Widespread roles for piRNAs and WAGO-class siRNAs in shaping the germline transcriptome of Caenorhabditis elegans

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ABSTRACT

Piwi-interacting RNAs (piRNAs) and small interfering RNAs (siRNAs) are distinct classes of small RNAs required for proper germline development. To identify the roles of piRNAs and siRNAs in regulating gene expression in Caenorhabditis elegans, we subjected small RNAs and mRNAs from the gonads of piRNA and siRNA defective mutants to high-throughput sequencing. We show that piRNAs and an abundant class of siRNAs known as WAGO-class 22G-RNAs are required for proper expression of spermatogenic and oogenic genes. WAGO-class 22G-RNAs are also broadly required for transposon silencing, whereas piRNAs are largely dispensable. piRNAs, however, have a critical role in controlling histone gene expression. In the absence of piRNAs, histone mRNAs are misrouted into the nuclear RNAi pathway involving the Argonaute HRDE-1, concurrent with a reduction in the expression of many histone mRNAs. We also show that high-level gene expression in the germline is correlated with high level 22G-RNA production. However, most highly expressed genes produce 22G-RNAs through a distinct pathway that presumably involves the Argonaute CSR-1. In contrast, genes targeted by the WAGO branch of the 22G-RNA pathway are typically poorly expressed and respond unpredictably to loss of 22G-RNAs. Our results point to broad roles for piRNAs and siRNAs in controlling gene expression in the C. elegans germline.

INTRODUCTION

Piwi-interacting RNAs (piRNAs) and small interfering RNAs (siRNAs) are discrete classes of small RNAs with largely non-overlapping genetic requirements, but which share certain biological functions, such as transposon silencing (1–3). The extent to which piRNA and siRNA pathways intersect is not well understood in most animals, but in Caenorhabditis elegans the two pathways are tightly connected (1,2). Caenorhabditis elegans piRNAs are 21 nucleotides (nt) long and contain a 5′ uracil (U) (4–7). Each piRNA is processed from its own autonomous transcript (8,9). piRNAs associate with the Piwi protein PRG-1 within the germline where they engage in mRNA surveillance (4–6). It is not known if piRNAs directly silence their targets; however, piRNAs act as a potent trigger for siRNA production from target mRNAs (6,10–14). Secondary siRNAs produced from piRNA targets are 22 nt long, contain a 5′ guanine (G) and associate with several Argonautes in the worm-specific WAGO subfamily, and are thus commonly called WAGO-class 22G-RNAs (15). WAGO-class 22G-RNA production is correlated with RNA silencing. Thus, piRNAs presumably orchestrate RNA silencing by triggering the production of WAGO-class 22G-RNAs from target mRNAs.

A second class of 22G-RNAs associates with the Argonaute CSR-1 and acts seemingly in opposition to piRNAs to promote germline gene expression (16–19). WAGO- and CSR-1-class 22G-RNAs share many of the same genetic requirements but differ in their dependency on mutator (mut) genes for their formation (15,16,20). WAGO-class 22G-RNAs are synthesized by an RNA-dependent RNA polymerase, which functions as part of a protein complex that is seeded by the intrinsically disordered protein MUT-16 at the cytoplasmic surface of the nuclear envelope in

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structures called Mutator foci (21). Mutator foci are adjacent to P granules, germ granules in which much of the piRNA machinery is housed. Thus, the WAGO-class 22G-RNA machinery and the piRNA machinery reside in close proximity to one another but appear to occupy largely distinct compartments (4,5,21).

The 22G-RNAs produced from piRNA targets can provide a molecular readout for piRNA activity (10,13). However, the presence of WAGO-class 22G-RNAs is not in and of itself indicative of an mRNA having been targeted by piRNAs, as there are other mechanisms that can trigger mRNA entry into the WAGO-class 22G-RNA pathway (22). Furthermore, in some instances, piRNAs initiate WAGO-class 22G-RNA production but are then dispensable for continued propagation of 22G-RNAs from an mRNA target, which can persist in the absence of the piRNA trigger for multiple generations (11,12,14). Recently, an improved understanding of piRNA-target recognition rules and biochemical experiments to identify PRG-1 interacting mRNAs have revealed that piRNAs interact with essentially all germline mRNAs (23,24). However, in addition to CSR-1-class 22G-RNAs, at least two other mechanisms exist, both involving cis-acting sequence elements, to counter piRNA-mediated gene silencing, and thus it is not clear to what extent piRNAs regulate germline gene expression (23,25). Neither piRNAs nor WAGO-class 22G-RNAs are essential for development at favorable growth temperatures but mutations in core factors in the pathways, such as prg-1 or mut-16, respectively, cause reduced fertility that is exacerbated at higher temperatures (4-6,20).

Attempts to identify the roles of piRNAs in regulating gene expression on a genome-wide scale have been limited in their scope and confounded by whole animal-based approaches that fail to account for the diminished germlines of piRNA-defective mutants (4,5,10,13,26,27). Furthermore, genomic approaches to identify the roles of WAGO-class 22G-RNAs in regulating gene expression are also needed to better understand RNA silencing in the germline. Here we explore the roles of piRNAs and WAGO-class 22G-RNAs in regulating gene expression in the adult germline through parallel mRNA and small RNA sequencing from dissected gonads of prg-1 and mut-16 mutants. The results provide a comprehensive analysis of gene regulation by piRNAs and WAGO-class 22G-RNAs, revealing extensive roles for the two classes of small RNAs in shaping the germline transcriptome and uncovering a complex relationship between small RNAs and mRNA expression.

MATERIALS AND METHODS

Strains

NL1810[mut-16(pk710) I] (28) and SX922[prg-1(n4357) I] (6) were outcrossed to wild type (N2) 1 × 1 just prior to expansion for gonad dissections and RNA sequencing. DUP178[glh-1{sam24} glh-1::gfp::3xFlag] prg-1[sam97] [TagRFP::3xFlag::PRG-1 I] (29) and USC717[mut-16{cmp3[mut-16::gfp::3xFLAG + foxP]} I] (30) were used to examine PRG-1 and MUT-16 expression in animals at the stage in which gonad dissections were done. TAM24[mut-16(ram18[ko(302–4051)]) I], containing a 3750 bp deletion in mut-16, and TAM22[prg-1[ram17[ko(615–2575)] I]] containing a 1,961 bp deletion in prg-1, were generated using CRISPR-Cas9 genome editing (31–33). Double strand breaks were induced on both the 5′ and 3′ ends of the respective genes by introducing a Cas9 ribonucleaseprotein complex containing an IDT Alt-R crRNAs (TAM24: ACACCACGAAAGACAUACGCAUCACGU; TAM22: UACAAUAUGAGCAUCUUGGC and GGUUCAACAGGUUCAACGUAG). Double strand breaks were presumably repaired through endogenous non-homologous end joining mechanisms. Candidates were screened for large deletions using PCR and Sanger sequencing. TAM40[prg-1[ram22[D583A]] I] was generated by introducing a Cas9 ribonucleaseprotein complex containing an IDT Alt-R crRNA (UACACAGACUGACAUUGAAG), resulting in a double strand break adjacent to the D583 residue of the DDH catalytic site. Double strand breaks were repaired from a single stranded donor oligonucleotide (IDT UltraDNA Oligo: CATTCCGGTTAAAACAACATG ATCGTCCGCTACGCCTGTACATGATTTACCAT TGAAAAGAAAACCTGCGTTTGCGGTC) which introduced a point mutation that converts the aspartic acid residue to alanine. Silent mutations were introduced into the donor oligonucleotide to prevent re-cutting at the locus. Candidates were screened using PCR and Sanger sequencing.

Gonad dissections

Gonads were dissected from gravid adults grown at 20°C for 68–70 h post L1 synchronization as described (34). The proximal arms of the gonads were discarded such that only the distal arms were captured.

RNA isolation

Whole animals and dissected distal gonads (~500 gonads per replicate, three replicates per strain) were collected into Trizol, flash frozen in liquid nitrogen, thawed, and subjected to two chloroform extractions followed by isopropanol precipitation overnight at −80°C.

mRNA-seq libraries

Total RNA was depleted of ribosomal RNA using the Ribo-Zero rRNA Removal Kit (Illumina). rRNA-depleted RNA was size selected (>200 nucleotides) to remove 5S rRNA and tRNA using RNA Clean & Concentrator-5 Kit (Zymo Research). Sequencing libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB). All cDNA and PCR products were purified with AMPure XP beads. Samples were sequenced on an Illumina NextSeq 500 (High Output Kit, single-end, 75 cycles).

mRNA-seq data analysis

Adapters and low-quality bases were removed from high-throughput sequencing reads using Trimmomatic v. 0.35 (35). Trimmed reads were mapped to the C. elegans genome.
(Wormbase release WS230) or transposon consensus sequences (36) using Star v. 2.5.0a (37). Reads from specific features were counted using RSEM v. 1.3.0 (38), except reads from transposon consensus sequences, which were counted with SAMtools (39). Differential expression analysis was done using DESeq2 v. 1.18.1 (40). In addition to the data reported here, RNA-seq libraries from henn-1(pk2295) mutant gonad samples were processed, normalized, and analyzed in parallel and reported in Svendsen et al. (41). A 1.3 fold-change cutoff and a corrected P-value cutoff of 0.05 were applied when filtering for differentially expressed genes. Venn diagrams were drawn with BioVenn (42) and InteractiVenn (43). The plots modeled after UpSet plots were drawn in Adobe Illustrator (44). All other plots were drawn in R, Excel and IGV (45). See Supplementary Table S1 for additional details.

Small RNA-seq libraries

16–30-nt RNAs were size selected on 17% polyacrylamide/urea gels. Purified small RNAs were treated with RNA polyphosphatase (Illumina) to reduce 5′ di- and triphosphates to monophosphates to enable 3′ adapter ligation to 22G-RNAs. Sequencing libraries were prepared using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (NEB). PCR amplicons were size selected on 10% polyacrylamide gels. Samples were sequenced on an Illumina NextSeq 500 (High Output Kit, single-end, 75 cycles).

Small RNA-seq data analysis

Small RNA sequences were parsed from adapters and trimmed reads with >1 base having a Phred quality score <30 were discarded. The remaining reads were mapped to the C. elegans genome (Wormbase release WS230) using CASHX v. 2.3 (46) or transposon consensus sequences (36) using Bowtie2 (47). Imperfectly matching reads were discarded. Reads from specific features were counted using custom Perl scripts and SAMtools (39). Small RNA features were classified as described (48). Differential expression analysis was done using DESeq2 v. 1.18.1 (40). In addition to the data reported here, RNA-seq libraries from henn-1(pk2295) mutant gonad samples were processed, normalized, and analyzed in parallel and reported in Svendsen et al. (41). A 1.3 fold-change cutoff and a corrected P-value cutoff of 0.05 were applied when filtering for differentially expressed small RNAs. Custom Perl and Python scripts, R, Excel and IGV were used for all other data analyses and for drawing plots. See Supplementary Table S1 for additional details. The HRDE-1 co-IP data analysis was described previously (49).

Imaging

Adult stage C. elegans expressing GLH-1::GFP and RFP::PRG-1 or MUT-16::GFP were imaged on a Zeiss Axio Imager Z2 microscope after immobilization in 25 mM sodium azide.

Quantitative RT-PCR

Total RNA from whole adult stage animals (72 h post L1 synchronization) was treated with Turbo DNase (ThermoFisher) and subjected to reverse transcription using SuperScript III (ThermoFisher) and random hexamer primers. qRT-PCR was done using iTaq Universal SYBR Green Supermix (Bio-Rad) and primers complementary to the his-10 family, which also includes his-14 and his-26 (CA TCCAAGGTATCACAAGCCG and GTATGTGACG GCATACGGATC), and the his-12 family, which also includes his-43 and his-16 (CCCAAGACATCTCAACTTGG CC and CTCCTCTTGGACCGATTTGTG). Because of the similarity in histone genes, we cannot rule out that additional histones with near perfect complementarity to the primer sequences were not also amplified. Average Ct values were calculated for three biological replicates with 3–6 technical replicate PCR amplifications done in parallel. Relative histone mRNA levels were calculated using the 2−ddCt method (50). rpl-32 levels were used for normalization.

Statistical analysis

Benjamin-Hochberg corrected P-values are reported for all differential expression analysis. An arbitrary 1.3 fold-change and false discovery rate of 0.05 was applied when interpreting differentially expressed features, unless otherwise indicated. A hypergeometric test was used to assess statistical significance in the overlap of gene lists. Two-sample t-tests were used when comparing total mRNA or small RNA reads between different histone families and a Bonferroni correction was applied to account for multiple comparisons. P-values for qRT-PCR assays were calculated using Tukey HSD tests assessing all possible pairwise comparisons. Only P-values for relevant comparisons are reported.

RESULTS

High-throughput sequencing of mRNAs and small RNAs from adult gonads

piRNAs and WAGO-class 22G-RNAs are both required for optimal fertility but their impact on endogenous mRNA expression is not well understood (4–6,20). To explore the roles of piRNAs and WAGO-class 22G-RNAs in regulating gene expression in the C. elegans germline, we isolated RNA from gonads dissected from adult wild type animals and from prg-1(n4357) and mut-16(pk710) mutants. Our samples contained the distal arms of the gonad that are comprised of both mitotic and meiotic germ cells but excluded the proximal arms that contain the oocytes and sperm (Figure 1A). Total RNA >200 nt long was depleted of ribosomal RNAs and subjected to high-throughput sequencing. In parallel, we also sequenced 16–30 nt small RNAs. To categorize mRNAs and small RNAs enriched or depleted in the dissected distal gonad arms relative to whole animals, we also subjected RNA from a subpopulation of our wild type whole adults to RNA-seq (Supplementary Table S1).

We then compared gene expression in our wild type gonad and whole animal libraries to identify mRNAs and small RNAs predominantly expressed in the distal germline. An arbitrary false discovery rate of 0.05 was
applied for reporting misregulated genes throughout this study. Additionally, a 1.3-fold-change cutoff was applied when reporting differentially expressed small RNAs and mRNAs, which excluded many misregulated genes based on a P-value cutoff of 0.05 but is more likely to reflect biologically relevant changes in expression. We identified 3206 annotated mRNAs and 1981 annotated 22G-RNA loci enriched in our distal gonad libraries, of which 1242 corresponded to a common set of genes (Figure 1B–D and Supplementary Tables S2 and S3). 8054 mRNAs were reduced in our distal gonad libraries relative to whole animals and are thus predominantly expressed in the soma or gametes (Figure 1B and C and Supplementary Table S4). The majority of miRNAs (181), and many WAGO-class 22G-RNAs (672) and piRNAs (1523), were depleted in the distal gonad samples, indicating that they are preferentially expressed in either somatic or gametic cells (Figure 1D and Supplementary Table S5). Given that piRNAs are primarily expressed in germ cells, it is likely that those that were depleted in distal gonads tend to be expressed more highly in sperm and oocytes. The vast majority (~95%) of small RNAs enriched in the distal gonad libraries were CSR-1 class 22G-RNAs, indicating that their expression is highest in non-gametic germ cells (Figure 1D and Supplementary Table S3).

These datasets enable parallel analysis of small RNA and mRNA expression in the distal gonad, thereby establishing a valuable framework for exploring the roles of small RNAs in gene regulation in the distal germline. The data can be visualized in Integrative Genomics Viewer and is available for download as a user-friendly standalone session at https://www.montgomerylab.org/resources.html (51).

**Gonad-seq on prg-1 and mut-16 mutants**

PRG-1 is the only known binding partner of piRNAs in *C. elegans*, and in prg-1 mutants, piRNAs are lost (4–6). Mutations in *mut-16*, a gene required for the formation of the RNA-dependent RNA polymerase complex that syn-

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**Figure 1.** Small RNA and mRNA sequencing on whole animals and dissected gonads. (A) Gonads were dissected and proximal gonad arms removed from wild type animals and prg-1(n4357) and mut-16(pk710) mutants. RNA from distal gonad arms, as well as a subpopulation of wild type whole animals, was subjected to small RNA and mRNA high-throughput sequencing. (B) Overlap between mRNAs and 22G-RNAs enriched in distal gonads or whole animals based on a corrected p-value cutoff of 0.05 and a 1.3-fold change cutoff. (C) Scatter plot displaying each mRNA as a function of average normalized reads in gonads (y-axis) versus whole animals (x-axis) (n = 3 biological replicates). (D) Scatterplot displaying each small RNA feature (miRNA, piRNA, WAGO-class 22G-RNA locus, and CSR-1-class 22G-RNA locus) as a function of average normalized reads in distal gonads (y-axis) versus whole animals (x-axis) (n = 3 biological replicates). (E) Model showing piRNAs bound to Piwi/PRG-1 directing their mRNA targets into the RNAi pathway in which an RNA-dependent RNA polymerase, in a complex with MUT-16 and other mutator proteins, synthesizes 22G-RNAs antisense to the mRNA target that will go on to bind WAGO subfamily Argonautes. (F) RFP::PRG-1 and MUT-16::GFP expression in adult animals at the same age as the animals used in the gonad dissections illustrated in (A). GLH-1::GFP is shown as a germ cell marker. The distal and proximal gonad arms are indicated. (G) mRNA and small RNA read distribution across a well-characterized piRNA and 22G-RNA target gene, *bath-45*, in wild type animals and prg-1(n4357) and mut-16(pk710) mutants. For simplicity, strandedness is not shown.
prg-misexpression in the distal gonads of piRNA and WAGO-
mut-16 Widespread gene misexpression in the distal gonads of
studys are also available for download and visualization at
https://www.montgomerylab.org/resources.html.

data faithfully reflects previous studies, thereby enabling us
to assess more broadly the roles of piRNAs and WAGO-
class 22G-RNAs in regulating gene expression in the distal
erm. As proof of principle, we examined small RNA and mRNA
read distribution across bath-45, a relatively well characterized piRNA target that produces
high levels of WAGO-class 22G-RNAs (10,11,13). Within our mRNA sequencing datasets, 2517
genes were uniquely upregulated in prg-1 and mut-16 mutants, whereas mRNA
levels were upregulated ∼10-fold (Figure 1G). Thus, our data faithfully reflects previous studies, thereby enabling us
to assess more broadly the roles of piRNAs and WAGO-
class 22G-RNAs in regulating gene expression in the distal
erm. The prg-1 and mut-16 datasets used in this study are also available for download and visualization at

Widespread gene misexpression in the distal gonads of prg-1 and mut-16 mutants

We first did a general analysis of small RNA and mRNA
misexpression in the distal gonads of piRNA and WAGO-
class 22G-RNA defective mutants, focusing initially on prg-
l and the piRNA pathway. In prg-1 (n4357) mutants, ∼66% of annotated WAGO targets were depleted of 22G-RNAs and nearly all piRNAs were lost, consistent with whole animal studies (Figure 2A and Supplementary Tables S6 and S7) (10,13). Within our mRNA sequencing datasets, 2517 genes were upregulated and 968 genes were downregulated in prg-1 mutants relative to wild type after applying an arbitrary 1.3-fold-change cutoff (P < 0.05) (Figure 2B and Supplementary Tables S8 and S9). By extension, ∼26% of the 13 367 distal germine expressed genes (mRNAs we captured with a base mean number of reads > 1) were misregulated in prg-1 mutants. Among the differentially expressed genes, the majority corresponded to predicted or validated coding genes, many of which are annotated as causing lethality or sterility when knocked down or mutated (Figure 2C). These results point to broad roles for piRNAs in shaping the germine transcriptome and suggest that their functions extend far beyond their well-known roles in silencing non-
self and aberrant genes.

We then assessed the role of mut-16 and thus the WAGO-
class 22G-RNA pathway in regulating gene expression in the distal gonad. As predicted based on previous studies exploring small RNA expression in whole animals, WAGO-
class 22G-RNAs were strongly depleted in mut-16 mutants (Figure 2D and Supplementary Tables S10 and S11) (20,21,52). However, there was also a modest reduction in 22G-RNAs levels for 341 CSR-1 target genes, possibly because of competition between the WAGO-class Argonautes and CSR-1 such that some mRNAs are targeted by both pathways (Figure 2D and Supplementary Table S10) (52). In our mRNA-sequencing libraries, we identified 1623 genes upregulated and 512 genes downregulated >1.3-fold in distal gonads dissected from mut-16 (pk710) mutants relative to wild type gonads (Figure 2E and Supplementary Tables S12 and S13). Similar to prg-1 mutants, most genes misexpressed in mut-16 mutants are annotated as protein
coding genes and many are annotated as being essential for survival or fertility (Figure 2C). We conclude that, like piRNAs, WAGO-class 22G-RNAs have widespread roles in regulating gene expression in the germine.

Next we compared the overlap in mRNAs and small RNAs misexpressed in prg-1 and mut-16 mutants. Because piRNAs trigger WAGO-class 22G-RNA production from target mRNAs, we predicted similar effects on gene expression in prg-1 and mut-16 mutants. Indeed, there was considerable overlap in the mRNAs upregulated or, to a lesser degree, downregulated in prg-1 and mut-16 mutants, although many genes were uniquely affected in one strain or the other (Figure 2F). It is not unexpected that mutations in mut-
16 would affect a subset of mRNAs not affected by prg-
l, as WAGO-class 22G-RNA production can be triggered through piRNA-independent mechanisms (22). However, it is surprising that ∼60% more mRNAs were misregulated in prg-1 mutants than in mut-16 mutants, given that piRNAs are thought to function exclusively through the WAGO-
class 22G-RNA pathway (10,13). It is possible that piRNAs function in two distinct modes, one of which is not dependent on the WAGO-class 22G-RNA pathway for target regulation. Nonetheless, consistent with the characterized role of piRNAs in triggering WAGO-class 22G-RNA production, ∼89% of loci depleted of 22G-RNAs in prg-1 mutants were also depleted in mut-16 (Figure 2G).

We were not able to identify any high-confidence features uniquely associated with the genes specifically upregulated in only one of the two strains. However, ∼10% of the genes uniquely upregulated in mut-16 mutants are annotated as transposons suggesting that mut-16 may be more broadly required for transposon silencing than prg-1. Several of the genes uniquely downregulated in prg-1 are associated with P

gene or function, including glh-2, meg-1, meg-
2, mex-1 and mes-1 (Supplementary Table S9). Additionally, many histone genes were strongly downregulated in prg-1 mutants, which we did not observe to the same extent in mut-16 mutants, although there was a modest reduction (<1.7-fold) in some histone mRNA levels in mut-16 (Supplementary Tables S9 and S13). In the following sections, we explore the common and unique roles for prg-1 and mut-16 in regulating gene expression in the distal germine.

Misregulation of spermatogenic and oogenic genes in prg-1 and mut-16 mutants

To identify common roles for the piRNA and WAGO-
class 22G-RNA pathways, we examined the genes misexpressed in both prg-1 and mut-16 mutants (Figure 2F). Most mRNAs misexpressed in either prg-1 or mut-16 mutants were depleted in our wild type libraries from distal gonads, which, as noted above, are comprised primarily of germ
cells but lack sperm and oocytes, and were enriched in our whole animal libraries (Figure 3A and B). This suggests that genes misregulated in prg-1 and mut-16 are preferentially expressed in the proximal gonad or in somatic cells.

PRG-1 and MUT-16 localize at or adjacent to P granules, ribonucleoprotein compartments that reside on the cytoplasmic surface of germ cell nuclei (4,5,21). P granules are implicated in silencing somatic genes in the germline and consequently it is possible that piRNAs and WAGO-class 22G-RNAs mediate somatic gene silencing (53,54). However, while most mRNAs misexpressed in the distal gonads of prg-1 and mut-16 mutants were enriched for expression in whole animals relative to gonads, none of the mRNAs that were expressed exclusively in whole animals and not in gonads, were misregulated in either prg-1 or mut-16 mutants (Figure 3A and B and Supplementary Table S14). Additionally, there was very little overlap between the mRNAs misregulated in prg-1 (∼7% overlap) or mut-16 (∼6% overlap)}
lap) mutants and the 1181 mRNAs previously classified as soma-specific by Knutson et al. (53). Instead, the majority of mRNAs misregulated in prg-1 and mut-16 mutant distal gonads were amongst the top 6,000 mRNAs captured in sperm or oocyte RNA-seq libraries (Supplementary Figure S1) (55,56). For example, ~64% of mRNAs downregulated in prg-1 mutants were amongst the top 6,000 expressed in oocytes and ~56% of mRNAs upregulated in prg-1 mutants were amongst the top 6000 expressed in sperm (Supplementary Figure S1A). Similarly, ~46% of mRNAs downregulated in mut-16 mutants were amongst the top 6,000 oocyte-expressed genes, whereas ~53% of mRNAs upregulated in mut-16 mutants were amongst the top 6000 sperm-expressed genes (Supplementary Figure S1A). Thus, it is likely that the genes misregulated in prg-1 and mut-16 mutants are predominantly expressed in gametes rather than

Figure 3. Misregulation of gametic genes in prg-1 and mut-16 mutant gonads. (A, B) UpSet plots displaying the overlap in mRNAs upregulated or downregulated in prg-1(n4357) (A) and mut-16(pk710) (B) mutants and mRNAs enriched in whole animals or dissected distal gonads (P < 0.05, fold-change > 1.3). (C-D) UpSet plots displaying the overlap in mRNAs upregulated or downregulated (P < 0.05, fold-change > 1.3) in prg-1(n4357) (C) and mut-16(pk710) (D) mutants and mRNAs enriched in spermatogenic or oogenic gonads. The percentages shown are for the gene sets upregulated in prg-1 or mut-16 mutants. (E) mRNA and small RNA read distribution across a cluster of spermatogenesis genes (gene names shown only for sperm genes) in wild type animals and prg-1(n4357) and mut-16(pk710) mutants. For simplicity, strandedness is not shown.
in somatic cells, indicating that other factors contribute to P granule-mediated silencing of somatic genes.

The elevated levels of genes expressed in sperm and the reduced levels of genes expressed in oocytes in the distal gonads of prg-1 and mut-16 mutants points to a possible role for prg-1 and mut-16 in regulating spermatogenesis and oogenesis. Therefore, to assess the role of piRNAs and WAGO-class 22G-RNAs in regulating spermatogenic and oogenic genes, we compared the mRNAs misregulated in our distal gonad libraries from prg-1 and mut-16 mutants with mRNAs enriched in oogenic or spermatogenic gonads (57). There was a slight underrepresentation in the 1732 genes enriched in oogenic gonads within our datasets of mRNAs upregulated in prg-1 (~3.3-fold underrepresentation, \( P < 0.0005 \)) and mut-16 (~1.7-fold underrepresentation, \( P < 0.0005 \)) relative to what would be expected by chance (Figure 3C and D). In contrast, there was overrepresentation of oogenic genes within our datasets of mRNAs downregulated in prg-1 (~7-fold enrichment, \( P < 0.0005 \)) and mut-16 (~3-fold enrichment, \( P < 0.0005 \)) (Figure 3C and D). The 2748 mRNAs enriched in spermatogenic gonads, ~62% were upregulated in prg-1 mutants (~5-fold overrepresentation, \( P < 0.0005 \)) and ~34% were upregulated in mut-16 mutants (~4-fold overrepresentation, \( P < 0.0005 \)) (Figure 3C and D). The median fold change in mRNAs upregulated in spermatogenic gonads was ~22-fold in prg-1 mutants and ~8-fold in mut-16 mutants, as illustrated by a cluster of spermatogenesis genes on chromosome II (Figure 3E). Not surprisingly, bath-45, the piRNA target described above (Figure 1G), is also enriched in the spermatogenic gonad (57).

 Gonads in this study were dissected from adult animals, at which time the hermaphroditic germline has normally fully transitioned from spermatogenesis to oogenesis. The upregulation of spermatogenic genes and downregulation of oogenic genes we observed is consistent with tiling array experiments involving whole adult prg-1 mutants (5) and suggests that prg-1 and mut-16 mutants may be defective in transitioning from spermatogenesis to oogenesis. To assess whether the effect on spermatogenic genes is directly related to 22G-RNA expression, we examined the relationship between the spermatogenic mRNAs upregulated or downregulated in prg-1 and mut-16 mutants and changes in 22G-RNA levels from these genes. There was a tendency for spermatogenic mRNAs upregulated in either prg-1 or mut-16 to have altered levels of 22G-RNAs (Supplementary Figure S2). However, ~48% (824) of spermatogenic mRNAs upregulated in prg-1 mutants and ~63% (596) upregulated in mut-16 mutants did not have detectable changes in 22G-RNA levels (Supplementary Figure S2). Many in fact had elevated levels of 22G-RNAs, contrary to what would be predicted if these mRNAs were directly regulated by piRNAs or WAGO-class 22G-RNAs (Supplementary Figure S2). This suggests that the impact of piRNAs and WAGO-class 22G-RNAs on gametic gene expression is at least partially indirect and may be caused by defects in cell specification or other abnormalities in the germlines of prg-1 and mut-16 mutants. It is also possible that mutations in prg-1 and mut-16 shift the balance away from WAGO-class 22G-RNAs towards the production of CSR-1-class 22G-RNAs. This may explain why large proportions of spermatogenic genes, particularly those upregulated in prg-1 mutants, have elevated levels of 22G-RNAs in prg-1 and mut-16 mutants.

**Transposon desilencing in prg-1 and mut-16 mutants**

piRNAs and siRNAs are well known for their roles in silencing transposons (1). However, in *C. elegans*, the extent to which piRNAs and siRNAs impact transposon expression is not clear. To explore the roles of piRNAs and WAGO-class 22G-RNAs in regulating transposons, we extracted reads mapping to each of the 152 transposon family consensus sequences within our mRNA and small RNA sequencing datasets from distal gonads of prg-1 and mut-16 mutants (36). Of the 152 transposon families, only 11 were upregulated >1.3-fold in prg-1 mutants, only one of which was depleted of 22G-RNAs (Figure 4A and Supplementary Table S15). Furthermore, only 21 transposon families were depleted of 22G-RNAs in prg-1 mutants, whereas 72 had elevated levels of 22G-RNAs, the reason for which is unclear (Figure 4B and Supplementary Table S15). In contrast, 34 transposon families had elevated mRNA levels in mut-16 mutants, 30 of which were depleted of 22G-RNAs in mut-16 mutants and are thus direct targets of the WAGO-class 22G-RNA pathway (Figure 4C and Supplementary Table S16). 22G-RNAs from 101 transposon families were depleted in mut-16 mutants, however, the corresponding mRNAs were upregulated >1.3-fold in only 30 of these, suggesting that loss of 22G-RNAs from most transposon families has little impact on their expression (Figure 4C and D and Supplementary Table S16).

The fertility defects in both prg-1 and mut-16 mutants are exacerbated at 25°C (4,5,20). Although it is not clear what causes the loss of fertility at 25°C, it is possible that elevated levels of transposon transposition is responsible. If so, we would predict that transposon mRNA levels would be elevated at 25°C relative to 20°C. To test this, we again sequenced mRNAs from wild type animals and prg-1(n4357) and mut-16(pk710) mutants, this time using whole adult animals grown at 20°C or 25°C. Surprisingly, there was very little difference in transposon misregulation in either prg-1 or mut-16 mutants when grown at 25°C versus 20°C, although we did observed modest differences in which transposons were affected (Supplementary Figure S3A–D and Supplementary Tables S15 and S16). This suggests that transposon misregulation is not responsible for the additional reduction in fertility that occurs in prg-1 or mut-16 mutants when grown at 25°C compared with 20°C.

The Tc3 and MIRAGE transposon families were previously shown to be upregulated in prg-1 mutants (5,6,26). We observed an ~1.7-fold increase in Tc3 levels in prg-1 mutants, similar to what was previously reported for this allele using quantitative RT-PCR, but substantially lower than the ~3-4-fold upregulation observed in other prg-1 alleles (Supplementary Figure S3E) (6). MIRAGE mRNA levels were upregulated ~1.2-fold in prg-1 mutants, which is below the 1.3 fold-change threshold we used for classifying differentially expressed genes, and substantially less than was previously shown in RNA-seq experiments using the same allele (Figure 4E) (26). However, both Tc3 and MIRAGE mRNA levels were upregulated ~4–15-fold in mut-16 mutants (Figure 4E and Supplementary Figure S3E).
Based on these results, we conclude that, in contrast to mut-16 and the WAGO pathway, prg-1 and the piRNA pathway have a modest role in maintaining transposon silencing in the distal germline, although it is possible that piRNAs have a role initiating transposon silencing that is maintained in the absence of prg-1. Alternatively, other features of transposons may direct their entry into the WAGO-class 22G-RNA pathway. Our results are consistent with a recent study showing that the frequency of transposon-induced double-strand breaks is much higher in mut-16 mutants than in prg-1 mutants (58).

Histone misexpression in prg-1 and mut-16 mutants

We next explored the roles of piRNAs and WAGO-class 22G-RNAs in regulating histone expression. Several histones were among the most highly downregulated genes in the distal gonads of prg-1 mutants (Supplementary Table S9). For example, histones within the chromosome II cluster, which contains representatives from each of the four core histone families, were downregulated ~10–20-fold in prg-1 mutants (Figure 5A and Supplementary Table S9). The majority of the 65 canonical replication-dependent histone genes, corresponding to H2A, H2B, H3 and H4, were downregulated in prg-1 mutants, although some of the core histone mRNAs were unchanged or upregulated in prg-1 mutants (Figure 5B). However, summing total mRNA reads for each histone family, only H2A and H4 families were downregulated at a Bonferroni corrected p-value cutoff of 0.05 in prg-1 mutants (Figure 5C). Coincident with the downregulation of histone mRNA levels was a dramatic increase in 22G-RNAs from histone genes, although most are not annotated as 22G-RNA loci (Figure 5A and D). With only two exceptions, 22G-RNA production from histone mRNAs was upregulated ~1.5–73-fold in prg-1 mutants relative to wild type animals (Figure 5D). Total 22G-RNA levels from H2A, the most strongly downregulated histone family at the mRNA level, were upregulated ~35-fold in prg-1 mutants (Figure 5E). H2B- and H3-derived 22G-RNAs were also upregulated >10-fold in prg-1 mutants (Figure 5E). In contrast to the core replication-dependent histone mRNAs, the H1 linker histone and the replication-independent histone variants H3.3 and H2A.Z were not downregulated, and in some instances were upregulated, in prg-1 mutants (Figure 5B).

Histone mRNA levels were only modestly affected in mut-16 mutants and there was not a clear trend in upregulated and downregulated genes (Figure 5A and Supplementary Figure S4A). Total mRNA levels from each of the core histone families were unchanged in mut-16 mutants (Supplementary Figure S4B). Furthermore, there was only a modest and inconsistent effect on histone-derived 22G-RNA levels in mut-16 mutants (Supplementary Figures S4C and D). However, 22G-RNAs derived from his-24, an H1 linker histone, which is not one of the core histones and which ranks 14 among all genes in terms of total siRNAs produced in wild type animals, were depleted ~93-fold in mut-16 mutants, indicating that it is likely a bona fide target of the WAGO pathway (Supplementary Figure S4C). mRNA and 22G-RNA levels from the other H1-like genes, hil-1-hil-8, which are not well characterized, were only modestly affected or unchanged in mut-16 mutants (Supplementary Tables S10–S13). Several other histones, particularly H4 family members, were also depleted of 22G-RNAs in mut-16 mutants, suggesting that the WAGO pathway may have a role in suppressing certain histone genes, consistent...
Figure 5. Histone misexpression in *prg-1* mutants. (A) mRNA and small RNA read distribution across a cluster of core histone genes in the distal gonads of wild type animals and *prg-1*(*n4357*) and *mut-16*(*pk7310*) mutants. For simplicity, strandedness is not shown. (B) Bar plot displaying each histone gene as a function of its log2-fold change in mRNA expression in *prg-1*(*n4357*) mutants relative to wild type distal gonads. Bars are colored by histone family as indicated in the key. (C) Total histone family mRNA levels in *prg-1*(*n4357*) mutants relative to wild type distal gonads. Error bars show standard deviation (n = 3 biological replicates). (D) Bar plot displaying each histone gene as a function of its log2-fold change in 22G-RNA expression in *prg-1*(*n4357*) mutants relative to wild type distal gonads. Bars are colored by histone family as indicated in the key. (E) Total histone family small RNA levels in *prg-1*(*n4357*) mutants relative to wild type distal gonads. Error bars show standard deviation (n = 3 biological replicates). (F-G) Histone-derived 22G-RNA enrichment in FLAG:HRDE-1 co-IPs relative to input cell lysates from whole animals wild type (*prg-1(+)) (F) or mutant (*prg-1(-)) (G) for *prg-1*. The *prg-1* mutant allele is *n4357*. (H) qRT-PCR assay of his-12 and his-10 expression in wild type whole animals and *prg-1*(*ram17*) and *mut-16*(*ram18*) single and double mutants. Error bars show standard deviation (n = 3 biological replicates). (I) qRT-PCR assay of his-12 and his-10 expression in wild type whole animals and *prg-1*(*n4357*) and *prg-1*(*ram22*) mutants. Error bars show standard deviation (n = 3 biological replicates). (J) qRT-PCR assay of his-12 and his-10 expression in wild type whole animals and *prg-1*(*ram17*) mutants at one generation of growth directly after generating the line and again at 10 generations. Error bars show standard deviation (n = 3 biological replicates).
with the modest upregulation of some histone mRNAs in mut-16 mutants (Supplementary Figure S4A and C).

**Histone mRNAs are misdirected into the HRDE-1 nuclear RNAi pathway in prg-1 mutants**

Because most histone small RNA and mRNA levels were only modestly affected or unchanged in mut-16 mutants, WAGO-class 22G-RNAs likely have a minor role in regulating histone genes under normal conditions. In contrast, the upregulation of 22G-RNAs derived from histone mRNAs and the reduction in histone mRNA levels in prg-1 mutants suggests a prominent role for piRNAs in protecting histone mRNAs from RNA silencing. If so, we would predict that histone-derived 22G-RNAs interact with the downstream silencing machinery upon loss of piRNAs. We therefore tested whether the 22G-RNAs produced from histones associate with HRDE-1, a nuclear WAGO Argonaute that binds WAGO-class 22G-RNAs and promotes trans-generational inheritance of piRNA-mediated gene silencing (10–12,14,59). To examine histone-derived 22G-RNA association with HRDE-1, we compared 22G-RNA enrichment in FLAG::HRDE-1 co-immunoprecipitates (co-IPs) from whole animals with wild type (+) or mutant (−) for prg-1 (49). With the exception of the subset of histones depleted of 22G-RNAs in mut-16 mutants (Supplementary Figure S4C), 22G-RNAs from most histones were underrepresented in FLAG::HRDE-1 co-IPs relative to cell lysates in prg-1 (+) animals, indicating that they are not normally routed into the HRDE-1 pathway (Figure 5F). In contrast, in prg-1 (−) animals, 22G-RNAs from each of the histone mRNAs were enriched in FLAG::HRDE-1 co-IPs (Figure 5G). This suggests that PRG-1 somehow prevents histone mRNAs from inappropriately entering the HRDE-1 nuclear RNAi pathway.

If mut-16 and the WAGO-class 22G-RNA pathway are required for the histone silencing we observed in prg-1 mutants, histone gene expression should be at least partially restored in prg-1 mut-16 double mutants. To test this, we did qRT-PCR to test histone gene expression in a series of prg-1 and mut-16 single and double mutant strains we generated using CRISPR-Cas9 to delete the coding regions of the two genes. With these newly generated alleles, we could simultaneously confirm that the histone silencing phenotype was not related to background mutations in the prg-1 (n4357) strain used in our RNA-seq experiments. Consistent with our RNA-seq results using the prg-1 (n4357) mutant, the two histone genes we analyzed by qRT-PCR, his-12 (H2A) and his-10 (H4), were downregulated ~14-15-fold in the prg-1 deletion mutant (P < 0.0005) (Figure 5H). In the prg-1 mut-16 double mutant, we observed an ~2.5-fold increase in his-12 and his-10 expression relative to the prg-1 single mutant (P-values < 0.01) (Figure 5H). However, his-12 and his-10 expression was still lower in the prg-1 mut-16 double mutant than in the mut-16 single mutant, indicating that inactivating mut-16 only partially rescues histone expression in prg-1 mutants (Figure 5H). Together, these results indicate that prg-1 protects histones from silencing by the WAGO-class 22G-RNA pathway, but also that other factors contribute to the strong loss of histone expression in prg-1 mutants and the modest loss in mut-16 mutants.

**PRG-1 is not directly involved in histone 3′ end cleavage**

The histones silenced in prg-1 mutants are predominantly canonical replication-dependent histones, which are unusual in that they lack poly(A) tails and instead contain a hairpin in their 3′ UTRs that promotes cleavage and 3′ end maturation (60). It is unclear how 3′ end maturation occurs in *C. elegans* as the U7 snRNA involved in cleaving histones in most metazoans is not found in worms (60). It is possible that PRG-1 promotes 3′ end cleavage and in its absence histone mRNAs are recognized as aberrant and thus routed into the HRDE-1 pathway for silencing. PRG-1 contains the catalytic triad of amino acid residues implicated in slicer activity and it is possible that PRG-1 cleaves histone mRNAs in place of the U7 associated machinery found in other metazoans (61). To test this, we introduced a mutation in one of the conserved catalytic residues of prg-1 using CRISPR-Cas9. We then tested whether his-12 and his-10 were silenced in the prg-1 catalytic mutant using qRT-PCR. We did not detect a difference in the levels of his-12 or his-10 in prg-1 catalytic mutant animals (prg-1<sup>1<i>OH</i></sup>)(P-values = 0.97 and 0.56, respectively), whereas in prg-1 (n4357) loss of function mutants both his-12 and his-10 were strongly silenced (P < 0.0005) (Figure 5I). Thus, it is unlikely that PRG-1 is directly involved in histone 3′ end maturation. It is possible that PRG-1 recruits other factors to promote histone maturation. However, we did not observe extended 3′ ends on histone mRNAs in our RNA-seq data, arguing against this possibility (Figure 5A). Nonetheless, histones were among the most highly represented genes in *in vivo* PRG-1-mRNA crosslinking experiments (median gene rank: 371 out of 20 204 genes; rank range: 6–7391), pointing to a direct interaction between the piRNA machinery and histone mRNAs (Supplementary Table S9) (24,62).

**prg-1 mutants display a transgenerational loss of fertility (27).** Because we analyzed his-12 and his-10 mRNA levels in the new CRISPR-Cas9 deletion strains used in this study directly after generating them, our results indicate that histone silencing occurs immediately upon loss of prg-1. It is possible, however, that the silencing becomes progressively stronger over multiple generations. To test this, we compared by qRT-PCR his-12 and his-10 expression in our fresh deletion allele of prg-1 as soon as it was possible to obtain a homozygous line and then again after 10 generations of growth on a continuous supply of food at 20°C. There was no detectable difference in either his-12 or his-10 expression between 1–10 generations (P-values = 0.89 and 0.99, respectively) (Figure 5J). Therefore, it is unlikely that histone silencing in prg-1 mutants is progressive over multiple generations, although it is still possible that it contributes to the transgenerational sterility of prg-1 mutants through cumulative effects of reduced histone activity on gene expression across generations.

**piRNA target site abundance is not correlated with mRNA silencing**

Two distinct approaches were recently used to identify piRNA targets in *C. elegans*. The first approach computed base-pairing rules for piRNA-target mRNA interactions to predict piRNA target sites genome-wide, and the second
Correlation between 22G-RNA production and mRNA silencing

The relationship between WAGO-class 22G-RNAs and target mRNA expression is not well understood. To explore the role of 22G-RNAs in regulating gene expression in the germline, we compared small RNA and mRNA expression from mut-16-dependent 22G-RNA loci in wild type and mut-16 mutants. Of the 2738 annotated gene loci depleted of 22G-RNAs by >1.3-fold in mut-16 mutants, ∼81% were represented at sufficient levels for statistical analysis in our mRNA sequencing libraries from distal gonads. Of these, ∼19% were upregulated and ∼17% were downregulated in mut-16 mutants (P < 0.05, no fold-change cutoff applied) (Figure 7A). For the remaining ∼64%, we did not detect a difference in mRNA levels in mut-16 mutants (Figure 7A).

The modest and bidirectional effect we observed on mut-16-dependent 22G-RNA target mRNAs could reflect low-level, inconsequential small RNA production from the majority of WAGO-class 22G-RNA targets. Therefore, we focused on the mut-16-dependent loci with the highest abundance of 22G-RNAs: the 294 loci that produced >1,000 normalized 22G-RNA reads on average in our wild type distal gonad libraries and that were depleted >3-fold in mut-16 mutant libraries. Surprisingly, only ∼46% of the mRNAs corresponding to the 294 22G-RNA loci were upregulated >1.3-fold in mut-16 mutants, and ∼13% were instead downregulated >1.3-fold (Figure 7B). The remaining ∼41% were unaffected in mut-16 mutants, despite loss of abundant 22G-RNAs (Figure 7B). The predicted coding gene T12G3.1, for example, produced very high levels of mut-16-dependent 22G-RNAs but its overall mRNA levels were not detectably changed in mut-16 mutants (Figure 7C). These results indicate that WAGO-class 22G-RNA abundance is not a reliable indicator of RNA silencing.

Finally, we examined more generally the relationship between siRNA production and mRNA expression in the distal germline, including both mut-16-dependent and mut-16-independent 22G-RNA loci. Of the 6121 genes that yielded >10 normalized 22G-RNA reads (10 reads per million total mapped reads, rpm) in wild type animals, ∼28% were depleted of 22G-RNAs by >2-fold in mut-16 mutants and are thus presumed to be WAGO targets (Supplementary Table S17). We observed a clear distinction in expression levels between mRNAs that produced 22G-RNAs depleted >2-fold in mut-16 mutants and those that did not (Figure 7D and E). From the presumptive WAGO targets that yielded >10 normalized reads (rpm), the median normalized mRNA read counts was only 27 (~4.75 on a log2 scale) (Figure 7D). In contrast, the median mRNA reads for mut-16-independent 22G-RNA loci that yielded >10 normalized and mut-16-independent loci (~43 versus ~47) (Figure 7D and E). These mut-16-independent 22G-RNA loci are presumably CSR-1 targets as this is the only other characterized class of 22G-RNAs. Consistent with the weak correlation between mut-16-dependent 22G-RNA production and mRNA silencing noted above, the median reads for mut-16-dependent 22G-RNA target mRNAs was increased by only ∼19% in the distal gonads of mut-16 mutants relative to wild type (Figure 7D and F). As predicted, the expression of genes yielding mut-16-independent 22G-RNAs, which are presumed to be CSR-1-class 22G-RNAs, was essentially unchanged in mut-16 mutants (Figure 7E and G). Lastly, we observed a strong positive correlation between 22G-RNA levels and corresponding mRNA levels for mut-16-independent loci (R² = 0.70) but to a much lesser extent for mut-16-dependent loci (R² = 0.29) (Figure 7D and E). This supports the proposed role for the mut-16-independent branch of the 22G-RNA pathway involving CSR-1 in promoting germline gene expression (16–18). From these results, we conclude that WAGO-class 22G-RNAs are typically derived from poorly expressed genes and have little impact on the expression of most target mRNAs.
Regulated (4). Nonetheless, our results demonstrating that undergoing spermatogenesis, spermatogenic genes are downstage at which wild type hermaphrodites are normally unmutant hermaphrodites (4). However, in L4 stage larvae, the are partially rescued by providing wild type sperm to spermatogenesis, and the fertility defects of prg-1

prg-1

Thereducedfertilityin

prg-1

transposons

Roles of piRNAs and WAGO-class 22G-RNAs in regulating adult animals (5). Upregulation of spermatogenic genes in results observed in tiling array experiments involving whole nadsofadult sperm-enrichedtranscriptsareupregulatedinthedistalgono-

tants (4–6, 20).

helptoexplainthereducedfertilityof

prg-1

mutants as the log2 number of predicted piRNA target sites it contains (y-axis) versus its log2 fold-change in

prg-1

mutants (x-axis). (B) Scatter plot displaying each gene misexpressed in the distal gonads of

prg-1

mutantsand22G-RNAlivelsformosttransposonswereupregulated, rather than downregulated as would be predicted if piRNAs had a role in directing mRNAs into the WAGO-class 22G-RNA pathway. Nonetheless, consistent with previous studies, Tc3 mRNA levels were modestly upregulated in

prg-1

mutants and it was previously shown that Tc3 transposition rates are substantially higher in

prg-1

mutants (6). MIRAGE transposon mRNA levels were marginally affected in our datasets but were substantially upregulated in another study involving the same allele of

prg-1

(26). Some transposons identified previously as being upregulated in

prg-1

using qRT-PCR were also not affected in our datasets (10). These results can be reconciled in a model in which transposon desilencing in

prg-1

mutants is somewhat stochastic, possibly resulting from inconstant inheritance of the WAGO-class 22G-RNAs that provide a transgenerational memory of piRNA activity (11, 12, 14). Rearing conditions and many rounds of propagation could exacerbate the effect.

Histone silencing in

prg-1

mutants

We observed a striking reduction in the levels of most histone mRNAs in

prg-1

mutants, which coincided with misrouting of histone mRNAs into the HRDE-1 nuclear RNAi pathway. Canonical replication-dependent histone mRNAs are distinct from most protein-coding mRNAs in that they are not thought to contain poly(A) signal sequences and poly(A) tails but rather contain a hairpin in their 3' UTRs that promotes cleavage and maturation of the 3' end (60). In

C. elegans,

the U7 snRNA implicated in cleaving histones in other metazoans is absent, and it is not known how histone 3' end maturation occurs (60). It is possible that PRG-1 promotes 3' end cleavage and in its absence histone mRNAs are recognized as aberrant and thus routed into the

Figure 6. Correlation between piRNA target site abundance and mRNA silencing. (A) Scatter plot displaying each gene misexpressed in the distal gonads of

prg-1

(n4357) mutants as the log2 number of predicted piRNA target sites it contains (y-axis) versus its log2 fold-change in

prg-1

mutants (x-axis). (B) Scatter plot displaying each gene misexpressed in the distal gonads of

prg-1

(n4357) mutants as the log2 number of PRG-1 binding sites it contains (y-axis) versus its log2 fold-change in

prg-1

mutants (x-axis). (C) Bar plot displaying the percentage of genes upregulated in the distal gonads of

prg-1

(n4357) mutants in bins of genes ranked by either the number of predicted piRNA target sites or the number of PRG-1 interacting sites they contain. The top 700 genes in each category are in sequential bins of 100.

DISCUSSION

Regulation of gametogenesis by piRNAs and WAGO-class 22G-RNAs

Through a genome-wide parallel analysis of mRNA and small RNA defects in the distal gonads of

prg-1

and

mut-16

mutants, we uncovered wide-ranging roles for piRNAs and WAGO-class 22G-RNAs in shaping the transcriptome of the C. elegans distal germline. Widespread misexpression of gametic genes in

prg-1

and

mut-16

mutants points to a role for both classes of small RNA in controlling germ cell fate. The extent to which this is a direct effect and is not caused by other developmental defects is not clear. Regardless, it may help to explain the reduced fertility of

prg-1

and

mut-16

mutants (4–6, 20).

prg-1

was previously implicated in regulating spermatogenesis, and the fertility defects of

prg-1

mutants are partially rescued by providing wild type sperm to

prg-1

mutant hermaphrodites (4). However, in L4 stage larvae, the stage at which wild type hermaphrodites are normally undergoing spermatogenesis, spermatogenic genes are downregulated (4). Nonetheless, our results demonstrating that spermatogenic transcripts are upregulated in the distal gonads of adult

prg-1

mutants are consistent with previous results observed in tiling array experiments involving whole adult animals (5). Upregulation of spermatogenic genes in the distal gonad, which lacks gametes and should be fully transitioned to oogenesis, points to incomplete shutoff of sperm transcripts during oogenesis in

prg-1

mutants. Given that spermatogenic genes are also upregulated in

mut-16

mutants, albeit to a lesser extent, the role of

prg-1

in regulating spermatogenesis is likely linked to its function in routing mRNA targets into the WAGO-class 22G-RNA pathway (10, 13).

Roles of piRNAs and WAGO-class 22G-RNAs in regulating transposons

The reduced fertility in

prg-1

and

mut-16

mutants could also be caused by elevated levels of transposon mRNAs and a subsequent increase in mutagenic transposition events. Our data supports a prevalent role for

mut-16

and WAGO-class 22G-RNAs in silencing transposons, but a far more limited role for piRNAs. There was a very modest effect on transposon mRNA levels in

prg-1

mutants and 22G-RNA levels for most transposons were upregulated, rather than downregulated as would be predicted if piRNAs had a role in directing mRNAs into the WAGO-class 22G-RNA pathway. Nonetheless, consistent with previous studies, Tc3 mRNA levels were modestly upregulated in

prg-1

mutants and it was previously shown that Tc3 transposition rates are substantially higher in

prg-1

mutants (6). MIRAGE transposon mRNA levels were marginally affected in our datasets but were substantially upregulated in another study involving the same allele of

prg-1

(26). Some transposons identified previously as being upregulated in

prg-1

using qRT-PCR were also not affected in our datasets (10). These results can be reconciled in a model in which transposon desilencing in

prg-1

mutants is somewhat stochastic, possibly resulting from inconstant inheritance of the WAGO-class 22G-RNAs that provide a transgenerational memory of piRNA activity (11, 12, 14). Rearing conditions and many rounds of propagation could exacerbate the effect.
HRDE-1 pathway. However, prg-1 is clearly not essential for histone 3' end formation, as many replication-dependent histone mRNAs were unaffected in prg-1 mutants. Furthermore, we did not observe a difference in histone mRNA 3' ends in our wild type and prg-1 mutant sequencing datasets. The slicer activity of PRG-1 was also presumably not required for proper histone expression, which argues against a direct role in processing. Nonetheless, it is possible that other factors are redundant with prg-1 in histone processing. Interestingly, histone mRNAs are also downregulated in csr-1 mutants. CSR-1 appears to have a direct but unclear role in histone maturation (63). Perhaps CSR-1 and PRG-1 function redundantly to process histone mRNAs, which would be rather unusual given their seemingly opposite roles in regulating gene expression otherwise. Other Argonates, such as the WAGOs, may also be involved in regulating histones, which could explain why we observed a modest reduction in some histone levels in mut-16 mutants.

Transcription of the core histones is coupled to the cell cycle and therefore it is possible that defects in germ cell proliferation in the germlines of prg-1 mutants is responsible for reduced histone mRNA levels (60). While this is certainly plausible, it does not explain why histone mRNAs are misrouted into the HRDE-1 RNAi pathway in prg-1 mutants, nor does it explain why histone mRNAs are directly targeted by PRG-1, as suggested by in vivo crosslinking experiments (24). Consequently, the role of PRG-1 in regulating histones is likely at least partially direct and may impact proliferation of germline stem cells, which could explain the diminutive germlines of prg-1 mutants.
Relationship between 22G-RNAs and mRNA expression

The role of 22G-RNAs in regulating gene expression in C. elegans is not well understood. Nearly all distal germline-expressed genes produce 22G-RNAs (Figure 7D and E) (16,64), yet there are two distinct classes of 22G-RNAs that seem to act in opposition to one another. mut-16-dependent WAGO-class 22G-RNAs are thought to silence gene expression, whereas mut-16-independent CSR-1-class 22G-RNAs are thought to promote gene expression (22). Our data demonstrates that high levels of mut-16-independent 22G-RNAs is directly correlated with high-level gene expression, supporting a role for the CSR-1 branch of the 22G-RNA pathway in licensing genes for expression, (17,18). In contrast, the relationship between mut-16-dependent 22G-RNA production and gene expression is relatively weakly correlated and the majority of WAGO targets are poorly expressed, even in mut-16 mutants. It is possible that the WAGO pathway imparts epigenetic modifications at target loci that somehow persist over multiple generations in the absence of 22G-RNAs. Alternatively, the WAGO pathway may selectively target poorly expressed genes as a means of combatting leaky transcription in the germline. Whatever the reason, these results point to a complex relationship between siRNA and mRNA expression and demonstrate that WAGO-class 22G-RNA production is not necessarily a good indicator of RNA silencing. A recent study exploring small RNA production in the C. elegans gonad concluded that 22G-RNA levels were inversely correlated with mRNA expression, which is not consistent with our results (64). The reason for this discrepancy may be that the authors relied on external mRNA sequencing datasets to complement their small RNA sequencing data, whereas our small RNA and mRNA data were generated from the same RNA samples.

Additional roles for piRNAs and WAGO-class 22G-RNAs in gene regulation

We identified hundreds of genes misregulated in prg-1 and mut-16 mutants that did not fall into any of the specific categories we explored. For example, several genes involved in RNA silencing pathways were misexpressed in prg-1 and mut-16 mutants. The piRNA trimmer par-1, for instance, was upregulated in prg-1 and mut-16 mutants, and the RNA helicase eri-6/7, required for ERGO-1-class 26G-RNA production, was strongly downregulated in mut-16 mutants (65,66). It will be important to investigate the roles of piRNAs and WAGO-class 22G-RNAs in regulating specific genes identified in this study as being misregulated in prg-1 and mut-16 mutants (see Supplementary Tables S8, S9, S12, and S13 for comprehensive lists of misregulated genes; see https://www.montgomerylab.org/resources.html to visualize the data in a genome browser).

Additional roles for piRNAs and WAGO-class 22G-RNAs in regulating gene expression in the germline will likely emerge from analysis of animals grown under non-optimal conditions. At 25°C, for example, the fertility defects of prg-1 and mut-16 mutants are exacerbated. Our characterization of transposon silencing in whole animals did not reveal any substantial differences in transposon silencing in prg-1 or mut-16 mutants grown at 25°C compared to animals grown at 20°C. However, we limited our analysis to transposons as prg-1 and mut-16 mutants grown at 25°C have developmental defects that could confound differential expression results and in particular increase the likelihood of false positives caused by indirect effects on gene expression.

This study provides a valuable framework for exploring the roles of small RNAs in regulating gene expression as it relates to development, genome defense, and epigenetic inheritance in C. elegans. The results will likely help to uncover shared and conserved roles for small RNAs in other animals as well.

DATA AVAILABILITY

All raw high-throughput sequencing data and counts tables described here have been deposited to the Gene Expression Omnibus (GEO) and is available under accession number GSE141243.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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