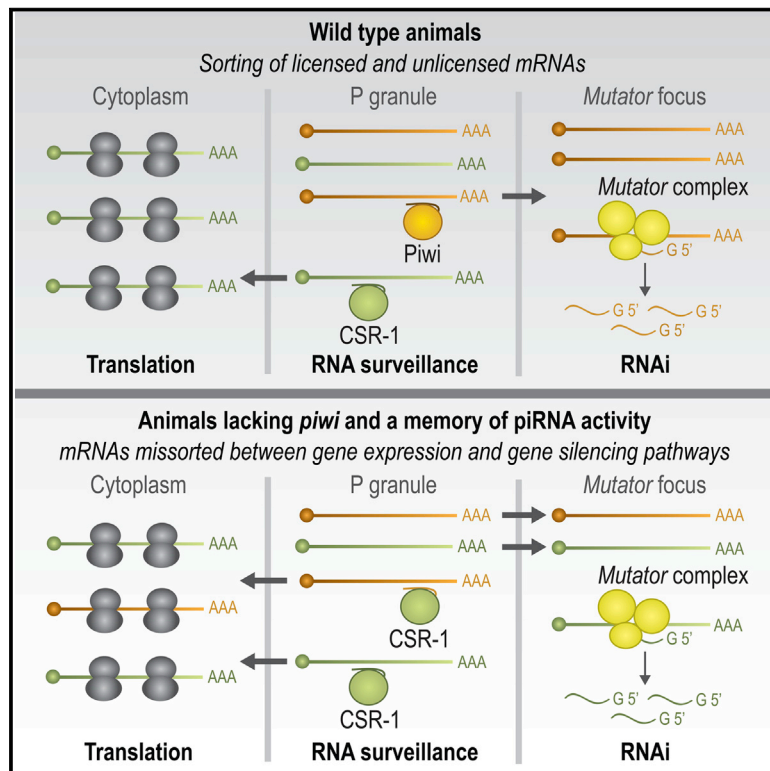


Developmental Cell

piRNAs and piRNA-Dependent siRNAs Protect Conserved and Essential *C. elegans* Genes from Misrouting into the RNAi Pathway

Graphical Abstract



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In Brief

By reanimating endogenous RNAi, Phillips et al. uncover an essential role for piRNAs and piRNA-dependent siRNAs in sorting mRNAs between gene-licensing and gene-silencing pathways. In the absence of piRNAs and a cellular memory of piRNA activity, essential and conserved genes are misrouted into the RNAi pathway.

Highlights

- Reanimating RNAi causes sterility in the absence of maternal piRNAs
- Maternal and paternal siRNAs provide an essential memory of piRNA activity
- Essential genes are targeted for RNAi in the absence of piRNA function
- piRNAs and piRNA-dependent siRNAs are required for proper sorting of mRNAs

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piRNAs and piRNA-Dependent siRNAs Protect Conserved and Essential *C. elegans* Genes from Misrouting into the RNAi Pathway

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SUMMARY

piRNAs silence foreign genes, such as transposons, to preserve genome integrity, but they also target endogenous mRNAs by mechanisms that are poorly understood. *Caenorhabditis elegans* piRNAs interact with both transposon and nontransposon mRNAs to initiate sustained silencing via the RNAi pathway. To assess the dysregulation of gene silencing caused by lack of piRNAs, we restored RNA silencing in RNAi-defective animals in the presence or absence of piRNAs. In the absence of piRNAs and a cellular memory of piRNA activity, essential and conserved genes are misrouted into the RNAi pathway to produce siRNAs that bind the nuclear Argonaute HRDE-1, resulting in dramatic defects in germ cell proliferation and function such that the animals are sterile. Inactivation of RNAi suppresses sterility, indicating that aberrant siRNAs produced in the absence of piRNAs target essential genes for silencing. Thus, by reanimating RNAi, we uncovered a role for piRNAs in protecting essential genes from RNA silencing.

INTRODUCTION

Piwi and its orthologs, the effector proteins that bind Piwi-interacting RNAs (piRNAs), constitute a subclass of Argonautes with essential roles in the proliferation and continuity of the germline (Luteijn and Ketting, 2013). *Caenorhabditis elegans* bearing mutations in the *piwi* ortholog *prg-1* are relatively healthy, and both males and hermaphrodites are fertile under normal growth conditions (Batista et al., 2008; Das et al., 2008; Simon et al., 2014; Wang and Reinke, 2008). *prg-1* is required for the initiation but not for the maintenance of transgenerational silencing of certain transgenes via a nuclear RNA-silencing pathway involving HRDE-1 (Ashe et al., 2012; Bagijn et al., 2012; Buckley et al., 2012; Lee et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). HRDE-1 is one of a set of more than a dozen WAGO clade

Argonautes, a subfamily that is highly diversified and unique to nematodes, that bind 22-nt small interfering RNAs (siRNAs) (WAGO class 22G-RNAs) produced through the secondary siRNA amplification branch of an RNA-silencing pathway involving the *mutator* class genes, including *mut-16*, *mut-14*, and *smut-1* (hereafter referred to as RNAi).

It is possible that the defects in germline development and fertility observed in other animals bearing mutations in *piwi* and its orthologs are not manifested under normal growth conditions in *C. elegans prg-1* mutants due to a transgenerational memory of WAGO class 22G-RNA production and RNA silencing (Ashe et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). This transgenerational memory continues in the absence of piRNAs for multiple generations, but is eventually lost unless an episode of starvation or low insulin-like signaling, which, in a manner not yet fully understood, allows the continued perdurance of normal RNAi without piRNAs (Simon et al., 2014). The eventual sterility of piRNA-deficient animals suggests that in the absence of piRNAs, siRNAs responsive to piRNAs become dysregulated to endogenous mRNA targets, leading to defects in germline function. Here we explore the aberrant siRNA responses that cause sterility in the absence of piRNAs.

RESULTS

piRNAs and Transgenerational RNAi Are Necessary for Fertility

To identify endogenous roles for piRNAs that may normally be masked by endogenous transgenerational RNAi, we developed a genetics-based approach to reset the cellular memory of RNA silencing. We crossed two strains bearing distinct homozygous recessive loss-of-function mutations (a *mut-14*^{-/-} *smut-1*^{-/-} double mutant and a *mut-16*^{-/-} mutant) that each cause severe defects in endogenous RNAi and the loss of associated siRNAs, as well as insensitivity to exogenous double-stranded RNAs, in order to reanimate RNAi in the heterozygous F1 progeny (*mut-16*^{+/-}; *mut-14*^{+/-} *smut-1*^{+/-}) (Phillips et al., 2014; Zhang et al., 2011). Both of the parental strains are relatively healthy except when grown at elevated temperatures. In parallel, we performed the same cross but with either or both parents also containing a mutation in *prg-1*, strains that are

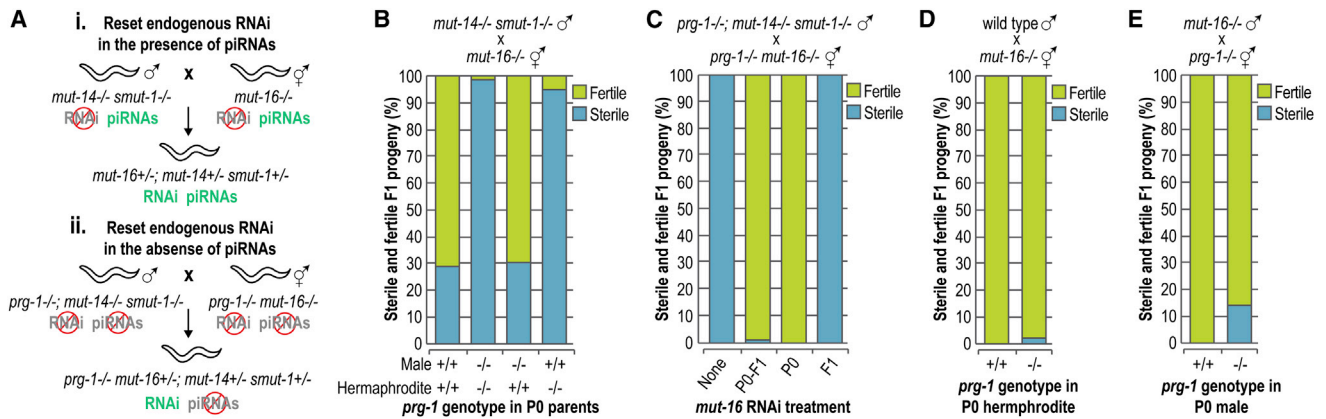


Figure 1. Resetting Endogenous RNAi in the Absence of piRNAs Causes Sterility

(A) Schematic illustrating a mating-based approach used to reset endogenous RNAi.

(B) The proportions of fertile and sterile animals after resetting RNAi in the presence or absence of *piwi/prg-1* and associated piRNAs.

(C) The proportions of fertile and sterile animals after resetting RNAi in the absence of piRNAs while treating with *mut-16* RNAi to prevent reactivation of the RNAi pathway. P0–F1, RNAi treatment began during the parental L4 larval stage and continued through the F1 generation. P0, RNAi treatment began during the parental L4 larval stage and then the F1 progeny were transferred as eggs off of RNAi. F1, RNAi treatment began in the F1 generation during the L1 larval stage.

(D) The proportions of fertile and sterile F1 animals after crossing wild-type males to *mut-16^{-/-}* or *prg-1^{-/-} mut-16^{-/-}* hermaphrodites.

(E) The proportions of fertile and sterile F1 animals after crossing *mut-16^{-/-}* or *prg-1^{-/-} mut-16^{-/-}* males to *prg-1^{-/-}* hermaphrodites.

See also Figure S1.

also relatively healthy despite lacking both piRNAs and WAGO class 22G-RNAs, in order to reset RNAi in the absence of piRNAs (Figure 1A). Thus, by first erasing all memory of piRNAs and RNAi and then restoring the RNAi pathway in the absence of piRNAs and an siRNA-based memory of piRNA function, we were able to assess the roles of piRNAs in transgenerational RNA silencing.

When both parents were homozygous mutant for *prg-1*, and thus lacked piRNAs, ~99% of the F1 progeny (*prg-1^{-/-} mut-16^{+/-}; mut-14^{+/-} smut-1^{+/-}*) in which the RNAi pathway was reactivated were sterile (Figure 1B). In contrast, only ~29% of F1 progeny (*prg-1^{+/+} mut-16^{+/-}; mut-14^{+/-} smut-1^{+/-}*) in which RNAi was reactivated were sterile when both parents were homozygous wild-type for *prg-1*, and thus contained piRNAs (Figure 1B). Although rare, fertile F1 animals in which RNAi had been reset in the absence of piRNAs produced F2 progeny that were either sterile or homozygous mutant for *mut-14* and *smut-1*, or *mut-16*, and were therefore RNAi defective (Figure S1A).

To confirm that a background mutation in one of the strains is not responsible for the sterility caused by resetting RNAi in the absence of piRNAs, we again crossed *prg-1^{-/-}; mut-14^{-/-} smut-1^{-/-}* males to *prg-1^{-/-} mut-16^{-/-}* hermaphrodites. However, to prevent efficient restoration of RNA silencing in the F1 progeny, we treated the P0 and F1 animals with *mut-16* RNAi (Figure 1C). Because both parental strains are RNAi defective, the RNAi treatment presumably takes effect in the F1 progeny after the paternal wild-type *mut-16* gene is expressed, which likely occurs during embryogenesis. Nearly 100% of F1 animals were fertile when treated with *mut-16* RNAi, whereas ~100% of F1 animals treated with a mock RNAi control were sterile (Figure 1C). To prevent sterility, *mut-16* RNAi treatment was only required in the P0 parental generation, indicating that double-stranded *mut-16* RNA and/or the primary siRNAs derived from it that mediate *mut-16* inactivation are deposited in the embryos, which contain a full complement of RNAi factors (Figure 1C). In contrast, F1 an-

imals treated with *mut-16* RNAi as L1 larvae while resetting RNAi in the absence of piRNAs were sterile (Figure 1C). These results indicate that reactivating RNAi at an early stage in germline development, prior to germ cell proliferation, which begins during the mid-L1 larval stage, causes sterility in the absence of piRNAs or piRNA-dependent siRNAs.

To determine whether the sterility caused by resetting RNAi in the absence of piRNAs is correlated with the severity of WAGO class 22G-RNA depletion prior to restoring RNAi, we crossed homozygous mutant *mut-14 smut-1* animals to RNAi-defective animals with either a very severe (*mut-16^{-/-}*), moderately severe (*mut-7^{-/-}*), or intermediate (*mut-15^{-/-}*) loss of WAGO class 22G-RNAs in the absence of piRNAs (*prg-1^{-/-}*) (Phillips et al., 2014). Similar to control crosses between *prg-1^{-/-}; mut-14^{-/-} smut-1^{-/-}* and *prg-1^{-/-} mut-16^{-/-}*, nearly 100% of the F1 progeny of crosses between *prg-1^{-/-}; mut-14^{-/-} smut-1^{-/-}* and *prg-1^{-/-}; mut-7^{-/-}* were sterile (Figure S1B). In contrast, only ~23% of the F1 progeny from crosses between *prg-1^{-/-}; mut-14^{-/-} smut-1^{-/-}* and *prg-1^{-/-}; mut-15^{-/-}* were sterile (Figure S1B). Thus, a near-complete lack of WAGO class 22G-RNAs in the parental strains is necessary in order to restore RNAi in the absence of piRNAs to cause sterility.

Maternal piRNAs Are Required for Fertility after Reanimating Endogenous RNAi

To test whether a maternal or paternal contribution of piRNAs is required for fertility after resetting RNAi, we again crossed the *mut-14^{-/-} smut-1^{-/-}* double mutant to the *mut-16^{-/-}* mutant but with only one parent containing a mutation in *prg-1*. If the hermaphrodite was mutant for *prg-1* and the male was wild-type for *prg-1*, ~95% of animals were sterile, whereas if the male was mutant for *prg-1* and the hermaphrodite was wild-type for *prg-1*, only ~30% of the F1 progeny were sterile despite the progeny of the two distinct crosses having the same genotype (*prg-1^{+/-}*

mut-16^{+/-}; *mut-14*^{+/-} *smut-1*^{+/-}) (Figure 1B). Thus, a maternal contribution of piRNAs is required for fertility after resetting RNAi.

Both Maternal and Paternal WAGO Class siRNAs Provide a Cellular Memory of piRNA Activity

To determine whether paternal siRNAs can provide the cellular memory of piRNAs necessary for fertility, we crossed an RNAi-competent male (wild-type) to an RNAi-defective hermaphrodite (*mut-16*^{-/-} or *prg-1*^{-/-} *mut-16*^{-/-}). Nearly 100% of the F1 progeny were fertile regardless of whether the hermaphrodite was wild-type or mutant for *prg-1*, indicating that paternally deposited WAGO class 22G-RNAs are sufficient for F1 fertility (Figure 1D). These paternally deposited siRNAs are not dependent on *alg-3* and *alg-4*, Argonautes that function during spermatogenesis to affect a transgenerational memory of germline gene expression (Figure S1C) (Conine et al., 2013). We then tested whether maternal siRNAs could also prevent sterility by crossing an RNAi-defective male (*mut-16*^{-/-} or *prg-1*^{-/-} *mut-16*^{-/-}) to an RNAi-competent hermaphrodite (*prg-1*^{-/-}). *prg-1* mutant hermaphrodites were used instead of wild-type hermaphrodites, because piRNAs deposited in the F1 progeny via the hermaphrodite are themselves sufficient to rescue fertility (Figure 1B). Approximately 100% of the F1 progeny from *mut-16*^{-/-} males crossed to *prg-1*^{-/-} hermaphrodites and 86% of the F1 progeny from *prg-1*^{-/-} *mut-16*^{-/-} males crossed to *prg-1*^{-/-} hermaphrodites, which each received only a maternal complement of WAGO class 22G-RNAs, were also fertile (Figure 1E). Thus, a maternal contribution of WAGO class 22G-RNAs is also sufficient to promote fertility in animals receiving a new complement of RNAi factors.

Continuity of the Germline Is Dependent on piRNAs and Transgenerational RNAi

Animals in which RNAi was reset in the absence of piRNAs displayed dramatic defects in germline proliferation and progression through meiosis, whereas the germlines of animals in which RNAi was reset in the presence of piRNAs developed normally (Figures 2A and 2B). Many animals in which RNAi was reset in the absence of piRNAs had germlines with few or no meiotic nuclei, as indicated by chromosome morphology and localization of the synaptonemal complex component HTP-3 (Figures 2A and 2B) (MacQueen et al., 2005). Germ cells that did enter meiosis rarely reached the pachytene stage (Figure 2A). In more severe cases, entire regions of the germline contained only somatic sheath cells, suggesting either a defect in germ cell proliferation or increased levels of germ cell apoptosis (Figure 2B). It is possible that germ cell apoptosis ensues as a result of increased DNA damage, as has been observed after inactivating *piwi* orthologs in other species (Carmell et al., 2007; Kuramochi-Miyagawa et al., 2004). Thus, we tested whether RNAi knockdown of key components of the DNA damage-checkpoint and apoptosis pathways could rescue the sterility caused by resetting RNAi in the absence of piRNAs. None of ten gene inactivations in the DNA damage and apoptosis pathways suppressed sterility after resetting RNAi in the absence of piRNAs (Figure S1D). These results indicate that, rather than protecting against DNA damage-induced apoptosis, piRNAs and piRNA-triggered transgenerational RNAi are required for the proliferation and progression of the *C. elegans* germline.

P granules, sites of RNA processing that lie adjacent to nuclear pores on the cytoplasmic face of the nuclear envelope of germ cells, contain several factors in the piRNA and siRNA pathways, including Piwi/PRG-1 and the gene-licensing Argonaute CSR-1 (Batista et al., 2008; Claycomb et al., 2009; Conine et al., 2010; Gu et al., 2009; Seth et al., 2013; Wang and Reinke, 2008; Wedeles et al., 2013). After resetting RNAi in the presence of piRNAs, the P granule component PGL-1 localized normally to the nuclear periphery of germ cells (Figure 2C). In contrast, in animals in which RNAi was reset in the absence of piRNAs, PGL-1 often failed to localize to the nuclear periphery and was instead distributed diffusely or in cytoplasmic aggregates, indicating that piRNAs or piRNA-dependent siRNAs are required for normal P granule assembly and integrity (Figure 2C).

Genome-wide Analysis of siRNAs after Reanimating Endogenous RNAi

To determine whether siRNA production is affected by reintroducing the RNAi machinery, we sequenced small RNAs from animals in which RNAi was reset in the presence or absence of piRNAs. In addition to WAGO class 22G-RNAs, *C. elegans* contains a distinct class of endogenous 22-nt-long germline siRNAs that are not dependent on *mutator* class genes, such as *mut-16*, *mut-14*, and *smut-1*, and bind to the Argonaute CSR-1 (Claycomb et al., 2009; Gu et al., 2009; Phillips et al., 2014; Zhang et al., 2011). CSR-1 class 22G-RNAs license many *C. elegans* genes for expression in the germline to protect, by a not yet understood mechanism, their transcripts from piRNAs and RNAi (Conine et al., 2013; Seth et al., 2013; Wedeles et al., 2013). Resetting RNAi in the absence of piRNAs caused an ~85% reduction in WAGO class 22G-RNA levels, relative to control crosses between wild-type animals (Figure S2A). We also observed an ~79% reduction in CSR-1 class 22G-RNA levels after resetting RNAi in the absence of piRNAs (Figure S2A).

Germline-less mutants are depleted of 22G-RNAs (Gu et al., 2009), and therefore the loss of both CSR-1 and WAGO class siRNAs is likely due, at least in part, to the diminished germlines caused by restoring RNAi in the absence of piRNAs (Figure 2). To control for the number of germ cells in each strain, we normalized siRNA levels to the germline-derived microRNA (miRNA) miR-35 (Figure S2A) (Wu et al., 2010). After normalizing to miR-35 levels, the majority of WAGO targets were depleted of siRNAs in the progeny of a control cross between *prg-1* mutants relative to a cross between wild-type animals, consistent with a requirement for piRNAs in directing mRNAs into the RNAi pathway (Figure 3A) (Bagijn et al., 2012; Lee et al., 2012). In animals in which we reset RNAi in the presence of piRNAs, siRNA production from WAGO targets returned to near normal, although most targets yielded slightly lower levels of siRNAs, possibly because siRNA production takes more than one generation to reach its full capacity after resetting RNAi (Figure 3B). In animals in which we reset RNAi in the absence of piRNAs, the vast majority of WAGO targets yielded reduced levels of siRNAs; however, the reduction in siRNA levels was not as severe as what was observed in the progeny of the control cross between *prg-1* mutants (Figures 3A and 3C).

After resetting RNAi in the presence of piRNAs, transposon siRNA levels returned to near wild-type levels (Figure S2B; Table S1). In animals in which RNAi was reset in the absence

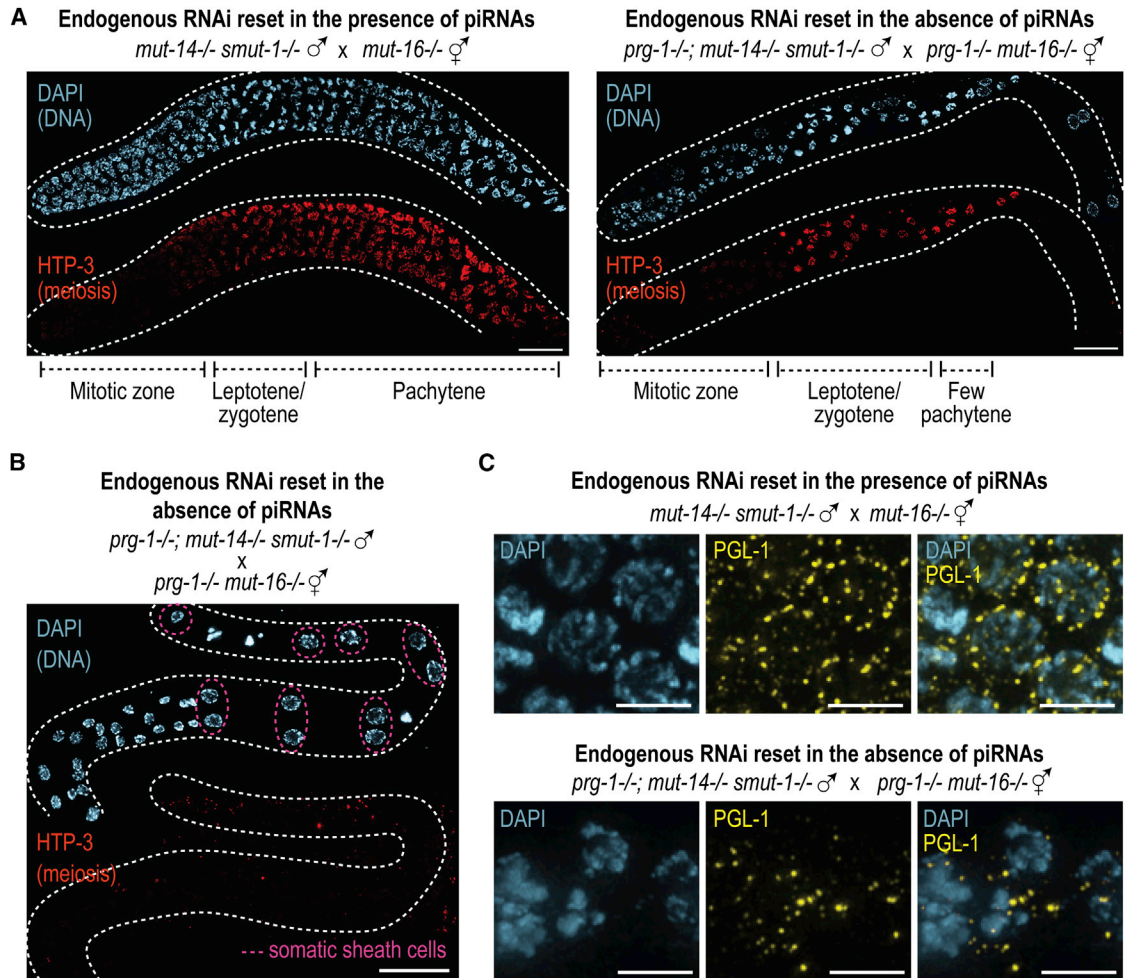


Figure 2. Resetting Endogenous RNAi in the Absence of piRNAs Disrupts Germline Development

(A and B) Germlines from adult animals showing modest (A) or severe (B) defects in germline proliferation and progression after RNAi was reset in the absence of piRNAs. Animals were dissected and immunostained for the synaptonemal complex component HTP-3, indicative of meiotic entry. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). Scale bars represent 20 μ m.

(C) Localization of the P granule component PGL-1 after resetting RNAi in the presence or absence of piRNAs. Scale bars represent 5 μ m. All images are projections of 3D images following deconvolution.

of piRNAs, many transposons yielded reduced levels of siRNAs, but many were unaffected or yielded elevated levels of siRNAs (Figure S2C; Table S1). Only 20 of the 93 transposon families analyzed were depleted of siRNAs by >3-fold after correcting for the number of germ cells by normalization to miR-35, the majority of which were also depleted of siRNAs in the progeny of the control cross between *prg-1* mutants (Figures S2C and S2D; Table S1). It is possible that defects in transposon silencing contribute to the sterility that we observe after resetting RNAi in the absence of piRNAs. However, that we do not observe gross defects in transposon siRNA production and that RNAi knock-down of genes in the DNA damage and apoptosis pathways does not improve fertility (Figure S1D) suggest that transposon desilencing is not the primary cause of sterility.

siRNA production from CSR-1 targets was largely unaffected in progeny from the *prg-1* control cross and in animals in which RNAi was reset in the presence of piRNAs, after correcting for the number of germ cells by miR-35 normalization (Figures 3A

and 3B). In animals in which RNAi was reset in the absence of piRNAs, CSR-1 targets were moderately affected, with some displaying elevated levels of siRNAs and others displaying reduced levels (Figure 3C). Together, these results suggest that resetting RNAi in the absence of piRNAs alters the balance of CSR-1 and WAGO class siRNAs.

HRDE-1 and the Nuclear RNAi Pathway Induce Sterility in the Absence of piRNA Function

HRDE-1 is one of several WAGO Argonautes that bind RNAi-associated 22G-RNAs in the germline. Unlike other WAGO Argonautes, HRDE-1 localizes to the nucleus to affect transcriptional gene silencing and transgenerational RNAi (Ashe et al., 2012; Buckley et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). Because our results show that piRNA-triggered heritable RNA silencing is necessary for fertility, we tested whether *hrde-1* knockdown would suppress the sterility caused by resetting RNAi in the absence of piRNAs. Inactivation of *hrde-1* while

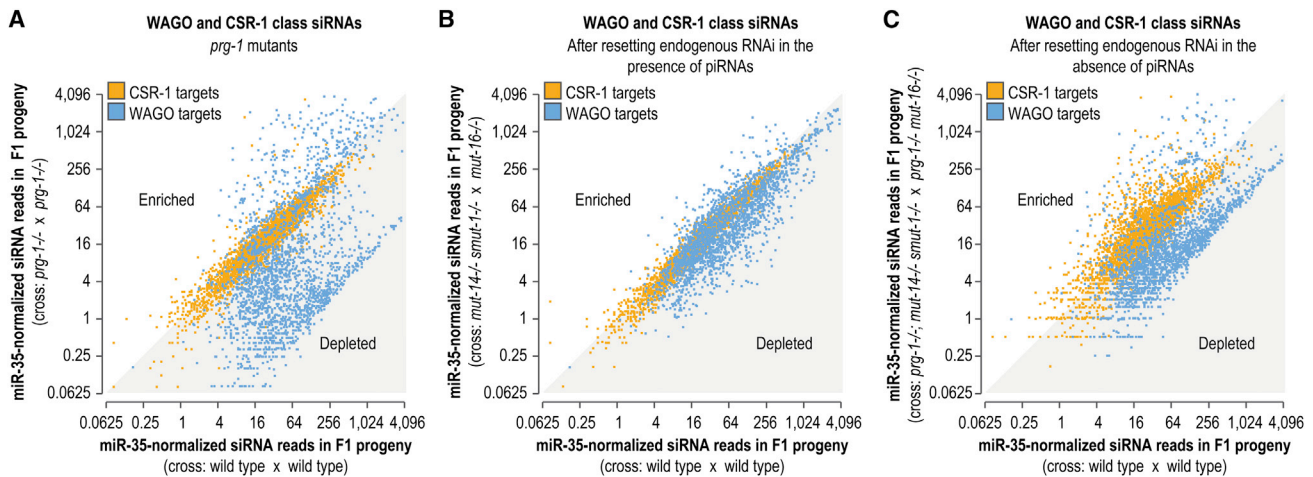


Figure 3. High-Throughput Sequencing of Small RNAs after Resetting Endogenous RNAi

Each CSR-1 and WAGO target is represented as the number of miR-35-normalized siRNA reads (reads per 10,000 miR-35 reads) to account for differences in the numbers of germ cells between strains.

(A) siRNA levels in the F1 progeny of *prg-1* males crossed to *prg-1* hermaphrodites (y axis) and in the F1 progeny of a control cross between wild-type animals (x axis).

(B) siRNA levels in the F1 progeny of animals in which RNAi was reset in the presence of piRNAs (y axis) and in the F1 progeny of a control cross between wild-type animals (x axis).

(C) siRNA levels in the F1 progeny of animals in which RNAi was reset in the absence of piRNAs (y axis) and in the F1 progeny of a control cross between wild-type animals (x axis).

See also [Figure S2](#) and [Table S1](#).

resetting RNAi in the absence of piRNAs rescued fertility by ~52% ([Figure 4A](#); [Figure S1D](#)). This suggests that HRDE-1 acts in the genetic pathway that causes sterility in the absence of piRNAs and piRNA-triggered transgenerational RNA silencing.

HRDE-1 Binds siRNAs from CSR-1 Targets in the Absence of piRNA Function

Disruption of the RNAi pathway, the piRNA pathway, or both pathways simultaneously does not cause sterility under standard *C. elegans* growth conditions. We hypothesized that the sterility that ensues when RNAi is reset in the absence of piRNAs is due to misdirection of mRNAs derived from endogenous essential genes, which are not normally subjected to RNA silencing, into the RNAi pathway. The majority, if not all, of core cellular and therefore essential germline genes produce siRNAs that normally bind to CSR-1 to license gene expression and do not bind to WAGOs, such as HRDE-1, which would instead mediate silencing of these mRNAs ([Claycomb et al., 2009](#)). Despite different genetic requirements, CSR-1 class 22G-RNAs cannot be distinguished on the basis of siRNA sequence alone from WAGO class 22G-RNAs. Therefore, it was not possible to distinguish gene-licensing siRNAs from gene-silencing siRNAs using the small RNA sequencing datasets from animals in which we reset RNAi in the presence or absence of piRNAs. However, if our hypothesis that essential genes are misrouted into the RNA-silencing pathway is correct, genes that are normally targeted by CSR-1 but not by the RNAi pathway should now produce siRNAs via the RNAi pathway that associate with HRDE-1. To test this, we sequenced small RNAs bound to FLAG::HRDE-1 after resetting RNAi in the presence or absence of piRNAs ([Figure 4B](#); [Figure S3A](#)). In addition to crosses in which we reset

RNAi using P0 male and hermaphrodite combinations that were either both wild-type or both homozygous mutant for *prg-1*, we set up a third cross in which the male was homozygous mutant for *prg-1* and the hermaphrodite was homozygous wild-type for *prg-1* (male *prg-1*^{-/-}; FLAG::*hrde-1*^{+/+}; *mut-14*^{-/-} *smut-1*^{-/-} crossed to hermaphrodite *mut-16*^{-/-}) ([Figure 4B](#)). This allowed us to select F2 animals in which RNAi was reset in the presence of piRNAs that had the same F2 genotype as animals in which RNAi was reset in the absence of piRNAs (*prg-1*^{-/-}; FLAG::*hrde-1*^{+/+}) ([Figure 4B](#)). To prevent sterility in animals in which RNAi was reset in the absence of piRNAs, we treated the P0 animals and their progeny with *mut-16* RNAi to inhibit reestablishment of the RNAi pathway while we set up the cross, selected for specific genotypes, and expanded populations for protein-RNA immunoprecipitation (IP) experiments. These animals were then removed from *mut-16* RNAi for multiple generations prior to FLAG::HRDE-1 IP, during which time they showed increased levels of sterility indicative of loss of *mut-16* knockdown and reestablishment of RNAi.

FLAG::HRDE-1 IPs from animals in which RNAi was reset in the presence of piRNAs, regardless of whether they were wild-type or mutant for *prg-1*, were enriched for siRNAs produced from the majority of WAGO targets and depleted for siRNAs normally produced from CSR-1 targets ([Figures 4C](#) and [4D](#); [Table S2](#), tabs 1 and 2). In contrast, FLAG::HRDE-1-bound small RNAs from animals in which RNAi was reset in the absence of piRNAs were enriched for siRNAs produced from the majority of both WAGO and CSR-1 targets ([Figure 4E](#); [Table S2](#), tab 3). Total CSR-1 class 22G-RNA levels were slightly depleted in FLAG::HRDE-1 IPs, relative to the input, from animals in which RNAi was reset in the presence of piRNAs but enriched ~5-fold in the FLAG::HRDE-1 IP from

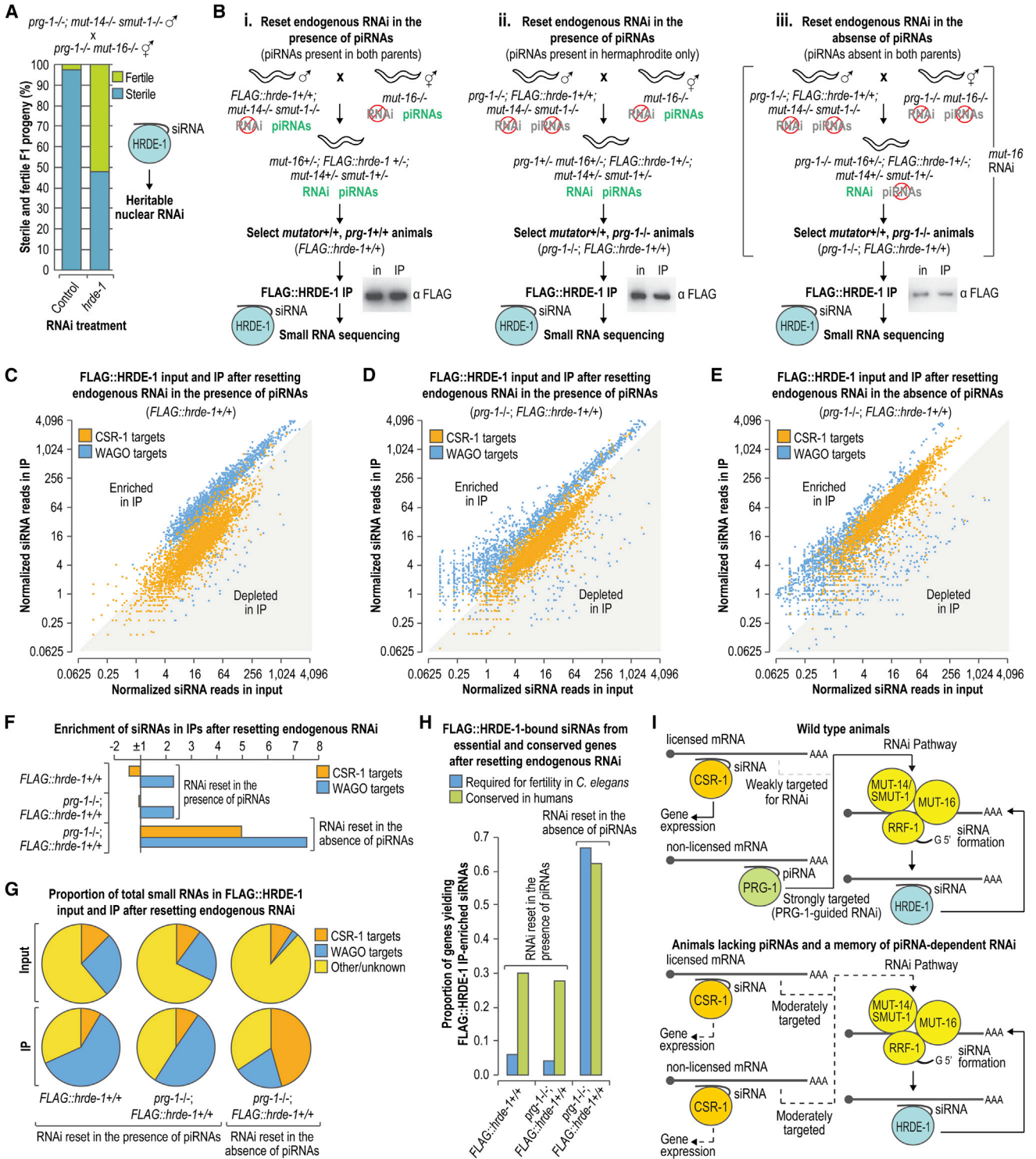


Figure 4. The Nuclear Argonaute HRDE-1 Binds siRNAs from Essential Genes after Resetting Endogenous RNAi in the Absence of piRNAs

(A) The proportions of fertile and sterile animals after resetting RNAi in the absence of piRNAs while treating with *hrde-1* RNAi or a mock RNAi control. Animals were treated with RNAi starting in the parental L4 larval stage and continuing through the F1 generation.

(B) Schematic illustrating the approach used to reset endogenous RNAi and then immunoprecipitate FLAG::HRDE-1 and sequence the associated small RNAs. Animals of the indicated genotype were selected for FLAG::HRDE-1 IP and small RNA sequencing. Animals in which RNAi was reset in the absence of piRNAs were treated with *mut-16* RNAi to prevent efficient reactivation of RNAi while they were genotyped and expanded. The western blots display FLAG::HRDE-1 protein in the input (in) and IP.

(legend continued on next page)

animals in which RNAi was reset in the absence of piRNAs (Figure 4F). WAGO class 22G-RNAs were enriched in FLAG::HRDE-1 IPs regardless of whether RNAi was reset in the presence or absence of piRNAs (Figure 4F).

Animals in which RNAi was reset in the absence of piRNAs had similar levels of CSR-1 class 22G-RNAs and reduced levels of WAGO class 22G-RNAs in the input fractions relative to animals in which RNAi was reset in the presence of piRNAs (Figure 4G). Because *mut-16* was inactivated while resetting RNAi in the absence of piRNAs in order to prevent sterility, populations from subsequent generations (F5+) used in these experiments contained a mixture of fertile and sterile animals due to the transgenerational persistence of exogenous RNAi and thus failure to completely restore RNA silencing. These animals did not display the dramatic loss of siRNAs from CSR-1 targets that we observed after resetting RNAi in the absence of piRNAs without *mut-16* inactivation, possibly because their germlines tended to be healthier due to incomplete reactivation of RNAi (Figure 4G; Figure S2A).

The majority of small RNAs bound by FLAG::HRDE-1 after resetting RNAi in the presence of piRNAs were WAGO class 22G-RNAs (Figure 4G). In contrast, after RNAi was reset in the absence of piRNAs, siRNAs from CSR-1 targets outnumbered siRNAs from WAGO targets in the FLAG::HRDE-1 IP by greater than 2-fold (Figure 4G). After resetting RNAi in the absence of piRNAs, FLAG::HRDE-1 continued to localize properly to the nucleus, suggesting that it maintained its normal function in transcriptional gene silencing (Figure S3B). Together, these results indicate that CSR-1 targets are misrouted into the RNAi pathway to produce siRNAs that bind HRDE-1 to affect nuclear RNA silencing in the absence of piRNAs and a memory of piRNA activity.

Essential and Conserved Genes Are Targeted for RNAi in the Absence of piRNA Function

There are approximately 1,200 gene inactivations, about 6% of all genes, that cause sterility in *C. elegans* (WormBase release WS220), the majority of which are CSR-1 targets (Figure S3C). Of these ~1,200 genes essential for fertility, ~67% produced siRNAs enriched in the FLAG::HRDE-1 IP after resetting RNAi in the absence of piRNAs (Figure 4H). In contrast, only ~5% produced siRNAs enriched in FLAG::HRDE-1 IPs after resetting RNAi in the presence of piRNAs (Figure 4H). Approximately 62% of the 7,663 *C. elegans* genes that have a likely ortholog in humans (Shaye and Greenwald, 2011), many of which are also CSR-1 targets (Figure S3C), produced siRNAs enriched in the FLAG::HRDE-1 IP from animals in which RNAi was reset in the absence of piRNAs (Figure 4H). This is twice the representation of these genes that we observed in FLAG::HRDE-1 IPs after resetting RNAi in the presence of piRNAs (Figure 4H).

To determine whether essential and conserved genes are downregulated after resetting RNAi in the absence of piRNAs, we did qPCR on pools of animals in which RNAi was reset in the presence or absence of piRNAs. In animals in which RNAi was reset in the absence of piRNAs, we observed a substantial reduction in the abundance of each of the six CSR-1 targets analyzed, each of which is essential and/or conserved, relative to animals in which RNAi was reset in the presence of piRNAs (Figure S3D). In contrast, the piRNA target *bath-20* was upregulated after resetting RNAi in the absence of piRNAs (Figure S3D). Every germline gene that we examined is targeted by either WAGO or CSR-1, and thus in our qPCR assay we were unable to control for the reduced number of germ cells caused by resetting RNAi in the absence of piRNAs. Therefore, it is possible that the substantial reduction in CSR-1 target mRNA levels that we observe might also reflect the loss of germ cells in these animals.

Several lines of evidence suggest that essential genes are subjected to RNA silencing after RNAi is reestablished in the absence of piRNAs: (1) sterility and germline proliferation defects point to reduced levels or impaired function of essential genes; (2) sterility is rescued by inactivating genes involved in RNA silencing at both the siRNA production step (*mutator* class genes) and the effector step (*hrde-1*); and (3) siRNAs from essential genes bind to HRDE-1, which indicates that these genes are routed into the RNAi pathway.

CSR-1 Binds siRNAs from WAGO Targets in the Absence of piRNA Function

Misrouting of CSR-1 targets into the RNAi pathway could result from reduced competition between CSR-1 and the RNA-silencing Argonautes, such as PRG-1 and HRDE-1. In wild-type animals, CSR-1 localizes to P granules at the nuclear periphery (Claycomb et al., 2009); however, after RNAi was reset in the absence of piRNAs, CSR-1 was diffusely cytoplasmic or formed cytoplasmic aggregates, although CSR-1 foci also still formed at the nuclear periphery of some germ cells (Figure S3E). We examined small RNAs bound to CSR-1 after resetting RNAi in the absence of piRNAs by subjecting GFP::CSR-1-associated small RNAs to high-throughput sequencing (Figure S3F). As in the FLAG::HRDE-1 IP from animals in which RNAi was reset in the absence of piRNAs, *mut-16* was inactivated by RNAi to prevent efficient reestablishment of RNA silencing and subsequent sterility during the cross and while expanding populations for IP (Figure S3F). GFP::CSR-1 IP was enriched for siRNAs produced from the vast majority of both CSR-1 and WAGO targets (Figure S3G). Of 53 genes containing high-confidence piRNA target sites (Bagijn et al., 2012), all but one had elevated levels of siRNAs, and in total these piRNA targets produced siRNAs that were enriched nearly 6-fold in the GFP::CSR-1 IP, relative to

(C–E) Each CSR-1 and WAGO target is represented as the number of normalized siRNA reads in FLAG::HRDE-1 input (x axis) and IP (y axis) fractions after resetting RNAi in the presence (C and D) or absence (E) of piRNAs.

(F) Enrichment or depletion of CSR-1 and WAGO class 22G-RNAs in FLAG::HRDE-1 IPs relative to the corresponding input fractions after resetting RNAi.

(G) The pie charts display the proportions of total small RNA reads in FLAG::HRDE-1 input and IP fractions after resetting RNAi.

(H) The proportions of conserved genes and genes required for fertility that produced siRNAs enriched by >2-fold in FLAG::HRDE-1 IPs relative to input fractions after resetting RNAi.

(I) Model depicting the role of Piwi/PRG-1 in directing the proper mRNAs into the RNAi pathway.

See also Figure S3 and Table S2.

the cell lysate (Figure S3H; Table S2, tab 4). In contrast, siRNAs produced from a distinct RNAi-related pathway involving the Argonaute ERGO-1, which are abundant in oocytes and somatic cells, were not substantially enriched, and miRNAs were strongly depleted (Figure S3H).

As with the FLAG::HRDE-1 IP in which RNAi was reset in the absence of piRNAs, GFP::CSR-1 was immunoprecipitated from a mixed population of animals in which there was variable reactivation of RNAi due to the persistence of *mut-16* RNAi. Thus, it is possible that the enrichment we observed in siRNAs produced from CSR-1 targets in the GFP::CSR-1 IP was an artifact caused by incomplete reactivation of RNAi in some animals. Nonetheless, the enrichment of siRNAs from CSR-1 targets in the FLAG::HRDE-1 IP and the enrichment of siRNAs from WAGO targets in the GFP::CSR-1 IP after resetting RNAi in the absence of piRNAs point to a defect in mRNA sorting between the CSR-1 and WAGO pathways. Thus, we conclude that in the absence of piRNAs to guide RNAi, the cellular machinery can no longer distinguish beneficial and harmful genes, ultimately leading to missorting of mRNAs between gene-licensing and gene-silencing pathways (Figure 4I).

DISCUSSION

It is possible that the mortal germline phenotype of *prg-1* mutants following multiple generations without starvation is a consequence of two distinct events that occur as the memory of piRNA activity is slowly lost over time: (1) upregulation of transposons and repetitive elements, as proposed by Simon et al. (2014), possibly due to misrouting into the CSR-1 pathway for gene licensing; and (2) downregulation of essential genes due to misrouting into the RNAi pathway for gene silencing. Misrouting of essential genes into the RNAi pathway is somewhat stochastic, which may explain why we observe a range of germline defects after resetting RNAi in the absence of piRNAs (de Albuquerque et al., 2015, in this issue of *Developmental Cell*). Despite mechanistic differences between piRNA pathways in *C. elegans* and other animals, our results suggest that they ultimately serve a similar essential function in preserving germ cells from one generation to the next.

EXPERIMENTAL PROCEDURES

C. elegans strains were cultured at 20°C. A list of strains used in the study is available in Supplemental Experimental Procedures. Fertility of the progeny from genetic crosses was determined by the presence of embryos in the uterus in adult animals 2 days after the L4 stage. Germlines were dissected, fixed in methanol, and immunostained with HTP-3 (MacQueen et al., 2005), PGL-1 (Strome and Wood, 1983), GFP (Life Technologies; A11122), or FLAG (Sigma; F1804) antibodies, as described (Phillips et al., 2009). FLAG::HRDE-1, GFP::CSR-1, and associated small RNAs were captured from adult animals 68 hr post L1 synchronization by coimmunoprecipitation with FLAG (Sigma; F1804) or GFP (Life Technologies; A11120) antibodies, followed by protein A agarose beads (Roche; 11134515001). Western blot assays were done using FLAG (Sigma; F1804) or GFP (Pierce; MA5-15256-HRP) antibodies. Total RNA from cell lysates or that copurified with FLAG::HRDE-1 or GFP::CSR-1 was extracted using TRIzol (Life Technologies) and chloroform and precipitated in isopropanol. RNAs (18- to 30-nt) were size selected on denaturing 17% polyacrylamide gels, and small RNA libraries were prepared as described (Montgomery et al., 2012). High-throughput sequencing was done using an Illumina HiSeq 2000. Sequences were parsed from adapters

and mapped to the *C. elegans* genome, WS230, using CASHX v. 2.3 (Fahlgren et al., 2009). Data analysis was done using R, Excel, and custom Perl scripts. Full methods are available in Supplemental Experimental Procedures.

ACCESSION NUMBERS

The accession number for the high-throughput sequencing data reported in this paper is GEO: GSE63114.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.devcel.2015.07.009>.

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