Nuclear RNAi maintains heritable gene silencing in *Caenorhabditis elegans*

Nick O. Burton, Kirk B. Burkhart, and Scott Kennedy[1]

Laboratory of Genetics, University of Wisconsin, Madison, WI 53706

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RNA interference (RNAi) is heritable in *Caenorhabditis elegans*; the progeny of *C. elegans* exposed to dsRNA inherit the ability to silence genes that were targeted by RNAi in the previous generation. Here we investigate the mechanism of RNAi inheritance in *C. elegans*. We show that exposure of animals to dsRNA results in the heritable expression of siRNAs and the heritable deposition of histone 3 lysine 9 methylation (H3K9me) marks in progeny. siRNAs are detectable before the appearance of H3K9me marks, suggesting that chromatin marks are not directly inherited but, rather, reestablished in inheriting progeny. Interestingly, H3K9me marks appear more prominently in inheriting progeny than in animals directly exposed to dsRNA, suggesting that germ-line transmission of silencing signals may enhance the efficiency of siRNA-directed H3K9me. Finally, we show that the nuclear RNAi (Nrde) pathway maintains heritable RNAi silencing in *C. elegans*. The Argonaute (Ago) NRDE-3 associates with heritable siRNAs and, acting in conjunction with the nuclear RNAi factors NRDE-1, NRDE-2, and NRDE-4, promotes siRNA expression in inheriting progeny. These results demonstrate that siRNA expression is heritable in *C. elegans* and define an RNAi pathway that promotes the maintenance of RNAi silencing and siRNA expression in the progeny of animals exposed to dsRNA.

transgenerational | epigenetics

Epigenetics is the study of changes in gene expression or phenotype that are not the result of changes in DNA sequence. The mechanistic underpinnings of many epigenetic phenomena appear to be related. For instance, many epigenetic processes are associated with the posttranslational modification of histones (1). In many cases, epigenetic information is thought to be mitotically, but not meiotically, stable; epigenetic information passes from parent cell to daughter cell but is erased at the start of each new generation (2, 3).

In some cases, epigenetic information can be transferred to progeny. For instance, the epigenetic phenomenon of paramutation in maize is meiotically stable. Two alleles of the *b1* gene (*B′* and *B-I*) are expressed differently, even though the underlying DNA sequence of these alleles is identical. *B-I* plants express the *b1* gene, but *B′* plants do not. In heterozygous *B′*/*B-I* plants, the *B′* allele converts (or paramutates) the *B-I* allele to a low expression state. The conversion of *B-I* to *B′* is heritable; virtually all progeny (which include *B-I*/*B-I* progeny) from *B′*/*B-I* self-crosses exhibit the *B′* phenotype (4). Thus, epigenetic information can be passed across generations.

RNAi is the ability of dsRNA to silence homologous cellular RNAs (5). dsRNAs are cleaved by the enzyme Dicer into siRNAs, which are bound by Argonaute (Ago) proteins (6, 7). Silencing occurs when siRNA/Ago complexes base pair and inhibit gene expression by inducing the degradation of complementary mRNAs (8–10). In plants, worms, and *Schizosaccharomyces pombe*, RNA-dependent RNA polymerases (RdRPs) amplify silencing signals by synthesizing secondary (2°) siRNAs, which themselves inhibit gene expression (11–15). dsRNA can silence nuclear-localized RNAs (nuclear RNAi) in *Caenorhabditis elegans* (16–18). Nuclear RNAi depends on at least four genes, termed nuclear RNAi defective 1–4 (*nrde*-1 to *nrde*-4) (16–18). The Ago NRDE-3 escorts 2° siRNAs into the nucleus, where NRDE-3 recruits NRDE-1 and NRDE-2 to nascent transcripts that exhibit homology to NRDE-3–bound 2° siRNAs (16, 17). After recruitment to pre-mRNA, NRDE-1 associates with chromatin via a process requiring NRDE-4 (18). Together, the Nrde factors inhibit RNA polymerase II elongation and direct the deposition of histone 3 lysine 9 methylation (H3K9me) marks at genomic sites targeted by RNAi (17, 18). siRNA-directed H3K9me was first observed in plants and *S. pombe* (19–21). In *S. pombe*, siRNA/Ago complexes associate with nascent transcripts and recruit the H3K9 methyltransferase enzyme Clr4 to genomic sites of RNAi. Clr4 catalyzes H3K9me, which contributes to transcriptional gene silencing (21). In *C. elegans*, the role of siRNA-directed H3K9me in gene silencing is unclear.

dsRNA can induce multigenerational gene silencing in *C. elegans*. The progeny of *C. elegans* exposed to dsRNA (henceforth referred to as "inheriting progeny") maintain the ability to silence genes that were targeted by RNAi in the previous generation (RNAi inheritance) (5, 22–24). Thus, RNAi is a multigenerational epigenetic process. In *C. elegans*, the RNAi inheritance signal is dominant and heritable even in the absence of the gene targeted by dsRNA (22). These data argue that RNAi inheritance in *C. elegans* is directed by a dominantly acting extragenic agent. Similarly, paramutation in plants acts dominantly and requires the RdRP mediator of paramutation 1 (MOP1), indicating that paramutation in maize is mediated by the RNAi machinery (25). These data show that RNAi is heritable in both plants and worms; however, the molecular nature of the RNAi inheritance signal and the mechanism(s) by which this signal is transmitted and maintained across generations are not known.

Here we show that RNAi in *C. elegans* induces the heritable expression of siRNAs and the heritable deposition of H3K9me chromatin marks in the progeny of animals exposed to dsRNA. In addition, we show that the nuclear RNAi pathway acts in inheriting progeny to maintain heritable RNAi silencing, siRNA expression, and H3K9me.

Results

Nuclear RNAi Maintains RNAi Silencing in the Progeny of Animals Exposed to dsRNA. We asked whether the nuclear RNAi pathway contributed to RNAi inheritance by testing animals defective for nuclear RNAi for their ability to inherit RNAi silencing. First, we built strains that harbored mutations in the nuclear RNAi factors *nrde-1*, *nrde-2*, *nrde-3*, or *nrde-4* and also expressed a transgenic copy of GFP driven by the *sur-5* promoter (*sur-5p::gfp*). *sur-5p::gfp* expresses GFP in all *C. elegans* cells, beginning in the later stages of embryogenesis and during all stages of larval development (26). As expected, WT animals exposed to *gfp*

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[1]To whom correspondence should be addressed. E-mail: sgkennedy@wisc.edu.

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dsRNA silenced *gfp* expression (Fig. 1*A*). The F1 progeny of animals exposed to *gfp* dsRNA also exhibited *gfp* silencing, demonstrating that RNAi silencing was heritable (Fig. 1 *A* and *B*). Heritable *gfp* silencing was seen in embryonic-, larval-, and adult-stage inheriting progeny (Fig. 1 *A* and *B*). F2 progeny did not exhibit *gfp* silencing. *nrde*(−) animals exposed directly to *gfp* dsRNA exhibited *gfp* silencing (Fig. 1*A*). Embryonic-stage progeny of *nrde*(−) animals exposed to dsRNA inherited *gfp* silencing, indicating that the animals defective for nuclear RNAi are able to inherit *gfp* silencing signals (Fig. 1*B*). During larval development, however, heritable *gfp* silencing was relieved; *nrde*(−) inheriting larvae did not exhibit *gfp* silencing (Fig. 1*A* and Fig. S1). Thus, *nrde*(−) animals inherit *gfp* silencing in embryos but fail to maintain *gfp* silencing during larval development.

These data suggest that nuclear RNAi promotes the maintenance of heritable silencing signals in the progeny of animals exposed to dsRNA. Consistent with this idea, we found that *nrde*(−) animals inherited silencing directed toward a gene that functions during early embryonic development but not toward a gene that functions later, during larval development. For instance, the *dpy-11* gene encodes a thioredoxin-like protein expressed in the hypodermal cells of larval-stage animals (27). RNAi targeting the *dpy-11* gene was heritable; *dpy-11* RNAi induced a dumpy (Dpy) phenotype in F1 larval progeny of animals exposed to *dpy-11* dsRNA (Fig. 1*C*). F2 progeny did not exhibit *dpy-11* silencing. F1 *nrde*(−) inheriting progeny did not exhibit a Dpy phenotype, indicating that the nuclear RNAi pathway is required for heritable *dpy-11* silencing (Fig. 1*C*). *pos-1* encodes a CCCH finger protein that is required for early embryonic cell-fate decisions (28). *pos-1* RNAi induces embryonic arrest in the F1 embryos of animals exposed to *pos-1* dsRNA (29). *nrde*(+) and *nrde*(−) animals respond similarly to *pos-1* RNAi, indicating that the nuclear RNAi pathway is not required for heritable *pos-1* silencing (Fig. 1 and Fig. S2). Thus, nuclear RNAi is not required for heritable silencing of a gene that functions during early embryogenesis but is required for heritable silencing of a gene that functions later during larval development. Altogether, these data indicate that nuclear RNAi maintains heritable RNAi silencing in the progeny of animals exposed to dsRNA (hereafter referred to as "RNAi maintenance").

Ago NRDE-3 Promotes RNAi Maintenance by Acting in the Nuclei of Inheriting Progeny. Nuclear RNAi could promote RNAi maintenance by acting in animals exposed directly to dsRNA or by

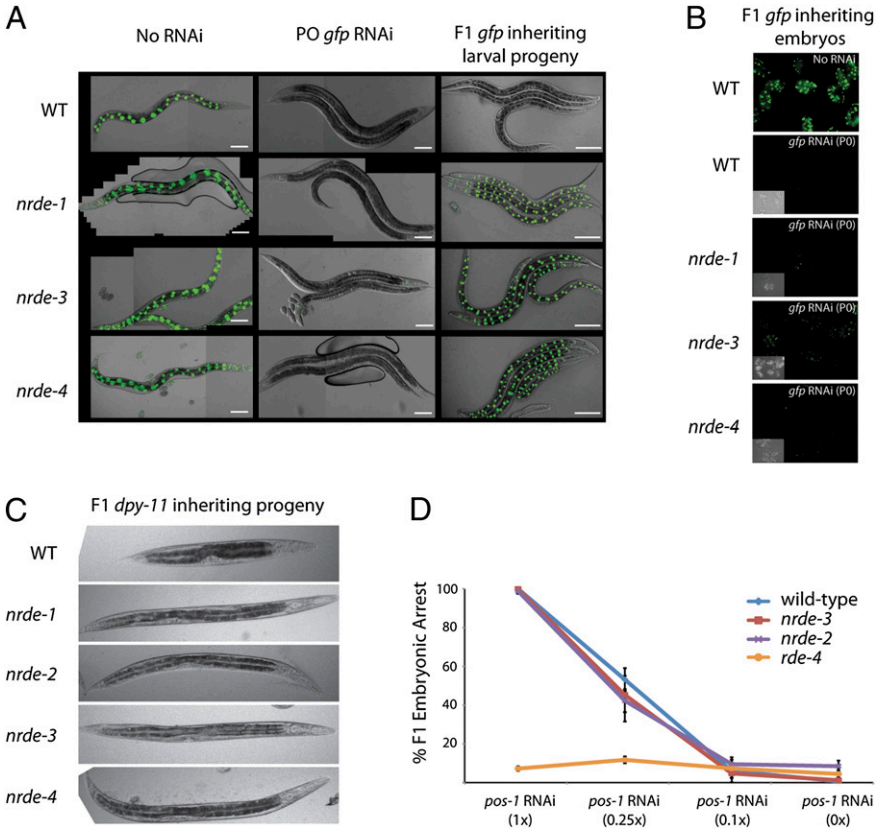

Fig. 1. Nuclear RNAi maintains heritable gene silencing. (*A*) Animals of the indicated genotypes expressing *sur-5p::gfp* were fed *Escherichia coli* expressing ± *gfp* dsRNA. A similar feeding RNAi approach is used for the remainder of the RNAi experiments in this article. Overlaid florescent and light images of representative adult animals not exposed to *gfp* dsRNA (column 1) or exposed to *gfp* dsRNA (column 2) are shown. Column 3 shows embryos from animals in column 2 that were isolated by hypochlorite treatment and grown in the absence of *gfp* dsRNA. Overlaid florescent and light images of larval-stage 3 (L3) progeny are shown. In all conditions, >98% of animals exhibited phenotypes similar to that shown in images. See Fig. S1 for *nrde-2 gfp* RNAi inheritance data. The following genotypes were used in *A–D*: *nrde-1*(*gg088*), *nrde-2*(*gg091*), *nrde-3*(*gg066*), and *nrde-4*(*gg129*). Unless otherwise indicated, these same *nrde* alleles are used for the remainder of the experiments in this article. (*B*) Florescent image of embryos obtained by hypochlorite treatment of animals treated ± *gfp* dsRNA. (*C*) Animals were exposed to *dpy-11* dsRNA. F1 progeny were grown in the absence of *dpy-11* dsRNA. Images show light microscopy of representative L3 progeny. In all conditions, >98% of animals exhibited phenotypes similar to that shown in images. (*D*) Animals of the indicated genotypes were grown on *pos-1* dsRNA expressing bacteria or *pos-1* dsRNA expressing bacteria diluted to the indicated concentration with *E. coli* not expressing *pos-1* dsRNA. Animals were allowed to lay a brood overnight (in the absence of dsRNA), and, 24 h later, the percentage of these F1 embryos that hatched were scored ($n = 4 \pm$ SD). *rde-4* is required for the initiation of RNAi silencing (22). The *rde-4* allele used was *ne301*. See Fig. S2 for *nrde-1* and *nrde-4* exposed to *pos-1* dsRNA data.

acting in their progeny. To address this issue, we exposed *nrde-3* (+/−) heterozygous animals to *dpy-11* dsRNA and scored the progeny of these animals for *dpy-11* RNAi inheritance and *nrde-3* genotype. We found that *nrde-3*(+/+) offspring inherited *dpy-11* silencing and *nrde-3*(−/−) offspring failed to inherit *dpy-11* silencing (Fig. 2*A*). These results show that NRDE-3 acts in the progeny of animals exposed to dsRNA to promote RNAi maintenance.

NRDE-3 escorts siRNAs from the cytoplasm to the nucleus (16). We asked whether the nuclear localization and the siRNA binding ability of NRDE-3 were required for RNAi maintenance. *nrde-3* encodes a bipartite nuclear localization signal (NLS) that is required for NRDE-3 to localize to the nucleus. A mutant variant of NRDE-3 harboring two point mutations in the *nrde-3* NLS [termed NRDE-3(*NLS)] expresses similarly to WT NRDE-3, associates with siRNAs similarly to WT NRDE-3, but fails to localize to the nucleus (16). NRDE-3(PAZ*) is a mutant variant of NRDE-3 that harbors point mutations in the Piwi/Argonaute/Zwille (PAZ) domain of NRDE-3, rendering NRDE-3(*PAZ) unable to bind siRNAs (16). WT NRDE-3 rescued the *dpy-11* RNAi inheritance defect associated with *nrde-3*(−) animals (Fig. 2*B*). NRDE-3(*NLS) (unable to localize to nucleus) and NRDE-3(*PAZ) (unable to bind siRNAs), however, failed to rescue *nrde-3*(−) (Fig. 2*B*). These data indicate that NRDE-3 must bind siRNAs and localize to the nucleus to promote RNAi maintenance.

RNAi Induces the Heritable Expression of siRNAs, Which Bind NRDE-3. The Ago NRDE-3, which binds siRNAs, acts in inheriting progeny to promote RNAi maintenance. We asked whether, after RNAi, siRNA were present in inheriting progeny and whether NRDE-3 associated with these siRNAs. We detected a >3,000-fold increase in NRDE-3–associated *dpy-11* siRNAs in animals exposed to *dpy-11* RNAi (Fig. 3*A*). *dpy-11* siRNAs were also detected in F1 inheriting progeny. F1 embryos expressed *dpy-11* siRNAs at a level slightly below that seen in animals exposed to *dpy-11* dsRNA, and F1 larvae expressed *dpy-11* siRNAs at a level similar to that seen in animals exposed to *dpy-11* dsRNA (Fig. 3*A*). We also isolated total RNA from the progeny of animals exposed to *dpy-11* dsRNA and detected *dpy-11* siRNAs (Fig. S3). We used three controls to ensure that our *dpy-11* siRNA assay was specifically detecting *dpy-11* siRNAs. First, an independent *dpy-11* siRNA assay also detected NRDE-3–associated *dpy-11* siRNAs in inheriting progeny (Fig. S3). Second, RDE-1 is an Ago that is required for the production of the majority of siRNAs that are expressed in *C. elegans* after

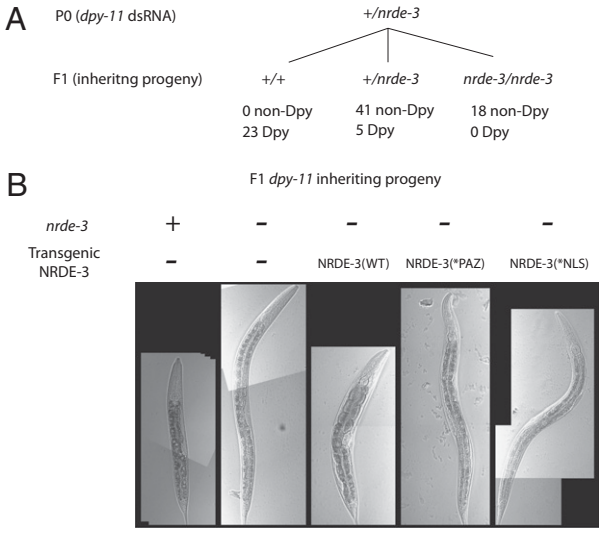

Fig. 2. The Ago NRDE-3 promotes RNAi inheritance by acting in the nuclei of inheriting progeny. (*A*) *unc-1* is 1.2 cM from *nrde-3*. *nrde-3*(*gg066*) males were crossed with *unc-1*(*e538*) hermaphrodites. Non-Unc (cross) progeny (defined as P0) were exposed to *dpy-11* dsRNA. F1 embryos were isolated and grown on *E. coli* not expressing *dpy-11* dsRNA. F1 progeny were scored for Dpy/non-Dpy phenotypes and Unc/non-Unc phenotypes. The *nrde-3* (*gg066*) genotype of the predicted *nrde-3*(+/+) and *nrde-3*(−/−) animals was verified by PCR analysis. *eri-1*(*mg366*) animals exhibit an enhanced RNAi phenotype (44). An *eri-1*(*mg366*) background was used to facilitate phenotypic analysis. Note that 41/46 *unc-1*/+ (*nrde-3*/+) F1 animals were not Dpy, indicating that *nrde-3*(*gg066*) is semidominant in this assay. (*B*) Animals of the indicated genotypes were exposed to *dpy-11* dsRNA. Representative light images of F1 L3 progeny are shown. In all conditions, >98% of animals exhibited phenotypes similar to that shown in images.

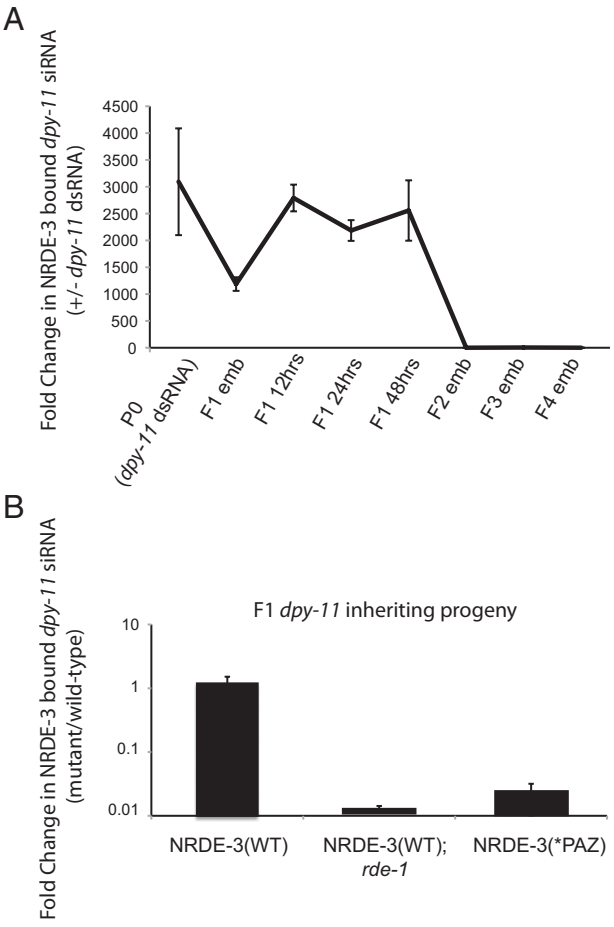

Fig. 3. RNAi induces the heritable expression of siRNAs, which bind NRDE-3. (*A*) NRDE-3::FLAG::GFP–expressing animals were exposed to dsRNA. NRDE-3::FLAG::GFP was immunoprecipitated with anti-FLAG antibody (Sigma) at the indicated times and from the indicated generations. NRDE-3 coprecipitating RNA was isolated, converted to cDNA, and quantified by quantitative real-time PCR coupled with a TaqMan probe set (*Materials and Methods*). From each sample, the amount of immunoprecipitated NRDE-3 was determined by quantitative Western blotting, and data were normalized to NRDE-3 levels and expressed as fold enrichment [(*dpy-11* RNAi)/(no RNAi)]. Signal detected in F4 embryos was arbitrarily defined as one ($n = 3 \pm$ SD). Similar results were observed when P0 embryos not exposed to *dpy-11* dsRNA were defined as one (Fig. S6). emb, embryo. (*B*) Animals of the indicated genetic backgrounds were exposed to *dpy-11* dsRNA, and NRDE-3 or NRDE-3(*PAZ) was immunoprecipitated from the L4 progeny of these animals. *dpy-11* siRNAs were quantified as described in *A*. The level of *dpy-11* siRNA bound to WT NRDE-3 in WT animals was defined as one. Data were normalized to the amount of NRDE or 3/NRDE-3(*PAZ) immunoprecipitated from each sample ($n = 3 \pm$ SEM). The *rde-1* allele used was *ne219*.

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RNAi (29, 30). In *rde-1*(−) animals, ∼100-fold less *dpy-11* siRNA associated with NRDE-3 (Fig. 3*B*). Third, NRDE-3(*PAZ) associated with ∼50-fold less *dpy-11* siRNAs than WT NRDE-3 did (Fig. 3*B*). These controls indicate that *dpy-11* siRNAs are expressed in inheriting progeny. Thus, *dpy-11* RNAi induces heritable *dpy-11* siRNA expression, and the Ago NRDE-3 associates with these heritable siRNAs.

Nuclear RNAi Promotes siRNA Perdurance in Inheriting Progeny. The Nrde factors are required for maintaining RNAi silencing in the progeny of animals exposed to dsRNA (Fig. 1). We asked whether the Nrde pathway influenced the heritability and/or perdurance of siRNAs in the progeny of animals exposed to dsRNA. Nrde mutant animals exposed directly to *dpy-11* dsRNA expressed NRDE-3–associated *dpy-11* siRNAs (Fig. 4*A*). F1 embryos from these Nrde animals inherited *dpy-11* siRNAs to a similar extent as WT animals did (Fig. 4). *dpy-11* siRNAs, however, were less abundant in larval-stage *nrde*(−) progeny, suggesting that siRNA expression is not maintained in *nrde*(−) inheriting progeny (Fig. 4). Similar nuclear RNAi-dependent siRNA maintenance was observed from total RNA preparations obtained from *dpy-11* inheriting progeny and when *gfp* siRNAs were quantified in the progeny of animals exposed to *gfp* dsRNA (Fig. S4). These data indicate that nuclear RNAi is not required for the inheritance of siRNAs but is required to maintain heritable siRNA expression in progeny of animals exposed to dsRNA.

Nuclear RNAi Directs Heritable H3K9me. In *S. pombe* and *C. elegans*, siRNAs direct the nuclear deposition of H3K9me marks at regions of the genome homologous to siRNAs (17, 18, 21, 31, 32). We used H3K9me chromatin immunoprecipitation (ChIP) to ask whether dsRNA-directed H3K9me was heritable. We observed an enrichment of H3K9me marks at the *dpy-11* locus in animals exposed to *dpy-11* dsRNA (Fig. 5*A*). In the progeny of these animals, we observed near-background levels of H3K9me during embryonic development (Fig. 5*A*). H3K9me marks reappeared after 24 h of F1 development and became more pronounced after 48 h of F1 development (Fig. 5*A* and Fig. S5). Interestingly, we found that H3K9me was significantly more pronounced in inheriting progeny than in animals exposed directly to dsRNA (Fig. 5*A*, Fig. S5, and *Discussion*). Previously, we showed that *dpy-11* siRNAs are expressed in inheriting embryos (Fig. 3*A*). Therefore, we conclude that siRNA-directed H3K9me marks are heritable but that siRNAs likely precede H3K9me.

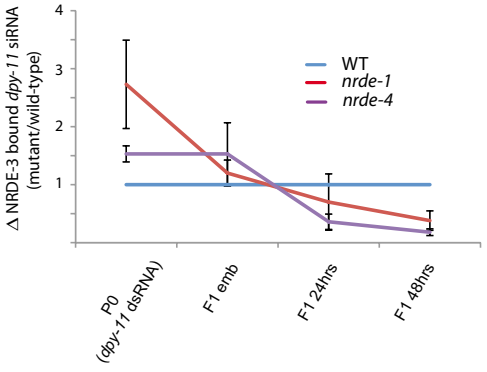

Fig. 4. Nuclear RNAi promotes siRNA perdurance in the inheriting generation. (*A*) Animals of the indicated genotypes expressing NRDE-3::FLAG::GFP were exposed to *dpy-11* dsRNA. NRDE-3 was immunoprecipitated at the indicated times and indicated generation, and *dpy-11* siRNAs were quantified as described in Fig. 3*A*. Data were normalized to the amount of NRDE-3 immunoprecipitated from each sample ($n = 3 \pm$ SEM). siRNA signal bound to WT NRDE-3 in WT worms was defined as one.

Finally, we asked whether the nuclear RNAi pathway promoted H3K9me inheritance. In *nrde-1*, *nrde-3*, and *nrde-4* animals, *dpy-11* RNAi did not induce heritable H3K9me (Fig. 5*B*). We conclude that nuclear RNAi is required for the establishment and/or maintenance of heritable H3K9me chromatin marks.

Discussion

Here we show that exposure of *C. elegans* to dsRNA results in the heritable transmission of siRNAs and H3K9me marks to progeny. The Ago NRDE-3 binds siRNAs in inheriting progeny and, acting in conjunction with the other components of the nuclear RNAi pathway, directs H3K9me at genomic sites targeted by RNAi in the previous generation. Finally, we show that nuclear RNAi maintains RNAi silencing and siRNA expression in the progeny of animals exposed to dsRNA.

We find that RNAi inheritance occurs normally in embryos of animals defective for nuclear RNAi; we observe *pos-1* and *gfp* RNAi inheritance in *nrde*(−) embryos (Fig. 1), and we detect normal levels of heritable siRNAs in *nrde*(−) embryos (Fig. 4). These data indicate that nuclear RNAi is not required for the initial inheritance of RNAi silencing signals or the initial gene-silencing events that occur in inheriting embryos. Over the course of development, however, animals defective for nuclear RNAi fail to maintain RNAi silencing and siRNA expression. How might nuclear RNAi promote RNAi maintenance? *S. pombe* that lack the H3K9 methyltransferase Clr4 express fewer siRNAs (21, 33). Conversely, *S. pombe* lacking the RNAi machinery fail to establish H3K9me (21, 34). Thus, a self-reinforcing

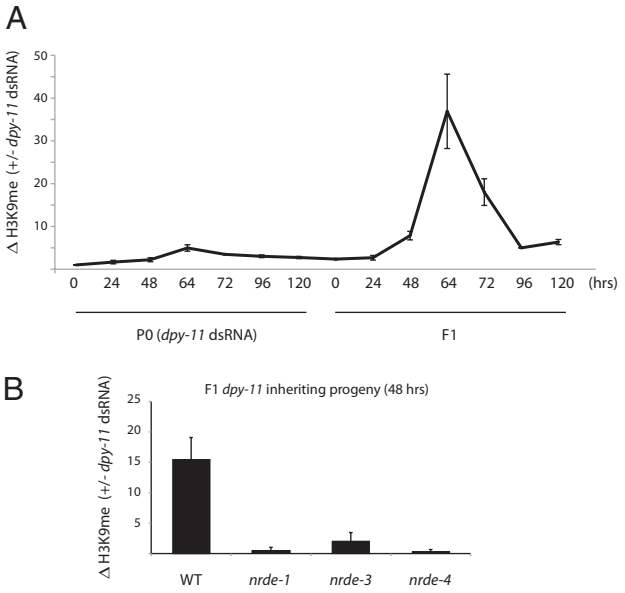

Fig. 5. Nuclear RNAi directs heritable H3K9me. (*A*) Animals were exposed to ± *dpy-11* dsRNA and subjected at the indicated times to ChIP of H3K9me. F1 embryos/animals were obtained from P0 animals exposed to *dpy-11* dsRNA for 70 h. Extracts were cross-linked with formaldehyde, and H3K9me was immunoprecipitated with anti-H3K9me antibody (Millipore). Coprecipitating DNA was quantified by quantitative PCR. Data were normalized to coprecipitating *eft-3* DNA. H3K9me levels in P0 animals (−RNAi) at the 0 time point was defined as one. Data are expressed as a ratio of H3K9me signal ± RNAi ($n = 3$ for 0–64 h; $n = 2$ for 72–120 h; data points ± SE). Animals from the 72-h, 96-h, and 120-h time points were grown on 5-fluoro-2′-deoxyuridine (F0503; Sigma) to prevent F1 (or F2) animals from interfering with the experiment. (*B*) Animals of the indicated genotypes were exposed to *dpy-11* dsRNA. L4 progeny (equivalent to 48-h time point) were subjected to H3K9me ChIP. Data were normalized to *eft-3* coprecipitating DNA, and *dpy-11* H3K9me signal in WT animals not exposed to RNAi was defined as one. Data are expressed as a ratio of H3K9me signal ± RNAi ($n = 3 \pm$ SEM).

loop of chromatin and RNAi machinery coordinately maintains siRNA expression and RNAi silencing in fission yeast. Here we show that disrupting the nuclear RNAi pathway in *C. elegans* blocks H3K9me inheritance and, concomitantly, causes a reduction in siRNA expression in inheriting progeny. (Note that our data do not differentiate between whether the nuclear RNAi pathway is required for the establishment and/or maintenance of H3K9me during RNAi inheritance.) Altogether, these data suggest that, similar to what has been seen in *S. pombe*, the *C. elegans* chromatin and RNAi machinery may form a self-reinforcing loop that coordinately maintains siRNA expression in the progeny of animals exposed to dsRNA.

We observed that the embryonic progeny of animals exposed to *dpy-11* dsRNA do not display *dpy-11* H3K9me marks but do express *dpy-11* siRNAs (Figs. 3*A* and 5*A*). Several questions arise from this observation. First, what is the primary silencing agent that is directly inherited from parental generations? Because we detect siRNAs in inheriting progeny before H3K9me, siRNAs are more likely to be the primary heritable agent than H3K9me marks are. Consistent with this idea, RNAi silencing in *C. elegans* has been shown to be heritable even in the absence of the gene toward which RNAi is targeted (22). NRDE-3 associates with 2° siRNAs produced by RdRPs acting upon mRNA templates (16). Thus, it is possible that the NRDE-3–associating 2° siRNAs are directly deposited into germ cells. These 2° siRNAs could engage currently unknown Agos (the worm genome encodes 27 Agos) to direct embryonic silencing. Later in development, these siRNAs could bind NRDE-3 to promote RNAi maintenance. Alternatively, another silencing signal could be deposited into germ cells that directs the subsequent production of NRDE-3 2° siRNAs. For instance, RNAi silencing is systemic in plants: silencing signals can spread from one cell to another. Primary ds siRNAs (produced by DICER-mediated cleavage of dsRNAs) are thought to be the active spreading agent in plants (35–37). It is possible that primary siRNAs are deposited into *C. elegans* germ cells and induce the synthesis of 2° siRNAs, which bind NRDE-3 to promote RNAi maintenance and H3K9me.

Another question that emerges from our data is why are H3K9me marks missing in inheriting embryos when siRNAs are clearly present? The transgenically expressed NRDE-3::GFP reporter is expressed in most, if not all, somatic cells beginning at the ~64-cell stage of development (16). Thus, one obvious explanation for the lack of H3K9me marks in early embryos is that NRDE-3 is not expressed in early embryos. In addition, NRDE-3 binds nascent transcripts that exhibit sequence homology to NRDE-3–associated siRNAs (16). *dpy-11* is not expressed during early embryogenesis, is expressed weakly during late embryogenesis, and is strongly expressed in hypodermal cells of larval-stage animals (27). Therefore, an alternative explanation for the lack of H3K9me marks in embryos is that NRDE-3 cannot efficiently localize to the *dpy-11* gene during embryogenesis because *dpy-11* is not robustly transcribed at this time. Later, when *dpy-11* becomes active, NRDE-3 can bind *dpy-11* pre-mRNA and direct H3K9me.

Interestingly, we find that H3K9me marks are more pronounced in the progeny of animals exposed to *dpy-11* dsRNA than in animals exposed directly to *dpy-11* dsRNA (Fig. 5*A* and Fig. S5). In Fig. 5*A* and Fig. S5, F1 embryos/animals were obtained from animals exposed to *dpy-11* dsRNA for 70 h. Thus, 48-h F1 animals were exposed to *dpy-11* silencing for a total of 118 h total (70 h in P0 and 48 h in F1). At 118 h after *dpy-11* RNAi, these F1 animals show a 35- to 40-fold elevation of H3K9me at *dpy-11*. In P0 animals, however, at 120 h after *dpy-11* RNAi exposure, we observe only a two- to threefold elevation in *dpy-11* H3K9me. These data suggest that the germ-line transmission of silencing (and not simply the time of exposure) may promote the ability of RNAi to induce H3K9me. In other words, inheriting progeny seem to "remember" the silencing event of the previous generation. What might this "memory" be? In the case of *dpy-11* RNAi inheritance, we detected similar levels of two NRDE-3–associated *dpy-11* siRNAs in both parents and progeny, suggesting that inheriting progeny do not simply express more *dpy-11* siRNAs (Fig. 3*B*). We cannot rule out the possibility, however, that levels of other *dpy-11* siRNAs that we did not measure differ between generations. Another possibility is that RNAi silencing signals in the germ line or early embryos (which are present in inheriting progeny but not in the previous generation) mark the *dpy-11* gene in such a way as to allow the more efficient coupling of siRNAs to H3K9me deposition later, during larval development. It will be of interest to further investigate the timing and extent of RNAi-directed chromatin modifications in the germ line and early-stage embryos.

The RNAi inheritance phenotypes described here were heritable for one generation; both *dpy-11* and *sur-5p::gfp* silencing were observed in F1 progeny. We did not observe evidence of silencing in F2 progeny. In addition, we detected heritable siRNAs in F1 progeny but not F2 progeny (Fig. 3*A*). In *C. elegans*, RNAi inheritance can last for more than one generation (22–24). For instance, GFP expressed in the germ line can be silenced for >20 generations by *gfp* dsRNA (24). Why are some RNAi silencing signals propagated across multiple generations whereas other silencing signals persist for only one generation? We note that the three previous reports showing more than one generation of RNAi inheritance in *C. elegans* used dsRNAs that targeted germ-line–expressed genes (22–24). In this article, we show that the somatically expressed *dpy-11* and *gfp* genes are silenced for one generation. It seems reasonable to speculate, then, that dsRNA-based silencing of germ-line–expressed genes induces multigenerational RNAi inheritance, and dsRNA targeting somatically expressed genes induces RNAi inheritance for one generation. Why might this be the case? RNAi silencing acts at the level of RNA; in worms, RNA serves as a template for RdRPs that amplify RNAi silencing signals (11, 12). Therefore, germ-line transcription may be required to provide the mRNA template for RdRP-mediated propagation of RNAi silencing signals across multiple generations. In the case of somatically expressed genes, lack of germ-line expression might deprive the germ-line RNAi machinery of the templates needed for long-term germ-line silencing. It will be important to ask whether nuclear RNAi is required for maintaining germ-line RNAi inheritance and, if not, to identify the germ-line factors that maintain epigenetic signals across multiple generations.

An important remaining question is why do systems for RNAi inheritance and RNAi maintenance exist? To date, *C. elegans* RNAi inheritance/maintenance has only been observed after the experimental introduction of dsRNA. Thus, it remains possible that this phenomenon only occurs in laboratory settings. Alternatively, *C. elegans* might use the RNAi machinery to transmit information between generations. There are many known examples of environmental signals that regulate transcription of specific genes (38). Antisense transcription appears to be widespread (39, 40). Perhaps environmental signals also regulate antisense transcription. If so, environmental signals might reasonably be expected to induce the production of dsRNAs, which could trigger RNAi, and influence gene expression programs across generations. Such a system might be used to prepare progeny for the harsh environmental conditions being experienced by the parents. Cataloguing small RNA populations in the progeny of animals exposed to differing environmental stimuli should be sufficient to test this admittedly speculative idea. Finally, many multigenerational epigenetic phenotypes have been documented (41–43). It will be of interest to test whether small RNAs are the vectors for transmitting and maintaining any of these multigenerational epigenetic signals.

Materials and Methods

Strains. The strains used were as follows: N2, (YY160) nrde-1(gg088), (YY186) nrde-2(gg091), (YY158) nrde-3(gg066), (YY453) nrde-4(gg129), (WM27) rde-1 (ne219), (YY174) ggIS1[nrde-3p::3xflag::gfp::nrde-3], (YY225) rde-1(ne219); ggIS1, (YY228) nrde-1(gg088); ggIS1, (YY229) nrde-2(gg091); ggIS1, (YY454) nrde-4(gg129); ggIS1, (YY525) ggIS17[nrde-3p::3xflag::gfp::nrde-3(*paz)], MH1046 [*unc-119*(*ed3*); *him-5*(*e1490*); *kuEx74*(*pTG96*; *unc-119*$^{+}$; *pBluescript-SK*)], (YY518) nrde-1(gg088); kuEx74, (YY519) nrde-2(gg091); kuEx74, (YY520) nrde-3(gg066); kuEx74, (YY521) nrde-4(gg129); kuEx74, (WM49) rde-1(ne301), (YY472) eri-1(mg366); unc-1(e538), (YY189) eri-1(mg366); nrde-3(gg066), (YY179) nrde-3(gg066); ggIS1, (YY265) nrde-3(gg066); ggIS17, (YY298) nrde-3 (gg066); ggIS24[nrde-3p::3xflag::gfp::nrde-3(*nls)].

RNAi. Hypochlorite-prepped embryos were placed onto HT115 bacteria expressing dsRNA against *pos-1*, *gfp*, or *dpy-11*. To obtain F1 animals from worms grown on *gfp* or *dpy-11* bacteria, embryos were hypochlorite-prepped and placed onto either an OP50 or HT115 bacteria expressing the L4440 empty vector food source. The *dpy-11* and *pos-1* bacterial clones were taken from the Ahringer RNAi library.

Genetic Cross. YY189 males were crossed with YY472 hermaphrodites. Adult hermaphrodites were placed on *dpy-11* RNAi plates; adult progeny from these worms were then moved to OP50, scored for the Dpy phenotype, and then genotyped for *nrde-3*(*gg066*) (Fig. 2*A*).

Total RNA Preparation. Total RNA was prepped as described previously (10).

siRNA Isolation. NRDE-3 immunoprecipitation was performed as described previously (10) with the following modifications. Larval-stage worms were dounced 10 times before sonication. FLAG::NRDE-3 protein was immunoprecipitated with anti-FLAG M2 antibody (A2220; Sigma).

TaqMan. *dpy-11* and GFP small RNAs were reverse-transcribed with microRNA reverse-transcription kits (4366596; Applied Biosystems). cDNA was quantified by RT-PCR using custom small RNA TaqMan assays from Applied Biosystems (4398987; assay IDs CSLJH0V, CSS060B, and CST946J). TaqMan assays were designed to quantify the following small RNA sequences: GFP, 5′-GUGUCCAAGAAUGUUUCCAUCUU-′3; Dpy-11-1, 5′-GUCAUUCUUGUCACGAGCUCCA-′3; and Dpy-11-2, 5′-GACAUCGAAAGUUUGAAGAAGA-′3.

ChIP. ChIP experiments were performed as described previously (10) with the following modifications. Larval-stage worms were dounced 12 times before sonication. Reverse cross-linking was performed at 75 °C. For 72-h, 96-h, and 120-h time points, 5-fluoro-2′-deoxyuridine was added to the agar plates at a concentration of 0.1 mg/mL. H3K9me3 antibody was from Upstate Biotechnology (07-523). Dpy-11 primers for real-time PCR were as follows: F1, 5′-TGTCTTCTCTTCACACCATAGGC-′3; R1, 5′-TCTGGAATTGACTTGTTTTATAGATG-′3; F2, 5′-TAATGTGTGTGCGTGTCTGC-′3; and R2, 5′-AGCGGAAGATTGAAAACGAA-′3.

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1. Bonasio R, Tu S, Reinberg D (2010) Molecular signals of epigenetic states. *Science* 330: 612–616.
2. Reik W, Dean W, Walter J (2001) Epigenetic reprogramming in mammalian development. *Science* 293:1089–1093.
3. Surani MA, Durcova-Hills G, Hajkova P, Hayashi K, Tee WW (2008) Germ line, stem cells, and epigenetic reprogramming. *Cold Spring Harb Symp Quant Biol* 73:9–15.
4. Arteaga-Vazquez MA, Chandler VL (2010) Paramutation in maize: RNA mediated trans-generational gene silencing. *Curr Opin Genet Dev* 20:156–163.
5. Fire A, et al. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391:806–811.
6. Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409:363–366.
7. Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 293:1146–1150.
8. Elbashir SM, Lendeckel W, Tuschl T (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* 15:188–200.
9. Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 404:293–296.
10. Tuschl T, Zamore PD, Lehmann R, Bartel DP, Sharp PA (1999) Targeted mRNA degradation by double-stranded RNA in vitro. *Genes Dev* 13:3191–3197.
11. Pak J, Fire A (2007) Distinct populations of primary and secondary effectors during RNAi in *C. elegans*. *Science* 315:241–244.
12. Sijen T, Steiner FA, Thijssen KL, Plasterk RH (2007) Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. *Science* 315:244–247.
13. Cogoni C, Macino G (1999) Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. *Nature* 399:166–169.
14. Dalmay T, Hamilton A, Rudd S, Angell S, Baulcombe DC (2000) An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* 101:543–553.
15. Smardon A, et al. (2000) EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. *Curr Biol* 10:169–178.
16. Guang S, et al. (2008) An Argonaute transports siRNAs from the cytoplasm to the nucleus. *Science* 321:537–541.
17. Guang S, et al. (2010) Small regulatory RNAs inhibit RNA polymerase II during the elongation phase of transcription. *Nature* 465:1097–1101.
18. Burkhart KB, et al. (2011) A pre-mRNA-associating factor links endogenous siRNAs to chromatin regulation. *PLoS Genet* 7:e1002249.
19. Volpe TA, et al. (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* 297:1833–1837.
20. Simon SA, Meyers BC (2011) Small RNA-mediated epigenetic modifications in plants. *Curr Opin Plant Biol* 14:148–155.
21. Moazed D (2009) Small RNAs in transcriptional gene silencing and genome defence. *Nature* 457:413–420.
22. Grishok A, Tabara H, Mello CC (2000) Genetic requirements for inheritance of RNAi in *C. elegans*. *Science* 287:2494–2497.
23. Alcazar RM, Lin R, Fire AZ (2008) Transmission dynamics of heritable silencing induced by double-stranded RNA in *Caenorhabditis elegans*. *Genetics* 180:1275–1288.
24. Vastenhouw NL, et al. (2006) Gene expression: Long-term gene silencing by RNAi. *Nature* 442:882.
25. Alleman M, et al. (2006) An RNA-dependent RNA polymerase is required for paramutation in maize. *Nature* 442:295–298.
26. Yochem J, Gu T, Han M (1998) A new marker for mosaic analysis in *Caenorhabditis elegans* indicates a fusion between hyp6 and hyp7, two major components of the hypodermis. *Genetics* 149:1323–1334.
27. Ko FC, Chow KL (2002) A novel thioredoxin-like protein encoded by the *C. elegans dpy-11* gene is required for body and sensory organ morphogenesis. *Development* 129:1185–1194.
28. Tabara H, Hill RJ, Mello CC, Priess JR, Kohara Y (1999) *pos-1* encodes a cytoplasmic zinc-finger protein essential for germline specification in *C. elegans*. *Development* 126:1–11.
29. Tabara H, et al. (1999) The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* 99:123–132.
30. Tabara H, Yigit E, Siomi H, Mello CC (2002) The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in *C. elegans*. *Cell* 109: 861–871.
31. Verdel A, et al. (2004) RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* 303:672–676.
32. Noma K, et al. (2004) RITS acts in *cis* to promote RNA interference-mediated transcriptional and post-transcriptional silencing. *Nat Genet* 36:1174–1180.
33. Motamedi MR, et al. (2004) Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. *Cell* 119:789–802.
34. Sugiyama T, Cam H, Verdel A, Moazed D, Grewal SI (2005) RNA-dependent RNA polymerase is an essential component of a self-enforcing loop coupling heterochromatin assembly to siRNA production. *Proc Natl Acad Sci USA* 102:152–157.
35. Carlsbecker A, et al. (2010) Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. *Nature* 465:316–321.
36. Dunoyer P, et al. (2010) Small RNA duplexes function as mobile silencing signals between plant cells. *Science* 328:912–916.
37. Molnar A, et al. (2010) Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. *Science* 328:872–875.
38. Yosef N, Regev A (2011) Impulse control: Temporal dynamics in gene transcription. *Cell* 144:886–896.
39. Katayama S, et al.; RIKEN Genome Exploration Research Group; Genome Science Group (Genome Network Project Core Group); FANTOM Consortium (2005) Antisense transcription in the mammalian transcriptome. *Science* 309:1564–1566.
40. Willingham AT, Gingeras TR (2006) TUF love for "junk" DNA. *Cell* 125:1215–1220.
41. Seong K-H, Li D, Shimizu H, Nakamura R, Ishii S (2011) Inheritance of stress-induced, ATF-2-dependent epigenetic change. *Cell* 145:1049–1061.
42. Ng SF, et al. (2010) Chronic high-fat diet in fathers programs β-cell dysfunction in female rat offspring. *Nature* 467:963–966.
43. Carone BR, et al. (2010) Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. *Cell* 143:1084–1096.
44. Kennedy S, Wang D, Ruvkun G (2004) A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans*. *Nature* 427:645–649.

Supporting Information

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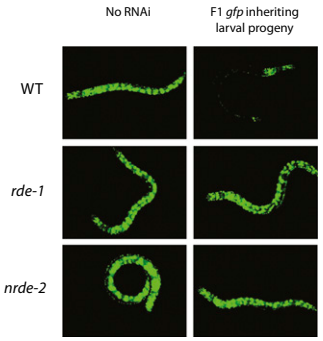

Fig. S1. NRDE-2 is required for RNAi maintenance. Animals of the indicated genotypes were exposed to *gfp* dsRNA. Florescent image are of larval-stage 3 (L3) progeny. The *nrde-2* allele used was *nrde-2*(*gg091*), and the *rde-1* allele used was *ne219*.

	pos-1 dsRNA (Embryonic Arrest)
control	+
rde-1(ne219)	-
nrde-1(gg088)	+
nrde-4(gg129)	+

Fig. S2. NRDE-1 and NRDE-4 are not required for *pos-1* RNAi inheritance. Animals of the indicated genotypes were fed bacteria expressing undiluted *pos-1* dsRNA. F1 progeny were scored as "+" (100% unhatched progeny) or "−" (0% unhatched progeny). A total of 50–200 animals were scored blind in each trial (n = 5). The genetic background was *eri-1*(*mg366*).

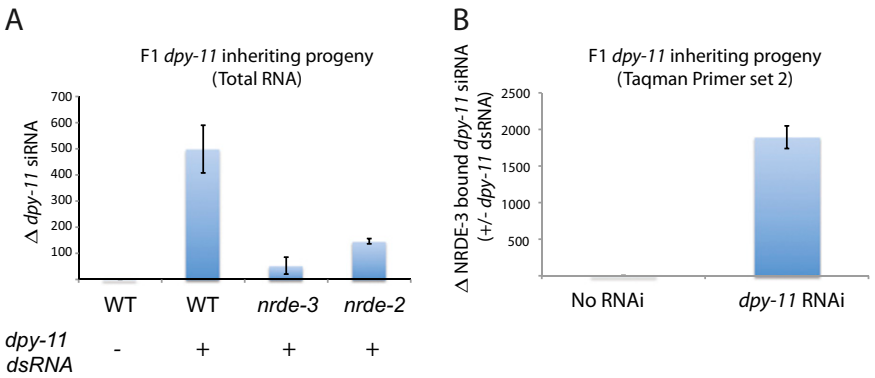

Fig. S3. dsRNA induces heritable siRNA expression in progeny. (*A*) Animals of indicated genotypes were exposed to *dpy-11* dsRNA. RNA was isolated from the L3/L4 progeny of these animals, and *dpy-11* siRNAs were measured as described in Fig. 3*A*. For each sample, data were normalized to *eft-3* mRNA levels (n = 3 ± SEM). (*B*) An independent *dpy-11* TaqMan primer set was used to quantify heritable NRDE-3–associating siRNAs, which were isolated as described in Fig. 3*A* (n = 3 ± SEM).

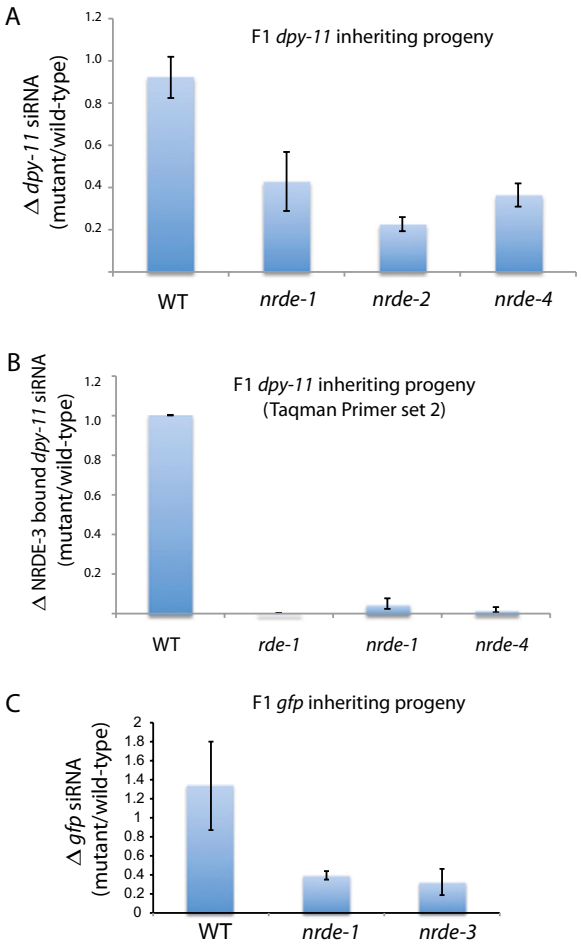

Fig. S4. NRDE-1, NRDE-2, and NRDE-4 are required for maintenance of siRNA expression in the progeny of animals exposed to dsRNA. (*A*) Animals of indicated genotypes were exposed to *dpy-11* dsRNA. RNA was isolated from the L3/L4 progeny of these animals, and *dpy-11* siRNAs were measured as described in Fig. 3*A*. For each sample, data were normalized to *eft-3* mRNA levels, and *dpy-11* siRNA levels in WT animals were defined as one ($n = 3 \pm$ SEM). (*B*) The 48-h siRNA preparations from Fig. 4 were assayed with a second TaqMan probe set. Signal from WT animals was defined as one ($n = 3 \pm$ SEM). (*C*) *sur-5::gfp*–expressing animals of the indicated genotype were exposed to *gfp* dsRNA. RNA was isolated from L4 progeny of these animals. A *gfp* TaqMan probe set was used in quantitative real-time PCR reactions to quantify *gfp* siRNAs. Data were normalized to *eft-3* mRNA levels ($n = 3 \pm$ SEM).

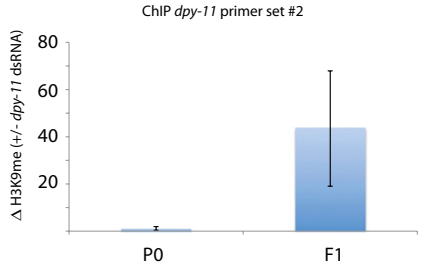

Fig. S5. *dpy-11* RNAi induces heritable histone 3 lysine 9 methylation (H3K9me). ChIP samples from F1 64-h and P0 64-h time points from Fig. 5*A* were queried with an independent *dpy-11* primer set. Data were normalized to *eft-3* DNA levels ($n = 3 \pm$ SEM).

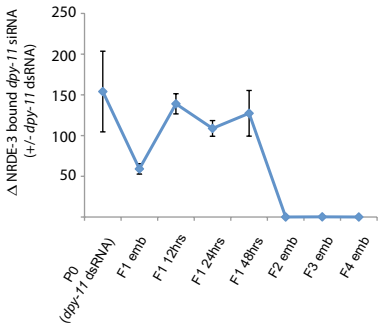

Fig. S6. These data are the same as those shown in Fig. 3*A*. In this analysis, the *dpy-11* siRNA signal from animals not exposed to *dpy-11* dsRNA was defined as one.