction with the doubly methylated histone H3 tail (Figure 2D). The K_D values measured by fluorescence and ITC for CMT3 binding to the doubly methylated H3 tail are in the range of 0.9–9 μM. Importantly, these K_D values are similar to those measured previously for HP1 and Pc chromodomains binding to their respective singly methylated H3 tails (4–5 μM) (Fischle *et al*, 2003b).

To test the role of the putative aromatic cage of CMT3 in binding methylated lysines, we mutated residue F382 to alanine and then tested this mutant chromodomain using fluorescence anisotropy binding studies (Figure 2C). This mutation resulted in a complete defect in binding to the doubly methylated H3 tail, confirming the importance of the predicted aromatic cage residues of the CMT3 chromodomain in target methyllysine binding.

Together, these data suggest that while the same aromatic cage in HP1, Pc, and CMT3 chromodomains may be required for target recognition, the CMT3 chromodomain is unique in its ability to cooperate via two subunits to bind to a doubly methylated H3 tail. As such, the chromodomain of CMT3 is reminiscent of the chromo shadow domain of HP1, which also forms a homodimer (Thiru *et al*, 2004). Since homodimerization does not occur with HP1 or Pc chromodomains despite their overall high sequence similarity with CMT3, we suggest that the six-residue insertions within the β-strand 1 segment of the chromodomain (Figure 2B), or nonconserved residues within the CMT3 chromodomain, contribute to a dimerization interface of this protein. Interestingly, the maize homolog, ZMET2 (Papa *et al*, 2001), also contains a similar insertion suggesting a related function for another plant chromodomain that resides within a DNA methyltransferase fold.

Colocalization of methyl H3K9 and K27 at heterochromatin

The *in vitro* CMT3 binding data suggest a model in which CMT3 targeting to chromatin involves a combinatorial double methylation signal. This model predicts that H3K27 methylation should be localized to loci controlled by CMT3. To test this, we performed chromatin immunoprecipitation (ChIP) experiments using antibodies to mono-, di-, and trimethyl H3K27 (Peters *et al*, 2003; Perez-Burgos *et al*, 2004), looking for enrichment at silent CMT3-controlled loci. We tested two loci found in pericentromeric heterochromatin that we previously found were affected in *cmt3* mutants, the *Ta3* retrotransposon and the 180 bp CEN repeat (Lindroth *et al*, 2001; Johnson *et al*, 2002). Both loci showed enrichment of mono- and dimethyl H3K27 over *ACTIN*, a euchromatic gene used for normalization (Figure 3A). The monomethyl H3K27 signal was stronger than the dimethyl signal, and we did not observe trimethyl signal over the no antibody controls. These results suggest that both H3K9 and H3K27 methylations are enriched at DNA methylated and silent loci in pericentromeric heterochromatin. Interestingly, while the *kyp* mutation reduced the H3K9 methylation at these loci, it did not affect the H3K27 methylation (Figure 3A).

To assess whether H3K27 methylation is generally associated with heterochromatin, we assayed the distribution of methyl H3K27 in different nuclear compartments using immunohistochemical staining of interphase nuclei (Figure 3B). We utilized the same mono-, di-, and trimethyl H3K27 antibodies to look for colocalization with DAPI staining chromocenters where most of the heterochromatin resides. H3K27m preferentially localized to chromocenters with very little staining in euchromatin. H3K27m^2 localized strongly to chromocenters and to a lesser extent to euchromatin. Trimethyl staining was observed in small speckles, not coinciding with DAPI-stained chromocenters. While the level of H3K9m^2 at chromocenters was reduced in the *kyp* mutant, all staining patterns with the H3K27 methyl antibodies were unaffected (Figure 3B). These results agree with the ChIP analyses and show that *KYP* is not responsible for the H3K27 methylation at silent heterochromatin.

Finally, we used ChIP analysis to access the levels of H3K27 methylation at two CMT3-controlled loci that are silent but reside in otherwise euchromatic regions of the genome, *SUPERMAN* and *FWA* (Figure 3C). We found that both H3K9 and H3K27 methylations were enriched at these loci. And again, while the *kyp* mutation reduced the H3K9 methylation, it did not reduce the levels of H3K27 methylation.

Histone methyltransferases affecting H3K27 methylation

It is currently unknown which genes in *Arabidopsis* are responsible for methylating H3K27 sites in heterochromatin. Our findings predict that mutations in these genes should reduce CMT3 recruitment to chromatin, and thus should mimic the *kyp* and *cmt3* mutants (reducing CNG methylation). In *Arabidopsis*, the most obvious candidate genes are the PcG related E(Z) homologs, since animal E(Z) proteins are known to methylate H3K27 (Cao *et al*, 2002; Czermin *et al*, 2002; Kuzmichev *et al*, 2002; Muller *et al*, 2002). The three plant E(Z) genes are *MEA*, *CLF*, and *SWN*. *MEA* is paternally imprinted and the maternal copy is only expressed

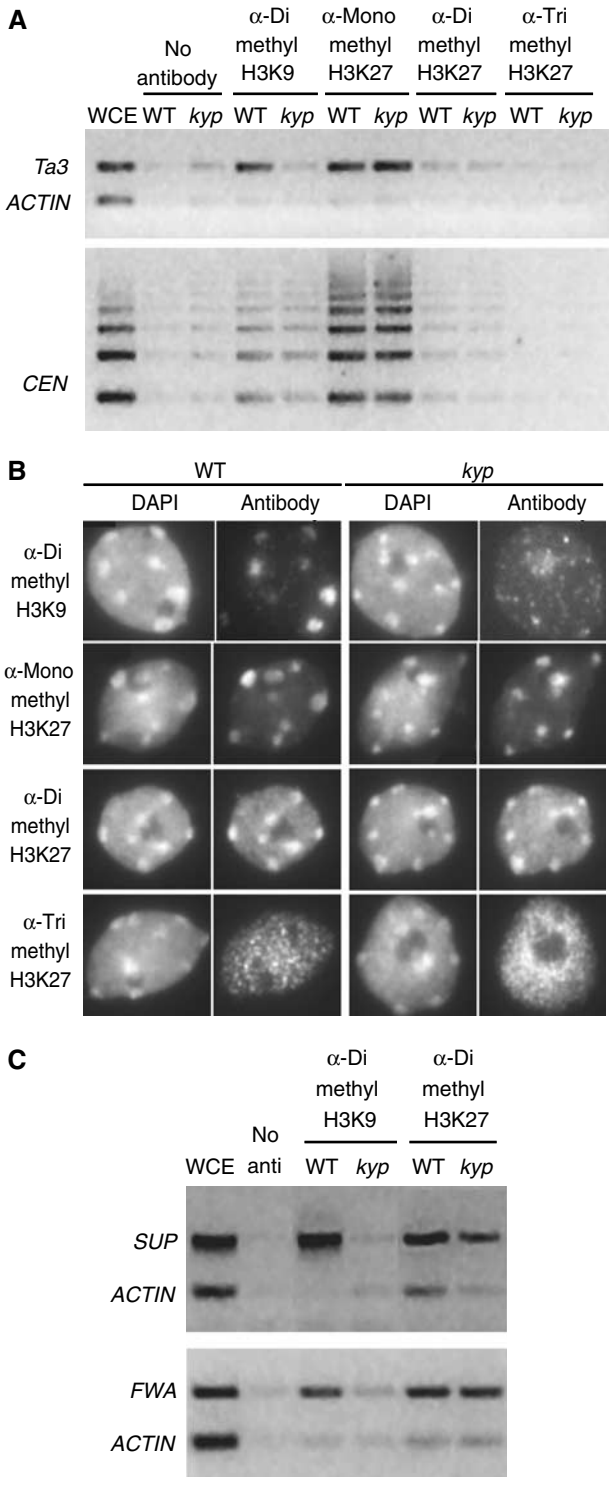

Figure 3 Colocalization of methyl H3K9 and H3K27 at heterochromatin. (**A**) ChIP analysis showing methyl H3K9 and H3K27 enrichment at the *Ta3* retrotransposon and *CEN* repeats, relative to *ACTIN*. *kyp* affects levels of methyl H3K9 but not mono-, di-, or trimethyl H3K27. *ACTIN* is used for normalization, and the amount of chromatin used in each sample (for both the *Ta3* and *CEN* experiments) was adjusted so that an equal amount of *ACTIN* was amplified. WCE, whole-cell extract. (**B**) Distribution of mono-, di-, and trimethyl H3K27 in interphase leaf nuclei. Mono- and dimethyl H3K27 are enriched at DAPI-stained chromocenters, while trimethyl H3K27 signals are more evenly distributed. *kyp* reduces dimethyl H3K9 at heterochromatin, but not H3K27 methylation. (**C**) ChIP analysis showing enrichment of both H3K9 and H3K27 methylation at *SUPERMAN* and *FWA* relative to ACTIN.

in the endosperm and not in the rest of the plant (Reyes and Grossniklaus, 2003). It is therefore very unlikely that *MEA* is involved in global H3K27 methylation in heterochromatin. The other two genes, *CLF* and *SWN*, are widely expressed, and are potentially good candidates for global H3K27 methyltransferases (Chanvivattana *et al*, 2004). We therefore generated a double loss-of-function *clf* and *swn* mutant. The *clf-50* allele is a complete deletion of the *CLF* locus, and the *swn-3* allele is a T-DNA insertion upstream of the catalytic SET domain (Chanvivattana *et al*, 2004). The *clf-50 swn-3* double mutant is viable but shows severe developmental abnormalities (Chanvivattana *et al*, 2004). Another *Arabidopsis* PcG member is *FIE*, a homolog of the *Drosophila* WD-40 protein Extra sex combs (ESC). Since *FIE* is a single-copy gene in *Arabidopsis*, *fie* mutants are likely to affect all H3K27 methylation targeted by E(Z) protein-containing complexes (Reyes and Grossniklaus, 2003). Although *fie* null mutants are embryonic lethal, likely due to the role of *FIE* in imprinting and early endosperm development, a transgenic allele was isolated, *fie TK114*, in which *FIE* is expressed only during very early embryo development, but not during later seedling development (Kinoshita *et al*, 2001). This allele causes ectopic expression of homeotic genes and a severe morphological phenotype that resembles that of the *clf-50 swn-3* double mutant (Kinoshita *et al*, 2001; Chanvivattana *et al*, 2004). We tested both the *clf-50 swn-3* double mutant and the *fie TK114* mutant for defects in H3K27 methylation at heterochromatin and for effects on DNA methylation.

In immunohistolocalization experiments, we analyzed the global distribution of H3K27 methylation in the *clf-50 swn-3* double mutant and the *fie TK114* mutant (Figure 4A). The levels of H3K27m were unaffected in these mutants. Using the antibody specific for H3K27m[2], we observed a reduction in the staining intensity in euchromatin, while labeling of the chromocenters was similar to the wild type. As noted above, the H3K27m[3] antibody usually gave signals that were more or less evenly distributed throughout the nucleus. However, in a fraction of the nuclei from the *clf-50 swn-3* double mutant and the *fie TK114* mutant, we observed reduced staining of the euchromatic areas and enhanced staining in chromocenters (Figure 4A).

Southern blot analysis using methylation-sensitive restriction enzymes showed that neither the *clf-50 swn-3* double mutant nor the *fie TK114* mutant affected levels of DNA methylation at the *Ta3* and *CEN* repeats, two loci where *cmt3* and *kyp* mutants show strong losses of CNG methylation (Figure 4B).

We also analyzed mutations in two additional *Arabidopsis* PcG genes, the SU(Z)12 homolog *EMF2* (Yoshida *et al*, 2001) and the E(Z) related gene *MEA*. Neither of these mutants altered the staining patterns using the different H3K27 methyl antibodies (Supplementary Figure S1), nor did they affect levels of DNA methylation at *Ta3* or *CEN* repeat loci (data not shown).

These results are consistent with the role of *Arabidopsis* PcG proteins in controlling histone methylation and transcriptional repression of developmentally important genes in euchromatin, and also show that the available H3K27 antibodies are useful reagents for these studies. However, our results suggest that the PcG proteins do not control H3K27 methylation at CMT3-controlled silent heterochromatin.

Discussion

Genetic studies of the cytosine DNA methyltransferase gene *CMT3* and the histone H3K9 methyltransferase gene *KYP* have identified a link between histone and DNA methylation, since mutations in both genes show a loss of DNA methylation, especially in CNG sequence contexts (Bartee *et al*, 2001; Lindroth *et al*, 2001; Jackson *et al*, 2002; Malagnac *et al*, 2002). This report addresses the possible mechanistic explanations for the resemblance of the *cmt3* and *kyp* mutant phenotypes. We propose a model in which the combinatorial presence of methyl H3K9 and methyl H3K27 acts to directly recruit the CMT3 DNA methyltransferase to target loci (Figure 5). This model is supported by the observation that the CMT3 chromodomain cannot bind to histone H3 peptides that are singly methylated at the H3K9 or H3K27 positions, but does bind efficiently to peptides that are doubly methylated at the H3K9 and H3K27 positions. This model is also supported by our findings that both H3K9 and H3K27 methylations colocalize to the sites of CMT3 action.

Function of Arabidopsis LHP1

From *Drosophila* to mammals, HP1 is a crucial silencing component in heterochromatin (Grewal and Elgin, 2002). However, our current genetic analyses, and those of a previous study (Malagnac *et al*, 2002), do not support a role for LHP1 in gene repression in heterochromatin. Remarkably, in *Neurospora*, an organism in which histone H3K9 methylation controls all DNA methylation (Tamaru and Selker, 2001), an HP1 homolog has been shown to be indispensable for DNA methylation (Freitag *et al*, 2004). However, in *Arabidopsis*, recent analysis suggests that LHP1 instead regulates euchromatic loci that are not subject to DNA methylation (Kotake *et al*, 2003; Takada and Goto, 2003; Tariq and Paszkowski, 2004). Thus, *Arabidopsis* HP1 may have evolved a different role than HP1 homologs in animals and fungi. Interestingly, while plants contain the conserved components of the PRC2 complex, including homologs of E(Z), ESC, and Suz (12), plants lack components of the PRC1 complex, such as the chromodomain protein Polycomb, which binds to histones methylated at the H3K27 position. Given that LHP1 mutants have several developmental phenotypes, it is thus possible that LHP1 has evolved to fulfill the role of the PRC1 complex.

Function of the CMT3 chromodomain

The CMT3 chromodomain binds to histone H3 peptides only when both K9 and K27 are simultaneously methylated. Interestingly, the immediate sequences surrounding the K9 and K27 sites in histone H3 are highly related, suggesting that they may be recognized in a similar manner. ITC studies indicate that two CMT3 chromodomains associate with the doubly methylated histone tail. This finding suggests that cooperation between subunits allows the productive binding of this sequence. As such, the simultaneous recognition of two post-translational modifications by the CMT3 chromodomain supports the notion of a combinatorial code in which two or more post-translational marks on a histone tail can influence each other (Strahl and Allis, 2000; Turner, 2000).

Chromodomains including those of CMT3, HP1, and Pc share three conserved aromatic residues that form a methylammonium recognition cage in the structures of the HP1 and Pc chromodomains in complex with their target peptides

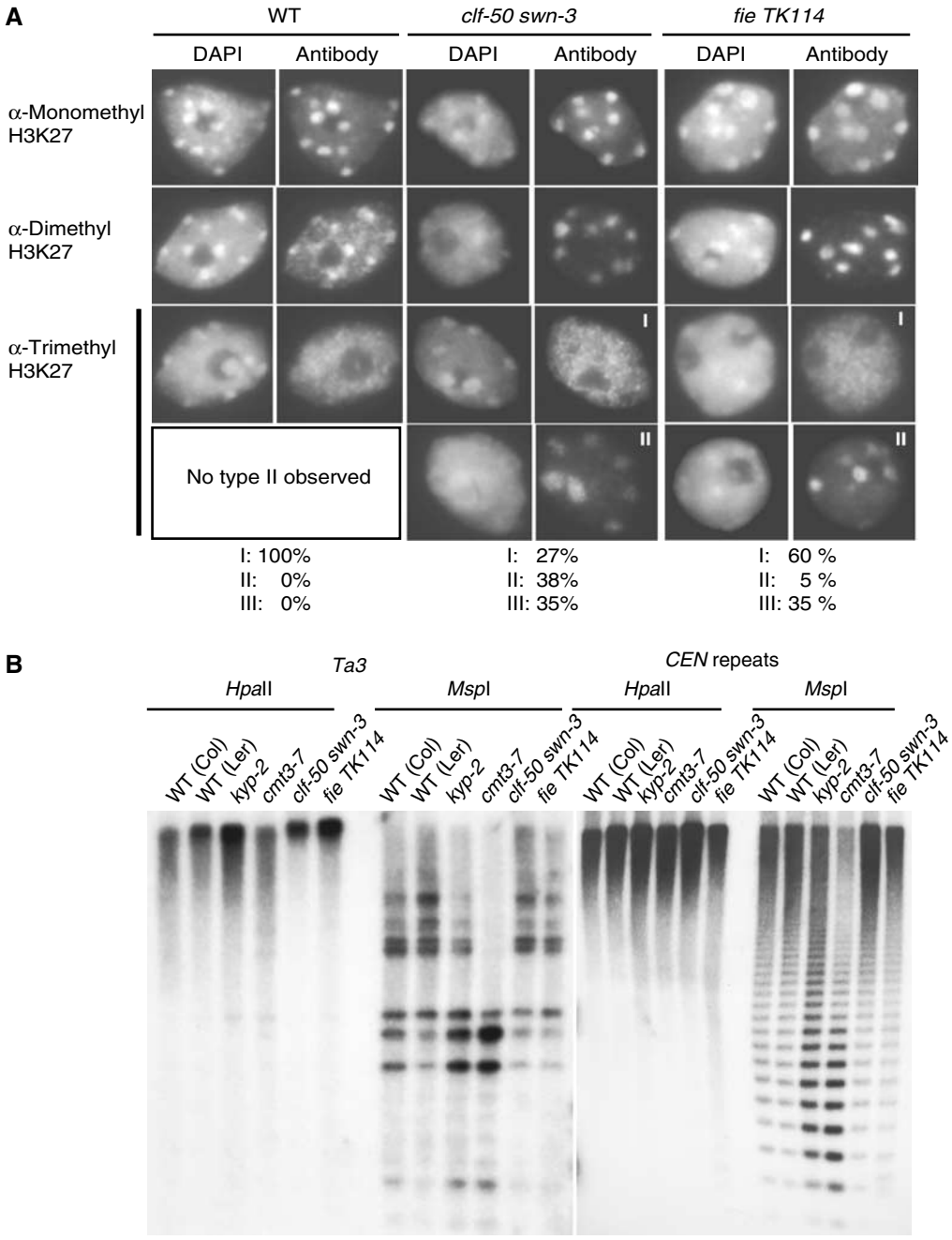

Figure 4 Effect of PcG mutations on the distribution of methyl H3K27 in leaf interphase nuclei and on DNA methylation. (**A**) Distribution of mono-, di-, and trimethyl H3K27 in the *clf-50 swn-3* double mutant and the *fie TK114* mutant. These mutants do not reduce heterochromatic H3K27 methylation, but do reduce euchromatic H3K27m^2. Whereas H3K27m^3 was evenly distributed in wild-type nuclei, in a fraction of *clf-50 swn-3* and *fie TK114* nuclei, signal was concentrated in chromocenters. The percent of nuclei showing three different staining patterns with the H3K27m^3 antibody is listed below (types I, II, and III). Type I is a pattern similar to wild type with signals evenly distributed within nuclei. Type II is H3K27m^3 signals concentrated at chromocenters (examples shown in the lower row of photographs). Type III is an intermediate pattern where H3K27m^3 was concentrated at only those chromocenters associating with nucleoli (not shown). At least 150 nuclei were evaluated for each genotype. (**B**) Southern blot analysis showing the lack of effect of the *clf-50 swn-3* double mutant and the *fie TK114* mutant on DNA methylation. The *cmt3* and *kyp* mutants are included as controls and show loss of CNG methylation (an increased band intensity of the low-molecular-weight bands as compared to the high-molecular-weight bands). Two wild-type controls are shown because the *cmt3* and *kyp* mutants are in the L*er* background, and the *clf-50 swn-3* and *fie TK114* mutants are in the Col background.

(Jacobs and Khorasanizadeh, 2002; Nielsen *et al*, 2002; Fischle *et al*, 2003b; Min *et al*, 2003). We found that mutagenesis of F382, one of these aromatic residues, completely abolished binding of the CMT3 chromodomain to methylated histone tail peptides. This is consistent with the expectation that the CMT3 chromodomain, like the HP1 and Pc chromodomains, similarly uses an aromatic cage structure for the recognition of each methyllysine.

Lastly, a comparison of CMT3 and maize ZMET2 with HP1 and Pc shows that the plant chromodomains contain a six-residue insertion of unknown function. Thus, while the aromatic residues of the CMT3 chromodomain are clearly

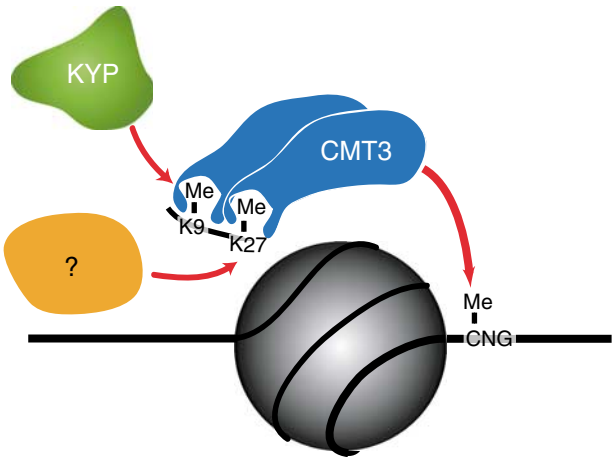

Figure 5 Model for the relationship of histone methylation and CNG DNA methylation. Histone H3K9 methylation through KRYPTONITE and H3K27 methylation through an unknown methyltransferase act to target the activity of the CMT3 DNA methyltransferase.

important for methyllysine binding, the way in which the CMT3 chromodomain interacts with doubly methylated histones may be different from the way the HP1 and Pc chromodomains interact with singly methylated histones. Interestingly, the chromodomain of CMT3 is nested between the conserved motifs present within the DNA methyltransferase catalytic domain of CMT3, and modeling of its location suggests that it is on the opposite face from the methyltransferase catalytic pocket (Henikoff and Comai, 1998). It is therefore possible that the binding of CMT3 to methylated histones might not only serve in recruitment but could potentially affect the catalytic activity of CMT3.

Targeting of CMT3 to methylated chromatin

Our model predicts that chromatin that is methylated at histone H3K9 and H3K27 should be a target for CMT3 action. However, the *Arabidopsis FLC* locus is methylated at both H3K9 and H3K27, but this gene contains no DNA methylation (Bastow *et al*, 2004; Sung and Amasino, 2004). This shows that H3K9 and H3K27 methylation is not sufficient for targeting DNA methylation, and suggests that other chromatin modifications may be involved *in vivo*. In addition to its chromodomain, CMT3 contains a second conserved domain of unknown function, the BAH domain (Callebaut *et al*, 1999), and one could imagine that this might confer upon CMT3 the ability to recognize an additional histone modification that allows the proper targeting of CMT3 to silent loci *in vivo*. A second possibility is that small RNAs are required to establish DNA methylation at heterochromatic silent loci, but these RNAs do not arise at loci such as *FLC*. Indeed, in addition to the *cmt3* and *kyp* mutations, we also isolated a mutation in the RNA silencing gene *ARGONAUTE4* in our screen for suppressors of a hypermethylated *superman* epiallele (Zilberman *et al*, 2003), showing that small RNAs are important in the targeting of CNG methylation. Small RNAs are known to affect DNA methylation in several other systems as well (Jones *et al*, 2001; Aufsatz *et al*, 2002; Melquist and Bender, 2003). Furthermore, mutation of *ARGONAUTE4* or of three other RNA silencing loci prevents the establishment of DNA methylation at the *FWA* locus (Chan *et al*, 2004).

Arabidopsis H3K27 methyltransferases

In order to find the enzyme responsible for methyl H3K27 in heterochromatin, we analyzed mutations in the *Arabidopsis E(Z)* homologs as well as mutations in other PcG genes. We found that these mutations reduced the level of H3K27 methylation in the euchromatic compartment of nuclei, and in some cases enhanced the H3K27 methylation signals at chromocenters. However, these mutants did not reduce H3K27 methylation at chromocenters and did not reduce DNA methylation at specific silent loci. These results are in line with the known phenotypes of PcG mutants, which include several specific homeotic and growth defects due in part to the misexpression of specific regulatory genes (reviewed in Hsieh *et al*, 2003). Thus, it is likely that the *Arabidopsis* PcG proteins control H3K27 methylation of developmentally important genes in euchromatin, while a separate enzyme controls H3K27 methylation in heterochromatin. The *Arabidopsis* genome contains at least 29 expressed SET domain-containing proteins, including many family members related to Su(var)3-9 and KYP. Reverse genetic analysis of these genes might prove useful in identifying the enzyme(s) responsible for methylating H3K27 at heterochromatin.

Materials and methods

Plant materials

The *terminal flower2* mutants, *tfl2-1* and *tfl2-2* (in this paper referred to as *lhp1-3* and *lhp1-4*, respectively), were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, USA), and the phenotype of these mutants was first described by Larsson *et al* (1998). We mapped the mutation of *lhp1-3* as being a nonsense mutation truncating the protein from amino-acid position 280. This mutant allele therefore lacks the chromo shadow domain. We found that the *lhp1-4* mutation is a complete null, since it represents a 9.9 kbp deletion including the entire LHP1 gene. Flanking sequences of the deleted gene can be PCR amplified with the primers JP1804 forw. 5′-TCCCAAACAGGAACTGGAGC-3′ and JP1717 rev. 5′-TTGGTCTTGGACATTGCGG-3′ to give a 2.5 kb product. The *lhp1-4* deletion was determined to include 1302 bp upstream of the initiation codon and 6219 bp downstream of the stop codon (Kotake *et al*, 2003). The *clark kent* strain (*clk*-st) used in this study has been described by Cao and Jacobsen (2002). We crossed *lhp1-4* (ecotype Columbia) two successive times to *clk-st* plants (ecotype Landsberg *erecta*) background, and then plants homozygous for *clk*-st and heterozygous for *lhp1*-4 were selfed, and their progeny were scored for *lhp1*and *clk* phenotypes and PCR genotyped. We identified and analyzed more than 50 independent *lhp1-4 clk-st* homozygous plants.

The mutants of *MEA* (Kinoshita *et al*, 1999), *FIE* (*fie TK*114; Kinoshita *et al*, 2001), and *emf2-3* (Moon *et al*, 2003) were a generous gift from R Fisher, T Kinoshita, and Z Renee Sung, respectively.

Southern blot analysis

DNA was isolated from 3- to 5-week-old rosette leaves of Landsberg *erecta*, Columbia, *lhp1-3*, *lhp1-4*, *cmt3-7*, and *fwa-1* (Soppe *et al*, 2000), as previously described (Lindroth *et al*, 2001). The amount of DNA used for the Southern blot analysis was 100 ng per sample for *Ta3* and *CEN*, and 2 μg for *FWA*. Here, *fwa-1* was used as a control for complete lack of methylation of the *FWA* gene. The CG- and CNG-sensitive endonucleases *Cfo*I and *Bgl*II were used to cut the DNA, respectively. For all the other loci tested, the DNA was cut with *Hpa*II (sensitive to CG and CNG methylation) and *Msp*I (sensitive to CNG methylation).

RT–PCR

First-strand cDNA synthesis was performed on 3 μg total RNA from the inflorescences of 3-week-old plants using oligo-dT priming and Superscript II reverse transcriptase (Invitrogen, Inc.). PCR was performed under semiquantitative conditions by limiting cycle number such that the reactions were within the linear range of amplification. By normalization with *ACTIN* PCR, the amount cDNA added was adjusted for comparison between the different samples. The primers for *ACTIN* were (JP771) 5′-ATA GCT GCA TTG TCA CCC GA and (JP1564) 5′-GGT TGT GTC AAG AAG TCT TGT GTA CTT TAG TTT TA-3′ and for *Ta3* (JP1565) 5′-GAT TCT TAC TGT AAA GAA CAT GGC ATT GAG AGA-3′ and (JP1566) 5′-TCC AAA TTT CCT GAG GTG CTT GTA ACC-3′. PCR conditions were 95°C, 15 s, −65°C, 30 s, and −72°C, 1 min for 25–49 cycles.

Nuclei isolation and histone immunolabeling

Nuclei isolation from young leaves or small seedlings (the *clf-50 swn-3* double mutant) and histone immunolabeling were performed as previously described (Jasencakova *et al*, 2003). Slides were evaluated using a Zeiss Axioplan 2 fluorescence microscope equipped with a cooled CCD camera (either CH250/A, Photometrics or Spot 2e, Diagnostic Instruments). The exposure times for wild-type and mutant samples were identical in the respective experiments. The images were not processed for contrast or intensity enhancement.

Preparation of peptides and protein and the binding assays

Synthetic peptides corresponding to the trimethyl H3K9 and trimethyl H3K27 regions include residues 1–32 and 15–32, respectively, of the H3 tail. The synthetic unmodified as well as doubly trimethylated peptide at K9 and K27 includes residues 1–32 of the H3 tail.

An *Escherichia coli* expression construct of CMT3 chromodomain (residues 376–439) with an N-terminal maltose binding protein tag was prepared by subcloning the corresponding DNA into the *Eco*RI/*Xba*I sites of the pMAL-c2 vector (New England Biolabs). Point mutation of the chromodomain was prepared using the QuikChange™XL Site-Directed Mutagenesis kit (Stratagene).

Fusion protein was expressed in *E. coli* strain BL21 (DE3) (Novagen) and purified to homogeneity by amylose-affinity chromatography (NEB). Protein concentration was determined by absorbance spectroscopy using predicted extinction coefficients at $280\,nm = 78\,660\,M^{-1}\,cm^{-1}$. The fusion protein was used in all binding assays using buffer conditions of 20 mM Tris pH 8, 25 mM NaCl, and 1 mM DTT at 10°C. To perform fluorescence anisotropy, 100 nM of fluorescein-labeled peptide was used and titration binding curves were measured and analyzed as previously described (Jacobs *et al*, 2004). ITC was carried out using similar buffer condition by 10-μl injections of a 400 μM H3 peptide (doubly methylated at K9 and K27) solution into 15 μM fusion protein sample. The ITC experiment was performed and analyzed as previously described (Jacobs *et al*, 2004).

Chromatin immunoprecipitation assay

Leaves of 3- to 4-week-old seedlings were harvested, crosslinked, and DNA was precipitated with methyl H3K9- and K27-specific antibodies, following procedures previously described (Johnson *et al*, 2002).

Antibodies

In the immunohistochemical analysis and ChIP assays in Figures 1, 3, and 4 and Supplementary Figure S1, we used the antibodies described by Perez-Burgos *et al* (2004) for detection of mono-, di-, and trimethyl H3K9 and 27, with the following exceptions: (1) in Figure 1E, we used antibodies from Upstate Biotechnology (07-212) recognizing dimethyl H3K9; (2) in Supplementary Figure S1, we used Upstate antibodies to recognize trimethyl H3K27 (catalog number 07-449).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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References

Aufsatz W, Mette MF, van der Winden J, Matzke AJ, Matzke M (2002) RNA-directed DNA methylation in *Arabidopsis*. *Proc Natl Acad Sci USA* **99:** 16499–16506

Bannister AJ, Zegerman P, Partridge JF, Miska EA, Thomas JO, Allshire RC, Kouzarides T (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410:** 120–124

Bartee L, Malagnac F, Bender J (2001) *Arabidopsis* cmt3 chromomethylase mutations block non-CG methylation and silencing of an endogenous gene. *Genes Dev* **15:** 1753–1758

Bastow R, Mylne JS, Lister C, Lippman Z, Martienssen RA, Dean C (2004) Vernalization requires epigenetic silencing of FLC by histone methylation. *Nature* **427:** 164–167

Callebaut I, Courvalin JC, Mornon JP (1999) The BAH (bromo-adjacent homology) domain: a link between DNA methylation, replication and transcriptional regulation. *FEBS Lett* **446:** 189–193

Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, Jones RS, Zhang Y (2002) Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* **298:** 1039–1043

Cao R, Zhang Y (2004) The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. *Curr Opin Genet Dev* **14:** 155–164

Cao X, Jacobsen SE (2002) Role of the *Arabidopsis* DRM methyltransferases in *de novo* DNA methylation and gene silencing. *Curr Biol* **12:** 1138–1144

Chan SW, Zilberman D, Xie Z, Johansen LK, Carrington JC, Jacobsen SE (2004) RNA silencing genes control *de novo* DNA methylation. *Science* **303:** 1336

Chanvivattana Y, Bishopp A, Schubert D, Stock C, Moon YH, Sung ZR, Goodrich J (2004) Interaction of Polycomb-group proteins controlling flowering in *Arabidopsis*. *Development*, (in press)

Czermin B, Melfi R, McCabe D, Seitz V, Imhof A, Pirrotta V (2002) *Drosophila* enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. *Cell* **111:** 185–196

Dutnall RN (2003) Cracking the histone code: one, two, three methyls, you're out!. *Mol Cell* **12:** 3–4

Elgin SC, Grewal SI (2003) Heterochromatin: silence is golden. *Curr Biol* **13:** R895–R898

Fischle W, Wang Y, Allis CD (2003a) Histone and chromatin cross-talk. *Curr Opin Cell Biol* **15:** 172–183

Fischle W, Wang Y, Jacobs SA, Kim Y, Allis CD, Khorasanizadeh S (2003b) Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. *Genes Dev* **17:** 1870–1881

Freitag M, Hickey PC, Khlafallah TK, Read ND, Selker EU (2004) HP1 is essential for DNA methylation in neurospora. *Mol Cell* **13:** 427–434

Gaudin V, Libault M, Pouteau S, Juul T, Zhao G, Lefebvre D, Grandjean O (2001) Mutations in LIKE HETEROCHROMATIN PROTEIN 1 affect flowering time and plant architecture in *Arabidopsis*. *Development* **128:** 4847–4858

Goodrich J, Puangsomlee P, Martin M, Long D, Meyerowitz EM, Coupland G (1997) A Polycomb-group gene regulates homeotic gene expression in *Arabidopsis*. *Nature* **386:** 44–51

Grewal SIS, Elgin SCR (2002) Heterochromatin: new possibilities for the inheritance of structure. *Curr Opin Genet Dev* **12:** 178–187

Hall IM, Shankaranarayana GD, Noma K, Ayoub N, Cohen A, Grewal SI (2002) Establishment and maintenance of a heterochromatin domain. *Science* **297:** 2232–2237

Henikoff S, Comai L (1998) A DNA methyltransferase homolog with a chromodomain exists in multiple polymorphic forms in *Arabidopsis*. *Genetics* **149:** 307–318

Hsieh TF, Hakim O, Ohad N, Fischer RL (2003) From flour to flower: how Polycomb group proteins influence multiple aspects of plant development. *Trends Plant Sci* **8:** 439–445

Jackson JP, Johnson L, Jasencakova Z, Zhang X, PerezBurgos L, Singh PB, Cheng X, Schubert I, Jenuwein T, Jacobsen SE (2004) Dimethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in *Arabidopsis thaliana*. *Chromosoma* **112:** 308–315

Jackson JP, Lindroth AM, Cao X, Jacobsen SE (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Nature* **416:** 556–560

Jacobs SA, Fischle W, Khorasanizadeh S (2004) Assays for the determination of structure and dynamics of the interaction of the chromodomain with histone peptides. *Methods Enzymol* **376:** 131–148

Jacobs SA, Khorasanizadeh S (2002) Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail. *Science* **295:** 2080–2083

Jacobs SA, Taverna SD, Zhang Y, Briggs SD, Li J, Eissenberg JC, Allis CD, Khorasanizadeh S (2001) Specificity of the HP1 chromo domain for the methylated N-terminus of histone H3. *EMBO J* **20:** 5232–5241

Jasencakova Z, Soppe WJ, Meister A, Gernand D, Turner BM, Schubert I (2003) Histone modifications in *Arabidopsis*—high methylation of H3 lysine 9 is dispensable for constitutive heterochromatin. *Plant J* **33:** 471–480

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

Jones L, Ratcliff F, Baulcombe DC (2001) RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. *Curr Biol* **11:** 747–757

Kinoshita T, Harada JJ, Goldberg RB, Fischer RL (2001) Polycomb repression of flowering during early plant development. *Proc Natl Acad Sci USA* **98:** 14156–14161

Kinoshita T, Yadegari R, Harada JJ, Goldberg RB, Fischer RL (1999) Imprinting of the MEDEA polycomb gene in the *Arabidopsis* endosperm. *Plant Cell* **11:** 1945–1952

Kotake T, Takada S, Nakahigashi K, Ohto M, Goto K (2003) *Arabidopsis* TERMINAL FLOWER 2 gene encodes a heterochromatin protein 1 homolog and represses both FLOWERING LOCUS T to regulate flowering time and several floral homeotic genes. *Plant Cell Physiol* **44:** 555–564

Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D (2002) Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes Dev* **16:** 2893–2905

Lachner M, O'Carroll D, Rea S, Mechtler K, Jenuwein T (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410:** 116–120

Larsson AS, Landberg K, Meeks-Wagner DR (1998) The TERMINAL FLOWER2 (TFL2) gene controls the reproductive transition and meristem identity in *Arabidopsis thaliana*. *Genetics* **149:** 597–605

Lehnertz B, Ueda Y, Derijck AA, Braunschweig U, Perez-Burgos L, Kubicek S, Chen T, Li E, Jenuwein T, Peters AH (2003) Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. *Curr Biol* **13:** 1192–1200

Lindroth AM, Cao X, Jackson JP, Zilberman D, McCallum CM, Henikoff S, Jacobsen SE (2001) Requirement of *CHROMOMETHYLASE3* for maintenance of CpXpG methylation. *Science* **292:** 2077–2080

Malagnac F, Bartee L, Bender J (2002) An *Arabidopsis* SET domain protein required for maintenance but not establishment of DNA methylation. *EMBO J* **21:** 6842–6852

Melquist S, Bender J (2003) Transcription from an upstream promoter controls methylation signaling from an inverted repeat of endogenous genes in *Arabidopsis*. *Genes Dev* **17:** 2036–2047

Min J, Zhang Y, Xu RM (2003) Structural basis for specific binding of Polycomb chromodomain to histone H3 methylated at Lys 27. *Genes Dev* **17:** 1823–1828

Moon YH, Chen L, Pan RL, Chang HS, Zhu T, Maffeo DM, Sung ZR (2003) EMF genes maintain vegetative development by repressing the flower program in *Arabidopsis*. *Plant Cell* **15:** 681–693

Muller J, Hart CM, Francis NJ, Vargas ML, Sengupta A, Wild B, Miller EL, O'Connor MB, Kingston RE, Simon JA (2002) Histone methyltransferase activity of a *Drosophila* Polycomb group repressor complex. *Cell* **111:** 197–208

Nakayama J, Rice JC, Strahl BD, Allis CD, Grewal SI (2001) Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* **292:** 110–113

Nielsen PR, Nietlispach D, Mott HR, Callaghan J, Bannister A, Kouzarides T, Murzin AG, Murzina NV, Laue ED (2002) Structure of the HP1 chromodomain bound to histone H3 methylated at lysine 9. *Nature* **416:** 103–107

Okamoto I, Otte AP, Allis CD, Reinberg D, Heard E (2004) Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science* **303:** 644–649

Papa CM, Springer NM, Muszynski MG, Meeley R, Kaeppler SM (2001) Maize chromomethylase Zea methyltransferase2 is required for CpNpG methylation. *Plant Cell* **13:** 1919–1928

Perez-Burgos L, Peters AH, Opravil S, Kauer M, Mechtler K, Jenuwein T (2004) Generation and characterization of methyl-lysine histone antibodies. *Methods Enzymol* **376:** 234–254

Peters AH, Kubicek S, Mechtler K, O'Sullivan RJ, Derijck AA, Perez-Burgos L, Kohlmaier A, Opravil S, Tachibana M, Shinkai Y, Martens JH, Jenuwein T (2003) Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. *Mol Cell* **12:** 1577–1589

Plath K, Fang J, Mlynarczyk-Evans SK, Cao R, Worringer KA, Wang H, de la Cruz CC, Otte AP, Panning B, Zhang Y (2003) Role of histone H3 lysine 27 methylation in X inactivation. *Science* **300:** 131–135

Reyes JC, Grossniklaus U (2003) Diverse functions of Polycomb group proteins during plant development. *Semin Cell Dev Biol* **14:** 77–84

Rice JC, Briggs SD, Ueberheide B, Barber CM, Shabanowitz J, Hunt DF, Shinkai Y, Allis CD (2003) Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. *Mol Cell* **12:** 1591–1598

Rougeulle C, Chaumeil J, Sarma K, Allis CD, Reinberg D, Avner P, Heard E (2004) Differential histone H3 Lys-9 and Lys-27 methylation profiles on the X chromosome. *Mol Cell Biol* **24:** 5475–5484

Schotta G, Ebert A, Krauss V, Fischer A, Hoffmann J, Rea S, Jenuwein T, Dorn R, Reuter G (2002) Central role of *Drosophila* SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. *EMBO J* **21:** 1121–1131

Soppe WJ, Jacobsen SE, Alonso-Blanco C, Jackson JP, Kakutani T, Koornneef M, Peeters AJ (2000) The late flowering phenotype of fwa mutants is caused by gain-of- function epigenetic alleles of a homeodomain gene. *Mol Cell* **6:** 791–802

Strahl BD, Allis CD (2000) The language of covalent histone modifications. *Nature* **403:** 41–45

Sung S, Amasino RM (2004) Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. *Nature* **427:** 159–164

Tachibana M, Sugimoto K, Fukushima T, Shinkai Y (2001) Set domain-containing protein, G9a, is a novel lysine-preferring mammalian histone methyltransferase with hyperactivity and specific selectivity to lysines 9 and 27 of histone H3. *J Biol Chem* **276:** 25309–25317

Tachibana M, Sugimoto K, Nozaki M, Ueda J, Ohta T, Ohki M, Fukuda M, Takeda N, Niida H, Kato H, Shinkai Y (2002) G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. *Genes Dev* **16:** 1779–1791

Takada S, Goto K (2003) Terminal flower2, an *Arabidopsis* homolog of heterochromatin protein1, counteracts the activation of flowering locus T by constans in the vascular tissues of leaves to regulate flowering time. *Plant Cell* **15:** 2856–2865

Tamaru H, Selker EU (2001) A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. *Nature* **414:** 277–283

Tamaru H, Zhang X, McMillen D, Singh PB, Nakayama J, Grewal SI, Allis CD, Cheng X, Selker EU (2003) Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in *Neurospora crassa*. *Nat Genet* **34:** 75–79

Tariq M, Paszkowski J (2004) DNA and histone methylation in plants. *Trends Genet* **20:** 244–251

Thiru A, Nietlispach D, Mott HR, Okuwaki M, Lyon D, Nielsen PR, Hirshberg M, Verreault A, Murzina NV, Laue ED (2004) Structural basis of HP1/PXVXL motif peptide interactions and HP1 localisation to heterochromatin. *EMBO J* **23:** 489–499

Turner BM (2000) Histone acetylation and an epigenetic code. *BioEssays* **22:** 836–845

Yoshida N, Yanai Y, Chen L, Kato Y, Hiratsuka J, Miwa T, Sung ZR, Takahashi S (2001) EMBRYONIC FLOWER2, a novel polycomb group protein homolog, mediates shoot development and flowering in *Arabidopsis*. *Plant Cell* **13:** 2471–2481

Zilberman D, Cao X, Jacobsen SE (2003) ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. *Science* **299:** 716–719