



Published in final edited form as:

Nat Genet. 2009 May ; 41(5): 630–634. doi:10.1038/ng.365.

## RNA Polymerase V transcription guides ARGONAUTE4 to chromatin

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### Summary

Retrotransposons and repetitive DNA elements in eukaryotes are silenced by small RNA-directed heterochromatin formation. In *Arabidopsis*, this process involves 24 nt siRNAs that bind to ARGONAUTE4 (AGO4) and facilitate the targeting of complementary loci<sup>1,2</sup> via unknown mechanisms. Nuclear RNA Polymerase V is an RNA silencing enzyme recently shown to generate noncoding transcripts at loci silenced by 24nt siRNAs<sup>3</sup>. We show that AGO4 physically interacts with these Pol V transcripts and is thereby recruited to the corresponding chromatin. We further show that DEFECTIVE IN MERISTEM SILENCING3 (DMS3), a Structural Maintenance of Chromosomes (SMC) hinge-domain protein<sup>4</sup>, functions in the assembly of Pol V transcription initiation or elongation complexes. Collectively, our data suggest that AGO4 is guided to target loci through base-pairing of associated siRNAs with nascent Pol V transcripts.

*Arabidopsis* Pol V, AGO4, DMS3 and the putative chromatin remodeller, DRD16 function in the silencing of siRNA-homologous loci at one or more steps downstream of siRNA biogenesis<sup>3,7–10</sup>. Recently, we showed that DRD1 facilitates Pol V transcription of noncoding RNAs at target loci, revealing a functional relationship between these two activities<sup>3</sup>. However, the functional relationships, if any between AGO4, DMS3 and Pol V transcription are unclear.

Mutations disrupting *NRPE1* (the Pol V largest subunit), *AGO4* or *DMS3* cause similar losses of RNA-directed DNA methylation at *AtSN1* retrotransposons, *INTERGENIC REGION 5* (IGN5) and a retroelement solo LTR locus (Figs. 1A and 1B). Likewise, histone H3 lysine 27 monomethylation (H3K27me1), a characteristic of silenced heterochromatin, is reduced at these loci in *nrpe1*, *ago4* and *dms3* mutants compared to wild-type plants (ecotype Col-0) (Fig 1C). These results indicate that Pol V, AGO4 and DMS3 collaborate in the establishment of repressive chromatin modifications. At the *solo LTR* locus transcribed by RNA polymerase II (Pol II), chromatin immunoprecipitation (ChIP) shows that levels of diacetylated histone H3 (H3Ac2; acetylated on lysines 9 and 14), a mark of active chromatin, increases in the mutants (Fig 1D), coincident with increased Pol II occupancy of the locus (Fig. 1E). At *IGN5* and *AtSN1*, which lack associated Pol II (Fig. 1E), no increase

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Author contributions: T. R. generated anti-AGO4 antibody; J.R.H. and T. R. assayed NRPE1-AGO4 interactions; J.R.H. produced Fig. 4D; A.T.W. performed all remaining experiments. A.T.W. and C.S.P. wrote the manuscript.

in histone H3 acetylation is observed in the mutants (Fig. 1D). *AtSN1* elements are thought to be transcribed by Pol III, therefore differences in H3 acetylation at the *solo LTR* and *AtSN1* loci may reflect the different polymerases involved.

AGO4 and Pol V colocalize in a nucleolus-associated Cajal body<sup>7,8</sup> that is distant from the target loci subjected to siRNA-mediated silencing. These observations have suggested that AGO4-siRNA complexes might guide Pol V to the target loci<sup>7,8</sup>. To test this hypothesis, we asked whether production of Pol V transcripts is AGO4-dependent. At intergenic regions IGN5 and IGN63, Pol V transcripts are lost or substantially reduced in the Pol V mutant (*nrpe1*) but not in the *ago4* mutant (Fig. 2A); in fact, IGN5 transcript levels increase by ~50% in *ago4* (Fig. 2B). This increase in transcript levels is Pol V-dependent, as shown by analysis of the *nrpe1 ago4* double mutant (Fig. 2A). In the *rdr2* (*rna-dependent rna polymerase2*) mutant, which abolishes 24 nt siRNA biogenesis<sup>11,12</sup>, or in an *rdr2 ago 4* double mutant, Pol V transcript levels are unaffected compared to wild-type (Col-0) plants. We conclude that AGO4-siRNA complexes are dispensable for Pol V transcription at target loci, arguing against the hypothesis that AGO4-siRNA complexes guide Pol V to target loci. The functional significance of AGO4 and Pol V colocalization in Cajal bodies is unclear but could reflect independent protein processing/assembly or storage functions that are unrelated to RISC (RNA-induced silencing complex) assembly.

To test an alternative hypothesis, that AGO4-siRNA complexes are recruited to chromatin in a Pol V-dependent manner, we assayed AGO4 associations with target loci using ChIP (Fig. 3). In wild-type (Col-0) plants, *solo LTR*, *IGN5*, *AtSN1* and *IGN6* loci are all enriched upon AGO4-ChIP, whereas only background levels are observed in *ago4* or *nrpe1* mutants or in control ChIP reactions lacking anti-AGO4 antibody (Fig. 3A). These findings indicate that AGO4 interacts with target locus chromatin, and does so in a Pol V-dependent manner. AGO4-chromatin interactions are not diminished by mutation of *DRM2* (Fig. 3A), the *de novo* DNA methyltransferase that carries out siRNA and AGO4-dependent cytosine methylation<sup>13,14</sup>. Collectively, these data indicate that Pol V, but not pre-existing DNA methylation, is required to recruit AGO4 to chromatin.

To test whether Pol V enzymatic activity is required for AGO4 binding to chromatin we examined AGO4-chromatin associations in *nrpe1* mutants that had been transformed with either a full-length, wild-type *NRPE1* transgene or an equivalent transgene bearing point mutations within the Metal A motif of the active site (*NPPE1 ASM* transgene). The active site point mutations do not affect NRPE1 stability or its association with the second-largest subunit but eliminate Pol V transcripts and Pol V biological activity<sup>3,15</sup>. Whereas the wild-type *NRPE1* genomic transgene (*NRPE1 wt*) restored AGO4 interaction with the *solo LTR*, *IGN5*, *AtSN1* and *IGN6* loci in the *nrpe1* mutant background (Fig. 3B), the active-site mutant (*NRPE1 ASM*) failed to do so. Immunoblotting ruled out the trivial explanation that AGO4 protein levels might be differentially affected by the *nrpe1* mutation or the *NRPE1* transgenes (Fig. 3C) and also demonstrates that the antibody specifically recognizes AGO4, which is absent in the *ago4* mutant. Collectively, the data indicate that Pol V transcriptional activity is required to recruit AGO4 to chromatin.

Base-pairing between AGO4-associated siRNAs and nascent Pol V transcripts could be a mechanism by which Pol V transcription recruits AGO4 to target loci. To test this hypothesis, we used RNA immunoprecipitation to ask whether AGO4 associates with Pol V transcripts *in vivo*. In wild-type (Col-0) plants, anti-AGO4 antibody immunoprecipitates *IGN5* and *IGN6* Pol V transcripts<sup>3</sup> (Fig. 4A). Important controls show that Pol V transcripts are not immunoprecipitated in the *ago4* or *nrpe1* mutant backgrounds. Anti-AGO4 immunoprecipitation of *IGN5* or *IGN6* RNAs was also reduced or eliminated in *rdr2* mutant plants, indicating that AGO4-Pol V transcript interactions are dependent on siRNAs. However, in the absence of siRNA biogenesis, as in the *rdr2*, *nrpd1*, *nrpd2/nrpe2* or *dcl2,3,4* mutants, AGO4 protein levels drop below the limits of immunoblot detection<sup>7,8</sup> (Fig. 4B–D). By contrast, AGO4 protein levels are unaffected in the *nrpe1* or *drm2* mutants, which act downstream of siRNA biogenesis (Fig. 4B–D). The instability of AGO4 in the absence of siRNAs complicates the interpretation of these results. Although we favor the hypothesis that siRNA-Pol V transcript base-pairing is responsible for AGO4 association with Pol V transcripts, we cannot rule out the possibility that AGO4 binds Pol V transcripts directly, with siRNAs merely being required for AGO4 stability.

*DMS3* was recently identified as a gene required for RNA-directed DNA methylation that acts at an unspecified step downstream of siRNA biogenesis<sup>4</sup>. The encoded protein shares sequence similarity with the hinge domain regions of Structural Maintenance of Chromosomes (SMC) proteins, such as the core proteins of cohesin and condensin complexes<sup>16</sup>, suggesting a chromatin-related function. We find that at *IGN5*, *IGN6* and *AtSN1* loci, Pol V transcripts are substantially reduced or absent in *dms3* mutant plants, as in *nrpe1* (Fig. 5A) or *drd1* mutants<sup>3</sup>. Likewise, transcriptional suppression of *AtSN1* and *solo LTR* elements is similarly disrupted in *dms3* and *nrpe1* mutants (Fig. 5B). Chromatin immunoprecipitation using an anti-NRPE1 antibody revealed that in the *dms3* mutant, Pol V-chromatin associations are reduced to background levels resembling the actin and *nrpe1* mutant controls (Fig. 5C). Collectively, the data of Figure 5 indicate that DMS3 is required for Pol V transcription, as shown previously for the chromatin remodeller, DRD13. The loss of detectable Pol V-chromatin association in *dms3* or *drd1* mutants suggests that these chromatin proteins participate in the assembly of Pol V transcription complexes.

Our results suggest that siRNAs and Pol V transcripts are produced by independent pathways that intersect to bring about heterochromatin formation and gene silencing (Fig. 6). In one pathway, Pol IV, RDR2 and DCL3 collaborate to produce 24 nt siRNAs that associate with AGO4<sup>1</sup>. Independent of this pathway, DRD1 and DMS3 facilitate noncoding Pol V transcription at target loci. AGO4's interaction with Pol V transcripts, and the fact that AGO4 association with chromatin requires the Pol V active site, suggests that siRNA-AGO4 complexes are guided to target loci by interacting with Pol V transcripts. It has also been reported that AGO4 can interact with the C-terminal domain (CTD) of NRPE1 *in vitro*<sup>7,17</sup> and *in vivo*<sup>7</sup>, suggesting that Pol V might recruit AGO4 directly, in an RNA-independent manner. However, we have been unable to detect AGO4-Pol V associations *in vivo* using immunoprecipitation and subsequent immunoblotting nor by mass spectrometric analysis of affinity purified Pol V (data not shown) suggesting that any interactions between AGO4 and

Pol V may be weak or transient. We suggest that AGO4 recruitment to chromatin is primarily an RNA-mediated process but may also involve protein-protein interactions.

In fission yeast, artificial tethering of the RNA Induced Transcriptional Silencing (RITS) complex to *ura4* pre-mRNAs is sufficient to induce heterochromatin formation at the normally euchromatic *ura4*<sup>+</sup> locus<sup>18</sup>. These, and other, results are consistent with the hypothesis that fission yeast silencing complexes are guided to chromatin via associations with nascent Pol II transcripts<sup>19</sup>. Our findings suggest that plants and yeast are fundamentally similar in their use of RNA guidance mechanisms for recruiting Argonaute-containing transcriptional silencing complexes to target loci. It is intriguing that plants should have evolved a unique RNA polymerase, Pol V whose specialized role appears to be the generation of noncoding RNAs that can serve as scaffolds for Argonaute recruitment.

## Methods

### Plant strains

*Arabidopsis thaliana nrpe1 (nrpd1b-11)* was described previously<sup>8</sup>. The *dms3-4* mutant (SALK\_125019C) of locus At3g49250 was obtained from the Arabidopsis Biological Resource Center. The *dcl2*, *dcl3*, *dcl4* triple mutant (*dcl234*) was provided by Todd Blevins. The *ago4-1* mutant (Ler ecotype background) was kindly provided by Steve Jacobsen (UCLA) and was introgressed into the Col-0 background by 3 rounds of backcrossing.

### Antibodies

Anti-Pol II (anti-NRPB2) was described previously<sup>20</sup>. Anti H3K27me1 antibody #883521 was provided by Thomas Jenuwein. Antibody against diacetyl-H3 (K9 and K14) was obtained from Millipore (cat. #06599, lot #JBC1349702). Rabbit anti-NRPE1 antibody has been described<sup>9</sup>. Rabbit anti-AGO4 was raised against a C-terminal portion of the protein (amino acids 573–924) expressed in bacteria.

### RNA and DNA analysis

RNA isolation, RT-PCR and real-time quantitative PCR were performed as described<sup>3</sup> except that real time quantitative PCR analysis of the IGN5 locus was performed using the following oligonucleotides: A195: ACATGAAGAAAGCCCAAACCA and A196: GGCCGAATAACAGCAAGTCCT. Densitometric analysis of DNA resolved by agarose gel electrophoresis was performed using ImageJ (<http://rsbweb.nih.gov/ij/>).

### ChIP and RNA IP

ChIP and RNA IP were performed as described<sup>3</sup> except that for ChIP with anti-AGO4, RNase A was added during IP, washes with TE buffer were omitted, immune complexes were eluted with 100 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS for 10 min at room temperature and a second elution at 65°C was performed. Crosslinking was reversed at 65°C for 1h in the presence of 40 µg Proteinase K (Invitrogen). DNA was purified by extraction with phenol:chloroform and ethanol precipitation. DNA recovery was assayed by PCR using 1.5u Platinum Taq (Invitrogen).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

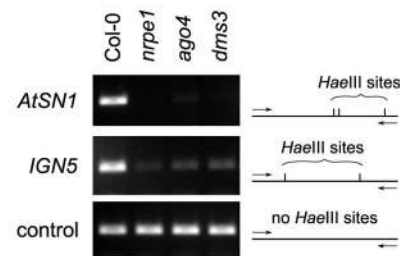
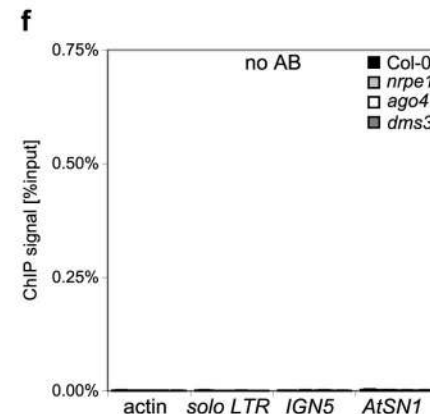
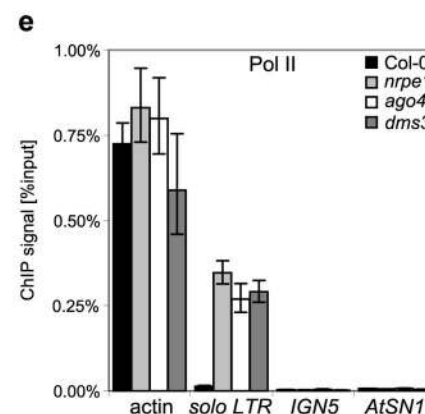
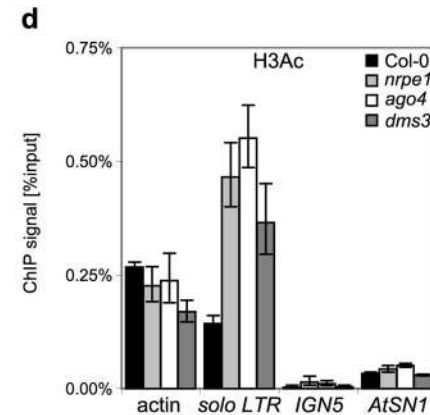
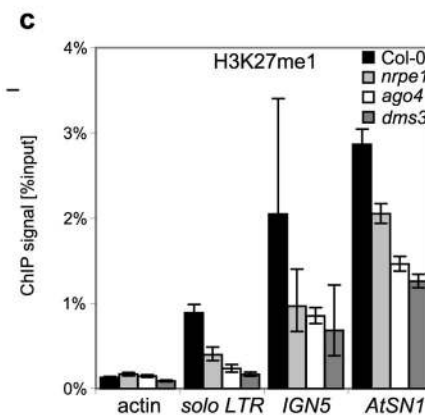
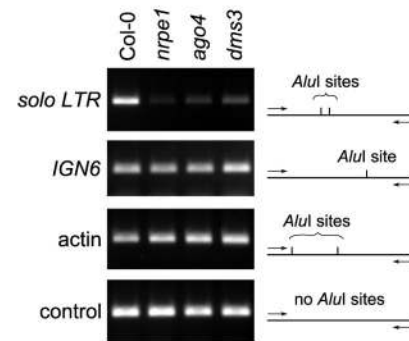
## Acknowledgments

Our work is supported by NIH grant GM077590. The content of the paper is the sole responsibility of the authors and does not necessarily reflect the views of the NIH.

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**a** *HaeIII* DNA methylation test**b** *AluI* DNA methylation test

**Figure 1. Pol V, AGO4 and DMS3 work non-redundantly in heterochromatin formation**  
 (A, B) DNA methylation analysis at the *AtSN1*, *IGN5* and *solo LTR* loci in *nrpe1*, *ago4* and *dms3* mutants. Genomic DNA was digested with *HaeIII* (A) or *AluI* (B) methylation-sensitive restriction endonucleases followed by PCR. Sequences lacking *HaeIII* sites (*actin* 2; panel A) or *AluI* sites (*IGN5*, panel B) served as controls to show that equivalent amounts of DNA were tested in all reactions.



(C–D) ChIP analysis of H3K27me1 (C) and H3Ac2 (D) levels in *nrpe1*, *ago4* and *dms3* mutants. Histograms show means  $\pm$  standard deviations obtained from three independent amplifications.

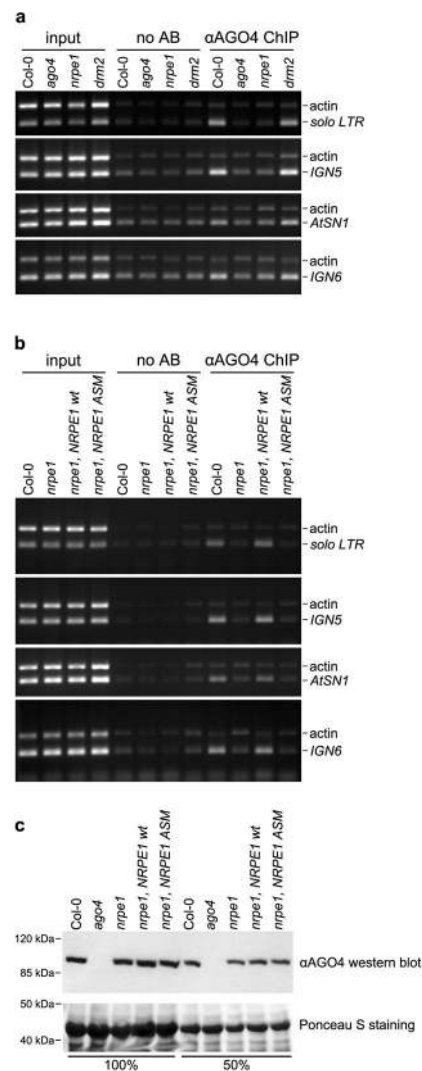
(E) ChIP analysis of Pol II binding to chromatin in *nrpe1*, *ago4* and *dms3* mutants. Histograms show means  $\pm$  standard deviations obtained from three independent amplifications.

(F) Control ChIP reactions performed in the absence of antibody reveal background signal levels.



(A) Strand specific RT-PCR of Pol V transcription at *IGN5* and *IGN6* in *ago4* and *rdr2* mutants as well as *nrpe1 ago4*, and *rdr2 ago4* double mutants. Wt sibling is a wild type sibling of the *ago4* mutant identified in a segregating family. Actin RT-PCR products and ethidium bromide-stained rRNAs resolved by agarose gel electrophoresis serve as loading controls. To control for background DNA contamination, a reaction using *IGN5* top strand primers but no reverse transcriptase (no RT) was performed. No RNA (0  $\mu$ g) controls are provided for all primer pairs.

Nat Genet. Author manuscript; available in PMC 2009 November 01.



**Figure 3. Pol V transcription is necessary for AGO4-chromatin interactions**

(A) ChIP data showing AGO4 binding to chromatin at *solo LTR*, *IGN5*, *AtSN1* and *IGN6* loci in *ago4*, *nrpe1* and *drm2* mutants. DNA purified from input chromatin samples, chromatin subjected to the immunoprecipitation procedure in the absence of antibody (no AB) and chromatin immunoprecipitated using anti-AGO4 antibody ( $\alpha$ AGO4) was amplified by PCR using locus-specific primers. Primers amplifying the *Actin 2* locus served as an internal control.

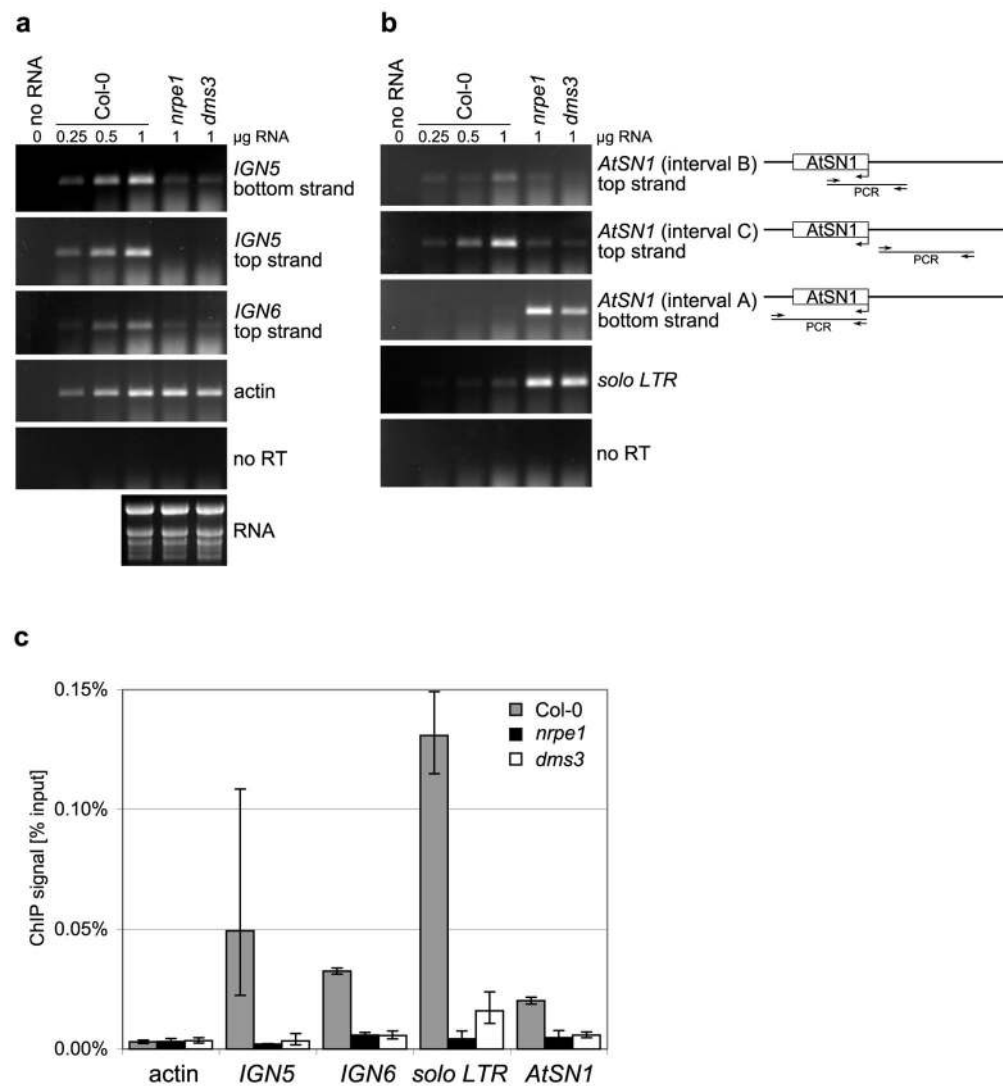
(B) ChIP data showing AGO4 binding to chromatin at *solo LTR*, *IGN5*, *AtSN1* and *IGN6* loci in *nrpe1* mutant, *nrpe1* mutant transformed with a wild type *NRPE1* transgene (*NRPE1 wt*) and *nrpe1* mutant transformed with an *NRPE1* active site mutant transgene (*NRPE1 ASM*).

(C) Immunoblot detection of AGO4 in protein extracts of wild-type (Col-0), *ago4*, *nrpe1*, or *nrpe1* transformed with either a wild type *NRPE1* transgene (*NRPE1 wt*) or an *NRPE1* active site mutant transgene (*NRPE1 ASM*). Ponceau S staining revealed equal loading of lanes. 100% and 50% sample loadings demonstrate that the assay is semi-quantitative.



(C) Immunoblot detection of AGO4 in protein extracts of wild-type (Col-0), *rdr2*, *dcl3*, *dcl234* or *nrpe1* mutants. Asterisks denote nonspecific bands.

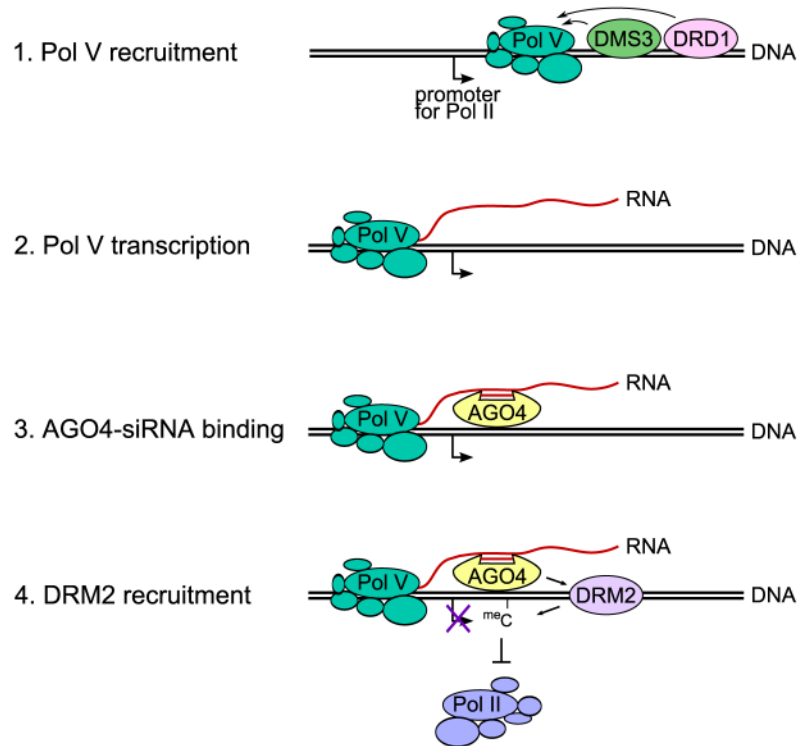
(D) Immunoblot detection of AGO4 in protein extracts of wild-type (Col-0), *nrpd1* (Pol IV) *nrpe1* (Pol V), *nrpd2/nrpe2* (shared subunit of Pol IV and Pol V), or *rdr2* mutants. Asterisks denote nonspecific bands.



**Figure 5. The SMC hinge-domain protein, DMS3 is required for Pol V transcription and detectable Pol V-chromatin interactions**

(A) Strand-specific RT-PCR detection of Pol V transcripts at *IGN5* and *IGN6* and *AtSN1* in wild-type (Col-0) and *nrpe1* and *dms3* mutants. Derepression of Pol II transcripts at the solo LTR and putative Pol III transcripts at *AtSN1* in the *nrpe1* and *dms3* mutants is shown in the right panel. Actin RT-PCR products and ethidium bromide-stained rRNAs resolved by agarose gel electrophoresis serve as loading controls. To control for background DNA contamination, a reaction using *IGN5* bottom strand and *AtSN1* (interval B) primers, but no reverse transcriptase (no RT) was performed. No RNA (0 µg) controls are provided for all primer pairs.

(B) ChIP with anti-NRPE1 antibody in Col-0 wild-type, *nrpe1* and *dms3* mutants followed by real-time PCR. Histograms show means  $\pm$  standard deviations obtained from three independent amplifications.

**Figure 6.**

A model for Pol V and siRNA-dependent heterochromatin formation. DMS3 and DRD1 mediate the assembly of Pol V initiation and/or elongation complexes and the production of Pol V transcripts. AGO4-siRNA complexes recognize target loci via base-pairing of siRNAs with nascent Pol V transcripts. AGO4 subsequently recruits chromatin modifying activities including the *de novo* DNA methyltransferase DRM2 and histone modifying enzymes via unknown mechanisms.