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Report

The *Drosophila* RNA Methyltransferase, DmHen1, Modifies Germline piRNAs and Single-Stranded siRNAs in RISC

Michael D. Horwich,[1,3] Chengjian Li,[1,3] Christian Matranga,[1,3] Vasily Vagin,[1,2] Gwen Farley,[1] Peng Wang,[1] and Phillip D. Zamore[1,*]

[1] Department of Biochemistry and Molecular Pharmacology
University of Massachusetts Medical School
Worcester, Massachusetts 01605

[2] Department of Animal Molecular Genetics
Institute of Molecular Genetics
Moscow 123182
Russia

Summary

Small silencing RNAs repress gene expression by a set of related mechanisms collectively called RNA-silencing pathways [1, 2]. In the RNA interference (RNAi) pathway [3], small interfering mRNA (siRNAs) defend cells from invasion by foreign nucleic acids, such as those produced by viruses. In contrast, microRNAs (miRNAs) sculpt endogenous mRNA expression [4]. A third class of small RNAs, Piwi-interacting RNAs (piRNAs), defends the genome from transposons [5–9]. Here, we report that *Drosophila* piRNAs contain a 2′-*O*-methyl group on their 3′ termini; this is a modification previously reported for plant miRNAs and siRNAs [10] and mouse and rat piRNAs [11, 12, 13]. Plant small-RNA methylation is catalyzed by the protein HEN1 [10, 14, 15]. We find that DmHen1, the *Drosophila* homolog of HEN1, methylates the termini of siRNAs and piRNAs. Without DmHen1, the length and abundance of piRNAs are decreased, and piRNA function is perturbed. Unlike plant HEN1, DmHen1 acts on single strands, not duplexes, explaining how it can use as substrates both siRNAs—which derive from double-stranded precursors—and piRNAs—which do not [8, 13]. 2′-*O*-methylation of siRNAs may be the final step in assembly of the RNAi-enzyme complex, RISC, occurring after an Argonaute-bound siRNA duplex is converted to single-stranded RNA.

Results and Discussion

Drosophila piRNAs Are 2′-*O*-methylated at Their 3′ Termini

In flies, both piRNAs (also known as repeat-associated siRNAs, rasiRNAs) and siRNAs, but not miRNAs, are modified at their 3′ termini [8, 16]. We selectively labeled (Figure S1 in the Supplemental Data available online) the terminal nucleotide of *Drosophila melanogaster* 0–2 hr embryo and mouse and bull testicular piRNAs. The resulting ^{32}P-radiolabeled nucleoside 2′ or 3′-monophosphates were resolved by 2D thin-layer chromatography (2D TLC) with a solvent system that can resolve nucleoside 2′ monophosphates, nucleoside 3′ monophosphates, and 2′-*O*-methyl nucleoside 3′ monophosphates (Figure S2). Modified nucleoside monophosphates derived from the 3′ termini of piRNAs were identified by comparison to modified and unmodified nucleoside 2′ and 3′ monophosphate standards (Figure 1A). The terminal nucleotide of the piRNAs of all three animals comigrated with 2′-*O*-methyl nucleoside 3′ monophosphate standards but not with any unmodified nucleoside monophosphate standard. Because mouse piRNAs were previously shown to contain 2′-*O*-methyl modified 3′ termini by both mass spectrometry [12] and a 2D TLC system [11] distinct from ours, we conclude that *Drosophila* and bull piRNAs also contain a 2′-*O*-methyl group at their 3′ termini.

DmHen1 Is Required for piRNA Modification In Vivo

In *Arabidopsis*, the RNA methyltransferase, HEN1, modifies the terminal 2′ hydroxyl group of small silencing RNAs. In *Drosophila*, predicted gene CG12367, whose 1559 nucleotide mRNA encodes a 391 amino acid protein with a 220 amino acid evolutionarily conserved methyltransferase domain [17], most closely resembles *Arabidopsis* HEN1 (Figure 1B) [18]. For simplicity, we call this gene *Drosophila melanogaster* (Dm) *hen1*. When homozygous, a piggyBac transposon insertion (PBac{WH}CG12367[f00810]) within the first intron of the fly *hen1* gene reduces the accumulation of *hen1* mRNA by 1000-fold in testes and by more than 40,000-fold in ovaries (Figure 2A) and can therefore be considered a null mutation, which we refer to as *hen1*^{f00810}.

We examined the 3′ termini of two types of highly abundant piRNAs in the germline of flies heterozygous or homozygous for *hen1*^{f00810}. In testes, the *Suppressor of Stellate* [*Su(Ste)*] locus produces 24–27 nucleotide rasiRNAs, a subclass of piRNAs that directs silencing of the selfish genetic element *Stellate*. *Su(Ste)* rasiRNAs, like other *Drosophila* piRNAs, are modified at their 3′ termini and therefore do not react with $NaIO_4$ [8]. In contrast, *Su(Ste)* rasiRNAs from *hen1*^{f00810}/*hen1*^{f00810} mutant testes reacted with $NaIO_4$ and could therefore be β-eliminated to remove the last nucleotide of the RNA, thereby increasing their gel mobility (Figure 2B) and indicating that in the absence of DmHen1 protein, they are not modified. Similarly, rasiRNAs that guide silencing of *roo*, the most abundant retrotransposon in *Drosophila melanogaster*, were not modified in *hen1*^{f00810} homozygous ovaries (Figure 2C). The *Su(Ste)* and *roo* rasiRNAs were also shorter in the *hen1*^{f00810} homozygotes. In contrast, the length and amount of miR-8, which is expressed in both the male and female germline, was unaltered in *hen1*^{f00810} homozygotes. For both *Su(Ste)* and *roo*, rasiRNAs were on average shorter and less modified even in *hen1*^{f00810} heterozygotes, compared to the wild-type, suggesting that the abundance of DmHen1 protein limits the stability or production of piRNAs in flies.

*Correspondence: phillip.zamore@umassmed.edu
[3] These authors contributed equally to this work.

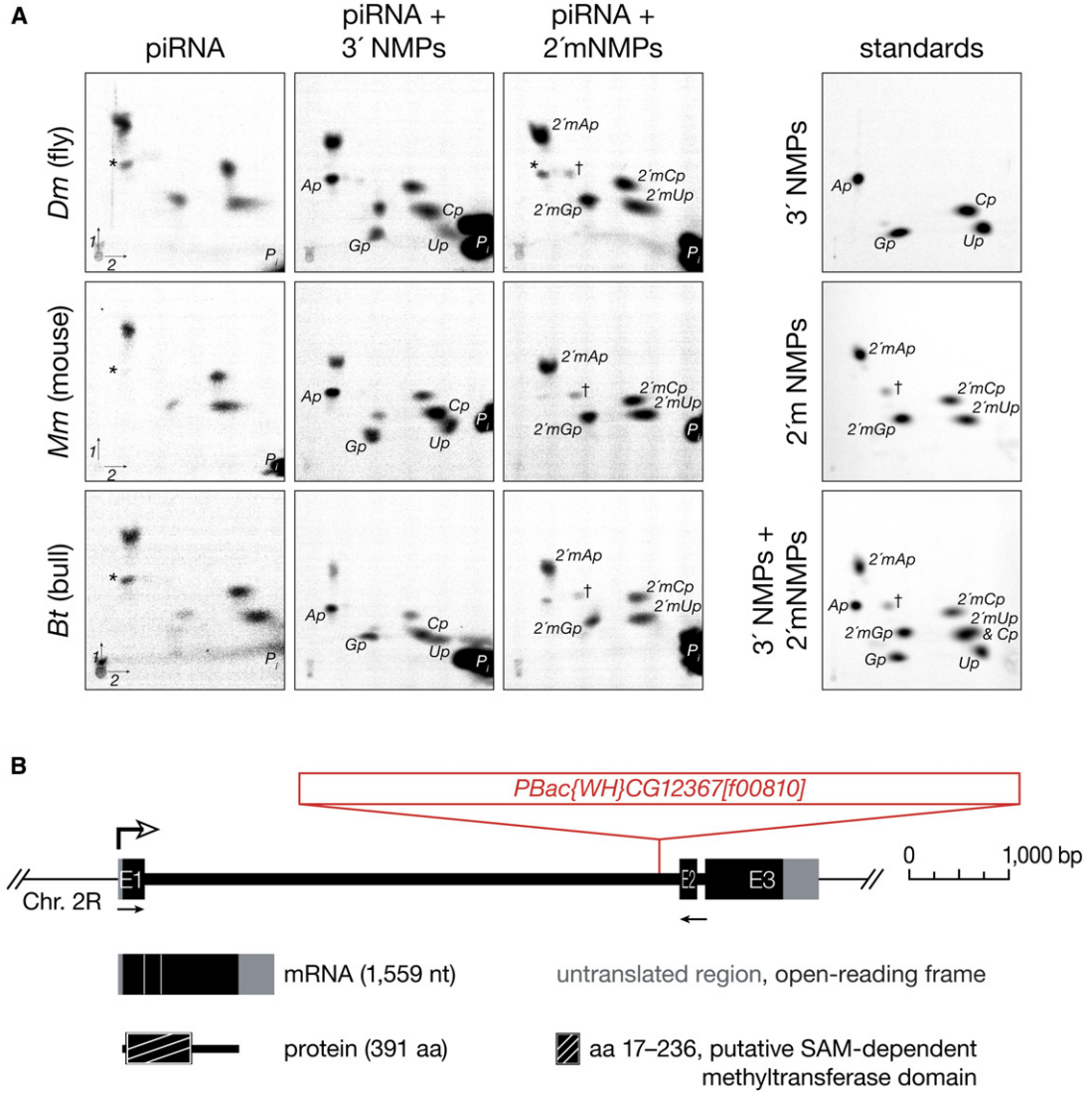

Figure 1. 2′-*O*-Methylation of piRNAs in *Drosophila*

(A) *Drosophila* piRNAs are 2′-*O*-methylated at their 3′ termini. The modified nucleotides on the 3′ termini of piRNAs from 0–2 hr fly embryos and mouse and bull testes were selectively ^{32}P radiolabeled. The radiolabeled modified mononucleotides from each species were resolved by 2D-TLC individually (piRNA), with ^{32}P-radiolabeled 3′ mononucleotide standards (piRNA + 3′NMPs), and with ^{32}P-radiolabeled 2′-*O*-methyl, 3′ phosphate mononucleotide standards (piRNA + 2′mNMPs). The modified nucleotides from the piRNA from all three animals comigrated with 2′mNMPs standards, but not with 3′NMPs standards. The following abbreviations are used: Ap, 3′AMP; Gp, 3′GMP; Cp, 3′CMP; Up, 3′UMP; 2′mAp, 2′-*O*-methyl AMP; 2′mGp, 2′-*O*-methyl GMP; 2′mCp, 2′-*O*-methyl CMP; 2′mUp, 2′-*O*-methyl UMP; and P_i, phosphate. The asterisk marks a contaminant, probably 3′ AMP, present in the [5′ ^{32}P] cytidine 5′,3′ bis-phosphate used to radiolabel the piRNA. The cross marks a contaminant present in the 2′-*O*-methyl, 3′ phosphate mononucleotide standards.

(B) *Drosophila hen1* gene (CG12367), mRNA, and protein. The piggyBac transposon, PBac{WH}CG12367[f00810], is inserted 207 bp upstream of the second exon. The open arrow indicates the predicted start of transcription. The closed arrows denote the position of the qRT-PCR primers used in Figure 2A. The first intron of *hen1* contains another gene, CG8878, that is transcribed in the opposite direction and whose expression is unaltered by the piggyBac insertion (Figure S3).

DmHen1 Is Required for piRNA Function In Vivo

Modification of the termini of *Drosophila* piRNAs plays a role in their function: mRNA expression from *HeT-A*, the element whose expression is most sensitive to mutations that disrupt piRNA-directed silencing in the female germline [8, 19, 20], quadrupled in *hen1*^{f00810} heterozygotes and was increased by more than 11-fold in homozygotes, relative to wild-type tissue (Figure 2D). We conclude that Hen1 protein is required for piRNA-directed silencing in the *Drosophila* germline.

DmHen1 Is Required for siRNA Modification

To test whether DmHen1 is required for modification of the 3′ termini of siRNAs, we depleted Hen1 by RNAi in cultured *Drosophila* S2 cells. We transfected the cells with long double-stranded RNA (dsRNA) targeting *hen1* on day 1 and day 5, then cotransfected them with both GFP dsRNA and *hen1* dsRNA on day 8. Total RNA was harvested on day 9, probed for modification with $NaIO_4/\beta$-elimination, and analyzed by Northern hybridization with a 5′ ^{32}P-radiolabeled DNA probe

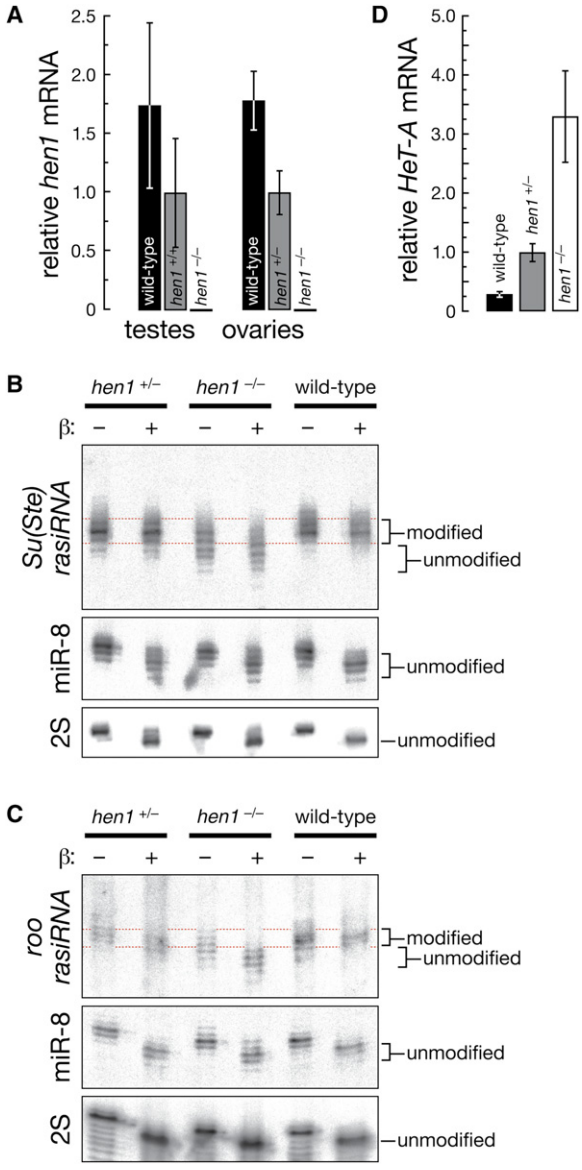

Figure 2. DmHen1 Is Required for Normal piRNA Biogenesis and Complete Silencing of the *HeT-A* Transposon

(A) Quantitative RT-PCR of *hen1* mRNA in testes and ovaries from wild-type or *hen1*^{f00810} flies, relative to *rp49*. Error bars report the average ± SD for at least four independent experiments.

(B and C) Northern hybridization to detect modification of small RNAs in wild-type and *hen1*^{f00810} testes (B) or ovaries (C). The same blot was probed sequentially to detect the most abundant *Su(Ste)* rasiRNA (B) or the three most abundant *roo* rasiRNAs (C), miR-8, and 2S ribosomal RNA.

(D) Quantitative RT-PCR of *HeT-A* transposon mRNA in ovaries. *HeT-A* mRNA levels were measured relative to *rp49* with total RNA prepared from wild-type and *hen1*^{f00810} heterozygous or homozygous ovaries. Error bars report the average ± SD for at least four independently prepared samples.

complementary to the most abundant GFP-derived siRNA (M.D.H., M. Ghildiyal, and P.D.Z., unpublished data). DsRNAs targeting two different regions of the fly *hen1* mRNA both reduced the amount of GFP siRNA modified at its 3′ terminus, whereas all the GFP siRNA remained modified when a control dsRNA was used (Figure 3A).

Surprisingly, RNAi-mediated depletion of Ago2, but not Ago1, prevented the GFP siRNA from being modified. This result suggests that Ago2, but not Ago1, plays a role in the modification of siRNAs by DmHen1. To test this idea, we examined the modification status of the 3′ terminus of miR-277, which partitions into both Ago1 and Ago2 complexes in vivo [21]. *Drosophila* miRNAs associate predominantly or exclusively with Ago1 [22] and have unmodified 3′ termini [8, 16, 23]. In contrast, approximately half the miR-277 in cultured S2 cells failed to react with $NaIO_4$ (Figure 3A), suggesting that approximately half of miR-277 is modified at its 3′ terminus. The fraction of miR-277 that was modified was reduced when two different dsRNAs were used to deplete DmHen1 by RNAi. When the cells were treated with dsRNA targeting *ago1*, all detectable miR-277 was modified, whereas all miR-277 became unmodified when dsRNA targeting *ago2* was used. In contrast, *bantam*, a miRNA that associates nearly exclusively with Ago1 [22], was unmodified under all conditions (Figure 3A).

siRNA Modification Correlates with Ago2-RISC Assembly In Vitro

siRNA modification can be recapitulated in lysates of embryos, ovaries, or cultured S2 cells. Modification of siRNA in vitro was inhibited by *S*-adenosyl homocysteine, but not by *S*-adenosyl methionine, consistent with DmHen1 transferring a methyl group from *S*-adenosyl methionine to the terminal 2′ hydroxyl group of the RNA, thereby generating *S*-adenosyl homocysteine as a product (Figure S4).

Our data from cultured S2 cells suggested that DmHen1 modifies that portion of miR-277 that enters the Ago2-RISC-assembly pathway, but not the population of miR-277 that assembles into Ago1-RISC. To further test the idea that small-RNA modification requires both Hen1 and the Ago2-RISC-assembly pathway, we prepared cytoplasmic lysates from dsRNA-treated cultured S2 cells. Lysate from control-treated cells modified the 3′ terminus of a 5′ ^{32}P-radiolabeled synthetic siRNA duplex but not lysate from *hen1*-depleted cells (Figure 3B). The addition of either of two different preparations of purified, recombinant DmHen1, expressed in *E. coli* as a ~74 kDa glutathione S-transferase fusion protein (GST-DmHen1; Figure S5), restored the ability of the lysates to modify the siRNA, indicating that loss of DmHen1 caused the loss of siRNA modification. Moreover, lysates depleted for Ago2, but not Ago1, could not modify the ^{32}P-siRNA in vitro (Figure 3B). These in vitro data, together with our S2-cell experiments, suggest that modification of the 3′ terminus of siRNAs and miRNAs is coupled to assembly into Ago2-RISC.

Dcr-2 and R2D2 act to load double-stranded siRNAs into Ago2. We prepared lysates from ovaries homozygous mutant for *hen1*, *dcr-2*, *r2d2*, and *ago2* by using alleles that were unable to produce the corresponding protein [22, 24, 25]. A 5′ ^{32}P-radiolabeled siRNA duplex was incubated in each lysate to assemble RISC. At each time point, we determined whether the siRNA was 3′ terminally modified by assessing its reactivity with $NaIO_4$ (Figure 3C). No modified siRNA accumulated when the duplex was incubated in *hen1*^{f00810}, *dcr-2*^{L811fsX}, *r2d2*^{1}, or *ago2*^{414} mutant lysate. Adding

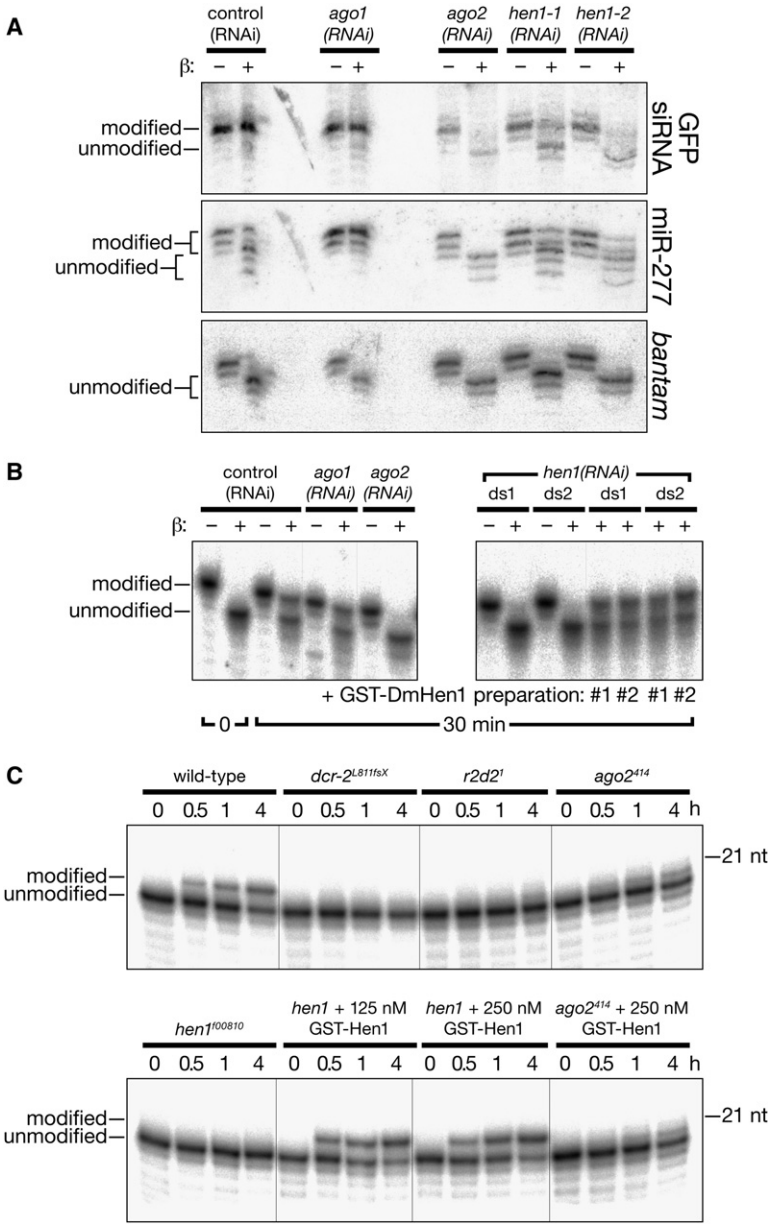

Figure 3. DmHen1 Modifies Ago2-Associated Small RNAs

(A) Modification of siRNAs and miRNAs in *Drosophila* S2 Cells. A stable S2-cell line expressing GFP was treated with the indicated dsRNA alone (day 1 and 5), then together with GFP dsRNA (day 8). Two nonoverlapping dsRNAs were used to target *hen1*. Total RNA was collected on day 9 and treated with $NaIO_4$/β-elimination; then, dsGFP-derived siRNA, miR-277, and *bantam* were detected by sequential Northern hybridization of the same blot.

(B) In vitro siRNA modification in dsRNA-treated S2-cell lysates.

(C) In vitro siRNA modification in *Drosophila* mutant ovary lysates.

250 nM purified, recombinant GST-DmHen1 restored siRNA modification to the $hen1^{f00810}$ but not the $ago2^{414}$ lysate. We conclude that the defect in $ago2^{414}$ reflects a requirement for Ago2 in small-RNA modification by DmHen1, rather than an indirect effect such as destabilization of DmHen1 in the absence of Ago2. GST-DmHen1 similarly rescued lysate from *hen1(RNAi)* but not *ago2(RNAi)*-treated S2 cells (Figure S6). Together, the results of our experiments using cultured S2 cells—a somatic-cell line—and ovaries, which comprise mainly germline tissue, suggest that a functional Ago2-RISC-assembly pathway is required for siRNA modification in *Drosophila*.

siRNAs Are Modified Only after Ago2-RISC Maturation

To test at which step in the Ago2-RISC-assembly pathway siRNAs become modified, we determined whether siRNAs are 2′-*O*-methylated by DmHen1 as single strands or as duplexes. In vitro, assembly of siRNAs into Ago2-RISC follows an ordered pathway in which the siRNA duplex first binds the Dicer-2/R2D2 heterodimer to form the RISC-loading complex (RLC). The RLC determines which of the two siRNA strands will become the guide for Ago2 and which will be destroyed (the passenger strand). The siRNA is then loaded into Ago2 as a duplex. In this pre-RISC complex, the passenger strand occupies the same position as future target RNAs [26]. Cleavage of the passenger strand by the Ago2 endonuclease domain converts pre-RISC to mature RISC [27–30]. No single-stranded guide or passenger RNA is produced prior to this maturation step. Thus, all single-stranded siRNA produced in vitro or in vivo [26] corresponds to mature RISC.

We assembled Ago2-RISC in vitro by using an siRNA designed to load only one of its two strands into Ago2

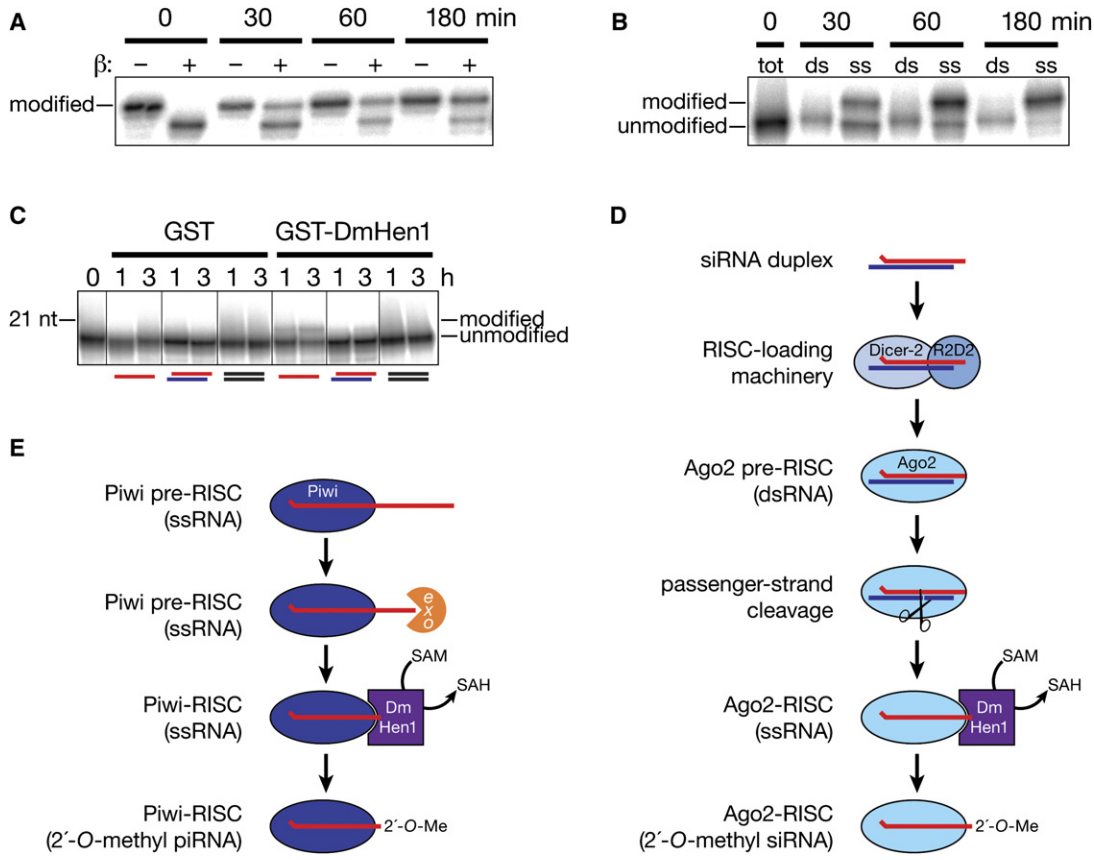

Figure 4. DmHen1 Modifies Single-Stranded RNAs in RISC

siRNAs are modified after the conversion of pre-RISC—which contains double-stranded siRNA—into mature RISC, which contains only single-stranded siRNA. siRNA duplexes with a 5′ ^{32}P-radiolabeled guide strand were incubated in *Drosophila* embryo lysate and then tested for the presence of a 3′ terminal modification.

(A) Total RNA from each time point in RISC assembly, without (−) and with (+) reaction with $NaIO_4$ and β-elimination.

(B) At each time in (A), single- and double-stranded siRNA were resolved and purified by native gel electrophoresis (Figure S7), then analyzed by denaturing electrophoresis separately for the presence of a 3′ terminal modification on the siRNA guide strand. (A) and (B) show the left and right halves of a single gel.

(C) Recombinant, purified GST-DmHen1, but not GST alone, can modify single-stranded 21 nt RNA, but not double-stranded siRNAs or blunt 21 nt RNA duplexes. However, in contrast to GST-DmHen1 incubated with *hen1*^{f00810} mutant ovary lysate (Figure 3C), the enzyme alone is inefficient. All samples were oxidized with $NaIO_4$, then β-eliminated.

(D) A model for 2′-*O*-methylation of siRNAs.

(E) A proposed role for 2′-*O*-methylation in piRNA biogenesis.

[31]. We then sampled the reaction over time, isolating the 5′ ^{32}P-radiolabeled siRNA under conditions previously demonstrated to preserve its structure [32], and separated single- from double-stranded siRNA by native gel electrophoresis (Figure S7). (Full-length siRNA duplexes and siRNA heteroduplexes comprising a full-length guide paired to a cleaved passenger strand comigrate as double-stranded siRNA in these gel conditions [28].) The RNAs were then isolated from the gel and tested for reactivity with $NaIO_4$ to determine the presence of modification at their 3′ termini (Figures 4A and 4B). At each time, total siRNA was analyzed in parallel. 3′ terminal modification increased over the course of RISC assembly and, at all times, was restricted to single-stranded siRNA: Within our limits of detection, all double-stranded siRNA was unmodified, even after 3 hr. We conclude that siRNA modification is coupled to RISC assembly and occurs only after the conversion of pre-RISC to mature RISC.

Recombinant DmHen1 Modifies Single-Stranded Small RNA

Whereas *Arabidopsis* HEN1 contains an N-terminal double-stranded RNA-binding motif [15], DmHen1 does not. To test whether DmHen1 modifies double-stranded small RNAs, we incubated purified, recombinant GST-DmHen1 with either single-stranded or double-stranded siRNAs. We detected modification, evidenced by loss of reactivity with $NaIO_4$, only for the single-stranded RNA, suggesting that DmHen1 modifies single-stranded substrates, but not siRNAs or blunt RNA duplexes (Figure 4C). A preference for single-stranded RNA would explain how DmHen1 could act on both siRNAs, which are born double stranded, and piRNAs, which are not. We note that the purified, recombinant GST-DmHen1 protein was more than 50-fold less active on its own than when supplemented with ovary lysate from *hen1*^{f00810} homozygous flies. We speculate that the Ago2-RISC machinery is required for Hen1 function in

flies, although we cannot yet exclude the possibility that the lysate contains a factor (e.g., a kinase) required for activating Hen1.

Modification of single-stranded siRNAs—that is, those loaded in fully mature Ago2-RISC but not double-stranded siRNAs (Figure 4D)—might allow cells to distinguish siRNAs loaded successfully into functional complexes from those that fail to assemble. For example, if a 3′-to-5′ nuclease acts to degrade single-stranded siRNAs, 2′-*O*-methylation of single-stranded siRNAs in Ago2 RISC may protect them from destruction. Moreover, such a nuclease might trim the 3′ end of piRNAs. 2′-*O*-methylation of the piRNA 3′ terminus may occur only when the length of RNA extending beyond the Piwi-family protein is short enough to permit the simultaneous binding of the final ribose sugar to the active site of DmHen1 and the interaction of DmHen1 with the Piwi protein itself (Figure 4E). Modification of the terminus of the trimmed piRNA would then block further 3′-to-5′ trimming of the small RNA, generating its Piwi-, Aubergine-, or Ago3-specific length. Our observation that piRNAs are shorter in *hen1*^{f00810} mutants supports this model.

We note that all 2′-*O*-methyl-modified small RNAs identified thus far are associated with RISC complexes that efficiently cleave their RNA targets—i.e., Ago1-associated plant miRNAs [33, 34], animal piRNAs [35], and Ago2-associated siRNAs in flies [36]—whereas *Drosophila* miRNAs are typically both unmodified and associated with Ago1 RISC, which does not catalyze mRNA target cleavage in vivo [21]. We speculate that DmHen1 is recruited to RISC complexes containing single-stranded small silencing RNAs according to the identity of their Argonaute protein. This model predicts that DmHen1 will bind only to complexes containing fly Ago2 or the three fly Piwi proteins, Piwi, Aubergine, and Ago3, but not Ago1. Clearly, future experiments will need to test this hypothesis.

Experimental Procedures

General Methods

Preparation of 0–2 hr embryo, ovary, and S2-cell lysates, in vitro RISC assembly and RNAi reactions, and Northern hybridization were as described [8, 37, 38]. A list of sequences of synthetic RNA and DNA oligonucleotides is available in the Supplemental Experimental Procedures section in the Supplemental Data online.

^{32}P-radiolabeled 3′ Mononucleotide Standards

Synthetic RNA oligonucleotides (Supplemental Experimental Procedures) were radiolabeled in a 20 μl reaction containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol, 10% (v/v) DMSO, 10 μg/ml BSA, 2 units/μl RNasin (Promega), 1.5 μCi/μl [5′ ^{32}P] cytidine 5′,3′ bis-phosphate ([5′ ^{32}P]-pCp; Perkin-Elmer), and 1 unit/μl T4 RNA Ligase 1 (New England Biolabs) at 4°C, overnight. The radiolabeled small RNAs were purified from a 15% denaturing urea-polyacrylamide sequencing gel and then digested with 1.5 U/μl micrococcal nuclease (Takara Mirus Bio) in a 40 μl reaction containing 20 mM Tris-HCl (pH 8.0), 5 mM NaCl, and 2.5 mM $CaCl_2$. 3′ ^{32}P-mononucleotides were further purified from a 22.5% denaturing urea-polyacrylamide sequencing gel.

2D-TLC

Small RNAs (21–29 nucleotides, containing both modified piRNAs and unmodified small RNAs) from 0–2 hr wild-type (Oregon R) fly embryos and small RNAs (26–31 nucleotdes, containing mostly modified piRNAs) from mouse and bull testes were purified from a 10% denaturing urea-polyacrylamide gel stained with SYBR Gold (Invitrogen). Approximately 100 pmol purified small RNAs were radiolabeled as described above, except in a 40 μl reaction with 3 μCi/μl [5′ ^{32}P]-pCp and 1 unit/μl T4 RNA Ligase 1, and then gel purified. The purified, ^{32}P-radiolabeled RNA was hydrolyzed in 200 mM Na_2CO_3 at 100°C for 1 hr, neutralized with an equal volume of 200 mM HCl, then dephosphorylated with 0.5 units/μl calf intestinal alkaline phosphatase (New England Biolabs) in a 200 μl reaction containing 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. Alkaline phosphatase was inactivated by extraction with phenol/chloroform; RNA in the aqueous phase was oxidized with 80 mM $NaIO_4$ in borax/boric-acid buffer (60 mM borax and 60 mM boric acid [pH 8.6]) at room temperature for 30 min and then β-eliminated with 200 mM NaOH at 45°C for 90 min. A total of 5 μl of this reaction was mixed with an equal volume of formamide loading buffer (98% deionized formamide, 10 mM EDTA [pH 8.0], 0.025% (w/v) xylene cyanol, and 0.025% (w/v) bromophenol blue) and resolved on a 22.5% denaturing urea-polyacrylamide sequencing gel. Equal intensities of modified mononucleotides and standards were spotted on 20 × 20 cm PEI-cellulose F glass TLC plates (EMD Chemicals) and separated first with isobutyric acid/25%ammonia/water (66:1:33, v:v:v) and then 0.1 M sodium phosphate (pH 6.8)/ammonium sulfate/1-propanol (100:60:2, v:w:v).

Analysis of RNA 3′ Termini

RNA was incubated for 30 min at room temperature with 25 mM $NaIO_4$ in borax/boric-acid buffer (60 mM borax and 60 mM boric acid [pH 8.6]), then 45.2 mM (f.c.) was NaOH added, and incubation continued for 90 min at 45°C (β-elimination). The reaction was stopped by the addition of 300 mM (f.c.) NaCl, 1 μg glycogen, and three volumes absolute ethanol. After 30 min on ice, the precipitated RNA was collected by centrifugation.

Recombinant *Drosophila* Hen1 Protein

DmHen1 coding sequence was amplified from *Drosophila* ovary cDNA and inserted into pEnt-D-Topo (Invitrogen). The entire *hen1* sequence was confirmed by sequencing. The entry plasmid was recombined with the N-terminal GST expression vector, pDest-15, with LR Clonase (Invitrogen). GST-Hen1 was expressed in BL21 Star DE3 cells (Invitrogen) grown at 37°C in LB broth containing 100 ug/ml ampicillin until to OD_{600} reached 0.50. The culture was then cooled to 25°C, and 0.4 mM IPTG was added to induce protein production. The culture was incubated at 25°C with vigorous shaking for 3 hr. The cells were harvested by centrifugation at 7,300 × g for 20 min, washed with PBS, and centrifuged again; then the cell paste was frozen in liquid nitrogen and stored at −80°C.

Hen1 fusion protein was purified with the GST Purification Kit (Clontech). Cells were resuspended in 40 ml of extraction/loading buffer and lysed by sonication (duty 30% for 6 min; Branson Sonificator II), with cooling in an ice bath. A total of 2 ml of clarified lysate was added to the column resin, and the column inverted several times to disperse the resin. The resin was then allowed to pack, and the remaining 38 ml passed through the column by gravity flow. Subsequent steps were in accordance with the manufacturer's directions.

Analysis of Double- and Single-Stranded siRNA

Double- and single-stranded 5′ ^{32}P-radiolabeled siRNA guide strands (10 nM; Figure S7 and Figure 4) were separated as described [32]. In brief, RISC-assembly reactions were stopped with 2× Proteinase K buffer, 2 mg/ml Proteinase K, 1 μg glycogen, and 250 nM unlabeled siRNA guide strand to prevent reannealing. After incubation for 30 min at 25°C, 3 volumes absolute ethanol were added, and the RNA precipitated for 30 min on ice. The precipitates were collected by centrifugation, washed with 80% (v/v) ethanol, then dissolved in 2 mM Tris-Cl (pH 7.5), 3% (w/v) Ficoll-400, 0.04% (w/v) bromophenol blue, 100 mM KCH_3CO_2, 30 mM HEPES-KOH, and 2 mM $Mg(CH_3CO_2)_2$ and resolved by electrophoresis through a 15% native polyacrylamide gel (19:1 acrylamide:bis; 89 mM Tris-Borate [pH 8.3], 2 mM Na-EDTA, and 2.5 mM $Mg[CH_3CO_2]_2$). The region of the native gel corresponding to double- or single-stranded siRNA was excised, and the RNA was eluted overnight in 1 M NaCl. A total of 1 μg glycogen and ethanol (60% final volume) was added to the eluate, the RNA collected with MegaClear filter cartridges (Ambion), eluted with H_2O, and then precipitated for 30 min on ice by

an addition of 500 mM (f.c.) $NH_4CH_3CO_2$ and 2.5 volumes absolute ethanol. The precipitate was collected by centrifugation and washed with 80% (v/v) ethanol, and the samples reacted with $NaIO_4$ and subsequent β-elimination (see above). The precipitated RNA was dissolved in 98% deionized formamide, 10 mM EDTA (pH 8.0), 0.025% (w/v) xylene cyanol, and 0.025% (w/v) bromophenol blue and then resolved on a 15% denaturing urea-polyacrylamide sequencing gel.

Supplemental Data

Additional Experimental Procedures and seven figures are available at http://www.current-biology.com/cgi/content/full/17/14/1265/DC1/.

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Note Added in Proof

This version differs slightly from the one published previously online in that on page 1 of the Supplemental Data, the dots have now been properly aligned beneath the sequences, whereas previously they were not.