LETTERS

Drosophila endogenous small RNAs bind to Argonaute 2 in somatic cells

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RNA silencing is a conserved mechanism in which small RNAs trigger various forms of sequence-specific gene silencing by guiding Argonaute complexes to target RNAs by means of base pairing[1,2]. RNA silencing is thought to have evolved as a form of nucleic-acid-based immunity to inactivate viruses and transposable elements. Although the activity of transposable elements in animals has been thought largely to be restricted to the germ line, recent studies have shown that they may also actively transpose in somatic cells, creating somatic mosaicism in animals[3]. In the *Drosophila* germ line, Piwi-interacting RNAs arise from repetitive intergenic elements including retrotransposons by a Dicer-independent pathway and function through the Piwi subfamily of Argonautes to ensure silencing of retrotransposons[4–9]. Here we show that, in cultured *Drosophila* S2 cells, Argonaute 2 (AGO2), an AGO subfamily member of Argonautes, associates with endogenous small RNAs of 20–22 nucleotides in length, which we have collectively named endogenous short interfering RNAs (esiRNAs). esiRNAs can be divided into two groups: one that mainly corresponds to a subset of retrotransposons, and the other that arises from stem–loop structures. esiRNAs are produced in a Dicer-2-dependent manner from distinctive genomic loci, are modified at their 3′ ends and can direct AGO2 to cleave target RNAs. Mutations in *Dicer-2* caused an increase in retrotransposon transcripts. Together, our findings indicate that different types of small RNAs and Argonautes are used to repress retrotransposons in germline and somatic cells in *Drosophila*.

Biochemical and mutation analyses have revealed the existence of multigene families encoding two key proteins—Dicer and Argonaute—in RNA silencing in *Drosophila*[10–13]. Dicer-1 generates *Drosophila* microRNAs (miRNAs) whereas Dicer-2 creates short interfering RNAs (siRNAs). Argonaute proteins directly bind small guide RNAs and either display endonucleolytic ('Slicer') activity or have a platform role for the assembly of silencing complexes[2]. The five *Drosophila* Argonaute proteins can be subdivided into two subfamilies: the ubiquitous AGO (AGO1 and AGO2) and the germline-specific Piwi (AGO3, Aubergine (Aub) and Piwi) subfamilies[2,14]. AGO1 is involved in the miRNA-dependent pathway that silences messenger RNA, whereas AGO2 functions in RNA interference (RNAi)[12] directed by exogenous siRNAs. Piwi proteins are involved in silencing retrotransposons through direct interaction with Piwi-interacting RNAs (piRNAs) in the germ line[4–9] by forming a Dicer-independent cycle that amplifies piRNAs[6,7]. In contrast, how RNA silencing might operate to repress retrotransposons in *Drosophila* somatic cells, and to what extent, remain unknown.

Unlike for the other Argonaute proteins in *Drosophila*, endogenous small RNA partners for AGO2 have not been identified. AGO2 could exist simply to lie in wait for small RNAs that exogenously come into contact with it, including those originating from infecting viruses that produce double-stranded RNAs (dsRNAs) as part of their replication cycle[15]. However, we surmised that AGO2 might also interact with endogenous siRNAs that are products of Dicer processing of dsRNAs, akin to endogenous siRNAs in *Arabidopsis thaliana*[1]. In animals, endogenous siRNAs have only been identified in *Caenorhabditis elegans*[16]. To test whether AGO2 exists in a complex with endogenous siRNAs, we immunopurified AGO2 with a specific monoclonal antibody from a cultured *Drosophila* somatic S2 cell line (Supplementary Fig. 1a, b) and examined its associated RNAs. AGO2 in S2 cells was predominantly associated with small RNAs of about 21 nucleotides in length (Supplementary Fig. 1c).

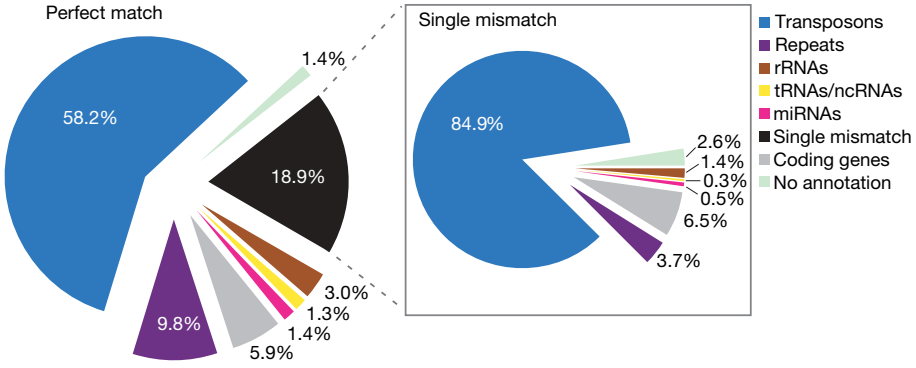

Figure 1 | Identification of endogenous small RNAs that bind AGO2. The left chart shows that the contents of AGO2-associated small RNAs perfectly match the *Drosophila* genome sequence (perfect match). Clones with single mismatches comprise 12,240 from a total of 64,588; these populations are shown on the right (single mismatch).

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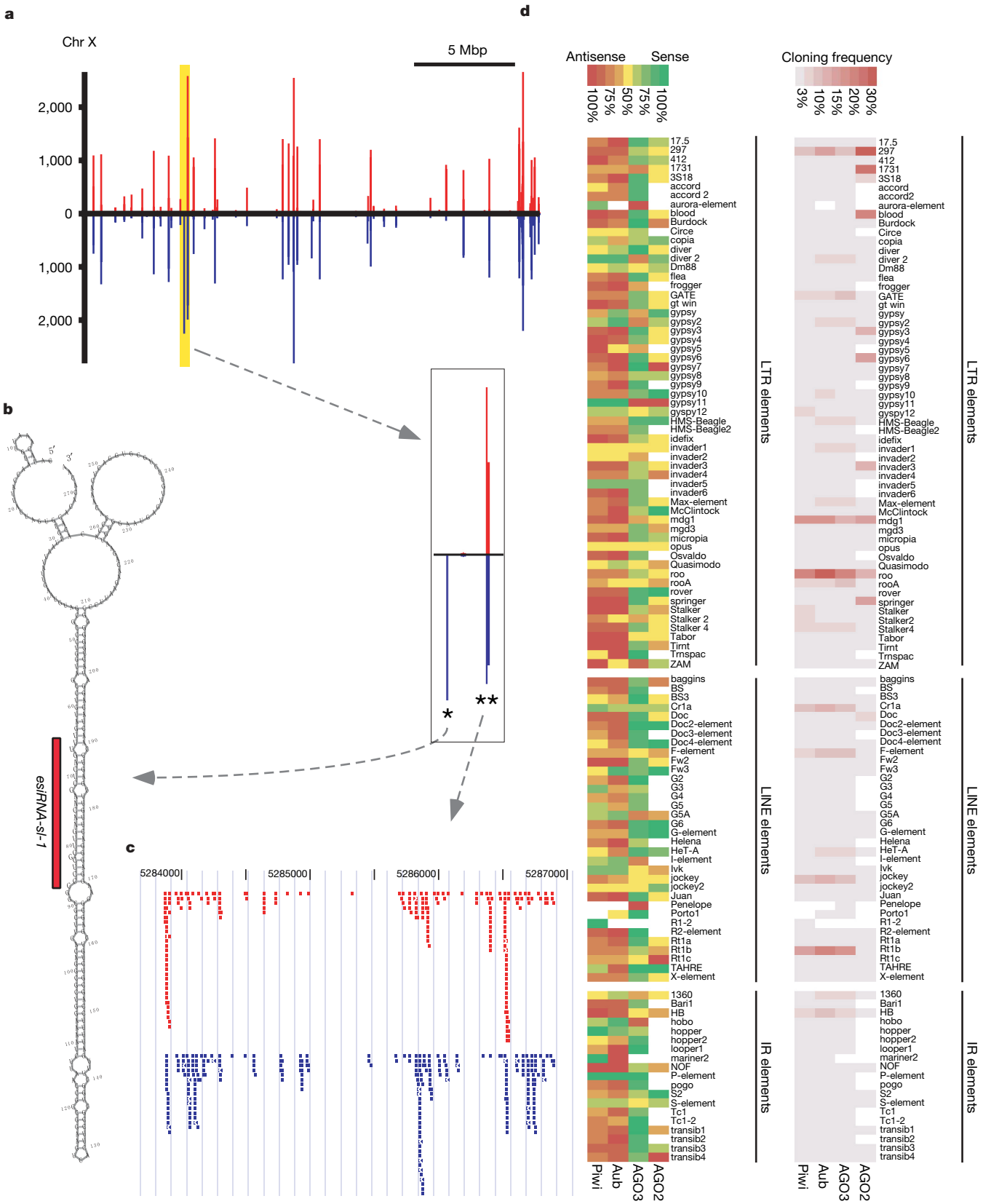

Figure 2 | esiRNA clusters. **a**, Frequency map of AGO2-associated small RNAs on chromosome X. The *x* and *y* axes represent the chromosomal position and the number of reads, respectively. The strand is represented by the colour of the bars: plus (red) and minus (blue). Two major hotspots indicated by the yellow box are enlarged in the rectangle below; one, indicated with the asterisk, expresses only from the minus strand, where 20 similar sequence patterns (~274 nt) are repeatedly encoded (Supplementary Fig. 5). Each fragment has a distinct stem–loop structure, as shown in **b**. The most abundant small RNA (*esiRNA-sl-1*) derived from this fragment is indicated by a red bar. The other hotspot (indicated with two asterisks) expresses esiRNAs from both strands, with details shown in **c**. **d**, The heat map (left panel) indicates the strand bias of cloned esiRNAs and piRNAs[7] with respect to canonical transposon sequences (indicated at the right). Transposons are grouped into long terminal repeat (LTR), long interspersed nuclear (LINE) and inverted repeat (IR) elements. The colour intensities indicate the degree of strand bias (green, sense; red, antisense; yellow, unbiased). Note that some transposons in the AGO2 lane are coloured either green or red, but the cloning frequency of these transposons was very low (see the right panel). The cloning frequencies of individual transposons in all four complexes (this study and ref. 7) are indicated as a heat map (right panel).

To characterize the small RNAs associated with AGO2, we constructed a complementary DNA library and performed high-throughput pyrosequencing[17] that generated 77,327 reads of the cDNA library. Consistent with the [γ-^{32}P]ATP-labelled result, the sizes of the small RNAs ranged from 20 to 22 nucleotides in length (Supplementary Fig. 1d), similar to the sizes of miRNAs but clearly different from the sizes of piRNAs (24–29 nucleotides). Most of these endogenous binding partners for AGO2 were a previously undescribed class of ~21-nucleotide small RNAs (see below), which we have collectively referred to as esiRNAs (also known as endo-siRNAs). Unlike many other small RNAs that bind Argonautes[1,18], esiRNAs do not display a nucleotide-bias at any particular position (data not shown). Among the 77,327 reads, 52,348 sequences matched the *Drosophila* genome with 100% identity over their entire length (Fig. 1). In addition, a large number of the AGO2-associated small RNAs showed single mismatches (12,168; Fig. 1). Adenosine-to-guanosine single mismatches were vastly overrepresented (Supplementary Fig. 1e), suggesting that a portion of the small RNAs may be adenosine-to-inosine RNA edited by ADAR (adenosine deaminase acting on RNA) enzymes[19].

Database searching revealed that a large number of the AGO2-associated small RNAs corresponded to transposons and other repetitive elements in the genome (Fig. 1). Pieces of protein-coding sense mRNAs (~6%) as well as ribosomal RNAs (~3%) were conspicuous in this screening, as is the case for piRNAs in fly[9] and mouse[20]. We also observed a small number of miRNAs (~1.4%) among the AGO2-associated small RNAs sequenced (Supplementary Table 1), including two previously unknown miRNAs (*miR-20071* and *miR-20072*) derived from introns of *prp8* and *cdc2*, respectively (Supplementary Figs 2 and 3).

We summed the number of unique small RNAs in a 5-kilobase (kb) sliding window and plotted this against the *Drosophila* draft assembly (Berkeley *Drosophila* Genome Project release 5). Clusters of small RNA production were observed (Fig. 2a, Supplementary Fig. 4 and Supplementary Table 2). Most esiRNAs could be divided into two types: those that matched retrotransposons and those that arose from long stem–loop structures from repetitive sequences located in an intergenic region on chromosome X (Fig. 2b, c and Supplementary Fig. 5). Populations of esiRNAs derived from retrotransposons are quite complex, with most RNAs being cloned only once (57.2% in perfect and single matches). Like *Drosophila* piRNAs[4–9], esiRNAs are derived from a subset of retrotransposons (Fig. 2d). Consistent with this, a number of esiRNAs (24.5%) overlap with registered piRNA sequences[6–9]; however, their sizes (20–22 nucleotides versus 24–27 nucleotides) and binding partners (AGO2 versus Piwi proteins) were clearly different. Thus, esiRNAs and piRNAs are distinct classes of endogenous small RNAs. Some retrotransposons including *1731* and *297* elements are overrepresented in the esiRNA populations. *Drosophila* piRNAs show a strong strand bias for sequences; Aub and Piwi mainly bind antisense-strand piRNAs, whereas AGO3 binds sense-strand piRNAs[6,7]. esiRNAs, however, seems to be unbiased (Fig. 2d and Supplementary Fig. 4). For example, one hotspot for small RNA production within a 7,600-nucleotide intergenic region on chromosome X was almost entirely covered by small RNAs on both strands (Fig. 2c). Thus, they must be derived from dsRNAs.

In contrast, esiRNAs arising from stem–loop structures (Fig. 2b) showed strand bias with exceptionally high cloning frequency (Supplementary Fig. 5). The stem–loop-generating region contains twenty ~274-nucleotide repeats and appeared to extend to the CG4068 gene (Supplementary Fig. 5). All of the esiRNAs from this region were found in a sense orientation with respect to the orientation of CG4068 (Supplementary Fig. 5). Northern blotting revealed that both types of esiRNAs specifically co-purified with AGO2, but not with AGO1 (Supplementary Fig. 6a–c). An *in vitro* Slicer assay also confirmed that AGO2–esiRNA complexes were capable of specifically cleaving target RNAs containing sequences perfectly complementary to esiRNAs (Fig. 3a). This also implied that esiRNAs can guide AGO2 to cleave complementary RNAs including retrotransposon transcripts in cells.

piRNAs and exogenous siRNAs in *Drosophila* are modified with 2′-*O*-methylation at their 3′ terminus, requiring an RNA methyltransferase, Pimet (also known as DmHen1 and CG12367) (refs 21 and 22). esiRNAs in S2 cells were resistant to periodate oxidation ($NaIO_4$) and β-elimination reactions (Fig. 3b, c), but esiRNAs from *DmHen1/Pimet* mutant ovaries could be β-eliminated (Fig. 3d). Thus, esiRNAs are likely to have the same 3′ modification, as is the case for piRNAs and exogenous siRNAs. These findings suggested that esiRNAs are produced by a pathway similar to that of exogenous siRNAs that bind AGO2 in S2 cells. Because exogenous siRNA could not displace esiRNAs from AGO2 (Supplementary Fig. 7a, b), 'empty' AGO2 (apo-AGO2) seems to exit to accommodate small RNAs from exogenous sources.

To test whether the production and normal accumulation of esiRNAs require Dicers and other known small-RNA-processing factors, we depleted each of these proteins from S2 cells by RNAi and monitored the abundance of small RNAs (Fig. 4a). A marked reduction of *esiRNA-sl-1*, one of the very abundant esiRNAs (Fig. 2b), was observed in Dicer-2-depleted S2 cells; this was observed less efficiently in AGO2-depleted S2 cells, and was not observed in Drosha-, Dicer-1- or AGO1-depleted S2 cells. We also confirmed

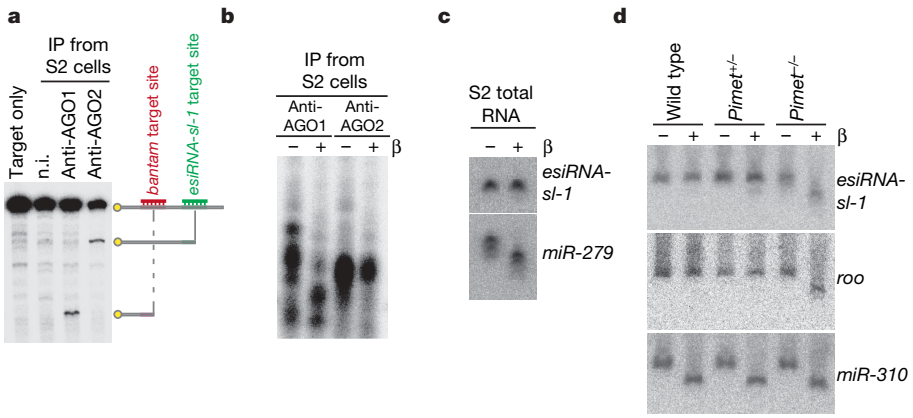

Figure 3 | Specific association and modification of esiRNAs. **a**, Target RNAs containing sequences complementary to *bantam* (red bar) and *esiRNA-sl-1* (green bar) are cleaved at each position with AGO1–miRNA and AGO2–esiRNA complexes, as expected. n.i.; non-immune IgG; IP, immunoprecipitation. **b**, Unlike miRNAs, esiRNAs show resistance to β-elimination. **c**, Total RNAs of S2 cells were probed for *esiRNA-sl-1* and *miR-279* before and after β-elimination. **d**, Loss of *Pimet* causes esiRNA to be sensitive to β-elimination, suggesting that esiRNAs are methylated by Pimet. *roo*, piRNA corresponding to the LTR retrotransposon *roo*.

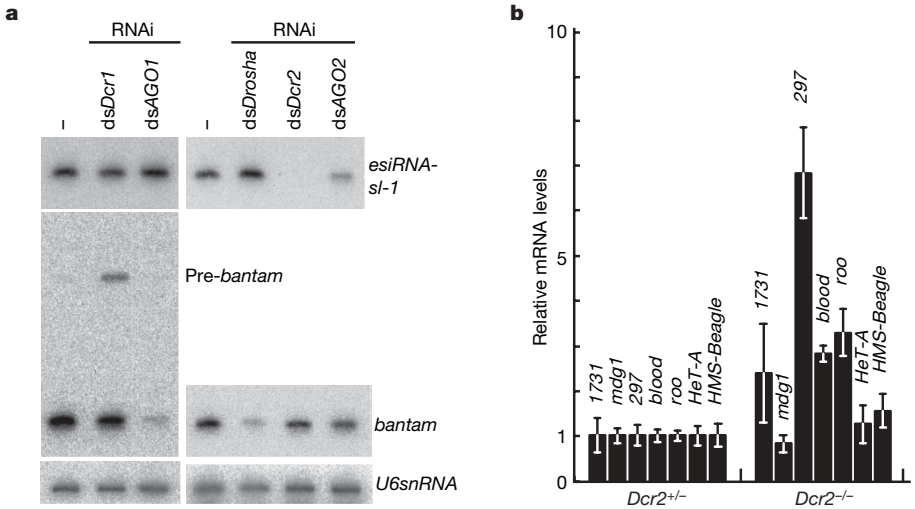

Figure 4 | esiRNAs are produced in a Dicer-2-dependent manner, and are involved in transposon repression in somatic cells. a, Dicer-2 (Dcr2) depletion causes a severe reduction of esiRNAs. **b**, Increase of retrotransposon transcript abundance in homozygous *Dcr2* mutants. The steady-state levels of transcripts from retrotransposons were measured by quantitative RT–PCR in carcasses (flies without testes) of heterozygous or homozygous *Dcr2* mutant males, relative to *rp49* (also known as *RpL32*). The error bars report the average ± s.d. for at least three independent experiments.

that esiRNAs were almost undetectable in the ovaries of *Dicer-2* mutants and were significantly reduced in *AGO2* mutants (Supplementary Fig. 8). Although the normal accumulation of piRNAs requires *Spindle-E* (*Spn-E*)[4], a lack of the *Spn-E* activity does not affect the levels of esiRNAs in the ovary (Supplementary Fig. 8). These results demonstrated that esiRNAs are produced in a Dicer-2-dependent manner.

We examined whether the somatic expression of retrotransposons is suppressed by the esiRNA pathway. Depletion of Dicer-2 or AGO2 coincided with higher levels of retrotransposon transcripts (Supplementary Fig. 9a, b). Mutations in *Dicer-2* also caused an increase in retrotransposon transcripts (Fig. 4b). We therefore concluded that the expression of a subset of retrotransposons was reduced by the esiRNA pathway in somatic cells.

Transposable elements are powerful mutator elements responsible for generating variations in the host genome, and therefore have a role as crucial factors shaping the genome through evolution[23]. Previous studies have suggested that expression of transposable elements in animal somatic cells is mainly silenced by DNA methylation at transcriptional levels[23]. However, we found that the expression of a subset of retrotransposons was reduced by the esiRNA pathway that requires Dicer-2 and AGO2 in *Drosophila* somatic cells. This was clearly distinct from the silencing of retrotransposons by the piRNA pathway that requires Piwi proteins but not Dicers in the germ line[4,6,7]. Our findings, in turn, suggested that some retrotransposons are actively transcribed in somatic cells, which are then processed into small RNAs by Dicer-2. Recent studies in the fission yeast *Schizosaccharomyces pombe* have shown that RNAi-mediated heterochromatin formation requires the production of RNA from heterochromatic regions to generate small RNAs and serve as part of a feed-forward loop required to maintain the heterochromatic state[24]. Thus, it is tempting to speculate that esiRNAs could not only guide AGO2 to cleave transcripts of retrotransposons but are also able to direct silencing complexes to specific chromosomal regions. Interestingly, a loss of *Dicer-2* or *AGO2* activity is correlated with defects in the formation of heterochromatin in *Drosophila*[25,26]. These results together suggest that changes in the activity of Dicer-2 and/or AGO2 could allow the expression or even transposition of retrotransposons that may in turn create quantitative genetic variation in gene expression within somatic cell populations.

METHODS SUMMARY

AGO1 and AGO2 were immunopurified from S2 cells using specific antibodies[27]. Cloning of small RNAs associated with AGO2 in S2 cells was carried out as described[9]. Genome mapping and annotation was performed as described in the Methods section. Northern blotting[5], the *in vitro* target-RNA cleavage assay[9], RNAi for S2 cells[12], periodixation/β-elimination[6,21] and RT–PCR analysis[4] were performed as described.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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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 Contributions Y.K., K.S., T.N.O. and M.C.S. performed AGO2 immunoprecipitations, northern blotting, RNAi, the *in vitro* cleavage assay, β-elimination and qRT–PCR, and prepared the AGO2-associated small RNA library. T.S. characterized and purified the AGO2 antibody. The bioinformatics analyses of AGO2-associated small RNAs were designed and carried out by T.K., K.S., Y.O. and K.A. M.C.S., K.S., Y.K. and H.S. designed the experiments, discussed the interpretation of the results and co-wrote the manuscript.

Author Information Small RNA sequences were deposited in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) under the accession number GPL6452. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to M.C.S. (siomim@sc.itc.keio.ac.jp) or H.S. (awa403@sc.itc.keio.ac.jp).

METHODS

***Drosophila* strains.** *Yellow white* (*y w*) was used as a wild-type strain. The strain bearing the *Spn-E*$^{100.37}$ mutation was provided by P. Zamore. The strain bearing the *Dcr-2*^{L811fsX} mutation[13] was provided by K. Nakahara. The mutant fly (*piggyBac*^{f00810}) was used as the *DmHen1/Pimet* mutant. Production of the *AGO2* mutant, *AGO2*^{414}, was described previously[12].

Cloning of small RNAs associated with AGO2 in S2 cells. AGO1 and AGO2 were immunopurified from S2 cells using specific antibodies against each of the proteins[27]. Cell lysis buffer was 1× PBS supplemented with 1 mM EDTA, 0.1 mM DTT, 1% Empigen (Calbiochem), 2 μg ml^{-1} pepstatin, 2 μg ml^{-1} leupeptin and 0.5% aprotinin. After immunoprecipitation, GammaBind beads (GE Healthcare) that were in a bound form with the immunoprecipitates were washed five times with cell lysis buffer. Total RNAs were isolated from the immunoprecipitates with phenol:chloroform and were precipitated with ethanol. RNAs were dephosphorylated with calf intestinal alkaline phosphatase (CIP) (NEB) and labelled with [γ-^{32}P]ATP with T4 polynucleotide kinase (Takara) for visualization. Cloning of small RNAs associated with AGO2 in S2 cells was carried out as described[5,6,9]. The deep sequencing of the libraries was performed on a GS20 system (Roche).

Genome mapping and annotation. Small RNA sequences were mapped to the *D. melanogaster* draft assembly[28] (Berkeley *Drosophila* Genome Project release 5) using the NCBI BLASTN program. The annotation of each small RNA was determined by examining the overlap between its mapped regions and other feature track data of the UCSC Genome Browser (http://genome.ucsc.edu/). A small RNA was assigned to a feature when the length of its overlap was longer than 90% of the small RNA. We defined the priority of the feature assignment to avoid any conflict of assignment. The assignment to miRNA, rRNA, transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) and other non-coding RNAs (ncRNAs) was performed by using the FlyBase[29] non-coding track. We used the FlyBase genes track for the annotation of coding genes. We also used our earlier data of piRNAs[22] for annotating piRNAs. RepeatMasker (http://www.repeatmasker.org) and the natural transposon tracks were used for annotating transposons. Repetitive elements other than transposons were used to assign repeat elements.

Northern blot analysis. Northern blot analysis was carried out essentially as described previously[5]. Total RNAs of S2 cells, fly embryos and adult male flies (carcasses, flies without testes) were isolated using ISOGEN (Invitrogen) according to the manufacturer's instructions. Total RNAs from the immunoprecipitates were isolated with phenol:chloroform and precipitated with ethanol. A DNA fragment for detecting retrotransposon transcripts was cloned into the pBS SK+ vector using primers, *1731*-forward and *1731*-reverse, for *1731* (GenBank accession number X07656). The sequences of the primers were as follows: *1731*-forward, 5′-TATACGGCTTGAAGCAGTCAGGCAG-3′; *1731*-reverse, 5′-ACGCCAAGTACCGGAGGATGTGCTT-3′. PCR products were used as templates. DNA probes were synthesized *in vitro* using a random prime labelling kit (Takara) in the presence of [α-^{32}P]dCTP. Probes used for *miR-279*, *miR-310, roo* piRNA, *bantam*, *esiRNA-1731-1* and *esiRNA-sl-1* were as follows; *miR-279*, 5′-ATGAGTGTGGATCTAGTCA-3′; *miR-310*, 5′-AAAGGCCGGG-AAGTGTGCAATA-3′; *roo* piRNA, 5′-TCGACTCAGTGGCACAATAAAT-3′; *bantam*, 5′-CAGCTTTCAAAATGATCTCAC-3′; *esiRNA-1731-1*, 5′-AAGGT-GTCGTCGCTGGTCTAC-3′; *esiRNA-sl-1*, 5′-GGAGCGAACTTGTTGGA-GTCAA-3′; and U6snRNA, 5′-GGGCCATGCTAATCTTCTCTGTA-3′. The DNA oligonucleotides were labelled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP.

***In vitro* target-RNA cleavage assay.** To make an RNA target harboring sequences completely complementary to *bantam* miRNA and *esiRNA-sl-1*—a short dsDNA fragment (a target sequence for *bantam*) produced from two oligonucleotide DNAs (5′- AGCTTGAGATCATTTTGAAAGCTGATT-3′ and 5′- AGCTAATCAGCTTTCAAAATGATCTCA-3′) by annealing was first inserted in a pBS SKII+ vector at the HindIII site (yielding pBS-ban). Following this, another DNA fragment (a target sequence for *esiRNA-sl-1*) produced from two DNA oligonucleotides (5′-GATCGGAGCGAACTTGTTGGA-GTCAA-3′ and 5′-GGCCTTGACTCCAACAAGTTCGCTCC-3′) was inserted into a pBS-ban (between BamHI and NotI). PCR was again performed using primers for the T7 and T3 promoter sequences, and the PCR products used as templates for *in vitro* transcription using a MEGAscript T7 kit (Ambion). The resultant RNAs were radiolabelled at their 5′ ends with [γ-^{32}P]ATP using T4 polynucleotide kinase and were gel-purified. The target-RNA cleavage assay was performed as described previously[27] using AGO1 and AGO2 immunoprecipitates from S2 in a buffer containing Empigen.

RNA interference. RNAi in S2 cells was performed essentially as described[12]. Production of dsRNAs was described previously[12]. dsRNAs used in RNAi were: ds*Drosha*, homologous to nucleotides 2701–3925 of the *Drosha* open reading flame (ORF) sequence (accession number AE013599); ds*Dicer-1*, 10–950 of the *Dicer-1* ORF sequence (accession number AE014297); ds*Dicer-2*, 4091–4888 of the *Dicer-2* ORF sequence (accession number NM_079054); ds*AGO1*, 1035–2042 of the *AGO1* ORF sequence (accession number NM_166021); ds*AGO2*, 1304–2282 of the *AGO2* ORF sequence (accession number NM_168626).

Periodate oxidation and β-elimination. Periodate oxidation and β-elimination of RNAs were performed as described[6,21]. RNAs isolated from AGO1 and AGO2 immunoprecipitates were labelled with [γ-^{32}P]ATP after CIP treatment. 10,000 counts per min of ^{32}P-labelled RNAs was used per reaction. Total RNAs of S2 cells and of fly embryos were subjected to perioxidation and β-elimination without ^{32}P labelling. Visualization of *esiRNA-sl-1*, *miR-279*, *roo* piRNA and *miR-310* was done by northern blotting.

Quantitative RT–PCR analysis. Total RNA (0.4 μg) was used to reverse transcribe target sequences using oligo(dT) primer and PrimeScript RT reagent Kit (Takara) according to the manufacturer's instructions. The resulting cDNA was analysed by quantitative RT–PCR in a LightCycler real-time PCR system (Roche Diagnostics) using the SYBR Premix Ex Taq (Takara). Relative steady-state mRNA levels were determined from the threshold cycle for amplification. Supplementary Table 3 lists the PCR primer sequences. Rp49 was used as an internal control.

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