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Involvement of a Jumonji-C domain-containing histone demethylase in DRM2-mediated maintenance of DNA methylation

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INTRODUCTION

Cytosine DNA methylation is an epigenetic modification that is conserved in all kingdoms of eukaryotes and is largely associated with heterochromatic regions undergoing transcriptional gene silencing. In the model plant Arabidopsis thaliana, at least three methylation pathways exist and each is associated with a specific methyltransferase. Methyltransferase 1 (MET1) is a homologue of mammalian DNA methyltransferase 1 (DNMT1) and maintains methylation in the CG dinucleotide context. Chromomethylase 3 (CMT3) is a plant-specific methyltransferase that preferentially deposits the methyl mark in CHG contexts (where H is adenine, thymine or cytosine). Finally, the mammalian DNMT3 homologue DRM2 (domains rearranged methyltransferase 2) performs de novo DNA methylation, and maintains CHH or asymmetrical methylation through a small interfering RNA (siRNA)-driven signal in a process known as RNA-directed DNA methylation (RdDM; Law & Jacobsen, 2010). At some loci, CMT3 and DRM2 act redundantly to control the maintenance of both CHG and CHH methylation, but DRM2 alone is responsible for de novo DNA methylation (Cao & Jacobsen, 2002a; Chan et al, 2004).

Methylation patterns are correlated with specific histone modification signatures. For example, genome-wide studies in Arabidopsis have shown that histone 3 Lys 9 dimethylation (H3K9m2) is a histone mark that often occurs with CHG methylation and endogenous clusters of siRNAs (Bernatavichute et al, 2008). H3K9m2 directed by the Kryptonite (KYP), SU (VAR) 3–9 homologue (SUVH) 5 and 6 histone methyltransferases is required for the maintenance of CHG DNA methylation (Jackson et al, 2002; Malagnac et al, 2002; Ebbs & Bender, 2006), probably through direct targeting of CMT3 (Lindroth et al, 2004). Conversely, histone 3 Lys 4 mono/ditrimethylation (H3K4m1/2/3) is strongly negatively correlated with DNA methylation at nongenic silent loci (Zhang et al, 2009).
The discovery in mammals of two classes of enzyme that are able to demethylate histones—lysin-specific demethylase 1 (LSD1; Shi et al., 2004) and Jumonji-C (JmC) domain-containing proteins (Klose et al., 2006)—revealed that active removal of methyl marks from histones is necessary for proper epigenetic regulation. Two plant homologues of the mammalian histone demethylase LSD1—LSD1-LIKE 1 (LDDL1) and 2 (LDDL2)—are required for H3K4 demethylation at the FLC and FWA loci (Jiang et al., 2007). Although FLC is not a DNA-methylated gene, FWA transcription is controlled by DNA methylation at the tandem repeats in its 5′-untranslated region (5′-UTR), and FWA hypomethylation results in ectopic expression and a late-flowering phenotype (Soppe et al., 2000). Interestingly, ldl1 ldl2 double mutants flower late, and molecular analysis showed hypomethylation at FWA. These data suggest that persistent H3K4 demethylation is required to maintain DNA methylation at some loci in the genome. To gain further insight into the relationship between active histone demethylation and DNA methylation at silent loci, we compiled a collection of homozygous transfer DNA insertion mutants in genes containing JmC domains in Arabidopsis. We show that JMJ14 is required to maintain full levels of non-CG methylation at sites controlled by DRM2. We also found that the loss of non-CG methylation in jmj14 mutants corresponded with increases in H3K4m3 marks, suggesting that JMJ14 targets DNA-methylated loci. Interestingly, jmj14 mutants had no effect on DRM2-mediated establishment of methylation of an incoming FWA transgene, which is in contrast to all other mutants that were tested in the DRM2 pathway (Chan et al., 2004; Johnson et al., 2008; Ausin et al., 2009; Law & Jacobsen, 2010). These results suggest that establishment and maintenance of methylation mediated by DRM2 can be differentially regulated, and that JMJ14 has a specific role in the maintenance of RdDM.

**RESULTS**

**jmj14 mutations affect non-CG maintenance methylation**

Arabidopsis contains 21 genes with domains homologous to JmC histone demethylases (Lu et al., 2008; Hong et al., 2009). To examine potential effects on DNA methylation, we analysed 17 JmC mutants for which null alleles were available, at the medea-intergenic subtelomeric repeat (MEA-ISR) locus by using Southern blotting (supplementary Table S1 online). The MEA-ISR is a set of seven tandem repeats downstream from the medea (MEA) gene. Both MET1 (CG methylation) and DRM2 (CHG and CHH methylations) maintain DNA methylation at MEA-ISR, and hypomethylation phenotypes can be observed after digestion with the methylation-sensitive enzyme MspI (Cao & Jacobsen, 2002a). By Southern blot analysis, we were able to observe a consistent reduction of MEA-ISR methylation in two null alleles of jmj14 (Fig 1A). JM14—also referred to as JM4 and putative lysine demethylase 7B (PKDM7B)—is the protein encoded by At4g20400 (Lu et al., 2008). To confirm the jmj14 methylation defect, we performed bisulphite sequencing at the MEA-ISR locus (Fig 1B). Data from this analysis showed a reduction in non-CG methylation, but CG methylation was unchanged compared with the wild-type control. This indicates that the jmj14 mutation interacts with the DRM2 pathway, but not the MET1 pathway.

To confirm the genetic interaction of JMJ14 with the DRM2 pathway, we examined the effect of the mutation on other RdDM targets. Analysis of the methylation state of the 5′UTR of FWA was performed by using bisulphite sequencing. FWA, similarly to MEA-ISR, is mainly targeted by MET1 and DRM2 (Cao & Jacobsen, 2002a). Similarly to the bisulphite data at MEA-ISR, we observed a reduction in non-CG methylation but no effect at CG sites at FWA (Fig 1C). Finally, to examine DRM2-dependent methylation at the transposable element AtSN1, DNA from both wild type and jmj14 mutants was digested with the restriction endonuclease HaeII that cleaves GGCC sequences, but not GgMCC. Digested DNA was analysed by real-time quantitative PCR using primers that amplify a region spanning three asymmetrically methylated restriction sites (Fig 1D). Relative quantification of uncut DNA in the digested samples showed a significant decrease in CHH methylation in jmj14 mutants compared with wild type, although not to the same extent as in drm2. To examine whether the jmj14 mutant defects were specific to the DRM2 pathway, we also analysed the methylation state of Ta3—a single-copy transposable element that is methylated by CMT3 but not DRM2 (Cao & Jacobsen, 2002a). We observed no effect on methylation in any context for jmj14 compared with the wild-type control (Fig 1E). This indicates that JMJ14 acts primarily in the DRM2 pathway.

**jmj14 affects chromatin at RdDM target loci**

To examine the localization of JMJ14, we created a carboxy-terminal epitope-tagged (9 × Myc) JMJ14 transgene driven by the endogenous JMJ14 promoter and showed that this transgene fully complements the early-flowering phenotype (Jeong et al., 2009) of the jmj14 mutant (Fig 2A,B). Immunostaining for the Myc epitope revealed strong nuclear staining, consistent with the function of JMJ14 as a histone demethylase. Interestingly, we observed a specific pattern in which staining was uniformly present throughout the nucleoplasm but not in the nucleolus and the chromocentres (areas of dense heterochromatin that are highly enriched for H3K9m2; Fig 2C). This pattern is similar to that found for DRM2 (Li et al., 2006), consistent with the hypothesis that JMJ14 acts in the DRM2 pathway.

Phylogenetic analyses have shown that the JMJ14 sequence is closest to human lysine demethylase 5/Jumonji/Arid-domain containing protein 1 family histone demethylases (Lu et al., 2008) that are able to specifically demethylate H3K4m1, H3K4m2 and H3K4m3 (Chrisenten et al., 2007; Iwase et al., 2007; Lee et al., 2007; Seward et al., 2007). A recombinant JMJ14 was shown to efficiently demethylate H3K4m3 in vitro and to a lesser extent H3K4m2 and H3K4m1 (Jeong et al., 2009; Lu et al., 2010; Yang et al., 2010). This H3K4 demethylase activity was confirmed by an in vivo assay in Nicotiana benthamiana in which overexpression of JMJ14 correlated with a strong reduction in H3K4m3 and H3K4m2 marks (Lu et al., 2010). Finally, in Arabidopsis, JMJ14 was shown to demethylate H3K4m3 and H3K4m2 at two loci involved in floral transition and not controlled by DNA methylation (Jeong et al., 2009; Yang et al., 2010).

This suggests that the defect in DNA methylation at non-CG sites was caused by an increase in H3K4m4 in jmj14 mutants. To confirm this hypothesis, we used chromatin immuno-precipitation (ChIP) analysis to assess the levels of H3K4m2 and H3K4m3 at silent loci analysed for DNA methylation in wild type and jmj14. We observed a consistent increase in H3K4m3 marks at AtSN1, FWA and MEA-ISR (Fig 3). The extent of this increase was similar to that which has been found in jmj14 mutants at the floral transition loci flowering locus T (FT) and twin sister of FT.
**jmj14 does not affect de novo DNA methylation**

All components of the RdDM machinery that have been tested thus far have been shown to be required both for DRM2-dependent non-CG maintenance DNA methylation at MEA-ISR and other loci, and for establishment of methylation in all sequence contexts on previously unmethylated sequences—or de novo methylation—of an incoming transgene (Chan et al., 2004; Johnson et al., 2008; Ausin et al., 2009; Law & Jacobsen, 2010). When FWA is introduced into wild-type plants, siRNAs are able to target the repeats in the 5′UTR and the incoming transgene becomes methylated, and thus silenced. However, in RdDM mutants, the transgene remains unmethylated in all sequence contexts and is expressed (Cao & Jacobsen, 2002b; Chan et al., 2004). As we had observed non-CG maintenance methylation phenotypes at known RdDM targets in jmj14, we used the FWA transgene system to test for a function of JMJ14 in de novo methylation. Ectopic FWA expression leads to a late-flowering phenotype that gives a quantitative readout of the methylation establishment phenotype.

The jmj14 mutant flowers earlier than the wild-type plants, which has previously been shown to be due to de-repression of FT (Fig 4A; Jeong et al., 2009; Lu et al., 2010; Yang et al., 2010). Surprisingly, FWA-transformed jmj14 continued to flower earlier than wild-type control plants (Fig 4A). We note that other mutants with weak RdDM phenotypes—such as dicer-like 3 (dcl3) which shows only partial losses of MEA-ISR methylation (equivalent to those of jmj14)—do show substantial effects on FWA de novo DNA methylation establishment, and thus flower later (Henderson et al., 2006). These results suggest that the jmj14 mutation does not affect FWA de novo DNA methylation.

To confirm these findings, we analysed the methylation state of the newly introduced FWA transgene by using bisulphite sequencing (Fig 4B). We observed in the FWA transgene that CG methylation levels of the jmj14 mutant were comparable with those of wild type; however, there was a significant decrease in non-CG methylation. By contrast, the dcl3 mutant shows substantially less de novo methylation than wild type in all three sequence contexts, even though it exhibited a similar non-CG maintenance phenotype (Henderson et al., 2006). These results show that the CG DNA methylation that is primarily responsible for silencing FWA is fully established in jmj14. Once CG methylation is established, it is maintained by the MET1 pathway.

![Fig 1](image-url)
independently of DRM2, whereas DRM2 maintains non-CG marks. Consistent with a function in DRM2-mediated maintenance of non-CG methylation, and similarly to the FWA endogene (Fig 1C), we observed that maintenance of CHG and CHH methylation at the FWA transgene was reduced in the jmj14 mutant (Fig 4B).

**DISCUSSION**

JMJ14 is required for the maintenance of DRM2-mediated non-CG DNA methylation. Consistent with our findings, a recent study described the identification of JMJ14 through a forward-genetic screen for mutants impaired in hairpin-induced transcriptional silencing of the phytoene desaturase endogene (Searle et al., 2010). We observed a moderate but consistent increase in H3K4m3 levels at RdDM targets analysed in jmj14, suggesting that active demethylation of H3K4 is required for proper DRM2-pathway function, perhaps due to competition between the active H3K4 methylation mark and repressive marks such as DNA methylation (Fig 5). The fact that two enzyme families—JmjC domain and LSD-like (Jiang et al., 2007)—have functions in the demethylation process further supports the importance of these enzymes in regulating DNA methylation levels.
of H3K4 methyl marks at silent loci/RdDM targets underlies the importance of removing those marks for the maintenance of proper DNA methylation patterns.

Interestingly, jmj14 mutants showed no effect on DRM2-mediated de novo methylation of an incoming FWA transgene. This is in contrast to all other mutants tested in the DRM2 pathway: nrpd1, nrpe1, dcl3, rdr2, ago4, drd1, suvh2, dms3 and idn2 (Chan et al., 2004; Johnson et al., 2008; Ausin et al., 2009; Law & Jacobsen, 2010). This indicates that JM14 is required to maintain non-CG methylation patterns, but is not involved in the initial targeting of DNA methylation. This is an interesting finding as it implies that the maintenance activity of DRM2 can be mechanistically distinguished from its de novo methylation establishment activity, suggesting that during the maintenance phase there is another level of regulation of DRM2 activity by histones. The relationship between DRM2 activity and H3K4 methylation status is also interesting in the light of activity mechanisms of the mammalian DRM2 homologue DNMT3A. DNMT3A is in part recruited to silent loci through interaction with a related protein (DNMT3L) that can bind to H3 specifically when Lys 4 is unmethylated (Jia et al., 2007; Ooi et al., 2007). Future analyses might determine how H3K4 methyl marks antagonize the DRM2 pathway in Arabidopsis.

**METHODS**

**Plant materials.** We used the following Arabidopsis strains: wild-type Col-0 and the recessive alleles dcl3-1 and drm2-2 in the Col-0 background. The list of alleles of JmjC mutants tested is presented in supplementary Table S1 online.

**Southern blotting and bisulphite analysis.** See the supplementary information online for details.

**HaeIII Chop–qPCR.** DNA from young flowers was extracted using a standard Cetyl trimethyl ammonium bromide protocol. A total of 200 ng of genomic DNA was digested overnight at 37°C with HaeIII side-by-side with samples containing buffer and no enzyme (undigested). Quantitative real-time PCR validation of uncut DNA after HaeIII digestion was performed using the Bio-Rad Synergy Brands Green SuperMix on a MX3000 Stratagene cycler. The PCR parameters are as follows: one cycle of 10 min at 95°C, 40 cycles of 30 s at 95°C, 1 min at 55°C and 1 min at 72°C. PCR primers sequences are listed in supplementary Table S2 online.

**FWA transformation.** See the supplementary information online for details.

**Flowering-time analysis.** We measured flowering time as the total number of leaves (rosette and cauline leaves) developed by a plant.

**Generation of epitope-tagged complementing lines.** Epitope-tagged protein constructs were made by cloning 1.6 kb of genomic DNA upstream from the JM14 open reading frame and including the entire open reading frame into pENTR. A 9 × Myc epitope tag was introduced at the C-terminus. The tagged construct was then recombined into a modified pDEST vector and introduced into Agrobacterium strain AGL1.
Protein immunofluorescence analysis. We prepared nuclei for immunofluorescent imaging as described in Li et al., 2006. See supplementary information online for more details.

ChiP. The ChiP experiments were performed as previously described (Bernatavichute et al., 2008; Johnson et al., 2008; Zhang et al., 2009). See supplementary information online for more details.

Supplementary information is available at EMBO reports online (http://www.emboreports.org).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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