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Meng Xie
University of Nebraska-Lincoln

Guodong Ren
University of Nebraska-Lincoln, gren2@unl.edu

Chi Zhang
University of Nebraska-Lincoln, zhang.chi@unl.edu

Bin Yu
University of Nebraska - Lincoln, byu3@unl.edu

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The DNA- and RNA-binding protein FACTOR of DNA METHYLATION 1 requires XH domain-mediated complex formation for its function in RNA-directed DNA methylation

Meng Xie†, Guodong Ren†, Chi Zhang and Bin Yu*

Center for Plant Science Innovation & School of Biological Sciences, University of Nebraska-Lincoln, Lincoln, NE 68588-0660, USA

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*For correspondence (e-mail byu3@unl.edu).
†These authors contributed equally to this work.

SUMMARY

Studies have identified a sub-group of SGS3-LIKE proteins including FDM1–5 and IDN2 as key components of RNA-directed DNA methylation pathway (RdDM). Although FDM1 and IDN2 bind RNAs with 5′ overhangs, their functions in the RdDM pathway remain to be examined. Here we show that FDM1 interacts with itself and with IDN2. Gel filtration suggests that FDM1 may exist as a homodimer in a heterotetramer complex *in vivo*. The XH domain of FDM1 mediates the FDM1–FDM1 and FDM1–IDN2 interactions. Deletion of the XH domain disrupts FDM1 complex formation and results in loss-of-function of FDM1. These results demonstrate that XH domain-mediated complex formation of FDM1 is required for its function in RdDM. In addition, FDM1 binds unmethylated but not methylated DNAs through its coiled-coil domain. RNAs with 5′ overhangs does not compete with DNA for binding by FDM1, indicating that FDM1 may bind DNA and RNA simultaneously. These results provide insight into how FDM1 functions in RdDM.

Keywords: SGS3-LIKE proteins, RNA-directed DNA methylation, small RNAs, epigenetics, Arabidopsis.

INTRODUCTION

In plants and animals, DNA methylation is often associated with transcriptional silencing, and is thought to play key roles in maintaining genome stability (Moazed, 2009; Feng *et al.*, 2010; Zhang and Zhu, 2011). In Arabidopsis, a class of approximately 24 nucleotide repeat-associated small RNAs (ra-siRNAs) direct *de novo* DNA methylation at their homologous loci through an RNA-directed DNA methylation pathway (RdDM) (Moazed, 2009; Feng *et al.*, 2010; Zhang and Zhu, 2011). The framework of RdDM has been established through identification and characterization of genes involved in this process (Moazed, 2009; Feng *et al.*, 2010; Zhang and Zhu, 2011). The RNAse III enzyme DICER-LIKE 3 produces ra-siRNAs from dsRNAs converted by RNA-dependent RNA polymerase 2 from single-stranded RNAs (Xie *et al.*, 2004), which may be produced by plant-specific DNA-directed RNA polymerase IV (Pol IV) from RdDM target loci (Herr *et al.*, 2005; Kanno *et al.*, 2005; Onodera *et al.*, 2005; Pontier *et al.*, 2005). ARGONAUTE 4 (AGO4) binds ra-siRNA to form an AGO4–ra-siRNA complex (Zilberman *et al.*, 2003; Zheng *et al.*, 2007; Havecker *et al.*, 2010), which is recruited to chromatin by interaction of AGO4 and plant-specific DNA-directed RNA polymerase V (Pol V) (El-Shami *et al.*, 2007) and/or base-pairing between siRNA and Pol V-dependent transcripts (Wierzbicki *et al.*, 2008, 2009). Recruitment of AGO4 to some low-copy-number loci also requires DNA-directed RNA polymerase II (Pol II) (Zheng *et al.*, 2009). After loading onto chromatin, AGO4 is thought to recruit the protein Domains Rearranged Methyltransferase 2 (DRM2) that catalyzes *de novo* cytosine DNA methylation at symmetric CG or CHG sites and asymmetric CHH sites, where H is adenine, thymine or cytosine (Cao and Jacobsen, 2002; El-Shami *et al.*, 2007; Wierzbicki *et al.*, 2009). The KOW-CONTAINING TRANSCRIPTION FACTOR 1/SPT5-LIKE protein (KTF1/SPT5L) is required for RdDM and interacts with AGO4 to assist in recruitment of DRM2 (Bies-Etheve *et al.*, 2009; He *et al.*, 2009). Recruitment of SPT5L to Pol V-dependent transcripts and chromatin is AGO4-independent (Rowley *et al.*, 2011). CLASSY 1 (CLSY1), a chromatin-remodeling protein, and SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1)/DNA-BINDING TRANSCRIPTION FACTOR 1 (DTF1) are essential for ra-siRNA accumulation and DNA methylation (Smith *et al.*, 2007; Law *et al.*, 2011;

Liu *et al.*, 2011). These three proteins are co-purified with Pol IV, indicating that they form a complex (Law *et al.*, 2011). DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1; a chromatin-remodeling protein), DEFECTIVE IN MERISTEM SILENCING 3 [DMS3; a protein containing a hinge domain of structural maintenance of chromosome (SMC) protein], and RNA-DIRECTED DNA METHYLATION 1 (RDM1; a methylated DNA-binding protein) are required for generation of Pol V-dependent transcripts and RdDM (Kanno *et al.*, 2004, 2008; Gao *et al.*, 2010; Law *et al.*, 2010). It has been shown that DRD1, DMS3 and RDM1 function as a complex in RdDM (Law *et al.*, 2010). RDM1 also interacts with AGO4 and DRM2, and may help recruit the silencing complex to chromatin (Gao *et al.*, 2010).

Recent studies reveal that six homolog proteins including FACTOR of DNA METHYLATION 1–5 (FDM1–5) and INVOLVED IN DE NOVO 2 (IDN2, also called RDM12) act redundantly in the RdDM pathway in Arabidopsis (Ausin *et al.*, 2009; Zheng *et al.*, 2010; Xie *et al.*, 2012). These proteins belong to the plant specific SGS3-LIKE protein family, whose founder members are Arabidopsis SGS3 and its rice homolog X1 (Mourrain *et al.*, 2000; Bateman, 2002). SGS3 is an essential component in post-transcriptional gene silencing (Mourrain *et al.*, 2000). It may stabilize RNA intermediates generated during *trans*-acting siRNA biogenesis by its RNA-binding ability (Peragine *et al.*, 2004). SGS3 contains an XS domain and a coiled-coil domain from N- to C-terminus (Bateman, 2002). In contrast, FDMs and IDN2 possess two additional domains, an N-terminal zinc finger domain and an XH domain (X-homolog domain with unknown function) (Ausin *et al.*, 2009; Zheng *et al.*, 2010; Xie *et al.*, 2012). Like SGS3, IDN2 and FDM1 bind dsRNAs with 5′ overhangs (Ausin *et al.*, 2009; Zheng *et al.*, 2010; Xie *et al.*, 2012). However, the *in vivo* substrates of FDM1 and IDN2 remain to be identified, although they have been proposed to stabilize the duplex generated by base pairing between ra-siRNA and Pol V-dependent transcript (Ausin *et al.*, 2009; Xie *et al.*, 2012).

In this study, we report that FDM1 acts as a complex in RdDM. FDM1 interacts with both itself and IDN2. Gel filtration analysis suggests that FDM1 exists as a homodimer in a heterotetramer complex that may contain IDN2 *in vivo*. The FDM1 complex formation depends on its XH domain. Mutant FDM1 protein lacking its XH domain fails to form a complex and is unable to complement the DNA methylation defects of *fdm1-1 fdm2-1*, demonstrating that XH domain-mediated complex formation by FDM1 is required for its function in RdDM. FDM1 binds DNA *in vitro* through its coiled-coil domain. RNAs with 5′ overhangs do not abolish the DNA-binding ability of FDM1, indicating that FDM1 may bind both DNA and RNA simultaneously. Through functional analyses of FDM1 protein domains, this study extends our understanding of the RdDM pathway.

RESULTS

FDM1 interacts with itself and IDN2

FDM1 and FDM2 share high identity (approximately 93% identity and 96% similarity). However, the *fdm1-1* mutation (null) but not the *fdm2-1* mutation (null) individually reduces DNA methylation, indicating that FDM1 may play a major role in RdDM. In addition, expression of FDM1 but not FDM2 in *fdm1-1 fdm2-1* is sufficient to restore DNA methylation to wild-type levels. This provides an advantage of being able to study FDM1 function without an effect of FDM2 *in vivo* by expression of FDM1 mutants. Thus, we focused on FDM1 in this study. In order to obtain insight into how FDM1 acts in RdDM, we tested the interaction of FDM1 with known RdDM components including DRM2, DMS3, RDR2, SPT5L, FDM1 and IDN2 using the pGBKT7/pGADT7 two-hybrid system. In this system, a protein of interest is fused with a DNA-binding domain in the pGBKT7 plasmid, while the potential interactor is fused with a transcriptional activation domain in the pGADT7 vector. If two proteins interact, the DNA-binding domain associates with the transcriptional activation domain after co-transformation into yeast cells. This activates expression of a report gene that produces adenine (Ade), and thus enables growth of the yeast strain in the absence of Ade. Co-transformation of pGADT7-FDM1/pGBKT7-FDM1 and pGADT7-FDM1/pGBKT7-IDN2 pairs enabled growth of yeast cells in the absence of Ade (Figure 1a). In contrast, yeast cells failed to grow on in the absence of Ade after co-transformation of pGADT7/pGBKT7-FDM1, pGADT7/pGBKT7-IDN2 or pGBKT7/pGADT7-FDM1 (Figure 1a). These results indicate that FDM1 may interact with itself and IDN2. This assay did not detect interaction of FDM1 with DRM2, DMS3, RDR2 or SPTL5 (Figure 1b). The FDM1–RDR2 interaction result from this assay is consistent with the pulldown results in Xie *et al.*, (2012).

The XH domain of FDM1 is necessary for FDM1–FDM1 and FDM1–IDN2 interactions

To identify the protein domains of FDM1 responsible for the interaction, we generated a series of truncation mutants of FDM1 in pGADT7 (Figure 2a): lacking the XH domain (FDM1-T1), the XH domain alone (FDM1-T2), lacking the XH and coiled-coil domains (FDM1-T3) and lacking the zinc finger and the XS domain (FDM1-T4). We tested the interaction of these truncated FDM1 mutants with full-length FDM1 and IDN2 using the yeast two-hybrid assay described above. FDM1-T2 and FDM1-T4 were able to interact with FDM1 and IDN2, respectively, because co-transformation of these pairs enabled yeast cell to grow in the absence of Ade (Figure 2b,c). In contrast, FDM1-T1 and FDM-T3 did not interact with FDM1 and IDN2, respectively. These results indicated that the XH domain of FDM1 is necessary for FDM1–FDM1 and FDM1–IDN2 interactions. However, the yeast cells containing FDM1/FDM1-T2 (XH domain alone)

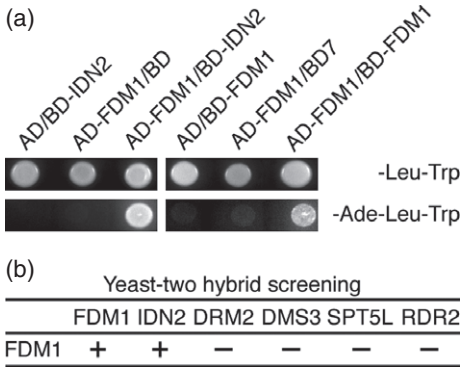

Figure 1. Determining the interaction of FDM1 with other components in RdDM

(a) Interactions of FDM1 with FDM1 and IDN2. Growth of yeast cells on adenine-deficient medium (–Ade–Leu–Trp) shows the interaction of FDM1 with FDM1 and IDN2. pGADT7 (AD) and pGBKT7 (BD) plasmids contain the activation and DNA-binding domains of GAL4, respectively. Paired AD and BD fusion constructs were co-transformed into yeast AH109 cells. The transformants were selected using synthetic dropout medium (–Leu–Trp) and spotted on adenine-deficient medium (–Ade–Leu–Trp).

(b) Summary of yeast two-hybrid analyses. '+' indicates interactions; '–' indicates non-interactions. FDM1 did not interact with DRM2, DMS3, SPT5L or RDR2.

and IDN2/FDM1-T2 grew more slowly than those containing FDM1/FDM1 and IDN2/FDM1, respectively (Figures 1 and 2). This result indicated that full-strength FDM1–FDM1 and FDM1–IDN2 interactions may require additional protein domains.

To validate the function of the XH domain in protein–protein interactions, we replaced the tryptophan at position 605 (W605) and the glutamic acid at position 617 (E617) with alanine (A) in the XH domain of FDM1 (FDM1-T5; Figure 2a). These two amino acids are conserved in XH domains and hence play important roles in mediating protein–protein interactions. As shown in Figure 2(d), FDM1-T5 did not interact with FDM1 and IDN2. This result confirmed that the XH domain of FDM1 is necessary for FDM1–FDM1 and FDM1–IDN2 interactions.

FDM1 pulls down FDM1 and IDN2 *in vitro*

To further confirm FDM1–IDN2 interaction, we performed an *in vitro* pulldown assay. We expressed recombinant IDN2 protein fused to a maltose-binding protein epitope at its N-terminus (MBP–IDN2), FDM1 fused to an N-terminal GST tag (GST–FDM1) or the controls MBP, GST and GST–FDM1ΔXH (FDM1 lacking the XH domain) in *Escherichia coli*. After expression, protein extracts containing MBP–IDN2 were mixed with extracts containing GST–FDM1, and reciprocal pulldown was then performed using amylose resin or glutathione beads. To avoid DNA- or RNA-mediated protein interactions, we treated the samples with micrococcal nuclease that digests both DNA and RNA. Enrichment of MBP–IDN2 in the GST–FDM1 complex and of GST–FDM1 in the MBP–IDN2 complex was detected using antibodies against MBP or GST, respectively (Figure 3a,b). In contrast, GST and GST–FDM1ΔXH failed to pull down MBP–IDN2, and MBP did not pull down GST–FDM1 (Figure 3a,b). To validate FDM1–FDM1 interaction, we mixed protein extracts containing YFP–FDM1 or YFP–FDM1ΔXH with extracts containing GST–FDM1 or GST, respectively, and performed a reciprocal pulldown assay. GST–FDM1 and YFP–FDM1 reciprocally pulled down each other (Figure 3c,d), but YFP–FDM1ΔXH and GST did not interact with GST–FDM1 and YFP–FDM1, respectively (Figure 3c,d).

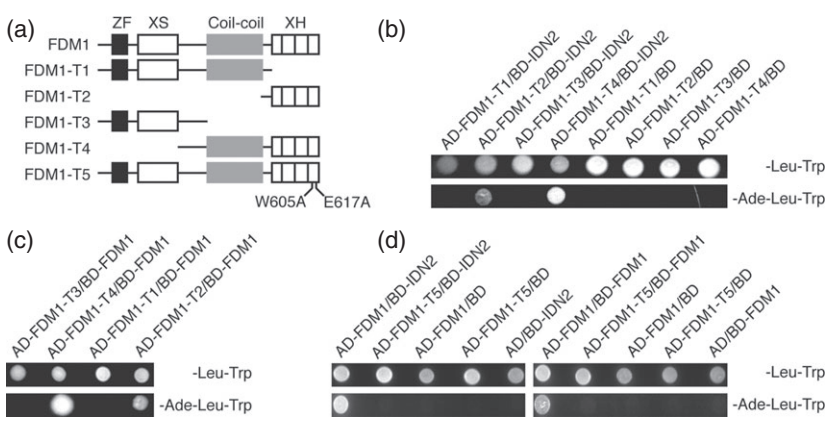

Figure 2. The XH domain mediates FDM1–FDM1 and FDM1–IDN2 interactions.

(a) Schematic structure of the full-length and truncated FDM1 proteins used for yeast-two hybrid assays. FDM1-T1, truncated FDM1 protein lacking the XH domain; FDM1-T2, XH domain alone; FDM1-T3, truncated FDM1 protein containing the zing finger and XS domain; FDM1-T4, truncated FDM1 protein containing the coiled-coil domain and XH domain; FDM1-T5, the tryptophan at position 605 (W605) and the glutamic acid at position 617 (E617) were replaced by alanine (A).

(b) Interaction analyses of truncated FDM1 proteins with IDN2 in yeast AH109 cells.

(c) Interaction analyses of truncated FDM1 with FDM1 in yeast AH109 cells.

(d) Interactions of FDM1 containing point mutations with FDM1 and IDN2. Mutated FDM1 was cloned into pGADT7 (AD). IDN2 or FDM1 were cloned into pGBKT7 (BD). The paired AD and BD fusion constructs were co-transformed into yeast. Positive clones selected on –Leu–Trp were spotted on –Ade–Leu–Trp medium.

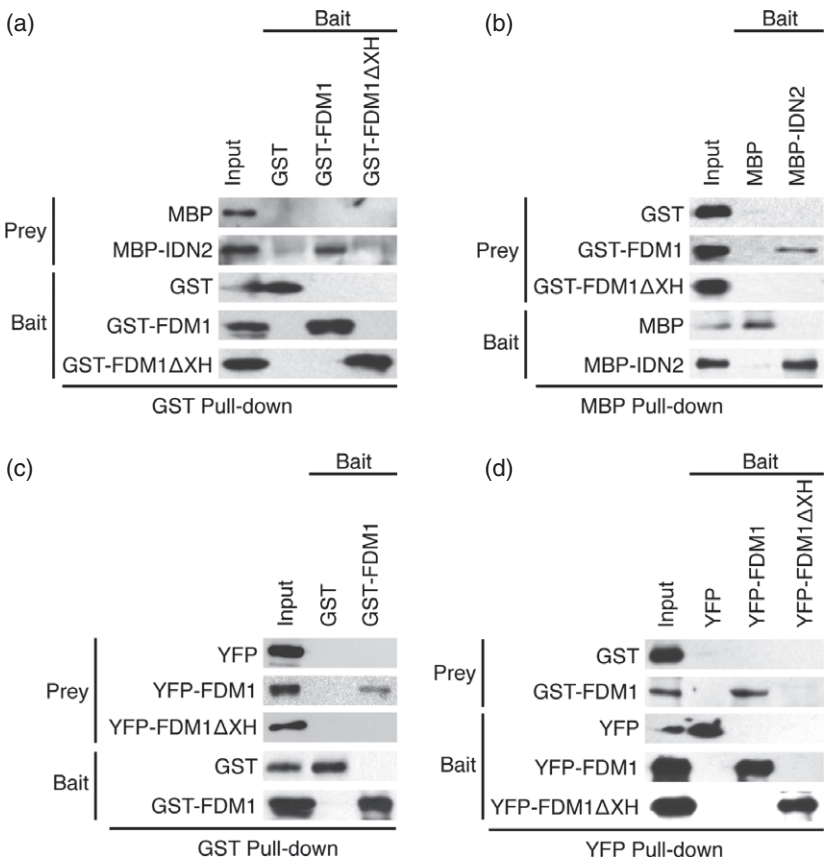

Figure 3. *In vitro* FDM1–FDM1 and FDM1–IDN2 interactions

(a) GST–FDM1 but not GST/GST–FDM1ΔXH pulls down MBP–IDN2 protein.

(b) MBP–IDN2 pulls down GST–FDM1 but not GST/GST–FDM1ΔXH.

GST, GST–FDM1 or GST–FDM1ΔXH extracts were separately mixed with MBP or MBP–IDN2 extracts, respectively, to generate GST/MBP, GST/MBP–IDN2, GST–FDM1/MBP, GST–FDM1/MBP–IDN2, GST–FDM1ΔXH/MBP or GST–FDM1ΔXH/MBP–IDN2 mixtures. Protein mixtures were incubated with glutathione Sepharose 4B beads or amylose resin to capture GST fusion proteins or MBP fusion proteins, respectively. MBP fusion proteins and GST fusion proteins were detected by Western blotting using MBP antibody and GST antibody, respectively. Bait: proteins captured by glutathione beads (a) or amylose resin (b). Prey: proteins associated with the bait.

(c) GST–FDM1 pulls down YFP–FDM1 but not YFP/YFP–FMD1ΔXH.

(d) YFP–FDM1 but not YFP–FMD1ΔXH pulls down GST–FDM1.

YFP, YFP–FDM1 or YFP–FDM1ΔXH extracts were separately mixed with GST–FDM1 or GST, respectively, to generate YPP/GST–FDM1, YFP/GST, YFP–FDM1/GST–FDM1, YFP–FDM1/GST, YFP–FDM1ΔXH/GST–FDM1 and YFP–FDM1ΔXH/GST mixtures. Protein mixtures were incubated with glutathione beads or anti-GFP antibody conjugated to agarose beads to capture GST fusion proteins or YFP fusion proteins, respectively. YFP fusion proteins and GST fusion proteins were detected by Western blotting. Bait: proteins captured by glutathione beads (c) or GFP antibody (d). Prey: proteins associated with the bait.

FDM1 forms a tetramer *in vitro*

The yeast two-hybrid and pulldown analyses suggest that FDM1 interacts with itself through its XH domain. Thus, we examined whether FDM1 forms a dimer or an oligomer complex. We first expressed recombinant FDM1 fused with a C-terminal 6 × His tag (FDM1-His) and a truncated FDM1-His lacking XH (FDM1ΔXH-His) in *E. coli*, and purified the resulting proteins. The FDM1-His or FDM1ΔXH-His proteins were then analyzed by size-exclusion HPLC. The elution fractions were then separated in SDS–PAGE and probed with antibodies recognizing the His tag. The column was calibrated using protein standards. We obtained information on the relative size of the FDM1 complex by comparing fractions of FDM1 with peak elution times of standard proteins. FDM1-His had a peak elution time of 114–118 min (Figure 4a), suggesting that FDM1-His may exist as a tetramer complex of approximately 300 kDa. In contrast, FDM1ΔXH-His eluted at between 144 and 148 min, corresponding to the size of the FDM1ΔXH monomer (approximately 60 kDa; Figure 4a). These analyses revealed that FDM1 forms a tetramer complex that requires the XH domain for its formation.

Because FDM1 also interacts with IDN2, we next tested whether incubation of IDN2 and FDM1 generates a larger complex or tetramer in order to obtain insight into the nature of the FDM1–IDN2 complex. We purified MBP–IDN2 and removed the MBP tag. However, incubation of IDN2 with

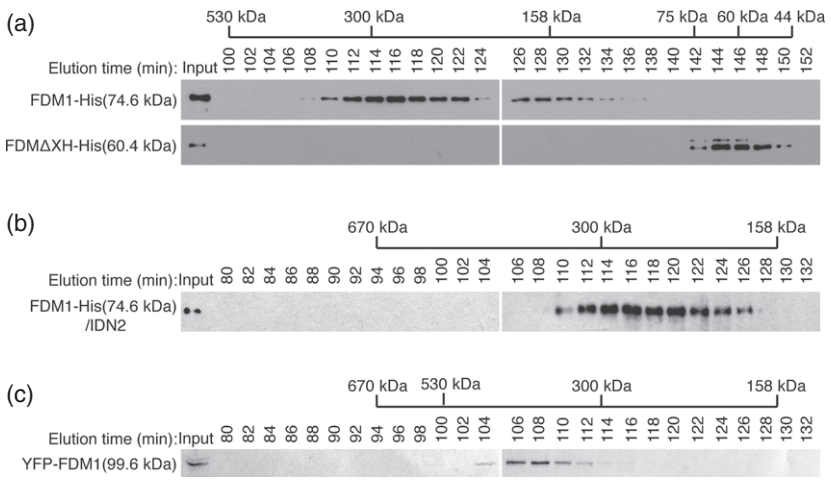

Figure 4. Gel filtration analysis of the FDM1 complex.
(a) XH domain-dependent tetramer formation of FDM1 *in vitro*.
(b) FDM1 exists in a tetramer complex *in vivo*.
(c) Effect of IDN2 on FDM1 complex formation.
Purified FDM1-His, FDM1-His/IDN2, FDM1ΔXH-His or Arabidopsis extracts containing YFP–FDM1 were separated by HPLC. Eluted fractions were separated by SDS–PAGE and detected by Western blotting using anti-His or anti-YFP antibodies. Elution times of protein standards are shown on the top of the gels.

FDM1-His still produced a tetramer (Figure 4b). This result indicates that FDM1 and IDN2 may form a tetramer *in vitro*. However, the copy numbers of FDM1 and IDN2 in the complex remain to be determined.

FDM1 protein exists as a dimer in a tetramer complex *in vivo*

To obtain information on the FDM1 complex *in vivo*, we analyzed Arabidopsis protein extracts containing YFP–FDM1 by size-exclusion HPLC. YFP–FDM1 complemented the DNA methylation defects in *fdm1-1*. Anti-YFP antibody detected the presence of YFP–FDM1 in a complex of approximately 350 kDa as calculated from a standard curve produced using the elution times of protein standards (Figure 4c). The calculated molecular mass for YFP–FDM1 is approximately 100 kDa, and that for untagged FDM proteins and IDN2 is approximately 75 kDa. Thus, the mass of approximately 350 kDa equals the molecular mass of two copies of YFP–FDM1 and two copies of other untagged FDM proteins or IDN2. This result suggests that FDM1 may exist as a homodimer in a heterotetramer complex.

The XH domain is required for the function of FDM1 in RdDM

Next, we examined whether the XH domain was required for FDM1 function in RdDM. We generated transgenic *fdm1-1 fdm2-1* plants expressing either *35S::YFP-FDM1* or *35S:YFP-FDM1ΔXH* lacking the XH domain. In a previous study, we showed that expression of *FDM1* under the direction of its native promoter is sufficient to complement the DNA methylation defects of *fdm1-1 fdm2-1* (Xie *et al.*, 2012). Using *fdm1-1 fdm2-1* enabled us to test the function of the XH domain of FDM1 without effects of FDM2, which has a 96% similarity with FDM1. The transcript levels of transgenes and their products were similar in all four transgenic lines (Figure 5a,b). We examined the methylation levels of two RdDM targets, AtSN1 and ING5, *AtSN1* and *IGN5* in two transgenic *fdm1-1 fdm2-1* lines harboring *35S::YFP-FDM1* and two transgenic *fdm1-1 fdm2-1* lines harboring *35S:YFP-FDM1ΔXH* using methylation-sensitive *Hae*III restriction enzyme digestion followed by PCR. Less DNA methylation at *AtSN1* and *IGN5* results in less PCR product after *Hae*III digestion because it cuts unmethylated but not methylated DNA. As shown in Figure 5(c), the *35S::YFP-FDM1* transgene restored the DNA methylation content of *AtSN1* and *IGN5* in *fdm1-1 fdm2-1* to wild-type levels. In contrast, the DNA methylation levels of *fdm1-1 fdm2-1* harboring *35S:YFP-FDM1ΔXH* were comparable with those in *fdm1-1 fdm2-1*. Consistent with this, silencing of *AtSN1* transcription was not restored in *fdm1-1 fdm2-1* harboring *35S:YFP-FDM1ΔXH* (Figure 5d). These results demonstrate that the XH domain is essential for the function of FDM1 in RdDM.

FDM1 binds unmethylated but not methylated DNA

Protein sequence analyses showed that the coiled-coil domain of FDM1 has approximately 50% similarity to a portion of the SMC (structural maintenance of chromosomes) protein from *Methanocaldococcus* sp. *FS406-22* (Figure 6a). As SMC proteins bind DNAs, this finding prompted us to test whether FDM1 binds DNA using a GST pulldown assay. This method reduces the background signal because it eliminates unbound probes. This method has been used previously to study protein–nucleic acid interactions (Jiao *et al.*, 2002; Yu *et al.*, 2008). We incubated purified GST–FDM1 with a 50 bp ^{32}P-labeled DNA fragment

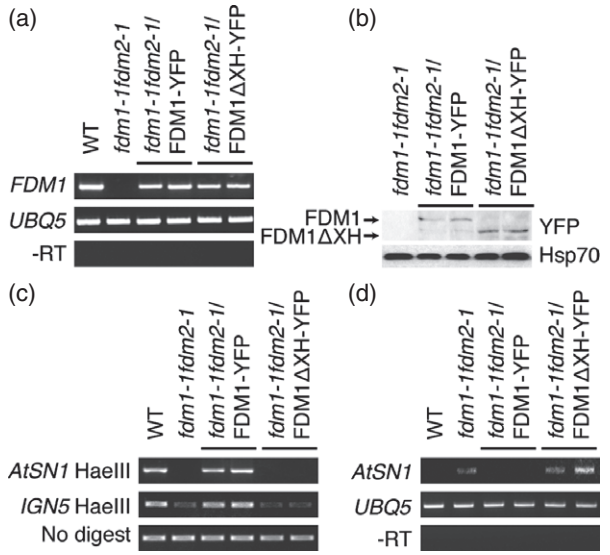

Figure 5. The XH domain is required for the function of FDM1 in RdDM.
(a,b) Deletion of the XH domain has no effect on expression of *FDM1*. The transcript levels of YFP–FDM1 and FDM1ΔXH were determined by RT-PCR. Amplification of *UBIQUITIN5* (At3g26650; *UBQ5*) with or without reverse transcription (–RT) is shown as a control. The protein levels of YFP–FDM1 and FDM1ΔXH were determined by Western blotting. Heat shock protein 70 (HSP70) was used as a loading control.
(c) Expression of the YFP–FDM1ΔXH construct does not rescue the DNA methylation defects at the *AtSN1* and *IGN5* loci in *fdm1-1 fdm2-1*. *Hae*III-digested genomic DNAs from various genotypes were used for PCR amplification of *AtSN1* and *IGN5*, and undigested genomic DNAs were used as loading controls.
(d) Expression of the YFP–FDM1ΔXH construct does not silence expression of *AtSN1* in *fdm1-1 fdm2-1*. *AtSN1* transcripts were detected by RT-PCR. Amplification of *UBQ5* with or without reverse transcription (–RT) is shown as a control.

and a 50 nucleotide ^{32}P-labeled single-stranded DNA (ssDNA) that corresponds to a fragment of *AtSN1* DNA (Figure 6b). After washing, the DNAs were extracted from beads and separated on a native PAGE gel. GST–FDM1 but not GST alone retained the 50 bp DNA fragment (Figure 6b). However, FDM1 was unable to bind the ssDNA (Figure 6b). Addition of unlabeled DNA with the same sequences eliminated the radioactive signals. These results indicated that FDM1 binds DNA (Figure 6b). FDM1 also bound a DNA fragment containing a poly(A) strand and a poly(T) strand (Figure 6d). This result suggests that DNA binding by FDM1 is not sequence-specific. However, FDM1 did not bind methylated DNA (Figure 6d). To identify protein domains required for the DNA-binding ability of FDM1, we expressed and purified a series of truncated FDM1 proteins fused to an N-terminal GST tag (Figure 6e,f). The truncated FDM1 protein lacking a portion of the coiled-coil domain but not other domains failed to bind DNA (Figure 6f). In addition, the coiled-coil domain itself was able to bind DNA (Figure 6f). Based on these results, we propose that the coiled-coil domain is necessary and sufficient for DNA binding of FDM1.

We have shown that FDM1 binds RNA with 5′ overhangs, and this depends on the XS domain. This raised a question of whether FDM1 can bind DNA and RNA simultaneously. To address this question, we examined whether addition of unlabeled RNAs with 5′ overhangs affects the DNA-binding ability of FDM1. If FDM1 binds DNA and RNA at the same time, addition of RNAs should not eliminate DNA binding of FDM1. As shown in Figure 6(c), addition of RNAs with 5′ overhangs did not affect DNA retention of FDM1.

DISCUSSION

Studies on FDM1 and IDN2 have suggested that they may act in downstream of RdDM, presumably by stabilizing the duplex of ra-siRNA–Pol V-dependent transcripts (Ausin *et al.*, 2009; Zheng *et al.*, 2010; Xie *et al.*, 2012). In this study, we demonstrate that FDM1 exists in a complex for proper function in RdDM, and is an RNA- and DNA-binding protein.

Yeast two-hybrid and *in vitro* protein pulldown experiments showed that FDM1 interacts with IDN2. Given its high similarity with FDM1, FDM2 most likely also interacts with IDN2. While this paper was in preparation, two other groups found that IDN2 complexes contain IDN2 PARALOG 1 (IDP1)/IDN2-LIKE1 (IDNL1) and IDP2/IDNL2 (Ausin *et al.*, 2012; Zhang *et al.*, 2012). IDP1/IDNL1 and IDP2/IDNL2 are synonymous with FDM1 and FDM2, respectively. These results demonstrate that FDM1/IDP1/IDNL1 and FDM2/IDP2/IDNL2 form a complex with IDN2. We detected a FDM1/IDP1–FDM1/IDP1 interaction in a yeast two-hybrid assay, but Zhang *et al.* (2012) did not. This discrepancy may be due to the fact that different yeast strains were used (PJ694A versus AH109). Protein pulldown (Figure 3) and gel filtration (Figure 4) experiments further confirmed the FDM1–FDM1 interaction. FDM1 forms a homotetramer *in vitro*, but may exist as a homodimer in a tetramer complex *in vivo* (Figure 4). Multidimensional protein identification technology (MudPIT) analysis showed that IDN2 may be the only partner of IDNL1/FDM1 (Ausin *et al.*, 2012). Crystal structure and yeast two-hybrid analyses revealed that IDN2 lacking the XH domain forms a homodimer *in vitro* (Ausin *et al.*, 2012; Zhang *et al.*, 2012). These results suggest that FDM1 and IDN2 form a heterotetramer containing an FDM1 dimer and an IDN2 dimer. FDM2 is present in the IDN2 complex and is highly similar to FDM1, indicating the presence of an IDN2–IDN2–FDM2–FDM2 tetramer. The presence of these two complexes is consistent with the functional redundancy of FDM1 and FDM2 (Xie *et al.*, 2012). However, it is possible that IDN2–IDN2–FDM1–FDM2 is present in low amounts such that MudPIT analysis cannot detect IDNL2/FDM2 in the IDNL1/FDM1 complex.

Whole-genome bisulfite sequence analysis revealed that DNA methylation patterns are similar in *idn2-1*, *idnl1-1 idnl2-1* (*fdm1 fdm2*) and *idn2-1 idnl1-1 idnl2-1*, indicating that IDN2, FDM1/IDNL1 and FDM2/IDNL2 mostly likely function together (Ausin *et al.*, 2012). IDN2, FDM1/IDNL1 and FDM2/

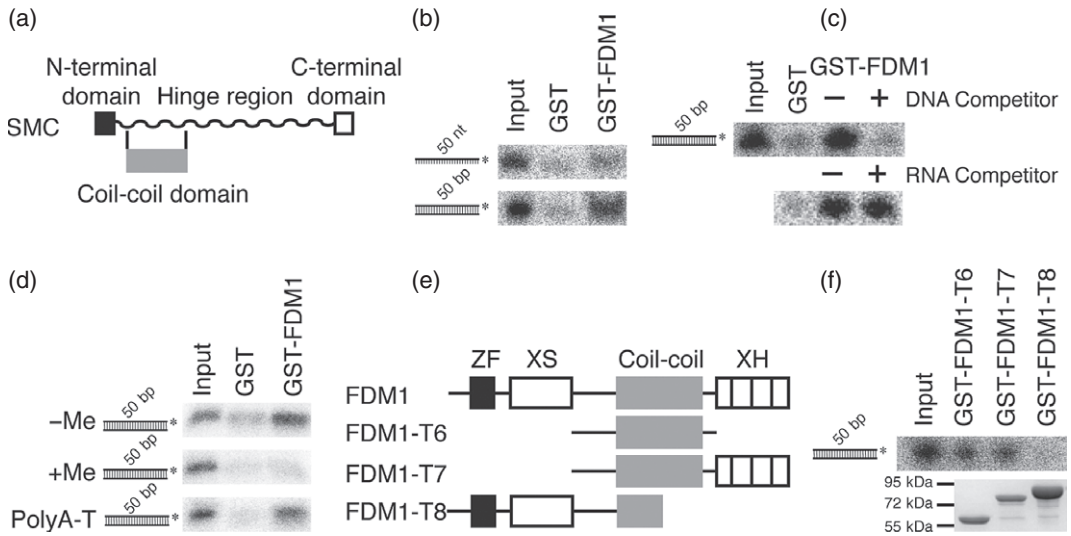

Figure 6. FDM1 binds DNA through its coiled-coil domain.
(a) Diagrams showing that the coiled-coil domain shares similarities with a portion of the SMC (structural maintenance of chromosomes) protein.
(b, c) FDM1 binds double-stranded DNA but not single-stranded DNA. The structure of various probes is shown on the right.
(d) DNA-binding specificity of FDM1. –me, unmethylated DNA control; +me, cytosine-methylated DNA; PolyA-T, DNA fragment containing a poly(A) strand and a poly(T) strand.
(e) Diagrams of truncated GST–FDM1 used for DNA-binding assays. FDM1-T6, coiled-coil domain of FDM1 alone; FDM1-T7, truncated FDM1 containing only the coiled-coil and XH domains; FDM1-T8, truncated FDM1 lacking the XH domain and a portion of coiled-coil domain.
(f) The coiled-coil domain is necessary and sufficient for DNA binding by FDM1. Purified proteins used in the binding assay were resolved by SDS–PAGE gel and stained with Coomassie Brilliant Blue and are shown below the DNA-binding gel. The protein molecular masses are indicated on the left.
Asterisks indicate radioactive labeled DNA strand. Approximately 50 μg protein was used for the binding assay. A 150-fold excess of unlabeled DNAs of the same sequence or RNAs with 5′ overhangs were used for the competition assay.

IDNL2 affect most DRM2 targets and few non-DRM2 targets, indicating that they mainly act in the RdDM pathway (Ausin *et al.*, 2012). The DNA methylation defect in *idn2-1*, *idnl1-1 idnl2-1* and *idn2-1 idnl1-1 idnl2-1* is weaker than that in *drm2*. This may be due to the redundant functions of homologs of IDN2, FDM1 and FDM2. Indeed, three FDM1 homologs, FDM3, FDM4 and FDM5, act redundantly with FDM1 (Xie *et al.*, 2012). Of these, FDM3 and FDM4 are in the IDN2 sub-family, whereas FDM5 is grouped with FDM1 and FDM2. The IDN2 complex does not contain FDM3, FDM4 and FDM5 (Ausin *et al.*, 2012; Zhang *et al.*, 2012), raising the possibility that other FDM complexes may exist. Perhaps the IDN2/FDM1 (FDM2) complex plays a major role in RdDM, while others have minor functions, because loss of function of either IDN2 or FDM1 alone causes a DNA methylation defect but loss of function of other FDM proteins alone does not (Xie *et al.*, 2012). Alternatively, they may play different roles to the IDN2/FDM1 complex.

The function of the XH domain was previously unknown. We found that FDM1 protein lacking the XH domain or harboring mutations in its XH domain failed to interact with itself or with IDN2 (Figures 2–4). The XH domain of FDM1 by itself interacts with FDM1 and IDN2, demonstrating that the XH domain of FDM1 functions in mediating protein–protein interaction. In addition, IDN2 without a functional XH domain fails to interact with IDP1/FDM1 (Zhang *et al.*, 2012). Both FDM1 and IDN2 lacking the XH domain failed to rescue DNA methylation defects in the corresponding mutants (Figure 5) (Zhang *et al.*, 2012), demonstrating that XH domain-mediated complex formation is essential for their function in RdDM.

It has been shown that FDM1/IDNL1 or FDM2/IDNL2 cannot replace IDN2 in their complexes because a strong *idn2-1* allele has similar DNA methylation defects to *idnl-1 idnl-2* and *idn2-1 idnl1-1 idnl2-1* (Ausin *et al.*, 2012). However, a weak *idn2-3* mutation reduced DNA methylation in *fdm1-1* and *fdm2-1*, respectively (Xie *et al.*, 2012), indicating the function of the IDN2/FDM1/FDM2 complex is further impaired in the double mutants. What causes the difference between FDM1 and IDN2? For both FDM1 and IDN2, the XH domain mediates protein–protein interaction and the XS domain binds dsRNAs with 5′ overhangs, indicating that they may not be the reasons for the differences between FDM1 and IDN2. We found that the coiled-coil domain of FDM1 binds DNA and is not required for FDM1–FDM1 and FDM1–IDN2 interactions. In contrast, the coiled-coil domain of IDN2 has been shown to mediate IDN2–IDN2 interaction (Ausin *et al.*, 2012; Zhang *et al.*, 2012). Thus, the coiled-coil domain of FDM1 is biochemically different from that of IDN2, and may be the factor that distinguishes FDM1 from IDN2. Given the high similarity between FDM1 and FDM2, this most likely is

the cause of the difference between FDM2 and IDN2 as well.

FDM1 and IDN2 bind RNAs with 5′ overhangs through their XS domains (Ausin *et al.*, 2009; Xie *et al.*, 2012; Zhang *et al.*, 2012). In addition, FDM1 binds DNA in a non-sequence-specific manner through the coiled-coil domain, and DNA binding of FDM1 is not be competed for by RNA, indicating that FDM1 may bind DNA and RNA simultaneously. These results have advanced the model for IDN2/FDM1 function (Ausin *et al.*, 2009, 2012; Zheng *et al.*, 2010; Xie *et al.*, 2012). The XS domain of FDM1 (FDM2) and IDN2 may bind the duplex of AGO4-bound ra-siRNA and Pol V-dependent transcripts (Ausin *et al.*, 2009, 2012; Zheng *et al.*, 2010; Xie *et al.*, 2012). This binding will recruit the FDM1–IDN2 or FDM2–IDN2 complex to RdDM loci. Subsequently, the coiled-coil domain of FDM1 (FDM2) binds the DNA. Binding of the FDM1–IDN2 complex to the RNA duplex and RdDM target loci may have two roles that are not mutually exclusive. One is to prevent potential cleavage of Pol V-dependent transcripts by the AGO4–ra-siRNA complex, which may disrupt the AGO4–chromatin interaction. However, the levels of Pol V transcripts are not affected by *fdm1 fdm2* and *idn2* mutations (Ausin *et al.*, 2012; Xie *et al.*, 2012), arguing against this possibility. The other is that the FDM1 complex may provide a marker for DRM2 to recognize. However, FDM1 does not bind methylated DNA, indicating that the FDM1 complex may be required for initiation but not reinforcement of DNA methylation. The yeast two-hybrid assay does not identify the FDM1–DRM2 interaction, suggesting that other factors may be involved. Clearly this model needs to be examined using an FDM1 mutant deficient in DNA and/or RNA binding.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Plants were grown at 22 °C under long-day conditions (16 h light/8 h dark). *fdm1-1* (SALK_075813), *fdm2-1* (SAIL_291_F01) and *fdm1-1 fdm2-1* are in the Columbia genetic background (Xie *et al.*, 2012).

Plasmid construction

YFP cDNA was cloned into binary vector pMDC32 (Curtis and Grossniklaus, 2003) to generate pMDC32-YFP. Then *FDM1* and *FDM1ΔXH* (lacking the XH domain) cDNAs were PCR-amplified and cloned into pMDC32-YFP to generate *p35S::FDM1-YFP* and *p35S::FDM1ΔXH-YFP* constructs, respectively. The *FDM1*, truncated *FDM1*, *IDN2*, *RDR2*, *DRM2*, *SPTL5* and *DMS3* cDNAs were PCR-amplified and cloned into the pGADT7 and/or pGBKT7 (Clontech, http://www.clontech.com/) vectors to produce constructions used for the yeast two-hybrid assay. The full-length *FDM1* and truncated *FDM1* cDNAs were PCR-amplified and cloned into pGEX-2TK(GE Healthcare, http://www3.gehealthcare.com/) or pET28(a), (EMD Millipore, http://www.emdmillipore.com/chemicals) vectors to generate GST or 6 × His fusion constructions. The *IDN2* cDNA was PCR-amplified and cloned into the pMAL-c5X(NEB, http://www.neb.com/nebecomm/default.asp) vector to generate the *MBP–IDN2* fusion construct. The primers used for plasmid constructions are listed in Table S1.

Plant transformation

p35S::FDM1-YFP and *p35S::FDM1ΔXH-YFP* were transformed into *fdm1-1* or *fdm1-1 fdm2-1*, respectively. The T_1 transgenic plants were selected by hygromycin resistance.

Yeast two-hybrid assay

Various plasmid pairs were co-transformed into yeast strain AH109. Selective drop-out medium lacking leucine and tryptophan was used to select yeast containing the plasmid pairs. The resulting clones were diluted in 50 μl water, and 5 μl was used for spot assays on selective drop-out medium lacking leucine, tryptophan and adenine. FDM1–FDM1 and FDM1–IDN2 interactions activate expression of Ade, which enables the growth of AH109 cells in the absence of Ade.

Protein expression

GST-, MBP- or His-tagged proteins were expressed in *E. coli*. BL21 and extracted as described previously (Xie *et al.*, 2012). The YFP–FDM1 and YFP–FDM1ΔXH constructs were transiently expressed in tobacco (*Nicotiana Benthamiana*) and extracted as described by Yu *et al.* (2008).

Protein pulldown assay

Protein extracts containing GST, GST–FDM1 or GST–FDM1ΔXH were mixed with equal volumes of protein extracts containing MBP–IDN2, YFP and YFP–FDM1, respectively. The mixed lysate was incubated with antibodies against GFP (and GFP variants) coupled to protein A agarose beads (Clontech), amylose resin (NEB) or glutathione Sepharose 4B beads (GE Healthcare) for 4 h.

The precipitates were washed five times (5min for each wash) with extraction buffer, separated by SDS–PAGE and blotted using antibodies recognizing the MBP, GST or YFP tag.

Gel filtration

FDM1-His and FDM1ΔXH-His were purified using Ni-resin (EMD Millipore) according to the manufacturer's instruction. After elution from Ni-resin, 100 μl protein solution was passed through a 0.22 μm filter and loaded onto the column. The gel filtration was performed on an HPLC system and a HiPrep 16/60 Sephacryl S-300 HR column (GE Healthcare) at a rate of 0.5 ml/min, and 0.5 ml fractions were collected every minute. For gel filtration of Arabidopsis protein extracts, collected fractions were precipitated with acetone at −20 °C overnight and resuspended in SDS loading buffer. Fractions were separated by 8% SDS–PAGE and analyzed by Western blotting using antibodies recognizing His or YFP. The protein standards (Bio-Rad, http://www.bio-rad.com/) used to calibrate the column contain five size standards, and the elution times for each peak are 94 min for 670 kDa, 129 min for 158 kDa, 150 min for 44 kDa, 173 min for 17 kDa, and 233 min for 1.35 kDa.

DNA methylation and RT-PCR analysis

The DNA methylation assay was performed as described previously (Xie *et al.*, 2012). Genomic DNAs extracted from flowers were digested with *Hae*III, and 5% of the digested DNA was used for PCR amplification of *AtSN1* and *IGN5*. Simultaneously, undigested genomic DNA was amplified as a quantity control. After DNase I treatment, 5 μg total RNAs from inflorescences were used to synthesize cDNA with SuperScript III (Invitrogen, http://www.invitrogen.com/) and oligo(dT) primers. The diluted cDNA was used to amplify *AtSN1* by PCR. Amplification of *UBQ5* was used as a loading control.

DNA-binding assay

GST–FDM1 and GST-tagged FDM1 mutants were purified as described by Xie *et al.* (2012). A 5′ overhanging dsRNA probe was generated as described previously (Xie *et al.*, 2012). A 50 nucleotide single-strand DNA fragment corresponding to a portion of *AtSN1* DNA was labeled at its 5′ end using T4 polynucleotide kinase (NEB) in the presence of [α^{32}P]ATP. Annealing this ssDNA to its complementary strand produced double-stranded DNA. The DNA- and RNA-binding assays were performed as previously described (Jiao *et al.*, 2002). Methylated DNA and its unmethylated control were synthesized by Integrated DNA Technologies (http://eu.idtdna.com/site) as described previously (Ito *et al.*, 2003).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer sequences.

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