Dependence of Heterochromatic Histone H3 Methylation Patterns on the *Arabidopsis* Gene *DDM1*

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The *Arabidopsis* gene *DDM1* is required to maintain DNA methylation levels and is responsible for transposon and transgene silencing. However, rather than encoding a DNA methyltransferase, *DDM1* has similarity to the SWI/SNF family of adenosine triphosphate–dependent chromatin remodeling genes, suggesting an indirect role in DNA methylation. Here we show that *DDM1* is also required to maintain histone H3 methylation patterns. In wild-type heterochromatin, transposons and silent genes are associated with histone H3 methylated at lysine 9, whereas known genes are preferentially associated with methylated lysine 4. In *ddm1* heterochromatin, DNA methylation is lost, and methylation of lysine 9 is largely replaced by methylation of lysine 4. Because DNA methylation has recently been shown to depend on histone H3 lysine 9 methylation, our results suggest that transposon methylation may be guided by histone H3 methylation in plant genomes. This would account for the epigenetic inheritance of hypomethylated DNA once histone H3 methylation patterns are altered.

SWI/SNF chromatin-remodeling complexes regulate gene expression by disrupting histone-DNA interactions and permit a variety of proteins to access DNA (*1*–*3*). The adenosine triphosphatase subunits of these complexes fall into at least five highly conserved subfamilies. Three subfamilies represented by yeast SWI2/SNF2, *Drosophila* ISWI, and mouse CHD1 have been implicated in both transcriptional activation and repression (*1*–*3*). DDM1 (*Arabidopsis*), Lsh (mouse), and YFR038W (yeast) represent a fourth subfamily that lacks a COOH-terminal SANT (SWI3, ADA2, N-CoR, TFIIIB) domain (found in ISWI) and AT hook domains (AT-rich DNA binding domains found in SWI2/SNF2) (*4*). Like *ddm1*, mutants in *Lsh* and patients with lesions in *ATRX* have altered DNA methylation patterns. However, human *ATRX* is a member of a fifth subfamily primarily involved in recombination and repair (*5*, *6*).

DDM1 is required to maintain gene and transposon silencing, as well as the associated DNA methylation (*7*). However, the mechanism by which DNA methylation is lost from *ddm1* is unknown (*8*, *9*). Hypomethylated DNA and associated epimutations (*10*) are inherited epigenetically in backcrosses, indicating that this loss is irreversible (*8*). It has been proposed that DDM1 allows DNA methyltransferases to gain access to condensed chromatin (*11*), either directly or by altering chromatin architecture (*4*). Methylation of lysine 4 and lysine 9 of histone H3 is typically associated with transcriptionally active and inactive chromatin, respectively (*12*). Given that *DDM1* potentially encodes a nucleosomal remodeling enzyme, and that it is involved in heterochromatin formation, we set out to test whether heterochromatic methylation of histone H3 is affected in *ddm1*.

We used antibodies raised against dimethyl histone H3 lysine 9 (H3mK9) and dimethyl histone H3 lysine 4 (H3mK4) peptides to show that *Arabidopsis* histone H3 has both modifications and that overall levels do not change appreciably in *ddm1-2* (Fig. 1). Next, we analyzed the pattern of histone H3 methylation in heterochromatic DNA from the knob of chromosome IV (*13*), which is methylated and transcriptionally quiescent (*9*). Chromatin immunoprecipitation (ChIP) (*14*) was carried out on 9-day-old wild-type (WT) and F4 inbred *ddm1-2* seedlings, using the antibodies described above and primer pairs from genes, upstream regions, and transposons from the heterochromatic knob (*14*). For each sequence, we scored histone association as either strong, absent, or weak, depending on whether specific products were amplified in two independent ChIP experiments, as well as mock precipitate and total extract controls. Among the 82 primer pairs, 12 were nonspecific, 11 failed to yield a product from the total extract, and 7 gave irreconcilable results between the two experiments (*14*). Our analysis is based on the remaining 52 primer pairs

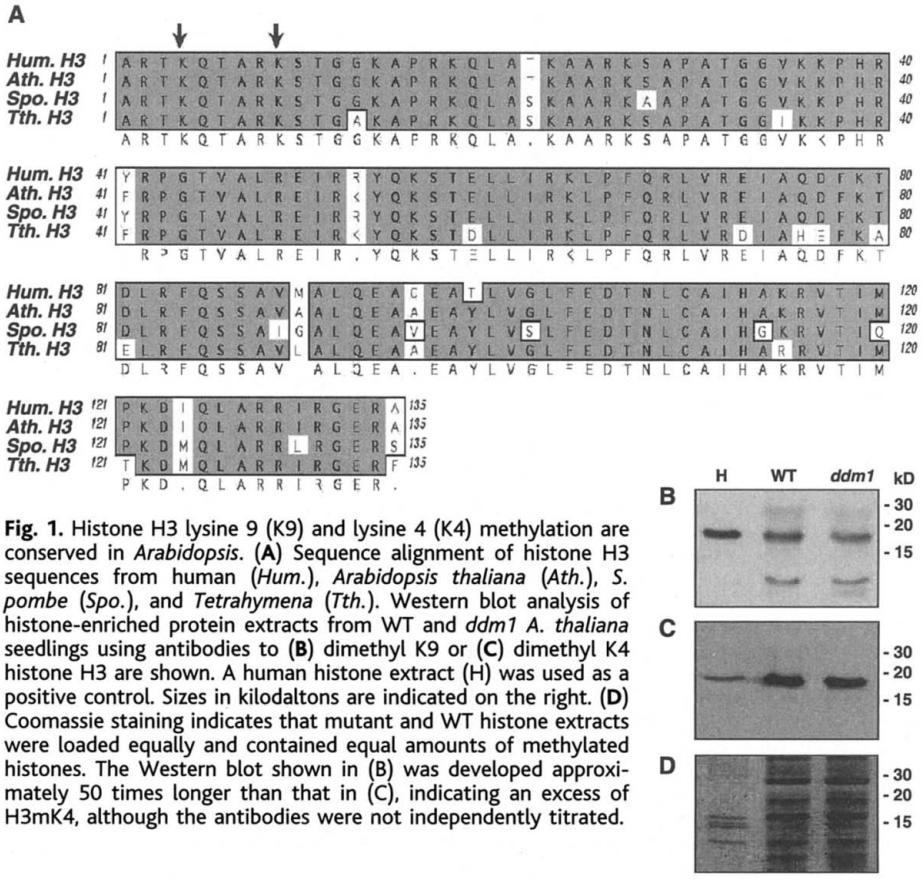

Fig. 1. Histone H3 lysine 9 (K9) and lysine 4 (K4) methylation are conserved in *Arabidopsis*. (**A**) Sequence alignment of histone H3 sequences from human (*Hum.*), *Arabidopsis thaliana* (*Ath.*), *S. pombe* (*Spo.*), and *Tetrahymena* (*Tth.*). Western blot analysis of histone-enriched protein extracts from WT and *ddm1 A. thaliana* seedlings using antibodies to (**B**) dimethyl K9 or (**C**) dimethyl K4 histone H3 are shown. A human histone extract (H) was used as a positive control. Sizes in kilodaltons are indicated on the right. (**D**) Coomassie staining indicates that mutant and WT histone extracts were loaded equally and contained equal amounts of methylated histones. The Western blot shown in (B) was developed approximately 50 times longer than that in (C), indicating an excess of H3mK4, although the antibodies were not independently titrated.

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(*15*), and representative gel patterns for each class are shown in Fig. 2.

In WT plants, all 15 transposons were associated with H3mK9, whereas four out of six known genes were associated with H3mK4. The methylation patterns of most hypothetical genes resembled transposons, which is consistent with their repetition in the *Arabidopsis* genome (*15*). In *ddm1*, a dramatic shift in the pattern of histone methylation was observed (Fig. 3). Thirty-two of the 41 sequences whose association with methylated histones could be detected either lost H3mK9 or gained H3mK4, or both. These included both known and hypothetical genes, transposons, and upstream regions, indicating that heterochromatin underwent a major restructuring in *ddm1* (*15*). Histone methylation patterns associated with the remaining nine sequences did not change appreciably, retaining H3mK9 or H3mK4 or both.

We also determined whether changes in histone H3 methylation correlated with changes in gene expression (Table 1). Reverse transcriptase polymerase chain reactions (RT-PCRs) were performed, using total RNA extracted from WT and *ddm1* seedlings (for examples, see Fig. 2). Up-regulated sequences included *Athila*, *Cinful*, *del-like*, and novel retrotransposons, as well as *MULE* and *CACTA* class II cut-and-paste transposable elements (*15*). In addition, one silent gene (encoding a phosphate translocator), and three other genes were up-regulated in *ddm1*, along with at least 10 hypothetical genes and two of their "upstream" regions (likely unannotated exons).

Tenfold or more overexpression in *ddm1* was observed with 26 sequences, and in 20 cases this was correlated with a decreased association with H3mK9, an increased association with H3mK4, or both. On the other hand, 16 sequences were expressed at similar levels in the wild type and mutant, whether or not they changed their histone methylation pattern. The remaining 10 sequences were not expressed in WT or *ddm1* seedlings and may be pseudogenes. No methylated histone association could be detected for 11 sequences, 9 of which were transcribed and 3 of which were up-regulated in *ddm1* (Table 1). Thus, although there was a clear correlation, histone methylation did not always change detectably in up-regulated sequences.

Overall levels of H3mK9 in the genome did not change appreciably in *ddm1* (Fig. 1). Rather, heterochromatin reverted to H3mK4, perhaps because H3mK9 is redistributed throughout the genome. Thus, either DDM1 actively eliminates H3mK4 from heterochromatin or it maintains a high concentration of heterochromatic H3mK9, presumably via displacement of modified nucleosomes during replication.

Most DNA methylation in plants and fungi is confined to transposons and repeats (*16*), and here we show that transposons are also associated with high levels of H3mK9 (Fig. 3) (*9*, *17*–*19*). Both types of modification depend on *DDM1*, although DNA methylation of rare sequences that retained H3mK9 in *ddm1* could not be assessed because of high copy number (*15*). DDM1 may act primarily on DNA methylation and only indirectly on histone methylation. DNA methylation is drastically reduced in *ddm1* (*8*), whereas overall levels of H3mK9

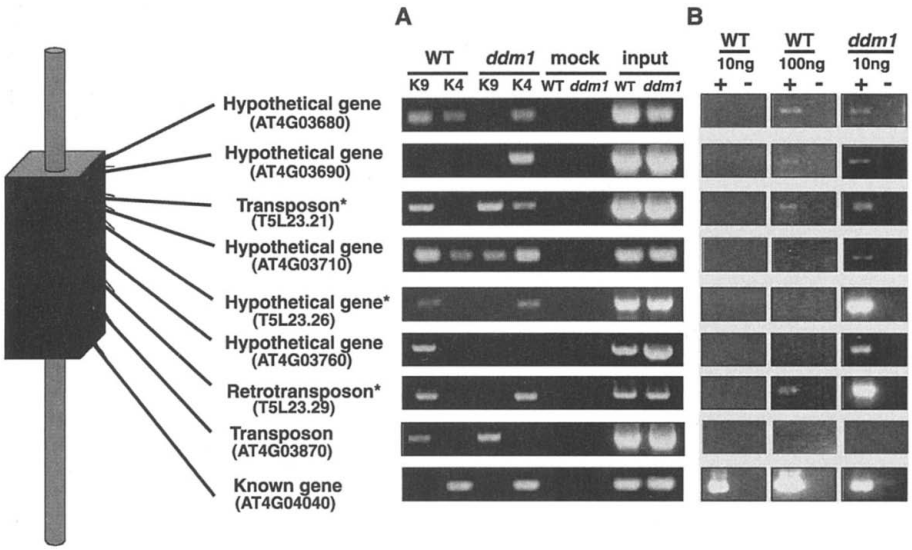

Fig. 2. Chromatin immunoprecipitation and RT-PCR analysis of selected sequences from *Arabidopsis* heterochromatin. Nine individual examples of genes and transposons from each of the classes described in Fig. 3 are shown. In each case, specific primers were used for chromatin immunoprecipitation and RT-PCR. (**A**) DNA was amplified from WT and mutant (*ddm1*) chromatin extracts that had been immunoprecipitated with antibody raised against histone H3 peptides methylated on lysine 9 (K9) and lysine 4 (K4). Total chromatin extract (input) and mock-precipitated extracts (to which no antibody was added) were used as controls. GenBank annotation for each feature is listed where known. Asterisk indicates annotation from (*9*). (**B**) Total RNA from WT and *ddm1* seedlings was amplified using RT-PCR to detect transcripts corresponding to each of the sequences shown in (A). Amplifications in the presence (+) and absence (–) of RT demonstrated that the PCR products were derived from RNA. Dilutions of input RNA (10 ng and 100 ng) were used to determine approximate levels of transcript in each genotype. A complete list of all the sequences assayed can be found in the supporting online material.

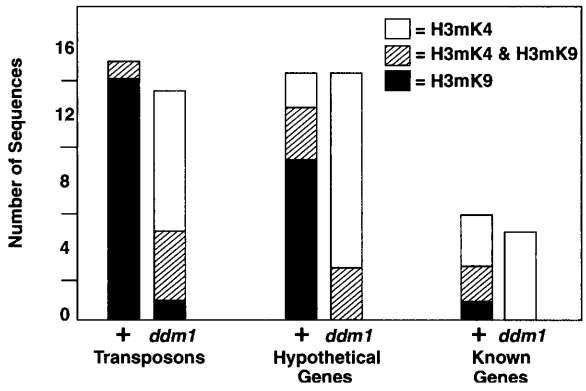

Fig. 3. The association of heterochromatic sequences with methylated histone H3 in WTand *ddm1* mutant *Arabidopsis*. Transposons and known genes were identified by homology. Hypothetical genes were identified by prediction algorithms alone and may include novel transposons. Sequences associated with methylated histone H3 in WT plants were scored according to their amplification from chromatin immunoprecipitation using H3mK9 and H3mK4 antibodies and specific primer pairs (Fig. 2). Five promoter regions up to 1 kb upstream were also amplified and behaved similarly to the adjacent gene (see supporting online data).

Table 1. Changes in gene expression are correlated with changes in histone H3 methylation patterns in *ddm1* mutants. Changes in the association of genes and transposons with methylated histone H3 in *ddm1* mutants were scored as "H3mK9 to H3mK4" if they lost association with H3mK9, gained association with H3mK4, or both (Fig. 3). Expression levels were estimated by RT-PCR (Fig. 2). Changes of 10-fold or greater were scored as an increase in *ddm1*. n.d., not detected.

Histone H3 methylation	Expression		
	Unchanged	Increased	n.d.
H3mK9 to H3mK4	8	20	4
Unchanged	2	3	4
n.d.	6	3	2

are maintained. However, when *ddm1* is back-crossed to the wild type, hypomethylated DNA is epigenetically inherited in subsequent generations (*8*). Further, epimutant alleles at the *SUPERMAN* locus arise at elevated frequencies in *ddm1*, but they are overmethylated rather than undermethylated (*20*).

We can account for these observations if DNA methylation during replication depends on a high concentration of H3mK9 in a given chromosomal domain. According to this scenario, redistribution of methylated histones in *ddm1* would lead to dilution of heterochromatic H3mK9 relative to H3mK4 and to subsequent loss of DNA methylation. When *ddm1* is crossed back to the wild type, de novo DNA methylation of transposons would fail to occur, because histone methylation patterns could not be restored once they were lost (*12*). The aberrant association of H3mK9 with euchromatic sequences in *ddm1* could also lead to sporadic hypermethylation of euchromatic genes such as *SUPERMAN*, and even to retargeting of transposable elements to euchromatic sites if transposons themselves recognize H3mK9 (*21*).

Recently, it has been shown that DNA methylation in *Neurospora* depends on *dim5* (*22*), which encodes a homolog of mouse *Su(var)3-9* and fission yeast *clr4* (*12*). These are K9-specific histone H3 methyltransferases characterized by a specific SET [Su(var)3-9, Enhancer-of-zeste, Trithotrax] domain. *Arabidopsis* has up to 15 genes that potentially encode this class of proteins (*23*), and *KRYPTONITE* (*KYP-1*) resembles *dim5* in that it is also required for DNA methylation, affecting *SUPERMAN* and a number of retrotransposons (*24*). H3mK9-dependent DNA methylation is conferred by the methyltransferase CHROMOMETHYLASE3, which binds H3mK9 indirectly via an HP1-like protein. However, overall DNA methylation losses are modest in *kyp-1* relative to *ddm1*, presumably because of gene redundancy.

Together these results provide a mechanistic basis for the loss of DNA methylation in *ddm1*, as a consequence of the reduced association of heterochromatin with H3mK9. However, DNA methylation could also reinforce histone methylation patterns via chromatin-remodeling complexes that bind methylated DNA (*25*). The pattern of histone tail modification at specific amino acid residues has been proposed to represent a "histone code," established by modifying enzymes and interpreted by nucleosome-binding proteins (*12*). According to this model, DDM1 and other chromatin remodeling enzymes would "typeset" the code to ensure accurate compartmentalization of modified nucleosomes after replication. It remains to be seen whether DDM1 and other SWI/SNF subfamilies interact specifically with methylated and otherwise modified nucleosomes (*26*).

In an accompanying paper, Volpe *et al.* (*27*) show that H3mK9 can be targeted to heterochromatic repeats in the fission yeast *Schizosaccharomyces pombe* by RNA interference (RNAi). In *Caenorhabditis elegans* and *Drosophila*, which lack DNA methylation, transposons and repeats are also a target of RNAi (*28*, *29*). In organisms that have DNA methylation, H3mK9 is both interpreted by DNA methyltransferases (*22*, *24*) and reinforced by histone deacetylase complexes that bind methylated DNA (*25*). Further, induction of H3mK9 by noncoding RNA has been implied in mouse X-inactivation (*30*). Taken together, these results suggest a model whereby RNAi initiates transposon methylation by imposing H3mK9, which is then maintained through chromatin remodeling by DDM1. These mechanisms may account for the preferential methylation of transposons in plants and other eukaryotes (*21*).

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Supporting Online Material

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Materials and Methods
Table S1
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A Photoactivatable GFP for Selective Photolabeling of Proteins and Cells

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We report a photoactivatable variant of the *Aequorea victoria* green fluorescent protein (GFP) that, after intense irradiation with 413-nanometer light, increases fluorescence 100 times when excited by 488-nanometer light and remains stable for days under aerobic conditions. These characteristics offer a new tool for exploring intracellular protein dynamics by tracking photoactivated molecules that are the only visible GFPs in the cell. Here, we use the photoactivatable GFP both as a free protein to measure protein diffusion across the nuclear envelope and as a chimera with a lysosomal membrane protein to demonstrate rapid interlysosomal membrane exchange.

Photoactivation, the rapid conversion of photoactivatable molecules to a fluorescent state by intense irradiation, can be used to mark and monitor selected molecules within cells (*1*). Previous efforts to develop a photoactivatable protein capable of high optical contrast when photoactivated under physiological conditions have had limited success (*2*–*5*). GFP's inherent brightness and suitability as a fusion protein in living cells (*6*, *7*) have prompted us to try to develop a variant that would allow selective marking of proteins through photoactivation.

Our efforts began with a codon-opti-