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Citation

Published Version
doi:10.1371/journal.pgen.1002616

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PIWI Associated siRNAs and piRNAs Specifically Require the Caenorhabditis elegans HEN1 Ortholog henn-1

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Abstract

Small RNAs—including piRNAs, miRNAs, and endogenous siRNAs—bind Argonaute proteins to form RNA silencing complexes that target coding genes, transposons, and aberrant RNAs. To assess the requirements for endogenous siRNA formation and activity in Caenorhabditis elegans, we developed a GFP-based sensor for the endogenous siRNA 22G sir-1, one of a set of abundant siRNAs processed from a precursor RNA mapping to the X chromosome, the X-cluster. Silencing of the sensor is also dependent on the partially complementary, unlinked 26G sir-O7 siRNA. We show that 26G sir-O7 acts in trans to initiate 22G siRNA formation from the X-cluster. The presence of several mismatches between 26G sir-O7 and the X-cluster mRNA, as well as mutagenesis of the siRNA sensor, indicates that siRNA target recognition is permissive to a degree of mispairing. From a candidate reverse genetic screen, we identified several factors required for 22G sir-1 activity, including the chromatin factors mes-4 and gff-1, the Argonaute ergo-1, and the 3′′ methyltransferase henn-1. Quantitative RT–PCR of small RNAs in a henn-1 mutant and deep sequencing of methylated small RNAs indicate that siRNAs and piRNAs that associate with PIWI clade Argonautes are methylated by HENN-1, while siRNAs and miRNAs that associate with non-PIWI clade Argonautes are not. Thus, PIWI-class Argonaute proteins are specifically adapted to associate with methylated small RNAs in C. elegans.


Received September 8, 2011; Accepted February 3, 2012; Published April 19, 2012

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Funding: This work is supported by National Institute of Health (GM44619) and Damon Runyon Cancer Research Foundation (DRG-2029-09 and DRG1988-08). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

MicroRNAs (miRNAs), PIWI-interacting RNAs (piRNAs) and small interfering RNAs (siRNAs) are distinct classes of ~20–30 nt regulatory RNAs. Each acts as a guide to direct an Argonaute-containing effector complex to target mRNAs [1]. The features required for small RNA-target interactions and the regulatory outcomes of these interactions are largely dictated by the Argonaute cofactor. There are three distinct clades within the Argonaute family [2], miRNAs associate with Argonautes in the AGO clade [1,3], whereas piRNAs associate with members of the PIWI clade [1,4,5], siRNAs associate with PIWI and AGOs in a variety of eukaryotes as well as several Argonautes in the expansive WAGO clade found only in nematodes [1,2,6–10]. Most eukaryotes contain multiple classes of small RNAs and Argonaute cofactors and thus require specialized mechanisms for sorting small RNAs and their target transcripts into the proper pathways [1]. Small RNA duplex structure, 5′ nt identity and length are important determinants for sorting small RNAs into specific effector complexes, although these features alone fail to account for some interactions [1].

In C. elegans, piRNAs (also called 21U RNAs) are broadly distributed throughout the genome but derive primarily from two clusters on chromosome IV [11]. They are almost exclusively 21 nt and contain a 5′ U [11]. At least some piRNAs are modified at their 3′ ends, presumably by 2′-O-methylation [10,11]. The PIWI PRG-1 and PRG-2 are the only proteins that have been shown to function in the C. elegans piRNA pathway. The specific roles of piRNAs in development are unclear, but mutations in prg-1 cause developmental defects including failure in spermatogenesis, abnormal germline development and sterility at elevated temperatures [4,5,12]. The only validated target of the piRNA pathway is the Tc3 DNA transposon family [4,5]. Increased Tc3 transposition may partially account for the defects observed in prg-1 mutants.

Endogenous siRNAs are processed from thousands of distinct loci, including transposons, pseudogenes and protein coding genes [7,13]. There are two types of endogenous siRNAs in C. elegans: 22G siRNAs which are 22 nt and bear a 5′ triphosphorylated guanine and 26G siRNAs which are 26 nt and bear a 5′ monophosphorylated guanine [14]. Processing of 26G, but not 22G siRNAs, requires the endoribonuclease Dicer [9,10,15–17]. Cleavage by Dicer generates RNAs containing 5′ monophosphates, whereas the nascent transcripts of RNA dependent RNA polymerases (RdRPs) are predicted to bear 5′ triphosphorylated nucleotides; this may account for the difference in 5′ phosphorylation state between 26G and 22G siRNAs. In addition to differences at their 5′ ends, siRNAs also differ at their 3′ ends, with a subset presumably having a 2′-O-methyl group [10,11]. Both 26G and 22G siRNA formation requires an RNA-dependent RNA Polymerase, but it is unclear if the nascent RdRP product is further processed to accommodate association with the ~20 to
30 nt cleft of an Argonaute. 26G siRNAs function as primary siRNAs to initiate formation of the more abundant secondary 22G siRNAs from target transcripts; however, the majority of 22G siRNAs are processed independent of a 26G siRNA trigger [8,10,18]. 26G and 22G siRNAs can be further classified by theirArgonaute binding partners. 26G siRNAs associate with the AGO clade Argonautes ALG-3 and ALG-4 during sperm development or with the PIWI clade Argonaute ERGO-1 during embryodevelopment [8–10]. 22G siRNAs associate with either CSR-1 to direct chromosome segregation or WAGO-1-WAGO-11 to guide RNA silencing [7,19,20]. At least a subset of 22G siRNAs also associate with the Argonaute NRDE-3 to block RNA polymerase II activity at target loci within the nucleus [21,22].

To identify the requirements for routing transcripts into RNA silencing pathways, we developed a GFP based sensor for endogenous siRNA activity in C. elegans. The responses of the siRNA sensor indicate that a single siRNA target site is sufficient to route a transcript into an RNA silencing pathway involving NRDE-3. Mutagenesis of the sensor siRNA target site revealed that siRNA target recognition and silencing of the sensor is permissive to some degree of mispairing. Additionally, we identify an endogenous gene that is targeted intrans by a partially complementary 26G siRNA to trigger 22G siRNA formation. Finally, from a candidate RNAi screen for gene inactivations that result in desilencing of the siRNA sensor, we identified the C. elegans ortholog henn-1. Together with Billi et al. [23] and Kamminga et al. [24], we show that henn-1 is required for proper accumulation of both piRNAs and siRNAs that associate with PIWIs, but not for miRNAs and siRNAs that associate with AGO or WAGO clade Argonautes.

Results

A Single siRNA Target Site Is Sufficient to Trigger RNA Silencing

To identify the requirements for siRNA directed RNA silencing, we developed a GFP based sensor for endogenous siRNA activity in C. elegans. The siRNA sensor ubl-1:GFP-siR-1-sensor contains a single target site for an abundant endogenous siRNA, 22G siR-1, embedded in the 3’ UTR of ubiquitin-like-1 (ubl-1) and expressed under the control of the ubl-1 promoter, which is presumably active in all tissues throughout development (Figure 1A). A control construct, ubl-1:GFP, lacks the siRNA target site, but is otherwise identical (Figure 1A). Each construct was introduced into C. elegans using Mos1-mediated single copy insertion [25]. GFP expression was ubiquitous in C. elegans containing the control, which lacks the 22G siR-1 target site, but was nearly absent in C. elegans containing the reporter with the 22G siR-1 sensor element in the 3’ UTR (Figure 1B).

22G siR-1 is derived from a cluster of 22G siRNAs on the X chromosome (termed the X-cluster [26]) that are dependent on ERGO-1 class 26G siRNA pathway components for their formation [10]. Thus, silencing of the siRNA sensor was predicted to require ergo-1 and other factors essential for ERGO-1 class 26G siRNA activity, as well as factors required for 22G siRNA formation and activity. To test this, RNAi against ergo-1 and several other validated and suspected RNAi factors was done in ubl-1:GFP-siR-1-sensor transgenic C. elegans. GFP expression was derepressed in C. elegans containing the siRNA sensor when treated with RNAi against ergo-1 and each of the other validated factors tested [7,9,10,13,27] (Figure 1C and Table 1). RNAi against several other factors implicated in RNAi [28], including the chromatin factors mes-4 and gfl-1, the ubiquitin ligase ncl-1, the transcription elongation regulators ter-1 and R03D7.4 and the spliceosome factor mps-2, also derepressed the siRNA sensor (Table 1). RNAi against many of the factors analyzed, including mutator (mut) class genes, causes desilencing of multicopy array based transgenes [29]; conceivably, the siRNA sensor, although a single copy transgene, is reporting on this phenomenon. However, loss of eri-6 or ergo-1 activity enhances silencing of tandem array transgenes and would therefore be expected to decrease GFP expression if the siRNA sensor was reporting on transgene desilencing [30]. In fact, eri-6 and ergo-1 were two of the strongest derepressors of the siRNA sensor, indicating that it is not reporting on transgene desilencing (Table 1 and Figure 1C). These results indicate that the genetic requirements for silencing the siRNA sensor reflect those of endogenous siRNA targets.

We assessed GFP mRNA and protein levels to identify the mode by which the siRNA sensor is silenced. GFP mRNA levels were much lower in the siRNA sensor strain than in the control strain, as determined by RNA blot assay (Figure 1D). RNAi against ergo-1 in C. elegans containing the siRNA sensor caused substantial increases in both GFP mRNA and protein levels (Figure 1E). GFP protein and mRNA levels were proportionally elevated ~8 fold in siRNA sensor-transgenic C. elegans treated with ergo-1 RNAi relative to control RNAi (p = 0.00003 and p<0.00001, respectively; Figure 1F), indicating that translational repression does not substantially contribute to GFP silencing. To determine if silencing of the siRNA sensor occurs cotranscriptionally via the nuclear RNAi pathway involving the Argonaute NRDE-3, we introduced the siRNA sensor or the control transgene into nde-3 mutant C. elegans. GFP expression from the siRNA sensor in the nde-3 mutant was derepressed to a level comparable to that of the control transgene, while GFP expression from the control transgene was unchanged between wild type and nde-3 mutants (Figure 1G and Figure S1). Thus, NRDE-3-mediated cotranscriptional gene silencing is the primary mode by which the siRNA sensor is silenced.

22G siR-1 Does Not Trigger siRNA Amplification and Spreading

Exogenous RNAi is initiated by low abundance primary siRNAs that recruit RdRPs and other factors to trigger formation of more abundant secondary siRNAs [31–33]. Endogenous ERGO-1 class 26G primary siRNAs are also expressed at relatively low levels compared to secondary 22G siRNAs derived from the same loci. Thus, an important role of at least some classes of siRNAs is to trigger siRNA amplification and spreading outside of the primary
siRNA target site. To determine if 22G siRNAs trigger production of siRNAs in the genomic vicinity of the initial target site, we deep sequenced small RNAs from *C. elegans* containing either the *ubl-1::GFP* or *ubl-1::GFP-siR-1-sensor* transgene. siRNAs derived from both the control and siRNA sensor transgene were predominantly 22 nt and contained 5′G (Figure 2A). The normalized siRNA levels (reads per million total small RNA reads) derived from the GFP mRNA were indistinguishable between the control and siRNA sensor strains (Figure 2B). siRNAs were uniformly distributed across both transgenes and were derived exclusively from coding and vector sequence and not from the *ubl-1* 5′ and 3′ untranslated regions (Figure 2C and 2D). Although a large peak was observed at the siRNA target site of the sensor, it likely corresponds to 22G siR-1, the target site was mutated to contain 1–3 mispairs or a single deletion or insertion, at various positions along the target sequence (Figure 3A). When introduced into *C. elegans*, mutations in the sensor that prevented or interfered with basepairing at the 5′ end of 22G siR-1 (*ubl-1::GFP-siR-1-sensor-1-3del, -4-5del, and -4del*), which includes the region analogous to the seed sequence of miRNAs, resulted in GFP expression similar to what was observed the control that lacks an siRNA target site (Figure 3A and 3B), indicating that near perfect complementarity is required between the 5′ end of an siRNA and its target for efficient silencing. Argonaute catalyzed endonucleolytic cleavage typically occurs between positions 10 and 11 on the target mRNA, relative to the 5′ end of the small RNA guide; mispairs at or near these positions inhibits cleavage [35]. We were unable to detect cleavage within the siRNA target site of the endogenous 22G siR-1 target transcript using 5′ RACE (Figure S2). Furthermore, most Argonautes that associate with 22G siRNAs in *C. elegans*, including NRDE-3, lack the conserved RNase H residues required for catalytic activity [2]. However, when we mutated positions 9–11 (*ubl-1::GFP-siR-1-sensor-9-11sub*) we did observe a modest increase in GFP expression from the siRNA sensor transgene (Figure 3A and 3B), indicating that these positions do play a role in siRNA target recognition.

Sequence Requirements for 22G siR-1 Target Recognition

The degree of sequence complementarity required for target recognition by miRNAs is relatively well characterized. Near perfect complementarity is required in the seed sequence (positions 2–8 of the miRNA, relative to its 5′ end), but generally not in the central or 3′ regions [34]. However, little is known about the requirements for siRNA target recognition, particularly in *C. elegans*. To determine the sequence requirements for target recognition of the siRNA sensor by 22G siR-1, the target site was mutated to contain 1–3 mispairs or a single deletion or insertion, at various positions along the target sequence (Figure 3A). When introduced into *C. elegans*, mutations in the sensor that prevented or interfered with basepairing at the 5′ end of 22G siR-1 (*ubl-1::GFP-siR-1-sensor-1-3del, -4-5del, and -4del*), which includes the region analogous to the seed sequence of miRNAs, resulted in GFP expression similar to what was observed the control that lacks an siRNA target site (Figure 3A and 3B), indicating that near perfect complementarity is required between the 5′ end of an siRNA and its target for efficient silencing. Argonaute catalyzed endonucleolytic cleavage typically occurs between positions 10 and 11 on the target mRNA, relative to the 5′ end of the small RNA guide; mispairs at or near these positions inhibits cleavage [35]. We were unable to detect cleavage within the siRNA target site of the endogenous 22G siR-1 target transcript using 5′ RACE (Figure S2). Furthermore, most Argonautes that associate with 22G siRNAs in *C. elegans*, including NRDE-3, lack the conserved RNase H residues required for catalytic activity [2]. However, when we mutated positions 9–11 (*ubl-1::GFP-siR-1-sensor-9-11sub*) we did observe a modest increase in GFP expression from the siRNA sensor transgene (Figure 3A and 3B), indicating that these positions do play a role in siRNA target recognition.
Basepairing in the region 3' of the bulged nucleotides of a miRNA, at positions 12–17, can enhance miRNA target recognition [34], suggesting that these positions could play an important role in target recognition by siRNAs. Three mispairs introduced at positions 12–14 of the siRNA target site of ubl-1::GFP-sir-1-sensor (ubl-1::GFP-sir-1-sensor-12-14sub) resulted in derepression of the siRNA sensor to a level similar to that of the control (Figure 3A and 3B). When we introduced a single mispair at position 13 we did not observe an increase in the levels of GFP expression (Figure 3A and 3B). Deletion of the paired nucleotide at position 13 (ubl-1::GFP-sir-1-sensor-13del), which would require the siRNA to loop out to accommodate binding to the 3' end of the siRNA, resulted in only a very modest increase in GFP expression from the siRNA target site within the siRNA sensor (Figure 3A and 3B). Deletion of the paired nucleotide at position 13 (ubl-1::GFP-sir-1-sensor-13del), which would require the siRNA to loop out at a single position somewhere between positions 13–15 to facilitate pairing with the 3' end of the siRNA, caused partial derepression of the siRNA sensor (Figure 3A and 3B). Finally, to determine if pairing at the 3' terminus of the siRNA is required for target recognition, we introduced three mispairs at positions 20–22 (ubl-1::GFP-sir-1-sensor-20-22sub) of the siRNA target site within the siRNA sensor (Figure 3A and 3B). GFP expression from the ubl-1::GFP-sir-1-sensor-20-22sub transgene was similar to that of the wild type siRNA sensor, indicating that basepairing at these positions is not essential for siRNA target recognition (Figure 3A and 3B). Although this study does not provide a comprehensive analysis of siRNA target recognition requirements, it demonstrates that a certain degree of mispairing is permissible for siRNA target recognition in C. elegans.

An Endogenous 26G siRNA Acts in trans to Trigger 22G sir-1 Formation

22G sir-1 and other 22G siRNAs derived from the X-cluster are dependent on the 26G siRNA pathway components, although the locus itself does not produce 26G siRNAs [10]. The X-cluster locus is unannotated but inspection of mRNA deep sequencing data [36] indicates that siRNAs are derived from an ~5 kb transcript produced directly upstream of an annotated coding gene, however, the annotated gene itself lacks evidence for transcription (Figure 4A). 22G sir-1 is the most abundant siRNA produced from the locus and is processed from a motif that is repeated multiple times within the cluster (Figure 4A). Given our finding that siRNAs do not require perfect complementarity for target recognition, we hypothesized that 22G siRNA formation from the X-cluster is initiated by a 26G siRNA derived from a distinct gene. To search for such an siRNA trigger, we aligned 26G siRNAs identified in a deep sequencing library enriched for ERGO-1 class 26G siRNAs [37] to the X-cluster transcript. We identified a 26G siRNA, 26G sir-O7, derived from the gene K02E2.11 that aligns with >69% nt complementarity at seven positions within the X-cluster region (Figure 4B and 4C). Aside from the 26G sir-O7 sequence, K02E2.11 does not share significant similarity to the X-cluster region. Interestingly, 26G sir-O7 aligns to the same repeated motif that gives rise to 22G sir-1 and shares perfect complementarity between positions 1–10 and 14–19, aside from 2 G:U pairs, and is mispaired at positions 11–13, relative to the 5' end of the siRNA (Figure 4C).
Because of the similarity between the 22G siR-1 target site within the siRNA sensor and the 26G siR-O7 target sites within the X-cluster, conceivably 26G siR-O7 could directly target the siRNA sensor (Figure 4G). To rule out this possibility we introduced the siRNA sensor of the control transgene into either an rde-2/mut-8 or rfy-1 mutant. The rde-2 mutation does not affect 26G siRNA levels, in particular 26G siR-O7, but it does result in a substantial, although not complete, loss of 22G siR-1 [13] (Figure S3). rfy-1 is an RNA-dependent RNA polymerase (RdRP) that produces 22G siRNAs, but it is not required for 26G siRNA formation [2,9]. An rfy-1 mutation by itself does not result in complete loss of 22G siRNAs due to redundancy with the RdRP ego-1 [7]. When introduced into either an rde-2 or rfy-1 mutant, GFP fluorescence from the siRNA sensor was substantially elevated relative to wild type, while GFP fluorescence from the control transgene was indistinguishable between rde-2 or rfy-1 mutants and wild type (Figure 4H). Furthermore, as described above, NRDE-3, which associates specifically with 22G siRNAs [21], is also required to silence the siRNA sensor (Figure 1G). Thus, although we cannot entirely rule out a modest or temporal primary contribution of 26G siR-O7, our data indicates that the siRNA sensor directly reports on 22G siRNA activity and indirectly on 26G siRNA activity.

henn-1 Is Required for 22G siR-1 Activity

In C. elegans, piRNAs and at least a subset of 26G siRNAs are modified at their 3’ ends, presumably by 2′-O-methylation, a common modification to small RNAs [39–44]. An ortholog of the 3′ methyltransferase HEN1 required for small RNA methylation [39] has not been described in C. elegans. The protein encoded by C02F5.6 is the only C. elegans gene with significant homology to Arabidopsis (p = 5 × 10^{-28}) and Drosophila (p = 2 × 10^{-17}) HEN1 proteins and is thus a likely ortholog. To determine if C02F5.6 is required for siRNA function, C. elegans containing the ubl-1::GFP-siR-1-sensor transgene were treated with RNAi against C02F5.6 (hereafter referred to as henn-1, where the extra n in the name indicates that it is the nematode ortholog of HEN1). When treated with henn-1 RNAi, a modest increase in GFP fluorescence was observed in C. elegans containing the siRNA sensor transgene, but not in C. elegans containing the control transgene that lacks an siRNA target site (Table 1 and Figure 5A). henn-1 RNAi resulted in a modest increase in GFP protein levels in the siRNA sensor strain compared to wild type, while GFP fluorescence from the siRNA sensor was substantially elevated relative to wild type, although possibly by affecting 26G siR-O7, the 26G siRNA that is an RNA-dependent RNA polymerase (RdRP) that produces 22G siRNAs, but it is not required for 26G siRNA formation [2,9]. An rfy-1 mutation by itself does not result in complete loss of 22G siRNAs due to redundancy with the RdRP ego-1 [7]. When introduced into either an rde-2 or rfy-1 mutant, GFP fluorescence from the siRNA sensor was substantially elevated relative to wild type, while GFP fluorescence from the control transgene was indistinguishable between rde-2 or rfy-1 mutants and wild type (Figure 4H). Furthermore, as described above, NRDE-3, which associates specifically with 22G siRNAs [21], is also required to silence the siRNA sensor (Figure 1G). Thus, although we cannot entirely rule out a modest or temporal primary contribution of 26G siR-O7, our data indicates that the siRNA sensor directly reports on 22G siRNA activity and indirectly on 26G siRNA activity.

henn-1 Functions in piRNA and ERGO-1 Class 26G siRNA Pathways

HEN1 is required for the stability of siRNAs in Arabidopsis and Drosophila [42,46]. To determine if henn-1 is required for the accumulation of piRNAs, miRNAs or siRNAs, RNA blot and qRT-PCR assays were done on RNA isolated from embryo, L4 larval and adult stage C. elegans. We also assessed by qRT-PCR the levels of several siRNAs and one piRNA target mRNAs. In embryos, the level of the piRNA 21UR-2921 was substantially reduced in henn-1 mutants, relative to wild type C. elegans (Figure 6A; data shown for one of three biological replicates). As
determined by qRT-PCR, the levels of three other piRNAs (21UR-1, 21UR-3442 and 21UR-3502) were reduced by 60–80% in henn-1 mutants, relative to wild type (p = 0.0002; Figure 6B). The requirement for henn-1 in piRNA stabilization is likely dependent on the developmental stage, as the levels of 21UR-1 were only modestly reduced in adults and unaffected in L4 stage henn-1 mutants, relative to wild type (Figure S4). The levels of two ERGO-1 class 26G siRNAs, 26G siR-O1 derived from C40A11.10 and 26G siR-O2 derived from E01G4.7, were depleted by 72% (p = 0.00001) and 45% (p = 0.03), respectively, in henn-1 mutants, relative to wild type (Figure 6A and 6B). Modest reductions in 26G siR-O1 and 26G siR-O2 levels were also observed in adult staged C. elegans (Figure S5). We also observed a modest reduction in the levels of 26G siR-O7 in henn-1 mutants, as determined by RNA blot assays (Figure 6A; data shown for one of three biological replicates). The levels of 22G siR-1, which is dependent on ergo-1 and 26G siR-O7 for its formation, were depleted by ~80% in henn-1, relative to wild type (p<0.00001; Figure 6A and 6B). An ergo-1-dependent 22G siRNA derived from E01G4.5 was also depleted in henn-1 mutants (Figure S5). In contrast, the levels of a 22G siRNA derived from fkb-8, which is not downstream of 26G siRNAs, were indistinguishable between henn-1 and wild type (Figure 6A). We also examined miR-35 and miR-58 using RNA blot assays. The levels of both miRNAs were unchanged between henn-1 mutant and wild type C. elegans (Figure 6A; data shown for one of three biological replicates). Consistent with the reduced levels of ERGO-1 class 26G siRNAs, the levels of three ERGO-1 class 26G siRNA target mRNAs, C40A11.10, E01G4.7 and E01G4.5, were elevated ~2–3 fold in henn-1 mutants, relative to wild type (p<0.0008; Figure 6C). The levels of two transposon mRNAs analyzed, Tc1 and Tc3, were unchanged in henn-1 mutants (p>0.8; Figure 6C).
Both Tc1 and Tc3 are targets of 22G siRNAs that are not dependent on 26G siRNAs. However, Tc3 is also the only validated piRNA target and its levels are modestly elevated in the absence of piRNAs [4,5]. That henn-1 mutants did not display elevated levels of Tc3 was somewhat puzzling. It is possible that there is residual activity of piRNAs in the absence of henn-1, which is consistent with the incomplete loss of piRNAs in henn-1 mutants.

In henn-1 mutant L4 larvae, which are enriched for ALG-3/4 class 26G siRNAs, the levels of three miRNAs (miR-1, miR-35 and miR-58) and an ALG-3/4 class 26G siRNA (26G siR-S5) derived from ssp-16 were each indistinguishable from wild type (Figure 6D and 6E). In contrast, 22G siR-1, which is expressed throughout development, was depleted similar to what was observed in embryos (Figure 6E). The levels of three ALG-3/4 target miRNAs, C04G2.8, ssp-16 and ZC168.6, were modestly depleted in henn-1 mutants in two independent experiments (Figure 6F).

Mutations in prg-1, the PIWI Argonaute that associates with piRNAs, result in reduced fertility, particularly at 25°C [4,5]. To determine if henn-1 mutants also display defects associated with reduced piRNA activity, the brood sizes of wild type and henn-1 mutants grown at either 20°C or 25°C were measured. At 20°C, a modest, but significant reduction in brood size was observed in henn-1 mutants (p<0.0001; Figure 6G). At 25°C, henn-1 mutants were nearly sterile, whereas wild type animals had only a modest reduction in brood size relative to those grown at 20°C (Figure 6G).

The reduced fertility of henn-1 mutants is likely caused by defects in piRNA activity and not ERGO-1 class 26G siRNA activity because ergo-1 mutants do not display obvious fertility defects [10]. Taken together, these results suggest that henn-1 is specifically required for the accumulation and activity of piRNAs, ERGO-1 class 26G siRNAs and ergo-1-dependent 22G siRNAs. The reduction in ergo-1-dependent 22G siRNAs in henn-1 mutants could be an indirect effect caused by reduced levels of the ERGO-1 class 26G siRNAs that trigger their formation.

Deep Sequencing of Methylated Small RNAs

To comprehensively identify methylated small RNAs in C. elegans and to determine if henn-1 is specifically required for methylated small RNAs, we deep sequenced both β-eliminated and untreated small RNAs isolated from wild type C. elegans. β-elimination is a chemical treatment that removes the 3′ nucleotide of RNAs that contain a 2′-O-methyl at the 3′ end, and leaves behind a 2′-P at the 3′ end which is incompatible with adapter ligation [47]. Thus, β-elimination can be used to enrich for methylated small RNAs in deep sequencing libraries [48]. Nearly every annotated piRNA was enriched and nearly every miRNA was depleted in the β-eliminated library, relative to the non-treated library (Figure 7A). ERGO-1 class 26G siRNAs were enriched in the β-eliminated library, whereas ALG-3/4 class 26G siRNAs were depleted (Figure 7A). The levels of normalized reads corresponding to piRNAs and ERGO-1 class 26G siRNAs were ~10 fold greater in the β-eliminated library relative to the non-treated library (Figure 7B). Each of the other classes of small RNAs was depleted in the β-eliminated library (Figure 7B). 22G siR-1 yielded ~1270 normalized reads (reads per million total) in the non-treated library and ~257 normalized reads in the β-eliminated library, amounting to an ~80% depletion of 22G siR-1 following β-elimination, indicating that 22G siR-1 is not methylated and thus indirectly affected by mutations in henn-1 (Figure S6). Interestingly, the methylated small RNAs, that is, piRNAs and ERGO-1 class 26G siRNAs, associate exclusively with Argonautes that are in the PIWI clade, while all other small RNAs in C. elegans are not methylated and associate with AGO and WAGO clade Argonautes (Figure 7C). Therefore, we conclude that HENN-1 specifically methylates small RNAs that associate with PIWIs in C. elegans.

Trimming and Tailing

In Drosophila, small RNAs that interact with perfect complementarity to target RNAs are subjected to trimming (3′-5′ shortening) and tailing (untemplated nucleotide additions) which marks them for degradation [49]. 3′ end methylation protects small RNAs from trimming and tailing in Drosophila and Arabidopsis [46,49]. Each class of siRNAs in C. elegans interacts with perfect or near perfect complementarity to their targets, whereas miRNAs generally interact with only partial complementarity, particularly at the 3′ end. It is unclear how piRNAs interact with their targets in C. elegans. We assessed which classes of small RNAs are tailed and trimmed in C. elegans by analyzing our deep sequencing libraries. miRNAs and piRNAs displayed relatively low proportions of trimmed and tailed sequences (Figure 7D). In contrast, each class of siRNAs showed relatively high proportions of trimmed and tailed sequences, although CSR-1 class 22G siRNAs and both classes of 26G siRNAs displayed the highest proportions (Figure 7D). Uridylation of certain siRNAs promotes their association with CSR-1, which at least partially explains the high
levels of trimming and tailing observed for this class of siRNAs [20]. It is interesting that although ERGO-1 class 26G siRNAs are presumably methylated, they are still subject to trimming and tailing at levels similar to the non-methylated ALG-3/4 class 26G siRNAs (Figure 7D).

**Discussion**

We developed a GFP-based sensor for endogenous siRNA activity in *C. elegans*. Using the siRNA sensor, we determined that endogenous 22G siRNAs, at least those that are dependent on *nrde-3*, do not trigger siRNA amplification or spreading from the target site and that a certain degree of mispairing is permissible for effective siRNA target recognition. We also show that 22G siRNA formation from an endogenous mRNA is initiated by a trans-acting 26G siRNA. This phenomenon is reminiscent of the trans-acting siRNA pathway in plants and the miR-243 pathway in *C. elegans*, in which one or more miRNAs or siRNAs trigger siRNA amplification from a distinct mRNA [50–53]. These findings are important to our understanding of RNA silencing pathways for two reasons. First, that endogenous siRNAs require only partial complementarity to their targets suggests that the hundreds of thousands of endogenous siRNAs in *C. elegans* have a multitude of potential targets distinct from the genes from which they are processed. Secondly, because our results suggest that endogenous 22G siRNAs do not trigger siRNA amplification, the effects of off-targeting may be negligible for all but the most abundant 22G siRNAs, as well as the 26G siRNAs.

From a candidate screen for endogenous siRNA factors, we identified a requirement for the *C. elegans* HEN1 ortholog *henn-1* in a specific endogenous siRNA pathway. Small RNA analysis in *henn-1* mutants and deep sequencing of methylated small RNAs revealed that ERGO-1 class 26G siRNAs and piRNAs are both methylated by HENN-1. Secondary 22G siRNAs that depend on ERGO-1 class 26G siRNAs also require *henn-1*, albeit indirectly, for their biogenesis. In *Drosophila*, small RNA methylation prevents degradation of small RNAs perfectly basepaired to their targets [49]. It is somewhat puzzling that although all siRNAs share perfect complementarity to their targets in *C. elegans* one class requires methylation but the others do not. One possibility is that
only ERGO-1 class 26G siRNA and piRNAs actually interact perfectly with their targets. Perhaps the 3' ends small RNAs are more easily liberated from the PIWI PAZ domains than from the AGO or WAGO PAZ domains, which accommodate the 3' ends of small RNAs [54–56], to interact with their targets. In this model, PIWI-associated methylated small RNAs bound at their 3' ends to target mRNAs would be protected by the 3'2'O-methyl group, while AGO- and WAGO-associated small RNAs would remain anchored to the PAZ domain and therefore inaccessible to nucleases. This might also explain why trimming and tailing levels are similar for ERGO-1 and ALG-3/4 class 26G siRNAs – both are equally protected, but by different means. Perhaps in the absence of HENN-1, ERGO-1 class 26G siRNAs would be hyper trimmed and tailed.

Given that only small RNAs that associate with PIWIs require hem-1, we propose that PIWIs are specifically adapted to associate with 3'-2'-O-methylated small RNAs and perhaps also with HENN-1 in C. elegans. An intriguing, but highly speculative possibility is that methylation is used as a sorting determinant to direct certain small RNA-Argonaute interactions. In vitro, the PAZ domains of the human PIWI clade Argonautes Hili and Hiwi preferentially bind methylated small RNAs, whereas the PAZ domain of a human AGO clade Argonaute Ago1 preferentially binds small RNAs lacking a 3'-2'-O-methyl group [56,57]. In animals, PIWIs associate with methylated small RNAs, while non-PIWI clade Argonautes associate with non-methylated small RNAs, with one exception: methylated siRNAs in Drosophila associate with the AGO clade Argonaute Ago2 [42]. In C. elegans, methylation of ERGO-1 class 26G siRNAs may prevent them from associating with ALG-3 and ALG-4 and lack of methylation on ALG-3/4 class 26G siRNAs may in turn prevent them from associating with ERGO-1. This model does conflict somewhat with findings in Drosophila that small RNAs are methylated only when bound to their Argonaute binding partner [42], but one could imagine that other features of the small RNA tag it for methylation before Argonaute loading and then upon loading methylation occurs. The presence or absence of methylation would then dictate whether or not the 3' end of the small RNA is stabilized within the Argonaute PAZ domain or if the small RNA is discarded.
ERGO-1 class 26G siRNAs function during oogenesis and trigger formation of 22G siRNAs that persists into adulthood [9,10,18], while piRNAs function during germline and sperm development [4,5,12]. Therefore, henn-1 is likely to have important roles in RNA silencing pathways throughout C. elegans development. It will be important to learn why henn-1 effects only specific sRNA pathways and why its activity seems to be dispensable for piRNA stabilization except at specific developmental stages.

**Methods**

**Transgenes and C. elegans Strains**

The ubl-1 upstream and downstream regulatory sequences were amplified from N2 genomic DNA using Phusion polymerase (Finnzymes) and the primers attB1-ubl-1p F and attB4-ubl-1p R or attB3-ubl-lu F and attB2-ubl-lu R. GFP was PCR amplified from plasmid DNA with the primers attB4r-GFP F and attB3r-GFP R. The 22G siR-1 target site was introduced by PCR into the ubl-1 3′ UTR using the primers X-motif-ubl-lu F and attB2-ubl-lu R. 22G siR-1 target site mutations were introduced by PCR using various forward primers in combination with attB2-ubl-lu R (Table S1). To generate the K02E2.11 mosDEL construct, a ~2.4 kb sequence of homology to K02E2.11 and sequence immediately downstream was PCR amplified from N2 genomic DNA using the primers attB1-K02E2.11 LH F and attB4-K02E2.11 LH R. A 2 kb sequence adjacent to the Mos1 insertion site in ttTi18384 was PCR amplified with attB3-K02E2.11 RH F and attB2-K02E2.11 RH R from genomic N2 DNA. The unc-119 rescue transgene was amplified from C. briggsae genomic DNA using attB4r-Cbr-unc-119 F and attB3r-Cbr-unc-119 R. PCR products were cloned into pDONR entry vectors using Gateway BP recombination (Invitrogen). Entry vectors were recombined into pCFJ178 or pCFJ151 modified to contain Gateway Pro LR recombination sites (pCMP2 and pCMP1, respectively). Constructs were sequence verified for accuracy. GFP constructs were introduced into C. elegans strain EG5003 using Mos1-mediated single copy insertion [25]. The K02E2.11 knockout construct was introduced into IE18384, which carries the Mos1 insertion ttTi18384, using Mos1-mediated deletion [38]. The henn-1 mutant strain, NL4415, contains the pk2295 allele; the rrf-1 mutant strain, NL2095, contains the pk1417 allele; and the rde-2 mutant strain, NL3531, contains the pk1657 allele [43]. The rde-3 mutant strain, WM156, contains the tm1116 allele. Each of the strains developed in this study are listed in Table S2. All primer sequences are listed in Table S1.

**Antibody Staining and C. elegans Imaging**

GFP antibody (Invitrogen, A-11122 and A-11034) and DAPI staining were done as described [58]. All imaging was done on a Zeiss AxiosImager.Z1 Microscope.

**RNA and Protein Preparation**

RNA was isolated from synchronized embryos, L4 larvae or adult C. elegans using Trizol (Invitrogen) followed by chloroform extraction and isopropanol precipitation. RNA samples were normalized to 1.0 or 2.0 µg/µl prior to blot assays, qRT-PCR assays and deep sequencing. Protein was extracted from synchronized L4 larvae using Laemmli buffer and normalized by Actin and the number of animals.

**RNA and Protein Blot Assays**

For small RNA Northern blots, 10 ug total RNA was separated on 17% denaturing polyacrylamide gels, transferred to positively charged Nitrocellulose membranes, crosslinked and probed with 32P-labeled LNA-modified (siRNA and piRNA probes) or unmodified (miRNA probes) DNA oligonucleotides antisense to each of the small RNAs analyzed (Table S1). For GFP mRNA blots, 2 µg total RNA was separated on denaturing 1.5% Agarose gels, transferred to positively charged nitrocellulose membranes, crosslinked and probed with a randomly labeled ~430 bp GFP DNA fragment. For Western blots, proteins were resolved on 4–12% Bis-Tris SDS polyacrylamide gels, transferred to nitrocellulose membranes and probed with GFP or Actin antibodies (Invitrogen, A-11122 and A-11034; Abcam, ab5290). Protein levels were quantified on a Typhoon phosphorimagery using the ImageQuant TL software (GE Healthcare Life Sciences). Actin levels were used for normalization across samples.

**Deep Sequencing and Data Analysis**

β-elimination was done as described [47]. 18–28 nt small RNAs were size selected on 17% denaturing polyacrylamide gels. Small RNAs were Tobacco Acid Phosphatase treated to reduce 5′ di- and triphosphate groups to monophosphates, ligated to 3′ and 5′ adapters and subjected to RT-PCR and gel purification of small RNA amplicons. A detailed protocol is available on request. For Illumina GAII sequencing (ubl-1:GFP and ubl-1::GFP-siR-1-sensor libraries), the 5′ adapter sequences were modified to contain barcodes (AAC and CCC, respectively) for multiplexing two libraries into one lane of a flowcell. For Illumina HiSeq sequencing, the TruSeq small RNA PCR Indexing primers RP11 and RP12 were used to introduce index sequences into each library and then multiplexed into one lane of a flowcell. Small RNA sequences were parsed and mapped to either the N2 reference genome (Wormbase release WS204) or ubl-1:GFP and ubl-1::GFP-siR-1-sensor transgene sequences using CASHX v. 2.0 and custom Perl programs [59]. Data analysis was done as described [13]. The small RNA trimming and tailing analysis was done as described [49] using annotated miRNA and piRNA sequences [4,60]. siRNAs were classified by their length and genomic locus [13].

**RNAi Assays**

Synchronized C. elegans were fed *E. coli* HT115 expressing dsRNA against target genes [61,62] beginning at L1 larval stage and scored and imaged at the L4 larval stage during the second generation of feeding at 23–25°C.

**qRT–PCR and 5′ RACE Assays**

Quantitative RT-PCR assays of small RNA (TaqMan, Life Technologies) and mRNA (SYBR Green, Bio-Rad) levels were done according to Life Technologies and Bio-Rad recommendations and as described [13]. For mRNA assays, spl-32 levels were used for normalization across samples. miR-1 or miR-55 levels were use for normalization of small RNA levels after determining their levels were unchanged using Northern blot assays. TaqMan probes were validated using mutants defective for each of the small RNAs analyzed. The 2−ΔΔCT method was used for comparing relative levels of small RNAs and miRNAs. 5′ RACE assays for siRNA-guided cleavage were done as described [63]. Primer and small RNA sequences are listed in Table S2.

**Statistics and Phylogenetics**

Statistical analysis was done in R and Excel. When comparing quantitative protein data, p values were calculated using two sample t-tests. For qRT-PCR data analysis, p values were calculated using ANOVA and Tukey’s HSD tests. P values for comparing wild type and henn-1 mutant brood sizes were calculated using the Mann-Whitney test. Bonferroni corrections were applied to account for multiple comparisons. Nucleic acid
sequence alignments were done with ClustalW v. 2.1. Argonaute protein sequences were aligned with ClustalW v. 2.1 using protein weight matrix Pam350 (Dayhoff) [64]. The phylogenetic tree was drawn with PHYLF v. 3.69.

Data Accession Numbers
The deep sequencing data reported here is available through the Gene Expression Omnibus database, www.ncbi.nlm.nih.gov/geo, via accession number GSE35550.

Supporting Information
Figure S1  *nrde-3* desilences GFP expression from the siRNA sensor. Protein blot assay of GFP from the control and siRNA sensor transgenes in either wild type or *nrde-3* mutants. Actin protein is shown as a loading control. (TIF)

Figure S2  5′ RACE assay of cleavage at the X-cluster locus. Gel image displays the PCR product generated by 5′ arrows. Indicate cleavage sites. The proportion of cloned 5′ ngaraved sites. The proportion of cloned 5′ RACE PCR products that indicate cleavage at each site is shown above the arrows. (TIF)

Figure S3  Small RNA defects in *rde-2* mutants. RNA blot assays of small RNAs in wild type and *rde-2* mutant adult *C. elegans*. EtBr stained rRNAs are shown as a loading control. (TIF)

Figure S4  piRNA defects in *henn-1* are stage specific. qRT-PCR assay of 21UR-1 levels in *henn-1* mutants relative to wild type *C. elegans*. T The proportion of cloned 21UR-1 reads (reads per million total) in small RNA libraries generated from wild type *C. elegans* and piRNAs act upstream of an endogenous siRNA pathway to suppress Tc3 and 21U-RNAs interact to form the piRNA complex required for fertility in *C. elegans*. Mol Cell 31: 67–78. (TIF)

Figure S5  Small RNA defects in *henn-1* mutants. qRT-PCR assay of individual small RNA levels in *henn-1* mutants relative to wild type adults. (TIF)

Figure S6  22G siR-1 is depleted by β-elimination. Normalized 22G siR-1 reads (reads per million total) in small RNA libraries generated from wild type *C. elegans* RNA that was either untreated or subjected to β-elimination. (TIF)

Table S1  Oligonucleotide sequences used in the study. Names and oligonucleotide sequences are shown. (XLSX)

Table S2  Transgenic strains used in the study. Names and descriptions of strains are shown. (XLSX)

Acknowledgments
Special thanks to Phil Zamore, Stefan Amares, and Ji-Hung Hung for providing the small RNA trimming and tailing data analysis; Rene Ketting for providing the *henn-1* mutant strain; John Kim for discussion and sharing unpublished data; and Maté Carre-Pierrat for providing the IE18384 strain. Thanks also to Noah Fahlgren, Jim Carrington, Chris Sullivan, Mark Borowsky, Kaleena Shirley, Stuart Levine, and Kevin Thai for computational and Illumina sequencing assistance.

Author Contributions
Conceived and designed the experiments: TAM GR. Performed the experiments: TAM Y-SR CZ RHD CMP SEJF. Analyzed the data: TAM. Wrote the paper: TAM GR.


