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 heterochromatin PcG 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.

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, m2, and m3), and recent evidence suggests that there may be functional differences between these methylation states (Dutnall, 2003). For instance, in mammalian cells, H3K9m3 is preferentially localized to pericentromeric heterochromatin, while H3K9m and H3K9m2 are localized to euchromatin (Peters et al, 2003; Rice et al, 2003). In Neurospora, silent loci are associated with H3K9m3 (Tamaru et al, 2003). However, in Arabidopsis, both H3K9m and H3K9m2 are found at silent loci, with H3K9m3 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 H3K27m2 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).
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 PAT 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 tf2 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
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 ($K_\text{D} = 0.9 \pm 0.1 \mu M$) (Figure 2C). This mode of binding suggests that the CMT3 chromodomain may form...
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: $K_D = 9.1 \pm 1 \mu M$, $\Delta H = -8.8 \text{ kcal/mol}$, $\Delta G = -6.5 \text{ kcal/mol}$, and $T \Delta S = -2.3 \text{ kcal/mol}$. 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 localized strongly to euchromatin and to a lesser extent to euchromatin. Trimethyl staining was observed in small speckles, not coinciding with DAPI-stained chromocenters. While the level of H3K9m2 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 methylation 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...
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). 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 H3K27m2, 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 H3K27m2 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 chromatocenters (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 H3K27m 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 methyl-ammonium recognition cage in the structures of the HP1 and Pc chromodomains in complex with their target peptides.
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 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 sun-3 double mutant and the fie TK114 mutant. These mutants do not reduce heterochromatic H3K27 methylation, but do reduce euchromatic H3K27m2. Whereas H3K27m3 was evenly distributed in wild-type nuclei, in a fraction of clf-50 sun-3 and fie TK114 nuclei, signal was concentrated in chromocenters. The percent of nuclei showing three different staining patterns with the H3K27m3 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 H3K27m3 signals concentrated at chromocenters (examples shown in the lower row of photographs). Type III is an intermediate pattern where H3K27m3 was concentrated at only those chromocenters associated 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 sun-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 Ler background, and the clf-50 sun-3 and fie TK114 mutants are in the Col background.
important for methyllsine 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 H3K72 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 chromodomain shadow domain. We found that the *lhp1-4* mutation is a complete null, since it represents a 9.9 kb deletion including the entire LHP1 gene. Flanking sequences of the deleted gene can be PCR amplified with the primers JPI1004 forw. 5′-TCCAAAACAGGAATCTGAGC-3′ and JP1717 rev. 5′-TGGTCTTGGACATTGCGG-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 dark 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* TK114; 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 *Ta5* 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 *CfoI* and *BglII* were used to cut the DNA, respectively. For all the other loci tested, the DNA was cut with *HpaII* (sensitive to CG and CNG methylation) and *MspI* (sensitive to CNG methylation).
RT-PCR
First-strand cDNA synthesis was performed on 3 μg total RNA from the inforesences 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 of cDNA added was adjusted for comparison between the different samples. The primers for ACTIN were (JP771) 5’TATA GCT GCA TTG TCA CCC GA and (JP1564) 5’GGT GTG 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’. 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 sun-3 double mutant) and histone immunolabeling were performed as previously described (Jasencakova et al., 2003). Slides were evaluated using a Zeiss Axioskop 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 to the binding assays
Synthetic peptides corresponding to the trimethyl H3K9 and trimethyl H3K27 regions include residues 1–32, 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 pMAL-c2 vector (New England Biolabs). Point mutation of the chromodomain was performed 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 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
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