Double-stranded RNA made in *C. elegans* neurons can enter the germline and cause transgenerational gene silencing

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An animal that can transfer gene-regulatory information from somatic cells to germ cells may be able to communicate changes in the soma from one generation to the next. In the worm *Caenorhabditis elegans*, expression of double-stranded RNA (dsRNA) in neurons can result in the export of dsRNA-derived mobile RNAs to other distant cells. Here, we show that neuronal mobile RNAs can cause transgenerational silencing of a gene of matching sequence in germ cells. Consistent with neuronal mobile RNAs being forms of dsRNA, silencing of target genes that are expressed either in somatic cells or in the germline requires the dsRNA-selective importer SID-1. In contrast to silencing in somatic cells, which requires dsRNA expression in each generation, silencing in the germline is heritable after a single generation of exposure to neuronal mobile RNAs. Although initiation of inherited silencing within the germline requires SID-1, a primary Argonaute RDE-1, a secondary Argonaute HRDE-1, and an RNase D homolog MUT-7, maintenance of inherited silencing is independent of SID-1 and RDE-1, but requires HRDE-1 and MUT-7. Inherited silencing can persist for >25 generations in the absence of the ancestral source of neuronal dsRNA. Therefore, our results suggest that sequence-specific regulatory information in the form of dsRNA can be transferred from neurons to the germline to cause transgenerational silencing.

epigenetics | mobile RNAs | soma to germline | Weismann barrier

The germline is separated from the rest of the body, or soma, during early development in most animals, consistent with the suggestion that environmental effects on soma throughout the lifetime of an animal cannot influence inheritance through the germline (1). However, some environmental changes can cause effects that last for three or more generations, even in the apparent absence of changes in the genotype (reviewed in ref. 2). These transgenerational epigenetic effects are presumably initiated either by direct changes within the ancestral germline or by the transfer of information from ancestral somatic cells to the ancestral germline. It is difficult to distinguish between these possibilities because complex ancestral changes that affect subsequent generations, such as diet (3–5) or endocrine disruption (6), perturb many genes in many tissues in ways that are as yet unclear. Manipulating the activity of a single gene in specific tissues and across generations can help distinguish between these possibilities. Such specific inactivation of a single gene can be achieved by using double-stranded RNA (dsRNA) to trigger RNA interference (RNAi) in the worm *Caenorhabditis elegans* (7).

As in most animals, the *C. elegans* germline is set aside early in development—after four cell divisions (8). Gene silencing initiated through RNAi-related mechanisms within the *C. elegans* germline can last for many generations (9–13). Such transgenerational silencing can be triggered by both injected dsRNA (14–16) and ingested dsRNA (16–19). However, both injection and ingestion can deliver dsRNA directly into the fluid-filled body cavity that surrounds the germline, without entry into the cytosol of any somatic cell (20, 21). Thus, it remains unknown whether somatic cells in *C. elegans* can export signals for delivery into the germline to cause transgenerational gene silencing.

The transfer of gene-specific information from one somatic tissue to another somatic tissue during RNAi has been observed in *C. elegans* (22). Such intertissue transfer of gene-regulatory information appears to occur through the transport of forms of dsRNA called mobile RNAs (23). Entry of these mobile RNAs into the cytosol requires the dsRNA-selective importer SID-1 (22, 24, 25). Consequently, when dsRNA is expressed in a variety of somatic tissues such as the gut, muscles, or neurons, SID-1–dependent silencing of genes of matching sequence is observed in other somatic tissues (20). Because gene silencing by mobile RNAs from neurons appears to be stronger than that by mobile RNAs from other somatic tissues (20), we examined whether neurons export mobile RNAs that can enter the germline to cause transgenerational gene silencing.

Here, we show that neuronal mobile RNAs can enter both somatic and germ cells to trigger gene silencing. Although silencing in somatic tissues is not detectably inherited despite multigenerational exposure to neuronal mobile RNAs, silencing in the germline is inherited for many generations after a single generation of exposure to neuronal mobile RNAs.

Results

Neuronal Mobile RNAs Can Enter Most Somatic Tissues and the Germline. Genetic analyses suggest that neuronal mobile RNAs are forms of dsRNA (23). Mobile RNAs generated from dsRNA expressed in neurons against the muscle gene *unc-22* can enter muscle cells through the dsRNA importer SID-1 and cause *unc-22* silencing (23). To examine silencing of a gene expressed in multiple

Significance

The germline, which produces sperm or oocyte, is separated from other cells that generate the rest of the body, the soma, during early development in most animals. Somatic cells experience and respond to the environment in each generation, and it is unknown whether they can transmit information to the germline for inheritance into subsequent generations. We found that neurons of the worm *Caenorhabditis elegans* can transmit double-stranded RNA to the germline to initiate transgenerational silencing of a gene of matching sequence. To our knowledge, these results demonstrate for the first time that a somatic tissue of an animal can have transgenerational effects on a gene through the transport of double-stranded RNA to the germline.

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tissues by a single source of dsRNA in neurons, we used animals that expressed cytosolic *gfp* (*Peft-3::gfp*) in all somatic tissues and *gfp–dsRNA* in all neurons (*Prgef-1::gfp–dsRNA*) (Fig. 1). GFP expression was detectably reduced in most somatic tissues (with the notable exception of the pharynx) in the presence of *Prgef-1::gfp–dsRNA* (Fig. 1*A*, *Top* vs. Fig. 1*A*, *Middle*), and this silencing was enhanced in the absence of the exonuclease ERI-1 (Fig. S1), consistent with ERI-1 acting to inhibit silencing by imported neuronal mobile RNAs (23). Silencing in all somatic tissues, even in the *eri-1(−)* background, was lost upon removal of the mobile RNA importer SID-1 (Fig. 1*A*, *Bottom*, and Fig. S1), suggesting that all observed silencing was due to mobile RNAs made in neurons.

To test whether the germline is susceptible to silencing by mobile RNAs, we examined silencing of GFP expression in animals that express *gfp* in the germline (*Pmex-5::gfp*) and neuronal mobile RNAs from a *Prgef-1::gfp–dsRNA* transgene. Like most somatic cells, the germline was susceptible to silencing by neuronal mobile RNAs, and the silencing was predominantly dependent on SID-1 (Fig. 1*B*). The silencing was sequence-specific and did not occur in animals with transgenic expression of a co-injection marker (Fig. S2*A*) or in animals with transgenic expression of *unc-22* dsRNA in neurons (Fig. S2*B*). Furthermore, silencing, as detected by the loss of GFP fluorescence within the germline (Fig. S3*A*), was correlated with a reduction in *gfp* mRNA levels (Fig. S3*B*). Consistent with mobile RNAs that are imported into the germline being forms of *gfp*–dsRNA, silencing was strongly dependent on the dsRNA importer SID-1 and the primary Argonaute RDE-1 that acts on short dsRNA (26), but independent of the RNA-dependent RNA polymerase RRF-1 that generates single-stranded secondary small RNAs in somatic cells (27) (Fig. S3*C*). The residual silencing observed in *sid-1(−)* and *rde-1(−)* animals may reflect additional *sid-1*– and *rde-1*–independent gene silencing mechanisms that can act in the germline (9–12). Because silencing of a germline target due to dsRNA expression in neurons is greatly reduced in the absence of SID-1 (Fig. 1*B*, *Bottom*, and Fig. S3*C*), we conclude that SID-1–dependent neuronal mobile RNAs can enter the germline.

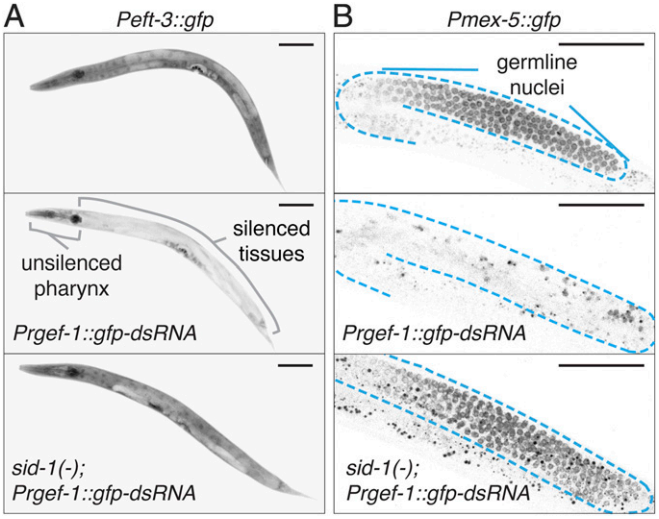


Fig. 1. Neuronal mobile RNAs can cause gene silencing in most somatic tissues and in the germline. (*A*) Representative fourth larval (L4)-staged animals that express GFP (black) in somatic tissues (*Peft-3::gfp*) in a wild-type (*Top*) background and animals that in addition express dsRNA in neurons against *gfp* (*Prgef-1::gfp–dsRNA*) in wild-type (*Middle*) or *sid-1(−)* (*Bottom*) backgrounds are shown. Silenced tissues and unsilenced pharynx are indicated (*Middle*). Detectable silencing was observed in 100% of wild-type animals (n = 135) and 0% of *sid-1(−)* animals (n = 115). (Scale bars, 50 μm.) Also see Fig. S1. (*B*) Representative L4-staged animals that express GFP (black) in the germline (*Pmex-5::gfp*; outlined in cyan) in a wild-type (*Top*) background and animals that in addition express *Prgef-1::gfp–dsRNA* in wild-type (*Middle*) or *sid-1(−)* (*Bottom*) backgrounds are shown. Because of the long exposure time required to acquire these images, variable and irregular autofluorescence due to gut granules was also detected. Detectable silencing was observed in 87% of wild-type animals (n = 54) and 27% of *sid-1(−)* animals (n = 59). (Scale bars, 50 μm.)

Together, our results suggest that neuronal mobile RNAs can enter most somatic tissues as well as the germline to silence genes of matching sequence. Because injection of in vitro-synthesized dsRNA can generate signals that are inherited in *C. elegans* (7, 14–16), our observations raise the possibility that neuronal mobile RNAs may also generate such inherited signals upon silencing a gene within the germline or upon silencing a gene in other somatic cells.

Silencing in the Germline by Neuronal Mobile RNAs Is Inherited for Many Generations. Injected or ingested dsRNA can cause transgenerational gene silencing of germline genes in *C. elegans* (10, 14–18). However, both forms of dsRNA delivery could result in the direct entry of dsRNA into the germline without entry into the cytoplasm of somatic cells. Ingested dsRNA is transcytosed across the gut into the body cavity that surrounds the germline (20, 21), and it is difficult to avoid spillage of injected dsRNA into the body cavity. These experimental considerations suggest that to test the possibility of somatic tissues initiating transgenerational gene silencing, it is necessary to express silencing triggers within somatic tissues and examine gene silencing within the germline. Although induction using heat shock of a transgene that encodes a viral genome in somatic tissues caused transgenerational silencing in *C. elegans* (13), such heat-shock induction also leads to expression within the germline (figure S5 in ref. 28). Therefore, because of the inherent difficulty in ensuring lack of expression within the germline from transgenes, only germline silencing that is reduced in the absence of the dsRNA importer SID-1 (Fig. 1*B*, *Bottom*, and Fig. S3*C*) can be interpreted as being caused by mobile RNAs.

To determine whether neuronal mobile RNAs that are imported into the germline can cause transgenerational silencing, we examined animals that lack the DNA for *gfp–dsRNA* but whose ancestors expressed neuronal dsRNAs. Because stable transgenic lines of extrachromosomal arrays are generated in *C. elegans* two generations after an animal [parental generation (P0)] is transformed with DNA (i.e., in the F2 generation) (29), we examined the silencing of GFP expression in wild-type animals of the F3 generation that lacked the *gfp*–dsRNA transgene and in their descendents (Fig. 2*A*, *Left*). Animals that lack the *gfp–dsRNA* transgene can be identified by the loss of a red fluorescent co-injection marker, the DNA for which is expected to be incorporated along with the DNA for *gfp–dsRNA* into a single extrachromosomal array upon cotransformation. All F3 animals without the extrachromosomal array showed silencing of GFP expression in the germline (Fig. 2*A*, *Right*). Inherited silencing due to the ancestral production of neuronal mobile RNAs persisted for >25 subsequent generations, despite unbiased passaging of worms from one generation to the next (Fig. 2*A*, *Right*, and Fig. S4). Consistent with the loss of the *gfp–dsRNA* transgene in animals that lack fluorescence from the co-injection marker, we failed to detect the *gfp–dsRNA* transgene in the DNA of worms that lacked the co-injection marker after 35 cycles of PCR amplification (Fig. 2*B*). These results suggest that neuronal mobile RNAs imported into the germline can initiate gene silencing that lasts for many generations in the absence of the ancestral source of neuronal dsRNA.

Transgenerational Silencing by Neuronal Mobile RNAs Has Distinct Genetic Requirements for Initiation and Maintenance. Although transgenerational silencing is reliably observed by using multiple transgenic sources of neuronal mobile RNAs (Fig. S4), the

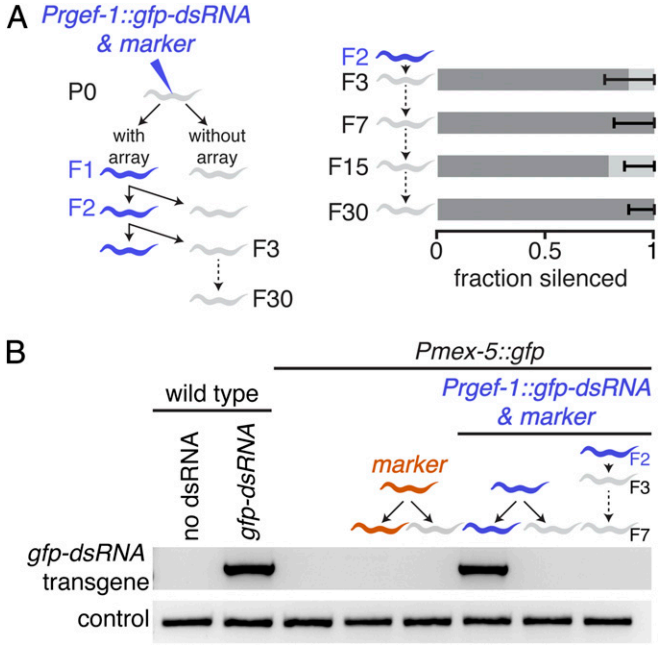


Fig. 2. Neuronal mobile RNAs can cause transgenerational silencing of a germline gene. (*A*) Inherited silencing in the germline lasts for >25 generations after the source of neuronal mobile RNAs is lost. (*A*, *Left*) *Pmex-5::gfp* animals (P0) were injected with constructs to express neuronal mobile RNAs (*Prgef-1::gfp–dsRNA*) along with a co-injection marker (*Pmyo-2::DsRed*) to generate F2 transgenic lines (blue worm). (*A*, *Right*) The proportions of animals that all lack fluorescence from the co-injection marker (gray worm) but that show either strong (dark gray bars) or weak (light gray bars) silencing in the F3 generation and in successive generations (F4–F30) were determined. Error bars indicate 95% CI and $n > 14$ L4-staged animals for each generation. Also see Fig. S4. Dark gray bars and light gray bars are as in Fig. S3C. (*B*) Animals that lack the co-injection marker also lack the *gfp–dsRNA* transgene. Genomic DNA from wild-type animals (no dsRNA), from wild-type animals that express *Prgef-1::gfp–dsRNA*, from *Pmex-5::gfp* animals, and from *Pmex-5::gfp* animals that either have or whose ancestors had extrachromosomal transgenes [i.e., *Pmex-5::gfp* animals that in addition express the co-injection marker alone (*marker*; orange worm) or along with *Prgef-1::gfp–dsRNA* (*Prgef-1::gfp–dsRNA & marker*; blue worm) or apparently lack these extrachromosomal transgenes (gray worm) but that were derived from ancestors that expressed these transgenes] were analyzed. Although the control gene was detected in all cases, a PCR product for the *gfp–dsRNA* transgene was detected only in wild-type animals with *gfp–dsRNA* and in *Pmex5::gfp* animals with *gfp–dsRNA* as evidenced by fluorescence from the co-injection marker.

number of generations that show silencing varied from one transgenic line to another, possibly due to differences in the levels of expression of dsRNA in different transgenic lines. To facilitate comparison of transgenerational silencing across multiple genetic backgrounds and to expose animals to mobile RNAs in defined generations, we chose a single extrachromosomal transgenic line that expresses neuronal mobile RNAs against *gfp* in wild-type animals and crossed it into animals that express *gfp* in the germline. This experimental scheme was then used to determine the genetic requirements for the initiation and maintenance of transgenerational gene silencing.

Using this experimental scheme, we found that exposure of a germline target gene to neuronal mobile RNAs for a single generation was sufficient to cause transgenerational silencing (Fig. 3*A*). Specifically, when animals with *Pmex-5::gfp* and animals with *Prgef-1::gfp–dsRNA* were mated, the F1 cross progeny that inherited the *Prgef-1::gfp–dsRNA* transgene could initiate transgenerational silencing. This silencing persisted for many generations, despite the loss of the source of neuronal mobile RNAs in the F2 generation (Fig. 3*A*).

To test whether a gene is required for germline silencing by neuronal mobile RNAs, we used the same experimental scheme as above, but with animals that also had a mutation in the gene being tested (Fig. 3*B*). For example, to test the requirement for *sid-1*, we mated *sid-1* null mutants [*sid-1(−)*] that express *Pmex-5::gfp* with *sid-1(−)* animals that express *Prgef-1::gfp–dsRNA* and examined silencing in *sid-1(−)* animals of a later generation that express both *Pmex-5::gfp* and *Prgef-1::gfp–dsRNA*. Germline

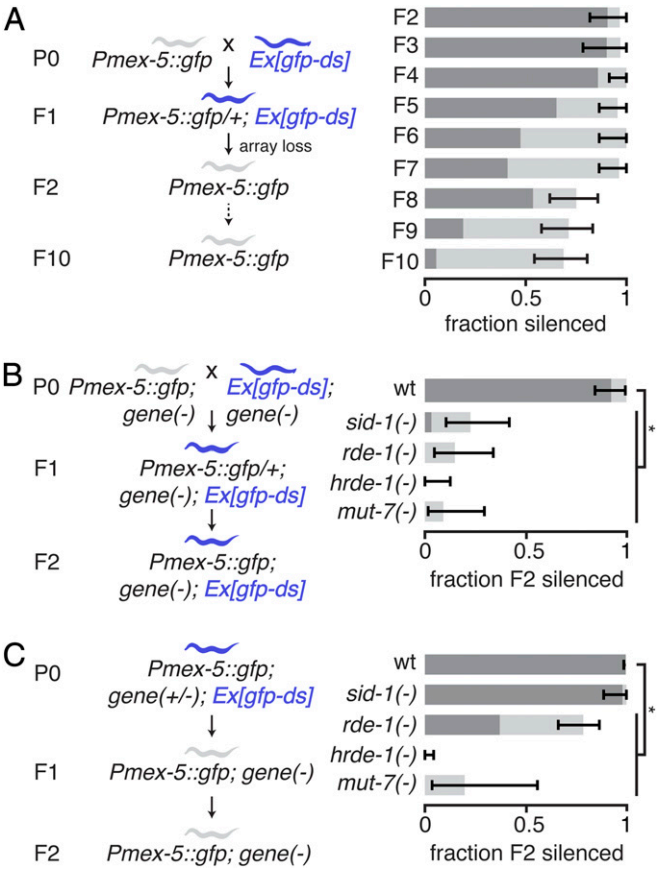


Fig. 3. Neuronal mobile RNAs have distinct requirements for the initiation and maintenance of transgenerational silencing. (*A*) Expression of neuronal mobile RNAs for one generation is sufficient to initiate multigenerational silencing. *Pmex-5::gfp* animals were crossed with animals that express neuronal dsRNA from an extrachromosomal array (*Ex[gfp–ds]*) and the proportions of animals that lack the extrachromosomal array (gray worm) but that show either strong (dark gray bars) or weak (light gray bars) silencing in the F2 generation and in successive generations (F3–F10) were determined. The loss of *Ex[gfp–ds]* was determined by the loss of the fluorescent co-injection marker. (*B*) Initiation of silencing by neuronal mobile RNAs requires *sid-1*, *rde-1*, *hrde-1*, and *mut-7*. Wild-type (wt), *sid-1(−)*, *rde-1(−)*, *hrde-1(−)*, or *mut-7(−)* animals that all express *Pmex-5::gfp* were mated with animals of identical genetic backgrounds that all express neuronal dsRNA (*Ex[gfp–ds]*), and the silencing in descendants that had both *Pmex-5::gfp* and *Ex[gfp–ds]* was measured as in *A*. (*C*) Maintenance of germline gene silencing by neuronal mobile RNAs requires HRDE-1 and MUT-7, but not SID-1 or RDE-1. Wild-type (wt), *sid-1(+/−)*, *rde-1(+/−)*, *hrde-1(+/−)*, or *mut-7(+/−)* animals that all had both *Pmex-5::gfp* and *Ex[gfp–ds]* were allowed to have progeny, and the silencing in wild-type (wt), *sid-1(−)*, *rde-1(−)*, *hrde-1(−)*, or *mut-7(−)* grand progeny animals that all had *Pmex-5::gfp* but that all lacked *Ex[gfp–ds]* was measured as in *A*. The analyzed grand progeny were progeny of animals that also lacked *Ex[gfp–ds]*. Error bars indicate 95% CI. $*P < 0.05$. $n > 19$ L4-staged animals, except for *mut-7(−)* animals in *C*, where $n = 10$ L4-staged animals. Dark gray bars and light gray bars are as in Fig. S3C.

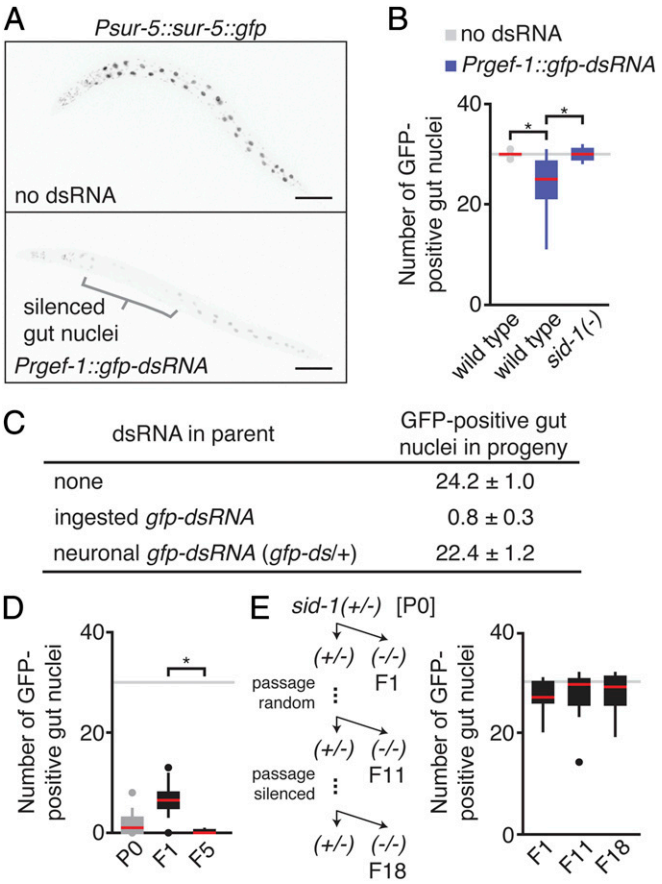


Fig. 4. Silencing of a somatic gene by neuronal mobile RNAs in parents is not detectably inherited by progeny. (*A*) Neuronal mobile RNAs can silence GFP expression in gut cells. Representative L4-staged animals that express GFP (black) in all somatic cells (*Psur-5::sur-5::gfp*) (*Upper*) or that in addition express dsRNA in all neurons (*Prgef-1::gfp–dsRNA*) (*Lower*) are shown. Brackets indicate strongly silenced gut nuclei. (Scale bars, 50 μm.) Also see Fig. S6. (*B*) Double-stranded RNAs expressed in neurons against *gfp* require SID-1 to silence GFP expression in gut cells. The numbers of GFP-expressing gut nuclei were counted in wild-type animals that do not express dsRNA against *gfp* (no dsRNA; gray) and in wild-type, or *sid-1(–)* animals that express *Prgef-1::gfp–*dsRNA (blue). Gray line indicates average number of gut nuclei in L4-staged animals, $n > 19$ L4-staged animals, and red bar in box plots indicates median. $^*P < 0.05$ (Student's *t* test). (*C–E*) An enhanced RNAi background [*eri-1(–)*] was used to maximize the ability to detect inherited silencing. (*C*) Unlike silencing by ingested dsRNA, silencing by neuronal mobile RNAs is not detectably inherited by progeny. Numbers of GFP-positive gut nuclei in genetically identical progeny of animals that were not exposed to *gfp–dsRNA* (none) or that were exposed to ingested *gfp–dsRNA* or that had one copy of an integrated transgene that expresses *Prgef-1::gfp–dsRNA* (*gfp–ds/+*) were counted. Errors indicate SEM. (*D*) Unbiased passaging of worms for multiple generations can lead to small differences in gene silencing. Worms that express *Prgef-1::gfp–dsRNA* (P0) were passaged for five generations (F1–F5) by picking a random worm at each generation, and the numbers of GFP-positive gut nuclei in animals of each generation were determined (see Fig. S8 for additional data). Gray line, *n*, red bar, and asterisks are as in *B*, except for F5, which had $n = 8$ L4-staged animals. (*E*) SID-1 is required for silencing by neuronal mobile RNAs even after 17 generations of ancestral silencing by neuronal mobile RNAs. (*E*, *Left*) Schematic of experimental design to test the requirement for SID-1 in each generation for silencing by neuronal mobile RNAs. At each generation, the numbers of GFP-positive gut nuclei in *sid-1(–/–)* animals were counted, and heterozygous [*sid-1(+/–)*] siblings of any *sid-1(–/–)* animal (F1–F11) or heterozygous siblings of the most silenced *sid-1(–/–)* animal (F12–F18) were passaged. (*E*, *Right*) The extent of silencing in F1, F11, and F18 are shown (see Fig. S9 for additional data). Gray line, red bar, and *n* are as in *B*.

silencing using this experimental scheme also required SID-1 and RDE-1, in agreement with the results obtained for silencing by transgenic lines that were independently generated in mutant backgrounds (Fig. S3*C*). Thus, germline silencing due to neuronal mobile RNAs likely relies on the import of forms of dsRNA through SID-1 and subsequent processing by the primary Argonaute RDE-1 within the germline. Further processing within the germline leads to the production of secondary single-stranded small RNAs. These secondary small RNAs eventually cause gene silencing through mechanisms that require many proteins (reviewed in ref. 30), including the secondary nuclear Argonaute HRDE-1 (18) and the RNase D homolog MUT-7 (31). We found that both HRDE-1 and MUT-7 were required for silencing by neuronal mobile RNAs, suggesting that silencing within the germline is executed by secondary small RNAs and downstream genes. For all genes tested above, the source of neuronal mobile RNAs was present in the animals that were tested. Therefore, the lack of silencing in *sid-1(–)*, *rde-1(–)*, *hrde-1(–)*, and *mut-7(–)* animals reflects a requirement for the corresponding genes in the initiation of germline silencing by neuronal mobile RNAs.

The observed genetic requirements for silencing by neuronal mobile RNAs are distinct from those observed for silencing by ingested or injected dsRNA. Whereas the requirement for SID-1 and RDE-1 is in agreement with the requirement for these genes when silencing is triggered using ingested and injected dsRNA, the requirement for HRDE-1 and MUT-7 is in contrast to the HRDE-1–independent silencing observed in response to ingested dsRNA (ref. 18 and Fig. S5) and the MUT-7–independent silencing observed in response to injected dsRNA (14). These differences might reflect differences in the dosage of dsRNA delivered into the germline using the different methods or the differential engagement of silencing machinery by the different sources of dsRNA used to trigger gene silencing.

To test whether a gene is required for the maintenance of transgenerational silencing by neuronal mobile RNAs, we examined silencing in animals that had mutations in the gene but were descendants of ancestors that had a wild-type copy of the gene during exposure to neuronal mobile RNAs (Fig. 3*C*). For example, to test the requirement for *sid-1* in the maintenance of transgenerational silencing, we examined silencing in *sid-1* null mutants [*sid-1(–)*] that were grand progeny of *sid-1(+/–)* heterozygous animals that were exposed to *Prgef-1::gfp–dsRNA*. Grand progeny were examined for silencing instead of progeny because maternal deposition of mRNA or protein from heterozygous parents can complicate interpretation of results in *sid-1(–)* progeny. We observed silencing in the *sid-1(–)* grand progeny of *sid-1(+/–)* heterozygous animals that were exposed to *Prgef-1::gfp–dsRNA,* which suggests that SID-1 is not required for the maintenance of transgenerational silencing. Similar experiments with null mutants of *rde-1*, *hrde-1*, and *mut-7* revealed that RDE-1, like SID-1, is dispensable for the maintenance of transgenerational silencing, but HRDE-1 and MUT-7 are required for the maintenance of transgenerational silencing.

In summary, our results suggest a model where mobile RNAs exported from neurons enter the germline through SID-1 to cause RDE-1–, MUT-7–, and HRDE-1–dependent silencing in the parent, which is subsequently maintained through a MUT-7– and HRDE-1–dependent, but SID-1– and RDE-1–independent mechanism. Because HRDE-1 has been shown to use secondary small RNAs to guide trimethylation of the histone H3 on lysine 9 (H3K9me3) at genes of matching sequence (17, 18), our results suggest that the initiation and maintenance of transgenerational silencing by neuronal mobile RNAs is associated with the deposition of H3K9me3 marks on genes of matching sequence. Although the response to ingested or injected dsRNA strongly suggests that secondary small RNAs are inherited (14, 17, 19), it is possible that in response to neuronal mobile RNAs chromatin marks are inherited across generations. Furthermore, although silencing of somatic genes has been reported to be inherited for

a few generations when the silencing is triggered by using ingested dsRNA (17, 19), it is unclear whether silencing of a somatic gene by neuronal mobile RNAs is inherited and whether transgenerational silencing by neuronal mobile RNAs within the germline can spread to somatic cells.

Silencing in Somatic Cells by Neuronal Mobile RNAs Is Not Detectably Inherited. To measure silencing by neuronal mobile RNAs in somatic cells, we used animals that have two different integrated transgenes—one that expresses nuclear-localized GFP (*sur-5::gfp*) under the control of a promoter that drives expression in all somatic cells (*Psur-5*) and one that expresses *gfp–dsRNA* under the control of a promoter that drives expression in all neurons (*Prgef-1*). Silencing due to neuronal mobile RNAs made from the *Prgef-1::gfp–dsRNA* transgene results in a reduction in fluorescence of nuclear-localized GFP made from the *Psur-5::sur-5::gfp* transgene (Fig. 4*A*). This silencing can be most easily observed in the large intestinal cell nuclei, and counting the number of GFP-positive gut nuclei provides a reliable measure of silencing that correlates with reduction in *gfp* mRNA levels (Fig. S6 *A* and *B*). Wild-type animals with neuronal mobile RNAs had, on average, fewer GFP-positive gut nuclei than did animals without neuronal mobile RNAs (Fig. 4*B*; 24.2 vs. 29.9 GFP-positive gut nuclei; $P < 0.05$). Consistent with silencing by neuronal mobile RNAs, this silencing was abolished in *sid-1(−)* animals (Fig. 4*B*) and not observed in wild-type animals that were merely cocultured with animals that express neuronal mobile RNAs (Fig. S6*C*).

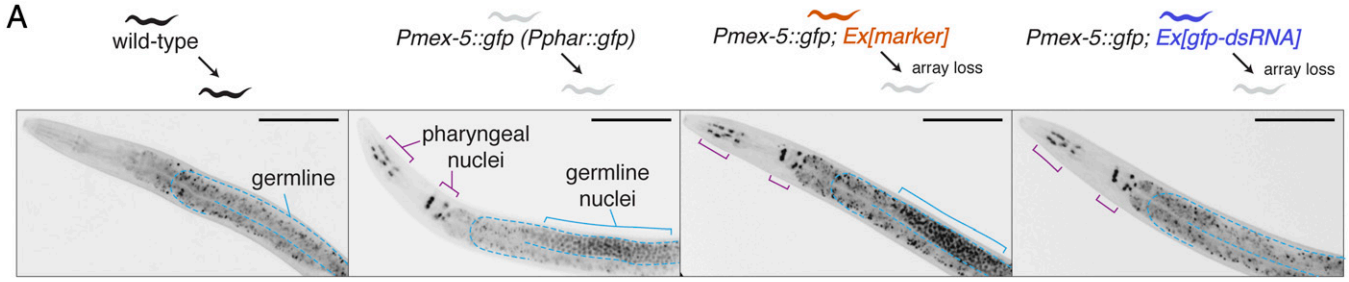
Because the initiation of inherited silencing occurs more frequently in animals that lack the exonuclease ERI-1 (16), we examined the ability of neuronal mobile RNAs to trigger inherited silencing in an *eri-1(−)* background, where trace amounts of dsRNA (32) and additional mobile RNAs (20) made from the multicopy *Psur-5::sur-5::gfp* transgene could also contribute to silencing. Using this sensitive genetic background, we did not detect inherited silencing by neuronal mobile RNAs (Fig. 4*C*), but did detect inherited silencing by ingested dsRNAs as reported earlier (Fig. 4*C* and ref. 19). We noticed a correlation between an increase in silencing by neuronal mobile RNAs and an increase in parental or ancestral exposure to mobile RNAs (Fig. S7). However, the increases in silencing were small and comparable to the small variations in silencing observed in successive generations when worms with *Prgef-1::gfp–dsRNA* of identical genotype were simply passaged (Fig. 4*D*). Furthermore, selection of the most silenced or most desilenced animal for four generations introduced marginal differences in silencing between the first and fifth generations (Fig. S8). Nevertheless, if marginal increases in inherited silencing accrued over many generations due to the presence of parental neuronal mobile RNAs, such inherited silencing might become independent of neuronal mobile RNAs and thus independent of SID-1 in later generations. However, we did not detect such SID-1–independent silencing, even after exposure to 17 generations of silencing by neuronal mobile RNAs (Fig. 4*E* and Fig. S9). The requirement for *sid-1* in every generation for silencing by neuronal mobile RNAs suggests that transport of neuronal mobile RNAs must occur in every generation to observe silencing in somatic cells.

The absence of robust inherited silencing by neuronal mobile RNAs of genes expressed in somatic cells could be either because somatic silencing does not generate signals for transmission to the next generation or because such signals require a template of matching sequence in the germline for stability. To test this latter possibility, we examined inherited somatic silencing by neuronal mobile RNAs in animals that express the target gene (*gfp*) in somatic cells as well as in the germline either from a single transgene (germline expression due to *Pmex-5::gfp* and pharyngeal expression due to an additional promoter in the *Pmex-5::gfp* transgene) (Fig. 5*A*) or from two separate transgenes (germline expression due to *Pmex-5::gfp* and gut expression due to *sur-5::gfp*) (Fig. 5*B*). In both cases, no inherited silencing was detected in somatic cells.

Together, our results suggest that neuronal mobile RNAs generate transgenerational silencing signals that have a strong effect on gene expression in the germline and a minimal effect, if any, on gene expression in somatic tissues.

Discussion

We found that neurons can transport forms of dsRNA into the germline to cause silencing that can last for many generations



B

P0 ♀	P0 ♂	F1 ♀	Number of GFP-positive gut nuclei in F1
Pmex-5::gfp; Ex[marker]	*Psur-5::sur-5::gfp*	*Pmex-5::gfp/+; Psur-5::sur-5::gfp/+*	28.9 ± 0.3
Pmex-5::gfp; Ex[gfp-dsRNA]	*Psur-5::sur-5::gfp*	*Pmex-5::gfp/+; Psur-5::sur-5::gfp/+*	29.1 ± 0.3

Fig. 5. Inherited silencing of a germline gene by neuronal mobile RNAs in the parent does not spread to the soma of the progeny. (*A*) Silencing of GFP expression within the germline by neuronal mobile RNAs does not cause detectable inherited silencing of GFP expressed from the same locus in the pharynx of progeny. *Pmex-5::gfp* animals and *Pmex-5::gfp* animals that in addition express an extrachromosomal source of either the co-injection marker (*Ex[marker]*) or neuronal mobile RNAs (*Ex[gfp–dsRNA]*) were passaged and L4-staged progeny that lack the extrachromosomal arrays were imaged under identical conditions. The pharyngeal expression of GFP (black) is from an additional uncharacterized promoter (*Pphar::gfp*) within the *Pmex-5::gfp* transgene and is absent in wild-type worms. Germline (outlined in cyan), GFP expression in the germline nuclei (cyan brackets), and GFP expression in pharyngeal nuclei (purple brackets) are indicated. (Scale bars, 50 μm.) (*B*) Silencing of GFP expression within the germline by neuronal mobile RNAs in the parent does not cause detectable inherited silencing of GFP expressed from a different locus in gut cells of progeny. *Pmex-5::gfp* animals (P0 hermaphrodite) that in addition expressed *Ex[marker]* or *Ex[gfp–dsRNA]* were crossed with *Psur-5::sur-5::gfp* animals (P0 male), and the numbers of GFP-positive gut nuclei were counted in the resulting F1 progeny that lack extrachromosomal arrays. Errors indicate SEM, and $n > 18$ L4-staged animals.

and that such transgenerational silencing is restricted to the germline with distinct genetic requirements for initiation and maintenance.

Mobile RNAs that enter the germline can provide an organism with the ability to transfer gene-specific regulatory information from somatic cells across generations and could be one mechanism by which the environment elicits transgenerational effects in animals. Although restricted to the germline, transgenerational silencing by mobile RNAs could underlie effects of the environment across generations in some cases. For example, expression of some genes within the germline can affect longevity (33), and transgenerational silencing of such genes might underlie the longevity that results from ancestral starvation in *C. elegans* (5). Thus, additional experiments are needed to determine the role of mobile RNAs, if any, in the transport of such experience-dependent information from somatic cells to subsequent generations in *C. elegans*.

The presence of a mammalian homolog of the dsRNA importer SID-1 that is also required for the uptake of dsRNAs into cells (34) raises the possibility that dsRNA generated from distant somatic cells—potentially in response to environmental influences—may be imported through SID-1 into the mammalian germline to trigger transgenerational epigenetic changes. Consistent with this possibility, small RNAs have been found in circulation in mammals (35); dsRNAs have been detected in mammalian germ cells (36–38); and injection of RNAs into the early mouse embryo can trigger epigenetic silencing (39). However, even if RNAs from somatic cells are transported to the germline in mammals, they may not always initiate transgenerational inherited effects because they have to escape mechanisms that reprogram epigenetic information in each generation (40). Additional studies are required to determine whether specific mechanisms prevent environmental influences from triggering transmission of information in the form of mobile RNAs from somatic cells to the germline.

Materials and Methods

All *C. elegans* strains were generated and maintained by using standard methods (41). Transgenic animals were generated by injecting PCR fragments or plasmids into the germline (29) of wild-type or mutant animals, and transgenes were also introduced into different genetic backgrounds through genetic crosses. Visible markers were used to balance *sid-1(–)* and to mark *Pmex-5::gfp*. Silencing of *Pmex-5::gfp* and *Peft-3::gfp* were measured by imaging under identical nonsaturating conditions using a Nikon AZ100 microscope. Silencing of *sur-5::gfp* was quantified by counting the number of gut nuclei that showed GFP expression above a fixed threshold of brightness. DNA of *Prgef-1::gfp–dsRNA* and *Pmex-5::gfp* transgenes were detected by using PCR in crosses and in inheritance experiments. Semiquantitative RT-PCR was used to determine relative mRNA levels by carrying out reverse transcription with gene-specific primers for *gfp* and *tbb-2* followed by <31 cycles of PCR. Inherited silencing by feeding RNAi (19) and statistical analyses (20) were performed as described earlier. Detailed procedures are provided in *SI Materials and Methods*.

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Supporting Information

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Strains Used. N2 wild type, AMJ2 *eri-1*(*mg366*) *nrIs20* (*Psur-5::sur-5::gfp*) *IV*; *sid-1*(*qt9*) *V*; *qtIs49* (*Prgef-1::gfp–dsRNA* and *pRF4*) *III*, AMJ154 *eri-1*(*mg366*) *nrIs20 IV*; *qtIs49 III*, AMJ265 *rrf-1*(*ok589*) *I*; *oxSi487* [*Pmex-5::gfp* and *unc-119*(+)] *II*; *unc-119*(*ed3*)*? III*, AMJ300 *nrIs20 IV*; *qtIs49 III*, AMJ301 *qtIs49 III*, AMJ310 *eri-1*(*mg366*) *nrIs20 IV*; *mIs10* (*Pmyo-2::gfp*) *V*, AMJ320 *nrIs20 IV*; *sid-1*(*qt9*) *V*; *qtIs49 III*, AMJ324 *oxSi487 II*; *unc-119*(*ed3*)*? III*; *sid-1*(*qt9*) *V* (generated by Julia Marré, A.M.J. laboratory, University of Maryland, College Park, MD), AMJ326 *oxSi487 II*; *unc-119*(*ed3*)*? III*; *rde-1*(*ne219*) *V* (generated by Julia Marré), AMJ349 *oxSi221* [*Peft-3::gfp* and *unc-119*(+)] *II*; *unc-119*(*ed3*)*? qtIs49 III*, AMJ361 *oxSi221 II*; *unc-119*(*ed3*)*? qtIs49 III*; *eri-1*(*mg366*) *IV*, AMJ363 *oxSi221 II*; *unc-119*(*ed3*)*? qtIs49 III*; *eri-1*(*mg366*) *IV*; *sid-1*(*qt9*) *V*, AMJ377 *oxSi487 II*; *unc-119*(*ed3*)*? III*; *eri-1*(*mg366*) *IV*, AMJ382 *oxSi221 II*; *unc-119*(*ed3*)*? III*; *eri-1*(*mg366*) *IV*, AMJ463 *oxSi487 II*; *unc-119*(*ed3*) *III ?*; *sid-1*(*qt9*) *V*; *jamEx131* (*pHC337* and *pHC448*), AMJ466 *oxSi487 II*; *unc-119*(*ed3*) *III*; *jamEx132* (*pHC337* and *pHC448*), AMJ502 *oxSi487 II*; *unc-119*(*ed3*) *III*; *jamEx145* (*pHC448*), AMJ471 *jamEx140* (*pHC337* and *pHC448*), AMJ533 *rde-1*(*ne219*) *V*; *jamEx140*, AMJ542 *sid-1*(*qt9*) *V*; *jamEx140*, AMJ577 *hrde-1*(*tm1200*) *III* [*4× outcrossed*], AMJ581 *oxSi487 dpy-2*(*e8*) *II*; *unc-119*(*ed3*)*? III* (generated by Samual Allgood, A.M.J. laboratory, University of Maryland, College Park, MD), AMJ585 *mut-7*(*ne4255*) *III* [*1× outcrossed*], AMJ586 *ox-Si487 dpy-2*(*e8*) *II*; *unc-119*(*ed3*)*? III*; *rde-1*(*ne219*) *V*, AMJ592 *hrde-1*(*tm1200*) *III*; *jamEx140*, AMJ593 *oxSi487 dpy-2*(*e8*) *II*; *unc-119*(*ed3*)*? III*; *sid-1*(*qt9*) *V*, AMJ595 *oxSi221 II*; *unc-119*(*ed3*)*? qtIs49 III*; *sid-1*(*qt9*) *V*, AMJ598 *oxSi487 dpy-2*(*e8*) *II*; *unc-119*(*ed3*)*? III*; *sid-1*(*qt9*) *V*; *jamEx140*, AMJ599 *oxSi487 dpy-2*(*e8*) *II*; *unc-119*(*ed3*)*? III*; *rde-1*(*ne219*) *V*; *jamEx140*, AMJ600 *ox-Si487 dpy-2*(*e8*) *II*; *unc-119*(*ed3*)*? III*; *jamEx140*, AMJ601 *oxSi487 dpy*(*e8*) *II*; *unc-119*(*ed3*)*? mut-7*(*ne4255*) *III*, AMJ602 *oxSi487 dpy-2*(*e8*) *II*; *unc-119*(*ed3*)*? hrde-1*(*tm1200*) *III*, AMJ603 *oxSi487 dpy-2*(*e8*) *II*; *unc-119*(*ed3*)*? III*; *qtEx136* (*Prgef-1::unc-22 dsRNA*) (1), AMJ620 *oxSi487 dpy-2*(*e8*) *II*; *unc-119*(*ed3*)*? hrde-1*(*tm1200*) *III*; *jamEx140 isolate 1*, AMJ621 *oxSi487 dpy-2*(*e8*) *II*; *unc-119*(*ed3*)*? hrde-1* (*tm1200*) *III*; *jamEx140 isolate 2*, AMJ628 *oxSi487 dpy-2*(*e8*) *II*; *unc-119*(*ed3*) *III?*; *jamEx147* (*pHC448*), AMJ639 *mut-7*(*ne4255*) *III*; *jamEx140*, AMJ643 *oxSi487 dpy-2*(*e8*) *II*; *unc-119*(*ed3*)*? mut-7*(*ne4255*) *III*; *jamEx140*, AMJ645 *oxSi487 dpy-2*(*e8*) *II*; *unc-119*(*ed3*)*? III*; *eri-1*(−) *IV*; *qtEx136*, EG6070 *oxSi221 II*; *unc-119*(*ed3*) *III*, EG6787 *oxSi487 II*; *unc-119*(*ed3*) *III*, GR1373 *eri-1*(*mg366*) *IV*, HC195 *nrIs20 IV*, HC196 *sid-1*(*qt9*) *V*, HC566 *nrIs20 IV*; *sid-1*(*qt9*) *V*, HC567 *eri-1*(*mg366*) *nrIs20 IV*, HC568 *eri-1*(*mg366*) *nrIs20 IV*; *sid-1*(*qt9*) *V*, HC780 *rrf-1*(*ok589*) *I* [*2× outcrossed*], and WM27 *rde-1*(*ne219*). The term dsRNA is used to refer to any form of base-paired RNA including hairpin RNA and double-stranded RNA for simplicity.

Transgenic Animals. Recombinant DNA fragments generated through overlap extension PCR using Expand Long Template polymerase (Roche) were purified by using the QIAquick PCR Purification Kit (Qiagen). Plasmids were purified by using the Plasmid mini kit (Qiagen). PCR products or plasmids were combined with a co-injection marker to transform *C. elegans* by using microinjection (2).

The plasmid pHC448 was used as a co-injection marker to express DsRed2 in the pharynx (1); pRF4 was used as a co-injection marker to express *rol-6(su1006)* (2); and pHC337 was used to express an inverted repeat of *gfp* in neurons (3), which is expected to generate a hairpin RNA (designated as *gfp–dsRNA*).

To express *gfp–dsRNA* in the neurons (*Prgef-1::gfp–dsRNA*): A 1:1 mixture of pHC337 (40 ng/μL) and pHC448 (40 ng/μL) in 10 mM Tris·HCl (pH 8.5) was microinjected into the wild-type strain N2 or into strains that express a single copy of *Pmex-5::gfp* in the germline as part of an operon (4) in wild-type [EG6787], *sid-1(−)* [AMJ324], *rde-1(−)* [AMJ326], *rrf-1(−)* [AMJ265], or *eri-1(−)* [AMJ377] backgrounds to generate three independent transgenic lines for each genetic background. In addition, pHC448 (40 ng/μL) in 10 mM Tris·HCl (pH 8.5) was injected into N2, EG6787, or AMJ377 to generate "no dsRNA" control transgenic lines.

Balancing *sid-1*. A transgene integrated on chromosome V [*mIs10* (*Pmyo-2::gfp*)] was used to balance *sid-1(qt9) V*. In Figs. 4*E* and S9, progeny of heterozygous *sid-1(qt9)/mIs10* animals were scored as homozygous mutants if they lacked GFP expression from *mIs10*. Tests using *rde-1* (~4.9 Mb from *sid-1*) suggest a low rate of recombination between *sid-1* and *mIs10*. Specifically, among the progeny of *rde-1(−)/mIs10* heterozygotes that lacked GFP expression from *mIs10*, ~94% (63/67) were found to be homozygous *rde-1(−)* by Sanger sequencing (determined by Edward Traver, A.M.J. laboratory, University of Maryland, College Park, MD).

Genotyping *Prgef-1::gfp–dsRNA*. The integrated transgene *qtIs49* was identified based on the cosegregation of the dominant Rol defect due to the pRF4 co-injection marker that is present along with *Prgef-1::gfp–dsRNA* (Figs. 1, 2*B*, 4, S1, and S6–S9). The DNA for *Prgef-1::gfp–dsRNA* in transgenes was detected by PCR using the primers GACTCAAGGAGGGAGAAGAG and GAGAGACCACATGGTCCTTC. A fragment of the *rrf-1* gene was amplified as a control by using the primers TGCCATCGCAGATAGTCC, TGGAAGCAGCTAGGAACAG, and CCGTGACAACAGACATTCAATC (Fig. 2*B*).

Feeding RNAi. Worms that were 24 h past the L4 stage were singled onto RNAi plates [NG agar plates supplemented with 1 mM IPTG (Omega) and 25 μg/mL carbenicillin (MP Biochemicals)] with 5 μL of *Escherichia coli* OP50. Twenty-four hours later, once eggs had been laid (typically, all OP50 was consumed by then), the parent worm was picked off the plate, and progeny were fed bacteria with a plasmid expressing *gfp*–dsRNA or with a control plasmid (L4440). For inherited silencing in somatic cells, 3 d later, gravid adults were treated with bleach (0.6% NaOCl and 1.5 M NaOH), and the silencing in progeny, which were protected by their egg shells, was measured when they reached the L4 stage by counting the number of GFP-positive gut nuclei (Fig. 4*C*) (adapted from ref. 5). For silencing in the germline, 2 d later, the germlines of L4-staged animals were imaged (Fig. S5).

Quantification of Silencing by Imaging. The silencing of GFP expressed from single-copy transgenes *oxSi221* (*Peft-3::gfp*) or *oxSi487* (*Pmex-5::gfp*) in different genetic backgrounds was compared by imaging L4-staged animals under nonsaturating conditions for the brightest strain being compared using a Nikon AZ100 microscope and a Photometrics Cool SNAP HQ^2 camera. When the extent of silencing was measured as a single proportion, 95% confidence intervals and *P* values for comparison of two proportions were calculated as described (3). For Fig. 1*B*, a Leica SP5X confocal microscope was used to measure GFP expression. All images being compared were adjusted identically by using Adobe Photoshop for display.

For Fig. 4*A*, GFP silencing in gut nuclei was measured by imaging L4-staged animals using a Nikon AZ100 microscope

under nonsaturating conditions and counting the number of GFP-positive gut nuclei that were above a fixed threshold of brightness. For all other figures, GFP silencing in gut nuclei was measured by counting the number of bright GFP-positive nuclei at a fixed magnification on an Olympus MVX10 fluorescent microscope. Comparison of this counting with measurements of fluorescence intensity (using Nikon AZ100 microscope and NIS Elements software) revealed that false calling of a GFP-positive nucleus as per the conservative criterion described in Fig. S6 occurred at most for one nucleus per animal. To measure fluorescence intensity in Fig. S6, an L4-staged worm was mounted on a slide after paralyzing the worm by using 3 mM levamisole (Sigma-Aldrich; catalog no. 196142). Fluorescence intensity in each nucleus of the worm was calculated by using the formula $A_n(I_n - I_b)$, where A_n = area of the nucleus; I_n = mean intensity within the nucleus; and I_b = mean intensity in an area of the slide outside the worm.

Semiquantitative RT-PCR. RNA from each strain was isolated by solubilizing 10 L4-staged animals in TRIzol (Ambion, catalog no. 15596-018) using three freeze–thaw cycles, followed by two cycles of chloroform extraction, and a final precipitation in 100% isopropanol with 10 μg of glycogen (Invitrogen, catalog no. 10814-010; Ambion, catalog no. AM9510) as a carrier. The RNA pellet was washed twice in 75% ethanol, resuspended in diethylpyrocarbonate-treated water, and treated with DNase I (New England Biolabs, catalog no. M0303S) for 60 min at 37 °C. The DNase was heat-inactivated for 10 min at 75 °C, and the concentration of RNA was measured (NanoVue). Within each biological repeat of the experiment, the same amount of total RNA was used as template for reverse transcription with SuperScript III (Invitrogen, catalog no. 18080-085) by using gene-specific primers designed to reverse-transcribe the sense strand (AGGGCAGATTGTGTGGACAG for *gfp* and TCGTCTTCGGCAGTTGCTTC for *tbb-2*). The resulting cDNA was used as a template for PCR (27 cycles for *sur-5::gfp*, 30 cycles for *Pmex-5::gfp*, and 30 cycles for *tbb-2*) using Taq polymerase and gene-specific primer pairs (AAGAGTGCCATGCCCGAAG and CCATCGCCAATTGGAGTATT for *gfp* and GACGAGCAAATGCTCAACG and TTCGGTGAACTCCATCTCG for *tbb-2*). Intensity of each band was calculated by using ImageJ (NIH) and the formula $A(I - I_b)$, where A = area of the band; I = mean intensity within the band; and I_b = mean intensity in an area of the gel just above the band. Pictures of the gels were linearly adjusted for display by using Adobe Photoshop without loss of data.

Genetic Crosses. Males with an extrachromosomal array were generated for each cross in Fig. 3 by mating hermaphrodites that express the extrachromosomal array in wild-type or mutant backgrounds with wild-type males or corresponding mutant males, respectively. For example, to generate *Ex[gfp–dsRNA]; sid-1(−)* males, *Ex[gfp–dsRNA]; sid-1(−)* hermaphrodites were mated with *sid-1(−)* males. For all crosses with *Pmex-5::gfp* animals in Fig. 3, *dpy-2(e8)* was used as a linked marker to identify the homozygosity of *Pmex-5::gfp*. Only 3% (6/200) of the Dpy progeny of *Pmex-5::gfp*/+ *dpy-2(e8)*/+ double-heterozygous parents were not homozygous for the *Pmex-5::gfp* transgene (determined by Samual Allgood).

1. Jose AM, Garcia GA, Hunter CP (2011) Two classes of silencing RNAs move between *Caenorhabditis elegans* tissues. *Nat Struct Mol Biol* 18(11):1184–1188.
2. Mello CC, Kramer JM, Stinchcomb D, Ambros V (1991) Efficient gene transfer in *C. elegans*: Extrachromosomal maintenance and integration of transforming sequences. *EMBO J* 10(12):3959–3970.
3. Jose AM, Smith JJ, Hunter CP (2009) Export of RNA silencing from *C. elegans* tissues does not require the RNA channel SID-1. *Proc Natl Acad Sci USA* 106(7):2283–2288.
4. Frøkjær-Jensen C, Davis MW, Ailion M, Jorgensen EM (2012) Improved Mos1-mediated transgenesis in *C. elegans*. *Nat Methods* 9(2):117–118.
5. Burton NO, Burkhart KB, Kennedy S (2011) Nuclear RNAi maintains heritable gene silencing in *Caenorhabditis elegans*. *Proc Natl Acad Sci USA* 108(49):19683–19688.

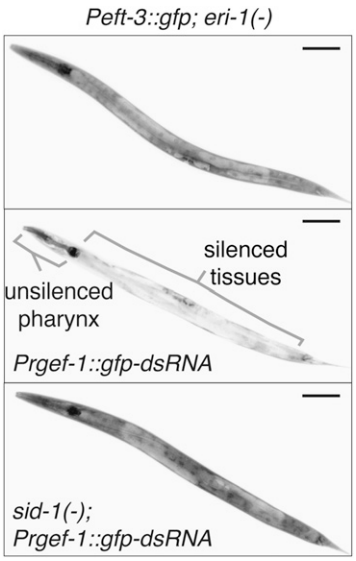

Fig. S1. Silencing by neuronal mobile RNAs is dependent on SID-1 even in an enhanced RNAi background. Representative L4-staged animals that express GFP (black) in all tissues (*Peft-3::gfp*) in an *eri-1(–)* (*Top*) background and animals that in addition express dsRNA in neurons against *gfp* (*Prgef-1::gfp–dsRNA*) in *eri-1(–)* (*Middle*) or *eri-1(–); sid-1(–)* (*Bottom*) backgrounds are shown. Detectable silencing was observed in 100% of *eri-1(–)* animals (n = 90) and 0% of *eri-1(–); sid-1(–)* animals (n = 88). Silenced tissues and unsilenced pharynx are indicated (*Middle*). (Scale bars, 50 μm.)

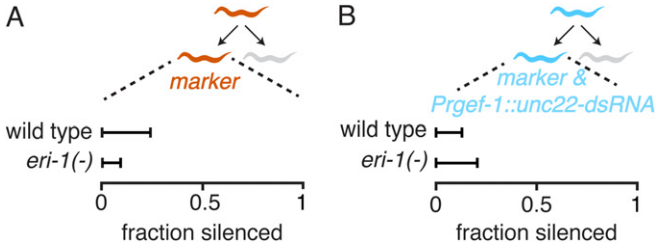

Fig. S2. Silencing in the germline by neuronal mobile RNAs is sequence-specific. (*A*) A repetitive transgene that lacks homology to *gfp* sequence does not cause silencing of GFP expression in the germline even in an *eri-1(–)* background. The proportions of animals that showed silencing of GFP expression in the germline were determined for *Pmex-5::gfp* or *Pmex-5::gfp; eri-1(–)* animals that express the co-injection marker alone (orange worm). (*B*) Neuronal mobile RNAs against the somatic gene *unc-22* do not cause silencing of GFP expression in the germline. The proportions of animals that showed silencing of GFP expression in the germline were determined for *Pmex-5::gfp* or *Pmex-5::gfp; eri-1(–)* animals that express neuronal *unc-22 dsRNA* (cyan worm). Error bars indicate 95% CI and $n > 20$ L4-staged animals.

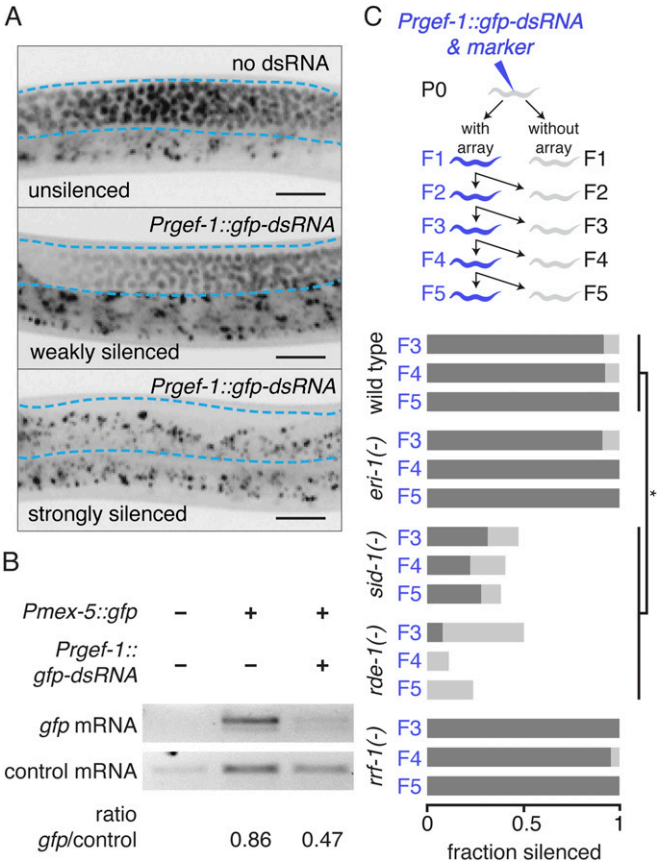

Fig. S3. Potent silencing by transgenes that express neuronal dsRNA requires neuronal mobile RNAs even when the transgenes are generated in animals that express the germline target gene. (*A*) Extent of germline silencing due to neuronal mobile RNAs can vary. Representative L4-staged animals that express GFP (black) from the *Pmex-5::gfp* transgene in the germline (outlined in cyan) (*Top*) and animals that in addition express dsRNA in neurons against *gfp* (*Prgef-1::gfp–dsRNA*) but show weak (*Middle*) or strong (*Bottom*) silencing are shown. Because of the long exposure time required for these images, variable and irregular autofluorescence of the gut granules was also detected. (Scale bars, 10 μm.) (*B*) Loss of GFP fluorescence in the germline is due to reduction in levels of *gfp* mRNA. Semiquantitative RT-PCR was used to detect *gfp* mRNA and *tbb-2* mRNA (control) in wild-type animals, *Pmex-5::gfp* animals, and *Pmex-5::gfp* animals that in addition express *Prgef-1::gfp–dsRNA*. The intensity of the *gfp* band was normalized to that of the *tbb-2* band in each sample. (*C*) Silencing of GFP expression in the germline by dsRNA expressed in neurons requires SID-1 and RDE-1, but not ERI-1 or RRF-1. Wild-type, *eri-1(–)*, *sid-1(–)*, *rde-1(–)*, or *rrf-1(–)* animals that express *Pmex-5::gfp* (P0 generation) were injected with constructs to express *Prgef-1::gfp–dsRNA* along with a co-injection marker (*Pmyo-2::DsRed*). For each genetic background, the proportions of worms with fluorescence from the co-injection marker (blue worm) that showed either strong (dark gray bars; as shown in *A*, *Bottom*) or weak (light gray bars; as shown in *A*, *Middle*) silencing of GFP expression in the germline were determined in the F3 (n = 11–24 L4-staged animals), F4 (n = 18–39 L4-staged animals), and F5 (n = 17–29 L4-staged animals) generations. *$P < 0.05$ (Student's t test).

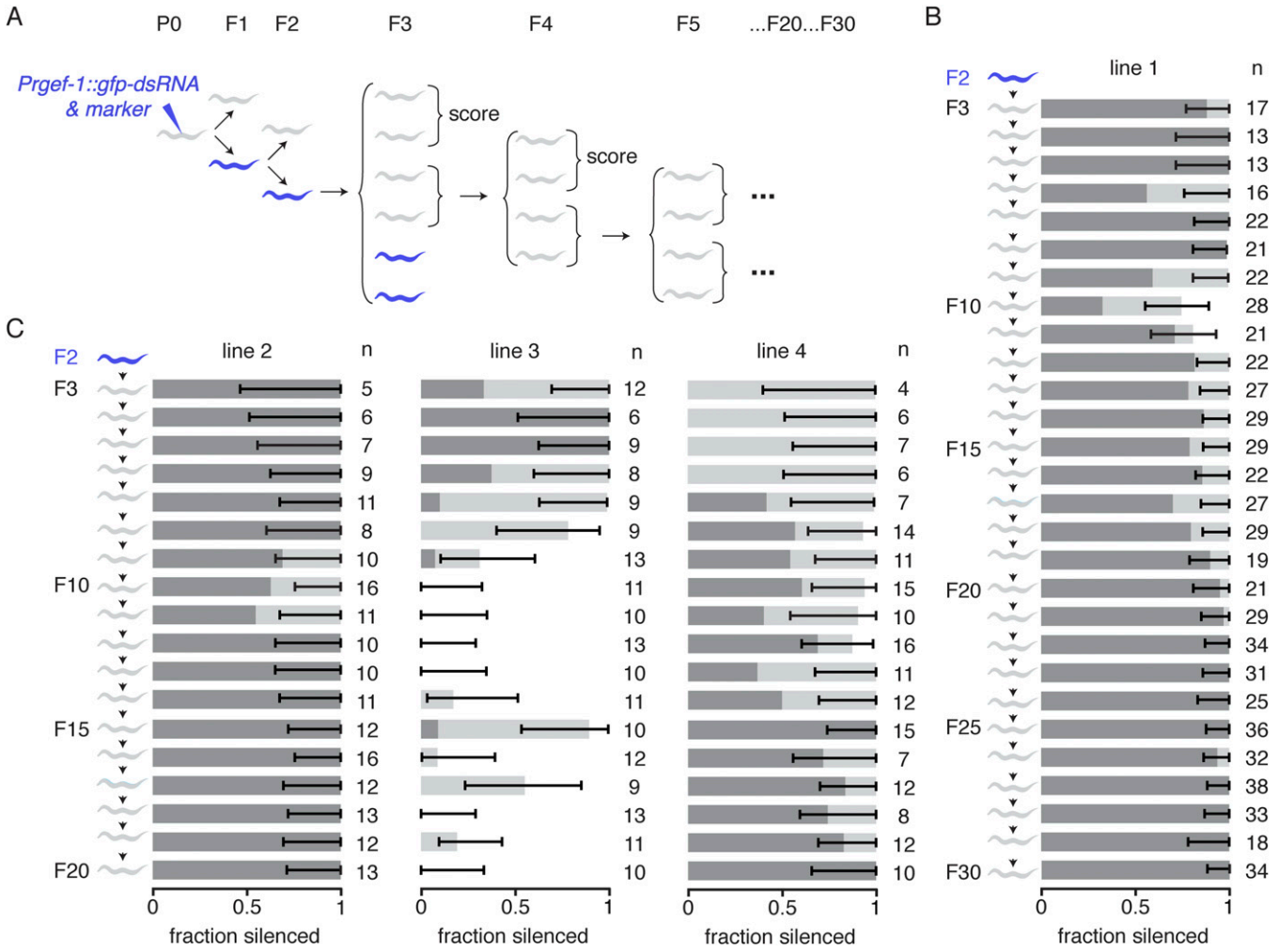

Fig. S4. Inherited silencing in the germline can persist for many generations after the source of neuronal mobile RNAs is lost. (*A*) Schematic of the assay for transgenerational silencing by neuronal mobile RNAs. *Pmex-5::gfp* animals (P0) were injected with constructs to express neuronal mobile RNA (*Prgef-1::gfp–dsRNA*) along with a co-injection marker (*Pmyo-2::DsRed*) to generate F2 transgenic lines (blue worm). F3 progeny and their descendants that lost the extrachromosomal array but were derived from F2 transgenic parents were scored for silencing by imaging the germline to detect the silencing of GFP. At each generation, the siblings of the scored animals were propagated to obtain the next generation. (*B* and *C*) The persistence of transgenerational silencing varies from one transgenic line to another. The proportions of animals that lack fluorescence from the co-injection marker (gray worm) but that show either strong (dark gray bar) or weak (light gray bar) silencing in the F3 generation and in successive generations (F4–F30 in *B* and F4–F20 in *C*) were determined for four independent transgenic lines (lines 1–4). Error bars indicate 95% CI, and *n* indicates number of L4-staged animals scored at each generation. Dark gray bars and light gray bars are as in Fig. S3C.

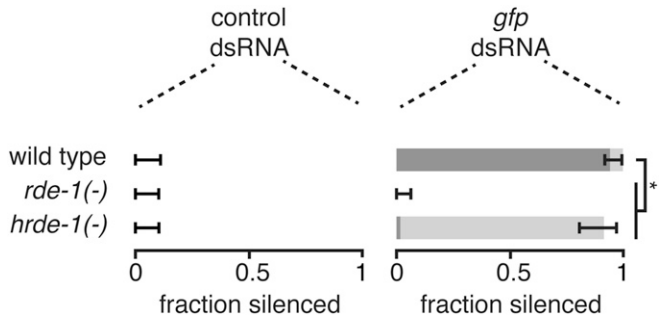

Fig. S5. Ingested dsRNAs can silence a gene within the germline independent of HRDE-1. Wild-type, *rde-1(–)*, or *hrde-1(–)* animals that all express *Pmex-5::gfp* were exposed for one generation to bacteria that have either the control L4440 plasmid (control dsRNA) or a plasmid that encodes dsRNA against *gfp* (*gfp* dsRNA) and silencing of GFP expression in the germline was measured. Error bars indicate 95% CI. **P* < 0.05. *n* > 35 L4-staged animals. Dark gray bars and light gray bars are as in Fig. S3C.

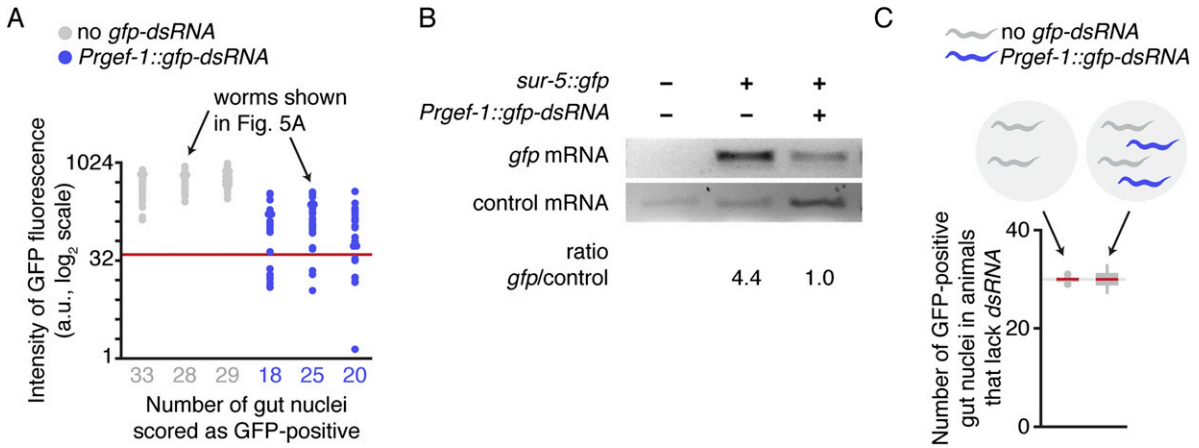

Fig. S6. Silencing by neuronal mobile RNAs against *gfp* reduces GFP fluorescence as well as *gfp* mRNA levels and is due to transport of mobile RNAs from neurons to other cells in animals that express dsRNA. (*A*) Nuclei counted as showing GFP silencing have several fold lower intensity of GFP fluorescence than even the dimmest nucleus in animals that do not show silencing. Intensity of GFP fluorescence in each gut nucleus of *sur-5::gfp* animals (no *gfp–dsRNA*, gray) or *sur-5::gfp* animals that express neuronal dsRNA (*Prgef-1::gfp–dsRNA*; blue) was measured and compared with the number of nuclei counted as not silenced for each worm (indicated along the *x* axis). Red line indicates threshold of expression below which a nucleus was scored as silenced in Figs. 4, 5*B*, S6*C*, and S7–S9. (*B*) Silencing of somatic GFP by neuronal mobile RNAs is due to reduction in mRNA levels. Semiquantitative RT-PCR was used to detect *gfp* mRNA and *tbb-2* mRNA (control) in wild-type animals, *sur-5::gfp* animals, and *sur-5::gfp* animals that in addition have *Prgef-1::gfp–dsRNA*. The intensity of the *gfp* band was normalized to that of the *tbb-2* band in each sample. (*C*) Animals that express neuronal mobile RNAs do not cause silencing in animals that lack neuronal mobile RNAs when grown together. The numbers of GFP-positive gut nuclei in animals that express *sur-5::gfp* were determined after growing the strain alone or after growing the strain for 4 d along with animals that contain both *Prgef-1::gfp-dsRNA* (marked with a dominant Rol defect) and *sur-5::gfp*. Gray line and red bar are as in Fig. 4*B*, and $n > 25$ L4-staged animals.

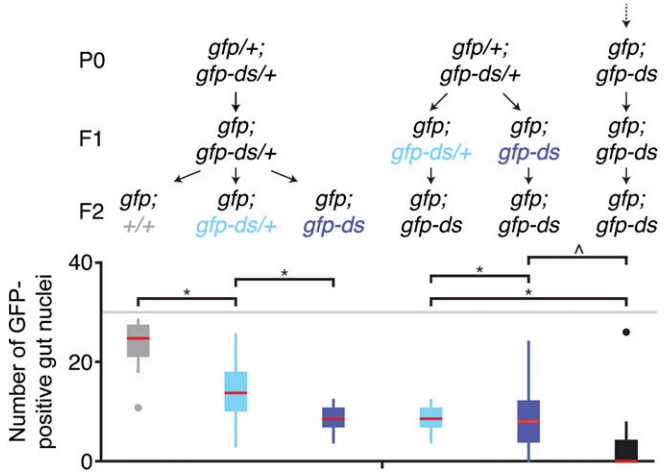

Fig. S7. Changes in parental mobile RNA silencing are correlated with small changes in mobile RNA silencing in progeny. Extent of RNA silencing in parents was varied, and inheritance of silencing was measured by comparing progeny of identical genotype in an *eri-1(–)* background. (*Left*) Dosage of dsRNA transgene in neurons against *gfp* dictates the level of silencing observed. Numbers of GFP-positive gut nuclei were counted in animals that either lack (gray) or that have one (cyan) or two copies (blue) of *Prgef-1::gfp–dsRNA* (*gfp–ds*) transgene. (*Right*) Increased mobile RNA silencing in parents is correlated with a small increase in mobile RNA silencing in progeny. Numbers of GFP-expressing gut nuclei were counted in animals that all expressed *Prgef-1::gfp–dsRNA* (*gfp–ds*) but that were progeny of parents that expressed one copy of *Prgef-1::gfp–dsRNA* (cyan), two copies of *Prgef-1::gfp–dsRNA* for one generation (blue), or two copies of *Prgef-1::gfp–dsRNA* for many generations (black). Gray line, red bar, and asterisks are as in Fig. 4*B*. ^$P = 0.054$. $n > 15$ L4-staged animals. These results are consistent with a small increase in silencing by mobile RNAs due to parental or ancestral silencing signals.

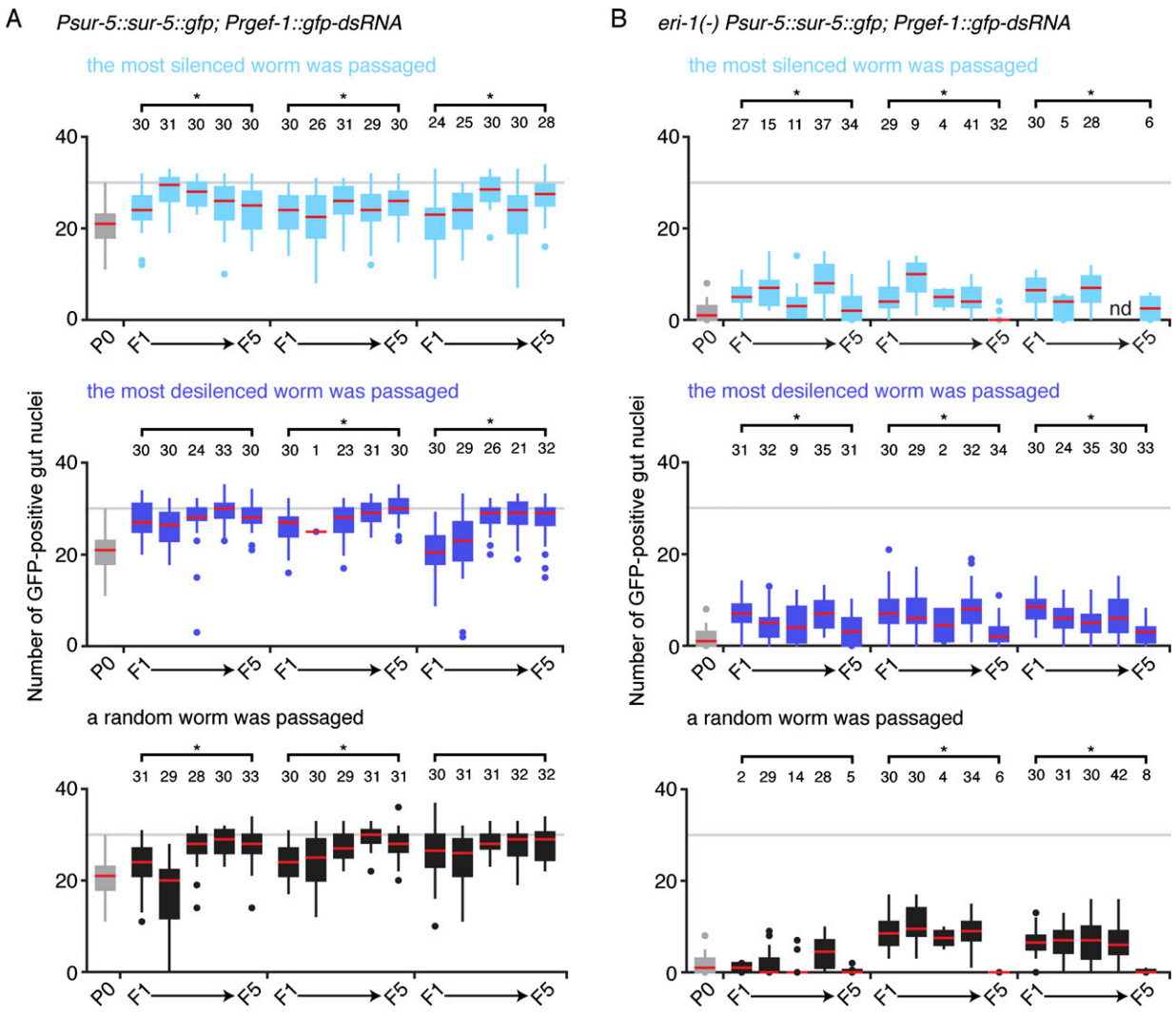

Fig. S8. Neuronal mobile RNAs can show small variations in silencing a somatic gene across generations. Animals that express neuronal mobile RNAs (*Prgef-1::gfp–ds RNA*) in *Psur-5::sur-5::gfp* (*A*) or in *eri-1(–) Psur-5::sur-5::gfp* (*B*) backgrounds were propagated for five generations (F1–F5) in triplicate by selecting at each generation the most silenced animal (cyan), the most desilenced animal (blue), or a random animal (black) from a starting population of animals (P0) and the numbers of GFP-expressing gut nuclei in L4-staged animals of each generation were counted. Gray line, red bar, and asterisks are as in Fig. 4*B*. The number of animals assayed in each generation varied from 1 to 42 and are indicated above each box plot.

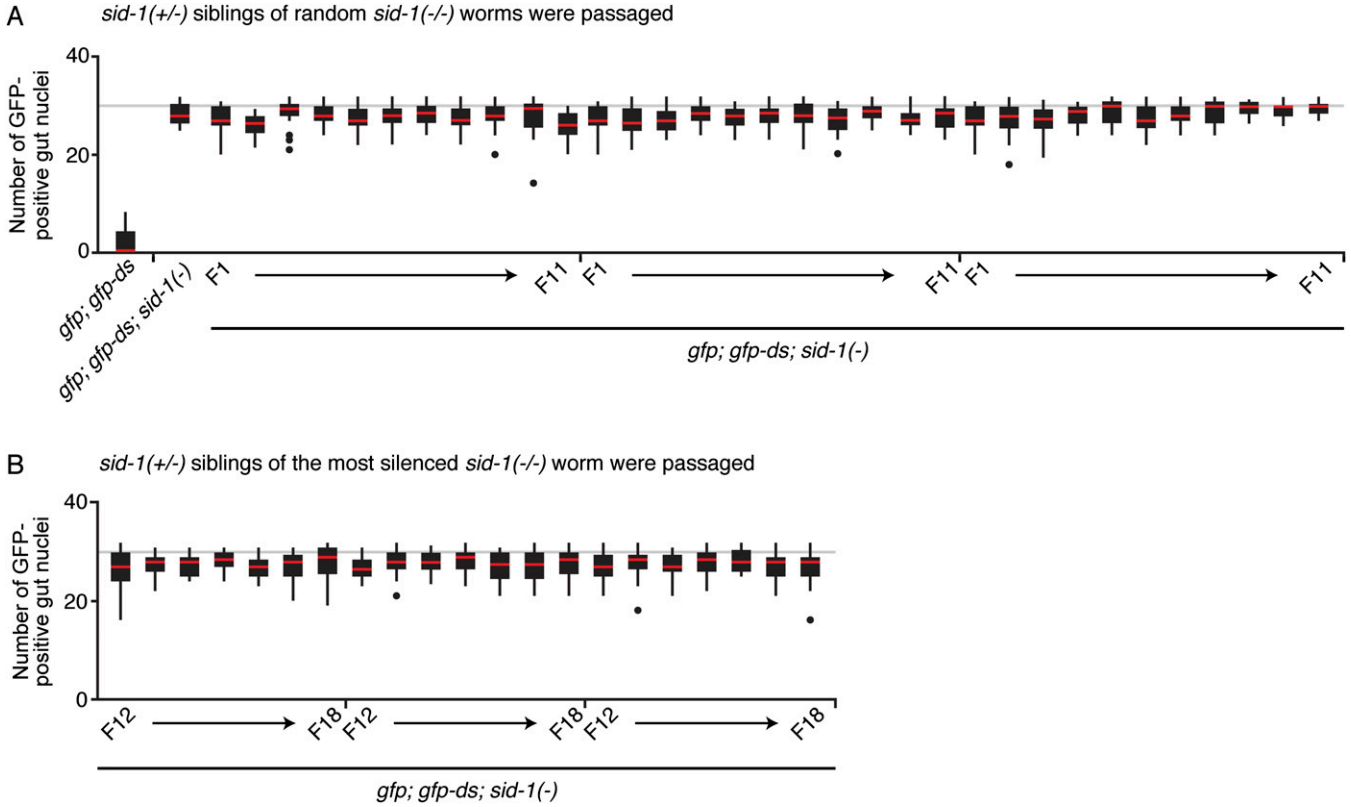

Fig. S9. SID-1 is required for silencing by neuronal mobile RNAs even after 17 generations of ancestral silencing. The numbers of GFP-positive gut nuclei were counted in animals that express neuronal mobile RNAs (*gfp–ds*) and nuclear-localized GFP in all somatic tissues (*gfp*) in an *eri-1(–)* background (*gfp; gfp–ds*) or in an *eri-1(–); sid-1(–)* background [*gfp; gfp–ds; sid-1(–)*]. By using the schematic described in Fig. 4*E*, *sid-1(–/–)* animals were generated after different numbers of generations of *sid-1(+/–)* animals that all had *gfp* and *gfp–ds*. The numbers of GFP-positive gut nuclei were counted in L4-staged *sid-1(–/–)* animals of each generation when *sid-1(+/–)* heterozygous siblings were passaged in triplicate without any selection for 11 generations (*A*) or when *sid-1(+/–)* siblings of the most silenced *sid-1(–/–)* animal were passaged in triplicate for seven more generations (*B*). Gray line, *n*, and red bars are as in Fig. 4*B*.