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The splicing factor SR45 affects the RNA-directed DNA methylation pathway in Arabidopsis

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Abbreviations: AGO4, ARGONAUTE 4; DCL3, DICER-LIKE 3; DRM2, DOMAINS REARRANGED METHYLTRANSFERASE 2; FLC, FLOWERING LOCUS C; FWA, FLOWERING WAGENINGEN; *MEA-ISR*, *MEDEA-INTERGENIC SUBTELOMERIC REPEATS*; *REP12*, *REPEAT 12*; RdDM, RNA-directed DNA methylation; siRNA, small interfering RNA; SR45, ARGININE/SERINE-RICH 45; UBQ10, UBIQUITIN 10

Cytosine DNA methylation is an epigenetic mark frequently associated with silencing of genes and transposons. In Arabidopsis, the establishment of cytosine DNA methylation is performed by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2). DRM2 is guided to target sequences by small interfering RNAs (siRNAs) in a pathway termed RNA-directed DNA methylation (RdDM). We performed a screen for mutants that affect the establishment of DNA methylation by investigating genes that contain predicted RNA-interacting domains. After transforming *FWA* into 429 T-DNA insertion lines, we assayed for mutants that exhibited a late-flowering phenotype due to hypomethylated, thus ectopically expressed, copies of *FWA*. A T-DNA insertion line within the coding region of the spliceosome gene *SR45* (*sr45-1*) flowered late after *FWA* transformation. Additionally, *sr45-1* mutants display defects in the maintenance of DNA methylation. DNA methylation establishment and maintenance defects present in *sr45-1* mutants are enhanced in *dcl3-1* mutant background, suggesting a synergistic cooperation between SR45 and DICER-LIKE3 (DCL3) in the RdDM pathway.

Results and Discussion

DRM2 carries out all known DNA methylation establishment—or de novo methylation—in *Arabidopsis thaliana*.[1] Given that DRM2 is guided by both siRNAs and long non-coding RNAs,[2,3] we screened a collection of homozygous lines carrying T-DNA insertion in genes containing known or predicted RNA-interacting domains.[4] For this screen we used *FWA* transgene silencing as a reporter system. *FWA* is a homeodomain transcription factor that has two tandem repeats in its promoter. In wild-type plants, the endogenous *FWA* repeats are stably methylated and *FWA* is silenced. However, hypomethylation in its promoter region leads to an ectopic expression and a late-flowering phenotype.[5] After *FWA* transformation, plants with an intact de novo methylation machinery are able to methylate and silence the transgenic *FWA*, while mutants affecting de novo methylation express the transgene and flower late.[6,7]

Following this strategy, we isolated a line containing a T-DNA insertion in ARGININE/SERINE-RICH 45 (*SR45*) that flowered slightly late before *FWA* transformation, but showed a major late flowering phenotype after transformation (**Figs. 1A and S1**). The *sr45-1* mutant has been reported to show a late flowering phenotype due to an increased expression of *FLOWERING LOCUS C* (*FLC*).[8] To assess whether the observed effect was mediated by *FLC* alone, we analyzed the methylation status of *FWA* in *sr45-1* mutants after transformation. Bisulfite sequencing analyses revealed that the endogenous *FWA* methylation was not affected in the CG-dinucleotide context, but did display a defect in non-CG methylation, which is consistent with other mutants in the DRM2 pathway.[9] However, the transgenic copy of *FWA* exhibited reduced methylation levels in every sequence context (CG, CHG and CHH; H being A, T or G) (**Fig. 1B**). This reduction in methylation is correlated with the late flowering phenotype and ectopic *FWA* expression in the transgenic plants (**Figs. S2A–C**). Moreover, the introduction of an *SR45* copy into *sr45-1*, partially restores the ability to methylate *FWA* to wild-type levels (**Figs. S2D and E**), ruling out that the sole increment in *FLC* levels accounted for the observed late flowering phenotype. It is worth noting that some individual mutant plants are able to establish methylation at or near wild-type levels, indicating a degree of stochasticity. However, on average, we can safely say that *sr45* mutant plants have impaired DNA methylation establishment capacity.

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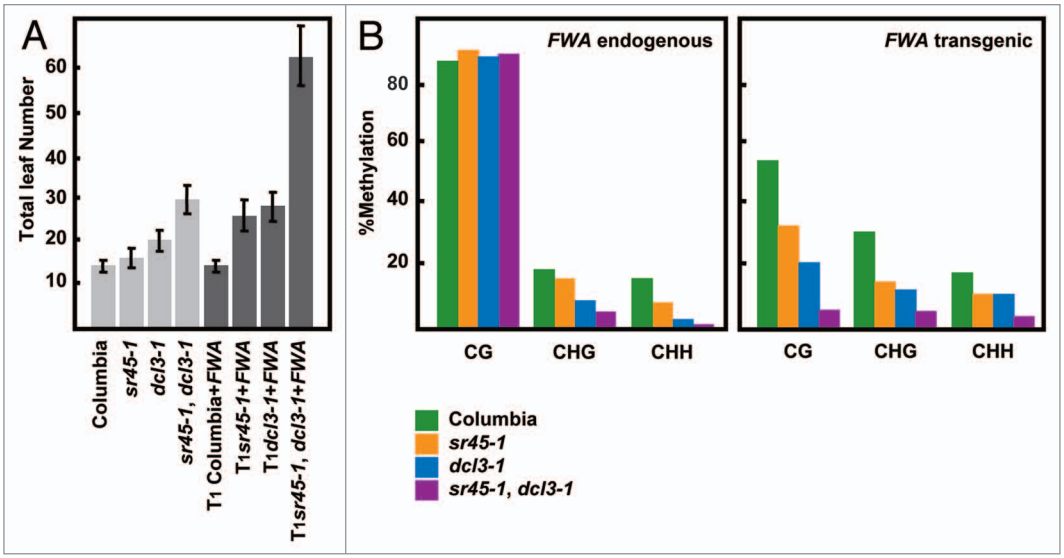


Figure 1. *sr45-1* de novo DNA methylation phenotype. (A) Flowering time of Columbia, *sr45-1*, *dcl3-1* and *sr45-1*, *dcl3-1* double before and after *FWA* transformation. Flowering time is measured as the total number of leaves at the time of flowering. (B) Methylation levels at endogenous and transgenic *FWA* after *FWA* transformation. The 494 base-pair repeated region in the 5' UTR was analyzed. The methylation state of the *FWA* endogene should remain unaltered by the presence of the *FWA* transgene. All samples were analyzed in the T1 generation.

We further analyzed the progression of *FWA* de novo methylation across subsequent generations in *sr45-1*. In wild type, the *FWA* transgene did not reach full levels of methylation until the T2 generation (**Fig. 2**). In *sr45-1* mutants, the levels of methylation at *FWA* transgene are reduced in the T1 generation, but in some cases complete methylation does not occur until the T3 generation or later (**Fig. 2**). Thus, the *sr45-1* mutant only partially impairs the de novo methylation machinery and wild type methylation levels are regained in later generations. These observations are consistent with the slow intergenerational silencing that has been reported in other transgene systems.[10]

To date, every mutant that has been shown to be defective in de novo methylation is also defective in the maintenance of non-CG methylation. We, therefore, examined the methylation status at known RdDM targets such as *FWA*, *MEA-ISR* and *AtSN1*. For this purpose we digested genomic DNA with methylation sensitive enzymes and performed either DNA gel blots or PCR, or we examined individual loci by sequencing following bisulfite treatment. Analysis revealed that the *sr45-1* mutant exhibits reduced non-CG methylation for all of the aforementioned loci (**Fig. 3A–C**). Due to the incompleteness of the phenotype, we generated double mutants with *dcl3-1*, which has been reported to display a weak DNA methylation phenotype.[11] We found that *sr45-1*, *dcl3-1* double mutants exhibited an additive effect in methylation phenotype. This enhancement was found at both RdDM target loci tested (**Fig. 3A–C**). Concordantly, *sr45-1 dcl3-1* double mutants also show an enhanced de novo methylation phenotype at *FWA* transgene (**Fig. 1**). Together this data indicate that SR45 and DCL3 could cooperate within the RdDM pathway.

To assess whether the methylation defect is specific to RdDM targets, we analyzed methylation and expression levels at *Ta3* and *REPEAT12* (*REP12*) loci by DNA gel blot and RT-PCR-two loci that are known to be methylated in a DRM2-independent manner.[12] We observed that *sr45-1* as well as *sr45-1, dcl3-1* double mutants showed no difference in methylation when compared with wild type, indicating that SR45 function is most likely confined to the DRM2 pathway (**Fig. 3D and E**).

In order to place SR45 within the context of the RdDM pathway, we analyzed *sr45-1* siRNA production by RNA gel blot. The 24-nucleotide siRNAs associated with RdDM are broadly grouped into two types: type I (dependent on both plant specifc RNA polymerases: Pol IV and Pol V) and type II (only dependent on Pol IV).[13] Regardless of type, the siRNAs abundance was reduced in *sr45-1* mutant plants (**Fig. 4A**), suggesting that SR45 acts in the pathway at steps prior to the production of small RNAs.

Previous studies have shown that ARGONAUTE 4 (AGO4) protein is destabilized in mutants upstream of siRNA biogenesis.[14] To test whether this holds true for *sr45-1*, we examined *AGO4* transcript levels by RT-PCR and RNA gel blots in three different tissues. We observed neither significant alteration in *AGO4* expression pattern nor major splicing variants in *sr45-1* relative to wild type (**Fig. 4B and C**). However, protein gel blot analyses revealed a slight, but reproducible decrease of AGO4 protein in *sr45-1* mutants. In addition, the effect of *sr45-1* on AGO4 levels was increased in *dcl3-1* background (**Fig. 4C**), reinforcing the hypothesis of those two genes cooperating in the regulation of the RdDM pathway. To further confirm the observed reduction of AGO4 levels, we analyzed the nuclear localization pattern of a complementing epitope-tagged version of AGO4 in the *sr45-1* background by immunoflourescence. Consistent with protein gel blot data, we observed a decrease in AGO4 abundance in *sr45-1*; nonetheless the localization pattern of AGO4 was similar to wild type (**Fig. 4D**).

The *FLC* locus, which is silenced by a DNA methylation-independent mechanism,[15] is also partially de-repressed in the *sr45-1*

mutant background (**Fig. S4**).[8] It is interesting to note that DCL3 has been previously reported to be required for *FLC* silencing despite the lack of transcriptional control by DNA methylation.[16] This DCL3 regulation of *FLC* is probably through small RNAs matching its 3' region.[17] Furthermore, the de-repression of *FLC* is enhanced in the *dcl3-1 sr45-1* double mutant (**Fig. S4**).

In sum, we have discovered a known spliceosome gene that is required for RdDM. It cannot be ruled out that SR45 may be involved in the splicing of an RdDM factor, thus the methylation phenotype is a secondary effect. Alternatively, given its small RNA phenotype, it potentially has a novel function in siRNA processing. It is worth noting that the nuclear cap-binding complex, which is involved in pre-mRNA splicing, has a role in a distinct DICER-LIKE1-dependent micro RNA pathway.[18] This suggests SR45, and perhaps other spliceosome factors, may indeed play a direct role in siRNA accumulation as well. We screened a number of known or putative spliceosome factors as part of our screen; however, *sr45* was the only one with an *FWA*-dependent flowering-time defect (**Table S1**). Interestingly, *sr45* shares a very similar phenotype as *dcl3*, even at the *FLC* locus which is not an RdDM target. This suggests that these two proteins likely work in concert to control RNA-mediated silencing.

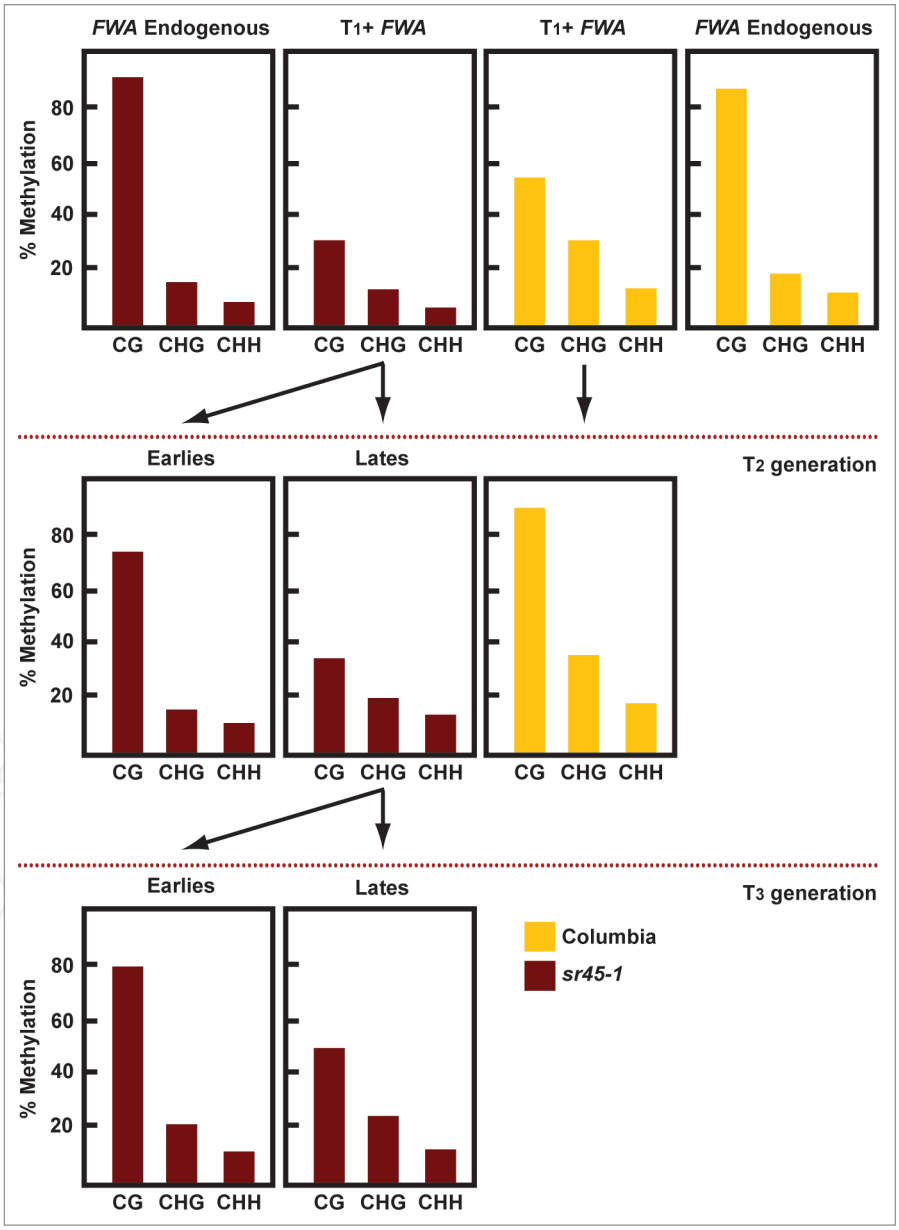


Figure 2. Analysis of the *FWA* transgene methylation generationally. Methylation levels at endogenous and transgenic *FWA* after *FWA* transformation. Endogenous *FWA* was only analyzed in the T1 generation. Transgenic *FWA* was analyzed during three generations after transformation.

Materials and Methods

Plant materials. We used the following Arabidopsisstrains:ThewildtypeColumbia; the recessive *sr45-1* (SALK_004132) and *dcl3-1* (SALK_005512); the Myc-tagged complementing AGO4 line used for immunofluorescence and protein gel blots is described in Li.[14]

***FWA* transformation and flowering-time analysis.** We performed *FWA* transformation using an AGL0 *Agrobacterium tumefaciens* strain carrying a pCAMBIA3300 vector with an engineered version of *FWA* in which an EcoRI site was converted into a BglII site. For selection, we sprayed the resultant T1 population with a 1:1,000 dilution of Finale™. We measured flowering time of resistant plants as the total number of leaves (rosette and cauline leaves) developed by a plant.

Bisulfite analysis. We performed sodium bisulfite sequencing using EZ DNA Methylation Gold (Zymo Research) reagents for conversion of plant genomic DNA extracted from floral tissue using a standard CTAB protocol. Following amplification of bisulfite treated DNA by PCR, we cloned the resulting PCR fragments into pCR2.1-TOPO (Invitrogen) and analyzed 15–22 clones per sample. The *FWA* transgene was distinguished from the endogene by BglII digestion prior to bisulfite treatment (see FWA Transformation methods) and elimination of any clones containing Col-0 polymorphisms from the data set after sequencing. All primers are listed in **Table S2**.

Southern blotting. DNA from young flowers was extracted using a standard CTAB protocol. One microgram of genomic DNA was digested overnight with *Msp*I. The digestion was run on a 1% agarose gel, transferred to Hybond N^+ membranes, blocked and washed according to manufacturer instructions

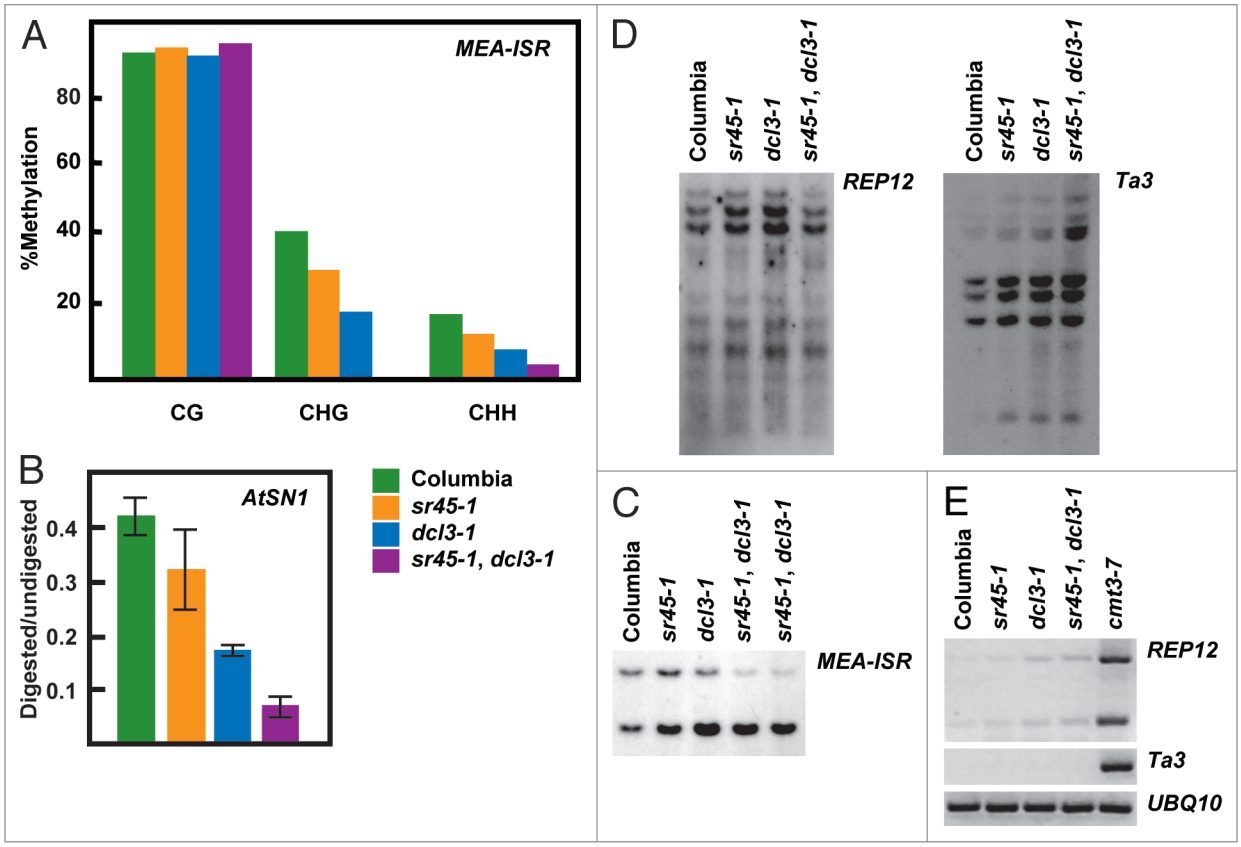
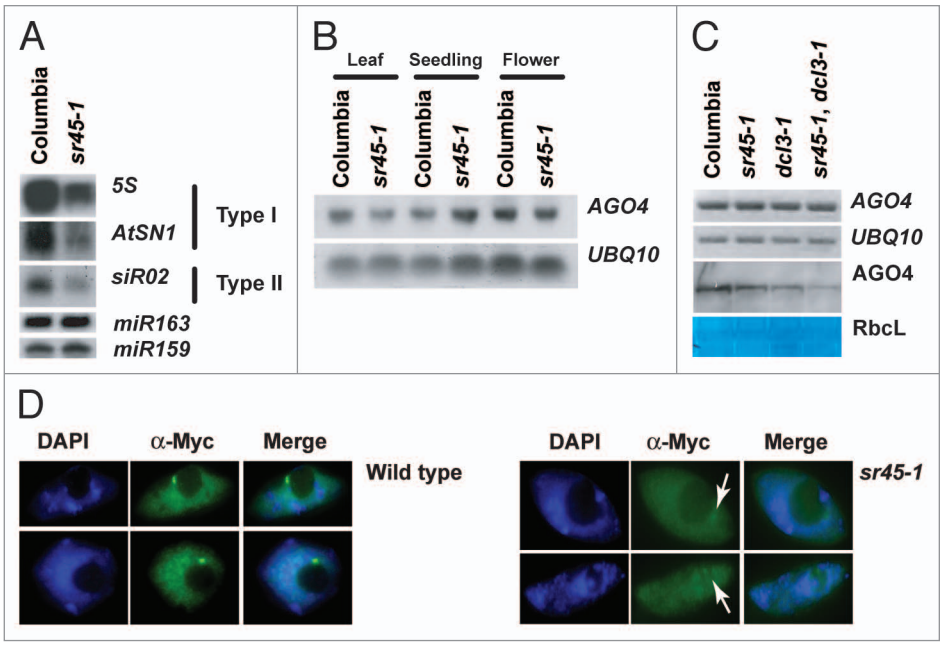


Figure 3. *sr45-1* maintenance DNA methylation phenotype. (A) Sodium bisulfite analysis of an 180 base-pair region of the *MEA-ISR* locus. (B) *AtSN1* Chop-qPCR assay. Genomic DNA was digested with the methylation sensitive enzyme HaeIII, which recognizes three sites in *AtSN1*. Amplification of *AtSN1* was quantified by Real Time PCR, and signal was normalized to undigested DNA. HaeIII, is blocked by C methylation in GGCC context. (C) *MEA-ISR* DNA gel blot. *Msp*I digested genomic DNA was probed with *MEA-ISR*. (D) *REP12* and *Ta3* DNA gel blot. *Msp*I digested genomic DNA was probed with *REP12* or *Ta3*. *Msp*I is blocked by methylation of the external C in CCGG context. (E) RT-PCR showing expression levels of *REP12* and *Ta3*. *UBQ10* expression is showed as a loading control.

Figure 4. Placement of SR45 in RdDM pathway. (A) RNA gel blots showing siRNAs abundance at both type I and II loci. Hybridization with *miR163* is shown as a loading control for *5S* siRNAs and hybridization with *miR159* is shown as a loading control for *AtSN1* and *siR02*. (B) RNA gel blot showing *AGO4* expression in leaves, seedlings and flowers. Hybridization with *UBQ10* is shown as a loading control. (C) RT-PCR and protein gel blot showing the expression and abundance of *AGO4*/AGO4. *UBQ10* expression is showed as a loading control for RT-PCR and amido black staining of the RUBISCO large subunit is shown as a loading control for the protein gel blot. (D) Immunofluorescent microscopy showing AGO4 localization in 4xmyc::AGO4 (Columbia) and 4xmyc::AGO4 (*sr45-1*) backgrounds. White arrows indicate the position of AGO4 in the *sr45-1* part.



(GE Healthcare). Membranes were probed with a PCR product radiolabeled with α ^{32}P-dCTP using the Megaprime DNA Labeling System. *MEA-ISR*, *Ta3* and *REP12* PCR products for probing were generated with primers listed in **Table S2**.

Small RNA northern blotting. Detection of small RNAs was performed exactly as described in Law.[19] Oligonucleotide sequences used for probing can be found in **Table S2**.

Immunofluorescent microscopy. Detection of Myc-tagged AGO4 protein was performed exactly as described in Li.[14] Primary mouse monoclonal anti-Myc (Covance 9E10) was used at a 1:200 dilution. Secondary anti-mouse FITC (Abcam) was used at a 1:200 dilution. DNA was stained using Vectashield mounting medium containing DAPI (Vector Laboratories).

RNA gel blotting. RNA was extracted from the indicated tissue using Trizol reagent (Invitrogen). RNA gel blots were performed as described in Henderson.[20] *AGO4* and *UBQ10* PCR products used for probing were generated with primers listed in **Table S2**.

Bisulfite cutting assay. DNA was extracted and bisulfite treated as described above. The cutting assay was performed exactly as described in Chan.[21]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental material can be found at:
www.landesbioscience.com/journals/epi/article/18782

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