LETTERS

Distinct catalytic and non-catalytic roles of ARGONAUTE4 in RNA-directed DNA methylation

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DNA methylation has important functions in stable, transcriptional gene silencing, immobilization of transposable elements and genome organization[1]. In *Arabidopsis*, DNA methylation can be induced by double-stranded RNA through the RNA interference (RNAi) pathway, a response known as RNA-directed DNA methylation[2]. This requires a specialized set of RNAi components, including ARGONAUTE4 (AGO4)[3–6]. Here we show that AGO4 binds to small RNAs including small interfering RNAs (siRNAs) originating from transposable and repetitive elements, and cleaves target RNA transcripts. Single mutations in the Asp-Asp-His catalytic motif of AGO4 do not affect siRNA-binding activity but abolish its catalytic potential. siRNA accumulation and non-CpG DNA methylation at some loci require the catalytic activity of AGO4, whereas others are less dependent on this activity. Our results are consistent with a model in which AGO4 can function at target loci through two distinct and separable mechanisms. First, AGO4 can recruit components that signal DNA methylation in a manner independent of its catalytic activity. Second, AGO4 catalytic activity can be crucial for the generation of secondary siRNAs that reinforce its repressive effects.

RNA-directed DNA methylation (RdDM) involves a class of siRNAs about 24 nucleotides (nt) in length, which are believed to confer sequence specificity on the process. *Arabidopsis* has evolved a set of RNAi components that are specialized for RdDM, including Dicer-like 3 (DCL3), RNA-dependent RNA polymerase 2 (RDR2), RNA polymerase IV and ARGONAUTE4 (AGO4)[3,4,7,8]. Mutations in these proteins can lead to decreased accumulation of siRNAs, decreased AGO4 stability[9], and decreased DNA methylation at many endogenous loci including transposons and repetitive elements[3–8,10]. It is highly probable that RNA polymerase IV, RDR2 and DCL3 are components of the siRNA biogenesis machinery. AGO4 is the prime candidate for the component of the effector complex that directs DNA methylation as guided by siRNAs.

We therefore tested whether AGO4 exists in a complex with siRNAs *in vivo*. We generated an *Arabidopsis* Landsberg *erecta* (La-*er*) transgenic line expressing a tandem affinity purification (TAP)-tagged AGO4 protein. This protein was recovered from whole plant extracts[11] and its associated RNAs were examined by SYBR-gold staining. TAP-AGO4 was associated predominantly with small RNAs about 24 nt in length (Fig. 1a). Parallel examination of AGO1 complexes showed prominent small RNAs about 21 nt in length[12,13] (Fig. 1a). Northern blotting revealed the binding of AGO4 to siRNAs originating from known transposons, specifically *AtSN1* (ref. 14), *AtMu1* (ref. 15) and a repeated sequence, *MEA-ISR* (ref. 16) (Fig. 1b), whose methylation is known to be controlled by RNAi[4].

To obtain a more complete catalogue of the small RNAs associated with AGO4, we cloned and subjected them to sequencing as described[17]. For comparison, the total small RNA population, ranging from 18 to 28 nt, and small RNAs associated with AGO1 were also sequenced. In all, 74,390 sequences were obtained for the whole population (total), and 55,497 and 193,167 sequences were obtained from AGO4 and AGO1 complexes, respectively. As a quality control, we mapped all candidate small RNA sequences to the *Arabidopsis* genome and found that 51,294 (total), 21,198 (AGO4) and 152,088 (AGO1) sequences perfectly matched at least one location (Supplementary Table S1). Only small RNAs passing this quality assessment were used in further analyses.

Small RNA sequences from the total population showed two discernible peaks at 21 and 24 nt. Most AGO4-associated RNAs were 23–24 nt in length, whereas AGO1-associated small RNAs were almost exclusively 21 nt (Fig. 1c); 10,058 (47%) of the AGO4-associated RNAs matched repetitive sequences in the genome, whereas only 15% of total and 3% of the AGO1-associated RNAs were repeat-derived (Supplementary Table S2). Such repeats comprise 17 of the 18 different types documented in Repbase[18] (Supplementary Table S3), with the top 30 families accounting for more than 50% of all matches (Supplementary Table S4). Genomic matches to AGO4-associated small RNAs were particularly dense in pericentromeric regions, reflecting their high concentration of repetitive sequences (Fig. 1d, and Supplementary Figs S1–S4). Although we cannot unambiguously determine whether a given small RNA was derived from a particular repeat copy, previous studies indicate that AGO4 complexes can act *in trans* to direct RdDM at matching loci[5,6]. One must therefore consider these plots as reflecting sites of possible action rather than sites of possible origin.

In all, 91% of AGO1-associated small RNAs matched known microRNAs (miRNAs) (Supplementary Table S5), a result consistent with the demonstrated role of AGO1 in miRNA-mediated control of plant development[12,13,19]. Only a small fraction of AGO4-associated RNAs (3%) matched microRNAs. However, a subset of the microRNAs found in AGO4 complexes showed preferential association with this protein (Supplementary Fig. S5). This implies selectivity in how individual microRNAs are processed and passed to specific AGO-containing RNA-induced silencing complexes (RISCs). Moreover, it implies that some microRNAs might need to act in concert with AGO4, perhaps in the nucleus, rather than with AGO1 in the cytoplasm. Many AGO4-associated small RNAs were also derived from the sense or antisense strand of genes, pseudogenes and intergenic regions (Supplementary Table S5), indicating that AGO4 might also have a previously unrecognized general role in regulating gene expression.

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ATREP2 (a *Helitron*-like DNA transposon with dispersed repeats in the genome) and *SIMPLEHAT2* (a DNA transposon) both matched abundant small RNAs that are associated with AGO4 (Supplementary Fig. S6a). Both the levels of these small RNAs and non-CpG methylation at these loci were reduced by genetic lesions in the AGO4 pathway (in *RDR2*, *DCL3* and *AGO4*; Supplementary Fig. S6b, c). Taken together, our data indicate that siRNAs associated with AGO4 direct it to target loci, where it can promote non-CpG (CpNpG and CpHpH) methylation.

It is not known how siRNAs act at target loci to direct RdDM. Opposing classes of models involve either pairing between an siRNA and a target DNA (RNA–DNA recognition) or pairing between an siRNA and a nascent RNA transcript (RNA–RNA recognition)[20]. However, methylation of *PHABULOSA* can be directed by a miR165/166 target site that crosses an exon–exon junction, strongly supporting an RNA–RNA recognition model[21]. A crucial question is whether this siRNA–RNA pairing leads to transcript cleavage and whether such cleavage functions in silencing[22].

Alignment of *Arabidopsis* AGO4 with known Slicers, human Ago2 (ref. 23) and *Arabidopsis* AGO1 (refs 12, 13), revealed the presence of the catalytic Asp-Asp-His (DDH) triad in all three proteins[24] (Fig. 2a). To test its catalytic potential directly, we incubated TAP-purified AGO4 with an RNA transcript containing a sequence complementary to identified ATREP2 siRNAs. The target RNA was cleaved by wild-type AGO4 protein (Fig. 2b) but not by mutants containing changes in essential catalytic residues (D660A, D742A and H874A substitutions, respectively; referred to hereafter as DDH mutants), despite similar expression levels and siRNA-binding capacity (Fig. 2c, and Supplementary Fig. S7). We could similarly detect target cleavage on incubation of AGO4 complexes with targets for two AGO4-interacting miRNAs, miR172 and miR390 (Supplementary Fig. S8).

To determine whether catalysis was important for RdDM, we turned to a system of epialleles that could be tracked through an obvious visual phenotype. SUPERMAN is required for proper floral development; when its activity is decreased, plants show an increased number of stamens (an average of ten in comparison with the normal six) and incompletely fused carpels (SUP phenotype). In addition to genetic mutants, there are also SUP epialleles (Clark Kent or *clk*)[25]. When the *clk-3* epiallele is placed in an AGO4-null background (*clk-3/ago4-1*, obtained from the *Arabidopsis* Biological Resource Center), most plants are wild-type (Fig. 3a), although some (20–30%) do retain the SUP phenotype. This indicates that there might be complex regulation of the locus and that SUP silencing in a subset of plants might be maintained in the absence of AGO4. However, when the *clk-3/ago4-1* plants were viewed as a population, CpNpG and CpHpH methylation at SUP were decreased substantially (Fig. 3b, and Supplementary Table S7) in comparison with *clk-st* (a stabilized Clark Kent allele in the presence of wild-type AGO4)[4].

AGO4 or each DDH mutant was expressed under the control of the AGO4 promoter in *clk-3/ago4-1* plants . Pooled samples from about 30 primary transformants (T_1 generation) showed that all proteins were expressed at similar levels (Supplementary Fig. S7). Essentially all T_1 plants transformed with wild-type AGO4 complemented *ago4-1* and displayed the SUP floral phenotype, whereas those with the empty vector did not (Fig. 3a). Intriguingly, all T_1 plants transformed with AGO4 DDH mutants also recovered the SUP phenotype

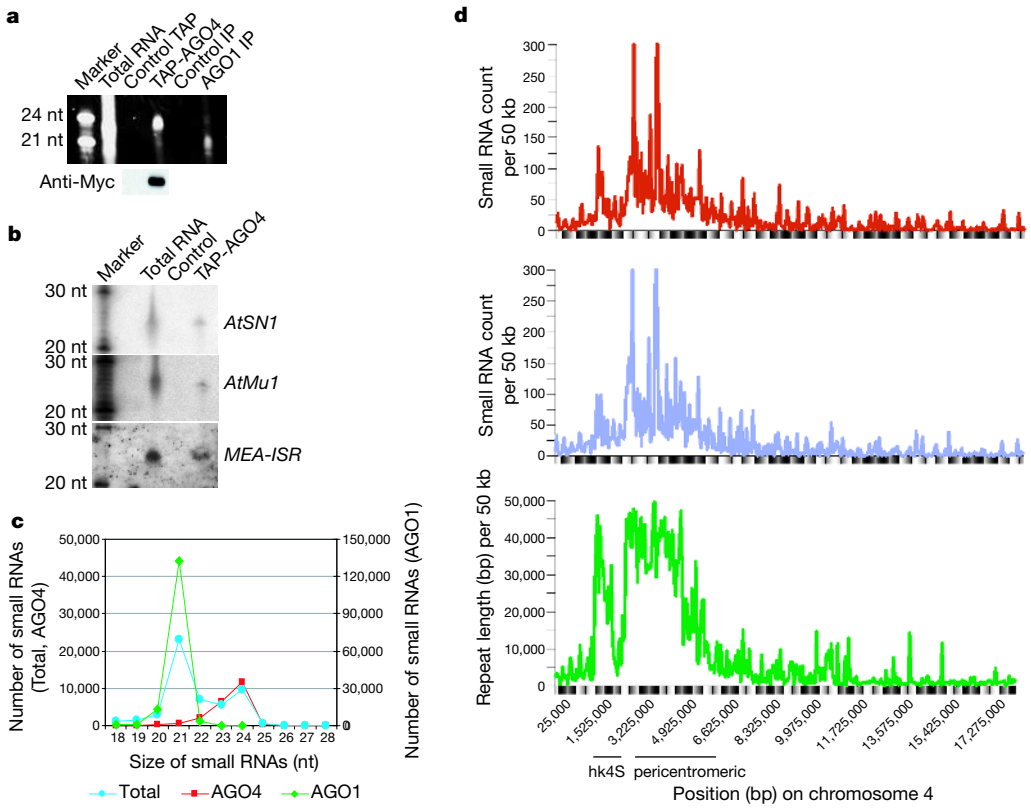

Figure 1 | A catalogue of AGO4-associated small RNAs. **a**, SYBR-gold was used to reveal small RNAs in total *Arabidopsis* RNA, TAP-AGO4 complexes, AGO1 complexes and control purifications (upper panel, as indicated). Western blotting with anti-Myc antibody detected AGO4 in the TAP-AGO4 purification but not in the control purification (lower panel). IP, immunoprecipitation. **b**, Northern blotting was used to detect small RNAs in total RNA, TAP-AGO4 complex and control purifications with the indicated probes. Radioactive RNAs of known sizes were included as markers. **c**, Size distribution of total (cyan), AGO4-associated (red) and AGO1-associated (green) small RNAs. The sets of redundant small RNAs were used to generate a histogram quantifying the number of sequences obtained for each size class. **d**, Chromosome-wide density analysis of the AGO4-associated small RNAs on chromosome 4. The density of small RNAs with perfect matches in the direct strand (upper panel) and the complementary strand (middle panel), and the density of repeats (presented as the total length of repeats (bp); lower panel) in a 50-kilobase sliding window, are plotted. The positions of the pericentromeric region and the heterochromatic knob *hk4S* are marked.

(Fig. 3a). Bisulphite sequencing of about 30 pooled seedlings of each genotype showed that non-CpG methylation was restored to approximately normal (*clk-st*) levels (Fig. 3b, and Supplementary Table S7). Although the regulation of SUP is likely to be complex, an intact AGO4 pathway reinforced silencing at the locus in a manner that did not depend on siRNA-directed RNA cleavage.

Examination of additional loci revealed a more complex picture (Fig. 4a, and Supplementary Table S7). At *AtMu1*, non-CpG methylation decreased in *ago4-1* plants[4] and was fully rescued by AGO4 or its DDH mutants. However, at three other loci, namely *MEA-ISR*, *ATREP2* and *SIMPLEHAT2*, wild-type AGO4 restored non-CpG methylation to normal levels, but the DDH mutants showed greatly decreased potency. For example, at *MEA-ISR*, introduction of AGO4 into *ago4-1* mutants led to a 3.2-fold increase in CpNpG and a 20.9-fold increase in CpHpH methylation. The DDH mutants had from no effect to a roughly 2.1-fold increase in CpNpG and a 2.2-fold to 4.2-fold increase in CpHpH methylation. Thus, the requirement in RdDM for AGO4 catalytic potential varies with the locus.

We next probed the correlation between non-CpG methylation, siRNA production and the effect of inactivating the AGO4 catalytic site. At *AtMu1*, a locus where DDH mutants complemented methylation defects efficiently, loss of AGO4 had no effect in itself on the abundance of *AtMu1* siRNAs, and these species were not increased on expression of any AGO4 variant (Fig. 4b). We were not able to examine the effect of DDH mutations on the accumulation of *SUP* siRNAs because these were below detection limits, as reported previously[4]. For the three repetitive elements for which the wild-type and DDH mutants showed differential effects, another pattern was observed. In all cases, siRNAs were substantially decreased in *ago4-1* mutant plants. Expression of wild-type AGO4 generally restored siRNA levels, but the DDH mutants had much less pronounced effects (Fig. 4b). For example, in the *MEA-ISR* locus, siRNAs in *ago4-1* mutants decreased to 18% of wild-type levels. Wild-type AGO4 restored this to 87% of normal, whereas the DDH mutants rescued siRNAs to only 27–38%. In comparison, siRNA02 was not decreased in *ago4-1* as described previously[6], and the introduction of either catalytic or non-catalytic AGO4 had no effect on overall siRNA02 abundance.

Thus, the catalytic activity of AGO4 was important both for efficient siRNA production and for non-CpG methylation at some loci. At others, where AGO4 loss had little effect on overall siRNA levels, AGO4 loss could still affect non-CpG methylation. However, in these cases a lack of catalytic potential was of little importance to the ability of ectopically expressed AGO4 to restore non-CpG methylation. All of these conclusions were based on multiple independent T_1 transgenesis studies and bisulphite experiments (up to five each), and all results were confirmed with two individual T_2 transgenic lines for

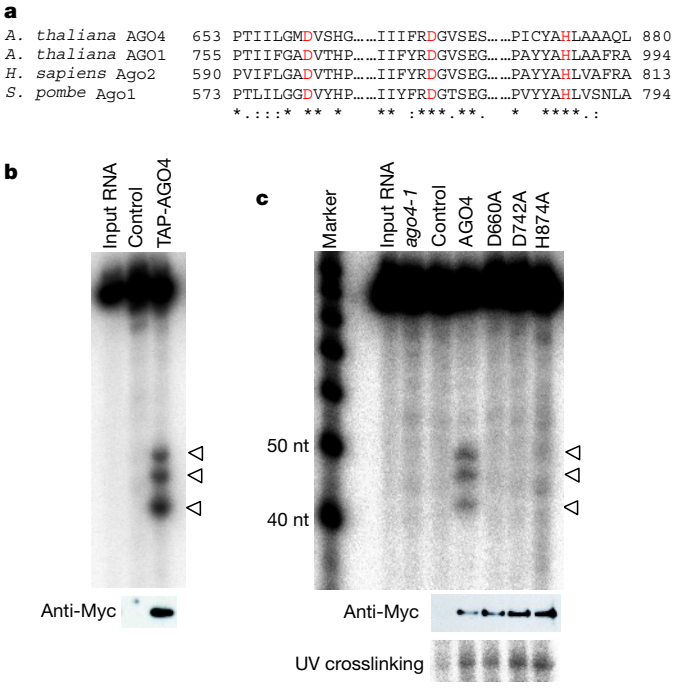

Figure 2 | AGO4 is a Slicer. a, A partial alignment of the PIWI domains of *Arabidopsis* AGO4, AGO1, human Ago2 and *Schizosaccharomyces pombe* Ago1 is shown. The residues forming the catalytic DDH motif are shown in red. The degree of similarity is indicated under the alignment: asterisk indicates identity in all sequences; colon indicates conservative substitutions, and full point indicates semi-conservative substitutions. The starting and ending positions of the sequences are as labelled. **b**, A ^{32}P-cap-labelled synthetic target RNA containing recognition sites for cloned ATREP2 siRNAs was incubated with TAP-purified AGO4 or a control purification. Positions of 5′ products of cleavages guided by three endogenous *ATREP2* siRNAs are indicated by the arrows (upper panel). Western blotting with anti-Myc antibody detected AGO4 in the TAP-AGO4 purification but not in the control purification (lower panel). **c**, The synthetic target RNA was incubated with immunopurified Myc-tagged AGO4 wild-type and DDH mutants (top panel). Decade RNA markers are shown for reference. Western blotting with an anti-Myc antibody detected AGO4 in the immunoprecipitates but not in the control purification (middle panel). AGO4 and control immunoprecipitates, as indicated, were incubated with single-stranded ^{32}P-labelled 24-nt siRNAs bearing photoreactive dT residues at the two 3′ positions[12]. Mixtures were irradiated with ultraviolet (UV) as described in Methods (bottom panel).

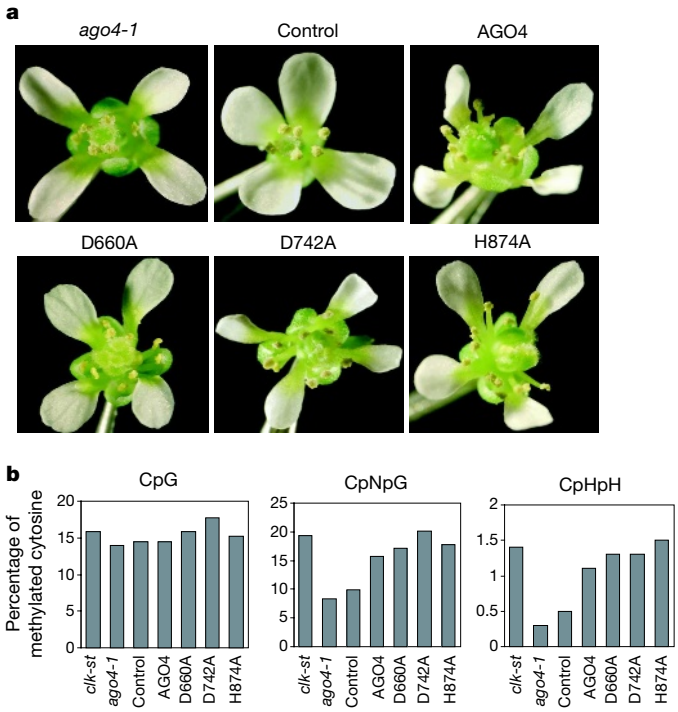

Figure 3 | Slicer activity is not required for non-CpG methylation and silencing at the *SUP* locus. a, Representative flowers from parental *clk-3*/*ago4-1* and plants of the same genotype transformed with vectors as indicated. About 70–80% of the *clk-3*/*ago4-1* plants and plants transformed with empty vector have wild-type flowers with six stamens and two fused carpels, with the remainder having the SUP phenotype (about ten stamens and three incompletely fused carpels; see the text). Essentially all flowers from plants transformed with AGO4 and DDH mutants display the *SUP* phenotype. **b**, CpG (left), CpNpG (centre) and CpHpH (right) methylation of the *SUP* gene was analysed by bisulphite sequencing of genomic DNA prepared from pooled T_1 seedlings. Data from two complete biological replicates were combined. The methylation level is shown by the percentage of methylated cytosine in all sequenced clones. The data in Supplementary Table S7 were used to generate the histograms.

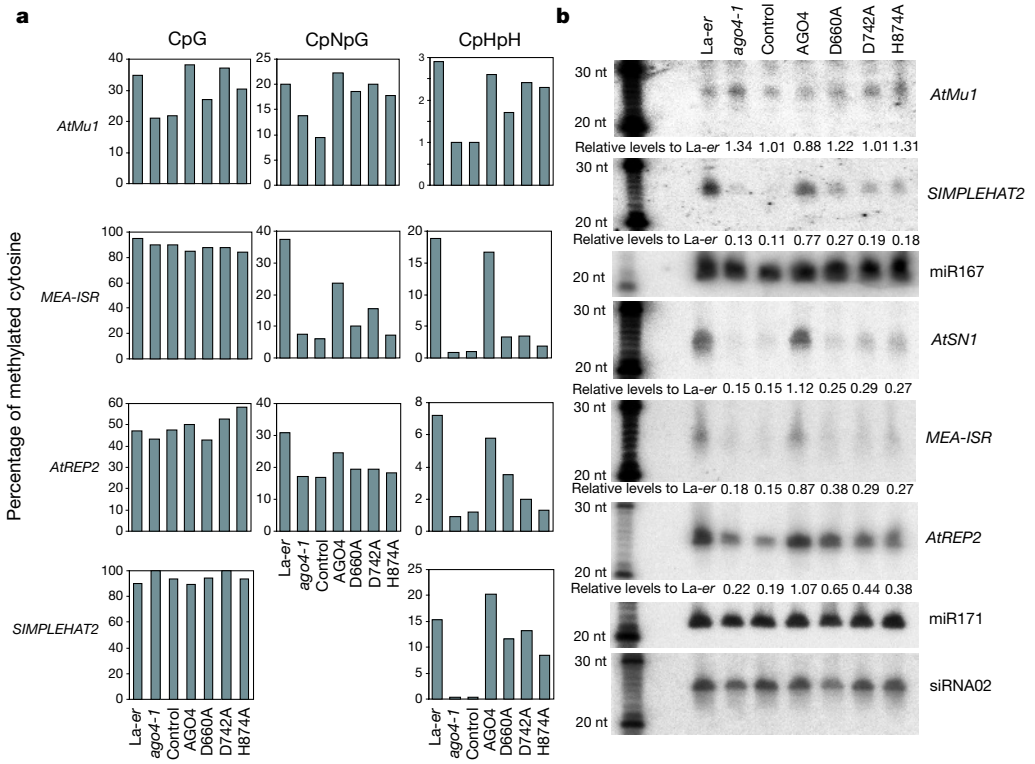

Figure 4 | Distinct effects of Slicer activity on DNA methylation and siRNA accumulation at endogenous repeats. a, CpG (left), CpNpG (middle) and CpHpH (right) methylation at *AtMu1*, *MEA-ISR*, *ATREP2* and *SIMPLEHAT2* loci were analysed by bisulphite sequencing. Data from two complete biological replicates were combined. Methylation levels are shown by the percentage of methylated cytosines in all sequenced clones. Data from Supplementary Table S7 were used to generate the histograms. **b**, Northern blotting was used to analyse siRNAs derived from *AtMu1*, *SIMPLEHAT2*, *AtSN1*, *MEA-ISR*, *ATREP2* and siRNA02 in RNA prepared from the indicated pooled T_1 plants. miR167 and miR171 were used as loading controls. The siRNA signals were normalized relative to miR167 (for *AtMu1* and *SIMPLEHAT2*) or miR171 (for other siRNAs), and the relative levels were calculated by comparison with those in La-*er* RNA (arbitrarily set to 1.00).

each construct (Supplementary Figs S9 and S10, and Supplementary Table S8).

Our data indicate that AGO4 can have two distinct functions in RdDM. First, AGO4 can direct chromatin remodelling factors to a target locus, probably through interactions between siRNAs and a nascent transcript. For this process, the catalytic activity of AGO4 is not required. Thus, given a source of siRNAs, the non-catalytic activity of AGO4 would be sufficient to sustain methylation and repression. Second, AGO4 has a function in which catalysis is required for efficient siRNA production. Cleavage may trigger the recruitment of RDR2-containing complexes to synthesize a double-stranded RNA using the cleaved transcript as template, with subsequent processing by DCL3 producing secondary siRNAs. This is reminiscent of the production of *Arabidopsis trans*-acting siRNAs, which is initiated by the cleavage of their precursor RNA by a miRNA-directed RISC[26]. Other, currently mysterious, mechanisms must also promote siRNA production from heterochromatic loci, because in this model an existing siRNA or miRNA would be required to initiate the cycle. siRNA accumulation and non-CpG DNA methylation of *AtMu1*, and by inference at *SUP*, are much less dependent on the catalytic activity of AGO4. This could simply indicate that another AGO protein functions redundantly with AGO4 at these sites[4] . Indeed, it was shown that DNA methylation at *AtMu1* is also controlled by AGO1 (ref. 10). It also remains possible that *AtMu1* and *SUP* might represent a subset of AGO4-dependent loci in which the role of siRNAs is less important, particularly considering that *SUP* siRNAs have yet to be detected.

Our results reveal a potentially general property of Argonaute proteins. A single Argonaute may simultaneously serve as a catalytic engine of RNA cleavage and as a flexible platform for the assembly of multiprotein complexes that trigger cleavage-independent repression. For AGO4, both of these functions act within a single silencing pathway to contribute to the management of repetitive sequences in the *Arabidopsis* genome.

METHODS

Complete details of methods used are given in Supplementary Information.

Construction of *Arabidopsis* transgenic lines. Transgenic plants were constructed with the use of a floral dip method[27] to transfer plasmids encoding a TAP-tagged or Myc-tagged wild-type or mutant AGO4 under the control of the AGO4 promoter.

Purification of AGO4 and AGO1 complexes. AGO1 and AGO4 complexes were purified from total extracts essentially as described described previously[11,12].

Small RNA cloning. Small RNAs were gel isolated and used to create libraries for 454 Life Sciences sequencing as described previously[17].

Slicer activity and siRNA-binding assays. TAP or immunopurified AGO4 complexes were incubated with target RNAs to examine the Slicer activity of AGO4 essentially as described[12]. The siRNA-binding assay was performed as described[12].

Bisulphite sequencing. Genomic DNAs were isolated from 3–4-week-old seedlings with the DNeasy Plant Mini kit (Qiagen). The EZ DNA Methylation-Gold kit (ZYMO Research) was used for bisulphite treatment of genomic DNA in accordance with the manufacturer's instructions.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information Sequences referred to in this study have been deposited in the GenBank database under accession numbers DQ927324–DQ972825. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to G.J.H. (hannon@cshl.edu).