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

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Histone demethylases—both lysine-specific demethylase 1 (LSD1) and Jumonji-C (JmjC) domain-containing proteins—are broadly implicated in the regulation of chromatin-dependent processes. In *Arabidopsis thaliana*, histone marks directly affect DNA methylation, and mutations in LSD1 homologues show reduced DNA methylation at some loci. We screened transfer DNA mutations in genes encoding JmjC domains for defects in DNA methylation. Mutations in *jmj14* result in reduced DNA methylation in non-CG contexts at targets of DRM2 (domains rearranged methyltransferase 2)-mediated RNA-directed DNA methylation (RdDM), which is associated with an increase in H3K4m3. Unlike other components of RdDM, JMJ14 is not required for *de novo* methylation of a transgene, suggesting that JMJ14 is specifically involved in the maintenance phase of DRM2-mediated RdDM.

Keywords: DNA methylation; epigenetics; Jumonji-C; histone demethylase; *Arabidopsis*

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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/di/trimethylation (H3K4m1/2/3) is strongly negatively correlated with DNA methylation at nongenic silent loci (Zhang *et al*, 2009).

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The discovery in mammals of two classes of enzyme that are able to demethylate histones—lysine-specific demethylase 1 (LSD1; Shi *et al*, 2004) and Jumonji-C (JmjC) 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 (LDL1) and 2 (LDL2)—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 JmjC 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 JmjC histone demethylases (Lu *et al*, 2008; Hong *et al*, 2009). To examine potential effects on DNA methylation, we analysed 17 JmjC 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 *Msp*I (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). JMJ14—also referred to as JMJ4 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 *Hae*III 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 (Christensen *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 H3K4 methylation in *jmj14* mutants. To confirm this hypothesis, we used chromatin immunoprecipitation (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*

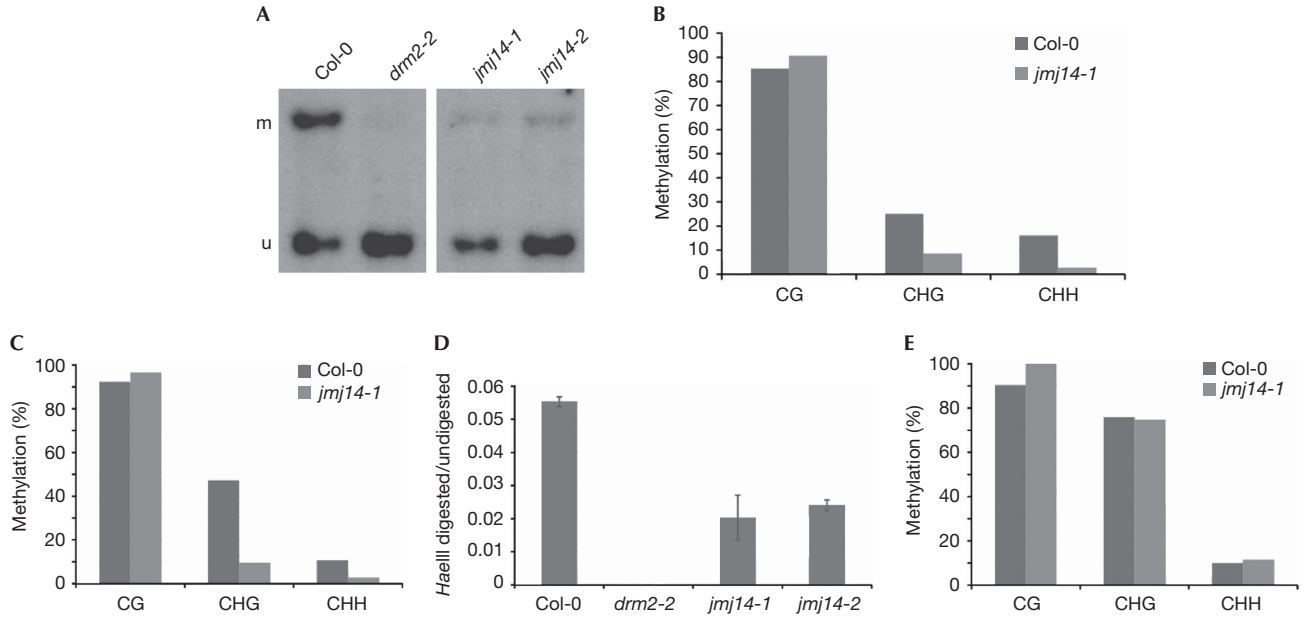

Fig 1 | DNA methylation analysis of *Jumonji 14* mutants. (**A**) *MEA-ISR* Southern blot. Genomic DNA was digested with the non-CG methylation-sensitive restriction endonuclease *Msp*I, and probed for *MEA-ISR*. The high-molecular-weight band (m) represents methylated DNA and the low-molecular-weight band (u) represents unmethylated DNA. Two alleles of *jmj14* show a methylation phenotype intermediate between wild type and the *drm2* mutant. (**B**) *MEA-ISR* bisulphite sequencing. Genomic DNA was treated with sodium bisulphite and amplified with primers specific for *MEA-ISR*. Sequencing shows an effect at non-CG sites compared with wild type, but not in the CG context. (**C**) *FWA* endogene bisulphite sequencing. The *FWA* locus has a similar pattern to *MEA-ISR* in the *jmj14-1* mutant. (**D**) *AtSN1 Hae*III Chop-qPCR. Genomic DNA was digested with non-CG methylation-sensitive restriction endonuclease *Hae*III. Digested DNA was quantified by using real-time qPCR with primers specific for a region of *AtSN1* spanning three restriction sites, and the signal was normalized to an undigested control. Two *jmj14* alleles had significantly more digestion compared with the wild-type control, thus there was less methylation. (**E**) *Ta3* bisulphite sequencing. The methylation state of *Ta3* shows no discernible defect in the *jmj14* mutant compared with wild type. *jmj14*, *Jumonji 14*; *MEA-ISR*, *medea*-intergenic subtelomeric repeats; qPCR, quantitative PCR.

(*TSF*; Jeong *et al*, 2009; Yang *et al*, 2010). We also saw a small but significant increase in H3K4m2 marks at the *FWA* locus, but not at *AtSN1* or *MEA-ISR* (Fig 3). The minor effects on H3K4m2 might be due to the redundant activity of other demethylases, such as LDL1 and LDL2 (Jiang *et al*, 2007). Overall, these results show that JMJ14 might directly target silent chromatin, and suggest that the active removal of H3K4 methyl marks at silent loci might be necessary for DRM2 to maintain proper DNA methylation patterns.

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

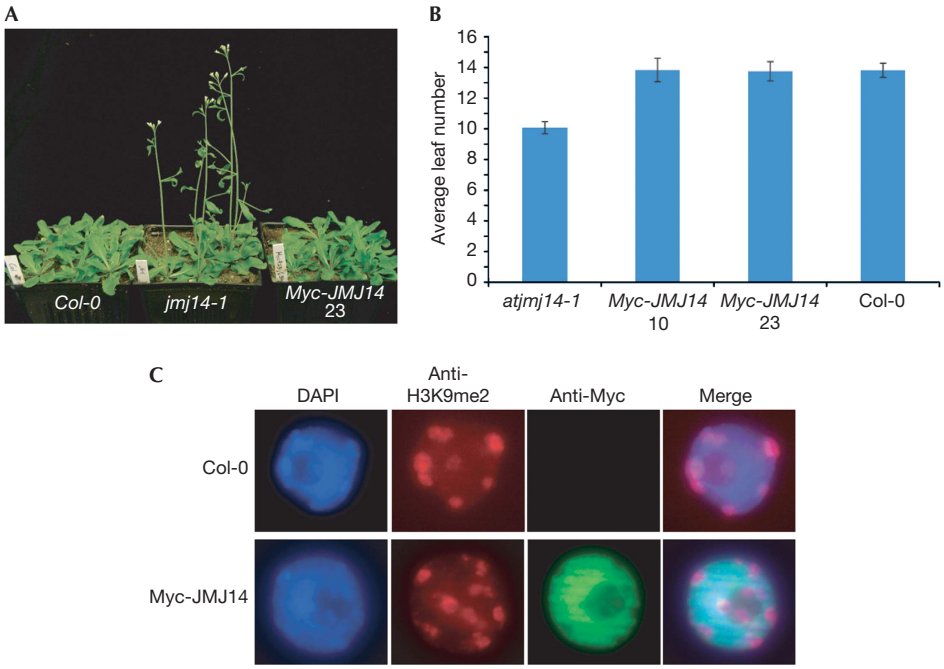

Fig 2 | Analysis of complementing Myc-tagged lines. (**A**) Myc-tagged JMJ14 constructs complement the early-flowering phenotype observed in the *jmj14-1* mutant background. (**B**) Flowering-time assay. Quantification of complementation for tagged JMJ14 lines. Note: Line 10 was used for immunofluorescence assay. (**C**) Immunolocalization of epitope-tagged JMJ14. A transgenic line expressing Myc-tagged complementing JMJ14 under its endogenous promoter was analysed by using fluorescent microscopy. JMJ14 is localized in the nucleus, but is depleted from the chromocentres (marked by histone 3 Lys 9 dimethylation enrichment and dense DAPI staining). DAPI, 4′,6-diamidino-2-phenylindole; JMJ14, Jumonji 14.

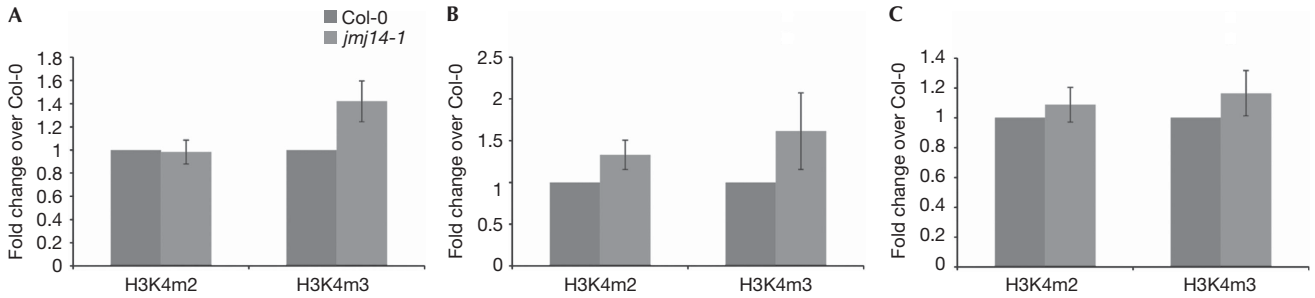

Fig 3 | Analysis of H3K4m2 and H3K4m3 state at RdDM targets by using chromatin immunoprecipitation. The immunoprecipitated DNA corresponding to (**A**) *AtSN1*, (**B**) *FWA* and (**C**) *MEA-ISR* was quantified by real-time PCR and normalized to an internal control—we used an intergenic region upstream from the isocitrate dehydrogenase gene-intergenic region (*ICDH-IGR*) and unlikely to be targeted by JMJ14. The fold enrichment in *jmj14-1* over wild type is shown at each locus (the wild type values were set to one). The values are the average ratio obtained from three independent ChIP experiments ± s.e. ChIP, chromatin immunoprecipitation; H3K4m2/m3, histone 3 Lys 4 dimethylation/trimethylation; ICDH, isocitrate dehydrogenase; JMJ14, Jumonji 14; RdDM, RNA-directed DNA methylation.

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

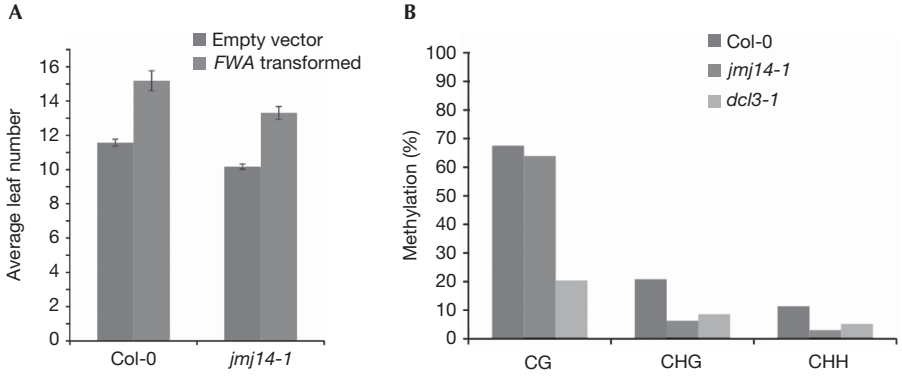

Fig 4 | The *de novo* DNA methylation analysis. (**A**) *FWA* flowering-time assay. Total leaf number on flowering was assessed for wild-type Col-0 and *jmj14-1* for both *FWA* and empty-vector transformants. (**B**) *FWA* transgene bisulphite sequencing. *jmj14-1* transformants had a minimal effect on CG methylation compared with *dcl3-1*. The effect on non-CG might be due to a maintenance defect after the initial methylation has been established. *jmj14-1*, *Jumonji 14-1*.

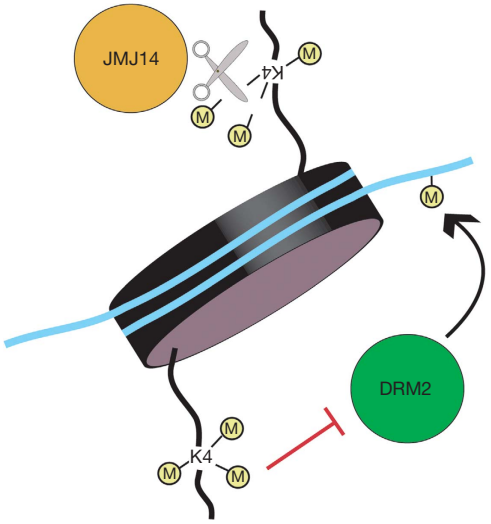

Fig 5 | Model for the role of Jumonji 14 in DRM2-mediated maintenance methylation. It is proposed that histone 3 Lys 4 methylation inhibits DRM2 pathway components. Active demethylation of the residue is needed for complete DRM2 maintenance activity. DRM2, domains rearranged methyltransferase 2; JMJ14, Jumonji 14.

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 JMJ14 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.

***Hae*III 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 *Hae*III side-by-side with samples containing buffer and no enzyme (undigested). Quantitative real-time PCR validation of uncut DNA after *Hae*III 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 *JMJ14* 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).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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