Lack of pairing during meiosis triggers multigenerational transgene silencing in *Caenorhabditis elegans*

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Single-copy transgenes in *Caenorhabditis elegans* can be subjected to a potent, irreversible silencing process termed small RNA-induced epigenetic silencing (RNAe). RNAe is promoted by the Piwi Argonaute protein PRG-1 and associated Piwi-interacting RNAs (piRNAs), as well as by proteins that promote and respond to secondary small interfering RNA (siRNA) production. Here we define a related siRNA-mediated silencing process, termed "multigenerational RNAe," which can occur for transgenes that are maintained in a hemizygous state for several generations. We found that transgenes that contain either GFP or mCherry epitope tags can be silenced via multigenerational RNAe, whereas a transgene that possesses GFP and a perfect piRNA target site can be rapidly and permanently silenced via RNAe. Although previous studies have shown that PRG-1 is typically dispensable for maintenance of RNAe, we found that both initiation and maintenance of multigenerational RNAe requires PRG-1 and the secondary siRNA biogenesis protein RDE-2. Although silencing via RNAe is irreversible, we found that transgene expression can be restored when hemizygous transgenes that were silenced via multigenerational RNAe become homozygous. Furthermore, multigenerational RNAe was accelerated when meiotic pairing of the chromosome possessing the transgene was abolished. We propose that persistent lack of pairing during meiosis elicits a reversible multigenerational silencing response, which can lead to permanent transgene silencing. Multigenerational RNAe may be broadly relevant to single-copy transgenes used in experimental biology and to shaping the epigenomic landscape of diverse species, where genomic polymorphisms between homologous chromosomes commonly result in unpaired DNA during meiosis.

epigenetics | epigenome | RNAe | transgenerational silencing | siRNAs

Small RNAs can repress expression of endogenous genes as well as parasites such as transposons or viruses. Small RNA-mediated repression has the potential to result in epigenetic silencing of genomic loci, which can yield a permanent, heritable state of expression in germ cells in metazoans.

RNA interference (RNAi) is a conserved biological process in which small noncoding RNA molecules promote gene silencing (1). RNAi was originally identified in *Caenorhabditis elegans*, but has been observed in a large number of eukaryotes ranging from fungi to plants to humans (1–6). Small interfering RNAs (siRNAs) can be produced from a variety of sources (7, 8) and include endogenous siRNAs, which are produced by genes, transposons, or aberrant transcripts, and exogenous siRNAs, which target foreign nucleic acids. siRNAs interact with Argonaute proteins that potentiate their functions (9). When exogenous double-stranded RNA is introduced into *C. elegans*, it is processed by the Dicer nuclease into primary 5′ monophosphorylated 22G siRNA duplexes that interact with the Argonaute RDE-1 whose slicer activity promotes degradation of one strand of the duplex (10). RDE-1 and associated primary siRNAs then interact with target mRNAs to recruit RNA-dependent RNA polymerases that synthesize secondary 5′ triphosphorylated siRNAs (11–15). Secondary Argonaute proteins then bind with the secondary siRNAs, and it is this effector complex that directly targets mRNA transcripts in the cytoplasm for degradation (16).

A second class of primary siRNAs in *C. elegans* is the Piwi-interacting RNAs (piRNAs) that are highly abundant in the germline and interact with the *C. elegans* Piwi Argonaute protein PRG-1 (17, 18). *C. elegans* piRNAs are termed 21U-RNAs as they are 21 nucleotides long and possess a 5′ uracil. PRG-1 and associated piRNAs target transposons and some genes, typically based on imperfect homology to their targets, which recruits RNA-dependent RNA polymerases to promote biogenesis of 22G secondary siRNAs that bear perfect homology to their targets (17, 18). These secondary 22G-RNAs interact with WAGO-class Argonaute proteins to promote transcriptional silencing of germline loci. The vast repertoire of *C. elegans* piRNAs and their ability to target nucleic acids with mismatches may allow them to target both endogenous loci as well as foreign nucleic acids such as transposons or viruses (19, 20). Although *C. elegans* piRNAs can target many endogenous transposons, *prg-1* mutants displayed transposition for only one of three DNA transposons tested, even though all three transposons become active if secondary siRNA populations are disrupted with *Mutator* gene mutations (21). These results indicate that small RNA-mediated epigenetic silencing of many transposons initially depends on PRG-1 and associated piRNAs, but then a downstream secondary siRNA system is capable of maintaining silencing of many transposon classes in the absence of piRNAs.

Significance

Transgenes can be permanently silenced in a single generation via a previously described small RNA-induced epigenetic silencing (RNAe) mechanism, which is promoted by the presence of a perfect Piwi-interacting RNA (piRNA) target site. In this study, we identify a previously unidentified mechanism capable of silencing single-copy transgenes that lack perfect piRNA target sites and that is triggered by a lack of chromosomal pairing during meiosis for multiple generations. Multigenerational RNAe can lead to reversible or permanent transgene silencing and may provide insight into variability in the expression of single-copy transgenes or single-copy genomic insertions, which are commonly used in experimental biology. Our analysis of "multigenerational RNAe" also offers new insights into potentially common epigenetic silencing events relevant to genome expression in the germline and embryo.

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A silencing process that relies on the secondary siRNA system of *C. elegans* is "cosuppression," where microinjection of plasmids into the germline leads to creation of repetitive extrachromosomal transgenic arrays that can be expressed in somatic cells but typically become rapidly and permanently silenced in the germline (22). In cosuppression, germline silencing can act *in trans* on the endogenous locus, leading to its silencing as well (23, 24). The diffusible transposon silencing factor depends on *Mutator* class proteins that promote secondary siRNA production, but not on proteins that initiate the response to exogenous double-stranded RNA such as RDE-1 or RDE-4 (23). Cosuppression was originally observed in plants in response to high copy number transgenes and was reported to be associated with a population of siRNAs targeting the transgenes, which foreshadowed the discovery of RNA interference (1, 25–27). Methylation-mediated silencing of multicopy transgenes has also been observed in zebrafish, where transgenes inserted as high copy concatemeric arrays become completely silenced in two to three generations (28, 29). Similar results are seen in mice, where copy number is inversely correlated to expression (30, 31). Therefore, epigenetic silencing of high copy number transgenes in the *C. elegans* germline is consistent with a natural defense response to foreign nucleic acids such as transposons that is seen in diverse organisms.

To stably express transgenes in *C. elegans*, an elegant method to create single-copy transgene insertions was developed with the aid of a unique copy of the *Drosophila melanogaster* transposon *MosI* to induce a chromosomal double-strand break that promotes site-specific recombination with a plasmid-derived template (32–35). Although single-copy transgenes can be stably expressed in the germline using this method, a *piRNA sensor* transgene that encodes *GFP* and also has a perfect piRNA target site in its 3′ UTR is expressed if created in a background that is mutant for *prg-1*, but the *piRNA sensor* is silenced if created in a background that is wild type for *prg-1* (17). If males containing a silenced *piRNA sensor* are crossed directly with *prg-1* mutant hermaphrodites, F2 lines that are homozygous for both the *piRNA sensor* and the *prg-1* mutation then display expression of GFP from the *piRNA sensor* (17). Thus, both initiation and maintenance of epigenetic silencing of the *piRNA sensor* transgene can depend on *prg-1*.

Three independent studies subsequently showed that the *piRNA sensor* transgene, as well as other transgenes possessing *GFP*, can be subjected to a permanent epigenetic silencing process in the germline, termed small RNA-induced epigenetic silencing (RNAe) where *prg-1* is required for initiation but not maintenance of transgene silencing (32, 35, 36). In the first study, the group that created the *piRNA sensor* transgene crossed it *in trans* to a *dpy-10 unc-4* balancer chromosome, and this transformed the *piRNA sensor* transgene into a permanently silent state whose maintenance was independent of PRG-1 (32). A second group showed that propagation of the *piRNA sensor* in a *henn-1* mutant background, which affects the stability of a subset of endogenous siRNAs (37), resulted in stochastic levels of RNAe, where initiation but not maintenance of transgene silencing depended on PRG-1 (35). A third group showed that certain single-copy *GFP* transgenes were prone to permanent silencing if created in a wild-type background, that this did not happen if they were created in a *prg-1* mutant background, and that once silenced via RNAe, these *GFP* transgenes failed to reactivate if combined with a *prg-1* mutation (36).

Together, the latter three studies suggest that transgenes can be subjected to a permanent silencing process, termed RNAe, where initiation of silencing depends on PRG-1/piRNAs, but subsequent maintenance of transgene silencing depends on Mutator class secondary siRNA proteins as well as nuclear RNAi and chromatin proteins (32, 35, 36). This implies that initial targeting of transgenes by PRG-1 and associated piRNAs elicits production of a secondary siRNA population whose maintenance, in conjunction with siRNA-directed histone marks, is sufficient to enforce a stable, heritable silent state.

The permanent silencing process termed RNAe occurs very rapidly, typically within a single generation (32, 35, 36). Herein we describe a distinct siRNA-mediated silencing process that we term "multigenerational RNAe." We identified single-copy transgenes that become silent if propagated in the hemizygous state for multiple generations. We found that multigenerational transgene silencing can either become permanent or, crucially, that it can be reversed, an unexpected result given the permanence of transgene silencing previously described for RNAe. Further, we identify molecular triggers that distinguish RNAe from multigenerational RNAe. Our study provides insight into the genesis and maintenance of epialleles, relevant not only to transgenes, transposons, and some viruses, but also to dynamic regulation of germline gene expression across generations for the vast majority of metazoans.

Results

Discovery of Multigenerational Transgene Silencing. The Protection of Telomeres-1 (POT-1) single-stranded telomere binding protein inhibits telomerase and forms discrete foci at *C. elegans* telomeres in vivo (38). We previously created three independent single-copy transgene insertions that express POT-1::mCherry, *ypSi1*, *ypSi2*, and *ypSi3* (38), each inserted via a transposon-induced double-strand break at the *MosI* locus *ttTi5605*, located near the center of chromosome *II* (Fig. 1*A*) (33, 34). POT-1::mCherry fluorescence can be detected throughout the germline and is particularly evident in meiotic pachytene germ cells as fluorescent punctae (Fig. 1 *B* and *E*) (38). Although several single-copy transgenes inserted in the *ttTi5605 MosI* locus are silenced in the F1 cross-progeny of a single outcross (36), we previously found that *pot-1::mCherry* transgenes remained robustly expressed following two successive crosses: one to combine *pot-1::mCherry* with a marker mutation and a second to cross in a mutation with relevance to telomere biology (38). However, when we crossed *pot-1::mCherry in trans* to the recessive marker mutation *rol-6 (e189)* for nine successive crosses—by crossing *pot-1::mCherry*/*rol-6* heterozygous males with *rol-6*$^{-/-}$ homozygous hermaphrodites and then selecting for *pot-1::mCherry* homozygotes in the F3—we found that POT-1::mCherry fluorescence was either weak or abolished ($n = 2$ crosses each for three independent *pot-1::mCherry* insertions). Further propagation of these strains for a number of generations revealed that POT-1::mCherry expression was robustly restored for *ypSi1.9* and *ypSi3.9*, but that the *ypSi2.9* transgene had become permanently silenced (Fig. S1). Neither nuclear nor telomeric POT-1::mCherry fluorescence was observed for the *ypSi2.9* strain during 3 years of culture in our laboratory (Fig. 1*E*), whereas the original noncrossed *ypSi2* strain always displayed robust expression during this time. The presence of the *pot-1::mCherry* transgene in *ypSi2.9* was confirmed by PCR from genomic DNA, and rtPCR indicated that *pot-1::mCherry* mRNA is present in the original *ypSi2* transgene, hereafter referred to as *pot-1::mCherry*, but not for the crossed *ypSi2.9* transgene, hereafter referred to as *pot-1::mCherry.9* (Fig. 1*C*). Together, these results led us to hypothesize that a partially penetrant multigenerational silencing process can occur when single-copy transgenes are repeatedly crossed in *C. elegans*. This process can ultimately result in a state of permanent transgene silencing that is stable for many generations.

We repeated the above cross by placing the *pot-1::mCherry* transgene *in trans* to the *rol-6* marker mutation and found that a fraction of *pot-1::mCherry*/*rol-6* animals were mCherry negative starting at cross 3, and transgene silencing was sustained for hemizygous *pot-1::mCherry*/*rol-6* animals until cross 7 (Fig. 1*F*). Notably, transgene silencing was never observed for the original *pot-1::mCherry* transgene homozygotes ($n > 200$), nor in the *pot-1::mCherry*/*rol-6* progeny from cross 1 or 2. These results confirmed that a partially penetrant multigenerational silencing

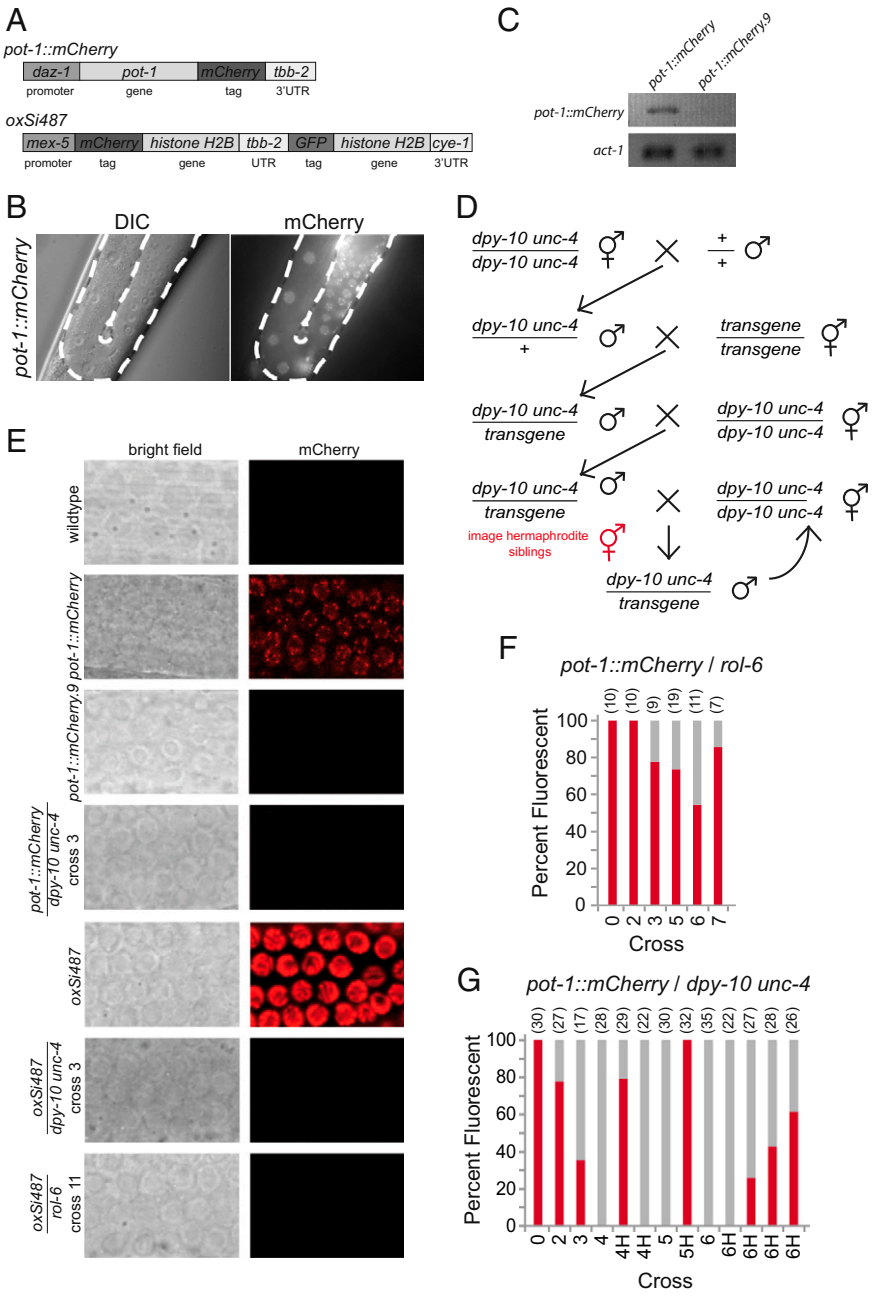

Fig. 1. mCherry-expressing transgenes can be silenced via multigenerational RNAe. (*A*) Structures of *pot-1::mCherry* and *oxSi487* transgenes. (*B*) DIC (*Left*) and mCherry fluorescence image (*Right*) of *pot-1::mCherry*. Dotted white line outlines the mitotic and meiotic germline. (*C*) RT-PCR of strains containing the *pot-1::mCherry* transgene. (*D*) Crossing schema used to cross chromosome *II* transgenes. (*E*) Confocal images of brightfield (*Left* column) and mCherry fluorescence (*Right* column) in the mitotic germline nuclei in various strains (60x). (*F* and *G*) Percent of population fluorescent at each round of crossing for *pot-1::mCherry* using either *rol-6* or *dpy-10 unc-4* marker strain. Numbers at *Top* in parentheses indicate number of worms scored. Bar 0 indicates original transgenic strain before crossing. H denotes homozygous F3 lines derived from parental heterozygous *transgene/marker*.

process occurs when the *pot-1::mCherry* transgene is crossed *in trans* to the *rol-6* marker mutation.

We next placed *pot-1::mCherry in trans* to the marker mutations *dpy-10 unc-4*, which flank the *pot-1::mCherry* transgene, and repeatedly crossed *pot-1::mCherry/dpy-10 unc-4* heterozygous males with *dpy-10 unc-4* hermaphrodites (Fig. 1*D*). A proportion of silent *pot-1::mCherry* heterozygotes were observed for crosses 2 and 3, followed by uniform silencing for crosses 4–6 (Fig. 1 *E* and *G* and Fig. S2*J*). F2 animals were singled and *pot-1::mCherry* homozygotes were identified by selecting against the *dpy-10 unc-4* balancer mutations, and a number of *pot-1::mCherry* homozygotes gave rise to F3 progeny that showed POT-1::mCherry expression (Fig. 1*G*). One *pot-1::mCherry* homozygous line remained completely silent (Fig. 1*G* column 6H). We therefore conclude that a single-copy transgene that is transmitted in the hemizygous state for multiple generations can be subjected to a powerful silencing process. Although transgene expression is often restored when the hemizygous transgene becomes homozygous, a fraction of *pot-1::mCherry* homozygotes remain permanently epigenetically silenced, as observed during our original outcrosses of *pot-1::mCherry in trans* to *rol-6*.

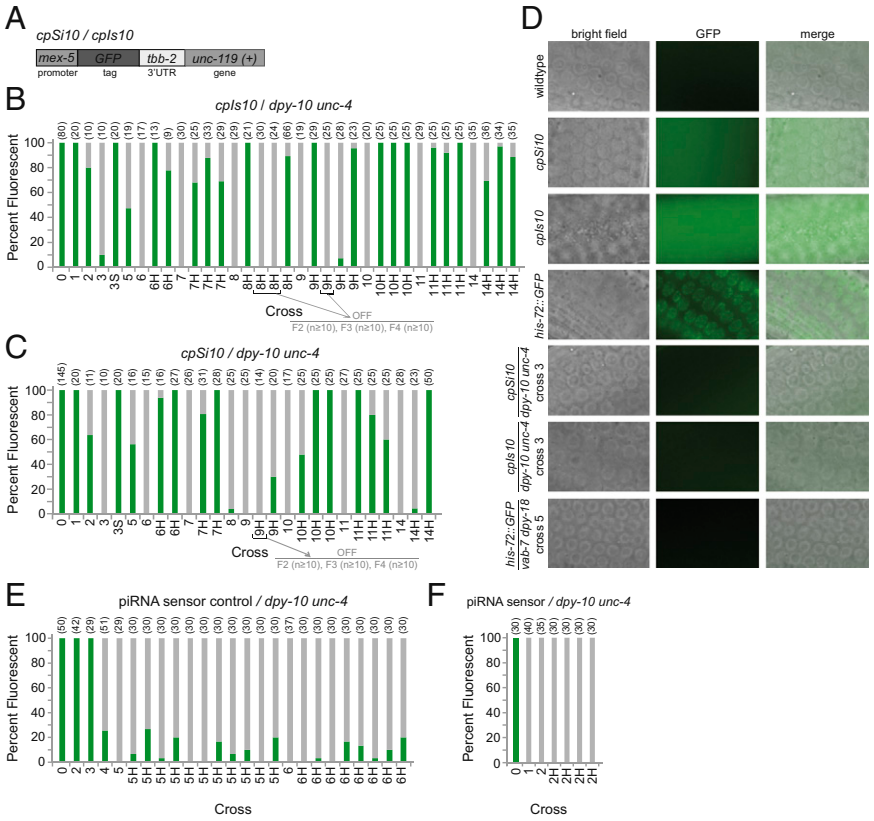

Fig. 2. Silencing of GFP-expressing transgenes via multigenerational RNAe. (*A*) Structures of *cpSi10* and *cpIs10* transgenes. *cpSi10* was inserted in an inverse orientation to that of *cpIs10*. (*B* and *C*) Percent of population fluorescent at each round of crossing for *cpIs10* and *cpSi10* using *dpy-10 unc-4* as a marker strain. Numbers at *Top* in parentheses indicate number of worms scored. Bar 0 indicates original transgenic strain before crossing. S denotes homozygous F3 worms derived from starved heterozygous *transgene/marker* F1. H denotes homozygous F3 worms derived from heterozygous *transgene/marker* F1. Call out indicates the number of generations for which silencing was scored. (*D*) Widefield microscopy images of the mitotic germline nuclei in brightfield (*Left* column), GFP fluorescence (*Center* column), and merged (*Right* column) (60x). (*E* and *F*) Percent of population fluorescent at each round of crossing for *piRNA sensor control* or *piRNA sensor* using a *dpy-10 unc-4* balancer chromosome. Note that the initial *piRNA sensor* strain was mutant for *prg-1* and that the status of the *prg-1* mutation was not followed during crosses *in trans* to *dpy-10 unc-4*. Numbers at *Top* in parentheses indicate number of worms scored. Bar 0 indicates original transgenic strain before crossing. H denotes homozygous F3 worms derived from heterozygous *transgene/marker* F.

Independent Transgenes Succumb to Multigenerational Silencing. We next tested multigenerational silencing of an independent single-copy transgene, *oxSi487*, which expresses both mCherry- and GFP-tagged proteins from an operon integrated into the *ttTi5605 MosI* locus, *Pmex-5::mCherry::H2B::tbb-2 3′UTR::gpd-2 operon::GFP::H2B::cye-1 3′UTR* (Fig. 1 *A* and *E*). The mCherry expression in this transgene was highly robust, whereas the GFP expression was substantially weaker; therefore, we used the mCherry fluorescence to score for transgene expression. Silencing was never observed in the original *oxSi487* strain ($n > 100$). However, crosses of *oxSi487 in trans* to a balancer chromosome containing *dpy-10* and *unc-4* mutations resulted in silencing for some progeny in cross 2 and complete silencing by cross 3 (Fig. 1*E* and Fig. S2*I*). We also crossed *oxSi487 in trans* to *rol-6* and found that a fraction of animals for four independent crosses were mCherry negative from crosses 4–11 (Fig. 1*E* and Fig. S2*H*). Together, the above *pot-1::mCherry* and *oxSi487* experiments indicate that multigenerational transgene silencing occurs for independent *mCherry* transgene strains with balancer chromosomes containing distinct marker mutations, that permanent transgene silencing can occur in either circumstance, and that weaker multigenerational silencing occurs when transgenes are crossed *in trans* to a balancer chromosome containing *rol-6*.

We next asked whether multigenerational transgene silencing also occurs for transgenes that only contain GFP-epitope tags, and whether transgene insertion via a double-strand break created by the *Drosophila MosI* transposon *ttTi5605* has any impact on transgenerational silencing. We therefore tested identical transgenes that express *Pmex-5::GFP::tbb-2 3′UTR* that were inserted either via *MosI*-mediated gene conversion at *ttTi5605*, *cpSi10 [Pmex-5::GFP::tbb-2 3′UTR + unc-119(+)]* or via CRISPR/Cas9-mediated gene conversion at the identical position on chromosome *II*, *cpIs10[Pmex-5::GFP::tbb-2 3′UTR + unc-119(+)]* (Fig. 2 *A* and *D*) (39). We found that GFP was expressed uniformly in worm populations from *cpSi10* and *cpIs10* stocks ($n > 100$), but that hemizygous *cpSi10* and *cpIs10* transgenes underwent multigenerational silencing with very similar kinetics (Fig. 2 *B* and *C*). Thus, the presence of the *Drosophila MosI* transposon at the *ttTi5605* locus before transgene insertion does not affect transgene silencing. When *cpSi10* and *cpIs10* were crossed *in trans* to our *dpy-10 unc-4* marker strain, *cpSi10* was silenced by cross 3 and *cpIs10* at cross 6 (Fig. 2 *B*–*D*). Propagating worms from starved cross plates yielded F3 progeny homozygous for the transgene, where transgene expression was fully restored (Fig. 2 *B*, column 3S and *C*, column 3S). To determine if starvation was necessary for restoration of transgene expression in transgene homozygotes, we singled unstarved F1 progeny from silent *cpSi10/ dpy-10 unc-4* and *cpIs10/ dpy-10 unc-4* heterozygotes and found that the majority of F3 *cpSi10* or *cpIs10* homozygotes expressed GFP in the absence of starvation (Fig. 2 *B* and *C*, columns marked H). Several of the homozygous F3 progeny that did not express GFP were isolated (Fig. 2 *B*, columns 8H and 9H

and *C*, column 9H). These lines remained GFP negative, and fully penetrant silencing was transmitted for at least five generations.

Because we saw variability in the kinetics of multigenerational silencing for *mCherry* transgenes using our *dpy-10 unc-4* and *rol-6* balancer chromosomes, we further examined the effect of balancer chromosome identity on multigenerational silencing of hemizygous *cpSi10* and *cpIs10* transgenes. We found that multigenerational transgene silencing could be promoted *in trans* to three distinct balancer chromosomes, one containing *dpy-2* and *unc-4* mutations (Figs. S2 *A* and *D* and S3), one carrying an inversion between *dpy-10* and *unc-4* mutations (Figs. S2 *B* and *E* and S3), and a new *dpy-10 unc-4* chromosome ordered directly from the *C. elegans* stock center (Figs. S2 *C* and *F* and S3). Furthermore, when transgene homozygotes were isolated from completely silent hemizygous populations, we once again saw desilencing of the transgene in some cases (Fig. S2 *A* and *D*). We conclude that a strong yet reversible multigenerational silencing process occurs for transgene hemizygotes *in trans* to a range of distinct balancer chromosomes, and that hemizygous transgene silencing is potent enough to become permanent when transgene homozygotes are isolated.

We next asked whether a CRISPR/Cas9-mediated GFP insertion at a distinct genomic location, the *his-72* locus on chromosome *III* (39), could also be subjected to multigenerational silencing. *his-72::GFP* was placed *in trans* to the marker mutations *vab-7 dpy-18* and crossed versus these mutations (Fig. S4). Similar to our other transgenes tested, a significant fraction of *his-72::GFP/vab-7 dpy-18* heterozygotes displayed transgene silencing for crosses 3–22 (Fig. 2*D* and Fig. S2*G*). Thus, multigenerational transgene silencing is not specific to single-copy transgenes at the *ttTi5605* locus on chromosome *II* and can regulate the expression of GFP cassettes inserted at endogenous genomic loci.

A piRNA Target Site Dictates RNAe Pathway Choice. It was previously observed that a "*piRNA sensor*" transgene could be subjected to fully penetrant, irreversible RNAe if crossed using *dpy-10 unc-4* as a balancer chromosome. To determine how this example of RNAe is related to the multigenerational RNAe process that we report here, we used the previously characterized *piRNA sensor* transgene that was prone to RNAe, as well as the "*piRNA sensor control*" transgene that was resistant to RNAe (32, 35, 36). The *piRNA sensor* contains the reverse complement of an endogenous piRNA sequence in its 3′ UTR, whereas the *piRNA sensor control* instead contains the piRNA sequence in opposite orientation. In agreement with published results, we saw expression of the *piRNA sensor* transgene in a *prg-1; piRNA sensor* strain, but a single cross *in trans* to a *dpy-10 unc-4* balancer chromosome resulted in silencing of the *piRNA sensor* (Fig. 2*F*) (32, 35, 36). We confirmed that isolation of *piRNA sensor* transgene homozygotes from *piRNA sensor/dpy-10 unc-4* hemizygotes resulted in fully penetrant silencing that reflects the rapid, irreversible RNAe process that was previously reported (Fig. 2*F*) (32). In contrast, when the *piRNA sensor control* transgene was outcrossed *in trans* to a *dpy-10 unc-4* balancer chromosome (Fig. 1*D*), we observed that it underwent multigenerational RNAe (Fig. 2*E*). Complete transgene silencing was observed for *piRNA sensor control* transgene hemizygotes at cross 3, and some transgene homozygotes derived from progeny of silent hemizygotes expressed the fluorescent transgene (Fig. 2*E*, columns H). Thus, one factor that distinguishes the previously described permanent RNAe process from the multigenerational RNAe process that we report is the presence of a piRNA target site.

PRG-1/piRNAs and Secondary siRNAs Promote Initiation and Maintenance of Multigenerational RNAe. RDE-2 is a component of the Mutator-class of RNA interference proteins that acts to promote biogenesis of secondary siRNA populations, which were previously shown to be required for maintenance of silencing of single-copy transgenes via RNAe (32, 35, 36) as well as for cosuppression-mediated silencing of repetitive transgenes (18, 23, 40–42). Consistently, we found that POT-1::mCherry fluorescence was partially restored when the silenced *pot-1::mCherry.9* transgene was placed in an *rde-2*–deficient background (Fig. 3 *A* and *B*).

The piRNA-interacting Argonaute protein PRG-1 is typically required for initiation but not maintenance of RNAe (17, 32, 35, 36). We found that independent alleles of *prg-1*, *tm872* and *n4357*, elicited partial desilencing of *pot-1::mCherry.9* (Fig. 3 *A* and *B*). As *prg-1* and *rde-2* mutations both resulted in partial desilencing of *pot-1::mCherry.9*, we constructed *rde-2 prg-1(tm872); pot-1::mCherry.9* and *rde-2 prg-1(n4357); pot-1::mCherry.9* strains and found that POT-1::mCherry fluorescence was fully restored in both cases (Fig. 3 *A* and *B*). Thus, proteins that play distinct roles in initiation or maintenance of single-generation single-copy transgene silencing via RNAe coordinately promote maintenance of multigenerational transgene silencing.

NRDE-1 is required for nuclear RNA interference, where it functions downstream of siRNAs to promote transcriptional gene silencing (32, 43). We found that when the silent *pot-1::mCherry.9* transgene was placed in a *nrde-1*–deficient background, silencing was completely abolished (Fig. 3 *A* and *B*). In contrast, the NRDE-3 Argonaute protein, which promotes transcriptional silencing in the soma in response to exogenous dsRNAs (44), was dispensable for *pot-1::mCherry* silencing (Fig. 3 *A* and *B*). These results are consistent with the possibility that a nuclear silencing process in the germline promotes maintenance of multigenerational RNAe.

As a control, we tested the RSD-6 Tudor domain protein, which has been previously described to promote spreading of RNAi from the soma to the germline and generation of robust secondary siRNA populations in response to exogenous dsRNA triggers, but is dispensable for single-generation silencing of the piRNA sensor transgene (32, 45, 46). *pot-1::mCherry* silencing was maintained when *rsd-6* was mutant (Fig. 3 *A* and *B*).

Having established roles for *prg-1* and *rde-2* in maintenance of multigenerational transgene silencing, we next asked whether these genes also played roles in initiation of multigenerational RNAe. We created strains with active *GFP* transgenes that have mutations in *prg-1* or *rde-2* and crossed these with a marker strain containing a *dpy-10 unc-4* balancer chromosome and either *prg-1* or *rde-2* mutations, respectively. Silencing of hemizygous *GFP* transgenes was not observed in either *prg-1* or *rde-2* mutant backgrounds (Fig. 4 *B–H*), indicating that both PRG-1/piRNAs and secondary siRNA biogenesis are required for initiation of multigenerational RNAe. Surprisingly, when we initiated these active transgene experiments by crossing either *prg-1* or *rde-2* mutations into *cpSi10* or *cpIs10* transgene backgrounds, we observed robust increases in GFP expression in comparison with the parental transgene strains (Fig. 4 *A* and *B*), which did not diminish during multigenerational RNAe, indicating that PRG-1 and RDE-2 promote a state of incomplete silencing for both of these active transgenes.

Lack of Chromosomal Pairing Induced Rapid Silencing. The ZIM proteins are a family of four related C2H2 zinc-finger domains proteins that promote pairing of specific chromosome homologs during meiosis (47). ZIM-1 promotes pairing of chromosomes *II* and *III*, ZIM-2 promotes pairing of chromosome *V*, and ZIM-3 promotes pairing of chromosomes *I* and *IV* (47). When mutated, these genes result in a loss of meiotic pairing of their respective chromosomes (48, 49). When *pot-1::mCherry; zim-1* males were crossed with *dpy-10 unc-4; zim-1* hermaphrodites, uniform mCherry silencing occurred in the progeny of cross 1 (Fig. 5*A*). The same result was observed for progeny of *oxSi487; zim-1* males crossed with *dpy-10 unc-4; zim-1* hermaphrodites (Fig. 5*A*). We confirmed that rapid transgene silencing in a *zim-1* mutant background was due to complete lack of pairing the chromosome containing the *pot-1::mCherry* transgene, chromosome *II*, by testing *zim-2* or *zim-3* mutations that promote pairing of distinct

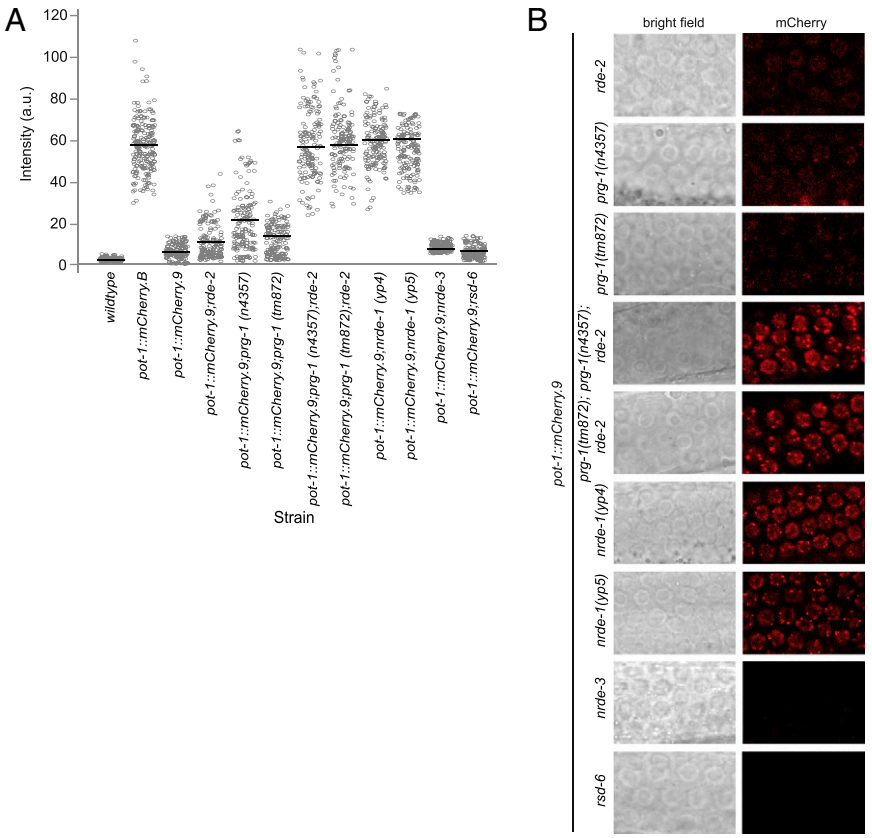

Fig. 3. *prg-1* and *rde-2* coordinately promote maintenance of the silencing of the *pot-1::mCherry* transgene. (*A*) Intensity measurements for individual nuclei ($n = 150$ for each genotype, five nuclei counted per animal). Solid horizontal bars indicate the mean intensity for each population. (*B*) Confocal images of both brightfield (*Left* column) and mCherry fluorescence (*Right* column) in the mitotic germline nuclei in various strains in the *pot-1::mCherry.9* transgenic background (60x).

autosomes. Neither *zim-2* nor *zim-3* promoted immediate transgene silencing (Fig. 5*A*), indicating that lack of pairing during meiosis promotes multigenerational transgene silencing.

Discussion

Lack of a pairing partner in meiosis is known to activate an RNA directed-silencing mechanism termed meiotic silencing of unpaired DNA (MSUD), which was first identified in *Neurospora crassa* (50). MSUD suppresses the expression of genes that fail to pair with their homologs during the first prophase of meiosis (51–53). MSUD requires small RNA factors, including an Argonaute protein and an RNA-dependent RNA polymerase, which likely respond to unpaired meiotic DNA to produce double-stranded RNAs that feed into an siRNA-mediated silencing system (50, 54–56). We found that transgene silencing occurred when hemizygous *single-copy transgene/marker mutation* heterozygotes were crossed with *marker mutation* homozygotes for multiple generations. Although potent, this multigenerational silencing process was reversible, as transgene activation was commonly observed when *transgene* homozygotes were isolated from silent *single-copy transgene/marker mutation* heterozygotes. These results imply that the trigger of multigenerational transgene silencing corresponds to unpaired transgene DNA during meiosis, which occurs if one homolog possesses a transgene and the other does not. In support of this model, we found that complete disruption of meiotic pairing of the chromosome containing a transgene normally targeted for multigenerational RNAe resulted in rapid transgene silencing in a single generation (Fig. 5*A*). These data imply that the molecular trigger of multigenerational transgene silencing is lack of pairing during meiosis (Fig. 5*B*), which is a physical hallmark of the siRNA-mediated genomic silencing process MSUD. Our study integrates the process of piRNA-mediated silencing, where targets are commonly identified based on imperfect homology to piRNAs (17), with small but nevertheless potent structural aberrations that can occur when meiotic chromosomes pair.

We observed silencing via multigenerational RNAe for *single-copy transgene/marker mutation* heterozygotes using various transgenes and marker mutations on different chromosomes (Figs. 1 and 2 and Fig. S2). However, for a single locus on chromosome *II*, we found that the kinetics and penetrance of multigenerational RNAe for independent transgenes was variable and dependent on the balancer chromosome containing the marker mutation used for the hemizygous transgene crosses (Figs. 1 and 2 and Fig. S2). Thus, although the hemizygous state represents the molecular trigger of multigenerational RNAe, factors on a balancer chromosome can influence the severity and rate of silencing. An interesting future line of investigation will be to identify the *trans*-acting factor on the *dpy-10 unc-4* balancer chromosome that promotes robust and completely penetrant multigenerational RNAe by crosses 3–5 (Figs. 1 *F* and *G* and 2 and Fig. S2).

The reversibility of multigenerational RNAe contrasts sharply with permanent transgene silencing of RNAe (32, 35, 36), indicating that multigenerational RNAe is a distinct, although related, form of siRNA-mediated genome silencing. RNAe was previously reported for a *piRNA sensor* transgene containing a perfect piRNA target site and for *GFP* transgenes that lack a perfect piRNA target site but are prone to silencing and are targeted at least in part by secondary siRNAs that may be generated by

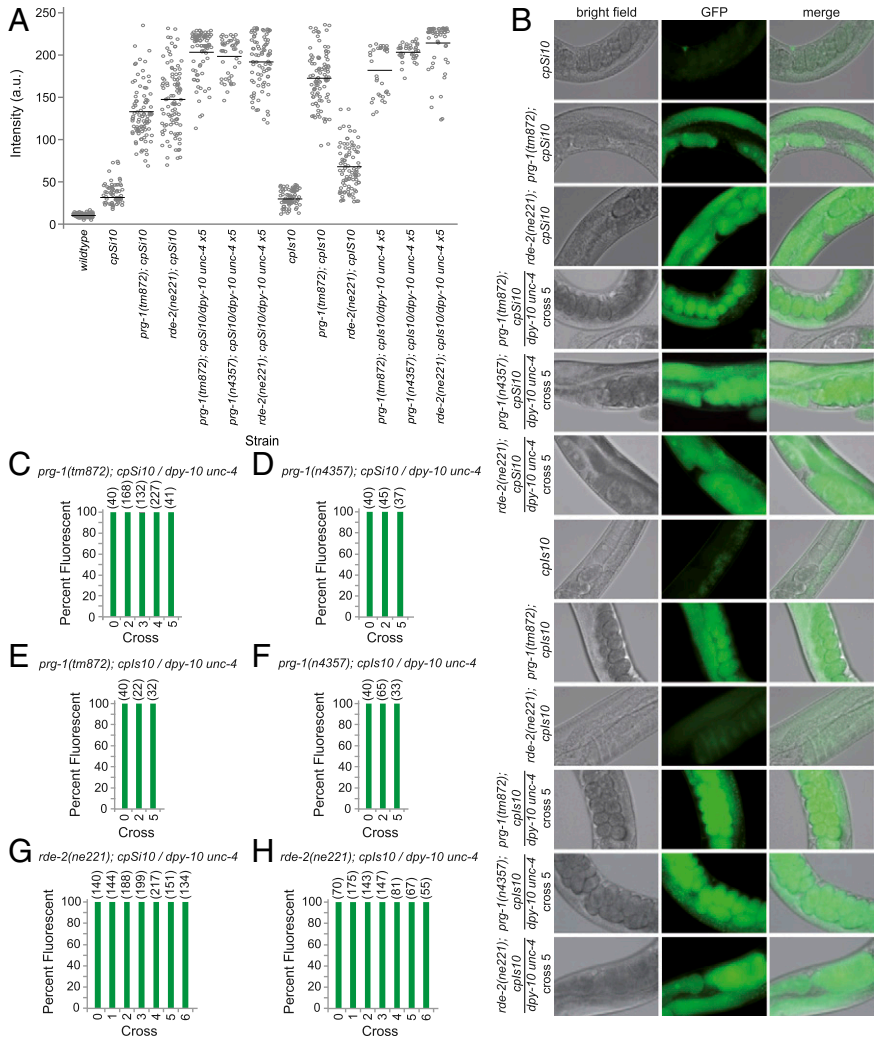

Fig. 4. *prg-1* and *rde-2* are not required for initiation of multigenerational RNAe. (*A*) Intensity measurements for germlines of various strains. ($n > 25$ for each genotype, three independent measurements of each germline). Solid horizontal bars indicate the mean intensity for each population. (*B*) Representative widefield microscopy images of the mitotic germline nuclei in brightfield (*Left* column), GFP fluorescence (*Center* column), and merged (*Right* column) (from *A*) (40x). Due to robust expression in *rde-2* and *prg-1* mutant backgrounds and uniform exposure time to accurately measure intensities, active transgene intensities appear extremely dim although they are significantly expressed. ($P < 0.0001$ wild-type vs. transgene unpaired *t* test). (*C–H*) Percent of population fluorescent at each round of crossing in *prg-1* or *rde-2* mutant backgrounds in either *cpSi10* or *cpIs10* using *dpy-10 unc-4* balancer chromosome. Numbers at *Top* in parentheses indicate number of worms scored. Bar 0 indicates original transgenic strain before crossing (combined data from three or more independent experiments).

piRNAs with imperfect homology to *GFP* (32, 35, 36). We found that the *piRNA sensor control* transgene, which lacks a piRNA target site but is otherwise identical to the *piRNA sensor* transgene (32, 35, 36), succumbs to multigenerational RNAe instead of RNAe (Fig. 2*F*). Because the *piRNA sensor* and the *piRNA sensor control* both contain *GFP* and likely possess imperfect piRNA target sites, we conclude that the presence of a perfect piRNA target site can be a decisive trigger of RNAe. The *piRNA sensor control* transgene contains a control piRNA target site that is in reverse orientation (on the opposite strand of transgene DNA in comparison with the *piRNA sensor*) and so is not expressed (17). Therefore, the perfect piRNA target site in RNA transcripts from the *piRNA sensor* transgene is crucial for interactions with PRG-1/piRNAs that promote RNAe-mediated transcriptional silencing of this transgene. Note that the hypothesis that a perfect piRNA target site can promote RNAe has not been rigorously tested. For example, it remains uncertain whether a perfect piRNA target site is sufficient to promote RNAe and how piRNA identity or piRNA target site homology might affect interactions with RNAe.

How might PRG-1/piRNAs promote multigenerational RNAe of the *piRNA sensor control* transgene that does not possess a perfect piRNA target site? We suggest that piRNAs scan *GFP* and possibly other segments of RNA expressed from a transgene to promote the synthesis and/or stability of secondary siRNA populations, specifically in response to lack of pairing of the transgene during meiosis (57). How PRG-1 and associated piRNAs specifically target RNA derived from unpaired meiotic DNA for silencing is presently unclear.

As the presence of a perfect piRNA target site can elicit rapid and permanent RNAe, we suggest that the degree of homology of a piRNA with its mRNA target could reflect the history of a piRNA. For example, if *C. elegans* piRNA genes are occasionally created in response to foreign nucleic acids and initially possess perfect homology to their targets, then when PRG-1 encounters a transcript that matches a piRNA perfectly, this could suggest the expression of a recently integrated foreign genetic element that has not accumulated mutations in its piRNA target site. We speculate that such

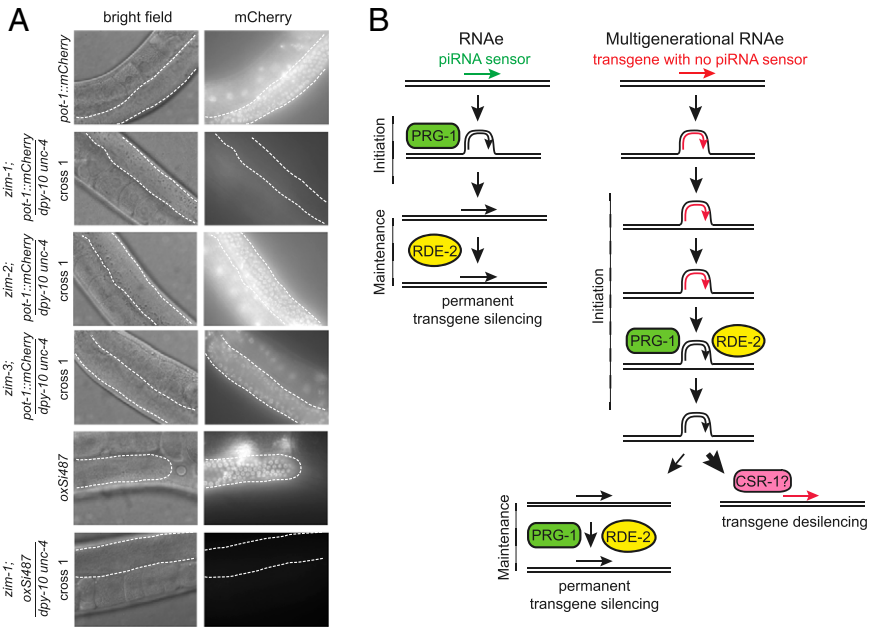

Fig. 5. Complete disruption of meiotic chromosome pairing leads to rapid transgene silencing. (*A*) Representative widefield microscopy images of brightfield (*Left* column) and mCherry fluorescence (*Right* column) of *pot-1::mCherry* ($n > 75$ expressed mCherry), *pot-1::mCherry/ dpy-10 unc-4; zim-1* ($n = 28$ were silent), *pot-1::mCherry/ dpy-10 unc-4; zim-2* ($n = 30$), *pot-1::mCherry/ dpy-10 unc-4; zim-3* ($n = 30$ were silent), *oxSi487* ($n > 75$ expressed mCherry), *oxSi487/ dpy-10 unc-4; zim-1* ($n = 21$; 19/21 were silent, 2/21 displayed faint mCherry fluorescence). Dotted white line outlines mitotic germline; all images are 40×. (*B*) Model depicting RNAe, as previously described, and multigenerational RNAe.

elements might be perceived as especially threatening and would then be targeted for immediate silencing via RNAe.

As we identified multiple *GFP* transgenes that succumb to multigenerational RNAe, we suggest that *GFP* transgenes that are prone to RNAe but lack a perfect piRNA target site (35) likely contain an additional factor that promotes RNAe-mediated transgene silencing. This factor could be an unusual piRNA target site or the expression of a transgenic protein that promotes RNAe-mediated silencing. For example, *GFP* transgenes that are prone to RNAe could repress an siRNA-mediated antisilencing system that was recently identified in *C. elegans*, which is mediated by the Argonaute protein CSR-1 and protects endogenous germline genes from silencing (58–61). Transgenes that are active in the germline and licensed by CSR-1 have been shown to elicit a multigenerational antisilencing effect, and active transgenes can act *in trans* via 22G-RNAs to awaken distinct silent transgenes (58, 60). The direct competition between CSR-1 and PRG-1 to mark a gene for either licensing and protection or for silencing and repression, respectively, is likely to potentiate both RNAe and multigenerational RNAe (35).

The interaction of pro- and antisilencing siRNA pathways is likely reflected by independent *GFP* transgenes that suffer from reduced levels of GFP expression due to PRG-1– and RDE-2–dependent silencing (Fig. 4), yet can be completely silenced via multigenerational RNAe (Fig. 2 *A–D*). We suggest that a significant number of transgenes that are routinely created in research laboratories may be incompletely silenced as a consequence of being hemizygous for several generations when they are created (32–35). Another general concern raised by our study is that once a transgenic strain has been created, the transgene may be subjected to transient or permanent silencing when manipulated in crossing schemes where it is hemizygous for several generations.

Previous reports have suggested that initiation and maintenance of transgene silencing are typically separable for single-copy *GFP* transgenes that are permanently silenced via RNAe, which can be initiated by the germline Argonaute protein PRG-1 but then maintained by secondary siRNA biogenesis proteins and small RNA-mediated nuclear silencing factors (36, 60). We show that maintenance of silencing for transgenes silenced by multigenerational RNAe can require both PRG-1 as well as the secondary siRNA protein RDE-2 (Fig. 3) and found that robust transgene desilencing occurred only when both *prg-1* and *rde-2* were disrupted. We conclude that multigenerational RNAe can lead to a state of epigenetic silencing where the maintenance phase of transgene silencing remains mechanistically coupled to PRG-1/piRNAs (Fig. 5*B*). Consistently, maintenance of silencing of the *piRNA sensor* transgene created in a wild-type background requires PRG-1/piRNAs (17), although outcrossing the *piRNA sensor* with a balancer chromosome will transform it into a permanently silent state that is no longer maintained by PRG-1/piRNAs (32, 35, 36). Overall, our results are consistent with genome silencing activities that can remain at least partially dependent on PRG-1 function, in contrast to the discrete role of PRG-1 in initiation, but not maintenance of silencing that has been typically reported for RNAe (32, 35, 36).

Our results are in consonance with a model derived from studies of epigenetic silencing in plants, where initiation and maintenance loops can promote transcriptional silencing mediated by small RNA-directed DNA methylation, for example in response to viral DNA (26, 62). Once silencing is established, the initiation loop can become dispensable for silencing, as is the case for PRG-1–initiated rapid silencing of a single-copy GFP transgene. However, the initiation loop can potentially contribute to maintenance of a silent state (26), as we show for PRG-1 in multigenerational silencing of the *pot-1::mCherry.9* transgene. These results are consistent with a known role for PRG-1 in maintenance of silencing for a subset of transposons—the Tc3 DNA transposon becomes desilenced and active when *prg-1* is mutant, but not Tc1 or Tc4 transposons, all of which become active when secondary siRNA biogenesis is disrupted (20). We further show that RDE-2 is required for initiation of silencing of active transgenes in multigenerational RNAe (Fig. 4), which is also consistent with examples in plants where initiation and maintenance phases of epigenetic silencing can be coupled (26). We note that the possibility that

secondary siRNA biogenesis is required for initiation of RNAe has not been formally tested.

The formation of epialleles in reaction to foreign DNA has been well documented in *Arabidopsis thaliana*. Although plants possess a system of DNA methylation, and *C. elegans* lacks this mechanism, other epigenetic alterations that affect chromatin states do occur in the nematode (63–65). Epigenetic modification in plants is accomplished via the RNA-directed DNA methylation pathway, where RNA-dependent RNA polymerases bind single-stranded RNAs and create small dsRNAs that are then cleaved by Dicer and loaded into an Argonaute complex (66–70), which target both CG and non-CG sites for methylation (71). Once established, the cytosine methyl marks are associated with an epigenetic state that is transmitted to future generations. Maintenance is mediated by specific DNA methyltransferases, which copy epigenetic information to the daughter strand of DNA during meiosis, resulting in the establishment of an epiallele that can be passed on from one generation to the next (66, 72).

Genome-wide association studies have revealed that DNA sequence polymorphisms explain only a minor fraction of variability for a number of common human traits such as type II diabetes. Some of this missing heritability may be due to epigenetic modification of the genome (73–75), as reflected by creation of numerous de novo epialleles in F2 lines derived from crosses between different *A. thaliana* ecotypes (66, 76). Although there are a number of explanations for creation of such epialleles, genomes of metazoans typically display numerous copy number changes between homologous chromosomes. For example, gain or loss of copies of a specific segment of the genome has been estimated to account for up to 5% of the variability between human individuals (77, 78), implying that significant levels of unpaired DNA occur during most meioses. We propose that the multigenerational epigenetic silencing process described in our study, likely triggered by lack of pairing during meiosis, can elicit both permanent and reversible forms of silencing that may be relevant to heritable epigenomic variation in many species, with pertinent yet complex implications for understanding and predicting heritability in the context of human disease.

Materials and Methods

Strains. All strains were cultured and maintained at 20 °C on nematode growth medium plates seeded with *Escherichia coli* OP50. Strains used include: Wild type (N2 Bristol ancestral strain), WM161 *prg-1(tm872) I*, SX922 *prg-1(n4357) I*, WM29 *rde-2(ne221) I*, *rsd-6(yp11) I*, GE1708 *dpy-2(e8) unc-4 (e120) II*, DR103 *dpy-10(e128) unc-4(e120) II*, *mIn1[unc-4(e120) dpy-10(e128)] II* derived from DR2054, YA1198 *ypIn3 [Pdaz-1:pot-1::mCherry::tbb-2utr]*, EG6787 *oxSi487 [Pmex-5::mCherry::H2B::tbb-2 3′UTR::gpd-2 operon::GFP::H2B::cye-1 3′UTR + unc-119(+)] II*, CB187 *rol-6 (e187) II*, SX1888 *prg-1(n4357) I; mjIs144 II*, *nrde-1(yp4) III*, *nrde-1(yp5) III*, LP135 *cpSi10 [Pmex-5::GFP::tbb-2 3′UTR + unc-119(+)] II; unc-119(ed3) III*, LP136 *cpIs10 [Pmex-5::GFP::tbb-2 3′UTR + unc-119(+)] II; unc-119(ed3) III*, *vab-7(e1562) dpy-18(e364) III*, LP148 *unc-119(ed3) his-72(cp10[his-72::gfp + LoxP unc-119 (+) LoxP]) III*, CA258 *zim-2(tm574) IV*, CA324 *zim-1(tm1813) IV*, *zim-3(yp8) IV*, YY158 *nrde-3 (gg66) X*, and SX1287 *mjIs145 II; unc-119 (ed3) III*. For *zim* experiments, males homozygous for a transgene and a *zim* mutation were created, crossed with *zim; dpy-10 unc-4* triple mutant hermaphrodites, and F1 cross-progeny were scored for mCherry fluorescence.

Microscopy. Live 1-d-old young adult worms were mounted on 2% (wt/vol) agarose pads in 7 μL of 2 mg/mL levamisole. Strains were examined in widefield using a Nikon Eclipse E800 microscope both under differential interference contrast (DIC) and a 595-nm excitation wavelength at 20×, 60×, or 100× magnifications. Confocal microscopy was performed using a Zeiss LSM710 laser-scanning microscope with a C-APO 40×/1.2 N.A. objective at 2.0× optical zoom. Images were obtained using a 561-nm diode laser for excitation, and emission was collected at 563–701 nm. Brightfield images were collected simultaneously.

Fluorescence Quantification. Animals were mounted as described above, and Z stacks were taken using confocal microscopy as described above, within 2 h of mounting. Zeiss ZEN 2009 software was used to collect and compile the stacks. Analysis of fluorescence in the nuclear volume (arbitrary units) was performed using ImageJ v1.45S and the "measure stack" volume measurement plugin (developed by R. F. Dougherty and available at www.optinav.com/imagej.html). For each genotype, 30 animals were selected and five nuclei were quantified from each animal. GFP transgenic lines were mounted and examined as described above. Pictures were taken at 40× with an exposure time of 300 ms. To determine intensities, background was subtracted out using NIS Elements software. In ImageJ, a circle (100 pixels high ×100 pixels wide) was used to measure the intensity of three independent sections of each germline. Each intensity measurement was plotted using an *x*-axis jitter.

rtPCR. Control and experimental animals were grown alongside each other under identical conditions. RNA extraction with TRIzol was performed using standard protocols. cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) with random hexamers. Primer pair sequences used are as follows: actin control (F-GATATGGAGAAGATCTGGCATCA and R-GGGCAAGAGCGGTGATT) and mCherry (F-TGGTCCAATTTCGTGGTTTATATCCTC and R-CTTTGCTCTTCGCCATTGTTTCC).

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