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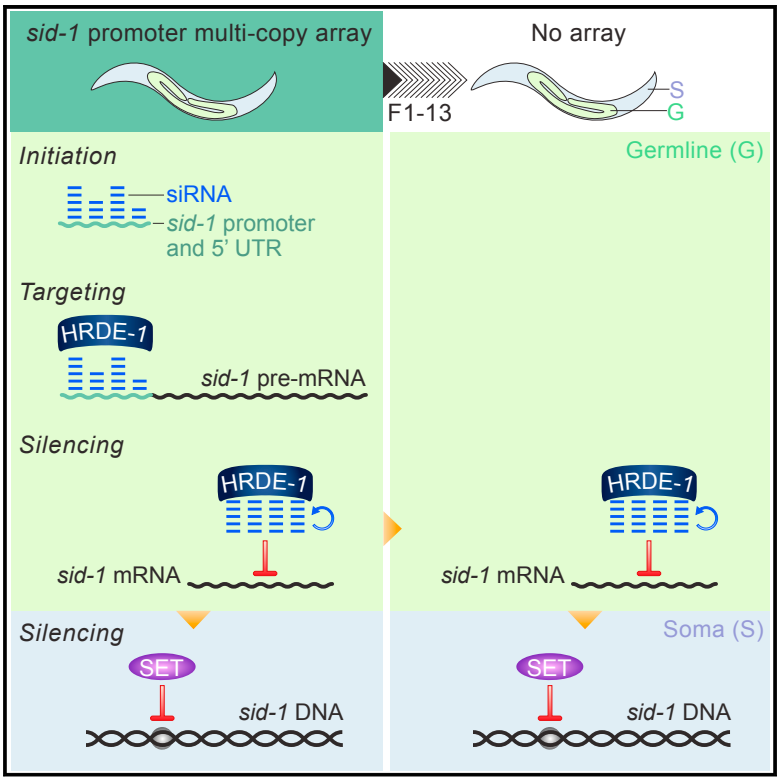
Molecular Cell

Stable Heritable Germline Silencing Directs Somatic Silencing at an Endogenous Locus

Graphical Abstract



Highlights

- A multi-copy array of the region upstream of *sid-1* silences *sid-1*
- Epigenetic *sid-1* silencing is transmitted to all progeny for up to 13 generations
- Small RNAs embody the inherited silencing signal in the germline
- Chromatin-modifying enzymes contribute to *sid-1* silencing in the soma

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In Brief

Epigenetic silencing of the endogenous *sid-1* locus is maintained by germline RNAi-mediated inheritance for up to 13 generations, while corresponding somatic silencing is dependent on chromatin-modifying enzymes.

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Stable Heritable Germline Silencing Directs Somatic Silencing at an Endogenous Locus

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SUMMARY

The importance of transgenerationally inherited epigenetic states to organismal fitness remains unknown as well-documented examples are often not amenable to mechanistic analysis or rely on artificial reporter loci. Here we describe an induced silenced state at an endogenous locus that persists, at 100% transmission without selection, for up to 13 generations. This unusually persistent silencing enables a detailed molecular genetic analysis of an inherited epigenetic state. We find that silencing is dependent on germline nuclear RNAi factors and post-transcriptional mechanisms. Consistent with these later observations, inheritance does not require the silenced locus, and we provide genetic evidence that small RNAs embody the inherited silencing signal. Notably, heritable germline silencing directs somatic epigenetic silencing. Somatic silencing does not require somatic nuclear RNAi but instead requires both maternal germline nuclear RNAi and chromatin-modifying activity. Coupling inherited germline silencing to somatic silencing may enable selection for physiologically important traits.

INTRODUCTION

Most inherited traits arise from pre-existing DNA sequence variation that, for recessive traits, must become sufficiently frequent in the population to produce homozygotes. In contrast, dominantly transmitted epigenetic traits can appear within a single generation and can spread rapidly in the population in response to environmental conditions. Mitotically propagated stable epigenetic traits have been described in microbes, plants, and animals (Jablonka and Raz, 2009). However, few transgenerational epigenetic phenomena have been mechanistically characterized and the precise nature of the trait-specific information that is transmitted between generations remains elusive.

Several well-characterized examples of inherited epigenetic silencing in *C. elegans* involve RNAi (Fire et al., 1998). Silencing of the endogenous germline-expressed gene *oma-1* by exogenously introduced double-stranded RNA (dsRNA) persists for up to four generations with selection (Alcazar et al., 2008), while dsRNA-initiated silencing of a germline-expressed *gfp* transgene can persist, with selection, for 80 or more generations (Ashe et al., 2012; Vastenhouw et al., 2006). Furthermore, endogenous piwi-interacting RNAs (piRNAs) can initiate silencing of a single copy germline *gfp* transgene in RNA-induced epigenetic silencing (RNAe) (Shirayama et al., 2012). Once initiated, transgenerational transgene silencing requires the germline-specific nuclear RNAi pathway as well as several putative histone-modifying enzymes (Ashe et al., 2012; Buckley et al., 2012; Shirayama et al., 2012). In germline nuclear RNAi, PRG-1-stabilized piRNAs or dsRNA-derived short interfering RNAs (siRNAs) direct the production of 22G siRNAs that are stabilized by the nuclear localized Argonaute HRDE-1 (Bagijn et al., 2012; Batista et al., 2008; Buckley et al., 2012; Lee et al., 2012; Sapetschnig et al., 2015). RNAi pathways can inhibit gene expression transcriptionally (Buckley et al., 2012; Burkhart et al., 2011; Burton et al., 2011; Guang et al., 2008, 2010) and post-transcriptionally (Montgomery et al., 1998; Sapetschnig et al., 2015; Tsai et al., 2015).

piRNA pathway-silenced loci are often paramutagenic (Ashe et al., 2012; de Vanssay et al., 2012; Hermant et al., 2015; Sapetschnig et al., 2015; Shirayama et al., 2012). In paramutation, an allele's expression state can stably alter the expression of a homologous locus in *trans* (Chandler, 2010). Silencing associated with a transgenic piRNA sensor can be transmitted to naive alleles, and single copy transgenes silenced in the germline produce a paramutagenic silencing signal in RNAe (Sapetschnig et al., 2015; Shirayama et al., 2012).

The import of RNAi-triggering dsRNA into cells and between generations ("systemic RNAi") requires the transmembrane protein SID-1 (Winston et al., 2002). Here we show that multiple copies of the *sid-1* upstream region initiate stable transgenerational silencing of the endogenous *sid-1* locus, resulting in animals that are resistant to RNAi in germline and somatic cells. The ability of a promoter and 5′ UTR transgene to silence a coding region has not been reported previously. In co-suppression, multi-copy transgenes must include the coding sequence to silence the endogenous gene (Dernburg et al., 2000; Ketting and Plasterk, 2000). Furthermore, epigenetic silencing at this endogenous locus is remarkably stable after loss of the initiating transgene, as silencing persists in the absence of selection and at near 100% penetrance for up to 13 generations in the germline and for at least four generations in the soma. Our study shows that the silenced state is only transmitted maternally, and provides evidence that small RNAs embody the inherited silencing

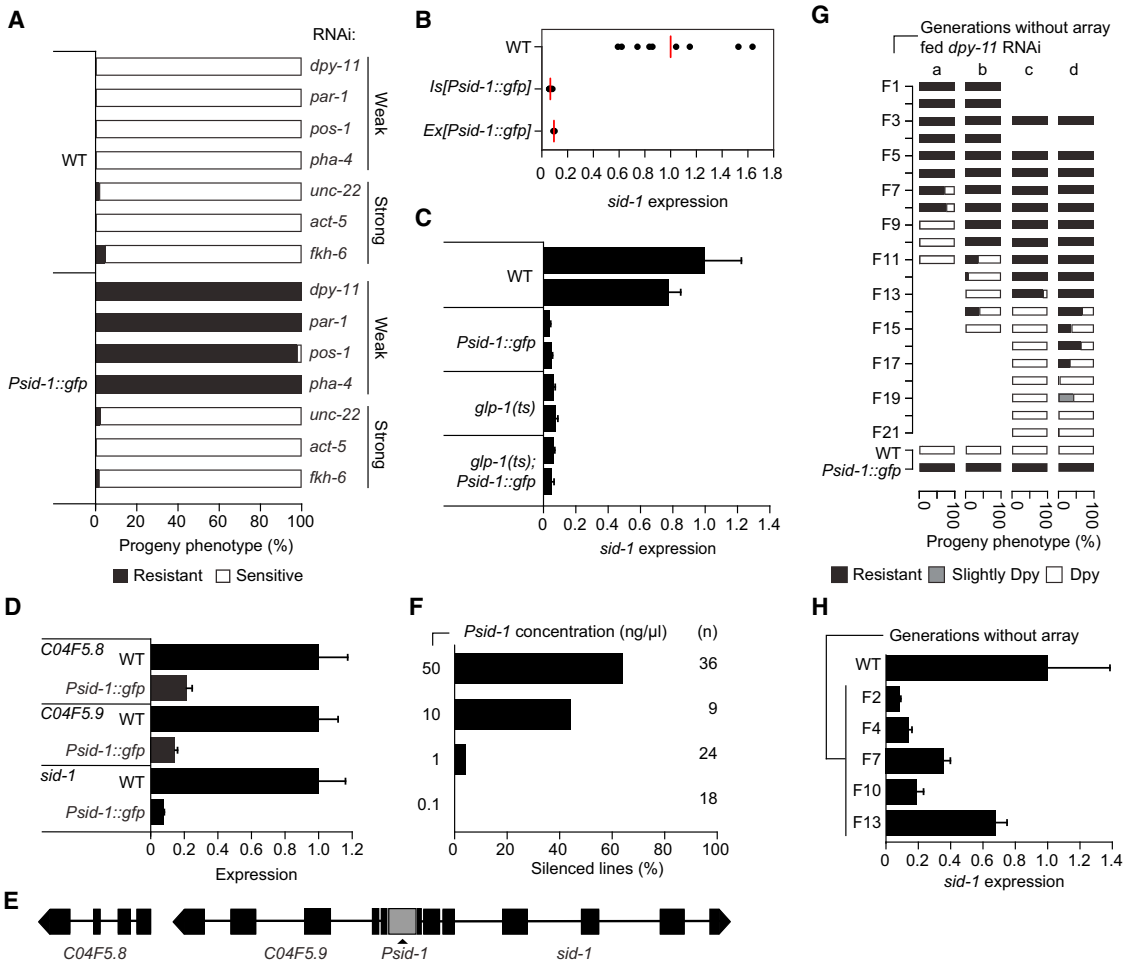

Figure 1. Transgenerational Epigenetic *sid-1* Silencing

(A) RNAi sensitivity of >100 progeny of L4 worms.

(B–D) mRNA expression in (B) mixed-stage worms (biological replicate average in red), (C) single young adults (25°C; each bar represents a single worm and two technical replicates), and (D) synchronized young adults.

(E) Silenced region.

(F) Fraction of *dpy-11* RNAi-resistant F2 *Psid-1* array lines (n).

(G) Inherited RNAi resistance of average 103 (a and b) or 870 (c and d) progeny from 3 (a and b) or 20 (c and d) L4 larvae fed *dpy-11* RNAi.

(H) *sid-1* expression in mixed-stage line b.

Expression is relative to *gpd-2/3*; wild-type is set to 1.0. Average ± SD of two technical replicates, unless otherwise noted. See also Figure S1.

signal. In addition, and in contrast to previous reports, germline silencing does not require the chromatin-modifying enzymes implicated in the piRNA pathway. Instead, these two histone-modifying methyltransferase homologs are required to silence *sid-1* specifically in the soma, suggesting that the mechanisms for transgenerational silencing in the germline and soma are distinct.

RESULTS

sid-1 Promoter Arrays Silence the sid-1 Locus

We previously constructed *sid-1* transcriptional reporter worms (Winston et al., 2002). Surprisingly, we found that animals harboring the multi-copy *Psid-1::gfp* array were defective for RNAi induced by ingested dsRNA (feeding RNAi) (Figure 1A). qRT-PCR showed a 10-fold reduction in *sid-1* mRNA levels in both extrachromosomal (Ex) and chromosomally integrated (Is) *Psid-1::gfp* array lines (Figure 1B). The majority of *sid-1* mRNA expression is confined to the germline in wild-type *C. elegans*. Consistent with gene expression analyses of isolated gonads and pre-transcriptional embryos, which indicate abundant germline-derived *sid-1* transcripts (Arnold et al., 2014; Baugh et al., 2003; Levin et al., 2012), *sid-1* mRNA levels are reduced 10-fold in germline-depleted (*glp-1*) young adults with or without the *Psid-1::gfp* array (Figure 1C). Thus, in adults, *sid-1* is primarily expressed in the germline and the *Psid-1::gfp* array strongly reduces this expression.

Psid-1::gfp-induced *sid-1* silencing results in feeding RNAi defects for some RNAi foods (Figure 1A). While *Psid-1::gfp* worms remain sensitive to potent RNAi foods that can silence the targeted gene in directly exposed animals, they are resistant to weak RNAi triggers that require two continuous generations of exposure for wild-type worms to show the expected phenotype (Figure S1A, available online). The specific resistance of *Psid-1::gfp* worms to weak RNAi foods that require maternal transmission of dsRNA is consistent with reduced *sid-1* germline expression.

To characterize the specificity of this unusual silencing, we sequenced all mRNA from synchronized wild-type and *Psid-1::gfp* young adults (Figure S1B). Only three genes showed significant silencing: *sid-1* and its two immediate upstream (5′) neighbors (Figures 1D and 1E). Thus, the *Psid-1::gfp* array specifically silences multiple genes in the vicinity of the endogenous *sid-1* locus.

The *Psid-1::gfp* transcriptional fusion contains the entire 716 bp intergenic region starting 10 bp upstream of the *sid-1* start codon. To determine whether the *sid-1* promoter is sufficient to silence *sid-1*, we injected into wild-type worms this 716 bp region along with a co-injection marker and DNA ladder to make complex arrays. In total, 23/36 *Psid-1* lines placed on *dpy-11* RNAi food produced only resistant progeny, indicating that the *sid-1* promoter array is sufficient to silence *sid-1* (Figures 1F and S1C). In a separate experiment, a similar proportion of lines were resistant after two generations, but six generations after injection nearly all lines produced only resistant progeny (Figure S1D). Additionally, diluting the *sid-1* promoter decreased the proportion of silenced lines (Figures 1F and S1C). These results confirm that *sid-1* promoter arrays induce *sid-1* silencing.

In feeding RNAi experiments, even the progeny that did not inherit the array from *Ex[Psid-1::gfp]* worms were resistant to *dpy-11* RNAi. Non-array worms remained resistant to *dpy-11* RNAi food for 8–13 generations after loss of the *Psid-1::gfp* array (Figure 1G). qRT-PCR analysis confirmed that *sid-1* expression correlated with resistance and sensitivity to *dpy-11* RNAi (Figure 1H). The upstream genes can also remain silenced over multiple generations after *Psid-1::gfp* array loss (Figure S1E). Therefore, exposure to the *Psid-1::gfp* array initiates a robust and stable epigenetic silenced state at the *sid-1* locus.

Transmission of the Silenced sid-1 State

To characterize the inheritance of the silenced *sid-1* state, we crossed *sid-1* silenced animals that no longer carry the *Psid-1::gfp* array to wild-type animals. The cross progeny of wild-type males and silenced hermaphrodites all silenced *sid-1*, and all F2 and F3 worms produced only resistant progeny on *dpy-11* RNAi food (Figures 2A, 2B, and S2A). Thus, when an expressed locus from a male is crossed to a silenced hermaphrodite, the naive locus from the male becomes stably silenced, indicating that the silenced state is dominant. Paramutation-like silencing can be transmitted via sperm and oocytes in *C. elegans* (Alcazar et al., 2008; Shirayama et al., 2012). However, we found that the cross progeny of wild-type hermaphrodites and silenced males all expressed *sid-1* and all F3 and F4 progeny were sensitive to *dpy-11* RNAi (Figures 2A and 2B). Thus, the silenced state at the *sid-1* locus is paramutagenic but transmitted only maternally (Figure S2C).

Epigenetic silencing in *C. elegans* is often associated with changes to chromatin structure (Ashe et al., 2012; Buckley et al., 2012; Burton et al., 2011; Guang et al., 2010; Vastenhouw et al., 2006). However, a heritable transgene-silencing signal can be transmitted in the absence of the silenced transgene (Sapetschnig et al., 2015). To determine whether the *sid-1* locus is required for transmission of the *sid-1* silenced state, we generated *Psid-1::gfp/+;sid-1+/nDf32* worms that contain a large deficiency (*nDf32*) that deletes over three megabases (>700 genes), including the *sid-1* locus (Winston et al., 2002). We then crossed wild-type males to silenced *sid-1+/nDf32* progeny (Figure S2D). All resulting cross progeny, whether they inherited the intact *sid-1* locus (*sid-1+/sid-1+*) or the deleted *sid-1* locus (*sid-1+/nDf32*), produced resistant progeny when placed on *dpy-11* RNAi food (Figures 2C and S2C). Thus, the *sid-1* locus and any associated chromatin marks are not required for transmission of silencing signals.

Epigenetic silencing in *C. elegans* is often associated with reduced transcription (Buckley et al., 2012; Burton et al., 2011; Guang et al., 2010; Shirayama et al., 2012). For example, comparison of pre-mRNA and mRNA levels indicates that both transcriptional and post-transcriptional mechanisms may contribute to dsRNA-mediated heritable silencing of a *gfp* transgene (Ashe et al., 2012), while silencing of *gfp* in RNAe is primarily transcriptional (Shirayama et al., 2012). If the 10-fold decrease in *sid-1* mRNA in *Psid-1::gfp* worms is due to transcriptional silencing, we also expect a 10-fold decrease in *sid-1* pre-mRNA in *Psid-1::gfp* worms. However, *sid-1* pre-mRNA levels, inferred by qRT-PCR of two *sid-1* introns, were indistinguishable in wild-type and *Psid-1::gfp* worms (Figure 2D). Although pre-mRNA levels are often difficult to measure, we found that our single-worm qRT-PCR protocol (see STAR Methods) results in reproducibly detectable levels of *sid-1* pre-mRNA. Thus, although we cannot rule out a minimal contribution of transcriptional silencing that may be undetectable by qRT-PCR or more complex mechanisms of regulation, this result suggests that silencing does not affect *sid-1* transcription or RNA splicing rates and is likely post-transcriptional.

22G siRNAs Target the Silenced sid-1 Locus

Small RNA pathways can regulate gene expression. Therefore, we sequenced two independent wild-type and four independent *Psid-1::gfp* small RNA libraries prepared from synchronized young adults. Normalized to library size, small RNAs antisense to the *sid-1* coding sequence in *Psid-1::gfp* worms increased 3-fold compared to wild-type (Figure 3B). In all libraries, the small RNAs targeting *sid-1* were highly enriched for 22 nt RNAs with a 5′ guanine, indicating that they are likely 22G secondary siRNAs (Figures 3C and 3D). The number of *sid-1*-aligned 22G siRNAs was dramatically reduced in *Psid-1::gfp* small RNA sequencing libraries prepared without phosphatase treatment, confirming that these small RNAs have a 5′ triphosphate, as would be expected of 22G siRNAs (Pak and Fire, 2007) (Figure S3A). Thus, *Psid-1::gfp*-dependent silencing is associated with an increase in secondary 22G siRNAs antisense to the *sid-1* mature transcript. Importantly, small RNA profiles are not

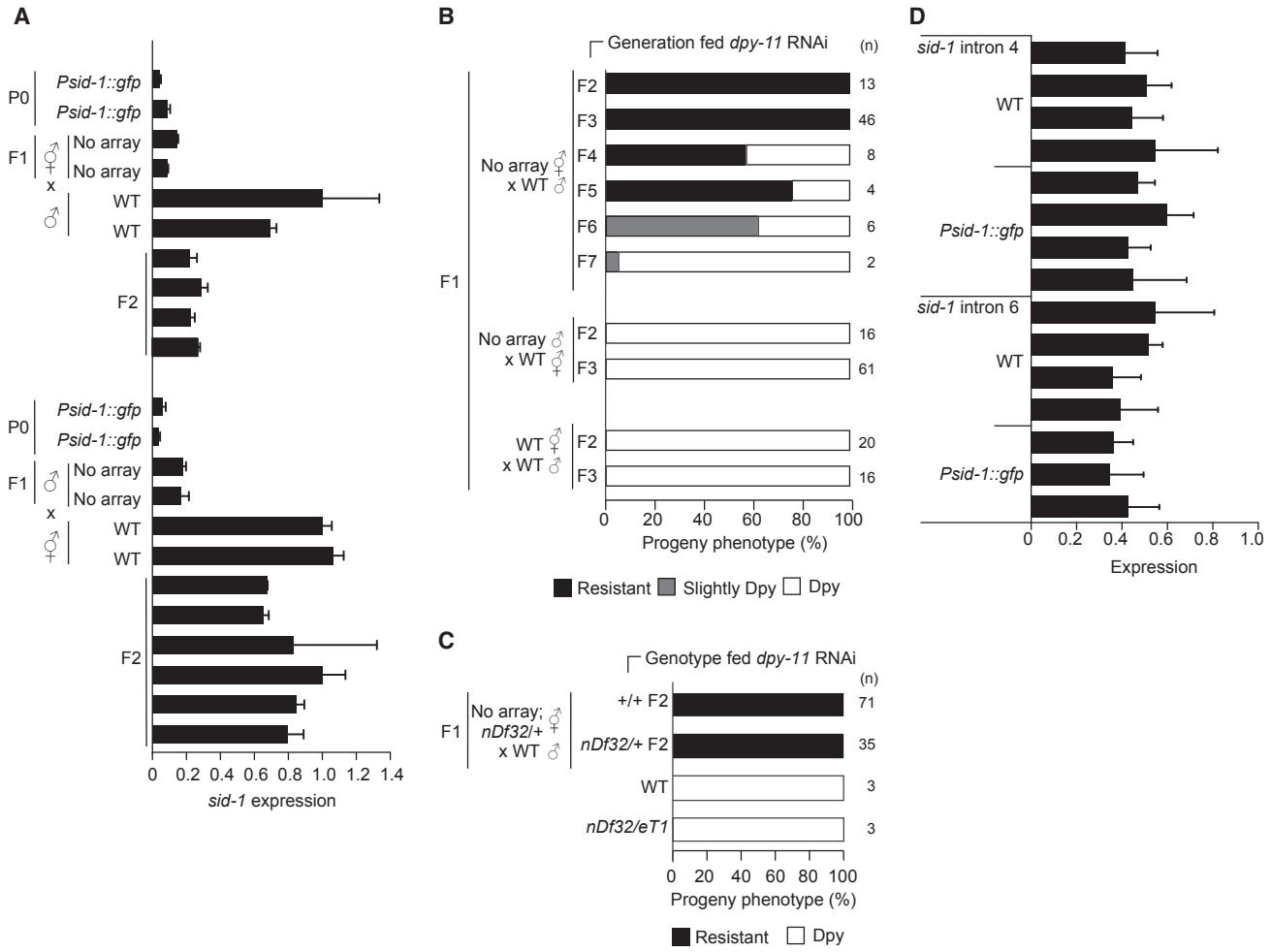

Figure 2. Maternally Transmitted Epigenetic Silencing

(A) Normalized *sid-1* mRNA levels (relative to *cpf-1*) in single silenced and non-silenced parents and progeny (see Figure S2B for non-normalized data). Average ± SD of at least two technical replicates.

(B) RNAi sensitivity of progeny (average of 62 per worm) from (n) F2 cross progeny described in (A) and their subsequent self-progeny fed *dpy-11* RNAi.

(C) RNAi sensitivity of progeny (average 68 per worm) from (n) F2 *sid-1*+/*nDf32* hemizygous or *sid-1*+/*sid-1*+ cross progeny fed *dpy-11* RNAi.

(D) qRT-PCR measurement of *sid-1* intron 4 and intron 6 expression in single worm adults relative to *cpf-1* intron 5 expression. cDNA used for qRT-PCR was generated using a gene-specific primer amplifying *sid-1* and *cpf-1*. Average ± SD of at least two technical replicates is shown.

See also Figure S2.

globally altered in *sid-1* silenced worms, indicating that the increased small RNAs at *sid-1* are not simply due to the perturbation of *sid-1* (Figure S3B).

In addition, in *Psid-1::gfp* libraries, small RNAs enriched for 22G RNAs also aligned to the *sid-1* promoter region (Figures 3A, 3C, 3D, and S3A). Promoter-aligned small RNAs were never detected in wild-type libraries. Our mRNA sequencing data indicate that *Psid-1::gfp* worms contain abundant transcripts that map to the *sid-1* promoter while no similar transcripts were detected in wild-type worms (Figure S3C), suggesting that the small RNAs that align to the *sid-1* promoter are templated by *Psid-1::gfp* array-associated transcripts.

To determine whether these *sid-1*-associated 22G RNAs persist in the absence of the *Psid-1::gfp* array, we sequenced small RNAs from lines c and d (Figure 1G) at 6 (silenced) and 21 (de-silenced) generations removed from a *Psid-1::gfp* array ancestor. No small RNAs aligned to the *sid-1* promoter in either line at either generation (Figure 3A). In contrast, in silenced worms (F6) we detected an increase in *sid-1* antisense small RNAs, while in de-silenced (F21) worms, small RNA levels were at wild-type levels (Figure 3B). These results extend the correlation between the coding-region 22G RNAs and persistent *sid-1* silencing.

The PRG-1/HRDE-1 Pathway Silences sid-1

We hypothesized that the small RNAs targeting the *sid-1* locus are generated and stabilized by one of the known *C. elegans* small RNA pathways. Previous cases of heritable transgene

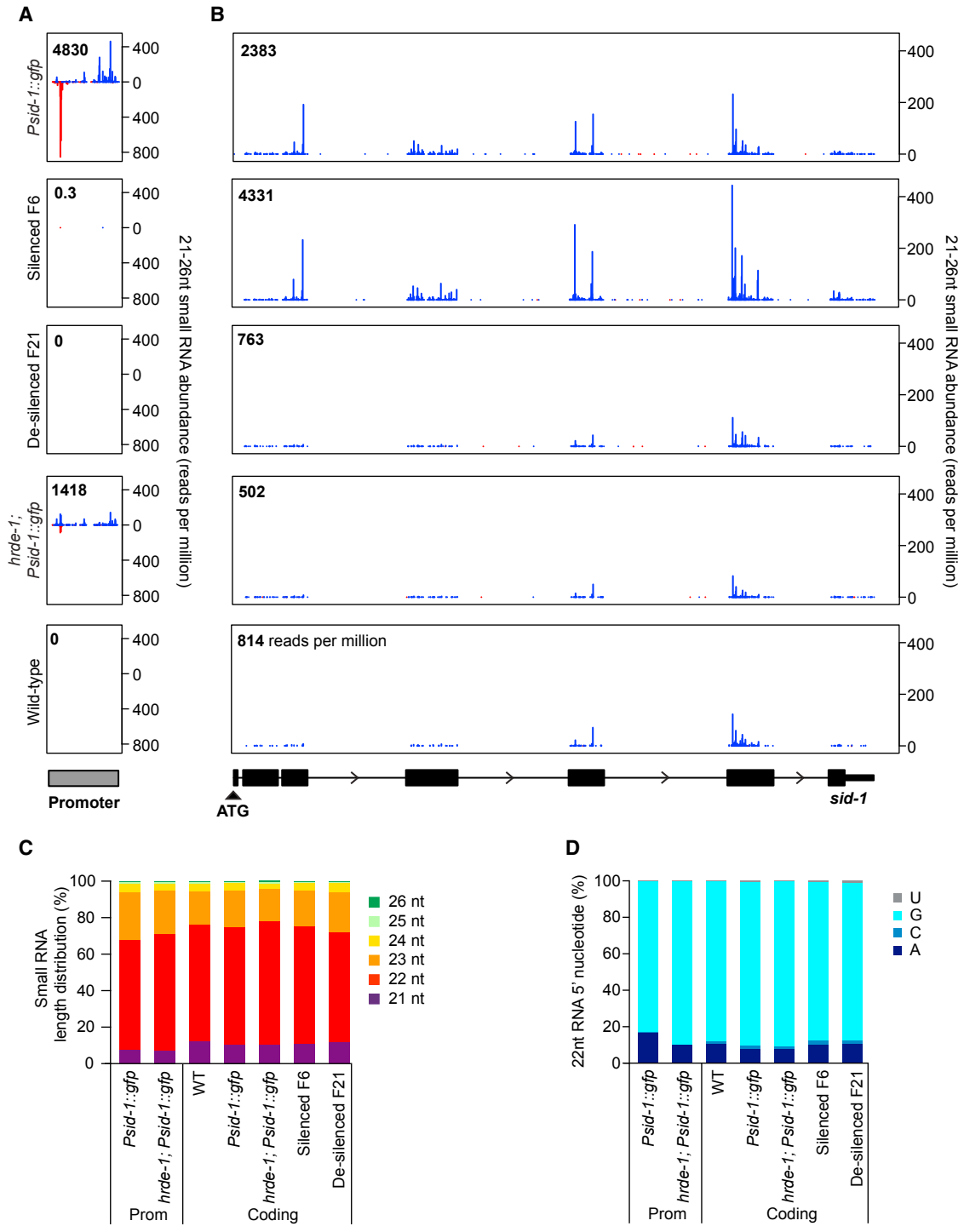

Figure 3. Transgenerational *sid-1* Silencing Is Associated with an Increase in HRDE-1-Dependent Small RNAs

(A and B) Frequency and distribution of sense (red) and antisense (blue) 21–26 nt small RNAs over the (A) *sid-1* promoter and (B) *sid-1* gene (start codon to 3′ UTR). Total read counts (upper corner) are normalized to all 21–26 nt reads that map to genes.

(C and D) Reads (both strands) are highly enriched for (C) 22 nt RNAs with (D) a 5′ guanine in all libraries.

Four *Psid-1::gfp* libraries and two libraries for all other genotypes were combined as replicates. See also Figure S3.

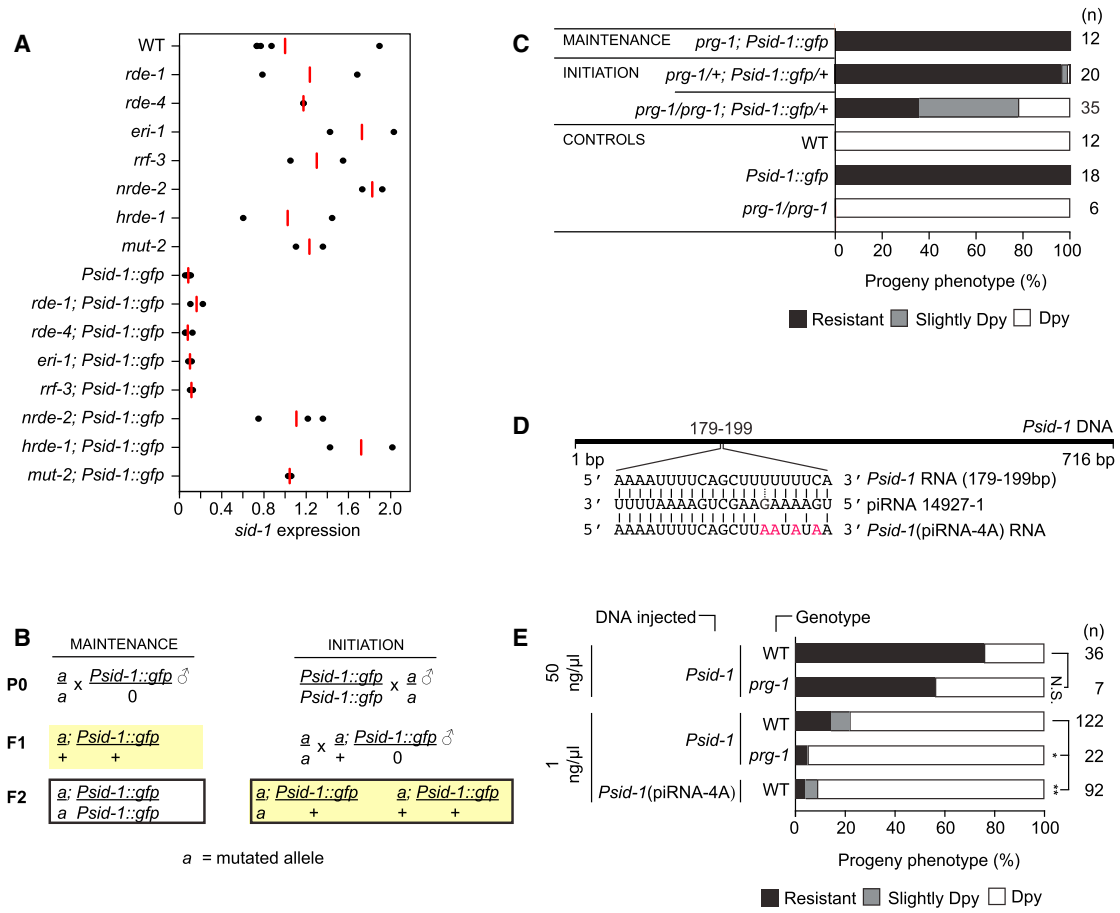

Figure 4. Genetic Requirements for *sid-1* Silencing

(A) *sid-1* mRNA expression in mixed-stage worms. Average (red bar) of biological replicates relative to *gpd-2/3*.

(B) Maintenance and initiation crosses. Silencing competent hermaphrodite germline is highlighted.

(C) RNAi sensitivity of progeny of (n) F2 L4 larvae produced by crosses in (B).

(D) Putative piRNA 14927-1 binding site and mutant *Psid-1*(piRNA-4A) sequence.

(E) RNAi sensitivity of progeny of (n) F2 lines produced by injected wild-type or piRNA-4A *Psid-1* DNA. N.S., not significant; *$p < 0.05$, **$p < 0.002$ (Mann-Whitney test). Resistant and slightly Dpy values were combined for statistics.

In (A)–(C), the *Psid-1::gfp* array is integrated on the X chromosome. See also Figure S4.

silencing in *C. elegans* require the nuclear RNAi pathway (Ashe et al., 2012; Buckley et al., 2012; Sapetschnig et al., 2015; Shirayama et al., 2012). We found that mutations in the nuclear RNAi factors *hrde-1* and *nrde-2*, and a mutation in *mut-2*, a putative nucleotidyltransferase required for siRNA accumulation (Zhang et al., 2011), also prevent promoter-mediated *sid-1* silencing (Figure 4A).

In addition to nuclear RNAi, the PIWI Argonaute PRG-1 has been implicated in heritable silencing of transgenes. While RNAe requires PRG-1 only to initiate silencing (Shirayama et al., 2012), another case of heritable transgene silencing also requires PRG-1 to maintain silencing (Bagijn et al., 2012). We found that PRG-1 is only partially required to initiate *sid-1* silencing. Specifically, *prg-1(n4357); Psid-1::gfp* animals that segregate from silenced *prg-1*/+ heterozygotes ("maintenance cross") remained fully silenced, showing that *prg-1* is not required to maintain established silencing (Figures 4B, 4C, and S4A). In contrast, crossing the array from a *prg-1(n4357)/+; Psid-1::gfp*/0 male into *prg-1(n4357)* homozygotes ("initiation cross") produced *prg-1(n4357); Psid-1::gfp* animals that incompletely silence *sid-1* (Figures 4B, 4C, and S4A). The dsRNA-dependent exogenous (*rde-1* and *rde-4*) and 26G small RNA-dependent endogenous (*rde-4*, *eri-1*, and *rrf-3*) RNAi pathways were not required to initiate (Figures S4B–S4D) or maintain (Figure 4A) *sid-1* silencing. Thus, *prg-1* uniquely participates in the initiation of promoter-mediated *sid-1* silencing, while *hrde-1* maintains silencing.

To characterize the *prg-1*-dependent initiation of *sid-1* silencing, we injected the *Psid-1* DNA fragment into wild-type and *prg-1(n4357)* mutant worms. High concentrations of the *Psid-1* DNA fragment caused *prg-1*-independent *sid-1* silencing, but at lower concentrations, the *Psid-1* DNA fragment was significantly better at silencing *sid-1* in wild-type worms than in *prg-1* mutants (Figures 4E and S4E). We then identified a 21 nt sequence

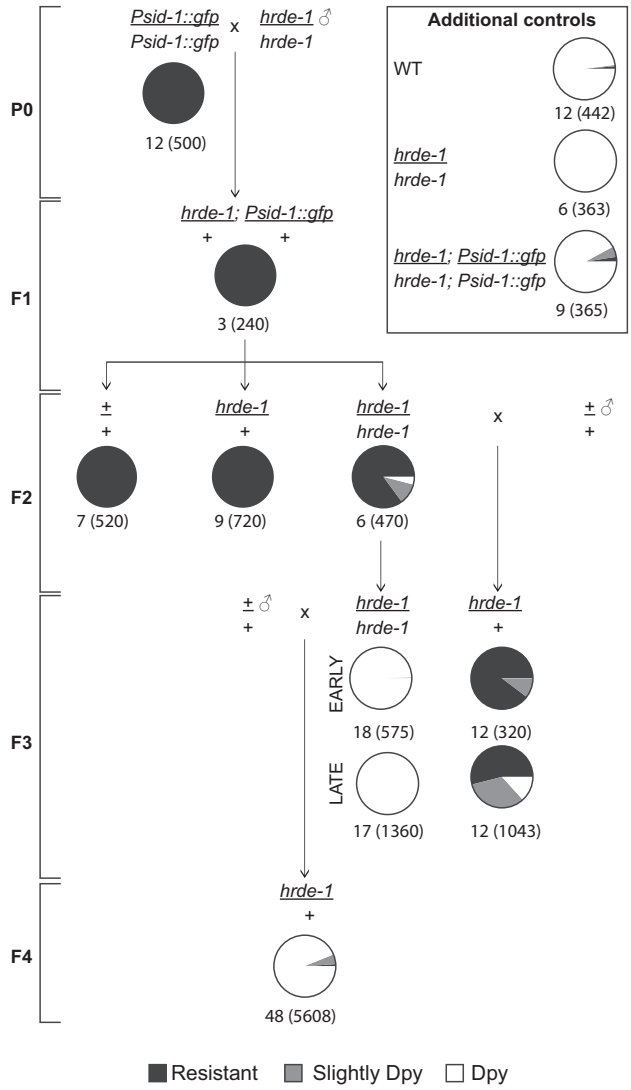

Figure 5. HRDE-1 Is Required to Execute Transgenerational Silencing

Crosses to test *hrde-1* necessity and sufficiency for transgenerational silencing. Pie charts show RNAi sensitivity of progeny of singled L4 larvae on *dpy-11* RNAi food. Below each pie chart is the number of L4 parents and the number of progeny scored (in parentheses). Early progeny are the first progeny laid; late progeny are progeny laid subsequently by F3 parent. See also Figure S5.

in the *sid-1* promoter region that could be targeted by the endogenous piRNA 14927-1 (Bagijn et al., 2012; Batista et al., 2008) and disrupted the putative piRNA binding site by four nucleotide changes ("*Psid-1*(piRNA-4A)"; Figure 4D). The efficiency of silencing induced by *Psid-1*(piRNA-4A) DNA fragment injection in wild-type worms was similar to that observed by injecting the wild-type fragment into *prg-1* mutant worms (Figures 4E and S4E). Thus, the requirement for *prg-1* to initiate efficient *sid-1* silencing likely reflects a requirement for piRNA 14927-1.

siRNAs Embody the Inherited Silencing Signal

HRDE-1 is a nuclear Argonaute required to silence *sid-1* in *Psid-1::gfp* worms (Figure 4A). To further characterize the contribution of HRDE-1 to transgenerational epigenetic inheritance of *sid-1* silencing, we generated *hrde-1(tm1200)/+; Psid-1::gfp/+* F1s and placed their F2 non-array progeny on *dpy-11* RNAi (Figures 5 and S5A). All *hrde-1(tm1200)/hrde-1(tm1200)* F2s produced non-Dpy F3 progeny, demonstrating that *sid-1* remained silenced. This likely represents maternal F1-produced, HRDE-1-deposited small RNAs in F2 embryos. In contrast, F3 worms fed *dpy-11* RNAi produced only Dpy F4 progeny (Figures 5 and S5A), indicating that HRDE-1 and/or silencing signal depletion results in the re-expression of *sid-1* in the F3 generation. If 22G RNAs targeting *sid-1* persist in the F3 but cannot execute silencing due to the depletion of HRDE-1, then re-introduction of HRDE-1 into the F3 progeny should restore *sid-1* silencing. Indeed, all tested F3 *hrde-1/+* worms placed on *dpy-11* RNAi food produced non-Dpy F4 progeny (Figures 5 and S5A). Thus, sequence-specific silencing information was transmitted from the heterozygous F1 grandparent, through the homozygous F2 mother, and into the heterozygous F3 grand-progeny, where the re-introduced wild-type HRDE-1 was sufficient to execute silencing. Importantly, introduction of a wild-type HRDE-1 a generation later cannot restore *sid-1* silencing, indicating that the silencing signal is impermanent and requires HRDE-1 for its production and/or maintenance. The restoration of silencing by wild-type HRDE-1 in the heterozygous F3 generation is strong evidence that small RNAs embody the inherited epigenetic information. Temporal analysis of silencing strength in F3 worms provides further evidence for this model. Cross progeny F3 worms laid earlier more efficiently silenced *sid-1* than cross progeny laid later (Figures 5 and S5A). This likely reflects dilution of silencing signals in the F2 germline such that early oocytes have more anti-*sid-1* siRNAs than do the late oocytes.

HRDE-1 is required for the transmission of the silencing signal across generations (Figure 5), and *sid-1* promoter and exon-associated small RNAs decrease significantly in *hrde-1(tm1200); Psid-1::gfp* worms compared to *Psid-1::gfp* worms (Figures 3A and 3B). Thus, in the presence of the *Psid-1::gfp* array, HRDE-1 is required for the accumulation and spread of 22G RNAs from the *sid-1* promoter region to the coding region.

How could small RNAs targeting array-produced promoter mRNA sequences direct HRDE-1-dependent accumulation of small RNAs to the *sid-1* coding region? *Psid-1::gfp* animals contain many small RNAs complementary to the abundant array-transcribed RNA corresponding to the *sid-1* upstream region (Figures 3A and S3C). Like many *C. elegans* transcripts, *sid-1* is *trans*-spliced, meaning that a 5′ UTR present in pre-mRNA is replaced by a splice leader (SL1) sequence in the mature transcript (Saito et al., 2013). While mRNA sequencing analysis detects only the abundant SL1 *trans*-spliced mature *sid-1* transcript, experiments designed to detect transcription start sites identify sites within the promoter region of *sid-1* (Chen et al., 2013; Saito et al., 2013) (Figure S5B). Thus, the abundant array-dependent small RNAs could target the *sid-1* primary transcript, enabling HRDE-1-dependent spread and accumulation of small RNAs targeting the *sid-1* exons.

Transgenerational Somatic sid-1 Silencing

Wild-type animals exposed to *dpy-11* RNAi food for two consecutive generations are strongly Dpy. This strongly Dpy phenotype

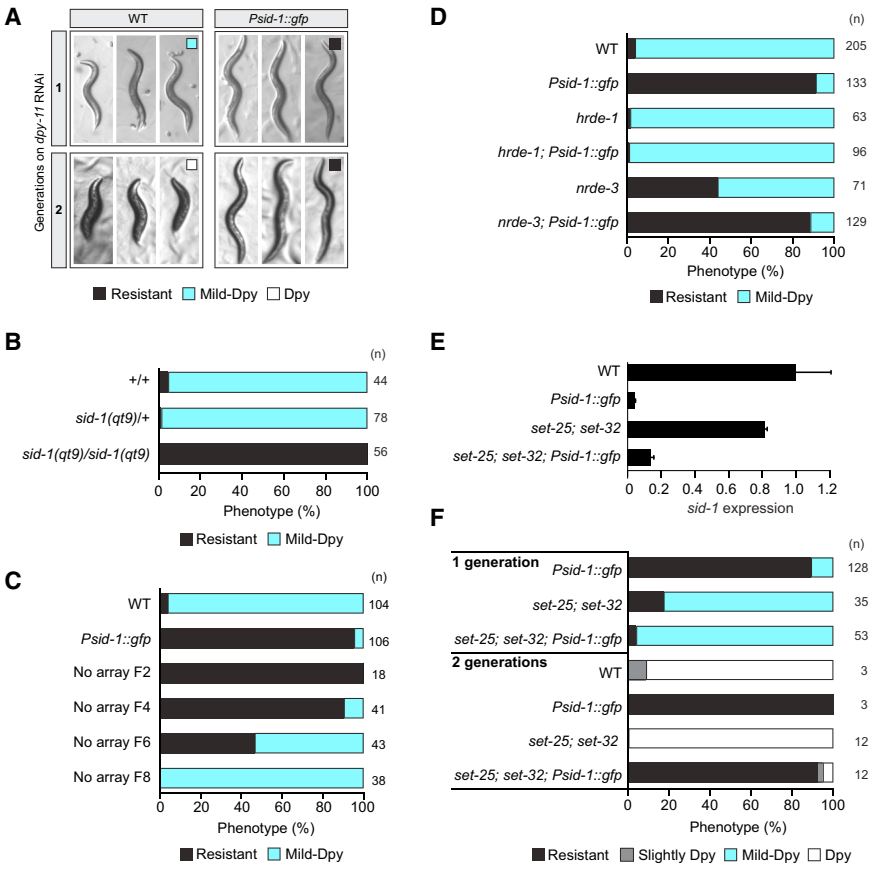

Figure 6. Transgenerational Somatic Silencing

(A) Progeny of wild-type or *Psid-1::gfp* larvae (two generations) or embryos (one generation) placed on *dpy-11* RNAi food.

(B–D) RNAi sensitivity of (n) worms hatched on *dpy-11* RNAi food, scored as adults. To determine *sid-1* genotype in (B), adults were fed *act-5* RNAi (L1 arrest).

(E) qRT-PCR analysis of *sid-1* mRNA levels (normalized, relative to *gpd-2/3*) in young adults. Average ± SD of at least two technical replicates.

(F) RNAi sensitivity of methyltransferase mutants after *dpy-11* dsRNA exposure for one (n worms scored) or two generations (average of 116 progeny from [n] L4 larvae scored).

See also Figure S6.

requires both maternal *sid-1* expression in the germline and somatic *sid-1* expression. However, wild-type animals exposed to *dpy-11* RNAi food for a single generation have a readily detectable and highly penetrant mild-Dpy phenotype (Figure 6A). To determine whether maternally inherited *sid-1* activity contributes to the single-generation mild-Dpy phenotype, we tested the progeny of *sid-1(qt9)/+* parents in the single-generation *dpy-11* RNAi assay. While *sid-1(qt9)/+* progeny developed the mild-Dpy phenotype as adults, *sid-1(qt9)/sid-1(qt9)* progeny were completely resistant adults (Figure 6B). Thus, maternal *sid-1* expression is not sufficient to produce the mild-Dpy phenotype. Further, the cross progeny of a non-array silenced worm and a wild-type male, which should express the non-silenced paternal *sid-1* allele, are mildly Dpy (Figure S6A). These results indicate that somatic *sid-1* expression is necessary and sufficient for the mild-Dpy phenotype. Therefore, the absence of this phenotype is a reliable indirect measure for somatic *sid-1* silencing. Because *Psid-1::gfp* worms are completely resistant in the single-generation *dpy-11* RNAi assay (Figure 6A), *sid-1* must be silenced in the soma in *Psid-1::gfp* worms.

The single-generation *dpy-11* RNAi assay accurately, sensitively, and specifically detects silencing of somatic *sid-1* expression. RT-PCR experiments comparing wild-type and *Psid-1::gfp* L1 larvae (~550 somatic cells and two germline precursors) also detect reduced *sid-1* transcripts (data not shown). However, unlike the single-generation *dpy-11* RNAi assay, these experiments cannot distinguish between maternal and zygotic *sid-1* transcripts. That is, if maternal *sid-1* transcripts persist in larval stages, then this measured decrease could reflect reduced maternal contribution rather than reduced zygotic expression. Thus, we used the single-generation *dpy-11* RNAi assay to specifically measure somatic *sid-1* expression.

Heritable silencing of a somatically expressed gene in *C. elegans* in response to exogenous RNAi has been reported. However, the silencing is transmitted at reduced penetrance (30%) and maternal (germline) contributions to the silencing were not investigated (Vastenhouw et al., 2006). To determine whether somatic *sid-1* silencing can occur and be inherited in the absence of the array, we tested the progeny of non-array silenced animals in the single-generation *dpy-11* RNAi assay. Not only did we find that *sid-1* somatic silencing can occur in the absence of the array, but the silencing is remarkably stable; somatic *sid-1* silencing is inherited, at nearly 100% penetrance, for four generations (Figures 6C and S6B).

The stability of somatic *sid-1* silencing allowed us to determine the genetic basis for heritable silencing in the soma. Unexpectedly, *sid-1* silencing in the soma did not require the somatically expressed HRDE-1 paralog NRDE-3 (Guang et al., 2008). *nrde-3(gg66); Psid-1::gfp* and *Psid-1::gfp* worms are equally and completely resistant to *dpy-11* RNAi (Figure 6D). In contrast, *hrde-1(tm1200); Psid-1::gfp* worms fail to silence *sid-1* in the soma (Figure 6D). We conclude that *sid-1* silencing in the soma is dependent on previous silencing in the maternal germline.

In contrast to other examples of heritable silencing (Ashe et al., 2012), promoter-mediated *sid-1* silencing in the germline does not require the putative histone H3 lysine 9 (H3K9) methyltransferases *set-25* and *set-32* (hereafter referred to as methyltransferases): *set-25; set-32; Psid-1::gfp* worms have reduced *sid-1* expression and when placed on *dpy-11* RNAi produce resistant progeny (Figures 6E and 6F). However, in single-generation

dpy-11 RNAi, *set-25; set-32; Psid-1::gfp* worms are mildly Dpy, indicating that both methyltransferases contribute to somatic *sid-1* silencing (Figure 6F). Thus, the requirement for HRDE-1 in both germline and somatic silencing, together with the requirement for SET methyltransferases exclusively in somatic silencing, suggests that small RNA-dependent silencing in the germline directs chromatin-dependent silencing in the soma.

DISCUSSION

Here we provide the first molecular and genetic analysis of heritable RNAi silencing at an endogenous locus in *C. elegans*. Silencing at the *sid-1* locus is initiated by multiple copies of the promoter and 5′ UTR in a dose-dependent process that is partially dependent on the piRNA-stabilizing protein PRG-1. Once initiated, the silencing is remarkably stable in the absence of the promoter array, persisting at 100% transmission for up to 13 generations without selection. The silencing is dependent on components of the germline heritable RNAi pathway, and HRDE-1-dependent small RNAs antisense to *sid-1* exons are associated with the silenced state. Our genetic analysis reveals that the silenced locus is not required for inheritance and that HRDE-1-responsive silencing information persists in the absence of HRDE-1 function over two generations.

Silencing in the context of an endogenous locus is more likely to reflect characteristics of evolutionarily relevant silencing than silencing of custom-designed transgenes. In fact, comparison of *sid-1* silencing to previously described multigenerational transgene silencing identifies numerous differences (Table S1). First, previously described cases of heritably silenced loci are initiated by either dsRNA or by endogenous piRNAs (Alcazar et al., 2008; Ashe et al., 2012; Buckley et al., 2012; Sapetschnig et al., 2015; Shirayama et al., 2012; Vastenhouw et al., 2006). In contrast, *sid-1* silencing is initiated by a multi-copy array containing only its promoter and 5′ UTR, which has not been reported for any other gene. An array-derived dsRNA intermediate is unlikely to be required for *sid-1* silencing because RDE-1 and RDE-4 are not required for silencing. Furthermore, although an endogenous piRNA can contribute to *sid-1* silencing, piRNAs are not required to initiate *sid-1* silencing. Second, *sid-1* is the first stably silenced endogenous locus, both in the germline and in the soma. In all other examples of endogenous silenced loci, silencing is passed on to only a fraction of progeny even one generation after removal of the initiating trigger (Alcazar et al., 2008; Vastenhouw et al., 2006). In contrast, after removal of the *Psid-1* array, *sid-1* silencing continues at 100% transmission, for up to 13 generations in the germline and for four generations in the soma. Third, while transgene silencing and silencing of the endogenous *oma-1* gene can be inherited through both the male and female germlines (Alcazar et al., 2008; Shirayama et al., 2012), silencing of *sid-1* is inherited exclusively through the maternal germline. As in other cases of heritable silencing (Ashe et al., 2012; Buckley et al., 2012; Sapetschnig et al., 2015; Shirayama et al., 2012), the nuclear Argonaute HRDE-1 is required for *sid-1* silencing in the germline. More surprisingly, two putative histone H3K9 methyltransferases that are required to silence transgenes in the germline (Ashe et al., 2012) are not required to silence *sid-1* in the germline. Instead, these methyltransferases are required to silence *sid-1* in the soma, suggesting a transition from post-transcriptional gene silencing (PTGS) in the germline to transcriptional gene silencing (TGS) in the soma.

The initiation of *sid-1* silencing likely reflects a two-step process: first, the accumulation of a persistent population of small RNAs that target the *sid-1* promoter region on the multi-copy array, followed by spreading of small RNAs from the endogenous promoter to the coding exons. There is precedent for spreading of siRNAs 3′ of the original trigger (Pak and Fire, 2007; Sapetschnig et al., 2015), and the efficient spreading of siRNAs along a spliced transcript is dependent on nuclear silencing (Zhuang et al., 2013; Sapetschnig et al., 2015). We also note that the presence of siRNAs at the expressed *sid-1* locus may facilitate the spreading of promoter siRNAs to the gene body. The delay in initial silencing likely reflects a below-threshold level of array promoter small RNAs. Consistent with this, injecting the promoter fragment showed dose-dependent efficiency of silencing (Figure 1F). At the lowest dose, the establishment of silencing is partially dependent on *prg-1* and the presence of a binding site for a specific piRNA (Figure 4E). Analysis of double mutants would be required to determine if any of the other known small RNA pathways are sufficient to initiate silencing in the absence of the piRNA pathway. Additionally, factors required for maintenance of silencing likely participate in the initiation of silencing, but this is not possible to test, as we cannot genetically isolate an initiation function for such factors.

Where tested, the Argonaute HRDE-1 is required for all RNAi-dependent transgenerational silencing described in *C. elegans*. Experiments comparing wild-type to *hrde-1* or *nrde-3* mutants undergoing RNAi support a role for TGS in nuclear RNAi (Buckley et al., 2012; Burkhart et al., 2011; Guang et al., 2008, 2010). However, we did not detect the expected TGS-associated decrease in *sid-1* pre-mRNA levels in worms that silence *sid-1* (Figure 2D), nor did we detect a requirement for the putative histone methyltransferases *set-25* and *set-32* in *sid-1* silencing in the germline (Figures 6E and 6F), supporting a PTGS mechanism for transgenerational *sid-1* silencing in the germline. Thus, *hrde-1*-dependent silencing can act through TGS and PTGS mechanisms in different contexts, as has been previously proposed due to the role of nuclear RNAi Argonautes in transitive RNAi (Sapetschnig et al., 2015; Zhuang et al., 2013).

Several groups have shown correlation between the presence or absence of siRNAs and silencing and non-silencing. These siRNAs could be a byproduct of an RNAi-independent silencing mechanism. For example, marked chromatin can trigger siRNA production in yeast (Bühler et al., 2006). Alternatively, these siRNAs could be the parentally provided sequence-specific cause of silencing in the progeny. In mammals, diet and stress influence the abundance of sperm-associated small RNAs that can alter gene expression and behavior in progeny (Chen et al., 2016; Gapp et al., 2014; Sharma et al., 2016). Our small RNA sequencing and multi-generational genetic analysis of *hrde-1* showed that *hrde-1* is required to stabilize siRNAs and execute *sid-1* silencing. *hrde-1*-dependent silencing signals can persist in *hrde-1* homozygotes for two generations, but cannot execute silencing unless wild-type *hrde-1* is re-introduced. Further, we showed that the *sid-1* physical locus is not required for transmission of silencing. Together, these results

strongly support the supposition that siRNAs physically embody the transgenerationally transmitted silencing information. Although our genetic analysis cannot discriminate between direct transmission of grandparental siRNAs acting in the germline of the grand-progeny versus a re-synthesis of siRNAs after the reintroduction of wild-type *hrde-1*, our results provide direct evidence for sustained RNA-directed transgenerational inheritance.

This study also provides the first example of stable, highly penetrant, transgenerational silencing in somatic cells. Multiple features of this somatic silencing were unexpected. First, not only was *nrde-3*, the somatically expressed homolog of *hrde-1*, not required for somatic *sid-1* silencing, but maternal *hrde-1* was required. Second, the putative methyltransferases *set-25* and *set-32* are specifically required to silence *sid-1* in the soma (Figures 6E and 6F). Low somatic *sid-1* expression levels relative to the germline expression levels likely masked any measurable effect on pre-mRNA in the soma (Figure 1C). Since both *hrde-1* and *set-25*/*set-32* are required for somatic silencing, they do not act redundantly and likely act in series. Thus, siRNA-mediated, *hrde-1*-dependent *trans*-acting germline silencing likely establishes chromatin-modifying, machinery-dependent *cis*-acting somatic silencing in the progeny.

Many multi-copy reporter construct arrays containing promoters have been made and analyzed and none are reported to epigenetically silence the endogenous locus. *sid-1* silencing is distinct from co-suppression, in which multi-copy arrays that include coding sequences can silence endogenous loci in the *C. elegans* germline (Dernburg et al., 2000; Ketting and Plasterk, 2000). First, co-suppression requires that the coding sequence be present in the multi-copy array; for example, a multi-copy array of the *fem-1* gene that includes the promoter, 5′ UTR, and coding sequence silences germline *fem-1* expression. However, a multi-copy array that contains the promoter and 5′ UTR only does not silence *fem-1* (Dernburg et al., 2000; Saito et al., 2013). The *Psid-1* fragment does not contain the *sid-1* coding sequence. The inclusion of a *trans*-spliced 5′ UTR in our *Psid-1::gfp* reporter construct does not sufficiently differentiate this array from other reporter constructs. In total, 70% of *C. elegans* transcripts are *trans*-spliced (Zorio et al., 1994) and transcriptional fusions often contain 5′ UTRs. However, they do not silence. Second, co-suppression-induced silencing is transmitted in the absence of the array weakly or not at all (Dernburg et al., 2000), whereas *sid-1* silencing can be transmitted for more than a dozen generations at 100% penetrance. Third, only germline genes can be silenced by co-suppression, whereas *sid-1* is also heritably silenced in the soma.

It is unlikely that *sid-1* is the only gene that can exhibit stable promoter-mediated epigenetic silencing. Indeed, the two germline-enriched genes upstream of *sid-1* can also be epigenetically silenced (Figures 1D and S1E) by an HRDE-1 dependent process, although we note that statistically significant changes in small RNAs targeting these genes are not consistently detected in silenced worms, perhaps because these small RNAs are present at very low levels (data not shown). Instead, *sid-1* may be special in our ability to readily detect epigenetic silencing. Unlike many germline-expressed genes, *sid-1* is not required for viability or fertility. If essential genes are silenced by a promoter multi-copy array, any silenced segregants would die out and only non-silenced progeny would persist. Additionally, like the two genes upstream of *sid-1*, loss of other epigenetically silenced germline genes may be undetectable unless measured intentionally. Loss of systemic RNAi is not only easily scored, but the strength, sensitivity, and target tissue(s) can be readily modified by the choice of RNAi food. This is particularly important as the level of *sid-1* silencing by the multi-copy promoter array is incomplete (90%). Had we only tested potent RNAi foods, we never would have detected the silencing. Additionally, the ability to infer small changes in *sid-1* expression levels from RNAi food phenotypes was crucial to recognize that *sid-1* was transgenerationally silenced in the soma.

Shared sequence-specific silencing between the germline and the soma potentially enables selection for advantageous phenotypes beyond fecundity. Several recent studies in *C. elegans* implicate *hrde-1*-dependent processes in physiologically induced transgenerational changes in gene expression (Rechavi et al., 2014; Schott et al., 2014). It remains unknown whether or how changes in expression of these genes contribute to a stress response. Because *hrde-1* is involved, there is presumably selection for genes like *sid-1* that are expressed both in the germline, to initiate and transmit heritable signals, and in somatic cells, to effect physiological phenotypes.

It remains unknown how silencing of endogenous genes is triggered in response to environmental stress. The initiation of *sid-1* silencing that we describe likely involves accumulation of the *Psid-1::gfp* array transcribed *sid-1* promoter and 5′ UTR RNA. We hypothesize that accumulation and export of this piRNA-targeted 5′ UTR exon to the cytoplasm initiates production of antisense 22G RNAs targeting the 5′ UTR, which can then be transported to the nucleus to initiate nuclear RNAi-dependent silencing. The endogenous *sid-1* 5′ UTR is efficiently *trans*-spliced prior to mRNA export to the cytoplasm; thus, the *sid-1* locus is not readily silenced in wild-type animals. However, it is plausible that *sid-1* or other loci could be similarly targeted for silencing if alternative splicing produces a transcript with an exon that contains a piRNA binding site. Because splicing is regulated by environmental conditions (Biamonti and Caceres, 2009), stress-responsive alternative splicing could trigger gene-selective epigenetic silencing in response to specific environmental conditions.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2017.01.034.

AUTHOR CONTRIBUTIONS

O.M. performed all experiments and analyzed the data. O.M. and C.P.H. designed experiments and wrote the paper.

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REFERENCES

Alcazar, R.M., Lin, R., and Fire, A.Z. (2008). Transmission dynamics of heritable silencing induced by double-stranded RNA in Caenorhabditis elegans. Genetics *180*, 1275–1288.

Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics *31*, 166–169.

Arnold, A., Rahman, M.M., Lee, M.C., Muehlhaeusser, S., Katic, I., Gaidatzis, D., Hess, D., Scheckel, C., Wright, J.E., Stetak, A., et al. (2014). Functional characterization of C. elegans Y-box-binding proteins reveals tissue-specific functions and a critical role in the formation of polysomes. Nucleic Acids Res. *42*, 13353–13369.

Ashe, A., Sapetschnig, A., Weick, E.-M., Mitchell, J., Bagijn, M.P., Cording, A.C., Doebley, A.-L., Goldstein, L.D., Lehrbach, N.J., Le Pen, J., et al. (2012). piRNAs can trigger a multigenerational epigenetic memory in the germline of C. elegans. Cell *150*, 88–99.

Bagijn, M.P., Goldstein, L.D., Sapetschnig, A., Weick, E.M., Bouasker, S., Lehrbach, N.J., Simard, M.J., and Miska, E.A. (2012). Function, targets, and evolution of Caenorhabditis elegans piRNAs. Science *337*, 574–578.

Batista, P.J., Ruby, J.G., Claycomb, J.M., Chiang, R., Fahlgren, N., Kasschau, K.D., Chaves, D.A., Gu, W., Vasale, J.J., Duan, S., et al. (2008). PRG-1 and 21U-RNAs interact to form the piRNA complex required for fertility in C. elegans. Mol. Cell *31*, 67–78.

Baugh, L.R., Hill, A.A., Slonim, D.K., Brown, E.L., and Hunter, C.P. (2003). Composition and dynamics of the Caenorhabditis elegans early embryonic transcriptome. Development *130*, 889–900.

Biamonti, G., and Caceres, J.F. (2009). Cellular stress and RNA splicing. Trends Biochem. Sci. *34*, 146–153.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71–94.

Buckley, B.A., Burkhart, K.B., Gu, S.G., Spracklin, G., Kershner, A., Fritz, H., Kimble, J., Fire, A., and Kennedy, S. (2012). A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. Nature *489*, 447–451.

Bühler, M., Verdel, A., and Moazed, D. (2006). Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. Cell *125*, 873–886.

Burkhart, K.B., Guang, S., Buckley, B.A., Wong, L., Bochner, A.F., and Kennedy, S. (2011). A pre-mRNA-associating factor links endogenous siRNAs to chromatin regulation. PLoS Genet. *7*, e1002249.

Burton, N.O., Burkhart, K.B., and Kennedy, S. (2011). Nuclear RNAi maintains heritable gene silencing in Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA *108*, 19683–19688.

Chandler, V.L. (2010). Paramutation's properties and puzzles. Science *330*, 628–629.

Chen, R.A.J., Down, T.A., Stempor, P., Chen, Q.B., Egelhofer, T.A., Hillier, L.W., Jeffers, T.E., and Ahringer, J. (2013). The landscape of RNA polymerase II transcription initiation in C. elegans reveals promoter and enhancer architectures. Genome Res. *23*, 1339–1347.

Chen, Q., Yan, W., and Duan, E. (2016). Epigenetic inheritance of acquired traits through sperm RNAs and sperm RNA modifications. Nat. Rev. Genet. *17*, 733–743.

de Vanssay, A., Bougé, A.-L., Boivin, A., Hermant, C., Teysset, L., Delmarre, V., Antoniewski, C., and Ronsseray, S. (2012). Paramutation in Drosophila linked to emergence of a piRNA-producing locus. Nature *490*, 112–115.

Dernburg, A.F., Zalevsky, J., Colaiácovo, M.P., and Villeneuve, A.M. (2000). Transgene-mediated cosuppression in the C. elegans germ line. Genes Dev. *14*, 1578–1583.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806–811.

Gapp, K., Jawaid, A., Sarkies, P., Bohacek, J., Pelczar, P., Prados, J., Farinelli, L., Miska, E., and Mansuy, I.M. (2014). Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. Nat. Neurosci. *17*, 667–669.

Guang, S., Bochner, A.F., Pavelec, D.M., Burkhart, K.B., Harding, S., Lachowiec, J., and Kennedy, S. (2008). An Argonaute transports siRNAs from the cytoplasm to the nucleus. Science *321*, 537–541.

Guang, S., Bochner, A.F., Burkhart, K.B., Burton, N., Pavelec, D.M., and Kennedy, S. (2010). Small regulatory RNAs inhibit RNA polymerase II during the elongation phase of transcription. Nature *465*, 1097–1101.

Hermant, C., Boivin, A., Teysset, L., Delmarre, V., Asif-Laidin, A., van den Beek, M., Antoniewski, C., and Ronsseray, S. (2015). Paramutation in Drosophila requires both nuclear and cytoplasmic actors of the piRNA pathway and induces cis-spreading of piRNA production. Genetics *201*, 1381–1396.

Jablonka, E., and Raz, G. (2009). Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. Q. Rev. Biol. *84*, 131–176.

Kamath, R.S., and Ahringer, J. (2003). Genome-wide RNAi screening in Caenorhabditis elegans. Methods *30*, 313–321.

Kennedy, S., Wang, D., and Ruvkun, G. (2004). A conserved siRNA-degrading RNase negatively regulates RNA interference in C. elegans. Nature *427*, 645–649.

Ketting, R.F., and Plasterk, R.H. (2000). A genetic link between co-suppression and RNA interference in C. elegans. Nature *404*, 296–298.

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S.L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. *10*, R25.

Lanjuin, A., VanHoven, M.K., Bargmann, C.I., Thompson, J.K., and Sengupta, P. (2003). Otx/otd homeobox genes specify distinct sensory neuron identities in C. elegans. Dev. Cell *5*, 621–633.

Lee, H.-C., Gu, W., Shirayama, M., Youngman, E., Conte, D., Jr., and Mello, C.C. (2012). C. elegans piRNAs mediate the genome-wide surveillance of germline transcripts. Cell *150*, 78–87.

Levin, M., Hashimshony, T., Wagner, F., and Yanai, I. (2012). Developmental milestones punctuate gene expression in the Caenorhabditis embryo. Dev. Cell *22*, 1101–1108.

Montgomery, M.K., Xu, S., and Fire, A. (1998). RNA as a target of double-stranded RNA-mediated genetic interference in Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA *95*, 15502–15507.

Pak, J., and Fire, A. (2007). Distinct populations of primary and secondary effectors during RNAi in C. elegans. Science *315*, 241–244.

Park, E.C., and Horvitz, H.R. (1986). Mutations with dominant effects on the behavior and morphology of the nematode Caenorhabditis elegans. Genetics *113*, 821–852.

Priess, J.R., Schnabel, H., and Schnabel, R. (1987). The glp-1 locus and cellular interactions in early C. elegans embryos. Cell *51*, 601–611.

Rechavi, O., Houri-Ze'evi, L., Anava, S., Goh, W.S.S., Kerk, S.Y., Hannon, G.J., and Hobert, O. (2014). Starvation-induced transgenerational inheritance of small RNAs in C. elegans. Cell *158*, 277–287.

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics *26*, 139–140.

Saito, T.L., Hashimoto, S., Gu, S.G., Morton, J.J., Stadler, M., Blumenthal, T., Fire, A., and Morishita, S. (2013). The transcription start site landscape of C. elegans. Genome Res. *23*, 1348–1361.

Sapetschnig, A., Sarkies, P., Lehrbach, N.J., and Miska, E.A. (2015). Tertiary siRNAs mediate paramutation in C. elegans. PLoS Genet. *11*, e1005078.

Schott, D., Yanai, I., and Hunter, C.P. (2014). Natural RNA interference directs a heritable response to the environment. Sci. Rep. *4*, 7387.

Sharma, U., Conine, C.C., Shea, J.M., Boskovic, A., Derr, A.G., Bing, X.Y., Belleannee, C., Kucukural, A., Serra, R.W., Sun, F., et al. (2016). Biogenesis and function of tRNA fragments during sperm maturation and fertilization in mammals. Science *351*, 391–396.

Shirayama, M., Seth, M., Lee, H.-C., Gu, W., Ishidate, T., Conte, D., Jr., and Mello, C.C. (2012). piRNAs initiate an epigenetic memory of nonself RNA in the C. elegans germline. Cell *150*, 65–77.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K.L., Parrish, S., Timmons, L., Plasterk, R.H., and Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. Cell *107*, 465–476.

Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., and Mello, C.C. (1999). The rde-1 gene, RNA interference, and transposon silencing in C. elegans. Cell *99*, 123–132.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., and Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. *7*, 562–578.

Tsai, H.-Y., Chen, C.-C.G., Conte, D., Jr., Moresco, J.J., Chaves, D.A., Mitani, S., Yates, J.R., 3rd, Tsai, M.-D., and Mello, C.C. (2015). A ribonuclease coordinates siRNA amplification and mRNA cleavage during RNAi. Cell *160*, 407–419.

Vastenhouw, N.L., Brunschwig, K., Okihara, K.L., Müller, F., Tijsterman, M., and Plasterk, R.H.A. (2006). Gene expression: long-term gene silencing by RNAi. Nature *442*, 882.

Winston, W.M., Molodowitch, C., and Hunter, C.P. (2002). Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. Science *295*, 2456–2459.

Zhang, C., Montgomery, T.A., Gabel, H.W., Fischer, S.E.J., Phillips, C.M., Fahlgren, N., Sullivan, C.M., Carrington, J.C., and Ruvkun, G. (2011). mut-16 and other mutator class genes modulate 22G and 26G siRNA pathways in Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA *108*, 1201–1208.

Zhuang, J.J., Banse, S.A., and Hunter, C.P. (2013). The nuclear argonaute NRDE-3 contributes to transitive RNAi in Caenorhabditis elegans. Genetics *194*, 117–131.

Zorio, D.A., Cheng, N.N., Blumenthal, T., and Spieth, J. (1994). Operons as a common form of chromosomal organization in C. elegans. Nature *372*, 270–272.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
5′ polyphosphatase	Epicentre	Cat#RP8092H
DNase I	Roche	Cat#4716728001
Critical Commercial Assays		
ThermoScript RT-PCR System	Invitrogen	Cat#11146-024
QuantiTect SYBR Green PCR Kit	QIAGEN	Cat#204145
Apollo 32 NGS Library Prep System: PrepX PolyA mRNA Isolation Kit	Wafergen	Cat#400047
Apollo 32 NGS Library Prep System: PrepX RNA-Seq Sample and Library Preparation Kit	Wafergen	Cat#400039
TruSeq RNA Library Preparation Kit v2	Illumina	Cat#RS-122-2001
Agencourt AMPure XP	Beckman Coulter	Cat#A63880
Deposited Data		
mRNA sequence data	This paper	GEO: GSE81708
Small RNA sequence data	This paper	GEO: GSE81708
Experimental Models: Organisms/Strains		
C. elegans: Strain HC125: *qtIs6[Psid-1::NLS::gfp::unc-54 3′ UTR] X*; referred to as "*qtIs6[Psid-1::gfp]*" below	Winston et al., 2002	HC125
C. elegans: Strain HC83: *qtEx6[Psid-1::NLS::gfp::unc-54 3′ UTR, pRF4]*	Winston et al., 2002	HC83
C. elegans: Strain WM27: *rde-1(ne219) V*	Tabara et al., 1999	WM27
C. elegans: Strain HC971: *rde-1(ne219) V; qtIs6[Psid-1::gfp] X*	This paper	HC971
C. elegans: Strain HC1011: *rde-4(ne301) III*	Tabara et al., 1999 and this paper	HC1011
C. elegans: Strain HC1012: *rde-4(ne301) III; qtIs6[Psid-1::gfp] X*	This paper	HC1012
C. elegans: Strain GR1373: *eri-1(mg366) IV*	Kennedy et al., 2004	GR1373
C. elegans: Strain HC1004: *eri-1(mg366) IV; qtIs6[Psid-1::gfp] X*	This paper	HC1004
C. elegans: Strain HC888: *rrf-3(pk2042) II*	Sijen et al., 2001 and this paper	HC888
C. elegans: Strain HC999: *rrf-3(pk2042) II; qtIs6[Psid-1::gfp] X*	This paper	HC999
C. elegans: Strain YY186: *nrde-2(gg91) II*	Guang et al., 2010	YY186
C. elegans: Strain HC972: *nrde-2(gg91) II; qtIs6[Psid-1::gfp] X*	This paper	HC972
C. elegans: Strain HC981: *nrde-3(gg66) X*	Guang et al., 2008 and this paper	HC981
C. elegans: Strain HC979: *nrde-3(gg66); qtIs6[Psid-1::gfp] X*	This paper	HC979
C. elegans: Strain YY538: *hrde-1(tm1200) III*	Buckley et al., 2012	YY538
C. elegans: Strain HC995: *hrde-1(tm1200) III; qtIs6[Psid-1::gfp] X*	This paper	HC995
C. elegans: Strain HC1001: *mut-2(ne298) I*	Tabara et al., 1999 and this paper	HC1001

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
C. elegans: Strain HC1000: *mut-2(ne298) I; qtIs6[Psid-1::gfp] X*	This paper	HC1000
C. elegans: Strain HC196: *sid-1(qt9) V*	Winston et al., 2002	HC196
C. elegans: Strain CB4037: *glp-1(e2141) III*	Priess et al., 1987	CB4037
C. elegans: Strain HC1040: *glp-1(e2141) III; qtIs6[Psid-1::gfp] X*	This paper	HC1040
C. elegans: Strain HC1038: *set-32(ok1457) I; set-25(n5021) III*	This paper	HC1038
C. elegans: Strain HC1039: *set-32(ok1457) I; set-25(n5021) III; qtIs6[Psid-1::gfp] X*	This paper	HC1039
C. elegans: Strain SX922: *prg-1(n4357) I*	Bagijn et al., 2012	SX922
C. elegans: Strain PY2417: *oyIs44[Podr-1::rfp] V*	Lanjuin et al., 2003	PY2417
C. elegans: Strain MT2583: *dpy-11(e224) nDf32 V/eT1(III;V)*	Park and Horvitz, 1986	MT2583
C. elegans: Strain HC992: *prg-1(n4357) I; qtIs6[Psid-1::gfp] X*	This paper	HC992
C. elegans: Ex[Psid-1, pHC183 (myo3::dsRed2)]	This paper	N/A
C. elegans: prg-1(n4357) I; Ex[Psid-1, pHC183 (myo3::dsRed2)]	This paper	N/A
C. elegans: Ex[Psid-1(piRNA-4A), pHC183 (myo3::dsRed2)]	This paper	N/A
E. coli HT115(DE3) with *dpy-11, par-1, pos-1, pha-4, unc-22, act-5, fkh-6* RNAi clones	Kamath and Ahringer, 2003	Source BioScience
Recombinant DNA		
Plasmid: pHC183 (*myo-3::dsRed2*)	Winston et al., 2002	N/A
Plasmid: pHC516 (*Psid-1*(piRNA-4A))	This paper	N/A
Sequence-Based Reagents		
Primers for qRT-PCR; see Table S3	This paper	N/A
Primers for cloning injection constructs; see Table S3	This paper	N/A
Software and Algorithms		
Tophat (v2.0.1)	Trapnell et al., 2012	N/A
Cufflinks (v2.2.1)	Trapnell et al., 2012	N/A
Bowtie2	Langmead et al., 2009	N/A
HTseq	Anders et al., 2015	N/A
edgeR	Robinson et al., 2010	N/A
Other		
Pippin Prep 3% Agarose, dye-free cassette	Sage Science	Cat#CDP3010

CONTACT FOR REAGENT AND RESOURCE SHARING

Please direct any requests for further information or reagents to the Lead Contact, Craig P. Hunter (cphunter@g.harvard.edu), Harvard University.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

C. elegans strains were maintained as previously described (Brenner, 1974). All experiments were performed at 20°C unless otherwise indicated. See Table S2 for strains and alleles.

METHOD DETAILS

Worm synchronization

To obtain synchronized young adult worms, young adults laid embryos for 3 hr. Adults were subsequently removed and staged embryos developed into synchronized young adult worms that were collected 64.5-65.5 hr after embryos were laid. For strains with more varied growth than N2, improperly staged worms were manually removed prior to collection.

RNAi assays

E. coli carrying IPTG-inducible vectors expressing *dpy-11, par-1, pos-1, pha-4, unc-22, act-5* and *fkh-6* dsRNA from the Ahringer RNAi library (Kamath and Ahringer, 2003) were grown for 15-17 hr in LB media with 100μg/mL carbenicillin. Cultures were seeded onto NGM plates containing IPTG and carbenicillin, and left at room temperature for 24 hr before use. Worms placed on RNAi food as embryos were scored as adults. The progeny of L4 larvae placed on RNAi food were continuously on RNAi and scored as adults. To avoid scoring the first progeny that may have received a lesser dose of dsRNA, L4 larvae placed on RNAi food were moved as adults the next day to a new RNAi plate. Only progeny laid for a day on this plate were scored unless otherwise noted. If all worms scored on RNAi were the same phenotype, the number of worms was often estimated to be greater than n worms, and n was used to calculate average number of worms scored. In the two generation assay on *dpy-11* RNAi, mild-Dpy worms were scored as resistant, since the two generation assay is used as an indirect measure for *sid-1* expression in the germline rather than the soma.

Transgenesis

Worms carrying the extrachromosomal and integrated *Psid-1::gfp* arrays were generated previously (Winston et al., 2002). To generate *Psid-1* worms, the *sid*-1 promoter was amplified from N2 genomic DNA (primers: 5′-GGTCATGAGAGGGTCGAGAG-3′, 5′-GGAAAAATGAGGAGTTTTAATTTC-3′) and gel purified (QIAquick Gel Extraction Kit, QIAGEN, 28704). To make complex extrachromosomal array lines, the germline of N2 (wild-type) worms was injected with 0.1-75ng/μl *Psid-1*, 15ng/μl pHC183 (*myo-3::dsRed2*) (Winston et al., 2002) and 25ng/μl DNA ladder (New England Biolabs, N3232S).

The *Psid-1*(piRNA-4A) fragment was amplified from genomic DNA in two pieces using a site directed mutagenesis strategy to introduce the appropriate mutations (See Table S3 for primers) and cloned (pHC516). *Psid-1*(piRNA-4A) fragments were amplified, gel purified and injected at a concentration of 50ng/μl or 1ng/μl into N2 or *prg-1(n4357)* worms with 15ng/μl pHC183 (*myo-3::dsRed2*) (Winston et al., 2002) and 25ng/μl DNA ladder (New England Biolabs, N3232S).

Genetics: maintenance and initiation crosses

To test for a requirement in the maintenance of silencing, *Psid-1::gfp* males were crossed to *mutant* hermaphrodites, resulting in *mutant/mutant; Psid-1::gfp/Psid-1::gfp* F2 or F3 worms. Mixed stage worms were collected for expression measurements, unless otherwise stated. To test for a requirement for *prg-1* in the initiation of silencing, *Psid-1::gfp* hermaphrodites were crossed to *prg-1;prg-1* males. Resulting *prg-1/+; Psid-1::gfp*/0 males were crossed to *prg-1/prg-1* hermaphrodites, and F2 cross progeny carrying the *Psid-1::gfp* array were placed on *dpy-11* RNAi. *prg-1/prg-1* worms were backcrossed prior to starting the experiment due to their mortal germline, and maintained at 15°C. The experiment was performed at 20°C. To test for a requirement for *rrf-3* and *rde-1* in the initiation of silencing, *Psid-1::gfp*/0 males were crossed to *mutant/mutant; Psid-1::gpf/Psid-1::gfp* hermaphrodites. F1 *mutant/+; Psid-1::gfp*/0 males were crossed to *mutant/mutant* hermaphrodites. For *rrf-3,* resulting F2s were placed on *dpy-11* RNAi as L4 larvae and their progeny were scored. F2 *rde-1/rde-1; Psid-1::gfp*/+ cross progeny self-fertilized and resulting *rde-1/rde-1; Psid-1::gfp/Psid-1::gfp* mixed staged worms were collected for RNA extraction and *sid-1* mRNA expression measurements because *rde-1* mutant animals are RNAi deficient. See Table S2 for strains and alleles.

Genetics: paramutation experiments

To test for maternal transmission, non-array hermaphrodites from *Ex[Psid-1::gfp]* parents were crossed to *Is[Podr-1::rfp]* males. To test for paternal transmission, non-array males from *Ex[Psid-1::gfp]* parents were crossed to *Is[Podr-1::rfp]* hermaphrodites. In both crosses, *Pod-1::rfp* was used as a marker to identify cross progeny. *sid-1* expression was measured in resulting single worm cross progeny directly as described below or by singling cross progeny onto *dpy-11* RNAi as described above.

To determine whether modified chromatin at the *sid-1* locus is required for transmission, *dpy-11(e224) nDf32* V/*eT1* (III;V) worms were crossed to N2 males. Resulting males were crossed to *Is[Psid-1::gfp]* worms. Progeny were singled (called "P0" for consistency with Figure 2) and *nDf32*/+*; Psid-1::gfp*/+ worms were identified by F1 phenotype (25% dead progeny, no Unc worms). Singled non-array F1 worms from *nDf32*/+*; Psid-1::gfp*/+ P0 parents were crossed to *Is[Podr-1::rfp]* males. F2 L4 cross progeny were placed on *dpy-11* RNAi and progeny were scored for the Dpy phenotype. Embryonic lethality in F3 was used to determine if F2 worms carried *nDf32* deficiency. See Figure S2D for further details.

Genetics: Non-array silenced and de-silenced worms

To directly compare non-array worms that silence *sid-1* to non-array de-silenced *sid-1* worms, two independent lines were established and maintained three generations after loss of the *Ex[Psid-1::gfp]* array. Synchronized *sid-1* silenced worms were collected

three generations later, at the 6th generation, and synchronized de-silenced *sid-1* worms were collected 18 generations later, at the 21st generation. To determine whether *sid-1* remained effectively silenced at each intervening generation, 20 L4 worms were placed on *dpy-11* RNAi food and their progeny were scored. Resistant worms indicate that *sid-1* is silenced, while Dpy worms indicate that *sid-1* is expressed.

Confirmation that non-array worms do not contain the Psid-1::gfp array

Four observations indicate that non-array worms do not surreptitiously carry a silenced array that maintains silencing. First, since the extrachromosomal array is normally maintained in only a fraction of progeny, to persist in 100% of the progeny for 8-13 generations it would need to be integrated in the genome and then when *sid-1* expression is restored coordinately lost in all or nearly all progeny over the course of 1-3 generations (Figure 1G). Second, while we can detect the array by PCR amplification in *Ex[Psid-1::gpf]* worms, we never detect this array by PCR from wild-type worms or from non-array F1 worms (picked as "non-array" from *Ex[Psid-1::gfp]* parent based on lack of *gfp* and *rol-6D* co-marker). Third, if the silencing was due to an undetected array, then we would expect "silenced non-array" males crossed to wild-type hermaphrodites to result in progeny that silence *sid-1*. However, silenced males cannot transmit the silencing signal (Figures 2A and 2B). Fourth, we detect very abundant promoter associated siRNAs in *Psid-1::gfp* array worms, but do not detect these siRNAs in silenced non-array animals (Figure 3A).

RNA extraction and first strand cDNA synthesis

To purify RNA from mixed stage or synchronized young adult worms, frozen worm pellets were extracted in Trizol/chloroform. The aqueous fraction was precipitated, resuspended in water, DNaseI treated for 1 hr (Roche, 04716728001) and purified (RNeasy Mini Kit, QIAGEN, 74106 or RNA Clean & Concentrator-5, Zymo Research, R1015 if small RNAs were required in downstream applications). To prepare RNA for 5′-independent small RNA sequencing, 5μg of RNA was treated with 5′ polyphosphatase (Epicenter, RP8092H) and re-purified (RNA Clean & Concentrator-5, Zymo Research, R1015).

First strand cDNA was synthesized using ThermoScript RT-PCR System (Invitrogen, 11146-024) with an OligodT primer and 125ng-1μg total RNA. Control cDNA synthesis reactions without the reverse transcriptase enzyme were included for each sample.

cDNA synthesis from single worms

A single adult worm was frozen in 5μl 10 mM Tris-Cl containing 90μg/mL proteinase K at −80°C for at least 10 min. Worms were lysed at 65°C for 10 min, followed by 95°C for 1 min to inactivate proteinase K. 2μl of lysis was used directly in a 20μl OligodT-primed cDNA synthesis reaction with and without reverse transcriptase (control) for measuring mRNA expression (Invitrogen, 11146-024). If measuring pre-mRNA levels, the lysis was treated with DNase I (Roche, 04716728001) for 10 min at 37°C and DNase I was inactivated for 10 min at 75°C prior to cDNA synthesis using a gene specific primer. See Table S3 for primers.

qRT-PCR

Quantitative RT-PCR analysis (QuantiTect SYBR Green PCR Kit, QIAGEN, 204145) was performed on an Eppendorf Mastercycler ep realplex 4. For expression measurements in a population of worms, 1/20th of the cDNA reaction was used in each qRT-PCR. qRT-PCRs were incubated at 95°C for 15 min followed by 35-40 cycles of 94°C 15 s, 50°C 30 s, 72°C 30 s. For single worm expression, 1/10th of the cDNA was used in each qRT-PCR and the extension time (72°C) was decreased to 15 s. qRT-PCR primers were designed and verified to amplify only cDNA and not genomic DNA, which was especially important for single worm expression in which the genomic DNA was not degraded prior to cDNA synthesis. Ct values were determined using noiseband quantification. Error bars represent standard deviation for at least two technical replicates unless otherwise stated. See Table S3 for primers used in qRT-PCR.

mRNA and small RNA library preparation

mRNA sequencing libraries were made from 1μg total RNA from synchronized young adult worms. RNA was PolyA purified using the Apollo 324 NGS Library Prep System with the PrepX PolyA 8 Protocol (Beta v1, Wafergen) and stranded mRNA sequencing libraries were made using the PrepX mRNA 8 Protocol (Beta v1, Wafergen). Resulting libraries were PCR amplified for 15 cycles and purified using the PCR Cleanup 8 Protocol (Apollo 324 NGS Library Prep System). Non-stranded mRNA sequencing libraries were prepared using the TruSeq RNA Library Preparation Kit v2 (Illumina, RS-122-2001). Libraries were fragmented after dsDNA synthesis on the Covaris instrument as described in Alternate Fragmentation Protocol (Illumina, RS-122-2001). Two biological replicates for each genotype were pooled and single end (stranded) or paired end (non-stranded) libraries were sequenced on the Illumina NextSeq 500 Mid output flow cell for 150 cycles.

Small RNA libraries were prepared from 1μg total RNA from synchronized young adult worms (5′ polyphosphatase treated for 5′-independent libraries as described above) using the PrepX Small RNA 8 Protocol on the Apollo 324 NGS Library Prep System. Libraries were amplified for 12 cycles and purified (QIAquick PCR Purification Kit, QIAGEN, 28106). Small RNAs were size selected on the Pippin Prep 3% dye free cassette (Sage Science, CDP3010) by collecting 126-160bp fragments. Size selected RNA libraries were purified and concentrated to 10μl (DNA Clean & Concentrator-5, Zymo Research, D4013). Libraries were pooled and sequenced on the Illumina NextSeq 500 High output flow cell for 75 cycles.

Library data processing

mRNA libraries were aligned to *C. elegans* genome WS235 using Tophat (v2.0.1) (Trapnell et al., 2012) with all parameters set to default except for minimum and maximum intron length (-i 20, -I 10000). Differential expression analysis was performed using Cufflinks (v2.2.1) (Trapnell et al., 2012) with default parameters, allowing a false discovery rate (FDR) of 0.05.

21-26 nucleotide reads were filtered from small RNA libraries and aligned to *C. elegans* genome WS235 using Bowtie2 (Langmead et al., 2009), allowing 0 mismatches (–score-min L,0,0) and up to four alignments per read (-k 4). Small RNA counts per gene were generated using HTseq (Anders et al., 2015) and normalized to total number of 21-26 nucleotide reads mapped to genes. Genes differentially targeted by small RNAs were identified with edgeR (Robinson et al., 2010) using the exact test and allowing an FDR of 0.05.

QUANTIFICATION AND STATISTICAL ANALYSIS

The Mann-Whitney test was used in Figure 4E because the data are independent of each other and the test does not assume a normal distribution. Values and n from Figures 4E and S4E are used in the statistical test, with Slightly Dpy and Dpy values combined. $p < 0.05$ was defined as significant.

Differential expression analysis for mRNA-seq data was performed using Cufflinks (v2.2.1) (Trapnell et al., 2012) with default parameters, allowing a false discovery rate (FDR) of 0.05. Genes differentially targeted by small RNAs were identified with edgeR (Robinson et al., 2010) using the exact test and allowing an FDR of 0.05.

All error bars represent Standard Deviation, as stated in figure legends.

DATA AND SOFTWARE AVAILABILITY

mRNA and small RNA sequence data have been deposited in the Gene Expression Omnibus under GEO: GSE81708.