

Export of RNA silencing from *C. elegans* tissues does not require the RNA channel SID-1

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Double-stranded RNA (dsRNA) triggers RNA interference (RNAi) to silence genes of matching sequence. In some animals this experimentally induced silencing is transported between cells, and studies in the nematode *Caenorhabditis elegans* have shown that the dsRNA channel SID-1 is required for the import of such transported silencing signals. Gene silencing can also be triggered by endogenously expressed RNAi triggers, but it is unknown whether such silencing is transported between cells. Here, we show that, in *C. elegans*, SID-1 is required for efficient silencing of multicopy transgenes, indicating that mobile silencing signals contribute to transgene silencing. Further, most tissues can transport silencing initiated by the tissue-specific transgenic expression of RNAi triggers to other tissues, consistent with expressed RNAi triggers generating mobile silencing signals. Whereas the import of silencing signals requires SID-1, we found that mobile silencing signals generated by transgene-expressed RNAi triggers are exported to other tissues through a SID-1-independent mechanism. Furthermore, when RNAi triggers are expressed in ingested *Escherichia coli*, silencing signals can be transported to internal tissues from the gut lumen across gut cells that lack SID-1. Thus, *C. elegans* can transport endogenous and exogenous RNA silencing signals between many different tissues via at least 2 SID-1 independent export pathways.

transcytosis | double-stranded RNA transport | intercellular signaling | non-cell autonomy | mobile silencing signals

Gene silencing mechanisms including RNA interference (RNAi), heterochromatin formation (1), and repeat-induced silencing (2) are associated with base-paired RNA structures. For example, double-stranded RNA (dsRNA) and hairpin RNA (hpRNA) can trigger RNAi to silence the expression of genes with matching sequence (3). These homology-driven silencing mechanisms are thought to protect the organism against viruses (4) and mobile repetitive elements such as transposons (5, 6). Consequently, these silencing mechanisms also reduce the expression of *Caenorhabditis elegans* transgenes that are present in multiple tandem copies (7). In plants, transgenic expression or introduction of an RNAi trigger locally within a tissue can silence the target gene or virus throughout the organism (8, 9). Similar systemic silencing can be triggered in some animals by ingestion, local introduction, or overexpression of RNAi triggers in some tissues (10, 11). In contrast to experimentally introduced RNAi triggers, whether RNAi triggers associated with endogenous gene silencing mechanisms (e.g., transcribed dsRNAs) are similarly transported between cells is unknown.

Several studies have provided insight into the distinct mechanisms used by plants and animals to transport experimentally introduced RNAi triggers between cells. Plants transport RNAi triggers between adjacent cells through dynamic intercellular bridges called plasmodesmata and between distant cells via the phloem vascular tissue (12, 8). In addition, genetic screens in plants have identified some of the proteins required for the generation or reception of transported silencing signals (13). Genetic screens in the nematode *C. elegans* have identified a

dsRNA channel called SID-1 as a key component required for the import of silencing signals into all cells sensitive to systemic RNAi (14). It is not known whether SID-1 is required for export of silencing signals. Most animals, except some insects, have at least one SID-1 homolog. In mouse, the SID-1 homolog sidT1 is required for the import of lipid-modified siRNA into hepatocytes (15), suggesting that the import of RNAi triggers through SID-1 is a broadly conserved process among animals. In cultured *Drosophila* S2 cells, which lack SID-1 homologs, import of RNAi triggers occurs through a slow and energy-dependent process (16, 17). Expressing *C. elegans* SID-1 in these cells, however, enables rapid energy-independent dsRNA import (17), suggesting that SID-1 functions as a dsRNA channel.

Here, we show that *C. elegans* requires SID-1 to efficiently reduce the expression of multicopy transgenes, suggesting that transgene silencing in one cell produces mobile silencing signals that function to initiate and/or maintain transgene silencing in another cell. Tissue-specific expression of either hpRNA or dsRNA in many tissues results in SID-1-dependent systemic silencing. We confirm that SID-1 is required for the import of these silencing signals, but show that tissues that lack SID-1 can export these silencing signals. Finally, when RNAi triggers are expressed in ingested *Escherichia coli*, silencing signals can be transported from the gut lumen to internal tissues via gut cells that lack SID-1. Therefore, we conclude that silencing signals are exported from *C. elegans* tissues through multiple SID-1-independent pathways.

Results

Efficient Transgene Silencing Requires SID-1. Transgene tandem arrays in *C. elegans* are subject to repeat-induced silencing that depends on genes required for RNAi and is more pronounced in mutants that show enhanced RNAi (e.g., *eri-1*, *rrf-3*) (18, 19). Repeat-induced silencing often occurs in a mosaic manner such that only a subset of cells containing the transgene tandem array shows silencing of transgene expression. It is unknown whether the silencing triggers, likely derived from aberrant expression of dsRNA and hpRNA from the tandem arrays, remain in the nucleus or are transported to the cytoplasm or between cells. If such RNAi triggers are transported between cells, then the extent of repeat-induced silencing should depend on SID-1, a dsRNA channel required for systemic RNAi (14).

To determine whether repeat-induced silencing spreads beyond the cells in which silencing is initiated we compared the extent of silencing in *sid-1(+)* and *sid-1(-)* strains. To induce and monitor spontaneous transgene silencing we used a transgene, *sur-5::gfp*, which expresses nuclear-localized green fluorescent

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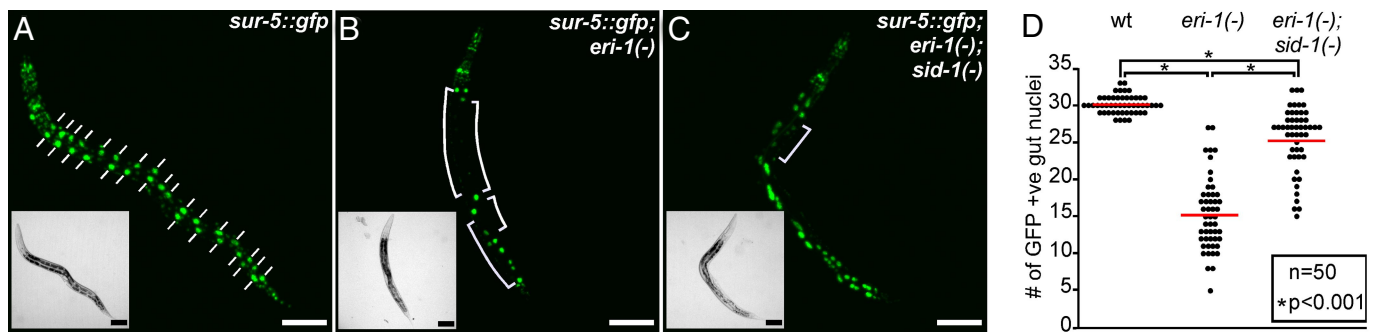


Fig. 1. The efficiency of transgene silencing depends on SID-1. Representative wild-type (A), *eri-1(-)* (B), and *eri-1(-);sid-1(-)* (C) animals of the fourth larval stage (L4) that express nuclear-localized GFP in all somatic cells (*sur-5::gfp*) are shown. (Insets): bright-field images. Large gut nuclei (dashes in A) and regions showing silencing in the gut (brackets in B and C) are indicated. (Scale bars, 100 μ m.) (D) Quantification of the extent of transgene silencing for each genotype shown in A–C. The number of GFP-positive gut nuclei in each of 50 L4 animals was counted. The average number (red line) for each genotype was significantly ($P < 0.001$; t test) different from that of the others.

protein (GFP) in all somatic cells. *sur-5::gfp* expression is detected in all somatic cells in a wild-type genetic background (Fig. 1A), whereas *sur-5::gfp* expression is silenced in many cells in an *eri-1(-)* background ((19) and Fig. 1B). This silencing is readily observed in the large nuclei of the gut cells (Fig. 1A, dashes vs. Fig. 1B, brackets). In a wild-type background, 30.1 ± 0.2 gut nuclei show bright GFP fluorescence, whereas in an *eri-1(-)* background, only 15.3 ± 0.7 gut nuclei show bright GFP fluorescence (Fig. 1D). Although *sur-5::gfp* expression was not detectably altered in a *sid-1(-)* background (data not shown), we found that in a *sid-1(-); eri-1(-)* double mutant background, 25.6 ± 0.6 gut nuclei show bright GFP fluorescence (Figs. 1C and D). This significant decrease in transgene silencing in the *sid-1(-); eri-1(-)* background compared with the *eri-1(-)* background ($P < 0.001$) shows that *sid-1* is required for efficient repeat induced silencing and that, like RNAi, such silencing produces mobile silencing signals. Further, the significant transgene silencing seen in the *sid-1(-); eri-1(-)* double mutant background compared with that in the wild-type background ($P < 0.001$) likely reflects the extent of cell-autonomous transgene silencing. Thus, to efficiently silence transgene tandem arrays, *C. elegans* requires the transport of silencing signals derived from expressed transgenes between cells and/or tissues.

Because the source for repeat-induced silencing triggers, the *sur-5::gfp* transgene, is expressed in all tissues, the observed *sid-1*-dependent silencing may reflect transport of silencing signals between cells within a tissue or may also involve transport between tissues. To determine whether silencing signals generated within a single tissue are transported between cells within that tissue, we examined silencing of the muscle-specific *myo-3::gfp* transgene (supporting information (SI) Fig. S1A). We found that this transgene is silenced in an *eri-1(-)* background (brackets in Fig. S1B), whereas *sid-1(-); eri-1(-)* animals appeared to have more body-wall muscle (bwm) cells that showed GFP fluorescence. To objectively compare the extent of silencing we measured the total GFP fluorescence per L4 animal for each genotype. This analysis showed that silencing is less efficient in the *sid-1(-); eri-1(-)* background than in the *eri-1(-)* background but more efficient than in the wild-type background (Fig. S1D). These results indicate that repeat-induced silencing signals are transported between cells within a single tissue.

Transgenic Expression of Either hpRNA or dsRNA Causes Transport of Silencing Between Multiple Tissues. Our analysis of transgene silencing suggests that silencing signals produced in the nucleus may be transported between cells; however, other studies have produced conflicting data. These studies examined systemic silencing from tissue-specific expression of either inverted-

repeat DNA constructs to produce hpRNA or a mix of sense- and antisense-oriented DNA constructs to produce dsRNA. For example, whereas the transport of RNAi from the pharynx to the bwm was detected when hpRNA was expressed in the pharynx (14), transport of RNAi from bwm cells to other tissues was detected only under certain environmental conditions when hpRNA was expressed in the bwm (20). Further, no transport of RNAi between cells was detected when hpRNA was expressed in the gut (21). Finally, RNAi induced by cell-specific expression of dsRNA was restricted to the cells expressing the dsRNA (22), suggesting that, in contrast to silencing triggered by transgenic expression of hpRNA, silencing triggered by transgenic expression of dsRNA may not be transported between cells. These observations suggest that the ability to export RNAi triggers may be restricted by cell type or RNA structure and may also be regulated by environmental conditions.

To directly compare systemic silencing triggered by dsRNA expression vs. by hpRNA expression, we expressed *gfp*-dsRNA in the pharynx, a tissue that shows readily detectable systemic silencing on *gfp*-hpRNA expression (14). We coinjected DNA constructs that express sense and anti-sense *gfp* RNA under the control of the pharynx-specific *myo-2* promoter (Fig. 2A) into *sid-1(+)* and *sid-1(-)* strains that express GFP in both the pharynx and the bwm. In *sid-1(+)* animals GFP expression was silenced in both the pharynx and the bwm (Fig. 2B, brackets in Middle), but in *sid-1(-)* animals GFP expression was silenced only in the pharynx (Fig. 2B, bracket in Right). Thus, the nuclear expression of *gfp*-dsRNA results in the SID-1-dependent transport of RNAi from the pharynx to the bwm cells. To examine the silencing of endogenous genes, we introduced similar constructs that express sense and antisense fragments of the bwm-specific *unc-22* gene in the pharynx. Animals that show *unc-22* silencing are easily observed as they twitch in response to the paralyzing drug levamisole (3). Expression of the *unc-22* sense and anti-sense constructs in the pharynx resulted in animals that show SID-1-dependent *Unc-22* twitching phenotypes (Fig. 2C), demonstrating that expressed dsRNA produces a mobile silencing signal in the pharynx that can move to the bwm. These results and the results from Winston *et al.* (14) show that pharyngeal expression of either hpRNA or dsRNA can produce effective mobile silencing signals.

To determine whether nonpharyngeal cells can export silencing signals, we transformed animals that expressed nuclear-localized GFP in all cells (Fig. 3A and A') with constructs that coexpressed both *gfp*-hpRNA and a red fluorescent protein, DsRed, in bwm cells or that coexpressed *gfp*-dsRNA, and DsRed in the gut. The resulting tandem arrays were not integrated into the genome and were thus mitotically unstable (23) such that

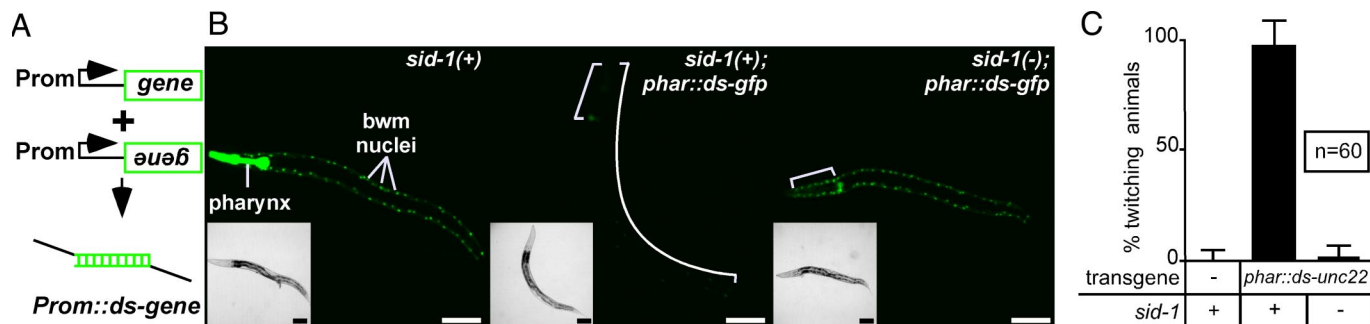


Fig. 2. Double-stranded RNA expressed in the pharynx requires SID-1 to silence target gene in the body-wall muscles (bwm). (A) Schematic showing expression of dsRNA under the control of a specific promoter to silence a target gene (Prom::ds-gene). (B) *gfp*-dsRNA expression in the pharynx and the bwm in either the wild-type background (Left, Middle) or *sid-1(-)* background (Right), and that in addition express *gfp*-dsRNA under the control of a pharynx-specific promoter (Middle, Right) are shown. Silenced pharynx and bwm (brackets) are indicated. (Insets): bright-field images. (Scale bar, 100 μ m.) (C) *unc-22*-dsRNA expressed in the pharynx requires *sid-1* to silence *unc-22* in the bwm. Wild-type animals and animals that express *unc-22*-dsRNA under the control of a pharynx promoter (*phar::ds-unc-22*) in either the wild-type or *sid-1(-)* backgrounds were scored for *unc-22* silencing (% twitching). Significant *unc-22* silencing was only detected in transgenic animals with a wild-type (*sid-1(+)*) genetic background ($P < 0.0002$). Error bars indicate 95% confidence intervals.

only a subset of cells expressed either DsRed and *gfp*-hpRNA (bwm) or DsRed and *gfp*-dsRNA (gut). Marking individual cells that express the RNAi trigger (red cells) within a tissue allows the detection of systemic silencing between cells within the same tissue and between cells in different tissues. We found that in addition to the silencing of GFP expression in the red gut and red bwm cells that express the RNAi trigger, silencing of GFP expression was also observed in nonred gut and nonred bwm cells, respectively (data not shown). Further, the muscle-expressed *gfp*-hpRNA and gut-expressed *gfp*-dsRNA constructs silenced GFP expression in cells of surrounding tissues (Fig. 3B and C). Consistent with systemic silencing, when these constructs were introduced into a *sid-1(-)* background, silencing was restricted to the cells that expressed the RNAi trigger (Fig. 3B' and C'). Similar results were obtained when *gfp*-dsRNA was expressed in the bwm (data not shown). These results show that the silencing in cells that do not express the RNAi trigger is because of SID-1-dependent transport of RNAi from the bwm cells or from gut cells. Thus, the ability to export silencing is not restricted to pharyngeal cells.

Neurons perform RNAi inefficiently (3) and some neurons (e.g., GABA-ergic motor neurons) do not show detectable RNAi-mediated silencing in wild-type animals. To determine whether neurons can export a silencing signal, we introduced constructs that coexpress *gfp*-hpRNA and DsRed in neurons into a strain that expresses nuclear-localized GFP in all cells. We found that GFP expression is silenced in most neurons that express *gfp*-hpRNA (Fig. 3D and D'), with the notable exception of the GABA-ergic motor neurons, which were not detectably silenced (data not shown). Although the nonred neurons continued to show bright GFP expression, there was robust silencing of GFP expression in all other tissues (Fig. 3D). As expected for a transported silencing signal, silencing in nonred cells was not detectable in *sid-1(-)* animals (Fig. 3D'). Thus, these results suggest that neurons, which perform RNAi inefficiently, can export silencing signals.

SID-1 Is Not Required for Export of RNAi Triggers. Because *sid-1* is not detectably expressed in neurons (14), the ability of neurons to efficiently export silencing signals suggests that *sid-1* may not be required for export. To determine whether SID-1 is required for the export of silencing signals, we generated mosaic *sid-1(-)* animals in which a tissue that lacks *sid-1* expresses *gfp*-hpRNA (exporting tissue) and monitored the silencing of *gfp* in another tissue that expresses a rescuing *sid-1(+)* cDNA (importing tissue). If *sid-1* is not required for export then silencing will be

observed in the *sid-1(+)* importing tissue, whereas, if *sid-1* is required for export, then silencing will not be observed in the importing tissue.

We first expressed *gfp*-hpRNA specifically in the pharynx of *sid-1(-)* animals that express *gfp* in the pharynx, gut, and body-wall muscle cells (Fig. 4A). In these animals, GFP expression is detectably silenced only in the pharynx. To determine whether silencing signals derived from the RNAi trigger expressed in the *sid-1(-)* pharynx were exported, we expressed *sid-1(+)* only in the gut of these animals. GFP expression was silenced specifically in the gut, whereas in the bwm cells, which were *sid-1(-)*, GFP expression was not detectably silenced (Fig. 4B). Thus, silencing signals derived from the expression of *gfp*-hpRNA are exported from the pharynx independent of SID-1.

To control for the possibility that export from the pharynx may have occurred through SID-1 because of ectopic SID-1 expression in the pharynx, we examined the SID-1 dependence of silencing in multiple tissues. When we introduced constructs that express *gfp*-hpRNA in all neurons of *sid-1(-)* animals that express GFP in a subset of neurons and in bwm cells, no silencing of GFP expression was observed. However, subsequent introduction of constructs that express *sid-1(+)* specifically in bwm cells resulted in robust silencing of GFP expression in bwm cells (Fig. S2), consistent with SID-1-independent export of silencing signals from neurons to bwm. Similarly, silencing signals derived from RNAi triggers expressed in *sid-1(-)* pharynx silenced GFP expression in *sid-1(+)* bwm cells (Fig. S2), consistent with SID-1-independent export of silencing signals from pharynx to bwm cells. Taken together, the data suggest that silencing signals derived from expressed RNAi triggers can be exported from tissues independent of SID-1.

Ingested RNAi Triggers Are Transported Across Gut Cells Independent of SID-1. A common method to deliver RNAi triggers to *C. elegans* is to feed them *E. coli* that expresses dsRNA or hpRNA. Ingestion of the RNAi trigger results in systemic silencing of the target gene in most tissues (24). Silencing in gut cells by ingested RNAi triggers requires SID-1 and SID-2, a transmembrane protein found almost exclusively on the gut luminal membrane (25). How the ingested RNAi triggers or a silencing signal derived from such triggers are delivered into the gut or transported from the gut to other internal tissues is unknown. To determine whether SID-1 is required in the gut for the transport of silencing signals from the lumen to internal tissues during feeding RNAi, we generated *sid-1(-)* mosaic animals where wild-type SID-1 was expressed only in bwm cells and examined

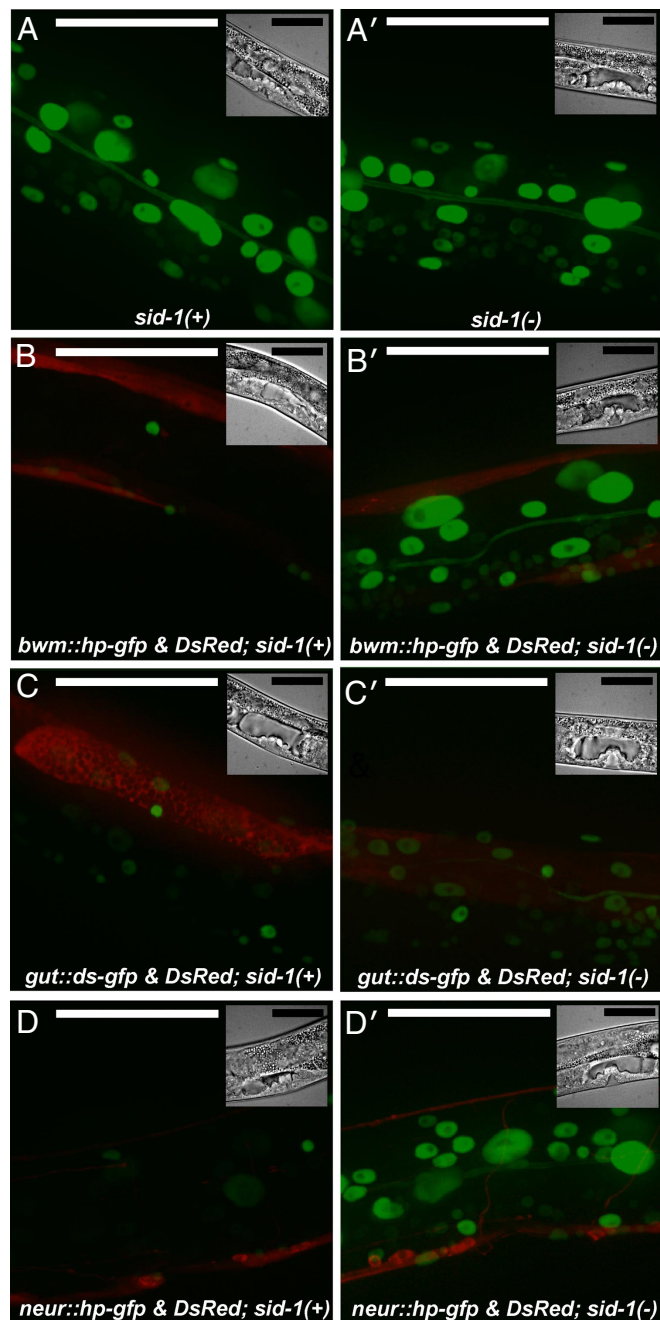


Fig. 3. RNAi triggers expressed in multiple tissues cause systemic silencing of target genes. *gfp* RNAi triggers expressed in the bwm, in the gut or in neurons show SID-1-dependent systemic silencing of GFP expression in adjacent tissues. Green channel images of representative L4 animals that express nuclear-localized *gfp* in all somatic cells (*sur-5::gfp*) in a wild-type (A) or a *sid-1(-)* (A') background; and merged green and red channel images of wild-type (B–D) or *sid-1(-)* (B'–D') L4 animals that in addition coexpress *gfp*-hpRNA and DsRed under the control of a bwm-specific promoter (B and B') or *gfp*-dsRNA and DsRed under the control of a gut-specific promoter (C and C') or *gfp*-hpRNA and DsRed under the control of a neuronal promoter (D and D') are shown. In addition to the silencing of *gfp* expression in red cells that express the RNAi trigger, silencing of *gfp* expression in nonred cells was seen in 100%, 80%, and 100% of wild-type animals that express RNAi triggers in the body-wall muscles, in the gut, and in neurons, respectively. Consistent with SID-1 dependent transport of silencing signals, in the *sid-1(-)* background, *gfp* silencing was restricted to red cells that express the RNAi trigger (100%). (Insets): bright-field images. (Scale bar, 50 μ m).

silencing of bwm-expressed genes in response to feeding RNAi. If SID-1 is not required in the gut for ingested silencing signals to pass through the gut cells to silence genes in the bwm, then the

RNAi trigger is likely transported from the gut lumen to the bwm cells without entry into the cytoplasm of gut cells. If SID-1 is required in the gut for ingested silencing signals to silence genes in the bwm, then the RNAi triggers likely enter the cytoplasm of gut cells before subsequent transport to the bwm cells.

To induce feeding RNAi of genes in the bwm, we used *E. coli* that express *gfp*-hpRNA with an *unc-22* loop, which triggers the silencing of both *gfp* and *unc-22* likely because of the production of *unc-22* dsRNA in the *E. coli* (14). When wild-type animals that express nuclear-localized GFP in all somatic cells (*sur-5::gfp*) were fed *E. coli* that express *gfp*-hpRNA with an *unc-22* loop, GFP expression was efficiently silenced in all nonneuronal cells (Fig. 5A, Left). This silencing was abolished in *sid-1(-)* animals. However, when a construct that expresses *sid-1(+)* in the bwm and a construct that expresses DsRed in the bwm were coinjected into these *sid-1(-)* animals, the resultant red *sid-1(+)* bwm cells were silenced in response to feeding RNAi (Fig. 5A, compare Middle and Right images). Consistent with a lack of ectopic SID-1 expression in the gut, GFP expression was not detectably silenced in the gut cells of *sid-1(-)* animals that express *sid-1(+)* in bwm cells. Thus, the ingested *gfp*-hpRNA is transported from the lumen of a *sid-1(-)* gut to *sid-1(+)* bwm. We then examined *unc-22* silencing in the above strains. Silencing *unc-22* results in animals that respond to the drug levamisole by twitching, and in a wild-type background, 100% of the animals twitched when fed *E. coli* that express *gfp*-hpRNA with an *unc-22* loop (Fig. 5B). This twitching was abolished in *sid-1(-)* animals (Fig. 5B, 0% twitching) but was rescued when *sid-1(+)* was expressed only in the bwm of *sid-1(-)* animals (Fig. 5B, 100% twitching). Thus, the ingested *gfp*-hpRNA with *unc-22* loop and *unc-22* dsRNA were exported from a *sid-1(-)* gut to silence *unc-22* in the *sid-1(+)* bwm. These results suggest that ingested RNAi triggers can be transported from the gut lumen into the animal independent of SID-1. To control for undetected misexpression of SID-1 in gut cells from the bwm-specific promoter, we isolated *sid-1* genetic mosaics by mitotic segregation of a transgene array that expresses GFP in all cell types and that expresses *sid-1* from its native promoter, which is active in all nonneuronal cells. Rare mosaic animals that had lost the array in all gut cells, as monitored by lack of gut GFP expression, but still expressed GFP in bwm and/or hypodermal cells were identified and placed as L4 larvae on *E. coli* that express *gfp*-hpRNA. Consistent with the analysis using tissue-specific promoters, compared with control animals, we observed modest silencing of GFP expression (8/8 animals). Therefore, during feeding RNAi, ingested RNAi triggers can be transported from the gut lumen to cells in other tissues without entry into the cytoplasm of gut cells.

Discussion

We found that SID-1-dependent mobile silencing signals contribute to transgene silencing in *C. elegans*. In addition, cells of all tested tissue types can export silencing signals derived from expressed RNAi triggers. Finally, we found that *C. elegans* cells can use SID-1-independent pathway(s) to export silencing signals upon transgenic expression of or ingestion of RNAi triggers.

Export of RNA Silencing from Cells That Express RNAi Triggers. Import of RNAi triggers into cells is readily observed in many organisms upon injection of dsRNA into the body cavity (11). For example, in adult *Drosophila*, intraabdominal injection of dsRNA can silence target genes in the central nervous system (26). In *C. elegans*, injection of dsRNA into the pseudocoelomic body cavity can cause silencing of target genes in most tissues (3). The ability of cells to import RNAi triggers, suggests that other cells within the organism may act as sources of RNAi triggers. Studies that used tissue-specific expression of RNAi triggers to examine the ability of cells to export silencing signals suggested that only a

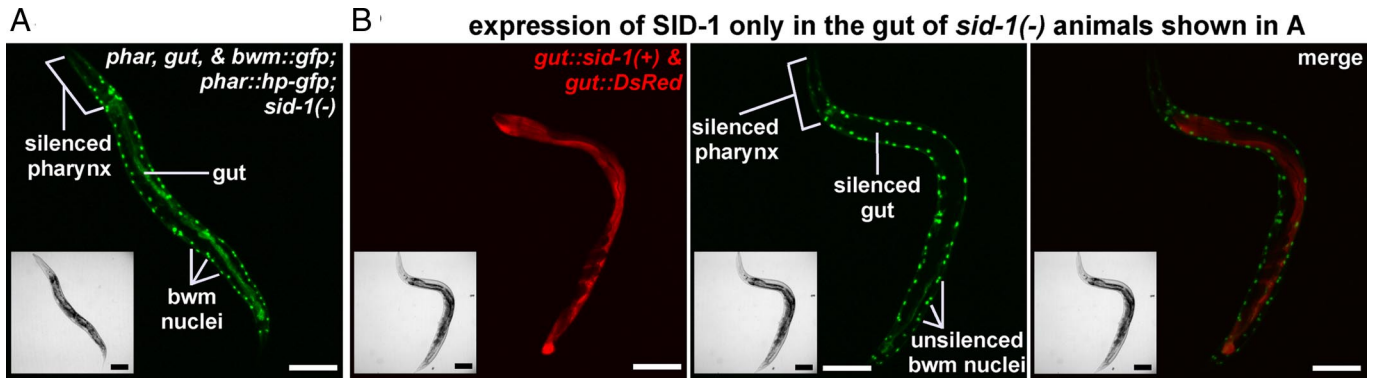


Fig. 4. Expression of RNAi triggers in *sid-1(-)* tissues can silence target genes in *sid-1(+)* tissues. (A) Representative *sid-1(-)* young adult that expresses GFP in the pharynx, gut, and bwm, but that expresses *gfp*-hpRNA only in the pharynx (*phar::hp-gfp*). Only GFP expression in the pharynx is silenced as indicated. (Inset): bright-field image. (B) Representative young adult of the same genotype as in A, but in which wild-type *sid-1* cDNA (*sid-1(+)*) and DsRed have been coexpressed in the gut. Red channel showing specific expression of *sid-1(+)* in the gut (Left); green channel showing specific silencing of *gfp* expression in the pharynx and in the gut (Middle); and merge of images from both channels (Right) are shown. (Insets): bright-field images. (Scale bar, 100 μ m.)

subset of cells within the organism could export silencing signals. For example, in the *C. elegans* gut and in *Drosophila*, silencing by transgenic expression of hpRNAs was apparently restricted to cells that express the RNAi trigger (21, 27). However, we found that expression of RNAi triggers in the cells of most *C. elegans* tissues, including gut cells and neurons, results in systemic silencing. Therefore, we propose that a mechanism to export silencing signals derived from expressed RNAi triggers exists in most tissues but may be active to different extents in different tissues.

SID-1-Independent Export. Although *C. elegans* tissues require SID-1 to import silencing signals, we found that they do not require SID-1 to export silencing signals (Fig. 4 and Fig. S2). Exported silencing signals may be produced in the nucleus or in the cytoplasm. Recent reports show that RNAi that occurs in the nucleus uses effector proteins distinct from those used in the cytoplasm (28), and similar specialized protein machinery may generate the transported silencing signal in either the nucleus or cytoplasm. Intriguingly, many of the mRNAs and microRNAs that are transported in vesicles between cultured mammalian

mast cells are not found in the cytoplasm of the exporting cell, consistent with a nuclear origin for these transported RNAs (29).

Why Do Animal Cells Export RNA Silencing? Numerous dsRNAs or hpRNAs are expressed as precursors to endogenous small RNAs that regulate gene expression (30). These small RNAs include small-interfering RNAs (siRNAs) from natural antisense transcripts, endogenous siRNAs, and microRNAs. Because our results show that RNAi triggers expressed from transgene tandem arrays are transported between animal cells, we propose that silencing signals derived from endogenously expressed dsRNAs or hpRNAs may be transported between cells. A possible function of such transport may be to directly coordinate the expression of target genes between cells in a multicellular organism. Additionally, transport of silencing signals may play a role in the strong silencing of transposons in the germ line (5, 6). However, because *sid-1(-)* mutants do not have an obvious developmental or behavioral phenotype (14), the transport of endogenous silencing signals may be regulated or may occur only under certain physiological or environmental conditions. For example, exposure to viruses may lead to the systemic transport

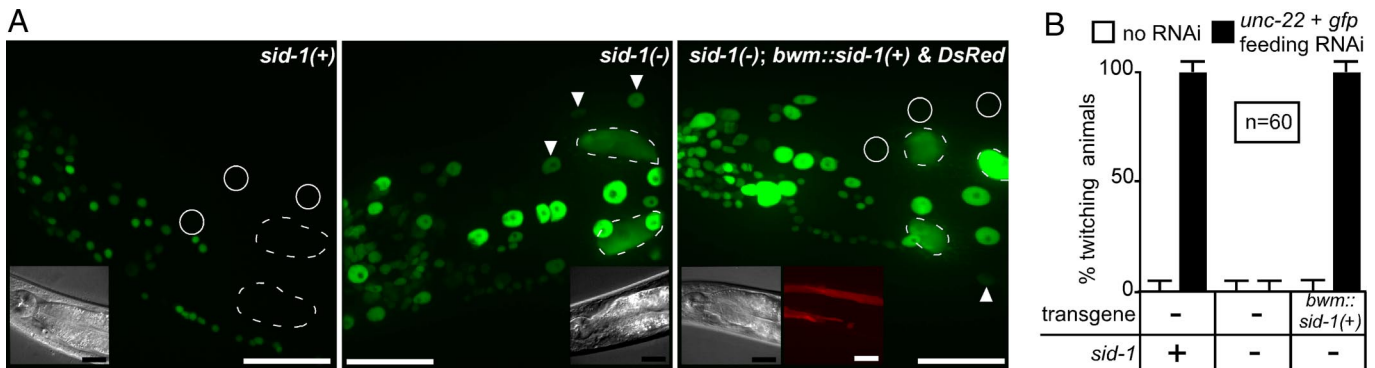


Fig. 5. Ingested RNAi triggers can silence target genes in animals that lack SID-1 in the gut. (A) Expression of *sid-1(+)* in bwm is sufficient to silence *gfp* expression in bwm of *sid-1(-)* animals by feeding RNAi. Representative adult animals that express nuclear-localized *gfp* in all somatic cells (*sur-5::gfp*) and that are wild-type (Left), *sid-1(-)* (Middle), or mosaic *sid-1(-)* animals that express *sid-1(+)* only in the bwm (Right) are shown. Animals were fed *E. coli* that express *gfp*-hpRNA with an *unc-22* loop sequence. bwm nuclei that express GFP (arrowheads) or that show silencing of GFP expression (circle) and gut nuclei (dashed outline), which do not show detectable silencing in *sid-1(-)* animals, are indicated. Most neuronal nuclei are not detectably silenced in all cases. (Insets): DIC and Red channel images. (Scale bar, 25 μ m.) (B) Expression of *sid-1(+)* in bwm is sufficient to silence *unc-22* expression in *sid-1(-)* animals by feeding RNAi. Wild-type, *sid-1(-)*, and mosaic *sid-1(-)* animals that express *sid-1(+)* under the control of a bwm promoter (*bwm::sid-1(+)*) were fed either *E. coli* (white bar) or *E. coli* that express *gfp*-hpRNA with an *unc-22* loop sequence (black bar) and were scored for *unc-22* silencing (% twitching). All animals also express *sur-5::gfp* (as in A). $n = 60$ animals, error bars indicate 95% confidence intervals.

of silencing signals to curtail the spread of the infection. In support of this possibility, virulence factors of some plant viruses specifically inhibit the transport of silencing signals between cells (4). However, evaluation of this hypothesis in *C. elegans* awaits the identification of a virus that infects *C. elegans* in nature.

Transcytosis of Ingested RNAi Triggers? In addition to the transport of silencing signals between cells within an organism, robust uptake of RNAi triggers from the environment into the organism is readily observed in some animals (10). For example, ingestion of *E. coli* that expresses RNAi triggers results in systemic silencing of the target gene in most tissues in *C. elegans* and in planaria. However, how silencing signals are exported to internal tissues from the gut lumen is unknown.

We found that ingested RNAi triggers apparently do not require SID-1 to pass through the *C. elegans* intestine to silence target genes in *sid1*(+) tissues that are not exposed to the environment (Fig. 5). SID-1 independent transport of RNAi triggers across the gut was more obvious in *sid-1*(-) animals where SID-1 was overexpressed in the body-wall muscles (bwm) by using a bwm-specific promoter than in *sid-1*(-) mosaic animals where SID-1 was expressed under its own promoter in nongut tissues. The difference in the level of silencing observed in the 2 experiments may reflect undetected SID-1 misexpressed in the gut or differences in SID-1 expression levels in target tissues. Knockdown of some vesicle transport proteins in *C. elegans* results in resistance to feeding RNAi (16), suggesting that ingested RNAi triggers are taken up into vesicles. A parsimonious model that explains the SID-1-independent import of ingested RNAi triggers is transcytosis (31) of the ingested RNAi triggers across the gut. Specifically, we propose that ingested RNAi

triggers are endocytosed into vesicles that bud off from the gut lumen, and that these vesicles then release their cargo into the pseudocoelomic cavity by fusing to the basal membrane of the gut. Because most tissues in *C. elegans* are exposed to the pseudocoelomic cavity, the ingested RNAi triggers can then be taken up through SID-1 into most tissues resulting in systemic silencing throughout the animal.

Materials and Methods

All *C. elegans* strains were generated and maintained using standard methods (32). Transgenic animals were generated by injecting DNA in 10 mM Tris-Cl pH 8.5 into the germ line by using standard methods (23), and at least 3 independent transgenic lines were analyzed for each experiment. Representative L4 or adult animals were imaged by using exposure times that avoid saturation of pixels by the brightest cells on an Axiovert 200 microscope (Zeiss) and all images within a figure were adjusted in the same way by using Photoshop (Adobe) for display and to enable comparison. Feeding RNAi and *unc-22* silencing measurements were adapted from standard procedures (33). By using Wilson's estimates and the proportion of pooled values, 95% confidence intervals for a single proportion and *P* values for comparison of two proportions were calculated, respectively (34). Detailed procedures are provided in the *SI Materials and Methods*, and a list of primers used to generate PCR fragments is available in [Table S1](#).

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Supporting Information

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SI Materials and Methods

Strains Used. N2 wild-type, HC46 *ccIs4251 [myo-3::gfp]*; *mIs11 [myo-2::gfp]*, HC114 *sid-1(qt9)*; *mIs11*; *ccIs4251*; *qtIs3 [myo-2::gfp-hpRNA]*, HC195 *nrIs20 [sur-5::gfp]*, HC196 *sid-1(qt9)*, HC565 *ccIs4251*; *mIs11*; *sid-1(qt9)*, HC566 *nrIs20*; *sid-1(qt9)*, HC567 *nrIs20*; *eri-1(mg366)*, HC568 *nrIs20*; *eri-1(mg366)*; *sid-1(qt9)*, HC570 *juIs73*; *ccIs4251*; *sid-1(qt9)*; *qtEx116[pRF4+pHC337]*, HC573 *juIs73*; *ccIs4251*; *sid-1(qt9)*, and HC575 *mIs11*; *ccIs4251*; *qtIs3*; *qtIs5 sid-1(qt9)*

Transgenes. In all cases except one (see below), transgenic animals were healthy and appeared morphologically normal.

Plasmids: pRF4 (23) or pHC183 (14) were used to obtain transgenic animals that roll or that express DsRed in the body-wall muscles (bwm), respectively.

To express *gfp-hpRNA* in the bwm: A 1:1 mix (0.035 mg/ml each) of pHC172 (14), which has the *gfp* coding region as an inverted repeat sequence under the control of the *myo-3* promoter, and pHC183 was injected into HC195 or HC566 animals to generate transgenic lines.

To express *gfp-hpRNA* in all neurons: The inverted-repeat sequence targeting *gfp* was subcloned from pHC172 under the control of the ≈ 3.4 kb promoter of the pan-neuronally expressed gene F25B3.3 (S1) to generate pHC337. A mix of pHC337 (0.04 mg/ml) and the coinjection marker PF25B3.3::DsRed (0.008 mg/ml) was injected into HC195 and HC566 animals to generate transgenic lines. A 1:1 mix of pHC337 and pRF4 (0.04 mg/ml each) was injected into HC573 animals to generate transgenic lines and a representative rolling transgenic line was designated as HC570.

To express *sid-1(+)* cDNA in the bwm: The SID-1 cDNA with a C-terminal FLAG tag was cloned as an NheI/KpnI fragment under the control of the *myo-3* promoter in pPD96.52 (gift of Andrew Fire, Stanford University) to generate pHC355. A 1:1 mix of pHC355 and pHC183 (0.04 mg/ml each) was injected into HC114, HC573, or rolling HC570 animals to generate transgenic lines. Some double transgenic animals produced by injecting rolling HC570 animals showed growth and morphological defects.

PCR fusion products: Promoter sequences were amplified with Expand Long Template (ELT) polymerase (Roche) and either noncoding sequences or coding sequences along with 3'UTR were amplified with PfuUltra II Fusion polymerase (Stratagene) by using primers that generate ≈ 50 bp overlap between the two PCR fragments. The fusion products were generated with ELT polymerase by using the amplified promoter and the coding sequences as template and nested primers. In some cases, the two PCR fragments were fused *in vivo*. The specific templates and primers used to generate the various PCR fusion products are detailed below. The sequences of all primers used (P1–P41) are provided in Table S1.

To make the coinjection marker Pmyo-2::DsRed: The *myo-2* promoter was amplified from pHC168 (14) with primers P1 and P3. The DsRed2 cDNA along with unc-54 3'UTR was amplified from pHC183 (14) with primers P4 and P5. The fusion product was generated with primers P2 and P6. Transgenic animals generated using this marker showed bright DsRed2 fluorescence in pharyngeal muscles and sometimes showed faint fluorescence in bwm, likely because of trace expression of pHC183, the template used to amplify DsRed cDNA.

To make the coinjection marker Psid-2::DsRed: The *sid-2* promoter was amplified from wild-type genomic DNA (gDNA)

with primers P23 and P25. The DsRed2 cDNA along with unc-54 3'UTR was amplified from pHC183 with primers P26 and P5. The two PCR products were mixed at 1:1 ratio (0.008 mg/ml each) for injection into animals to generate transgenic lines where the PCR products would be fused *in vivo*.

To make the coinjection marker PF25B3.3::DsRed: The F25B3.3 promoter was amplified from gDNA with primers P39 and P40; and DsRed2 cDNA along with unc-54 3'UTR was amplified from pHC183 with primers P41 and P5. The two PCR products were mixed at 1:1 ratio (0.008 mg/ml each) for injection into animals to generate transgenic lines where the PCR products would be fused *in vivo*.

To express *gfp-dsRNA* in the pharynx: (a) Pmyo-2::gfp-sense: The *myo-2* promoter was amplified from gDNA with primers P1 and P7. *gfp* coding sequence was amplified from pHC168 (14) with primers P8 and P9. The fusion was generated with primers P2 and P10. (b) Pmyo-2::gfp-antisense: The *myo-2* promoter was similarly amplified with primers P1 and P12 and *gfp* coding sequence was similarly amplified with primers P11 and P13. The fusion product was generated with primers B2 and P14. A 1:1 mix of Pmyo-2::gfp-sense and Pmyo-2::gfp-antisense (0.025 mg/ml each) along with Pmyo-2::DsRed (0.008 mg/ml) was injected into HC46 or HC565 animals to generate transgenic lines.

To express *unc-22-dsRNA* in the pharynx: (a) Pmyo-2::unc22sense: The *myo-2* promoter was amplified from pHC168 with primers P1 and P15. 589 bp of *unc-22* coding sequence was amplified from gDNA with primers P16 and P17. The fusion product was generated with primers P2 and P18. (b) Pmyo-2::unc22antisense: The *myo-2* promoter region was similarly amplified with primers P1 and P20; and the 589 bp of *unc-22* coding sequence was similarly amplified with primers P19 and P21. The fusion product was generated with primers C2 and P22. A 1:1 mix of Pmyo-2::unc22sense and Pmyo-2::unc22antisense (0.024 mg/ml each) along with Pmyo-2::DsRed (0.008 mg/ml) was injected into N2 or HC196 animals to generate transgenic lines.

To express *gfp-dsRNA* in the gut: (a) Psid-2::gfp-sense: The *sid-2* promoter was amplified from gDNA with primers P23 and P27. *gfp* coding sequence was amplified from pHC168 with primers P28 and P9. The fusion product was generated with primers P24 and P10. (b) Psid-2::gfp-antisense: The *sid-2* promoter region was similarly amplified with primers P23 and P30 and *gfp* coding sequence was similarly amplified with primers P29 and P13. The fusion product was generated with primers E2 and P14. A 1:1 mix of Psid-2::gfp-sense and Psid-2::gfp-antisense (0.025 mg/ml each) along with Psid-2::DsRed (0.008 mg/ml) was injected into HC195 or HC566 animals to generate transgenic lines.

To express *gfp-dsRNA* in the bwm: (a) Pmyo-2::gfp-sense: The *myo-3* promoter was amplified from gDNA with primers P31 and P33. *gfp* coding sequence was amplified from pHC168 with primers P34 and P9. The fusion product was generated with primers P32 and P10. (b) Pmyo-3::gfp-antisense: The *myo-3* promoter region was similarly amplified with primers P31 and P36 and *gfp* coding sequence was similarly amplified with primers P35 and P13. The fusion product was generated with primers P32 and P14. A 1:1 mix of Pmyo-3::gfp-sense and Pmyo-3::gfp-antisense (0.034 mg/ml each) along with pHC183 (0.034 mg/ml) was injected into HC195 animals to generate transgenic lines.

To express *sid-1(+)* cDNA in the gut: The *sid-2* promoter was amplified from gDNA with primers P23 and P37; and *sid-1(+)* cDNA along with unc-54 3'UTR was amplified from pHC355 with primers P38 and P5. A 1:1 mix of the two PCR products

(0.008 mg/ml each) along with Psid-1::DsRed (0.008 mg/ml) was injected into HC575 to generate transgenic lines where the *sid-2* promoter is fused to *sid-1(+)* cDNA in vivo.

Microscopy. For Fig. 1D, similar distributions were obtained when the number of brightly fluorescent gut nuclei per worm was counted independently by two researchers (data not shown). For Fig. 3, 30 transgenic animals from each of three transgenic lines (total of 90 animals) were analyzed per genotype. Animals were scored as showing silencing of GFP expression in nonred tissues if GFP expression was detectably dimmer than in control animals when scored at a fixed magnification. For Fig. S1, 37 confocal slices that encompass the entire worm were collected using exposure times that avoid saturation of pixels by the brightest cells on an Axiovert 200 microscope (Zeiss) and the total fluorescence was measured by using Image J (National Institutes of Health). A maximum of three L4 worms were analyzed per slide and 10 animals were analyzed per genotype. The background fluorescence for each slide was calculated as the total

fluorescence in 37 slices in a region where there were no worms. Background fluorescence of the slide was subtracted from the total fluorescence for each worm to obtain its average total fluorescence plotted in Fig. S1D.

Feeding RNAi. L1-L2 staged animals were fed *E. coli* that express *gfp*-hpRNA with an *unc-22* loop (pPD126.25). The animals were grown at 20 °C and were assayed 4 days later for GFP expression and *unc-22* silencing. *E. coli* that do not express any hpRNA or dsRNA was used as control. Among animals that express GFP and that were >3 days past L2, ≈30%–60% showed reduced GFP fluorescence in the gut even when fed control OP50 *E. coli*.

Unc-22 Silencing. For Fig. 2, Starved animals were transferred via an agar chunk to a plate with OP50 *E. coli* and 1.5 days later, young adults were assayed. Transgenic or control animals that twitch in response to 1 μl of 3 mM Levamisole (in water) within 10 seconds were scored as twitching. Sixty animals per genotype or 20 animals for each of 3 independent transgenic lines (total 60) were scored.

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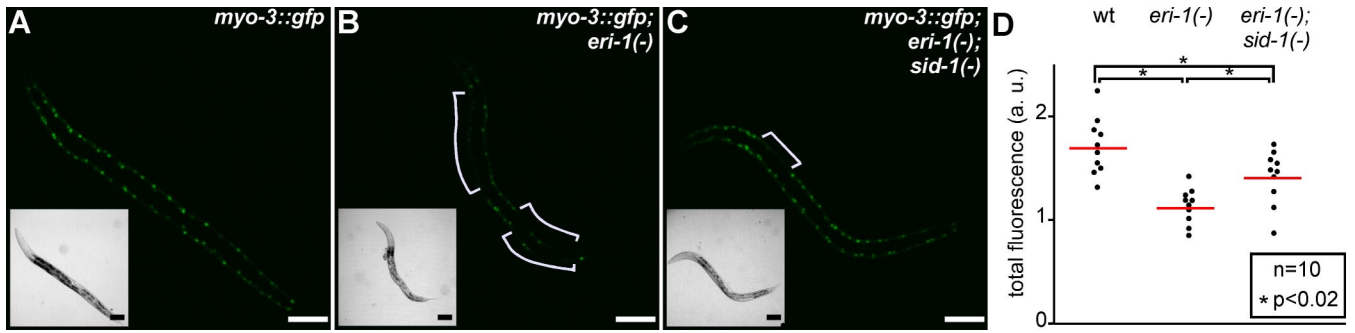


Fig. S1. The extent of silencing of a tissue-specific transgene depends on SID-1. Representative wild-type (A), *eri-1(-)* (B), and *eri-1(-);sid-1(-)* (C) animals of the fourth larval (L4) stage that express GFP in body-wall muscles (*bwm::gfp*) are shown. *Insets*: bright-field images. Regions showing silencing in the bwm (brackets in B and C) are indicated. Scale bar: 100 μ m. (D) Quantification of the extent of transgene silencing for each genotype shown in A–C. Total fluorescence per animal was measured for 10 L4 animals. The average fluorescence per animal (red line) for each genotype was significantly ($P < 0.02$; *t* test) different from that of the others.

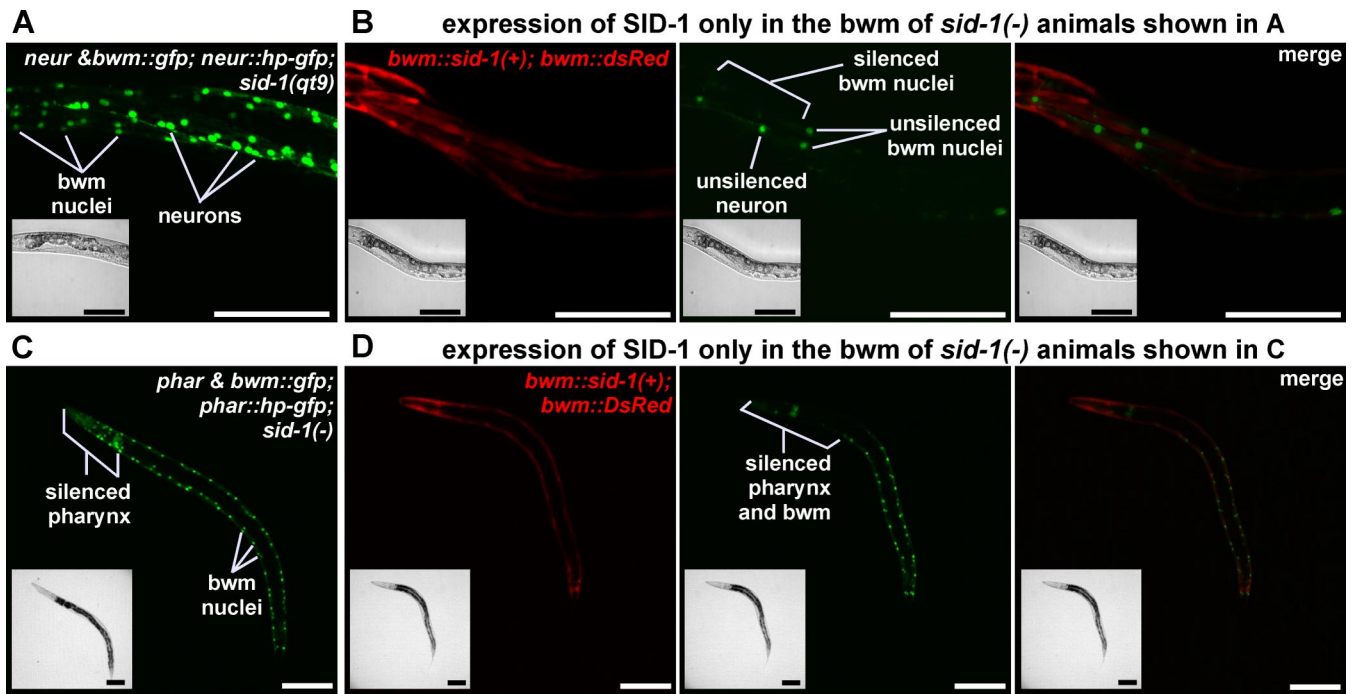


Fig. S2. Expression of *gfp*-hpRNA in either neurons or pharynx of *sid-1(-)* animals can silence *gfp* in *sid-1(+)* bwm. (A) Representative *sid-1(-)* L4 animal that expresses GFP in a subset of neurons and in the bwm, but that expresses *gfp*-hpRNA only in all neurons (*neur::hp-gfp*). No silencing of GFP expression is detected. *Inset*: bright-field image. (B) Representative L4 animal of the same genotype as in (A), but in which wild-type *sid-1* cDNA (*sid-1(+)*) and DsRed have been coexpressed in some of the bwm cells. Red channel showing specific but mosaic expression of *sid-1(+)* in the bwm (*Left*); green channel showing specific silencing of GFP expression only in the bwm cells that express *sid-1(+)* (*Middle*); and merge of images from both channels (*right*) are shown. *Insets*: bright-field images. Scale bars: 100 μm . (C) Representative *sid-1(-)* L4 animal that expresses GFP in the pharynx and bwm, but that expresses *gfp*-hpRNA only in the pharynx (*phar::hp-gfp*). Only GFP expression in the pharynx is silenced as indicated. *Inset*: bright-field image. (D) Representative L4 animal of the same genotype as in (C), but in which wild-type *sid-1* cDNA (*sid-1(+)*) and DsRed have been coexpressed in the bwm. Red channel showing specific expression of *sid-1(+)* in the bwm (*Left*); green channel showing silencing of GFP expression in the pharynx and the anterior half of bwm (*Middle*); and merge of images from both channels are shown. *Insets*: bright-field images. Scale bars: 100 μm .

