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Piwi and piRNAs Act Upstream of an Endogenous siRNA Pathway to Suppress Tc3 Transposon Mobility in the *Caenorhabditis elegans* Germline

Partha P. Das,[1,2,3] Marloes P. Bagijn,[1,4] Leonard D. Goldstein,[4,5,7] Julie R. Woolford,[1,2] Nicolas J. Lehrbach,[1,2] Alexandra Sapetschnig,[1,2] Heeran R. Buhecha,[1,2] Michael J. Gilchrist,[1] Kevin L. Howe,[5] Rory Stark,[5] Nik Matthews,[5] Eugene Berezikov,[6] René F. Ketting,[6] Simon Tavaré,[4,5,7] and Eric A. Miska[1,2,*]

[1]Wellcome Trust Cancer Research UK Gurdon Institute, University of Cambridge, The Henry Wellcome Building of Cancer and Developmental Biology, Tennis Court Road, Cambridge CB2 1QN, UK
[2]Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1GA, UK
[3]PhD Program, Faculty of Biology, GZMB, Georg-August-Universität Göttingen, Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany
[4]Department of Oncology, University of Cambridge, Hills Road, Cambridge CB2 2XZ, UK
[5]Cancer Research UK, Cambridge Research Institute, Li Ka-Shing Centre, Robinson Way, Cambridge CB2 0RE, UK
[6]Hubrecht Institute, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands
[7]Department of Applied Mathematics and Theoretical Physics, University of Cambridge, Centre for Mathematical Sciences, Wilberforce Road, Cambridge CB3 0WA, UK
*Correspondence: eam29@cam.ac.uk
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SUMMARY

The Piwi proteins of the Argonaute superfamily are required for normal germline development in *Drosophila*, zebrafish, and mice and associate with 24–30 nucleotide RNAs termed piRNAs. We identify a class of 21 nucleotide RNAs, previously named 21U-RNAs, as the piRNAs of *C. elegans*. Piwi and piRNA expression is restricted to the male and female germline and independent of many proteins in other small-RNA pathways, including DCR-1. We show that Piwi is specifically required to silence Tc3, but not other Tc/mariner DNA transposons. Tc3 excision rates in the germline are increased at least 100-fold in *piwi* mutants as compared to wild-type. We find no evidence for a Ping-Pong model for piRNA amplification in *C. elegans*. Instead, we demonstrate that Piwi acts upstream of an endogenous siRNA pathway in Tc3 silencing. These data might suggest a link between piRNA and siRNA function.

INTRODUCTION

Piwi proteins are part of a superfamily of Argonaute proteins that is defined by the presence of PAZ and Piwi domains (Cerutti et al., 2000). The PAZ domain has been shown to bind to 3′ ends of short RNAs, and the Piwi domain is similar to the catalytic domain of RNase H. The Argonaute superfamily can easily be divided into two clades according to sequence similarity (Carmell et al., 2002). The AGO clade is found in fission yeast, plants, and animals, whereas the Piwi clade is found in ciliates, slime molds, and animals. Members of the AGO clade directly bind to siRNAs and miRNAs, are involved in many aspects of transcriptional (TGS) and posttranscriptional gene silencing (PTGS) in many species, and are generally ubiquitously expressed (Joshua-Tor, 2006).

The expression of proteins of the Piwi clade might be restricted to germ cells in vertebrates (Carmell et al., 2007; Deng and Lin, 2002; Houwing et al., 2007; Kuramochi-Miyagawa et al., 2001, 2004), but not in *Drosophila*, where Piwis are also found in somatic cells associated with the germline (Brennecke et al., 2007; Cox et al., 1998; Gunawardane et al., 2007; Saito et al., 2006). In flatworms, Piwis are expressed in germ cells and somatic stem cells (Reddien et al., 2005). A number of functions have been assigned to Piwi proteins: *Drosophila* Piwi is required for oogenesis and stem cell maintenance (Cox et al., 1998, 2000; Lin and Spradling, 1997); a *Tetrahymena* Piwi protein, Tiwi, is required for DNA elimination in the macronucleus (Mochizuki et al., 2002); in planaria the Piwi protein SMEDWI-1 is required for stem cell regulation (Reddien et al., 2005); and, in vertebrates, Piwi proteins are required for germline development in the zebrafish (Houwing et al., 2007) and male germline development in the mouse (Aravin et al., 2006; Deng and Lin, 2002; Girard et al., 2006; Grivna et al., 2006; Kuramochi-Miyagawa et al., 2001). Piwi proteins are also required for transposon silencing in the *Drosophila* germline (Aravin et al., 2001; Savitsky et al., 2006; Vagin et al., 2006), and several studies suggest that many of the phenotypes observed in *piwi* mutants might be due to loss of germline integrity (Chen et al., 2007; Klattenhoff et al., 2007; Pane et al., 2007).

Recently, a class of 24–30 nucleotide RNAs has been found to interact with Piwi proteins in *Drosophila*, zebrafish, mice, and rats and has been named *Pi*wi-interacting *RNAs* or piRNAs (Klattenhoff and Theurkauf, 2008). piRNA populations are complex; there are hundreds of thousands of unique piRNAs in mammals. The identification of piRNAs has improved our understanding of Piwi function, in particular with regard to transposon silencing: for example, in *Drosophila* the piRNAs of the *flamenco* locus control the *gypsy* retrotransposon (Brennecke et al., 2007; Desset et al., 2003; Prud'homme et al., 1995).

The best-studied transposable elements of *C. elegans* are the DNA transposons of the Tc/*mariner* superfamily (Moerman and Waterston, 1984), in particular Tc1 and Tc3 (van Luenen et al., 1994; Vos et al., 1996). Tc1 and Tc3 are also the most abundant transposons of the Tc family in the N2 Bristol strain of *C. elegans* with 31 and 22 copies, respectively (*C. elegans* Sequencing Consortium, 1998). Tc1 and Tc3 are autonomous elements encoding a transposase specific to each element (van Luenen et al., 1993; Vos et al., 1993).

Here we demonstrate a role for *C. elegans* Piwi in germline development and germline transposon silencing. We identify the recently named 21U-RNAs as the piRNAs of *C. elegans*. We demonstrate that in *C. elegans* piRNAs act upstream of an endogenous siRNA pathway for Tc3 silencing. These data shed light on piRNA function.

RESULTS

Piwi Is Required for Normal Germline Development

To investigate the roles of Piwi proteins in *C. elegans* we generated mutants lacking Piwi function. The *C. elegans* genome encodes two *P*iwi-*r*elated *g*enes, *prg-1* and *prg-2*. These genes are likely the result of a recent gene duplication, as the genomes of the related nematode species *C. briggsae* and *C. remanei* each contain a single *prg* gene. PRG-1 and PRG-2 are 91% identical at the amino acid level, which suggests that they might act redundantly. We generated two deletion alleles each for *prg-1* (*n4357*, *n4503*) and *prg-2* (*n4358*, *nDf57*) and generated double mutant strains after outcrossing of the single mutants (see the Supplemental Data available online). For brevity, we will refer to PRG-1 and PRG-2 as Piwi, and we will refer to *prg-1; prg-2* double mutants as *piwi* mutants, i.e., *piwi(n4357; n4358)* instead of *prg-1(n4357); prg-2(n4358)*. *prg-1* and *prg-2* single mutants and *piwi* mutants were homozygous viable and showed neither a defect in exogenous RNAi in either the soma or germline nor defects in miRNA biogenesis or function (data not shown). However, all mutant strains showed reduced fertility (Figure S1A). Of the single mutants, *prg-1* mutant animals showed the most pronounced effect, with fertility reduced to 25% of that of wild-type animals (Figure S1A). The fertility defect is enhanced in *piwi* mutants (Figure S1A). These observations agreed with previous studies of *prg-1* using RNAi and an independent *prg-1* allele (Cox et al., 1998; Yigit et al., 2006). As *prg-1* RNAi had suggested a role for PRG-1 in spermatogenesis (Cox et al., 1998), we counted hermaphrodite sperm in wild-type and *prg-1* and *prg-2* mutant animals. Sperm counts were reduced to approximately 50% in both single and double mutants (Figure S1B). However, fertility of *piwi* mutants was not restored to wild-type levels by introducing wild-type sperm through mating, suggesting that Piwi function is not restricted to spermatogenesis (Figure S1C). Finally, *piwi* mutant germlines showed abnormal mitotic to meiotic transitions (data not shown). These data confirm a conserved role for PRG-1 and PRG-2 in *C. elegans* germline development (Cox et al., 1998).

The piRNAs of *C. elegans* Are 21 Nucleotide RNAs

In *Drosophila*, zebrafish, mice, and rats, Piwi proteins are associated with 24–30 nucleotide piRNAs (Aravin et al., 2006; Brennecke et al., 2007; Girard et al., 2006; Grivna et al., 2006; Gunawardane et al., 2007; Houwing et al., 2007; Lau et al., 2006; Saito et al., 2006; Vagin et al., 2006; Watanabe et al., 2006). Searching for piRNAs in *C. elegans*, we were unable to identify an abundant class of RNAs in this size range (data not shown). We therefore searched for piRNAs among the small RNAs previously identified in *C. elegans*: miRNAs, tncRNAs, endogenous siRNAs, and 21U-RNAs (Ambros et al., 2003; Lau et al., 2001; Lee and Ambros, 2001; Lim et al., 2003; Ruby et al., 2006). To identify candidate piRNAs, we tested if any of these short RNAs were dependent on Piwi. Surprisingly, we found that a 21U-RNA, 21UR-1, which was detected in RNA from wild-type animals, was absent in RNA from two independent *piwi* mutants by northern blotting (Figure 1A). In contrast, expression of a ubiquitous miRNA, miR-52, was unaffected. To test if 21U-RNAs were generally absent in *piwi* mutants we generated libraries of 5′ monophosphate small RNAs from wild-type and *piwi(n4357; n4358)* mutants. High-throughput sequencing identified 1398 out of 5454 previously known 21U-RNAs (Ruby et al., 2006). We also identified a large number of candidate 21U-RNAs (data not shown). 21U-RNAs were either absent or dramatically underrepresented in the *piwi* mutant library as compared to the wild-type library (Figure 1B). The most abundant 21U-RNA in the *piwi* sample had eight reads as compared to 2127 reads in wild-type. We also assessed the expression levels of other small RNAs in wild-type versus *piwi* mutants and found no differences in miRNA expression, tncRNA expression, or a number of siRNA species including a 26 nucleotide siRNA (Figure 1C and data not shown). These data suggest that 21U-RNAs might be the piRNAs of *C. elegans*. To test this hypothesis directly, we generated a rabbit polyclonal antibody against PRG-1. After immunoprecipitation of PRG-1 using αPRG-1 serum from wild-type *C. elegans* adult whole-cell extracts, we detected 21U-RNAs by RT-PCR but were not able to detect them from *piwi* mutant extracts or when using preimmune serum (Figure 1D). Overall, 21U-RNAs are 100-fold enriched in PRG-1 immunoprecipitates (Batista et al., 2008 [this issue of *Molecular Cell*]; see Discussion). As the high-throughput sequencing data suggested that 21U-RNAs were dramatically reduced in *piwi* mutants, we independently quantified the expression of seven 21U-RNAs by quantitative RT-PCR. As shown in Figure 1E, while the expression of a number of 21U-RNAs is dramatically reduced, some 21U-RNAs, including 21UR-1, were still detected in *piwi* mutants. For 21UR-1 we verified that the signal was specific by cloning and sequencing of RT-PCR products (data not shown). Taken together, these data suggest that the 21U-RNAs are the piRNAs of *C. elegans*, and we will refer to them as piRNAs below.

piRNA Biogenesis Is Independent of Many Genes in Other Small-RNA Pathways

To identify additional genes involved in piRNA pathways in *C. elegans*, we tested a panel of genes using mutants and RNAi for their effect on piRNA expression by northern blotting or quantitative RT-PCR (Figure 2 and Table S1). First, we checked piRNA expression in *prg-1* and *prg-2* single mutants. Using three independent alleles and RNAi experiments, we found that only PRG-1 but not PRG-2 is required for piRNA

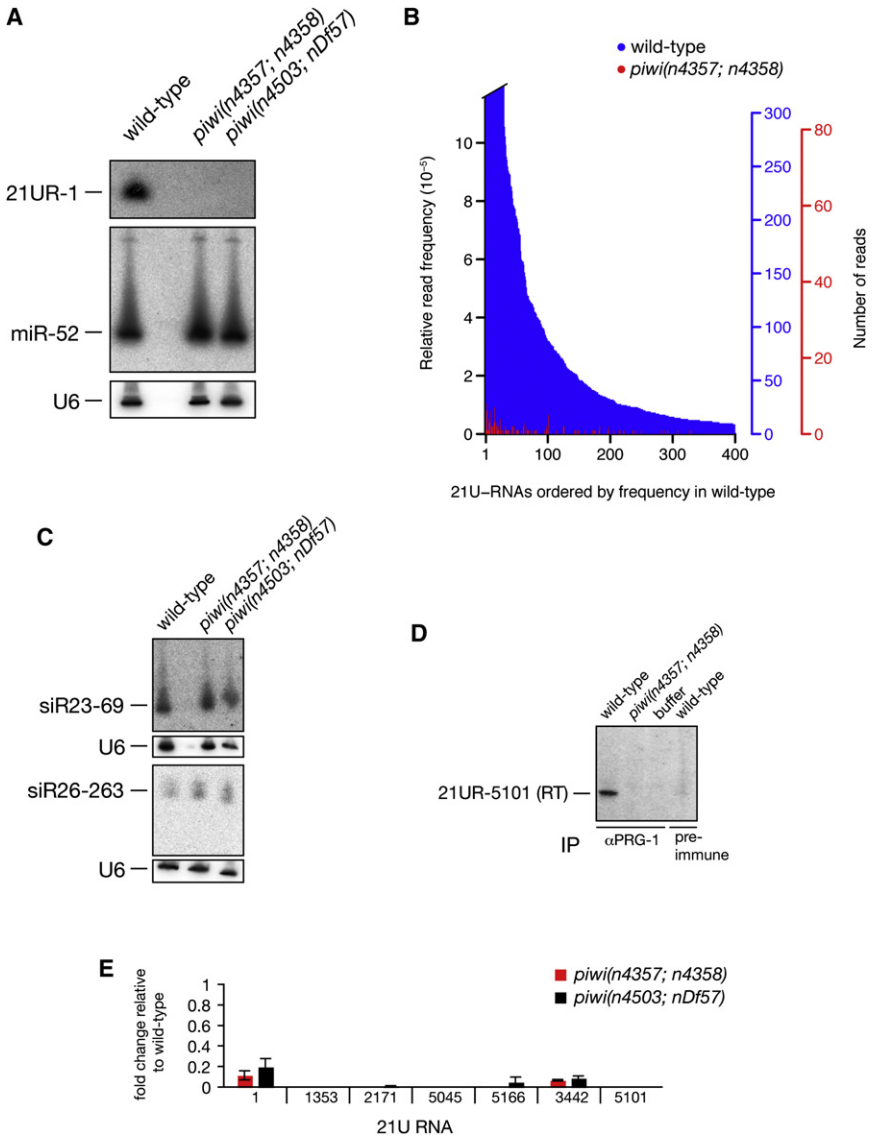


Figure 1. The 21U-RNAs Are *C. elegans* piRNAs

(A) Northern blot showing that 21UR-1 is not detected in RNA (40 μg) isolated from two independent *piwi* double mutants: *piwi(n4357; n4358)* and *piwi(n4503; nDf57)*, whereas miR-52 is expressed in both mutants (same blot reprobed). Antisense DNA probes were used for 21UR-1 and miR-52. A U6 northern blot is shown as loading control. *piwi(n4357; n4358)* is an abbreviation for *prg-1(n4357); prg-2(n4358)*. *piwi(n4503; nDf57)* is an abbreviation for *prg-1(n4503); prg-2(nDf57)*.

(B) High-throughput sequencing reveals that the expression of many 21U-RNAs is dramatically reduced in *piwi* mutants. 21U-RNAs cloned from 5′ dependent wild-type and *piwi(n4357; n4358)* mutant libraries. Frequencies are shown for wild-type (blue) and *piwi* mutant (red) for the 400 most abundant 21U-RNAs in wild-type, plotted in the order of their wild-type frequency. Read frequencies were obtained by dividing the number of reads for a given 21U-RNA by the total number of reads from the same library (left-hand y axis). The corresponding absolute number of reads are indicated in the right-hand y axes. 21U-RNAs for which frequencies are shown include the 21U-RNA with most reads in the *piwi* mutant library (21UR-3224), which was sequenced eight times in the *piwi* mutant and 2127 times in wild-type.

(C) Expression of a 23 nucleotide antisense RNA (siR23-69) and a 26 nucleotide antisense RNA (siR26-263) is not affected in *piwi* mutants (northern blotting, 40 μg total RNA, antisense DNA probes).

(D) Immunoprecipitation followed by RT-PCR for 21UR-5101 reveals that 21U-RNAs are associated with PRG-1 in *C. elegans* extracts.

(E) Quantitative RT-PCR of seven 21U-RNAs demonstrates that Piwi is not essential for 21U-RNA biogenesis. Total RNA was extracted from 12 hr adult *C. elegans*. Expression levels shown are relative to levels in wild-type RNA. miR-52 expression was used as an internal control. Data are from three independent biological replicates. Error bars represent standard error of the mean.

expression (Figure 2 and Table S1). Next, we tested other Argonaute proteins; these included RDE-1, which is required for exogenous RNAi (Tabara et al., 1999), ALG-1 and ALG-2, which are redundantly required for miRNA function (Grishok et al., 2001), ERGO-1, SAGO-1, SAGO-2, and a group of five "MAGO" Argonaute proteins associated with endogenous siRNA pathways (Yigit et al., 2006). None of these Argonaute proteins were required for piRNA expression, emphasizing the specificity of the requirement for PRG-1. In addition, we tested a number of other proteins involved in small-RNA biology in *C. elegans*, including all four known RNA-dependent RNA polymerases (RdRPs), RRF-1, RRF-2, RRF-3, and EGO-1 (Simmer et al., 2002; Smardon et al., 2000), none of which were required for piRNA expression. We also tested if the RNase III enzyme DCR-1, which is essential for the generation of siRNAs and germline development (Grishok et al., 2001; Ketting et al., 2001; Knight and Bass, 2001), is required for piRNA expression. As *dcr-1* mutants are sterile, these experiments were carried out using homozygous mutant animals derived from heterozygous mothers or using RNAi. We found that DCR-1 is not required for piRNA expression (Figure 2B; Table S1). These data were also confirmed by high-throughput sequencing in *dcr-1* mutants (Figure 2C). These data suggest that piRNAs are independent of many proteins involved in other small-RNA pathways, including DCR-1.

Piwi and piRNAs Are Restricted to the Male and Female Germline

We found that 21UR-1 expression is developmentally regulated with highest expression detected in young adults and adults by northern blot (Figure 3A). Additional piRNAs showed a similar expression pattern, as determined by quantitative RT-PCR (e.g., 21UR-5101; Figure 3B). Interestingly, *prg-1* and *prg-2* mRNAs show a similar pattern (Figure 3C; Figure S2). The observed

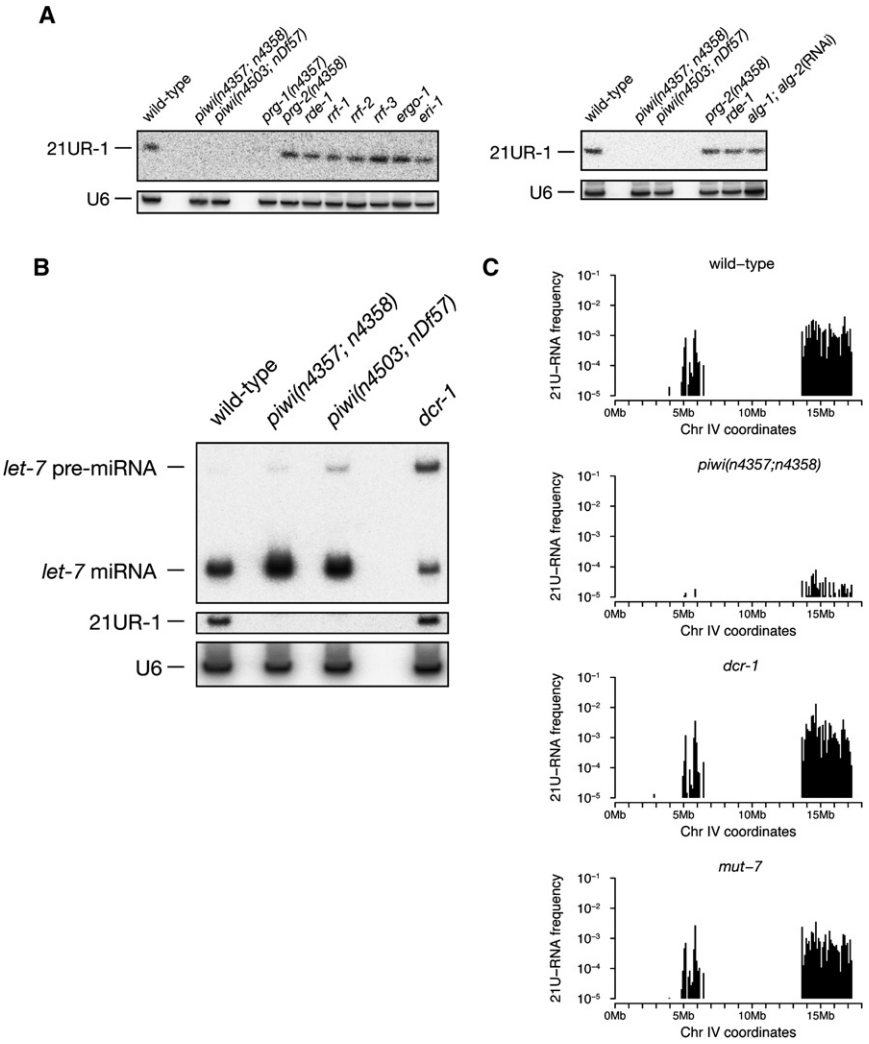


Figure 2. piRNA Biogenesis Does Not Require Many Known Small-RNA Pathway Proteins

(A) 21UR-1 northern blotting of total RNA of wild-type and mutant young adult *C. elegans*. In the case of *alg-1; alg-2*(RNAi), *alg-1* mutant L1 larvae were transferred to *alg-2* RNAi feeding plates, and young adult animals were harvested. A U6 northern blot is shown as loading control. See Table S1 in the Supplemental Data for quantification of these results.

(B) 21UR-1 northern blotting of total RNA of wild-type and mutant young adult *C. elegans*. *dcr-1* mutant animals used were homozygous animals derived from heterozygous mothers. To test for loss of DCR-1 activity in *dcr-1* mutants, *let-7* miRNA and pre-miRNA is shown. A U6 northern blot is shown as loading control.

(C) Distribution of 21U-RNAs on chromosome IV as detected by high-throughput sequencing of 5′ dependent wild-type, *piwi(n4357; n4358)*, *dcr-1*, and *mut-7* mutant libraries. Frequencies for a given 21U-RNA and locus were obtained by correcting the number of reads for multiple alignments and dividing by the total number of reads from the same library. Cumulative frequencies were plotted for nonoverlapping 100 kb windows along chromosome IV.

expression pattern for piRNAs and *piwi* mRNA is consistent with expression in the germline. As Piwi proteins and piRNAs are thought to be exclusively expressed in germ cells in vertebrates (Carmell et al., 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004; Kuramochi-Miyagawa et al., 2001), but not in *Drosophila* (Brennecke et al., 2007; Cox et al., 1998; Gunawardane et al., 2007; Saito et al., 2006), we decided to test their germline restriction in *C. elegans* using a set of temperature-sensitive mutants (Figure 3D). 21UR-1 was absent from *glp-4(bn2*ts*)* and *glp-1(e2144*lf*)* mutant animals at the restrictive temperature, which are devoid of germ cells (Beanan and Strome, 1992). However, 21UR-1 levels were unchanged in *glp-1(ar202*gf,ts*)* mutants at the restrictive temperature, which are highly enriched in germ cells that have not yet entered meiosis (Pepper et al., 2003). 21UR-1 RNA was also detected in RNA from *fem-1(hc17*ts*)* (Kimble et al., 1984; Nelson et al., 1978) and *fem-3(q22*sd,ts*)* (Barton et al., 1987) mutants at the restrictive temperature that are devoid of sperm and oocytes, respectively. The same restricted pattern was also observed for *piwi* mRNA (Figure 3E and Figure S2). Together these data suggested that *C. elegans* Piwi and piRNAs are restricted to the male and female germline.

Piwi Is Specifically Required for Silencing of Tc3 DNA Transposons

As Piwi proteins are required for transposon silencing in *Drosophila* (Aravin et al., 2001; Savitsky et al., 2006; Vagin et al., 2006), we examined transposon silencing in *piwi* mutants in *C. elegans*. We focused on the two most abundant DNA transposons in *C. elegans*, Tc1 and Tc3 (Figures 4A and 4B) (*C. elegans* Sequencing Consortium, 1998). First, we examined Tc1 transposase expression by quantitative RT-PCR using primers specific for 15 Tc1 loci (Figure 4C). We observed an approximately 50-fold increase in Tc1 transposase mRNA levels in *mut-7* mutants, which show an elevated rate of transposition of Tc/mariner elements in the germline (Ketting et al., 1999). We did not, however, observe an increase in Tc1 transposase mRNA in *piwi* mutants. Surprisingly then, we found increased Tc3 transposase mRNA in two independent *piwi* mutants and three independent *prg-1* mutants using primer pairs specific for 20 Tc3 loci (Figure 4D). Next, we assayed the germline excision rate of Tc1 and Tc3 transposons directly by the phenotypic reversion of *unc-22* transposon insertion alleles (Ketting et al., 1999). As shown in Table 1, *unc-22* reversion rates of Tc1, Tc3, or Tc4 insertion alleles were less than 10^{-6} in an otherwise wild-type background. In *mut-7* mutant animals the reversion rate was increased 100-fold, as reported previously (Ketting et al., 1999). Strikingly, in *prg-1* and *piwi* mutants Tc1 and Tc4 excision rates were not affected, but reversion of the Tc3 allele was increased 100- and 1000-fold, respectively (Table 1). These

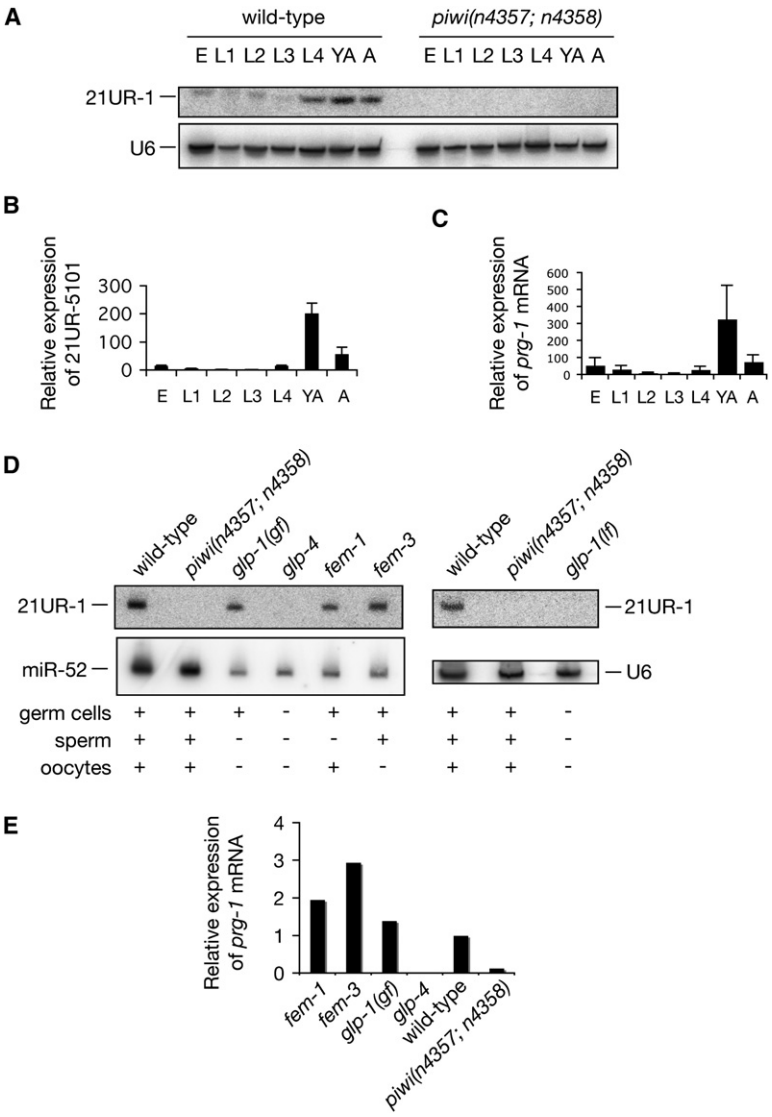


Figure 3. Piwi and piRNAs Are Restricted to the Male and Female Germline

(A) Profile of 21U-R1 expression during development. E, embryo. L1–L4, larval stages 1–4. YA, 12 hr adult. A, 24–48 hr adult. A U6 northern blot is shown as loading control.

(B) Quantitative RT-PCR of 21UR-5101. miR-52 expression was used as an internal control. Data are from three independent biological replicates. Error bars represent standard error of the mean.

(C) Quantitative RT-PCR of *prg-1* mRNA. Actin mRNA was used as an internal control. Data are from three independent biological replicates. Error bars represent standard error of the mean.

(D) Northern blot showing that piRNA expression is restricted to the male and female germline. *glp-1(gf)*, *glp-1(lf)*, *glp-4*, *fem-1*, and *fem-3* L1 larvae were grown to 12 hr adult stage at 25°C. Total RNA (20 μg) was loaded in each lane. U6 and miR-52 northern blots are shown as loading controls.

(E) Quantitative RT-PCR of *prg-1* mRNA. Actin mRNA was used as an internal control.

data suggest that PRG-1 and Piwi are powerful and specific suppressors of Tc3 transposition in the germline of *C. elegans*. As we found that Piwi and MUT-7 were both involved in germline transposon silencing, we wondered if these proteins shared additional functions. We therefore tested if Piwi, like MUT-7, is required for germline transgene silencing (Kim et al., 2005). However, we found that germline transgene silencing is intact in *piwi* mutants (Figure S3). These data suggest that Piwi and MUT-7 have overlapping and distinct roles in the *C. elegans* germline.

Piwi Acts Upstream of an Endogenous siRNA Pathway

Previous work demonstrated a role for MUT-7 (Ketting et al., 1999) and endogenous siRNAs (Sijen and Plasterk, 2003) in germline transposon silencing. Our findings suggested a role for Piwi and piRNAs in the same process. We therefore wanted to determine how these two pathways related to each other. First, we tested if MUT-7 and endogenous siRNAs might act upstream of piRNA expression. However, we found that piRNA levels were not affected in *mut-7* mutants by northern blotting and high-throughput sequencing (Figure 2C; Figure 5A). Next, we tested if Piwi and piRNAs might act upstream of MUT-7 and endogenous siRNAs. We searched for piRNAs mapping to Tc/mariner transposons and found that no known piRNAs mapped directly to Tc1 or Tc4. However, a single piRNA, 21UR-139, mapped to the sense strand of the transposase gene of 20 out of the 22 Tc3 insertions in the N2 genome (Table S2; Figure 5B). A second 21U-RNA in the Tc3 locus has also been identified (Batista et al., 2008; see Discussion). To identify endogenous siRNAs mapping to Tc transposons, we generated 5′ independent small-RNA libraries from wild-type and *piwi* mutants, as we expected these RNAs to carry 5′ di- or triphosphates (Pak and Fire, 2007; Ruby et al., 2006; Sijen et al., 2007). High-throughput sequencing identified a large number of endogenous small RNAs that map to Tc1 and Tc3 loci (Figure 5B; Supplemental Data). Interestingly, small RNAs mapping to the terminal inverted repeat (TIR) and the transposase open reading frame (ORF) of Tc3 were nearly absent in *piwi* mutants, but those mapping to Tc1 were unaffected. We confirmed these observations for small RNAs antisense to the transposase transcripts using an RNase protection assay (Figure 5C). While Tc1 and Tc3 antisense siRNAs were dependent on MUT-7, only Tc3 siRNAs were also dependent on Piwi. Unfortunately, we were unable to detect endogenous small RNAs from the TIRs using the same assay (data not shown). These data suggest that Piwi and piRNAs act upstream of a MUT-7-dependent endogenous siRNA pathway for Tc3 silencing. This finding raised the possibility that additional piRNAs are functionally linked to endogenous siRNAs. To test this hypothesis we examined piRNA loci for nearby endogenous siRNAs. Interestingly, we observed a local positional

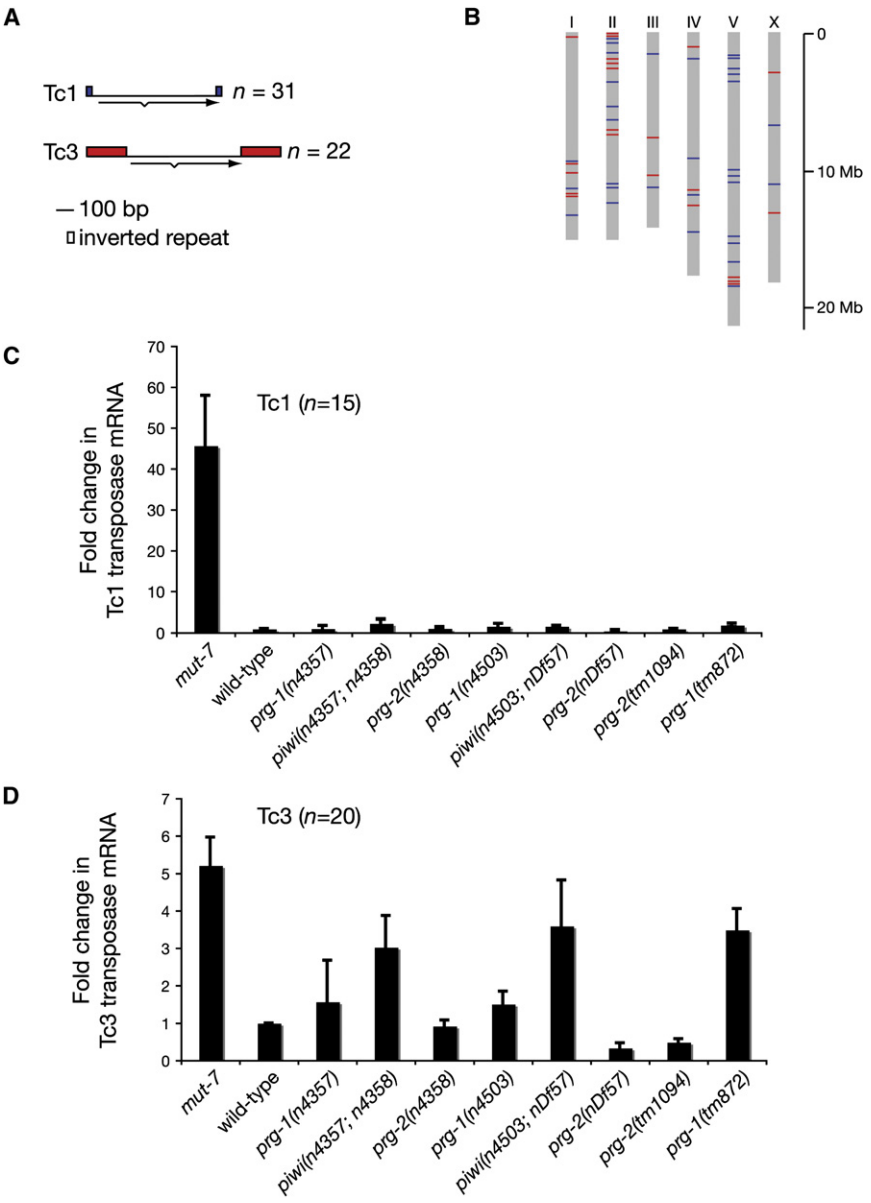


Figure 4. Piwi Is Required to Inhibit Tc3 Transposase Expression

(A) Diagram of the genomic structure of the two most common DNA transposons in *C. elegans*, Tc1, and Tc3. Tc1 and Tc3 are flanked by inverted repeats and encode a single spliced transcript for transposase. bp, base pairs. *n*, number of copies in the wild-type strain N2.

(B) Distribution of Tc1 and Tc3 transposons in the *C. elegans* genome.

(C and D) Quantitative RT-PCR of Tc1 or Tc3 transposase mRNA. As the genomic copies of Tc1 and Tc3 have minor sequence variations, the number of transposon loci amplified by each qRT-PCR primer pair are shown (*n*). Actin mRNA was used as an internal control. Expression levels shown are relative to levels from wild-type RNA. Data are from three independent biological replicates. Error bars represent standard error of the mean.

Table 1. PIWI Is Required to Inhibit Tc3 Transposition in the Germline

	unc-22::Tc1	*unc-22::Tc3*	*unc-22::Tc4*
Wild-type	$<10^{-6}$ [a]	$<10^{-6}$ [a]	$<10^{-6}$ [a]
prg-1(n4357)	10^{-6}	10^{-4}	10^{-6}
prg-1(tm872)	n.d.	10^{-3}	n.d.
piwi(n4357; n4358)	10^{-6}	10^{-3}	10^{-6}
piwi(n4503; nDf57)	n.d.	10^{-3}	n.d.
mut-7	10^{-4}	10^{-4}	10^{-4}

Transposition rates were estimated by scoring *unc-22* reversion rates. Germline excision was verified by scoring the progeny of revertants. Animals were grown at 20°C.

[a] No *unc-22* reversions were observed. n.d., not determined.

bias of antisense siRNAs downstream of piRNA loci, and their accumulation appears to be dependent on Piwi (Figure 6A; see Discussion).

DISCUSSION

21U-RNAs Are piRNAs with Surprising Features

We identify the piRNAs of *C. elegans* as 21 nucleotide RNAs (Figure 1). Our observations are strongly supported by the finding that PRG-1 immunoprecipitates are 100-fold enriched for 21U-RNAs (Batista et al., 2008). The *C. elegans* 21U-RNAs/piRNAs share several features with piRNAs of other species. First, *C. elegans* piRNAs have 5′ uridines. This bias is common to piRNAs from *Drosophila* and vertebrates (Klattenhoff and Theurkauf, 2008). Second, several *C. elegans* piRNAs have a 5′ monophosphate and a 3′ modification blocking the 2′ or 3′ oxygen, likely a 2′-*O*-methyl group (data not shown; Ruby et al., 2006). This modification is shared by piRNAs from *Drosophila* (Horwich et al., 2007; Saito et al., 2007; Vagin et al., 2006), zebrafish (Houwing et al., 2007), and mice (Kirino and Mourelatos, 2007; Ohara et al., 2007). However, 3′ ends with 2′-*O*-methyl groups are not a unique feature of piRNAs, as plant miRNAs and siRNAs and *Drosophila* siRNAs also carry this modification (Ghildiyal et al., 2008; Li et al., 2005).

The piRNAs of *Drosophila*, zebrafish, mice, and rats are 24–30 nucleotides in length, and as such are distinct from miRNAs or endogenous siRNAs (Klattenhoff and Theurkauf, 2008). The reason for the size difference between 21U-RNAs and piRNAs of other organisms is unclear. Another distinction of *C. elegans* piRNAs is their genomic location and organization: while

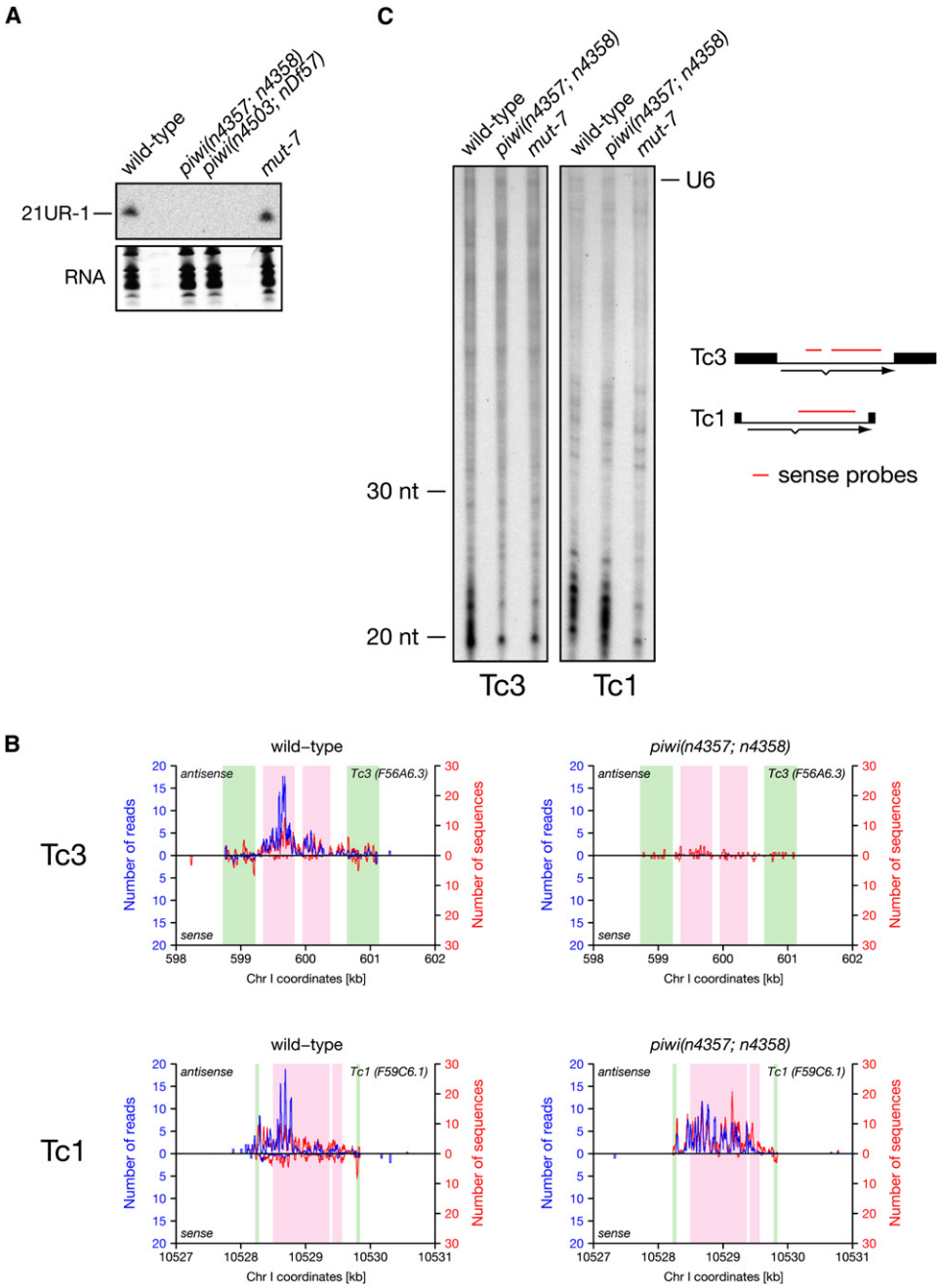
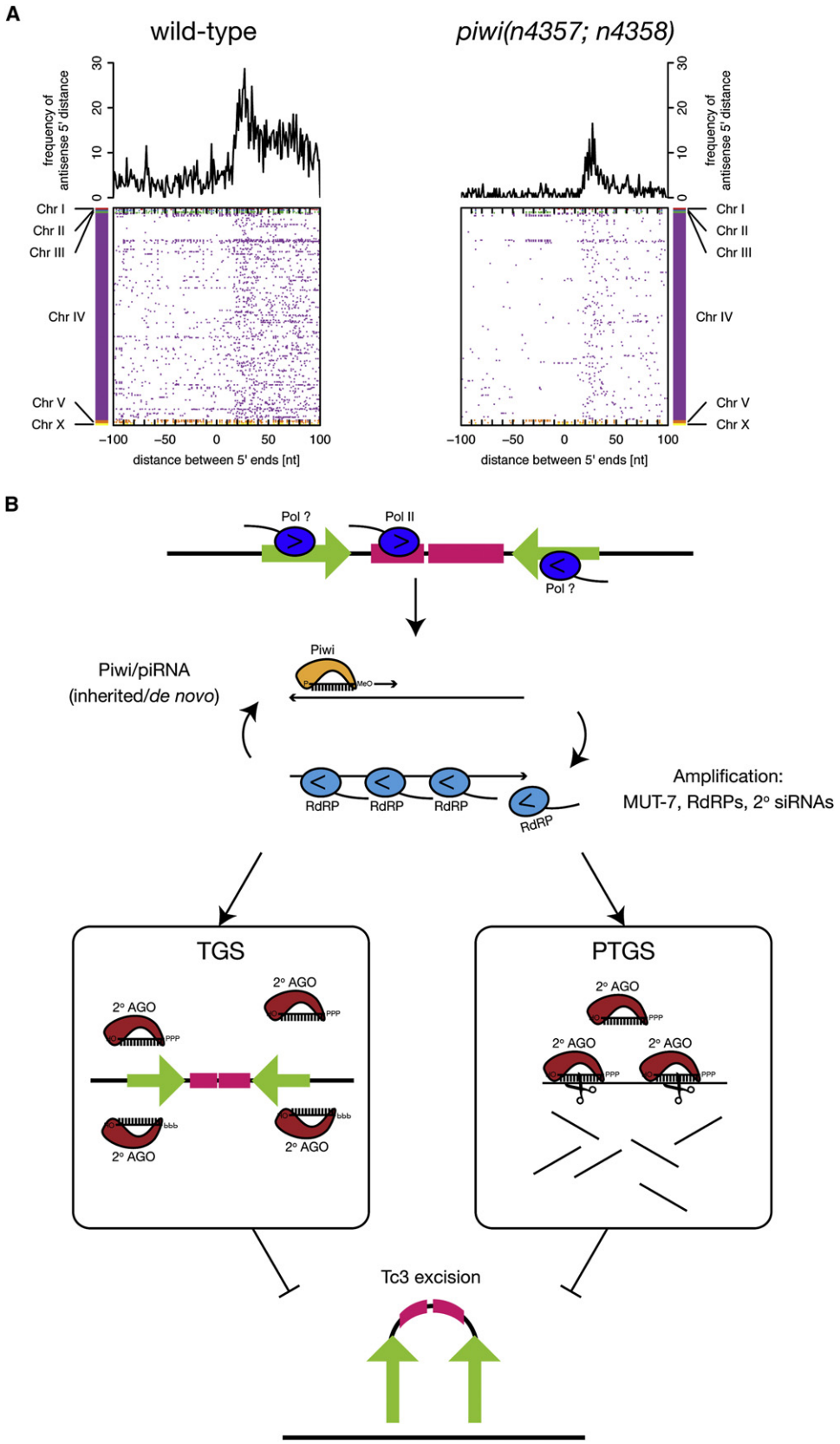


Figure 5. Piwi Acts Upstream of Endogenous siRNAs in Tc3 Silencing

(A) piRNA expression is independent of MUT-7. 21UR-1 northern blotting of total RNA of wild-type and mutant young adult *C. elegans*. Total RNA is shown as loading control (GelStar).

(B) Tc3-associated small RNAs are absent in *piwi* mutants. Small RNAs mapping to the loci of Tc3 (top) and Tc1 (bottom) on chromosome I as identified by high-throughput sequencing of 5′ independent wild-type (left) and *piwi* mutant (right) libraries. Inverted repeat and exon sequences are indicated in green and pink, respectively. The number of aligned sequence reads (blue) and number of aligned unique sequences (red) were plotted for each base pair position, with the top and bottom graph in each panel corresponding to the antisense and sense strand relative to the transposase transcript. Read and sequence counts were corrected for multiple alignments to the genome. The total number of reads from wild-type and *piwi* mutant libraries were comparable (2,963,895 and 3,017,027 of reads with perfect matches to the reference genome, respectively).

(C) Tc3 transposase antisense siRNAs are dramatically reduced in *piwi* mutants. RNase protection assay using sense fragments of Tc1 and Tc3 transposase. Sense siRNAs were not detected above background levels using this assay (see Figure S4 in the Supplemental Data). U6 was used as an internal control, but its concentration had to be titrated down due to some interference with small-RNA detection (Figure S5).


A
wild-type
piwi(n4357; n4358)
frequency of antisense 5' distance
0
10
20
30
Chr I
Chr II
Chr III
Chr IV
Chr V
Chr X
−100
−50
0
50
100
distance between 5' ends [nt]
B
Pol ?
Pol II
Pol ?
Piwi
Piwi/piRNA
(inherited/*de novo*)
MeO
RdRP
RdRP
RdRP
RdRP
Amplification:
MUT-7, RdRPs, 2° siRNAs
TGS
PTGS
2° AGO
2° AGO
2° AGO
2° AGO
2° AGO
2° AGO
PPP
bbb
Tc3 excision

Drosophila and vertebrate piRNAs map to regions devoid of protein-coding genes (Klattenhoff and Theurkauf, 2008), some *C. elegans* piRNAs are interspersed with protein-coding genes (Ruby et al., 2006).

Biogenesis of piRNAs

The striking difference in genomic organization of *C. elegans* piRNA clusters compared to vertebrates and *Drosophila* might suggest a divergent mechanism of biogenesis. We found that *C. elegans* piRNAs are Dicer independent (Figure 2B), as are *Drosophila* and zebrafish piRNAs (Houwing et al., 2007; Vagin et al., 2006). Taken together with the strong local piRNA strand bias in all three species (Brennecke et al., 2007; Houwing et al., 2007; Ruby et al., 2006; Vagin et al., 2006), it is unlikely that piRNAs are generated through a double-stranded RNA intermediate. Instead, piRNAs might be generated directly as primary transcripts. In *C. elegans*, this could be achieved by an RdRP. However, two observations argue against this hypothesis. First, we found no evidence for the dependence of piRNA expression on RdRPs (Table 1), although we cannot exclude redundancy. Second, piRNAs lack 5′ triphosphates, a hallmark of other RdRP-derived small RNAs. We therefore favor a model in which piRNAs are derived from longer primary transcripts as is the case for miRNAs (Bartel, 2004). Interestingly, we show that Piwi is not essential for piRNA biogenesis, but that piRNA levels are dramatically reduced in *piwi* mutants (Figure 1E). This is analogous to *C. elegans* miRNAs, whose levels are reduced but are not absent in *alg-1; alg-2* Argonaute mutants (Grishok et al., 2001). We therefore postulate that Piwi is not an essential component of the piRNA biogenesis pathway but is required for piRNA accumulation or stability.

The piRNA genomic organization in *Drosophila* suggests that whole piRNA clusters could be transcribed as single primary transcripts and then processed into many mature piRNAs (Brennecke et al., 2007). Our finding that 21U-RNAs are piRNAs now suggests an alternative model. Many 21U-RNA loci are associated with a conserved upstream motif containing an eight nucleotide core consensus sequence CTGTTTCA (Ruby et al., 2006; see Supplemental Data). This motif is located approximately 38 bases upstream of the base corresponding to the 5′ uridine of the 21U-RNA. Ruby et al. have postulated that this motif is part of a 21U-RNA promoter. We conclude that piRNAs might be transcribed as individual transcripts in *C. elegans* and perhaps other species. Alternatively, these motifs might also be involved posttranscriptionally in piRNA biogenesis. Having linked a conserved motif to piRNAs in *C. elegans*, it will be important to search for such motifs in other species. Perhaps such a motif might be found only for a subset of "primary" piRNAs in *Drosophila* or vertebrates.

No Evidence for Ping-Pong in *C. elegans*

Recently, a compelling model for piRNA production in *Drosophila* named the Ping-Pong amplification loop has been put forward (Brennecke et al., 2007; Gunawardane et al., 2007). In this model Piwi/Aub is bound by a primary piRNA with a 5′ uridine. Target slicing by this complex followed by 3′ end processing results in the generation of a secondary piRNA that is antisense to the primary piRNA, overlaps by ten complementary nucleotides, and has an adenine at position 10. The loop is completed by AGO3, the third Piwi protein of *Drosophila*, which binds the secondary piRNA and, given a suitable template, in turn generates piRNAs with 5′ uridines. This model is supported by the finding that Piwi/Aub-bound piRNAs have a strong bias for 5′ uridine and AGO3-bound piRNAs have a strong bias for adenine at position 10 (Brennecke et al., 2007; Gunawardane et al., 2007). To test the Ping-Pong hypothesis in *C. elegans*, we analyzed the overlap among 21U-RNAs and between 21U-RNAs and other *C. elegans* small RNAs (Figure 6A). We found few cases of overlap and no adenine peak at position 10 (Figure S6). We conclude that there is currently no evidence for a role of Ping-Pong to generate piRNAs or to couple piRNAs to endogenous siRNA pathways in *C. elegans*.

Piwi and piRNAs Act Upstream of Secondary siRNA Pathways in Tc3 Silencing

We suggest that *C. elegans* and *Drosophila* share primary piRNAs, but while amplification of small RNAs in *Drosophila* might be achieved through a Ping-Pong amplification loop, in *C. elegans* amplification might be achieved through secondary siRNAs and RdRPs (Figure 6B). We demonstrate that Tc3 transposon silencing and associated siRNAs depend on Piwi and MUT-7. However, we cannot directly implicate a specific piRNA in Tc3 transposon silencing. One attractive candidate might be 21UR-139, which maps to the Tc3 transposase ORF and might act in *cis*. A second 21U-RNA also maps to the Tc3 locus (Batista et al., 2008). Both are sense relative to the transposase gene. We propose a speculative model to explain how sense piRNAs might be required for antisense siRNA production at the Tc3 locus. Piwi loaded with sense piRNAs might stimulate RdRP activity using Tc3 antisense transcripts as templates, in a manner analogous to siRNAs in RNAi amplification (Baulcombe, 2007). The sense transcripts would be targeted by antisense siRNAs and act as a template for a second round of small RNA-stimulated RdRP activity (Figure 6B). Such a loop would maintain high levels of

Figure 6. Piwi and piRNAs May Act Upstream of siRNAs

(A) Small RNAs mapping to the opposite strand of nearby 21U-RNA loci show a preference for locations downstream of the 21U-RNA locus and were reduced in the *piwi* mutant (right) as compared to wild-type (left). Proximate small RNAs on the same strand as the 21U-RNA were also reduced in the *piwi* mutant (Figure S7). (Bottom) Rows correspond to 6021 21U-RNA loci, ordered by genomic position with colors representing different chromosomes. For a given row (21U-RNA locus), dots correspond to the relative position of nearby antisense small RNAs as defined by the distance of the 5′ end of the cloned small RNA relative to the 21U-RNA 5′ end. (Top) Shown is the frequency of distances between 5′ ends of 21U-RNAs and antisense small RNAs. Frequencies were based on the normalized number of loci for 5′ unique sequences.

(B) A speculative model of the role of Piwi in Tc3 silencing. TGS, transcriptional gene silencing. PTGS, posttranscriptional gene silencing. See Discussion for an explanation.

Tc3 siRNAs. In the case of the Tc3 transposase, this might lead to PTGS against the mRNA. This is in agreement with the low number of siRNAs mapping to the intron as compared to the two exons of the Tc3 transposase (Figure 5B). In the case of the Tc3 TIR, this might involve chromatin-mediated TGS. If the TIR-associated siRNAs are functionally distinct from the siRNAs mapping to the Tc3 transposase ORF, these two pathways might be separable. Indeed, Batista et al. (2008) observed that *prg-1* mutants lack only TIR-associated siRNAs, but not siRNAs associated with the transposase ORF (Batista et al., 2008). Together, these data suggest that PRG-1 and PRG-2 might have functionally distinct roles in Tc3 silencing. This is particularly intriguing as PRG-1, but not PRG-2, is required for piRNA accumulation (Figure 2A).

The amplification loop we propose is sequence independent, given an initiating 21U-RNA. Therefore the link between piRNA and siRNA pathways might not be restricted to Tc3. Indeed, we observe a positional bias for antisense siRNAs close to 21U-RNAs to map downstream of the 21U-RNA locus (Figure 6A). piRNA and siRNA pathways might be linked in other species too. *Drosophila* and vertebrates have lost secondary siRNAs and RdRPs, which are found in plants, yeast, and *C. elegans*. However, recently endogenous siRNAs in mouse oocytes were shown to map to the same loci as piRNAs (Tam et al., 2008).

Other Roles of Piwi in *C. elegans*?

We demonstrate a role for Piwi in inhibiting Tc3 mobility. However, it seems unlikely that the defects in germline development observed in *piwi* mutants are solely due to Tc3 activity. It has been proposed that mobile element excision and excessive DNA breaks result in meiotic catastrophe in *piwi* mutants in *Drosophila* (Klattenhoff and Theurkauf, 2008). We cannot exclude the possibility that other mobile elements in addition to Tc3 are hyperactivated in *piwi* mutants in *C. elegans*, with similar consequences. Alternatively, Piwi/piRNAs might be involved in gene regulation during germline development in *C. elegans*. Surveying whole-animal gene expression in wild-type and *piwi* mutant adult *C. elegans* using Affymetrix mRNA profiling, we did not observe striking changes in gene expression (data not shown). However, changes might only be revealed by studying gene expression in germ cells. Indeed, a recent study suggests that the expression of a subset of mRNAs expressed during spermatogenesis is deregulated in male gonads isolated from *prg-1* mutants (Wang and Reinke, 2008). The striking clustering of piRNAs on chromosome IV (Ruby et al., 2006) raises a number of interesting questions. Do piRNAs act in *cis*, or do they act in *trans*, analogous to miRNA function? If piRNAs act in *trans*, what are the rules for piRNA:piRNA target interaction? Do piRNAs always act through siRNA pathways as we have demonstrated for Tc3? Having identified piRNAs in *C. elegans*, we hope to address these questions using the powerful genetic tools that this organism offers.

EXPERIMENTAL PROCEDURES

C. elegans Strains and Culture

See the Supplemental Data and Tables S3 and S4 for strain information and culture conditions.

High-Throughput Sequencing and Data Analysis

We generated 5′-dependent libraries for N2 (2,719,949 reads), *piwi(n4357; n4358)* (764,960 reads), *mut-7* (3,475,722 reads), and *dcr-1* (149,997 reads) and 5′-independent libraries for N2 (2,963,895 reads) and *piwi(n4357; n4358)* (3,017,027 reads) that were sequenced using the Illumina/Solexa platform. See the Supplemental Data for additional information.

RNAi Experiments

RNAi experiments were carried out as reported previously (Fire et al., 1998; Timmons et al., 2001). Bacterial strains carrying plasmids expressing double-stranded RNA for the gene of interest were obtained from the Ahringer Laboratory genome-wide RNA library (Fraser et al., 2000; Kamath et al., 2003). All RNAi library inserts were confirmed by sequencing.

Transposon Excision Experiments

Transposon excision assays were carried out as described previously (Ketting et al., 1999).

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, eight figures, and five tables and can be found with this article online at http://www.molecule.org/cgi/content/full/31/1/79/DC1/.

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