SID-1 regulates a retrotransposon-encoded gene to tune heritable RNA silencing

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ABSTRACT

RNAs in circulation carry sequence-specific regulatory information between cells in plant, animal, and host-pathogen systems. Such RNA can cross generational boundaries, as evidenced by double-stranded RNA (dsRNA) from somatic cells of the nematode *C. elegans* silencing genes of matching sequence in the germline and in progeny. Here we dissect the intergenerational path taken by dsRNA from parental circulation and discover that cytosolic entry in the parental germline and/or developing progeny through the dsRNA importer SID-1 varies based on developmental time and dsRNA substrates. Temporary loss of SID-1 causes changes in the expression of the ***s**id-1*-**d**ependent **g**ene *sdg-1* that can last for more than 100 generations. The SDG-1 protein is enriched in perinuclear Z granules required for heritable RNA silencing, but it is expressed from a retrotransposon targeted by such RNA silencing. This auto-inhibitory loop reveals how retrotransposons could persist over evolutionary time by hosting genes that regulate their own silencing.

Key words: RNAi, transport, heritable silencing, stochastic, germline, gene expression

MAIN TEXT

RNAs released into circulation can act as intercellular messages that are used for gene regulation in distant cells. Examples include secretion of exosomal small RNAs in response to pathogenic fungal infection in *Arabidopsis*[1], virus-like proteins with their coding mRNAs in developing *Drosophila*[2] and mice[3], microRNAs from adipose tissue in mice[4] and small RNAs from the epididymis in mice[5-8]. Such extracellular RNAs have also been detected in humans, but their roles in gene regulation remain unclear despite their use as diagnostic markers for diseases (reviewed in ref.[9]). Furthermore, the recent development of double-stranded RNA (dsRNA)-based drugs (reviewed in ref.[10]; refs.[11-12]) that can silence genes of matching sequence through RNA interference[13] has heightened interest in understanding the import of dsRNA into cells. A conserved dsRNA-selective importer, SID-1[14-16], is required for the import of extracellular dsRNA into the cytosol of any cell in the nematode *C. elegans*. SID-1 has two homologs in mammals – SIDT1 and SIDT2. Although similar cytosolic entry of dsRNA through these mammalian homologs of SID-1 is supported by studies in mice reporting entry of viral dsRNA through SIDT2[17] enhanced dsRNA uptake when SIDT1 is overexpressed *in vitro*[18], and uptake of ingested dsRNA into cells through SIDT1[19], alternative roles for SIDT1 and/or SIDT2 in the uptake of cholesterol have also been proposed[20].

Secretion of dsRNA from *C. elegans* tissues expressing dsRNA from transgenes has been inferred based upon the SID-1-dependent silencing of matching genes in other tissues[14,21]. Secreted dsRNA from neurons can silence genes of matching sequence in most somatic cells[22] and within the germline[23]. Extracellular dsRNA delivered into parental circulation by injection or ingestion also enters the germline and can cause silencing of matching genes in progeny[13,24-27]. Such intergenerational transport of RNA is an attractive mechanism for explaining endogenous,

gene-specific effects in progeny that could occur in response to changes in somatic tissues of parents. However, which conditions induce transport of dsRNA into the germline, when during development this transport occurs, and what regulatory consequences ensue in progeny upon uptake of extracellular dsRNA from parents are all unknown.

Here, we dissect the intergenerational transport of extracellular dsRNA and discover a role for this mechanism in modulating RNA regulation within the germline using a gene that is located within a retrotransposon. Extracellular dsRNA is transported with developmental and substrate specificity from parental circulation to progeny, and its accumulation from neurons can be enhanced using oxidative damage. Blocking dsRNA import into the cytosol of all cells revealed heritable changes in gene expression and led to the identification of ***s****id-1*-**d**ependent **g**ene-1 (*sdg-1*). Although *sdg-1* is located within a retrotransposon that is a target of RNA silencing in the germline, the SDG-1 protein colocalizes with regulators of RNA silencing in perinuclear granules within the germline and dynamically enters the nucleus in proximal oocytes and during early cell cycles in developing embryos. Thus, we propose that import of dsRNA through SID-1 could regulate genes like *sdg-1* to tune RNA silencing in the germline across generations.

Requirements for the entry of extracellular dsRNA into the germline change during development

A convenient method for the delivery of extracellular dsRNA into *C. elegans* at various times during larval development is the expression of dsRNA in the bacteria that the animals ingest as food[24]. Using this approach, we found that changes during development from early

larval stages to adulthood impact the entry of dsRNA into the germline and/or subsequent silencing.

To determine when ingested dsRNA can enter the germline and cause silencing, we exposed developing animals with a ubiquitously expressed protein (GTBP-1) tagged with GFP to bacteria that express *gfp*-dsRNA. Silencing was detectable within the germline from the second larval stage (L2) onwards (Figures 1A and S1A), but either exposure to ingested dsRNA beyond the fourth larval stage (L4) (Figure 1A) or injection of dsRNA into the 1-day old adult germline (Figure S1A) was required to observe silencing in the germline of 3-day old adults. The need for exposure to dsRNA during late development to observe persistent silencing suggests recovery of expression within the germline despite detectable silencing until the L4-stage. Combined with the need for exposure to dsRNA after the L4 stage for silencing in progeny[26-27], these observations suggest that heritable RNA silencing is not effectively initiated during early development of the germline despite dsRNA entry and subsequent silencing. However, a 24-hour pulse of dsRNA exposure beginning at the L4 stage was sufficient for heritable silencing (Figure S3A and ref.[26]). This early window for heritable silencing likely relies on entry of dsRNA into the proximal germline because (1) silencing of a somatic gene in progeny after parental ingestion of dsRNA required RME-2 (Figure S3A), which is enriched in the proximal germline (Figure S3B and ref.[28]), and (2) some *gtbp-1::gfp* animals exposed to *gfp*-dsRNA until the first day of adulthood showed selective silencing in the proximal germline (Figures S1C and S1D).

These results reveal three periods of germline development that can be broadly distinguished based on response to extracellular dsRNA: (1) from the first larval to the third larval stage when exposure to dsRNA does not result in maximal silencing within the germline in adults; (2) from the fourth larval stage to early adulthood when entry of dsRNA primarily occurs

in the proximal germline through RME-2; and (3) later adulthood when entry can be independent of RME-2 (Figure S3A and ref.[27]) and germline silencing by ingested dsRNA is maximal. These differences in the entry of ingested dsRNA into cells and/or subsequent silencing could be driven by a variety of changes during development. These include changes in the uptake of dsRNA into the intestine, distribution of dsRNA to other tissues from the intestine, import of dsRNA into the germline, and subsequent gene silencing within the germline.

Oxidative damage in neurons expressing dsRNA enhances silencing in the germline by neuronal dsRNA

Another approach for delivering extracellular dsRNA into the germline is the secretion of dsRNA from neurons[23]. We found that extracellular dsRNA preferentially enters the proximal germline and that oxidative damage in neurons likely increases the amounts of extracellular dsRNA, resulting in more silencing within the germline.

We modulated the secretion of dsRNA from somatic cells into parental circulation during development by adapting an approach for damaging somatic cells[29]. Specifically, we generated animals that express the mini singlet oxygen generator (miniSOG) protein in neurons and exposed them to blue light. While animals expressing miniSOG from a single-copy transgene did not show an appreciable defect when compared with wild-type animals, those expressing miniSOG from a multi-copy transgene were paralyzed (Figures S1E and S1F, *top*) and had visibly damaged neurons (Figure S1F, *bottom*). Using this system, we induced oxidative damage in the neurons of animals that expressed dsRNA under the control of a neuronal promoter and evaluated silencing of target genes with matching sequence expressed in other tissues (Figure 1C). By exposing animals to blue light for 60 minutes at different times during development (Figure S1G), we observed SID-1-dependent enhancement in the silencing of the hypodermal

gene *bli-1* at the adult stage by neuronal *bli-1*-dsRNA, with maximal silencing when oxidative damage occurred during mid-to-late larval development (Figure S1H, light exposure from 42 to 66 hours post L4-stage of parent; Figure S1I, ~2-fold increase from 14.9% to 29.1% in a background with **e**nhanced **R**NA **i**nterference (*eri-1(-)*) and ~6-fold increase from ~1.6% to ~9.8% in a wild-type background). A similar period of maximal SID-1-dependent enhancement of silencing was also observed when neurons expressing *gfp*-dsRNA were damaged and silencing of a two-gene operon that expresses two fluorescent proteins, mCherry::H2B and GFP::H2B, in the germline was measured (Figures 1D, 1E, 1F and S1J – 48 to 60 hours post L4-stage of parent; *sid-1(-)* allele (*jam80[non]*) is depicted in Figure S2). While silencing of *gfp::h2b* was observed throughout the germline, silencing of the other cistron *mCherry::h2b* was often restricted to regions of the germline. Silencing of *mCherry::h2b* was most frequent in the proximal germline and was not observed in any other region without silencing in the proximal germline (proximal germline - 57%, distal germline - 47%, sperm - 29%, Figure 1F), likely due to reduction of *mCherry::h2b::gfp::h2b* pre-mRNA[30]. The pattern of *mCherry::h2b* silencing is similar to the spatial pattern observed for the RME-2-dependent entry of dsRNA delivered into the parental circulation[26] and is consistent with the pattern of target mRNA degradation in the germline by extracellular dsRNA[31].

Thus, by modulating the secretion of dsRNA we revealed two insights into the intercellular transport of dsRNA: (1) oxidative damage of neurons during particular periods in development increases the amount of dsRNA and/or changes the kinds of dsRNA in circulation either because of specific enhancement of secretion or nonspecific spillage; and (2) there is a preference for the entry of neuronal dsRNA into the proximal germline. These temporal and/or spatial preferences for silencing could be because of unknown characteristics of the exported

neuronal dsRNA (e.g., modifications, lengths, structures, etc.) that influence import or subsequent silencing – a hypothesis that is also supported by the different requirements for silencing by neuronal *gfp*-dsRNA compared to other sources of *gfp*-dsRNA[22]. Alternatively, these preferences could reflect universal constraints for any extracellular dsRNA in *C. elegans*.

Extracellular dsRNA in parental circulation can be transported through multiple routes to cause silencing in progeny

While the characteristics of extracellular dsRNA imported into the germline from ingested bacteria or from neurons are unknown, delivery of chemically defined dsRNA into the extracellular space in *C. elegans* can be accomplished using microinjection[13,26]. We found that the route taken by dsRNA from parental circulation to progeny depends on the characteristics of the dsRNA.

We examined differences, if any, in the entry of *in vitro* transcribed dsRNA into the germline during the L4 and adult stages as evidenced by silencing in progeny. Silencing was comparable regardless of whether wild-type or *rme-2(-)* parents were injected as L4-staged or adult animals (Figures 2A and S3C, *left*; also reported for adults in ref.[27]), although a weak requirement for RME-2 was discernable when lower concentrations of dsRNA were used (Figure S3C, *right*). The difference in RME-2 requirement between injected dsRNA and ingested dsRNA (Figure S3A) could reflect parental circulation accumulating different amounts of dsRNA (e.g., more upon injection than upon ingestion) and/or different kinds of dsRNA (e.g., because of modifications in bacteria or upon transit through the intestine). However, these possibilities could not be easily distinguished because sensitive northern blotting[32] revealed that both bacterial and *in vitro* transcribed dsRNA consist of a complex mix of dsRNAs (Figures S3D, S3E and S3F; consistent with refs.[33-34]), hereafter called mixed dsRNA. In contrast, when

synthesized *gfp*-dsRNA of a defined length (50 bp) with a fluorescent label was injected into circulation in adult animals, no entry into the germline was observed in the absence of RME-2[26]. We found that silencing of *unc-22* in progeny by similarly synthesized but unlabeled 50-bp *unc-22*-dsRNA with a 5' OH delivered into parental circulation also showed a strong requirement for RME-2, unlike mixed dsRNA (Figure 2A). Further comparison between the two forms of dsRNA revealed that silencing in progeny by 50-bp dsRNA injected into parental circulation was detectably less efficient in somatic cells (Figures 2B, S4A and S4B, *left*), even when ~14X more 50-bp dsRNA was delivered into parental circulation (Figure S4B, *right*), and was also less efficient in the germline (Figures 2B, S4A and S4C). Given that both 50-bp dsRNA and mixed dsRNA rely on the nuclear Argonaute HRDE-1[35] for silencing within the germline (Figures S4A and S4C) and can silence independent of the nuclear Argonaute NRDE-3[30] in somatic cells (Figures S4A and S4C), the observed difference in the extent of silencing could be the result of differences in the stability and/or intergenerational transport of 50-bp dsRNA versus mixed dsRNA. In addition to the diversity of lengths (Figure S3), a relevant feature shared by mixed dsRNA generated in bacteria or *in vitro*, is the presence of 5' triphosphates instead of the 5' OH in synthesized 50-bp dsRNA. In support of the impact of 5' phosphates on transport and/or silencing, addition of 5' monophosphates to synthesized 50-bp dsRNA injected into parental circulation reduced the dependence on RME-2 for silencing in progeny (Figures S3G and S3H). Thus, the requirements for entry into the germline and subsequent silencing vary for different lengths and/or chemical forms of dsRNA.

Fluorescently labeled 50-bp dsRNA delivered into parental circulation localized within intestinal cells in progeny (Figure 2C, *top left*), as has been observed for vitellogenin proteins[36] and fluorescent dyes[37]. Accumulation of fluorescently-labeled dsRNA was also detected at the

apical membrane of the intestine, which could reflect exocytosis of dsRNA into the lumen of developing intestinal cells. However, separation of the fluorescent label from dsRNA catalyzed by cellular enzymes cannot be excluded. Therefore, to dissect differences, if any, between the transport of short dsRNA (synthesized 50-bp with 5'OH) and mixed dsRNA (mixture transcribed *in vitro* using ~1 kb DNA template) we injected *unc-22*-dsRNA into animals with mutations in genes that play roles in the import of dsRNA. We found that maternal SID-1 was required for silencing by short dsRNA in progeny (Figure 2C, *bottom*, left bars), suggesting that the SID-1-dependent entry of short dsRNA into the cytosol likely occurs in the injected parent or during early development in progeny. Uptake of dsRNA from the intestinal lumen requires SID-2, a transmembrane protein located in the apical membranes of intestinal cells[38-39]. We found that SID-2 was not required for most silencing in progeny by short or mixed dsRNA injected into parental circulation (Figure 2C, *top right* and *bottom*). Exit of dsRNA from intracellular vesicles requires SID-5, a transmembrane protein located in endolysosomal membranes[40]. Silencing in wild-type animals was comparable to silencing in *sid-5(-)* animals (Figure 2C, *top right*). However, when animals that lacked SID-1 were injected, SID-5 was required in progeny for silencing by mixed dsRNA from parental circulation (Figure 2C, *bottom*, right bars; as also reported in ref.[27]). Since dsRNA is expected to be present in vesicles upon entry through RME-2 in the absence of SID-1[26-27], this observation suggests that SID-5 is required for the release of mixed dsRNA from inherited vesicles in progeny.

In summary, extracellular dsRNA can enter the germline in parents and be transmitted to progeny through two routes with different substrate selectivity. One route is preferentially used by short dsRNA and relies on RME-2-mediated endocytosis of dsRNA into oocytes, where early exit from vesicles is required for silencing in progeny as evidenced by the need for maternal

SID-1 (Figure 2D, blue). The other route appears to exclude short dsRNA, but allows mixed dsRNA entry into the cytosol in the parental germline through SID-1 and exit from inherited vesicles in progeny through a process that requires both zygotic SID-1 and SID-5 (Figure 2D, grey; ref.[27]).

Expression of SID-1 is consistent with a role in intergenerational transport of extracellular dsRNA

The proposed model (Figure 2D) for dsRNA transport into the germline and to progeny suggests developmental variation in the expression pattern of SID-1. Previous attempts at observing SID-1 localization relied on multi-copy transgenes[14], which can become silenced within the germline[41] and could produce a variety of tagged and untagged proteins[42]. When using multi-copy transgenes to express a SID-1 fusion protein tagged at the C-terminus with DsRed or GFP (Figure S5A) under the control of a promoter that drives expression within body-wall muscles, we observed intracellular localization of SID-1::DsRed or SID-1::GFP (Figure S5B, *top*) along with rescue of gene silencing by ingested dsRNA in body-wall muscles (Figure S5B, *bottom*). However, similar tagging to express SID-1 fusion proteins from either a single-copy transgene expressed in the germline (SID-1::DsRed) or the endogenous locus (SID-1::wrmScarlet) did not enable gene silencing by ingested dsRNA (Figure S5C), suggesting that the C-terminal fusions of SID-1 were likely non-functional and that apparent function when using multi-copy transgenes reflects production of untagged variants. In support of our rationale, a recent prediction of SID-1 structure[43-44] suggests that the C-terminus is sequestered, a feature that may be disrupted by the addition of C-terminal fluorophores, potentially leading to misfolded proteins that are degraded. Consistently, we found that internal tagging of the *sid-1* gene using Cas9-mediated genome editing to express SID-1::mCherry (Figure 3A) resulted in a

fusion protein with detectable function (Figures 3B and S5D). Therefore, we analyzed fluorescence from this fusion protein expressed from the endogenous locus under the control of native regulatory sequences (Figures 3C, 3D, S5E, S5F and S5G). Fluorescence from SID-1::mCherry progressively increased during development with tissue-specific enrichments in the developing embryo (Figures 3C, *left*, and S5G), becoming ubiquitous in hatched L1 larvae (Figures 3C, *middle*, and 3E). SID-1::mCherry was not easily detectable in the germline during larval development (Figure 3C, *middle* and *right*), but was visible in the proximal and distal regions of the adult germline (Figure 3D). Similarly, endogenous RME-2 was most abundant in the proximal oocytes of the adult germline (Figure S3B and ref.[28]). These expression patterns are consistent with the entry of most dsRNA from circulation of adult animals into the proximal germline[26] and subsequent activity of transport mechanisms in developing embryos that inherit parental dsRNA (Figure 2).

To determine if acute induction rather than developmental expression of SID-1 would be sufficient for the import of dsRNA into the germline, we engineered the endogenous *sid-1* gene to transcribe a fusion transcript with an aptamer-regulated ribozyme (Figure S6A, *left*) that cleaves itself when not bound to tetracycline (Figure S6A, *right*) (based on ref.[45]). Exposing these animals to tetracycline enabled silencing by dsRNA in somatic tissues (hypodermis: Figure S6B, *left*; body-wall muscles: Figure S6B, *right*), indicative of stabilization of *sid-1* mRNA, production of SID-1 protein, and subsequent dsRNA import in somatic cells. However, such tetracycline-induced silencing was not detectable in the germline (Figures S6C, S6D, S6E and S6F). Yet, similar tagging of the ubiquitously expressed gene *gtbp-1::gfp* results in detectable rescue of expression within the germline by tetracycline (Figure S6G). A possible explanation for the poor rescue of SID-1 activity within the germline is that post-transcriptional mechanisms

targeting *sid-1* mRNA in the germline but not the soma interfere with tetracycline-dependent stabilization of the *sid-1* transcript (e.g., piRNA-based regulation of *sid-1* mRNA[46-47]), or that acute stabilization of the *sid-1* transcript does not override developmental regulation of SID-1 translation.

Additional attempts to tag the SID-1 protein guided by structure and to modulate *sid-1* transcripts guided by post-transcriptional regulatory interactions could improve control of dsRNA transport between cells. Nevertheless, the developmentally regulated expression observed for both SID-1 and RME-2 in the germline is consistent with intergenerational or transgenerational effects of dsRNA from parental circulation after development of the adult germline.

Temporary loss of *sid-1* causes a transgenerational increase in the levels of mRNA from two germline genes

To understand how the dsRNA importer SID-1 might be used in endogenous gene regulation across generations, we searched for *sid-1*-dependent changes in gene expression that could be heritable (Figures 4, S2 and S7). We initially analyzed polyA+ RNAs extracted from wild-type animals, two available *sid-1* loss-of-function mutants[14,48] (*sid-1(-)*) and one available rescue strain where *sid-1(-)* was rescued with a transgene that overexpresses *sid-1(+)*[48], but found that pairwise comparisons between wild-type and mutant samples with otherwise similar genetic backgrounds did not yield any significantly misregulated genes present in both comparisons (Figures S7A and S7B). Strains with similar genotypes (*sid-1(+)* or *sid-1(-)*) did not cluster together when using principal component analysis (Figure S7A), suggesting that other differences (e.g., genetic background) obscure or misrepresent differences between *sid-1(+)* and *sid-1(-)* animals. To ameliorate this problem, we used Cas9-mediated genome editing to delete

the entire *sid-1* coding sequence (*del*) or introduce a nonsense mutation (*non*) in cohorts of the same wild-type animals. When comparing polyA+ RNA from this wild type with that of the newly generated *sid-1(jam113[del])* (Figure S7C) or *sid-1(jam80[non])* (Figures 4A and 4B) animals, we found that 26 genes were significantly ($q < 0.05$) misregulated in *sid-1(jam113[del])* (Figure S7D) and 6 in *sid-1(jam80[non])* (Figure 4B, *top*), both including *sid-1*. The most upregulated gene in *sid-1(jam113[del])*, *F14F9.5*, which is located immediately 3' to *sid-1* in the genome, was only misregulated in the deletion mutant *sid-1(jam113[del])* and not in the nonsense mutant *sid-1(jam80[non])* (Figure S7F, *left*). Both mutants, however, were equally defective for silencing by ingested dsRNA (Figure 3B). This observation suggests that while both mutations result in loss of SID-1 protein, the deletion of *sid-1* also changes local regulatory sequences (potentially explaining upregulation of the neighboring gene *F14F9.5*) and eliminates *sid-1* mRNA, which could participate in RNA-based regulatory interactions within the germline[46-47]. Nevertheless, we could detect two genes that were upregulated in both *sid-1(jam113[del])* and *sid-1(jam80[non])* animals (red in Figures 4B, *top*, and S7D): the identical loci *W09B7.2/F07B7.2* (Figure S7F, *middle*), and *Y102A5C.36* (Figure S7F, *right*). Both genes have been reported[49] to be expressed within the germline (Figure S7G, *left*) and regulated by endogenous small RNAs (Figure S7G, *middle* and *right*). Spliced mRNA levels measured at a later generation using RT-qPCR demonstrated that both transcripts were upregulated in *sid-1(jam80[non])* animals compared to wild-type animals as expected (Figure 4C), but no upregulation was detectable in *sid-1(jam113[del])* animals (Figure S7E), further supporting the existence of complex effects caused by deletion of *sid-1* DNA (e.g., *F14F9.5* overexpression, loss of *sid-1* mRNA, etc.) that could be independent of SID-1 protein function.

To determine if changes in *W09B7.2/F07B7.2* and *Y102A5C.36* expression were heritable, we reverted the *sid-1* nonsense mutation to wild-type sequence using Cas9-mediated genome editing. This immediately restored silencing by ingested dsRNA (Figure 3B) with concomitant recovery of *sid-1* mRNA to wild-type levels (Figure 4C, *top*). In contrast, changes in both *W09B7.2/F07B7.2* and *Y102A5C.36* expression persisted (Figure 4C, *middle* and *bottom*) even after one year of passaging the reverted animals (*sid-1(jam86[rev])*) (i.e., after >100 generations, Figure 4C, *middle* and *bottom*). Thus, the *sid-1*-dependent accumulation of mRNA from these two germline genes persisted for many generations, likely through mechanisms that maintain heritable epigenetic changes. We hereafter refer to these ***s**id-1-**d**ependent **g**enes (sdg)* that show heritable epigenetic changes in response to temporary loss of SID-1 as *sdg-1* (*W09B7.2/F07B7.2*) and *sdg-2* (*Y102A5C.36*).

The *sid-1*-dependent gene *sdg-1* is affected by many factors that regulate RNA silencing in the germline

To determine if expression of *sdg-1* and *sdg-2* is regulated by other proteins that play a role in RNA silencing within the germline, we examined 21 published datasets[35,47,50-60] that reported changes that depend on such proteins (Figure 4). For each dataset, we determined if the lists of genes reported as showing significant changes in mutants compared to the respective wild types included *sdg-1* and/or *sdg-2* (Figure 4D). This analysis revealed that changes in mRNA and/or antisense small RNAs of *sdg-1* were detected in 20 of the 21 datasets while changes in *sdg-2* were observed in 9 of 21 (Figure 4D). When detected, changes in *sdg-2* were in the same direction as changes in *sdg-1*, suggestive of similar regulation of both genes.

RNAs transcribed in the germline can be recognized as they exit the nuclear pores by piRNAs bound to the Argonaute PRG-1, which recruits them for regulation by antisense small

RNAs called 22G RNA made by proteins organized within perinuclear germ granules (reviewed in ref.[61]). Interaction with piRNAs was detected for RNA from *sid-1*, *sdg-1*, and *sdg-2*, and the control gene *tbb-2* using crosslinking, ligation, and sequencing of hybrids[55] (Figure 4D), consistent with their germline expression. Depletion of downstream 22G RNAs targeting both *sid-1* and *sdg-1* was detectable upon loss of the germ granule component MUT-16[53] (Figure 4D). Both genes were among the top 500 genes targeted by 22G RNAs bound by the secondary Argonaute HRDE-1/WAGO-9[35] (Figure 4D), suggesting similar downregulation of both genes using 22G RNAs. Furthermore, multiple datasets support downregulation of *sdg-1* within the germline by HRDE-1/WAGO-9-bound 22G RNAs in the absence of PRG-1. One, loss of HRDE-1/WAGO-9 increased *sdg-1* RNA in whole animals[60] (Figure 4D) and in dissected gonads[59] (Figure 4D). Two, loss of PRG-1 decreased *sdg-1* RNA (Figure 4D) and increased 22G RNAs that are antisense to *sdg-1* (Figure 4D) in dissected gonads[54]. Three, although animals that lack PRG-1 become progressively sterile, the increase in *sdg-1* 22G RNA persisted in near-sterile animals (Figure 4D, near-sterile in ref.[54]), and this increase was eliminated upon additional loss of HRDE-1/WAGO-9 (Figure 4D, near-sterile in ref.[54]). One hypothesis suggested by these findings is that the *sdg-1* transcripts are protected from HRDE-1/WAGO-9-mediated silencing by PRG-1-bound piRNAs.

As expected for *sid-1*-dependent downregulation of *sdg-1*, multiple datasets reveal an inverse relationship between the two genes. In animals lacking PRG-1, *sid-1* RNA levels increased and *sid-1* 22G RNAs decreased[57] (Figure 4D), but both *sdg-1* RNA and *sdg-2* RNA levels decreased along with an increase in 22G RNAs[53-58] (Figure 4D). This inverse relationship between *sid-1* and *sdg-1* RNA regulation is also observed when many components of germ granules are mutated as indicated by changes in 22G RNA upon loss of the embryonic P granule

components MEG-3/-4[47] (Figure 4D), the PRG-1 interactor DEPS-1[53] (Figure 4D), or the Z granule component ZSP-1[52] (Figure 4D; also known as PID-2[62]).

In addition to the above studies, pioneering studies that used microarrays identified *sdg-1* as upregulated in animals lacking the germ granule component DEPS-1[51] (Figure 4D) and in animals lacking the dsRNA-binding protein RDE-4 (Figure 4D; second-most upregulated in ref.[50]), which recruits dsRNA imported through SID-1 for processing and eventual gene silencing. Animals that lack RDE-4 show a ~47.5-fold increase in *sdg-1* RNA[50]. A reduction in RDE-4 activity could also contribute to the ~11.6-fold increase in *sdg-1* RNA seen in *deps-1(-)* animals because these animals also show a ~3.2-fold decrease in *rde-4* RNA[51] (one of 13 downregulated genes). These observations support changes in *sdg-1* RNA through both piRNA-mediated regulation via germ granule components such as DEPS-1 and dsRNA-mediated regulation via SID-1 and RDE-4.

In summary, the levels of *sdg-1* RNA are detectably regulated by the dsRNA-selective importer SID-1, the dsRNA-binding protein RDE-4, and the piRNA-binding Argonaute PRG-1. Presence of dsRNA-mediated regulation or loss of piRNA-mediated regulation enhances MUT-16-dependent production of secondary small RNAs that bind the secondary Argonaute HRDE-1/WAGO-9. Consistent with downregulation of these *sid-1*-dependent transcripts by SID-1, disruption of many components of germ granules result in opposite effects on these transcripts and *sid-1* RNA. Intriguingly, a search of protein interaction studies revealed that the SDG-1 protein is among the interactors of two germ granule components: PID-2 by immunoprecipitation[62] (also known as ZSP-1[52]) and DEPS-1 by proximity labeling[63]. Thus, one possibility suggested by these observations is that reduction of *sdg-1* RNA via SID-1 reduces the

amount of SDG-1 protein, which could interact with components of germ granules to tune RNA regulation within the germline.

Regulation of *sdg-1* RNA is susceptible to epigenetic changes that last for many generations

SDG-1 is encoded by a gene located within a retrotransposon (Figure S8A) that is within a duplicated ~40 kb region and has two recognizable paralogs (Figure S8B). To facilitate analysis of SDG-1 expression, we tagged both loci that encode SDG-1 with *mCherry* coding sequences lacking piRNA-binding sites[64-65] (*mCherry*Δ*pi*) (Figures S8C and S8D), thereby preventing possible silencing of *mCherry* as a foreign sequence. This tagging resulted in the expression of *sdg-1::mCherry*Δ*pi* mRNA being ~16-fold higher than *sdg-1* mRNA (Figure S8E, *bottom*), potentially because of the reduction in the overall density of piRNA-binding sites per transcript, the additional introns included in *mCherry*Δ*pi* (based on refs.[66-67]), and/or other unknown factors. Fluorescence from SDG-1::mCherry was observed in the germline of adult animals (Figure 5A). However, animals showed dramatic variation in SDG-1::mCherry expression between their two gonad arms (Figure 5A, *middle* shows bright anterior (20% of animals) and *right* shows bright posterior (6% of animals)). A contributing feature for the observed stochasticity could be the location of *sdg-1* within a duplicated region (Figure S8A), as suggested by similar stochastic RNA silencing of multi-copy transgenes but not single-copy transgenes[42]. Despite this variation, unbiased passaging of self-progeny for more than 18 generations continuously preserved SDG-1::mCherry expression in an otherwise wild-type background (Figure 5B). In contrast, mating, which can perturb RNA regulation within the germline in cross progeny[65], caused dramatic changes in *sdg-1* expression that persisted in descendants (Figure 5C). Mating animals that express SDG-1::mCherry with wild-type animals resulted in heritable changes along lineages that express *sdg-1::mCherry*Δ*pi* mRNA or that

express *sdg-1* mRNA (Figures 5C and S9A). While mating-induced silencing could make decreases in fluorescence uninterpretable when using genetic crosses for mutant analysis (Figure 5D, potentially in *sid-2(-)*, *sid-5(-)* and *eri-1(-)* animals), we observed an increase in SDG-1::mCherry fluorescence in animals lacking RME-2 or MUT-16 (Figure 5D), supporting a role for these two proteins in downregulating SDG-1 expression.

To avoid mating-induced perturbations of RNA regulation within the germline, we used Cas9-mediated genome editing to introduce mutations into animals that express SDG-1::mCherry in an otherwise wild-type background. Use of this approach to mutate a control gene with no known roles in RNA regulation within the germline resulted in similar levels of SDG-1::mCherry fluorescence in multiple isolates of animals with and without the mutation (Figure S9B). In contrast, mutating *sid-1* using Cas9-mediated genome editing caused a range of expression levels in different isolates when compared with *sid-1(+)* animals (Figure 5E, 6 isolates lower, 2 isolates comparable, and 1 isolate higher), which differs from the persistent increase in *sdg-1* mRNA observed upon SID-1 loss in otherwise wild-type animals (Figure 4). One explanation for these observations is that the ~16-fold overexpression of *sdg-1::mCherry*Δ*pi* mRNA perturbs RNA-mediated epigenetic regulation, potentially indicative of a role for the SDG-1 protein in such regulation. Mutating *sid-3* also lowered the levels of SDG-1::mCherry in one isolate, but caused no detectable change in another (Figure 5F). While both isolates with loss of RDE-1 showed lower levels of SDG-1::mCherry, both isolates with loss of the germ granule component DEPS-1 showed higher levels of SDG-1::mCherry. Thus, loss of SID-3, SID-1 or RDE-1 can cause a reduction in the expression of SDG-1::mCherry, but loss of RME-2, MUT-16, or DEPS-1 increases the expression of SDG-1::mCherry. The opposing effects of SID-1 loss and of RME-2 loss on SDG-1::mCherry expression suggests that factors other than dsRNA

imported through RME-2 (e.g., lipids, proteins, etc.) contribute to changes in the levels of SDG-1::mCherry expression. Similarly, increases in the absence of MUT-16 and DEPS-1 suggest that germ granules contribute to changes in the levels of SDG-1::mCherry expression. Once the levels of SDG-1::mCherry were reduced upon loss of SID-1, downregulation persisted across generations even after restoration of wild-type SID-1 (Figure 5G), just as the upregulation of untagged *sdg-1* mRNA also persisted (Figure 4). Despite >100 generations of such persistent silencing, the expression of SDG-1::mCherry could be restored by mutating *deps-1* (Figure 5G), implicating small RNA-based regulation and germ granules in the maintenance of new epigenetic states established upon loss of SID-1.

Together, these results suggest that one or both *sdg-1* loci are subject to heritable changes upon loss of SID-1-dependent gene regulation, the direction of change might depend upon the levels of *sdg-1* mRNA, and maintenance of these changes requires a DEPS-1-dependent mechanism. The expression states observed for SDG-1::mCherry and SDG-1 (Figures 4 and 5) could reflect a role for the SDG-1 protein in piRNA-mediated regulation, potentially through interactions with the Z-granule component ZSP-1/PID-2[62] and/or the germ granule-associated protein DEPS-1[63]. Specifically, high levels of SDG-1::mCherry protein produced from *sdg-1::mCherry*Δ*pi* as a result of reduced piRNA-mediated silencing of *mCherry* as a foreign sequence[64] could result in a state of enhanced piRNA-mediated regulation within the germline. This state when perturbed either through mating (Figure 5C) or through a block in competing silencing by extracellular dsRNA (Figures 5D, 5E and 5F) is sufficient for initiating heritable RNA silencing. Silencing of the *sdg-1::mCherry*Δ*pi* fusion transcript once initiated is stable, requires DEPS-1, and is presumably mediated by the piRNA-binding sites located within the *sdg-1* sequence.

SID-1-dependent genes, including SDG-1, could alter RNA-mediated regulation in the germline

If *sid-1*-dependent genes such as *sdg-1* encode proteins that interact with regulators of RNA silencing (e.g., ZSP-1/PID-2, DEPS-1, etc.), loss of SID-1 could change intracellular RNA regulation in addition to preventing the entry of extracellular dsRNA into the cytosol. In support of this possibility, intracellular delivery of dsRNA through injection into the syncytial germline of *sid-1(-)* animals showed a defect in silencing that was detectable in later progeny (Figure 2D in ref.[26] and Figure 1 in ref.[27]). To examine if changes in the levels of SDG-1 alone could account for such a defect in silencing by intracellular dsRNA in the germline, we used a sensitive assay to examine animals that lack *sdg-1* (3 different deletions of the *sdg-1* open reading frame generated using Cas9-mediated genome editing) and animals that overexpress *sdg-1* (i.e., with *sdg-1::mCherry*Δ*pi*). Specifically, a ~16 hr pulse of dsRNA matching the germline gene *pos-1* was used to cause intermediate levels of silencing in wild-type animals. The extent of silencing both in animals that lacked *sdg-1* and in animals that overexpressed *sdg-1* were comparable to that in wild-type animals (Figure 6A), suggesting that changes in *sdg-1* alone are not sufficient to account for the previously reported defect in the response to intracellular dsRNA in *sid-1(-)* animals[26-27]. Alternatively, the SID-1-dependent regulation of the response to dsRNA within the germline could be through the promotion of competing piRNA-mediated gene regulation because loss of PRG-1 enhances heritable RNA silencing by dsRNA[68]. To test if SDG-1-mediated inhibition of silencing by dsRNA occurs through positive regulatory interactions of SID-1-dependent genes with competing piRNA-mediated silencing, we used an experimental system which initiates piRNA-mediated silencing of the two-gene operon described in Figure 1 through mating, independent of externally-provided dsRNA[65]. We found that *sid-1(-)* animals exhibited enhanced mating-induced silencing (Figure 6B*, top right*: ~50% off in *sid-1(+)* vs

100% off in *sid-1(qt9[non])*) while animals lacking *sdg-1* showed a small reduction in mating-induced silencing that is not statistically significant (Figure 6B*, bottom right*, ~40% off in *sdg-1(+)* vs ~30% off in *sdg-1(jam232[del])*). Taken together, these results support the model that loss of SID-1 weakly inhibits silencing by intracellular dsRNA but enhances silencing by piRNAs within the germline potentially through the action of multiple SID-1-dependent genes that promote piRNA-mediated gene regulation.

RNA regulation within the germline using piRNAs relies on phase-separated granules within the germline (reviewed in ref.[69]). To determine if the identification of SDG-1 as a potential interactor of the Z-granule component PID-2[62]/ZSP-1[52] and in proximity to the P-granule-adjacent protein DEPS-1[63] could be seen as colocalization *in vivo*, we examined the localization of SDG-1::mCherry within the cytoplasm at higher resolution using AiryScan imaging[70]. SDG-1::mCherry was enriched in perinuclear foci in many animals (Figure 6C, *top*; 7 of 9 animals) and these sites of enrichment colocalized with the Z-granule marker GFP::ZNFX-1 (Figure 6C, *bottom*; 100% colocalized in 10 of 12 animals with enrichment). Time-course imaging revealed re-localization of SDG-1::mCherry into the nucleus from the cytoplasm of the -1 oocyte, which will be the first to be fertilized (Figure 6D and Movie S1). Dynamic entry into the nucleus also occurred during early cell divisions in the developing embryo (Figure 6D and Movies S1, S2, S3 and S4). The timing of nuclear entry and exit of SDG-1::mCherry coincides with the nuclear envelope breakdown (NEBD) events that occur during fertilization and early development[71]. The *sdg-1* coding sequence was required for regulated nuclear entry as deletion of the *sdg-1* open reading frame in *sdg-1::mCherryΔpi* animals resulted in mCherry expression throughout the germline in both the cytoplasm and nuclei (Figure 6E). Nuclear localization of the SDG-1 protein in the -1 oocyte is similar to that of the essential Argonaute CSR-1b[72], thought to

play a role in protecting transcripts from silencing, and is consistent with a role for the SDG-1 protein in promoting the PRG-1/piRNA-dependent protection of transcripts (including *sdg-1*) from HRDE-1/WAGO-9-dependent silencing (Figure 4D).

Together these results support a model (Figure 6F) where the levels of *sdg-1* RNA are reduced by dsRNA imported through SID-1 and protected by piRNAs bound to PRG-1. However, increasing the levels of the SDG-1 protein alters the outcome of both dsRNA- and piRNA-mediated regulation of the *sdg-1* gene. The enrichment of the SDG-1 protein in perinuclear foci like Z-granules and its regulated entry into the nucleus suggest active roles for this protein in RNA regulation within the germline despite being encoded by a gene located within a retrotransposon.

DISCUSSION

Our analyses elucidate pathways for the transport of extracellular dsRNA from parents to progeny and reveal a potential role for this mode of intergenerational gene regulation. We show that dsRNA from neurons with oxidative damage can accumulate in parental circulation and that such extracellular dsRNAs are transported with developmental and substrate specificity to the next generation. Blocking all dsRNA import into the cytosol through the loss of the conserved dsRNA importer SID-1 disrupts RNA regulation within the germline through changes in a gene that is located within a retrotransposon, which is itself a target of RNA silencing. This *sid-1*-dependent gene encodes a regulator of RNA silencing within the germline, suggesting a regulatory architecture where SID-1-dependent silencing by extracellular dsRNA modulates the extent of heritable RNA regulation.

While the physiological conditions that promote secretion of dsRNA are not known, the discovery that oxidative damage of neurons can enhance the secretion of dsRNA suggests that

disruption of cell structures by oxidative damage (e.g., membrane integrity) or initiation of cellular processes that repair oxidative damage (e.g., through ejection of damaged macromolecules[73]) also promote the release of dsRNA. Pathologies of the central nervous system in humans, including cancer, stroke, multiple sclerosis, neurodegenerative disease, and brain injury, have been associated with extracellular RNAs detected in circulation (reviewed in ref.[74]), although their origins and regulatory consequences, if any, remain unknown. The gene regulatory effects of neuronal dsRNA released upon oxidative damage of neurons provide convenient readouts that can be analyzed to understand neuronal damage and its consequences in animals.

The trafficking of extracellular dsRNA from parent to progeny has spatial specificity, as evidenced by more silencing within the proximal germline (Figure 1), temporal specificity, as evidenced by the need for dsRNA beyond the fourth larval stage[26-27] (Figure 1), and substrate specificity, as evidenced by the differential requirements for 50-bp dsRNA with 5' OH versus a mix of longer dsRNAs with 5' triphosphates (Figure 2). One possible explanation for these constraints could be that proteins mediating dsRNA transport differ in their availability during development and in their affinities for different substrates. For example, SID-1, which was not detected in the developing larval germline but was detected in the adult germline (Figure 3), has an extracellular domain that binds dsRNA[75] and could prefer dsRNA molecules with 5' phosphates. Although the selectivity uncovered here could apply to all dsRNA delivered into the extracellular space of *C. elegans* from any source, the chemistry of the delivered dsRNA could be modified by as yet unidentified enzymes *in vivo* to overcome these requirements. Tracking labeled dsRNA with diverse chemistries from parental circulation to progeny could allow

correlation of differences observed in progeny silencing to differences in intergenerational trafficking.

The germline is a major site of dsRNA import in *C. elegans* as evidenced by the expression of SID-1 in the germline (Figure 3), heritable misregulation of germline genes in *sid-1(-)* animals (Figures 3 and 4), and accumulation of fluorescently-labeled dsRNA from the extracellular space in the germline[26-27]. As a result, *sid-1(-)* animals could have a defect in the germline that is detectable only under conditions that promote dsRNA transport (e.g., oxidative damage). Multiple physiological defects in the germline and soma of *sid-1(-)* animals have been reported but have not been widely reproduced, have only been characterized within single generations, or have not been attributed to any specific *sid-1*-dependent gene(s). These include defects in animals with some misfolded proteins in the endoplasmic reticulum[76], in animals exiting the dauer stage[77-78], in animals exposed to pathogenic *P. aeruginosa*[79-81], in animals exposed to odor[82], in intestinal cells that develop in the presence of a multi-copy transgene[83], and in animals that overexpress α-synuclein[84]. RNA-seq experiments in this study suggest that genetic background-dependent changes can obscure genuine *sid-1*-dependent changes (Figure S7), raising caution in the interpretation of putative *sid-1*-dependent defects. Comparing multiple *sid-1* mutants generated using genome editing with animals in which the mutated sequence has been reverted to wild-type sequence in the same genetic background could provide a firmer basis for the identification of *sid-1*-dependent processes.

Genes expressed within the germline are likely regulated by positive feedback loops required to continually produce factors for maintaining germline immortality and for preserving form and function across generations[85-86]. Thus, germline genes could be particularly vulnerable to heritable epigenetic changes, where deviations in the expression levels of a gene that is

regulated by or is part of such feedback loops has the potential to become permanent in descendants. Our analysis of *sdg-1* expression suggests that it is part of a regulatory architecture that is susceptible to heritable epigenetic changes through the perturbation of RNA regulation (Figures 4, 5 and 6). Such architectures within the germline could be exploited by 'selfish' genetic elements such as retrotransposons to persist across evolution if one of these elements also include genes encoding a regulator. In support of a wider use of such a strategy, a paralog of SDG-1, ZK262.8, is also encoded by a gene located within a retrotransposon and its loss along with that of the miRNA-associated Argonaute ALG-2 was reported to be synthetic lethal[87]. To buffer against heritable epigenetic changes, levels of gene expression would need to be maintained within a particular range for a given regulatory context. Given the association of SDG-1 protein with germ granule components ZSP-1/PID-2 and DEPS-1, and the maintenance of heritable changes in *sdg-1* expression by DEPS-1, buffering against changes in gene expression could involve both RNA- and protein-based regulation that tunes the function of perinuclear granules. We therefore speculate that one role for extracellular RNAs that enter germ cells in other systems (e.g., tRNA fragments in mammals[5-6,8]) could be to similarly buffer against heritable changes in gene expression.

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Author contributions

N.S., A.S., and A.M.J designed the research. N.S., A.S., A.L.Y., W.M.C., J.A.M., and A.M.J. performed all experiments, and collected and analyzed data. Contributions from J.A.M., now at the U. S. Food and Drug Administration, were made while in graduate school at the University of Maryland, College Park. N.S., A.S., and A.M.J. prepared the manuscript with contributions from all authors.

Data and Materials availability

All data and code are available in the manuscript or the supplementary materials. RNA-seq data has been deposited to Gene Expression Omnibus (GEO) with the accession number GSE185385.

Declaration of interests

The authors declare no competing interests.

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FIGURES AND LEGENDS

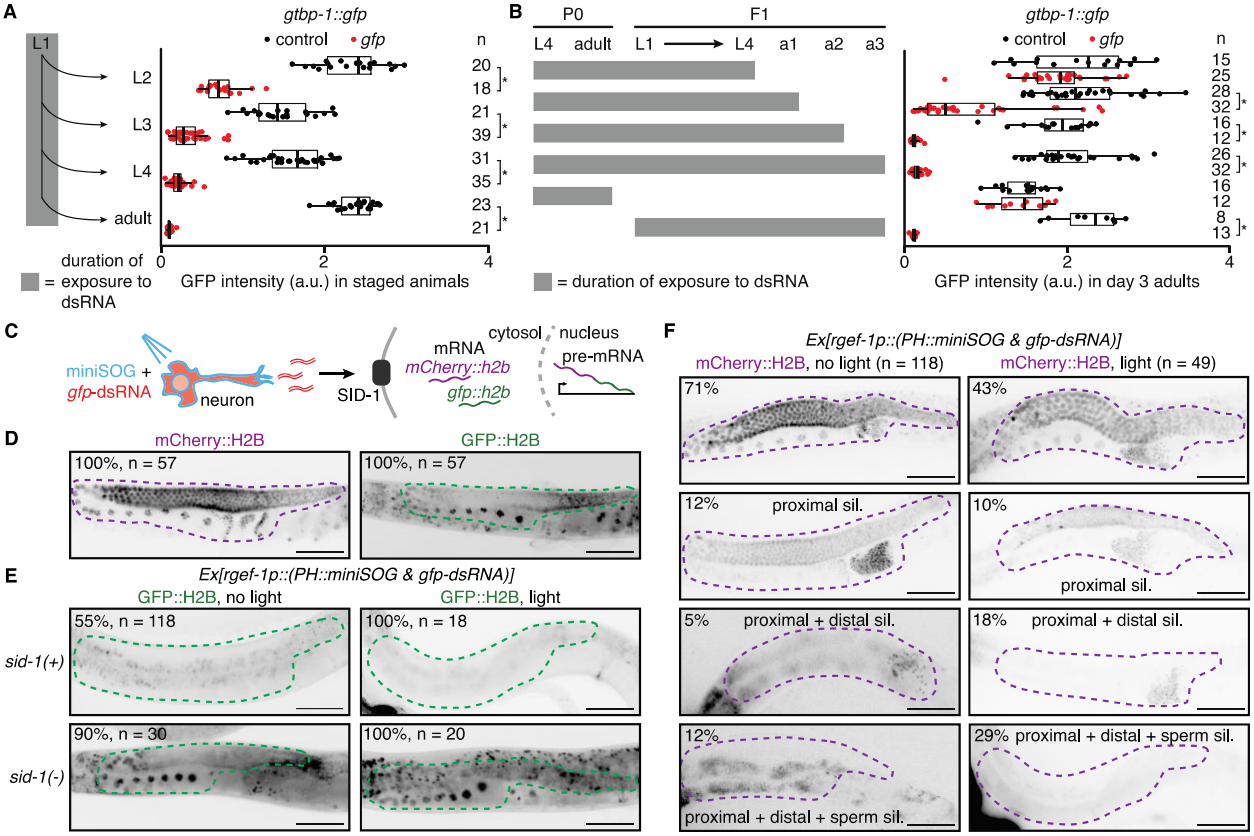

Figure 1. Timed delivery of ingested or neuronal dsRNA suggests spatiotemporal differences in germline entry. (A and B) Silencing in the germline after exposure of *gtbp-1::gfp* animals to bacteria expressing dsRNA and imaging of separate cohorts at indicated stages (A) or day 3 of adulthood (B). (A, *left* and B, *left*) Schematics depicting stages and durations of exposure to dsRNA. (A, *right* and B, *right*) GFP intensity (a.u.) in *gtbp-1::gfp* animals quantified in germ cells (larvae) or eggs *in utero* (adults) (A) or in day 3 adult (a3) animals (B) after exposure to control dsRNA (black) or *gfp*-dsRNA (red). The numbers of animals scored at each stage (n) are indicated. Asterisks in (A) and (B) indicate $P < 0.05$ with Bonferroni correction using Mann-Whitney U test for two-sided comparisons between animals exposed to control or *gfp*-dsRNA. Also see Figure S1. (C) Schematic illustrating exposure of animals expressing a singlet oxygen generator (miniSOG) and *gfp*-dsRNA in neurons to blue light and subsequent

release of dsRNA. Such extracellular dsRNA is expected to enter the cytosol of the germline through the dsRNA importer SID-1 and silence *gfp::h2b* mRNA from a two-gene operon that expresses *mCherry::h2b* and *gfp::h2b* as part of a single pre-mRNA. (D, E and F) Images of single gonad arms in adult animals with the two-gene operon (*mex-5p::mCherry::h2b::gfp::h2b*) showing fluorescence (black) of mCherry::H2B (magenta outline) or of GFP::H2B (green outline). Punctate autofluorescence from the intestine can also be seen. Numbers of animals assayed (n) and percentages of adult animals with the depicted expression patterns are indicated. Scale bars, 50 μm. (D) mCherry::H2B fluorescence is seen throughout the germline (*left*) and GFP::H2B fluorescence is seen in the oocytes and in the distal gonad (*right*). (E) GFP::H2B fluorescence in *sid-1(+)* and *sid-1(-)* animals expressing membrane-localized miniSOG (*PH::miniSOG*) and *gfp*-dsRNA driven by a neuronal promoter (*rgef-1p*) from a multi-copy transgene (*Ex*, *jamEx214*) without (*left*) or with (*right*) exposure to blue light at 48 hours post L4-stage of parent. (F) mCherry::H2B fluorescence in *sid-1(+)* animals with the transgene *Ex*. Silencing of mCherry is enhanced in the distal gonad (third row) and sperm (fourth row) after exposing animals to blue light at 48 hours and 54 hours post L4-stage of parent. Also see Figures S1 and S2.

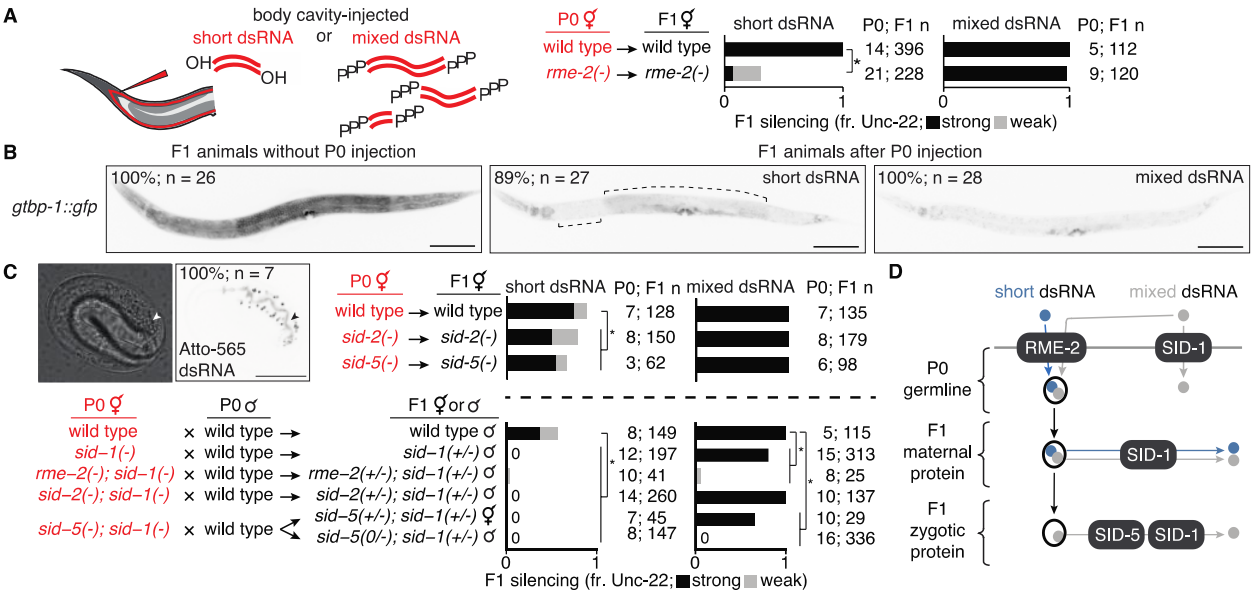

Figure 2. Transport of dsRNA from parental circulation to progeny occurs through two routes with distinct substrate selectivity. (A) Hermaphrodite animals of indicated genotypes (in red) were injected in the body cavity with 50-bp *unc-22*-dsRNA synthesized with a 5'-OH (short dsRNA, left bars) or *unc-22*-dsRNA with a 5' triphosphate transcribed from a ~1.1 kb template (mixed dsRNA, right bars). Hermaphrodite self-progeny of injected animals were scored for *unc-22* silencing (fr. Unc-22: strong, black; weak, grey). Numbers of injected parents and scored progeny (P0; F1 n) are indicated. Also see Figures S2 and S3. (B) Fluorescence images of progeny from animals with a *gfp* tag of the ubiquitously expressed gene *gtbp-1* (*gtbp-1::gfp*) that were not injected (*left*), injected with 50-bp *gfp*-dsRNA (short dsRNA injection, *middle*), or injected with dsRNA transcribed from a ~730-bp template (mixed dsRNA injection, *right*). Complete silencing is not observed in neurons or in the developing vulva; brackets indicate additional regions with dim GFP fluorescence. Numbers of animals assayed (n) and percentages of L4-staged animals with the depicted expression patterns are indicated. Scale bar, 100 µm. Also see Figure S4. (C) Requirements for intergenerational transport of extracellular dsRNA. (*top left*) Differential Interference Contrast (DIC) and fluorescence images of a

developing embryo from an animal injected in the body cavity with 50-bp dsRNA of the same sequence as in (B) and labeled at the 5' end of the antisense strand with Atto-565. Accumulation within the intestinal lumen (arrowhead), number of embryos imaged (n), and percentage of embryos with depicted pattern of fluorescence are indicated. Scale bar, 20 μm. (*top right* and *bottom*) Hermaphrodite animals of the indicated genotypes were injected with short dsRNA (left bars) or mixed dsRNA (right bars) and self-progeny (*top right*) or cross progeny after mating with wild-type males (*bottom*) were analyzed as in (A). Cases of no observable silencing are indicated with '0'. (D) Schematic summarizing requirements for transport of dsRNA from parental circulation to developing progeny. See text for details. Asterisks in (A) and (C) indicate $P < 0.05$ with Bonferroni correction using χ^2 test.

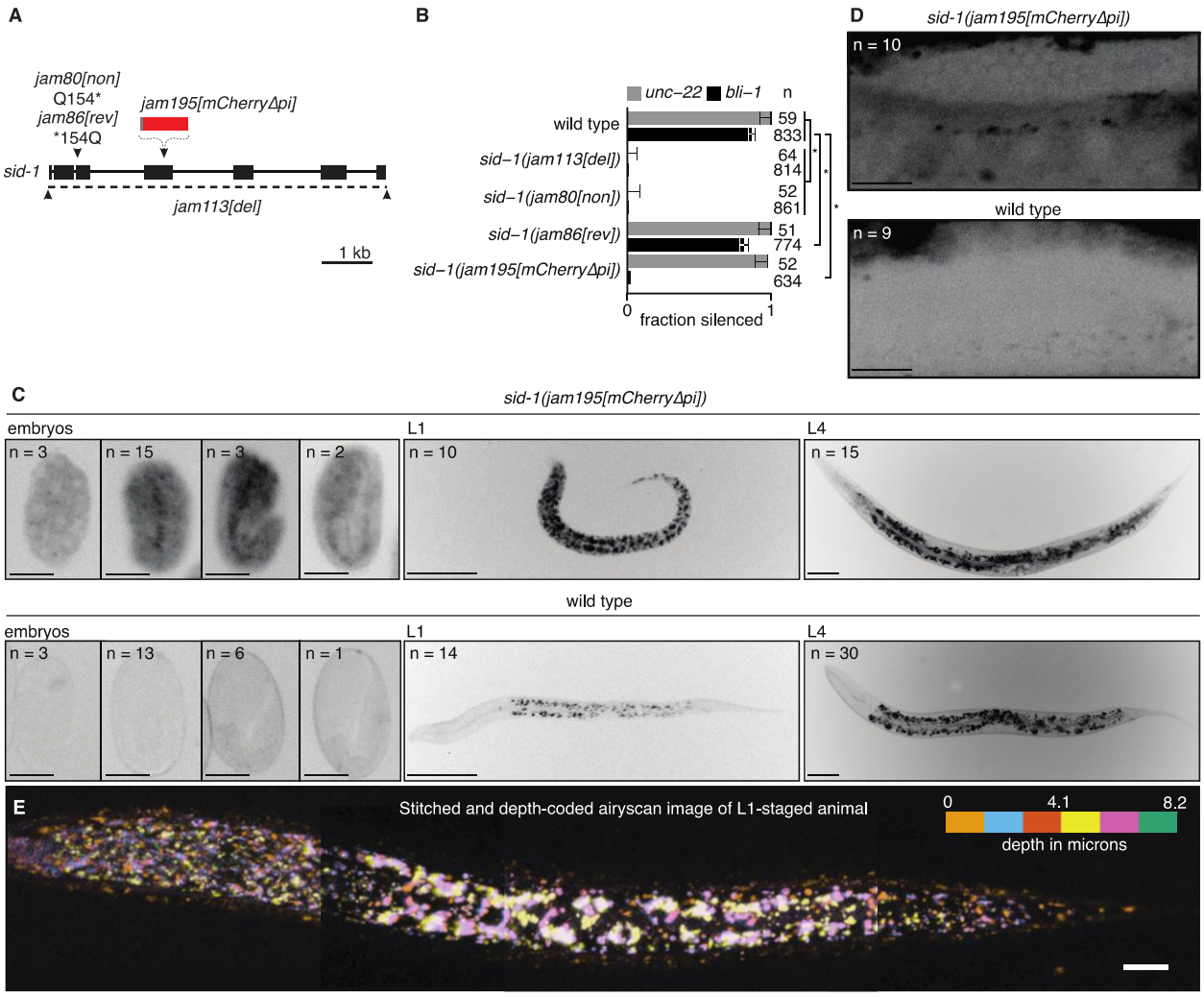

Figure 3. The expression pattern of SID-1 varies during development. (A) Schematic of modifications at the *sid-1* gene generated using Cas9-mediated genome editing. Deletion of the entire coding sequence (*jam113[del]*), a nonsense mutation (*jam80[non]*), its reversion to wild-type sequence (*jam86[rev]*), and insertion of *mCherry* sequence that lacks piRNA binding sites[64-65] (*jam195[mCherryΔpi]*) are depicted. (B) Fractions of animals of the indicated genotypes that show silencing in response to *unc-22*-dsRNA (grey) or *bli-1*-dsRNA (black). Tagging SID-1 with mCherry (*sid-1(jam195[mCherryΔpi])*) likely results in a partially functional SID-1::mCherry fusion protein because the rescue of *unc-22* silencing is robust but that of *bli-1* silencing is minimal (only 6 of 634 animals showed the Bli-1 defect). Numbers of

animals scored (n), significant differences using two-tailed test with Wilson's estimates for single proportions (asterisks, $P < 0.05$ with Bonferroni correction) and 95% CI (error bars) are indicated. (C and D) Representative images showing fluorescence from SID-1::mCherry (black) in (C) developing embryos (*left*), L1-stage animals (*middle*), L4-stage animals (*right*) or (D) the adult gonad arm of *sid-1(jam195[mCherryΔpi])* animals (*top*) compared to no detectable fluorescence in wild-type animals of the same stages (*bottom*). Numbers of embryos of each stage (C, *left*), L1 animals (C, *middle*), L4 animals (C, *right*) and adult gonad arms (D) imaged (n) are depicted and 100% of animals exhibited the depicted expression patterns. For animals imaged in (D), the distal germline was obstructed by the intestine in 1/10 *sid-1(jam195[mCherryΔpi])* and 5/9 wild-type animals. (E) Airyscan image of an L1-staged animal assembled by stitching depth-coded maximum projections of four Z-stacks, illustrating the expression of SID-1::mCherry throughout the worm. Scale bar for embryos in (C) and adult gonad arms in (D), 20 μm; scale bar for larvae in (C), 50 μm and in (E), 10 μm. Also see Figure S5.

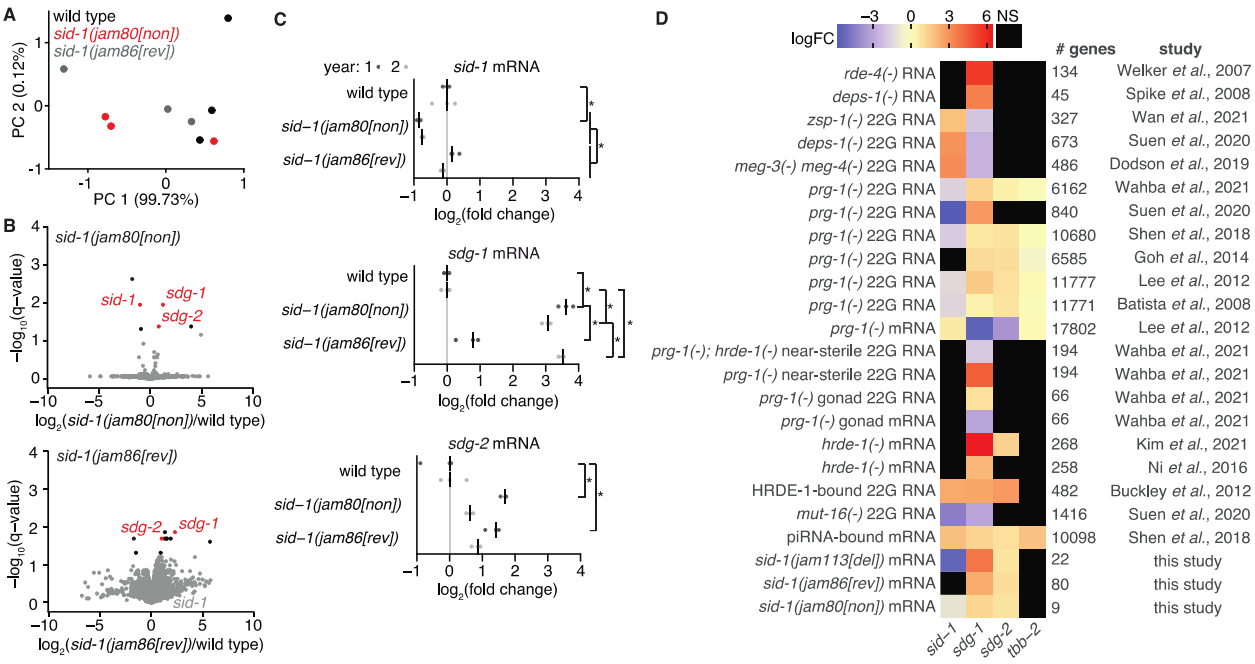

Figure 4. Ancestral loss of SID-1 causes transgenerational changes in the mRNA levels of two germline genes that are subject to RNA regulation. (A) Principal components explaining the variance between wild type (black), *sid-1(jam80[non])* (red), and *sid-1(jam86[rev])* (grey) polyA+ RNA samples. Almost all of the variance between samples is explained by PC 1. (B) Volcano plots of changes in the abundance of polyA+ RNA in *sid-1(jam80[non])* (*top*) and *sid-1(jam86[rev])* (*bottom*) animals compared with wild-type animals (black, $q < 0.05$; red, both $q < 0.05$ and change in the same direction in *sid-1(jam80[non])* and *sid-1(jam113[del])*; see Figure S7). While *sid-1* transcript levels in *sid-1(jam86[rev])* are comparable to that in wild type (grey), *sdg-1* (*W09B7.2*/*F07B7.2*) and *sdg-2* (*Y102A5C.36*) transcript levels remain elevated in *sid-1(jam86[rev])* (red). (C) Levels of spliced *sid-1*, *sdg-1* and *sdg-2* transcripts measured using RT-qPCR. The median of three technical replicates is plotted for each of three biological replicates (bar indicates median) assayed before and after 1 year of passaging animals (year 1, dark grey; year 2, light grey). Asterisks indicate $P < 0.05$ with Bonferroni correction using two-tailed Student's t-test. (D) Heatmap showing changes in the levels of transcripts (RNA and/or mRNA)

and antisense small RNAs (22G RNA) from *sid-1*, *sdg-1*, *sdg-2*, and *tbb-2* (abundant germline transcript for comparison). Fold changes (expressed as LogFC, indicating $\log_2$ for RNA, $\log_{10}$ for piRNA binding, and $\log_{10}$ for 22G RNA) were deduced by integrating reports (studies indicated) of 21 experiments that identify subsets of genes as being subject to RNA-mediated regulation within the germline (# genes). These prior studies include comparisons of RNA or 22G RNA from wild-type animals with that from mutant animals (e.g., *mut-16(-)* 22G RNA), biochemical detection of piRNA binding to transcripts (piRNA-bound mRNA), and biochemical detection of 22G RNA binding to an Argonaute (HRDE-1-bound 22G RNA). 'NS' indicates cases where changes, if any, were not significant based on the criteria used in the study. A conservative value of 2-fold is assigned to all genes reported as changing >2-fold in ref.[60].

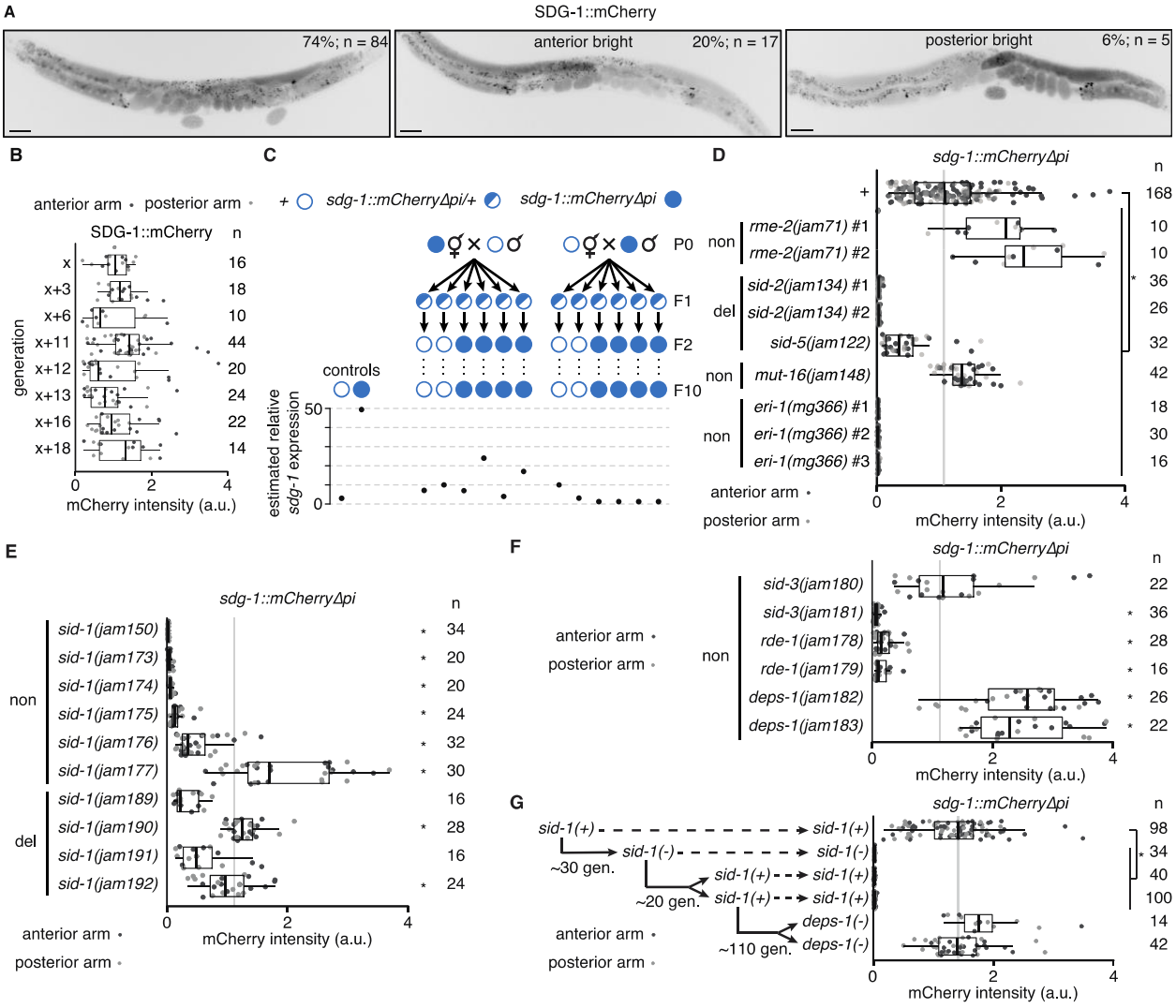

Figure 5. The *sdg-1* gene is prone to stochastic changes in gene expression that can become heritable. (A) Representative images showing fluorescence of SDG-1::mCherry (black) in a wild-type background. While most animals showed symmetric expression in the germline (*left*), animals with >2-fold difference in fluorescence between both gonad arms (bright anterior, *middle* and bright posterior, *right*) were also observed. Punctate fluorescence in the intestine likely represents autofluorescence. Scale bar, 50 µm. (B) Quantification of SDG-1::mCherry fluorescence intensity (arbitrary units, a.u.) in adult gonad arms (anterior arm, dark grey; posterior arm, light grey) of *sdg-1(jam137[mCherryΔpi])* animals starting in one generation (x)

and continuing in successive generations as indicated. Numbers of gonad arms quantified (n) is indicated. Expression in one generation was not significantly different when compared to that in the previous tested generation using Mann-Whitney U test for two-sided comparisons and Bonferroni correction. (C) Lineages and estimated relative *sdg-1* expression 10 generations after mating wild-type (open circle) males with *sdg-1::mCherry*Δ*pi* (filled circle) hermaphrodites and vice versa, and isolating *sdg-1(+)* or *sdg-1::mCherry* animals from F1 heterozygotes (half-filled circle). Expression of *sdg-1* in the F10 generation was measured by RT-qPCR of *sdg-1* mRNA purified from pooled wild-type animals of mixed stages or by quantification of SDG-1::mCherry fluorescence in gonad arms of adult *sdg-1::mCherry*Δ*pi* animals. Relative levels of *sdg-1* mRNA and SDG-1::mCherry fluorescence intensity were converted to units of estimated relative *sdg-1* expression (see STAR Methods) for comparison. See Figure S9A for raw data. (D, E and F) Fluorescence intensity measurements (quantified as in (B)) in adult animals with *sdg-1::mCherry*Δ*pi* (+) and additionally with mutations in genes introduced through genetic crosses (in regulators of dsRNA import *rme-2*, *sid-2* or *sid-5*, or in regulators of RNA silencing *mut-16* or *eri-1*) or through genome editing (in regulators of dsRNA import *sid-1* or *sid-3*, or in regulators of RNA silencing *rde-1* or *deps-1*). Asterisks indicate $P < 0.05$ with Bonferroni correction using Mann-Whitney U test for two-sided comparisons between animals with *sdg-1::mCherry*Δ*pi* (+) and animals with additional mutations. Nonsense mutations (non) or deletions (del) introduced through genetic crosses (isolate numbers #1, #2, etc. in (D)) or genome editing (different alleles in (E) and (F)) and numbers of gonad arms (n) quantified for each isolate are indicated. Mutations in genes required for dsRNA import or subsequent silencing resulted in fewer animals with asymmetric fluorescence between gonad arms (a combined proportion of 21/197 for *sid-1*, *sid-3*, *rde-1* and *deps-1* mutants versus 22/84 for wild type, $P =$

0.0009 using two-tailed test with Wilson's estimates for single proportions). Animals with at least one gonad arm brighter than the dimmest wild-type gonad arm in (A) and with asymmetric gonad arms were found in different genotypes (anterior bright: *sid-1(-)* – 5/122, *sid-3(-)* – 1/29, *rde-1(-)* – 2/22, *deps-1(-)* – 4/24, and posterior bright: *sid-1(-)* – 6/122, *rde-1(-)* – 2/22, *deps-1(-)* – 1/24). (G) Fluorescence intensity measurements as in (B) of animals with *sdg-1::mCherry*Δ*pi* that show loss of fluorescence when a nonsense mutation is introduced in *sid-1* using genome editing ~30 generations (gen.) later remain changed despite reversion of *sid-1* nonsense mutation to wild-type sequence after ~20 additional generations. Subsequent mutation of *deps-1* after another ~110 generations restored SDG-1::mCherry fluorescence to wild-type levels. Also see Figures S8 and S9.

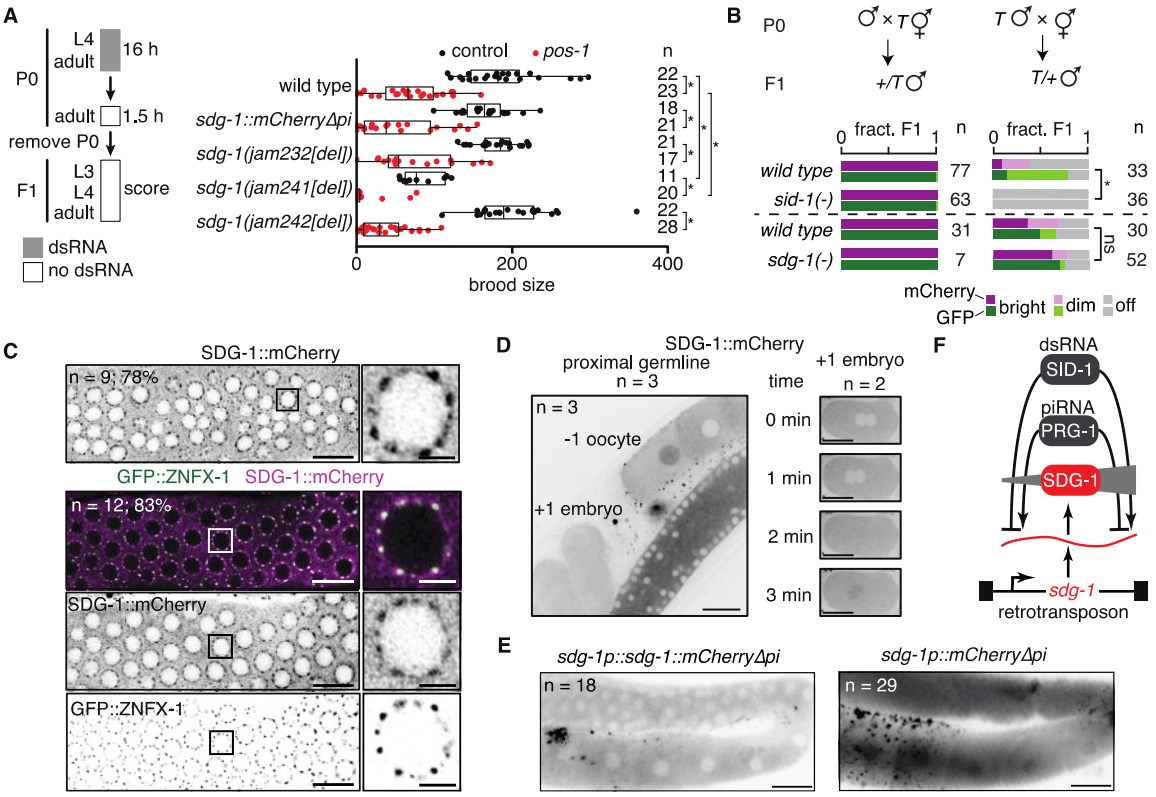

Figure 6. SID-1 modifies RNA regulation within the germline, potentially through *sdg-1* and other *sid-1*-dependent genes. (A, *left*) Schematic of assay for sensitive detection of *pos-1* silencing by ingested dsRNA. (A, *right*) Numbers of developed progeny (> 3rd larval stage) laid by wild-type animals, animals with a deletion (Δ) in *sdg-1* (*jam232*, *jam241*, *jam242*) or animals with overexpression (*sdg-1::mCherryΔpi*) of *sdg-1* exposed to *pos-1* dsRNA (red) or control dsRNA (black) for 16 hours are plotted. Asterisks indicate $P < 0.05$ using Mann-Whitney U test for two-sided comparisons with Bonferroni correction. (B) Cross progeny males that inherited the *mex-5p::mCherry::h2b::gfp::h2b* transgene (*T*)[65] (Figure 1) from maternal (*left*) or paternal (*right*) parents, both of wild-type, *sid-1(-)*, or *sdg-1(-)* background, were scored for expression of mCherry and GFP (bright, dim, off). Wild-type data for top set (n = 77 and n = 33) are replotted from ref. [65] for comparison. Dashed line separates independent experiments. Asterisk indicates $P < 0.05$ using χ^2 test with Bonferroni correction; n.s. indicates not significant. (C) Representative

AiryScan images of the distal germline (*left*; scale bar, 10 µm) or single germline nuclei (*right*; scale bar, 2 µm) showing SDG-1::mCherry alone (*top*) or with GFP::ZNFX-1 (*bottom*, merge and single channel images). The number of animals imaged (n) and the percentage that show enrichment of SDG-1::mCherry in perinuclear foci are indicated. Sites of SDG-1::mCherry enrichment coincide with GFP::ZNFX-1 localization. Boxes in left mark the nuclei shown in right. (D) Representative images showing entry of SDG-1::mCherry into the nucleus in -1 oocytes (*left*) and upon pronuclear fusion in early embryos during the time course indicated (*right*). Numbers of germlines and embryos imaged are indicated. Scale bars, 20 µm. Also see Movies S1, S2, S3 and S4. (E) Representative image of the hermaphrodite germline in animals with a translational (*left*) or transcriptional (*right*) reporter of *sdg-1*. Scale bars, 20 µm. Apparent extracellular punctae of SDG-1::mCherry and mCherry surrounding the proximal germline requires further study, but could be non-specific because similar localization is observed in animals with other promoters driving mCherry expression, but not GFP expression, in the germline (data not shown). The numbers of animals with the depicted fluorescence pattern are indicated. (F) Model for RNA-mediated regulation of *sdg-1*. The *sdg-1* RNA is inhibited by dsRNA imported through SID-1 and protected by piRNAs bound to PRG-1; increasing the levels of SDG-1 protein (grey triangle) can alter the outcome of both dsRNA- and piRNA-mediated gene regulation. See text for details.

STAR METHODS

Strains and oligonucleotides

All strains (listed in Table S1) were cultured on Nematode Growth Medium (NGM) plates seeded with 100 µl of OP50 *E. coli* at 20ºC and strains made through mating were generated using standard methods[88]. Oligonucleotides used are in Table S2 (for genotyping *sid-1(qt9)*: P1-P2, *ttTi5605*: P3-P5, *eri-1(mg366)*: P6-P7, *sid-1(tm2700)*: P8-P10, *hrde-1(tm1200)*: P11-P13, and *nrde-3(tm1116)*: P14-P16). Strains made through mating existing mutant strains and genotyping using the above primers are listed below.

To create *gtbp-1::gfp* animals with *hrde-1(tm1200)* in the background: AMJ577[23] was crossed with JH3197 males to create AMJ1220 and one other independent isolate.

To create *gtbp-1::gfp* animals with *nrde-3(tm1116)* in the background: JH3197 was crossed with WM156 males to create AMJ1383.

Transgenesis

Animals were transformed with plasmids and/or PCR products using microinjection[89] to generate extrachromosomal arrays or single-copy transgenes. All plasmids were purified from bacterial culture using QIAprep Spin Miniprep Kit (Qiagen) and all PCR products were generated with Phusion® High-Fidelity DNA Polymerase (New England BioLabs) and purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel).

To express *sid-1::DsRed* in the muscle from an integrated array: pAJ53a (*myo-3p::sid-1::DsRed::unc-54 3'UTR*, made by AMJ while in Hunter Lab, Harvard University) was generated by amplifying part of *sid-1* cDNA from pHC355[21] with primers P27 and P18, *DsRed* and *unc-54 3'UTR* from pHC183[14] with primers P17 and P30, fusing the fragments using PCR

with primers P30 and P31, and then cloning the fusion product into the pHC355 vector backbone using the restriction enzymes NruI and EagI. pAJ53a (40 ng/µl) was then injected into HC196 and animals expressing DsRed were isolated. AMJ3 was isolated as a spontaneous integrant. AMJ3 males were then crossed with AMJ308 hermaphrodites to generate AMJ327.

To express *sid-1::DsRed* in the germline from a single-copy transgene: The *mex-5* promoter was amplified from pJA252 (Addgene #21512) using the primers P19 and P20. The *sid-1* gene was amplified from N2 genomic DNA using the primers P21 and P22. The *DsRed* gene was amplified from pAJ53a (*myo-3p::sid-1(+)::DsRed::unc-54 3'UTR*; made by AMJ and Tessa Kaplan while in Hunter Lab, Harvard University) using the primers P23 and P24. The *sid-1* 3'UTR was amplified using the primers P25 and P26. Using NEBuilder® HiFi DNA Assembly (New England BioLabs), these four amplicons were placed into pCFJ151 (Addgene #19330) digested with AflII (New England BioLabs) and treated with CIP (New England BioLabs) to generate pJM10. pJM10 (50 ng/µl) and the coinjection markers pCFJ601 (50 ng/µl), pMA122 (10 ng/µl), pGH8 (10 ng/µl), pCFJ90 (2.5 ng/µl), and pCFJ104 (5 ng/µl) (plasmids described in ref.[90]) were injected into the germline of adult EG4322 animals. One transgenic line was isolated as described previously[90] and crossed with HC196 males to generate AMJ576. The integration of *mex-5p::sid-1(+)::DsRed::sid-1 3'UTR* in AMJ576 was verified by genotyping with primers P3-P5 and Sanger sequencing of the insertion.

To express *sid-1::gfp* in the muscle from an extrachromosomal array: pTK2 (*myo-3p::sid-1::gfp*, made by AMJ and Tessa Kaplan while in Hunter Lab, Harvard University) was constructed by amplifying part of *sid-1* cDNA from pHC355[21] with primers P27 and P28, *gfp* and *unc-54 3'UTR* from pPD95.75 (Addgene #1494) using primers P29 and P30, and then fusing the fragments using PCR with primers P30 and P31 and cloning the product into the pHC355

vector backbone using the restriction enzymes NruI and EagI. pTK2 (10 ng/µl) was injected into HC196 and animals expressing GFP were isolated as AMJ706.

To express *PH::miniSOG* in neurons from an extrachromosomal array: pNMS03 (*rgef-1p::PH::miniSOG::unc-54 3'UTR*) was generated by amplifying the vector backbone of pHC337 excluding the *gfp*-dsRNA hairpin sequence using primers P35 and P36, and assembling it with *PH::miniSOG(Q103L)* amplified from pCZGY2851 (gift from Andrew Chisholm) with primers P33 and P34 using NEBuilder® HiFi DNA Assembly (New England BioLabs). pNMS03 (40 ng/µl) was injected into N2 animals with pHC448[91] (*myo-2p::DsRed2::unc-54 3'UTR*; 40 ng/µl) as a coinjection marker to create AMJ837 and two other isolates.

pNMS03 (40 ng/µl) was also injected into N2 animals with PCR products forming *rgef-1p::DsRed* (40 ng/µl) generated previously[91] as a coinjection marker to create AMJ936 and two other isolates.

To express *PH::miniSOG* in neurons from a single-copy transgene: pNMS05 (*rgef-1p::PH::miniSOG::unc-54 3'UTR* with *ttTi5605* homology arms and *Cbr-unc-119(+)*) was generated by amplifying the transgene *rgef-1p::PH::miniSOG::unc-54 3'UTR* from pNMS03 with primers P37 and P38 containing AvrII restriction sites and cloning the fragment into pCFJ151 after AvrII (New England BioLabs) digestion. pNMS05 (50 ng/µl) and the coinjection markers pCFJ601 (50 ng/µl), pMA122 (10 ng/µl), pGH8 (10 ng/µl), pCFJ90 (2.5 ng/µl), and pCFJ104 (5 ng/µl) (plasmids described in ref.[90]) were injected into the germline of adult EG4322 animals. One transgenic line was isolated as described previously[90] and designated as AMJ1019. The integration of *rgef-1p::PH::miniSOG::unc-54 3'UTR* in AMJ1019 was verified by genotyping with primers P3-P5 and Sanger sequencing of the insertion.

To express *PH::miniSOG* with *bli-1*-dsRNA in neurons from an extrachromosomal array: pNMS03 (40 ng/µl) was injected with *rgef-1p::bli-1-sense* (40 ng/µl) and *rgef-1p::bli-1-antisense* (40 ng/µl) PCR products generated previously[92] into GR1373 animals with pHC448 (*myo-2p::DsRed2::unc-54 3'UTR*) as a coinjection marker (40 ng/µl) to create AMJ1007 and one other independent isolate. AMJ1007 was crossed with HC731 males to create AMJ1108 and two other isolates. AMJ1108 was crossed with HC196 males to create AMJ1114 and one other isolate. AMJ1007 was crossed with N2 males to create AMJ1123 and one other isolate. AMJ1123 males were crossed with 3X outcrossed FX02700 (designated as AMJ1153) to create AMJ1151 and two other isolates. AMJ1151 was crossed with GR1373 males to create AMJ1173 and two other isolates.

To express *PH::miniSOG* with *gfp*-dsRNA in neurons from an extrachromosomal array: pNMS03 (40 ng/µl) and pHC337 (*rgef-1p::gfp-dsRNA::unc-54 3'UTR*; 40 ng/µl) were injected into AMJ819[65] with pHC448 (*myo-2p::DsRed2::unc-54 3'UTR*; 40 ng/µl) as a coinjection marker to create AMJ1009 and one other independent isolate. AMJ1009 was crossed with N2 males to create AMJ1134. AMJ1159 was crossed with AMJ1134 males to create AMJ1312 and two other isolates.

All other transgenes were generated previously (*ccIs4251*[13]; *oxSi487*[90]; *tmIs1005*[48]; *jamEx140*[23]; *qtEx136*[22]).

Cas9-mediated genome editing

Genome editing was performed by injecting nuclear-localized Cas9 (PNA Bio) preincubated at 37ºC for 10 min with either a single guide RNA (sgRNA) generated by *in vitro* transcription (SP6 RNA Polymerase, New England BioLabs) or hybridized crRNA/tracrRNA

(IDT), as well as an oligonucleotide or PCR-amplified homology repair template, into the *C. elegans* distal gonad. Screening for plates with successfully edited F1 animals was performed using either *dpy-10* co-CRISPR[93-94] or the pRF4 plasmid used as a co-injection marker[95]. All plasmids were purified from bacterial culture using QIAprep Spin Miniprep Kit (Qiagen) and all PCR products were generated with Phusion® High-Fidelity DNA Polymerase (New England BioLabs) and purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). Alleles generated by genome editing are schematized in Figures 3A (*sid-1*), S2 (*deps-1*, *mut-16*, *sid-2*, *rme-2*, *sid-1*, *rde-1*, *sid-5*, and *sid-3*), S3B (*rme*-2), S5A (*sid-1*), and S8C (*W09B7.2/F07B7.2* (*sdg-1*)), and oligonucleotides used are in Table S2.

To delete the *rme-2* coding sequence: Two sgRNAs targeting the start and end of the *rme-2* coding sequence were *in vitro* transcribed from a SP6 transcription template amplified from pDD162 (Addgene #47549) using primers P42 (start sgRNA) or P43 (end sgRNA) as forward primers and P40 as a universal reverse primer. An sgRNA targeting *dpy-10* for co-CRISPR was also *in vitro* transcribed using a similar template amplified from pDD162 with primers P39 and P40. All sgRNAs were purified using organic extraction, were precipitated using ethanol, and resuspended in water prior to injection. Injection into HC196 with all sgRNAs, Cas9 and the homology repair templates for *rme-2* (P44) and *dpy-10* (P41), and screening for edited animals were performed as described above. Genotyping for *rme-2(del)* was performed using a triplex PCR with primers P45-P47 to isolate AMJ1120 and one other isolate and the *rme-2* deletion was verified using Sanger sequencing. AMJ1120 was crossed with N2 males to isolate AMJ1131.

To delete the *sid-1* coding sequence: Injection of crRNAs targeting the start (P59) and end (P52) of the *sid-1* coding sequence (IDT), tracrRNA, Cas9, a *sid-1(del)* homology repair

template (P60) and pRF4 into N2 and AMJ1372, and subsequent screening were performed as described above. Genotyping for *sid-1(del)* was performed using triplex PCR with primers P9, P54 and P55 to isolate AMJ1324 and one other independent isolate from N2 and AMJ1479-AMJ1482 from AMJ1372. The *sid-1* deletion was verified by Sanger sequencing in all isolates. AMJ1324 was crossed with AMJ1131 males to create AMJ1366.

To delete the *sid-2* coding sequence: Injection of crRNAs targeting the start (P71) and end (P72) of the *sid-2* coding sequence (IDT), tracrRNA, Cas9, a *sid-2(del)* homology repair template (P73) and pRF4 into N2, and subsequent screening were performed as described above. Genotyping for *sid-2(del)* was performed using triplex PCR with primers P74-P76 to isolate AMJ1368 and one other independent isolate. The *sid-2* deletion was verified by Sanger sequencing in both isolates. AMJ1368 was crossed with AMJ1324 males to create AMJ1380.

To delete the *sid-5* coding sequence: Injection of crRNAs targeting the start (P61) and end (P62) of the *sid-5* coding sequence (IDT), tracrRNA, Cas9, a *sid-5(del)* homology repair template (P63) and pRF4 into N2, and subsequent screening were performed as described above. Genotyping for *sid-5(del)* was performed using duplex PCR with primers P64 and P65 to isolate AMJ1332 and three other independent isolates. The *sid-5* deletion was verified by Sanger sequencing in all four isolates. AMJ1332 was crossed with AMJ1324 males to create AMJ1367.

To introduce a nonsense mutation into *sid-1* coding sequence: An sgRNA was designed to introduce into *sid-1* a nonsense mutation mimicking the *qt9* allele[14]. This sgRNA was *in vitro* transcribed from a SP6 transcription template amplified from pDD162 (Addgene #47549) using primers P48 and P40. An sgRNA targeting *dpy-10* for co-CRISPR was also *in vitro* transcribed using a similar template amplified from pDD162 with primers P39 and P40. Both sgRNAs were purified using organic extraction and were precipitated using ethanol prior to injection. Both

sgRNAs, Cas9 and the homology repair templates for *sid-1(non)* (P49) that includes a missense mutation (S155P) and nonsense mutation (R156*) downstream that would prevent recutting of edited DNA by Cas9, and for *dpy-10* (P41) were injected into N2. Screening for edited animals was performed as described above. Genotyping for *sid-1(non)* was performed using a duplex PCR with primers P1 and P2 followed by restriction digestion with HpyCH4V to isolate AMJ1159. The nonsense mutation was confirmed using Sanger sequencing. AMJ1159 males were crossed with AMJ581[23] to create AMJ1504 and two other independent isolates.

Injection of a crRNA with the same target sequence (P88) (IDT) as the sgRNA described above, tracrRNA, Cas9, the same *sid-1(non)* homology repair template (P49) and pRF4 into N2 and AMJ1372 and subsequent screening were performed as described above. Genotyping for *sid-1(non)* was performed using duplex PCR with primers P1 and P2 followed by restriction digestion with HpyCH4V to distinguish AMJ1399 from N2, and AMJ1389 and AMJ1442-AMJ1446 from AMJ1372. The nonsense mutation was verified using Sanger sequencing in all isolates.

To revert the mutation in *sid-1(non)* animals: An sgRNA was designed to revert the nonsense mutation described above back to wild-type *sid-1* sequence. The sgRNA was *in vitro* transcribed from a SP6 transcription template amplified from pDD162 (Addgene #47549) using primers P50 and P40. An sgRNA targeting *dpy-10* for co-CRISPR was also *in vitro* transcribed using a similar template amplified from pDD162 with primers P39 and P40. Both sgRNAs were purified using organic extraction and were precipitated using ethanol prior to injection. Injection into AMJ1159 with both sgRNAs, Cas9 and the homology repair template for *sid-1(rev)* (P51), which also reverted the missense mutation (S155P) and nonsense mutation (R156*) downstream of *sid-1(rev)* to wild-type sequence, and *dpy-10* (P41). Screening for edited animals was

performed as described above. Genotyping for *sid-1(rev)* was performed using duplex PCR with primers P1 and P2 followed by restriction digestion with HpyCH4V to isolate AMJ1217 and two other independent isolates. The revertant was verified using Sanger sequencing in all isolates.

Injection of a crRNA with the same target sequence (P93) (IDT) as the sgRNA described above, tracrRNA, Cas9, a *sid-1(rev)* homology repair template (P51) and pRF4 into AMJ1389 and AMJ1399, and subsequent screening were performed as described above. Genotyping for *sid-1(rev)* was performed using duplex PCR with primers P1 and P2 followed by restriction digestion with HpyCH4V to distinguish AMJ1412 and AMJ1413 from AMJ1389, and AMJ1405-AMJ1410 from AMJ1399. The revertant was verified using Sanger sequencing in all isolates.

To tag *W09B7.2/F07B7.2* with *mCherry*: Injection of a crRNA with the target sequence listed as P80 (IDT), tracrRNA, Cas9, an *mCherry* sequence lacking piRNA binding sites amplified using primers P81 and P82 from pSD6[65] as a homology repair template with homology arms to the C-terminus of *W09B7.2/F07B7.2*, and pRF4 into N2, and subsequent screening were performed as described above. Genotyping for identical tags *W09B7.2::mCherryΔpi* and *F07B7.2::mCherryΔpi* in isolate AMJ1372 was performed using triplex PCR with primers P79, P83 and P84. Tagging of both loci is evident in Figure S8D. The *mCherryΔpi* insertion was verified by Sanger sequencing. AMJ1372 hermaphrodites and males generated by heatshock were mated with N2 males and hermaphrodites, respectively, to examine expression in cross progeny and in homozygosed wild-type and *W09B7.2/F07B7.2(jam137[mCherryΔpi]*) animals across generations in six independent F1 lineages from each cross. See Figures 5C and S9A for associated data. YY916 males were crossed with AMJ1372 to generate AMJ1662. The *3xflag::gfp::znfx-1* locus was genotyped using primers P153, P154, and P155.

To introduce a nonsense mutation into *rde-1* coding sequence: Injection of a crRNA with the target sequence listed as P94 (IDT), tracrRNA, Cas9, a *rde-1(non)* homology repair template (P95) mimicking *rde-1(ne300)*[96], and pRF4 into AMJ1372 and subsequent screening were performed as described above. Genotyping for *rde-1(non)* was performed using duplex PCR with primers P96 and P97 and restriction digestion with NlaIII to isolate AMJ1447 and AMJ1448. The nonsense mutation was verified by Sanger sequencing for all isolates.

To tag *sid-1* with *wrmScarlet* at the 3' end: Injection of a crRNA with the target sequence listed as P52 (IDT), tracrRNA, Cas9, a *sid-1::wrmScarlet13* homology repair template with the beginning (1) and end (3) but not the middle (2) of the coding sequence[97] (P53), and pRF4 into N2 and subsequent screening were performed as described above. Genotyping for *wrmScarlet13* was performed using duplex PCR with primers P54 and P55 to isolate AMJ1280. The *wrmScarlet13* insertion was verified by Sanger sequencing. Subsequent injections were performed into AMJ1280 with a *wrmScarlet13* specific crRNA with the target sequence listed as P56 (IDT), a complete *wrmScarlet* coding sequence amplified from pSEM89 (made in Boulin Lab – gift from Kevin O'Connell) with primers P57 and P58 and the same components as described previously. After similar screening, genotyping for full *wrmScarlet* insertion was performed using duplex PCR with primers P54 and P55 to isolate AMJ1282 and one other independent isolate. The full *wrmScarlet* insertion was verified by Sanger sequencing. AMJ1282 was crossed with AMJ577 males to create AMJ1365.

To tag *rme-2* with *wrmScarlet* at the 3' end: Injection of a crRNA with the target sequence listed as P67 (IDT), tracrRNA, Cas9, a *rme-2::wrmScarlet13* homology repair template with the beginning (1) and end (3) but not the middle (2) of the coding sequence[97] (P69), and pRF4 into N2 and subsequent screening were performed as described above. Genotyping for

wrmScarlet13 was performed using duplex PCR with primers P70 and P47 to isolate AMJ1281. The *wrmScarlet13* insertion was verified by Sanger sequencing. Subsequent injections were performed into AMJ1281 with a *wrmScarlet13* specific crRNA with the target sequence listed as P77 (IDT), a complete *wrmScarlet* coding sequence amplified from pSEM89 (made in Boulin Lab – gift from Kevin O'Connell) with primers P57 and P58 and the same components as described previously. After similar screening, genotyping for full *wrmScarlet* insertion was performed using duplex PCR with primers P54 and P55 to isolate AMJ1284 and two other independent isolates. The full *wrmScarlet* insertion was verified by Sanger sequencing.

To tag *sid-1* internally with *mCherry*: Injection of a crRNA with the target sequence listed as P110 (IDT), tracrRNA, Cas9, an *mCherry* lacking piRNA binding sites amplified from pSD6[65] as a homology repair template with homology arms to exon 4 of *sid-1* with primers P111 and P112, and pRF4 into N2 and subsequent screening were performed as described above. Genotyping for *mCherry*Δ*pi* was performed using triplex PCR with primers P113, P114 and P79 to isolate AMJ1438 and one other isolate from the same lineage. The *mCherry*Δ*pi* insertion was verified by Sanger sequencing. Subsequent injections were performed into AMJ1438 with a crRNA targeting the 5'-end of *mCherry*Δ*pi* (P115) (IDT), a homology repair template containing a 45-nt linker sequence (P116) and the same components as described previously. After similar screening, genotyping for the linker insertion was performed using duplex PCR with primers P113 and P79 to isolate AMJ1485 and two other independent isolates. Insertion of the linker was verified by Sanger sequencing in all three isolates.

To introduce a nonsense mutation into *sid-3* coding sequence: Injection of a crRNA with the target sequence listed as P66 (IDT), tracrRNA, Cas9, a *sid-3(non)* homology repair template (P85) mimicking *sid-3(qt31)*[98] and pRF4 into AMJ1372 and subsequent screening were

performed as described above. Genotyping for *sid-3(non)* was performed using duplex PCR with primers P86 and P87, and restriction digestion with StyI to isolate AMJ1449 and AMJ1450. The nonsense mutation was verified by Sanger sequencing for both isolates.

To introduce a nonsense mutation into *deps-1* coding sequence: Injection of a crRNA with the target sequence listed as P68 (IDT), tracrRNA, Cas9, a *deps-1(non)* homology repair template (P137) mimicking *deps-1(bn124)*[51] and pRF4 into AMJ1372 and AMJ1412 and subsequent screening were performed as described above. Genotyping for *deps-1(non)* was performed using allele specific PCR with primers P138 and P139 amplifying the wild-type sequence and primers P140 and P141 amplifying the mutant allele to isolate AMJ1451-AMJ1452 from AMJ1372 and AMJ1574-AMJ1575 from AMJ1412. The nonsense mutation was verified by Sanger sequencing for both isolates.

To insert the tetracycline K4 aptazyme into the 3'UTR of *sid-1*: Injection of a crRNA with the target sequence listed as P52 (IDT), tracrRNA, Cas9, a *sid-1::tetracycline-K4-aptazyme* homology repair template (P78) and pRF4 into N2 and subsequent screening were performed as described above. Genotyping for insertion of the aptazyme sequence was performed using duplex PCR with primers P54 and P55 to isolate AMJ1323. The aptazyme insertion was verified by Sanger sequencing. AMJ1323 hermaphrodites was crossed with AMJ477[22] males to create AMJ1330 and with AMJ471[23] males to create AMJ1350. AMJ1323 males were crossed with JH3197 to create AMJ1355.

To insert the tetracycline K4 aptazyme into the 3'UTR of *gtbp-1(ax2053[gtbp-1::gfp])*: Injection of a crRNA with the target sequence listed as P89 (IDT), tracrRNA, Cas9, a *gtbp-1::gfp::tetracycline-K4-aptazyme* homology repair template (P90) and pRF4 into JH3197 and subsequent screening were performed as described above. Genotyping for insertion of the

aptazyme sequence was performed using duplex PCR with primers P91 and P92 to isolate AMJ1542. The aptazyme insertion was verified by Sanger sequencing.

To introduce a missense mutation into *dpy-10* coding sequence: Injection of crRNA with the target sequence listed as P142 (IDT), tracrRNA, Cas9, and a *dpy-10(mis)* homology repair template (P41) mimicking *dpy-10(cn64)*[99] into AMJ1372 was performed as described above and heterozygous F1 animals were screened for by passaging "rolling" animals. Animals that appeared wild-type and those that appeared Dpy (homozygous *dpy-10(-)*) were isolated from three independently edited F1 animals. See Figure S9B for associated data.

To delete the *W09B7.2/F07B7.2* coding sequence: Injection of crRNAs targeting the start (P143) and end (P144) of the *W09B7.2/F07B7.2* coding sequence (IDT), tracrRNA, Cas9, a *W09B7.2/F07B7.2(del)* homology repair template (P145) and pRF4 into N2, and subsequent screening were performed as described above. Genotyping for *W09B7.2/F07B7.2(del)* was performed using triplex PCR with primers P146-P148 to isolate AMJ1577, AMJ1612, and AMJ1613. Deletion of both *W09B7.2/F07B7.2* loci was verified by absence of wild-type band by PCR (see Figure S8D) and Sanger sequencing in all three isolates.

To delete the *W09B7.2/F07B7.2* coding sequence from *W09B7.2/F07B7.2::mCherry*Δ*pi*: Injection of crRNAs targeting the start (P143) and end (P149) of the *W09B7.2/F07B7.2* coding sequence (IDT), tracrRNA, Cas9, a *W09B7.2/F07B7.2(del)* homology repair template (P150) and pRF4 into AMJ1372, and subsequent screening were performed as described above. Genotyping for *W09B7.2/F07B7.2(del)* was performed using triplex PCR with primers P148, P151, and P152 to isolate AMJ1615, AMJ1616, and AMJ1617. Deletion of both *W09B7.2/F07B7.2* loci was verified by absence of wild-type band by PCR (see Figure S8D) and Sanger sequencing in all three isolates.

Light-induced damage of neurons

Optimizing duration of light exposure: 20-30 animals expressing PH::miniSOG in neurons (multi copy, AMJ837; single copy, AMJ1019) were placed on an unseeded NGM plate and exposed to blue light (470 nm wavelength) at a distance of approximately 7.5 cm from an LED (Cree Xlamp XP-E2 Color High Power LED Star – Single 1 UP, LED supply) producing light at a power of ~2 mW/mm^2 flashing at a frequency of 2 Hz for different durations of time. Animals were then scored for movement defects immediately after light exposure, OP50 was seeded onto the plate, and animals were scored again 24 h post light exposure (Figure S1E). Wild-type animals were exposed to blue light for the same durations as control. Representative widefield images of unparalyzed (wild type) and paralyzed (coiled, AMJ837) animals were taken using a Nikon AZ100 microscope and Photometrics Cool SNAP HQ^2 camera (Figure S1F, *top*). Confocal images of animals expressing PH::miniSOG and DsRed in neurons (AMJ936) with and without 30 minutes of blue light exposure were taken using a Leica TCS SP8 DLS microscope with HyVolution using a 40X oil objective lens. DsRed was excited using a 638 nm laser and fluorescence was collected through a 598 nm emission filter (Figure S1F, *bottom*). Images were adjusted for display using Fiji[100] (NIH).

Silencing by *bli-1*-dsRNA: Five L4 animals with an extrachromosomal array expressing PH::miniSOG and *bli-1*-dsRNA in neurons were placed on seeded NGM plates and allowed to lay progeny for 24 h. P0 animals were then removed and F1 progeny were exposed to blue light as described above for 60 min at different time points after initial P0 L4 animals were passaged. 96 h post light exposure F1 progeny with the array were scored for *bli-1* silencing (presence of blisters) in gravid adults (Figures S1G, *top*, S1H and S1I).

Silencing by *gfp*-dsRNA: L4 animals with the *mex-5p::mCherry::h2b::gfp::h2b* transgene (*oxSi487*) (Figure 1D) were mated with L4 male animals with an extrachromosomal array expressing PH::miniSOG and *gfp*-dsRNA in neurons (Figure 1C). After 36 h of mating and laying progeny, P0 animals were removed from plates and F1 progeny were exposed to blue light as described above for 60 min at different time points after initial P0 L4 animals were mated. 96 h after mating, F1 cross progeny hermaphrodites with the array were imaged as adults (Figure S1G, *bottom*) under a coverslip in 10 µl of 3 mM levamisole on a 2% agar pad using a Nikon AZ100 microscope and Photometrics Cool SNAP HQ^2 camera. A C-HGFI Intensilight Hg Illuminator was used to excite GFP (filter cube: 450 to 490 nm excitation, 495 dichroic, and 500 to 550 nm emission) and mCherry (filter cube: 530 to 560 nm excitation, 570 dichroic, and 590 to 650 nm emission). Animals were scored as bright if fluorescence was easily detectable without adjusting levels, dim if fluorescence could be observed after level was adjusted to saturation, and not detectable if fluorescence was still not observed after level adjustments (Figure S1J). Representative images were adjusted in Adobe Photoshop to identical levels for presentation (Figures 1D, 1E and 1F).

Sensitive northern blotting

Double-stranded RNA was *in vitro* transcribed from a PCR amplicon using T7 RNA Polymerase (New England BioLabs) (Figure S3F) or expressed in HT115 *E. coli* after IPTG induction during exponential growth (Figures S3D and S3E) and extracted using TRIzol (Fisher Scientific). RNA was then separated by size using fully denaturing formaldehyde polyacrylamide gel electrophoresis (FDF-PAGE)[101] wherein 10 µg RNA samples were heated with formaldehyde to disrupt dsRNA duplexes and run on a 4% denaturing polyacrylamide gel next to 1-kb and 100-

bp DNA ladders for size comparison. After migration, the ladder lanes were stained with ethidium bromide and imaged, and the RNA lanes were transferred to a positively charged nitrocellulose membrane using a Trans-Blot® Turbo™ Transfer System (Bio-Rad) and crosslinked using 120 mJ/cm^2 UV radiation. Blots were then exposed to 2.5 pmol of 40-nt HPLC purified DNA oligonucleotides conjugated to digoxigenin (DIG) using the DIG Oligonucleotide Tailing Kit (Roche) hybridized to the nitrocellulose membrane at 60°C overnight (42°C for 2 h for 5S rRNA) in ULTRAhyb™ buffer (Invitrogen™) to probe the sense or antisense strands of *unc-22* (Figures S3D and S3F) or *gfp*-dsRNA (Figure S3E) at different positions (adapted from ref.[32]). After hybridization, the membrane was washed and blocked using the DIG Wash and Block Buffer Set (Roche), incubated with Anti-DIG-AP, Fab fragments (Roche) and developed with CSPD (Roche) at 37°C for 15 min. Chemiluminescence from the AP/CSPD reaction was imaged using a LAS-3000 (Fujifilm) or iBright™ CL1000 (Invitrogen™) imager. Blots were compared to ethidium bromide-stained ladders after imaging to visualize fragment size. Blots were stripped using two washes with 5% SDS (Sigma Aldrich) and two washes with 2X SSC (Sigma Aldrich) and the hybridization, blocking and development procedures were repeated for each probe (5S RNA probe: P118; *unc-22* probes: P119-P124; *gfp* probes: P125-P130).

Injection of dsRNA

Injection of synthetic dsRNA: RNA oligonucleotides were purchased from IDT and resuspended in IDT Duplex Buffer (*unc-22*: P131 and P132; *gfp*: P133 and P134; fluorescently-labeled *gfp*: P135 and P136). 1 µg each of HPLC purified 50-nt sense and antisense oligonucleotide was diluted to 100-350 ng/µl with IDT Duplex Buffer at a final volume of 10 µl. Alternatively, *unc-22* single-stranded RNA was treated with polynucleotide kinase and annealed

in equal proportion at a final concentration of ~97 ng/µl of *unc-22*-dsRNA in IDT Duplex Buffer (Figures S3G and S3H). This mixture was heated to 95ºC for 1 min and cooled at a rate of 1ºC/min to a final temperature of 25ºC. The mix was centrifuged at 16500 x g for 20-30 min and loaded into a microinjection needle. Young adult animals were injected 24 h after the L4 stage in the body cavity just beyond the bend of the posterior gonad arm[26]. Injected animals were recovered with M9 buffer and isolated onto NGM plates and allowed to lay progeny. In cases where animals were mated with N2 males post injection, two adult N2 males were placed on each plate with an injected hermaphrodite.

Injection of *in vitro* transcribed dsRNA: Templates for transcription were amplified from RNAi vectors using one common primer specific to the T7 promoter sequence (P117). PCR products were purified using column purification (Macherey-Nagel, ref. 740609.50) and subsequently used for transcription by T7 RNA Polymerase (New England BioLabs). Many transcription reactions were pooled and purified using one column to produce concentrated RNA samples. Annealing, centrifugation, and injection into the body cavity of animals staged as L4s (injected between pharynx and anterior intestine) or young adults were performed as described for synthetic dsRNA with identical concentrations unless otherwise indicated in figure legends. In cases where animals were mated with N2 males post injection, two adult N2 males were placed on each plate with an injected hermaphrodite.

Scoring of gene silencing: For scoring silencing by *unc-22* dsRNA, 10-30 L4 animals were passaged into 10 µl of 3 mM levamisole and scored for twitching, observed as rapid movement of the head and/or tail (as in ref.[26]), 3-4 days after injection for progeny of *rme-2(+)* parents and 4-5 days after injection for progeny of *rme-2(-)* parents with no appreciable difference between days in which animals were scored post injection. Weak and strong twitching

were scored as in Movies S1, S2, S3 and S4 of ref.[26]. Numbers of silenced animals and total animals scored were summed across all days of scoring and experimental replicates.

When scoring silencing of *gfp*, animals were either scored by eye in comparison to animals injected with duplex buffer only (i.e. buffer; Figure S1B) or were mounted in 10 µl of 3 mM levamisole on a 2% agar pad and imaged under a coverslip as P0 adults (2 days post injection) or F1 L4s (3 days post P0 injection) using a Nikon AZ100 microscope and Photometrics Cool SNAP HQ^2 camera. A C-HGFI Intensilight Hg Illuminator was used to excite GFP (filter cube: 450 to 490 nm excitation, 495 dichroic, and 500 to 550 nm emission). Representative images for *gfp* expression in F1 animals after P0 injection were adjusted to identical levels in Adobe Photoshop for presentation (Figures 2B, S1C and S1D). See "Imaging and quantification of reporters using widefield microscopy" for other methods of scoring *gfp* expression after imaging.

Imaging of fluorescently labeled dsRNA: Embryos were imaged 22 hours post P0 injection with labeled dsRNA. Laid embryos were picked off plates and placed into 5 µl of 3 mM levamisole on a coverslip for at least 5 minutes before placing on a 2% agarose pad on a slide. Embryos were imaged using the Eclipse Ti Spinning Disk Confocal (Nikon) with the 60X objective lens. Atto 565 was excited using a 561 nm laser and fluorescence was collected through a 415-475 nm and 580-650 nm emission filter. Images were adjusted for display using Fiji[100] (NIH).

Feeding RNAi

P0 and F1 feeding: *E. coli* (HT115) expressing dsRNA was cultured in LB media with 100 µg/µl carbenicillin overnight at 250 rpm. 100 µl of cultured bacteria was then seeded onto

RNAi plates and incubated at room temperature for approximately 24 h. L4 animals were passaged onto seeded RNAi plates and progeny were scored for silencing by bacteria expressing dsRNA targeting *unc-22* (twitching in levamisole), *bli-1* (blisters), *pos-1* (dead eggs) or expressing L4440 as an empty vector control.

P0 only feeding: RNAi bacteria were cultured and seeded as described above. L4-stage or young adult-stage (24 h post L4) animals were passaged onto seeded RNAi plates and cultured at 20ºC for approximately 24 h. Animals were then picked into 1 ml of M9 buffer and washed four times to remove any residual bacteria (as in ref.[26]). After washing, animals were resuspended in 200 µl of remaining M9 buffer and placed onto a seeded NGM plate. 1 h later, animals were isolated onto single NGM plates and their progeny were scored for silencing as described above.

Limited P0 only feeding: RNAi bacteria were cultured and seeded as described above. L4-stage animals were passaged onto seeded RNAi plates and cultured at 20ºC for approximately 16 h. Animals were then passaged onto NGM plates seeded with *E. coli* (OP50) and cultured for 1.5 h at room temperature. Animals were then again passaged to new OP50 seeded plates (1 animal on each plate) and progeny (only L3 larvae, L4 larvae and adults) were counted after 4 days of being cultured at 20ºC (~96 hours after moving to new OP50 plates).

F1 only feeding: L4-staged animals were passaged onto RNAi plates seeded with 10 µl of *E. coli* (OP50). Animals were allowed to develop into adults and lay eggs over 24 h at 20ºC and then removed from plates. Plates with eggs were then seeded with RNAi bacteria cultured and seeded as described above and further cultured at 20ºC. Hatched progeny were imaged throughout development or as adults 3 days after being staged as L4 animals (day 3 adults).

Tetracycline-induced expression

For animals cultured with OP50 *E. coli*: 81.6 µl of a 500 µM solution of tetracycline in water was added to 4 mL NGM plates previously seeded with OP50 *E. coli* (at least two days earlier) to create plates with ~10 µM tetracycline (concentration based on ref.[45]). Volumes of 166.7 µl and 444.4 µl of tetracycline solution were used to create plates with final concentrations of ~20 µM or ~50 µM, respectively (see Figure S6D). Control plates were also made by adding the same amount of water to seeded NGM plates without tetracycline. Tetracycline plates and control plates were incubated at room temperature out of direct light overnight to allow any remaining liquid to dry. Animals were passaged to tetracycline or water plates with or without previous injection of 10 µM tetracycline or water into adult gonads. Progeny expressing neuronal *unc-22* or *gfp*-dsRNA were scored for silencing on the first day of adulthood. In the case of silencing of *gtbp-1::gfp* by neuronal *gfp*-dsRNA, animals with the array expressing *gfp*-dsRNA were passaged as L4s onto new tetracycline or water plates to be imaged as day 1 adults. The brood size of animals cultured on OP50 with 10 µM tetracycline or water was scored by staging single L4 animals on NGM plates with 10 µM tetracycline or water and moving animals every 24 h to new 10 µM tetracycline or water plates. Progeny laid on each of the four days were counted after growing to adulthood, continuously cultured under either condition.

For animals cultured on HT115 *E. coli* expressing dsRNA: Bacteria expressing *bli-1*-dsRNA, *gfp*-dsRNA, *pos-1*-dsRNA or L4440 control vector were cultured overnight to a maximum time of 24 hours (for *gfp*-dsRNA and L4440 only) and 100 µl of bacteria was seeded onto RNAi plates. Plates were incubated for 1-2 days at room temperature to allow for growth and drying of bacteria. 10 µM tetracycline or water was added to newly seeded plates as described above. After drying of tetracycline and water, P0 animals were added to plates and F1

animals were scored for silencing by *bli-1*-dsRNA or *gfp*-dsRNA as adults in the next generation. Silencing by *pos-1*-dsRNA was scored by measuring the brood of three L4 animals staged on a single RNAi plate with *pos-1*-dsRNA and 10 µM tetracycline or water. Brood size over four days was measured after moving all P0 animals every 24 h to new 10 µM tetracycline or water plates and scoring adult progeny cultured under either condition.

In all experiments, animals expressing *unc-22*-dsRNA in neurons were exposed to the same tetracycline and water solutions used and scored for *unc-22* silencing as adults as a control for effectiveness of tetracycline (see summary of data in Figure S6B).

Imaging and quantification of reporters using widefield microscopy

All animals and embryos expressing fluorescent reporters were imaged in 10 µl of 3 mM levamisole on a 2% agar pad using a Nikon AZ100 microscope and Photometrics Cool SNAP HQ[2] camera. A C-HGFI Intensilight Hg Illuminator was used to excite mCherry (filter cube: 530 to 560 nm excitation, 570 nm dichroic, and 590 to 650 nm emission), GFP or other autofluorescent molecules in the green channel (filter cube: 450 to 490 nm excitation, 495 nm dichroic, and 500 to 550 nm emission) and autofluorescent molecules in the blue channel (filter cube: 325 to 375 nm excitation, 400 nm dichroic, 435 to 485 nm emission). Intensity of GFP and mCherry were quantified in Fiji[100] (NIH) using the methods described below. Representative images were adjusted in Fiji[100] (NIH) and/or Adobe Photoshop to identical levels for presentation (Figures 3C, 3D, 5A, 6E, S5E, S5F, S5G, S6C, S6D, S6E and S6G).

For GTBP-1::GFP quantification post dsRNA injection: Somatic *gfp* expression was quantified between the pharynx and anterior gonad arm by drawing a circle or ventral to dorsal line within the boundaries of the animal (Figure S4A) on a brightfield image, creating a mask,

imposing that mask onto the GFP channel image and measuring average intensity or intensity along the line, respectively. To measure background fluorescence, the same circle or a new circle was used to measure average intensity outside of the animal. Germline GFP expression was quantified by freely selecting part of the distal or proximal region of the anterior or posterior gonad arm (Figure S4A) excluding the intestine to avoid intestinal autofluorescence. Selection was performed using a brightfield image, a mask was created and imposed onto the GFP channel image and average intensity was measured. To measure background fluorescence, the same selection boundary was moved outside of the animal and average background intensity was measured. To plot average GFP intensity measured by a circle or free selection, average background intensity was subtracted from GFP intensity for each image and plotted with a box plot (Figure S4C). To plot GFP intensity along the ventral to dorsal axis in the anterior soma, the average intensity in each tenth of the axis was calculated for each animal and plotted with a shaded region representing 95% confidence intervals (Figure S4B, *top*). To calculate differences in intensity between the interior and exterior of animals, the average intensity of the 0.4-0.6 region of the axis was divided by the average intensity of the 0.1 and 0.9 points of the axis. These values were calculated and shown for each animal as a box plot (Figure S4B, *bottom*). All plotting was done using custom R scripts.

For GTBP-1::GFP quantification post dsRNA feeding or neuronal expression: Animals fed L4440 or *gfp*-dsRNA for different durations of the P0 and/or F1 generation were scored for silencing in the germline and soma at different stages during the F1 generation (Figures 1A, 1B, and S1A). Somatic GFP intensity (a.u.) was quantified in the tail region by drawing a ventral to dorsal line within the boundaries of the animal (Figures S6C and S6E) on a brightfield image, creating a mask, imposing that mask onto the GFP channel image and measuring average

intensity or intensity along the line. To measure background fluorescence, a circle was used to measure average intensity outside of the animal. Germline GFP intensity (a.u.) was measured by free selection of germ cells while avoiding intestinal cells at each stage, selecting a region around the primordial vulva in L2 animals, in one of two extending gonad arms in L3 and L4 animals, in the proximal or distal gonad in young adults, and of eggs *in utero* in gravid adults. To measure background fluorescence, the same selection or a new selection was used to measure average intensity outside of the animal. To plot average GFP intensity measured by free selection, average background intensity was subtracted from GFP intensity for each image and shown as a box plot (Figures 1A, 1B, S1A, S6C, and S6E). All plotting was done using custom R scripts.

For adjustment of fluorescence images of *sid-1::mCherryΔpi* animals for comparison to images of wild-type animals: Representative images of *sid-1(jam195[linker::mCherryΔpi])* and wild type animals at different stages were adjusted to the same maximum and minimum displayed values of intensity using Fiji[100] (NIH) to highlight each region of interest below saturation (Figures 3C, 3D, S5E, S5F and S5G).

For SDG-1::mCherry quantification: Germline mCherry intensity was quantified by freely selecting part of the distal or proximal region of the anterior or posterior gonad arm excluding the intestine to avoid quantifying intestinal autofluorescence. Selection was performed using a brightfield image, a mask was created and imposed onto the mCherry channel image and average intensity was measured. To measure background fluorescence, the same selection boundary was moved outside of the measured gonad arm and average background intensity was measured. To plot average mCherry intensity, average background intensity was subtracted from mCherry intensity for each gonad arm and shown as a box plot using custom R scripts (Figures 5B, 5C, 5D, 5E, 5F, 5G and S9). In Figure 5C, SDG-1::mCherry intensity measurements,

adjusted by subtracting background intensity and intensity measurements made in a wild-type animal lacking mCherry, were normalized to RT-qPCR measurements by multiplying each median intensity value by a conversion factor. This conversion factor was calculated by dividing the median SDG-1::mCherry intensity in AMJ1372 animals by the median relative *sdg-1* mRNA level in AMJ1372 RNA samples. All estimated relative *sdg-1* expression values were then normalized to those of wild-type animals by dividing all values by the wild-type value.

Imaging and quantification of reporters using confocal microscopy

For *sid-1* reporters expressed from multi-copy transgenes: L4 animals expressing *myo-3p::sid-1 cDNA::DsRed* and *myo-3p::sid-1::gfp* were placed in 10 µl of 3 mM levamisole and imaged using the Eclipse Ti Spinning Disk Confocal (Nikon) with the 100X objective lens. DsRed was excited using a 561 nm laser and fluorescence was collected through a 415-475 nm and 580-650 nm emission filter. GFP was excited using a 488 nm laser and fluorescence was collected through a 500-550 nm emission filter. Images were adjusted in Fiji[100] (NIH) and Adobe Photoshop to identical levels for presentation (Figure S5B).

For the endogenous gene tag *sid-1::mCherryΔpi*: SID-1::mCherry fluorescence from an L1-staged animal was imaged using LSM 980 Airyscan 2 Laser Scanning Confocal (Zeiss) with a 63X oil objective lens after paralyzing the worm as above. mCherry was excited using a 561 nm laser and fluorescence was collected through a 422-477 nm and 573-627 nm emission filter. For Figure 3E, after removing noise using a 3D gaussian blur with 2.0 sigma in X, Y, and Z, depth-coded maximum intensity projections of Z-stacks were stitched together for display as described earlier[22].

For the endogenous gene tag *W09B7.2/F07B7.2::mCherryΔpi*: Adult animals were placed in 10 µl of 3 mM levamisole and imaged using the Eclipse Ti Spinning Disk Confocal (Nikon) with a 60X objective lens or the LSM 980 Airyscan 2 Laser Scanning Confocal (Zeiss) with a 63X oil objective lens. GFP was excited using a 488 nm laser and fluorescence was collected through a 499-557 nm and 659-735 nm emission filter, and mCherry fluorescence was excited and collected as described above. Images and movies were adjusted in Fiji[100] (NIH) and Adobe Photoshop to identical levels for presentation (Figures 6C, 6D and Movies S1, S2, S3 and S4).

RNA sequencing, principal component analysis and differential expression analysis

For analysis of previously generated *sid-1(-)* alleles: Mixed-stage animals were washed from 10 plates in biological duplicate 5 days after passaging L4-staged animals. Total RNA was extracted from pellets using TRIzol (Fisher Scientific). PolyA+ RNAs were purified and converted to DNA libraries by the University of Maryland Genomics Core using the Illumina TruSeq Library Preparation Kit. FASTQ files were processed[102] using the command "`cutadapt -j 0 -a AGATCGGAAGAGCACACGTCTGAACTCCAGTCA -m 20 -q 20 -o cutread.gz fasta1.gz`". Reads were assigned transcript IDs and counted[103] using the command "`salmon quant -i celegans.index -l A -r cutread.gz -p 8 –validateMappings -o quant_file`". For conversion of transcript IDs to gene IDs, a table of matching transcript and gene IDs was generated from a GTF file using the command "`grep "^[^#]" Caenorhabditis_elegans.Wbcel235.101.gtf | awk '{if($3 == "transcript"){print}}' | awk '{print $12,$14}' | tr -d '";' > transcript_id_gene_id.tsv`". Conversion was then made using this table

with tximport[104] in R, whereafter only genes with more than 0.1 counts per million for at least 2 samples were used in subsequent analyses with pairs of sample types (*sid-1(qt9[non])* vs. wild type and *sid-1(tm2700[del]); tmIs1005[sid-1(+)* vs. *sid-1(tm2700[del])*). After normalizing samples using the trimmed mean of M-values method[105], principal component analysis was performed in R by comparing samples based on the 500 genes with the largest standard deviations in their $\log_2$-fold change between each set of samples (Figure S7A). Differential expression analysis was performed using limma(voom)[106] in R (example available at AntonyJose-Lab/Shugarts_et_al_2023 on github). Volcano plots of differential expression for all genes compared were plotted using custom R scripts with genes having an adjusted *p*-value threshold (*q*-value) less than 0.05 in black and those greater than 0.05 in grey (Figure S7B).

For analysis of newly generated *sid-1(-)* alleles: Total RNA >200 nt was extracted using RNAzol (Sigma-Aldrich) from 200 µl pellets of mixed-stage animals collected from 6 non-starved but crowded plates in biological triplicate for each strain. PolyA+ RNAs were purified and converted to DNA libraries using the Illumina TruSeq Stranded mRNA Library Preparation Kit. Library quality was assayed using TapeStation (Agilent) and libraries were sequenced using a HiSeq X10 (Illumina) by Omega Bioservices. FASTQ files were processed[102] using the command "`cutadapt -j 0 -a AGATCGGAAGAGCACACGTCTGAACTCCAGTCA -A AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT -m 20 -q 20 -o cutread1.gz -p cutread2.gz read1.gz read2.gz`". Reads were assigned transcript IDs and counted[103] using the command "`salmon quant -i celegans.index -l A -1 cutread1.gz -2 cutread2.gz -p 8 –validateMappings -o quant_files`". For conversion of transcript IDs to gene IDs, a table of matching transcript and gene IDs was generated as described above. Conversion was then made using this table with tximport[104] in R, whereafter

only genes with more than 0.1 counts per million for at least 3 samples were used in subsequent analyses. Normalization, principal component analysis (Figures 4A and S7C) and differential expression analysis (example available at AntonyJose-Lab/Shugarts_et_al_2023 on github) were performed as described above. Volcano plots of differential expression were plotted as described above (Figures 4B and S7D). Genes that were similarly misregulated in Figures 4B and S7D are in red.

For analysis of data from Reed et al., 2020: FASTQ files were processed[102] using the command "`cutadapt -j 0 -a AGATCGGAAGAGCACACGTCTGAACTCCAGTCA -m 20 -q 20 -o cutread.gz fasta1.gz`". Reads were assigned transcript IDs and counted[103] using the command "`salmon quant -i celegans.index -l A -r cutread.gz -p 8 –validateMappings -o quant_file`". For conversion of transcript IDs to gene IDs, a table of matching transcript and gene IDs was generated as described above. Conversion was then made using this table with tximport[104] in R. Normalization and differential expression analysis (example available at AntonyJose-Lab/Shugarts_et_al_2023 on github) were performed as described above. Volcano plots of differential expression were plotted as described above with *sid-1*, *sdg-1* (*W09B7.2/F07B7.2*) and *sdg-2* (*Y102A5C.36*) in red and all other genes in grey (Figure S7G).

Genome mapping and visualization of sequencing reads for sid-1-dependent genes

After RNA sequencing samples were processed as described above, reads were mapped to the *C. elegans* genome[107] using the command "`hisat2 -p 8 -x Celegans98index -1 cutread1.gz -2 cutread2.gz -S sam1`". The SAM file outputs were then converted to BAM files[108] using the command "`samtools view -b sam1 | samtools`

`sort -> bam1.bam`" and BAM index files were created for visualization using "`samtools index bam1.bam`". Reads for the *sid-1* and *F14F9.5* locus, *W09B7.2/F07B7.2* locus, and *Y102A5C.36* locus were plotted using custom R scripts and axes were normalized for each sample based on its total mapped reads, calculated using the command "`samtools view -c -F 4 bam1.bam`" (Figure S7F).

Comparisons with published datasets

Datasets in 21 published studies were collected and compared based on the gene names to identify changes in *sid-1*, *sdg-1*, *sdg-2* and *tbb-2* (control), if reported (Figure 4D). After standardizing the names across all datasets, the fold-changes reported, if available, were used to plot a heatmap. Cases where fold-changes were not available were set conservatively as $\log_2$(fold change) = 2. The R script used is available at AntonyJose-Lab/Shugarts_et_al_2023 on github.

Reverse transcription and quantitative PCR

Total RNA was extracted using TRIzol (Fisher Scientific) from 200 µl pellets of mixed-stage animals collected from 3-6 non-starved but crowded plates in biological triplicate for each strain. The aqueous phase was then washed with an equal amount of chloroform and precipitated overnight on ice with 100 µl of 3 M sodium acetate, 1 ml of 100% ethanol and 10 µg glycogen (Invitrogen). RNA pellets were washed twice with 70% ethanol and resuspended in 22 µl nuclease free water. RNA samples were then Dnase-treated in Dnase buffer (10 mM Tris-HCl pH 8.5, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$) with 0.5 U Dnase I (New England BioLabs) at 37°C for 60 minutes followed by heat inactivation at 75°C for 10 minutes. RNA concentration was measured and 1 µg of total RNA was used as input for reverse transcription using 50 U

SuperScript III Reverse Transcriptase (Invitrogen) (+RT) or no reverse transcriptase as a negative control (-RT) (RT primers: *tbb-2* (P98), *sid-1* (P101), *W09B7.2/F07B7.2* (P104), *Y102A5C.36* (P107)). For qPCR, each +RT biological replicate was assayed in technical triplicate for each gene target, along with a single -RT sample for each corresponding biological replicate using 2 µl cDNA and a no template control (NTC) with the LightCycler® 480 SYBR Green I Master kit (Roche). Ct values were measured with the Bio-Rad C1000 CFX96 Real-Time System and Bio-Rad CFX Software (qPCR primers: *tbb-2* (P99 and P100), *sid-1* (P102 and P103), *W09B7.2/F07B7.2* (P105 and P106), *Y102A5C.36* (P108 and P109)). To calculate relative change in mRNA abundance compared to wild type, we calculated $\log_2(2^{(-(\text{gene Ct} - \text{tbb-2 Ct}))})$ using the median of technical replicates for the biological triplicates of each genotype. Ct values were only used if they were lower than corresponding -RT and NTC Ct values. The median value of wild-type biological replicates was then subtracted from the value for each sample to plot calculated values with respect to wild-type levels (Figures 4C, S7E, S8E and S9A).

BLAST searches and protein alignment

BLAST (NCBI) searches were performed using the W09B7.2/F07B7.2 (SDG-1) amino acid sequence with default parameters and any homologs identified were aligned to SDG-1 using Clustal Omega[109] with default parameters. Alignments produced are shown in Figure S8B with residues shared by two proteins (grey highlight) or all three proteins (black highlight) indicated.

Annotation of the Cer9 retrotransposon containing W09B7.2/F07B7.2

The *Cer9* retrotransposon containing *W09B7.2/F07B7.2* (*sdg-1*) was annotated using sequence features from UCSC Genome Browser and amino acid sequences obtained from ref.[110].

The 5' and 3' LTR sequences were identified using RepeatMasker and were confirmed to have TC and GA dinucleotides at the beginning and end of each sequence, respectively[110]. Amino acid sequences from ref.[110] corresponding to *gag* and *pol* (PR: protease, RT: reverse transcriptase, RH: RNaseH, IN: integrase) elements of *Cer9* were used in tblastn (NCBI) searches to determine their positions in the *Cer9* retrotransposon sequence that also contains *sdg-1*.

Mating-induced silencing

Mating-induced silencing was assayed by crossing males with the transgene labeled *T* (*oxSi487*) encoding *mex-5p::mCherry::h2b::gfp::h2b* to hermaphrodites lacking the transgene, both in otherwise wild-type backgrounds or indicated mutant backgrounds. Reciprocal control crosses were performed in parallel where hermaphrodites with *T* were crossed to males lacking *T*. Animals were imaged and scored as described for this transgene in the "Light-induced damage of neurons" section.

Rationale for inferences

Prior models and assumptions: All dsRNA are trafficked similarly. Entry of dsRNA into the germline can initiate transgenerational RNA silencing of some but not all genes. No SID-1-dependent germline genes are known, suggesting that SID-1 could be used solely in response to viral infection by analogy with roles of other members of RNA interference pathways.

Evidence supporting key conclusions: Temporal selectivity of dsRNA transport was probed using three approaches for delivery of dsRNA (damage-induced release from neurons, ingestion, and injection). Spatial selectivity was inferred based on differences in the frequency of patterns of silencing within the germline. Substrate selectivity of dsRNA transport pathways was

probed using genetic mutants and dsRNA of different lengths and 5' chemistry. Diversity of dsRNAs made in bacteria and upon *in vitro* transcription was visualized using Northern blotting. Analysis of *sid-1* mutants and a revertant was used for better control of genetic background, aiding in the identification of ***s****id-1*-**d**ependent **g**enes (*sdg*). Separate measurement of *sdg-1* expression in descendants of independently edited isolates, along different lineages after perturbations, and in different gonads within single animals demonstrated stochasticity in gene expression and revealed establishment of different heritable epigenetic states. Co-localization of SDG-1::mCherry in perinuclear foci with the Z-granule marker GFP::ZNFX-1, its reported association with the Z-granule component ZSP-1/PID-2 and DEPS-1, and its dynamic nuclear localization similar to CSR-1b was used to propose that SDG-1 plays a role in small RNA regulation while also being a target of the dsRNA importer SID-1.

SUPPLEMENTARY FIGURES AND FIGURE LEGENDS

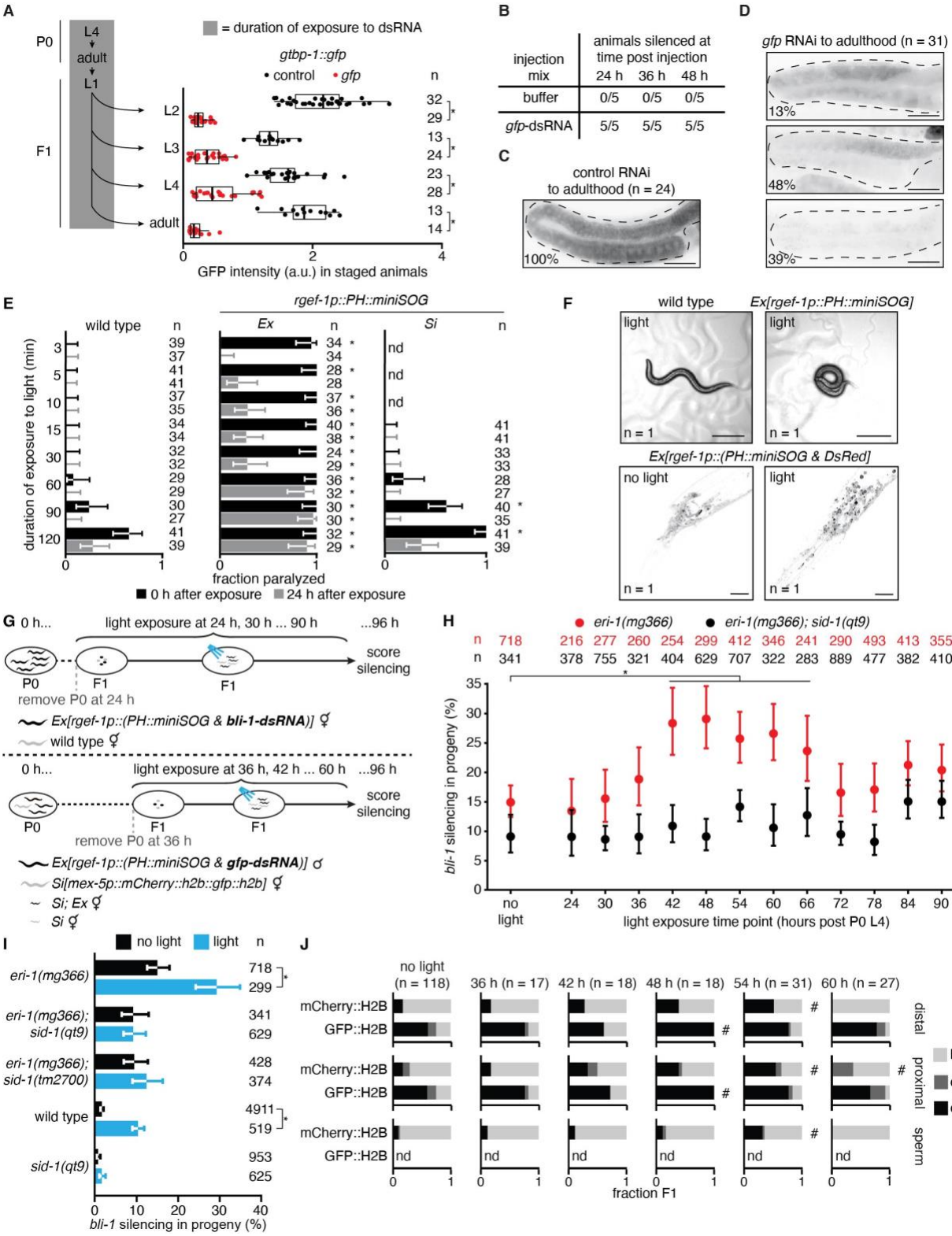

Figure S1. Timed delivery of extracellular dsRNA reveals temporal specificity in gene silencing in the soma and germline. (A, *left*) Schematic depicting continuous exposure of *gtbp-1::gfp* P0 animals, starting at the L4 stage, and their F1 progeny to bacteria expressing dsRNA, followed by imaging of animals at the indicated stages. (A, *right*) Quantification of GTBP-1::GFP intensity (arbitrary units, a.u.) in representative germ cells (larvae) or embryos *in utero* (adults) of F1 animals at indicated stages after P0 and F1 exposure to control (dark grey) or *gfp*-dsRNA (red). Numbers of animals scored at each stage (n) are indicated. Asterisks indicate $P < 0.05$ with Bonferroni correction using Mann-Whitney U test for two-sided comparisons between animals exposed to L4440 or *gfp*-dsRNA. (B) Silencing of *gtbp-1::gfp* in animals injected with duplex buffer (buffer) or *in vitro* transcribed *gfp*-dsRNA in duplex buffer during the first day of adulthood and scored for silencing 24, 36 and 48 h post injection. The numbers of animals out of 5 injected with each injection mix that exhibited silencing of both gonad arms are indicated for each time point. Animals injected with buffer never exhibited silencing in either gonad arm. (C and D) Representative fluorescence images of GTBP-1::GFP (black) in the germlines (dashed outline) of day 3 *gtbp-1::gfp* adult animals after P0 and F1 ingestion of control dsRNA (C) or *gfp*-dsRNA (D) up to the first day of adulthood. Numbers of animals imaged (n) and the percentages of animals exhibiting the depicted expression patterns are shown. Scale bars, 50 μm. (E) Wild-type animals (*left*) and animals expressing membrane-tethered mini singlet oxygen generator protein (*PH::miniSOG)* from an extrachromosomal array (*Ex*, *middle*) or a single-copy transgene (*Si*, *right*) under a pan-neuronal promoter (*rgef-1p*) were exposed to blue light for different durations (minutes) and animals were scored for paralysis immediately after exposure (0 h, black) and 24 hours later (24 h, grey). (F) Functional and anatomical evidence for oxidative damage in neurons. (*top*) Widefield images of animals without (*left*) and with (*right*) *Ex[rgef-1p::PH::miniSOG]* after 5 minutes of blue light exposure. Animals paralyzed in (E) often appear coiled (*right*). Scale bar, 100 μm. (*bottom*) Confocal fluorescence images of neurons in the head region of animals with *Ex[rgef-1p::(PH::miniSOG & DsRed)]* without (*left*) and with (*right*) 30 minutes of blue light exposure showing light-induced changes (black, DsRed fluorescence). Scale bar, 20 μm. (G) Schematic of assay for measuring the impact of oxidative damage in neurons at different times during development on silencing by neuronal dsRNA. For measuring silencing in the hypodermis (*top*) or germline (*bottom*), cohorts of animals with *Ex[rgef-1p::(PH::miniSOG & bli-1-dsRNA)]* (*top*), or *Ex[rgef-1p::(PH::miniSOG & gfp-dsRNA)]* obtained by mating males with the array and hermaphrodites with *Si[mex-5p::mCherry::h2b::gfp::h2b]* (*bottom*) were exposed to blue light as indicated and scored for *bli-1* silencing (*top*) or imaged (*bottom*) as stage-matched adults (at ~96 hours after the fourth larval stage of parent animals). (H) Percentages of *eri-1(mg366)* (red) or *eri-1(mg366); sid-1(qt9)* (black) animals silenced when assayed as described in (G, *top*). Silencing in the absence of exposure to blue light (no light) was also measured for comparison. (I) Percentages of stage-matched animals of the indicated genetic backgrounds with *Ex[rgef-1p::(PH::miniSOG & bli-1-dsRNA)]* that show *bli-1* silencing without (black) or with (blue) a 1-hour exposure to blue light 48 hours after the fourth larval stage of parent animals. The 48 hr time point from (H) is replotted to facilitate comparison. (J) Fractions of animals exhibiting bright (light grey), dim (dark grey) or not detectable (black) mCherry::H2B or GFP::H2B fluorescence in the distal gonad (*top*), proximal gonad (*middle*) or sperm (*bottom*) when assayed as described in (G, *bottom*). Silencing in the absence of exposure to blue light (no light) was used as the reference. Numbers of animals scored (n), measurements that were not done (nd), significant differences using two-tailed Wilson's estimates for single proportion compared to wild type (asterisks in (E))

or no light condition (asterisks in (H) and (I)) or χ^2 test compared to no light condition (hashes in (J); $P < 0.05$ with Bonferroni correction), and error bars (95% CI) are indicated.

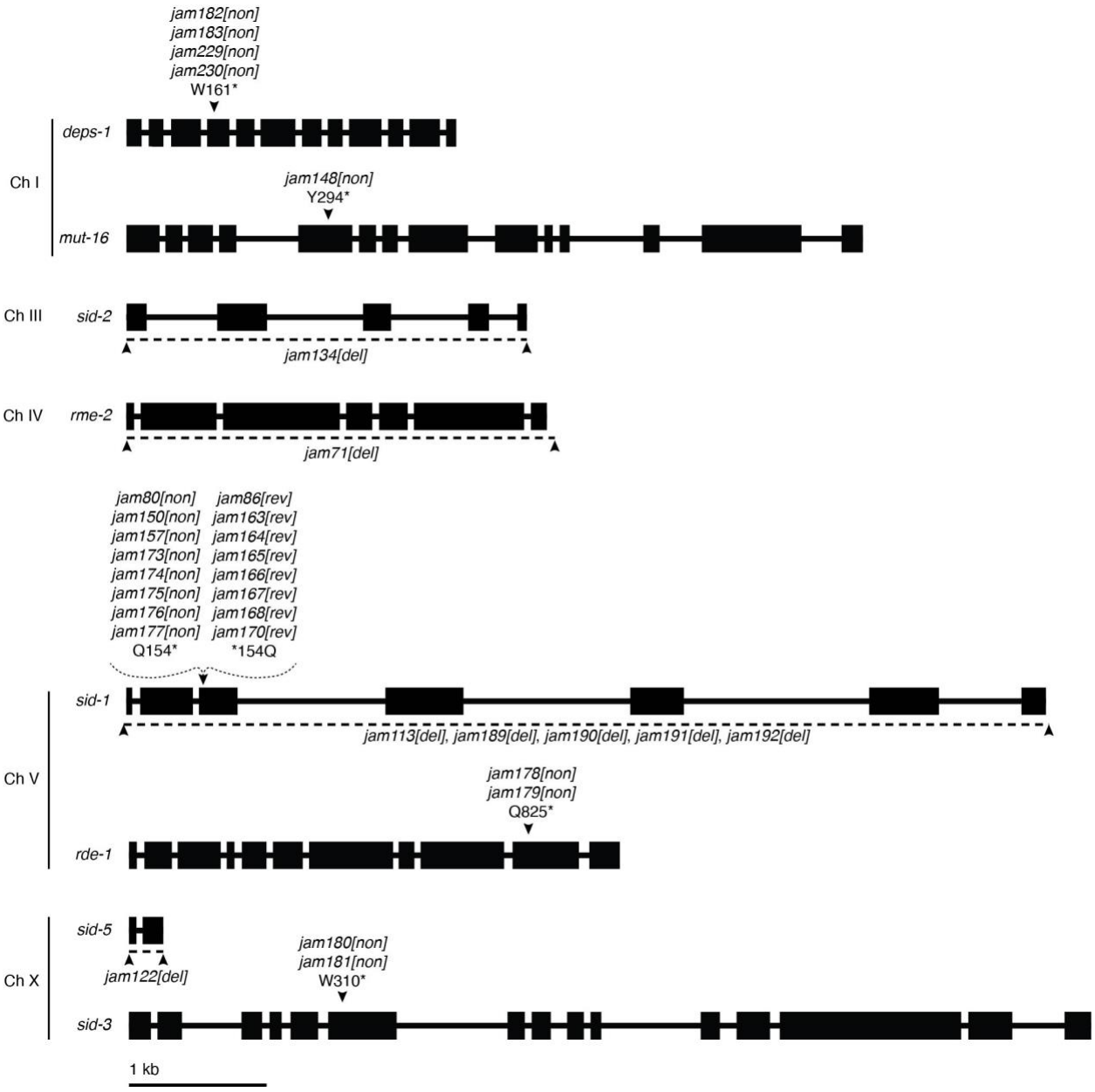

Figure S2. Schematics depicting mutations generated in this study. Structures (boxes, exons; lines, introns) and chromosomal locations of genes with mutations generated using Cas9-mediated genome editing. Nonsense mutations (e.g., *jam182[non]*) with associated amino acid changes (e.g., W161* for tryptophan at position 161 to stop) are indicated with black arrowheads and deletions of coding regions (e.g., *jam134[del]*) are indicated with a dashed line (deleted region) and flanking black arrowheads. Scale bar, 1 kb.

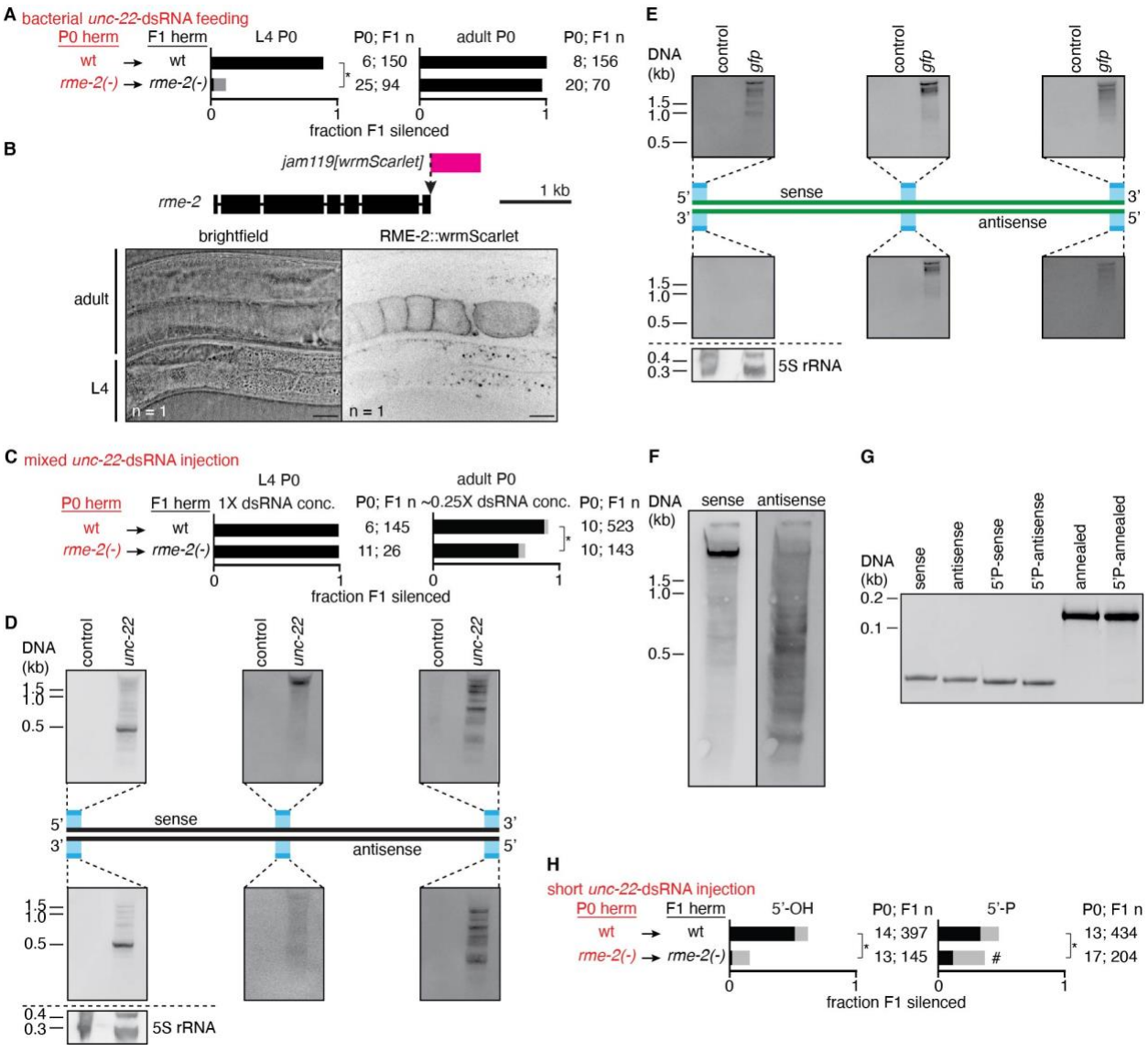

Figure S3. Requirement of RME-2 for silencing in progeny by parental dsRNA depends on source, concentration, length, and 5' modification of dsRNA. (A) Hermaphrodite animals of the L4 stage (left bars) or young adult stage (24 hour post L4, right bars) of the indicated genotypes were fed *unc-22*-dsRNA expressed in bacteria for 24 hours (red font). Hermaphrodite self-progeny of fed animals were scored for *unc-22* silencing (strong, black; weak, grey). Numbers of injected P0 parents and scored F1 progeny (P0; F1 n) are as indicated. Previously generated *rme-2(-)* animals were used in this assay (DH1390). (B) Expression of RME-2. (*top*) Schematic showing insertion of *wrmScarlet* (*rme-2*(*jam119[wrmScarlet]*)) at the *rme-2* locus. Scale bar, 1 kb. (*bottom*) Brightfield and fluorescence images of a *rme-2(jam119[wrmScarlet])* L4-stage and adult animal (n = 1 confocal image). Scale bars, 20 µm. (C) Hermaphrodite animals of indicated genotypes were exposed to *unc-22*-dsRNA (red font) and unexposed F1 progeny animals were scored as in (A). (*left*) L4-staged hermaphrodites were injected with transcribed *unc-22*-dsRNA in the body cavity at the same concentration as in Figure 2A (1X). (*right*) Young adult-staged hermaphrodites were injected with transcribed *unc-22*-dsRNA at ~0.25X of concentration in Figure 2A. Newly generated *rme-2(-)* animals were used in this assay

(AMJ1131). (D and E), Northern blots of bacterial *unc-22*-dsRNA (*unc-22*, (D)) or *gfp*-dsRNA (*gfp*, (E)) separated alongside empty vector control RNA using fully-denaturing formaldehyde polyacrylamide gel electrophoresis (FDF-PAGE)[101]. 40-nt digoxigenin (DIG)-labeled oligonucleotides (in blue) were used to probe the 5' end, middle and 3' end of the sense (*top*) and antisense (*bottom*) strands of the *unc-22* (D) and *gfp* (E) sequences present in the bacterial vectors. A 1-kb DNA ladder was used as a size reference and 5S rRNA was probed as a control for equal loading of total RNA. (F) Northern blot of *unc-22*-dsRNA transcribed from a ~1.1-kb template, separated using FDF-PAGE as in (D) and (E), and probed using 40-nt DIG-labeled oligonucleotides complementary to the sense (*left*) or antisense (*right*) strands of the *unc-22* gene. (G) Polyacrylamide gel stained with ethidium bromide showing 50-nt single-stranded (sense, antisense, 5'P-sense, 5'P-antisense) and 50-bp double-stranded *unc-22*-RNA (annealed, 5'P-annealed). A 100-bp DNA ladder was run alongside for rough size estimation. 5'-phosphate (5'P) was added using a polynucleotide kinase. (H) Young adult-staged hermaphrodites were injected with short *unc-22*-dsRNA with 5'-OH (left bars) or with 5'-phosphate added using a polynucleotide kinase (right bars) and self-progeny were scored as in (A). Newly generated *rme-2(-)* animals were used in this assay (AMJ1131). Comparisons with $P < 0.05$ after Bonferroni correction using χ^2 test between genotypes within conditions (asterisks in (A), (C), and (H)) or between conditions in *rme-2(-)* animals (hash in (H)) are indicated.

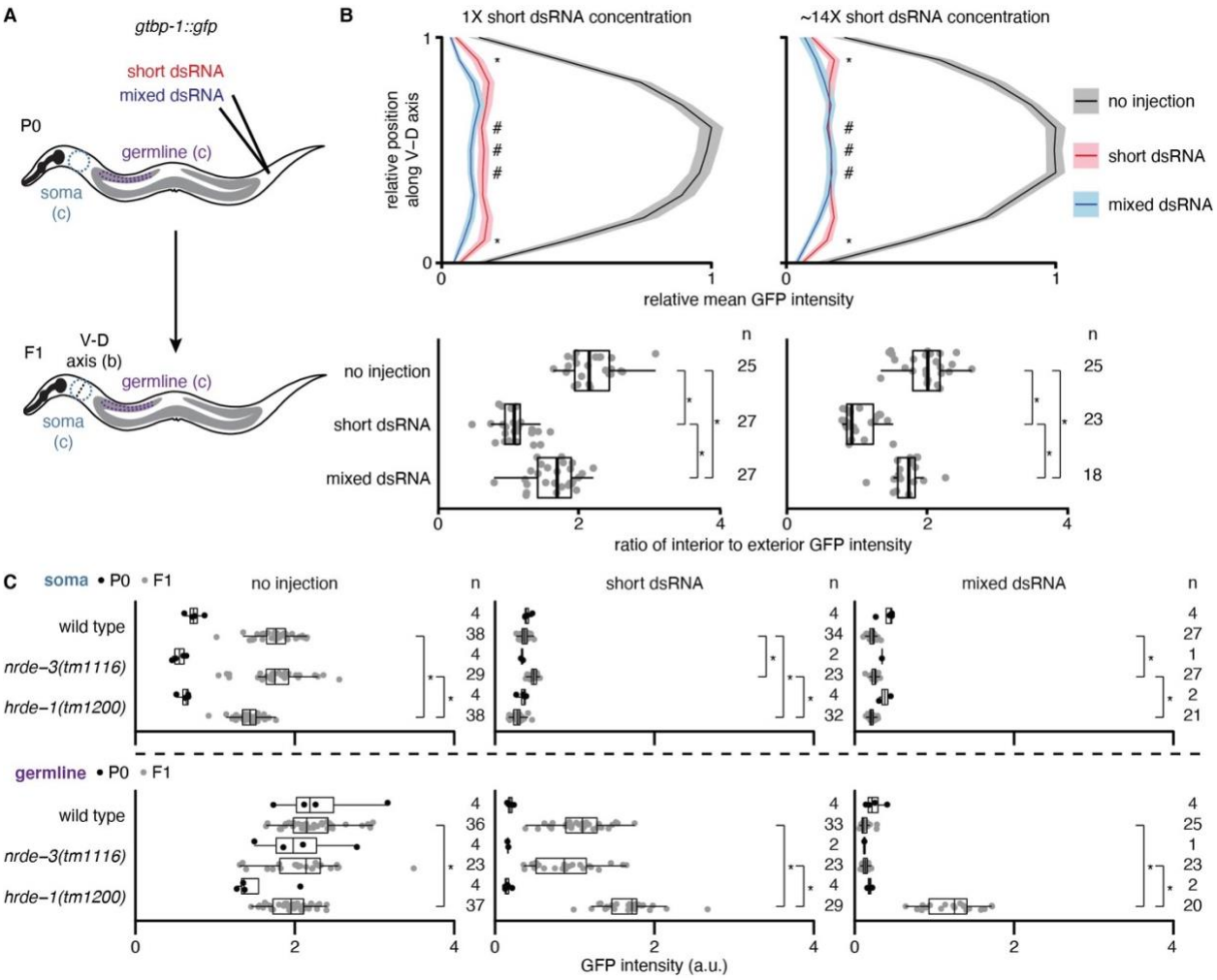

Figure S4. Extent of silencing in progeny by short or mixed dsRNA injected into parental circulation varies between tissues, but has similar nuclear Argonaute requirements. GTBP-1::GFP fluorescence from the ubiquitously expressed gene *gtbp-1::gfp* in the F1 progeny of uninjected P0 animals (no injection) or of P0 animals injected into the body cavity with synthetic 50-bp *gfp*-dsRNA (short dsRNA) or *gfp*-dsRNA transcribed from a ~730-bp DNA template (mixed dsRNA) was analyzed. (A) Schematic illustrating injection site (red) and scoring scheme. For the soma, a region between the pharynx and anterior gonad arm within a circle (blue, data in (C)) or along a ventral to dorsal (V-D) axis (black, data in (B)) was quantified. For the germline, a gonadal region that excluded the intestine (purple, data in (C)) was quantified. (B) Quantification of F1 progeny after injection of two different concentrations of short dsRNA (1X, 350 ng/µl, *left*; ~14X, 4977 ng/µl, *right*) into the body cavity of P0 animals. (*top*) The relative mean intensity profile of fluorescence along the V-D axis for progeny of uninjected animals (black), animals injected with short dsRNA (red), or animals injected with mixed dsRNA (blue). Shaded bands indicate 95% CI. (*bottom*) Ratios of mean intensities within interior points (hashes in *top*) to those of the exterior points (asterisks in *top*) are depicted for each imaged animal. (C) Quantification of P0 (black) and F1 (grey) wild-type, *nrde-3(tm1116)* or *hrde-1(tm1200)* animals. Regions within the soma and the germline were quantified as indicated in (A). The numbers of P0 and F1 animals quantified (P0; F1 n) are indicated. For each genotype, F1 progeny after no injection, short dsRNA injection, or mixed dsRNA injection into P0 animals

showed significantly different fluorescence values from each other ($P < 0.05$ after Bonferroni correction using Mann-Whitney U test for two-sided comparisons). Similarly significant differences between treatments across genotypes are indicated (asterisks).

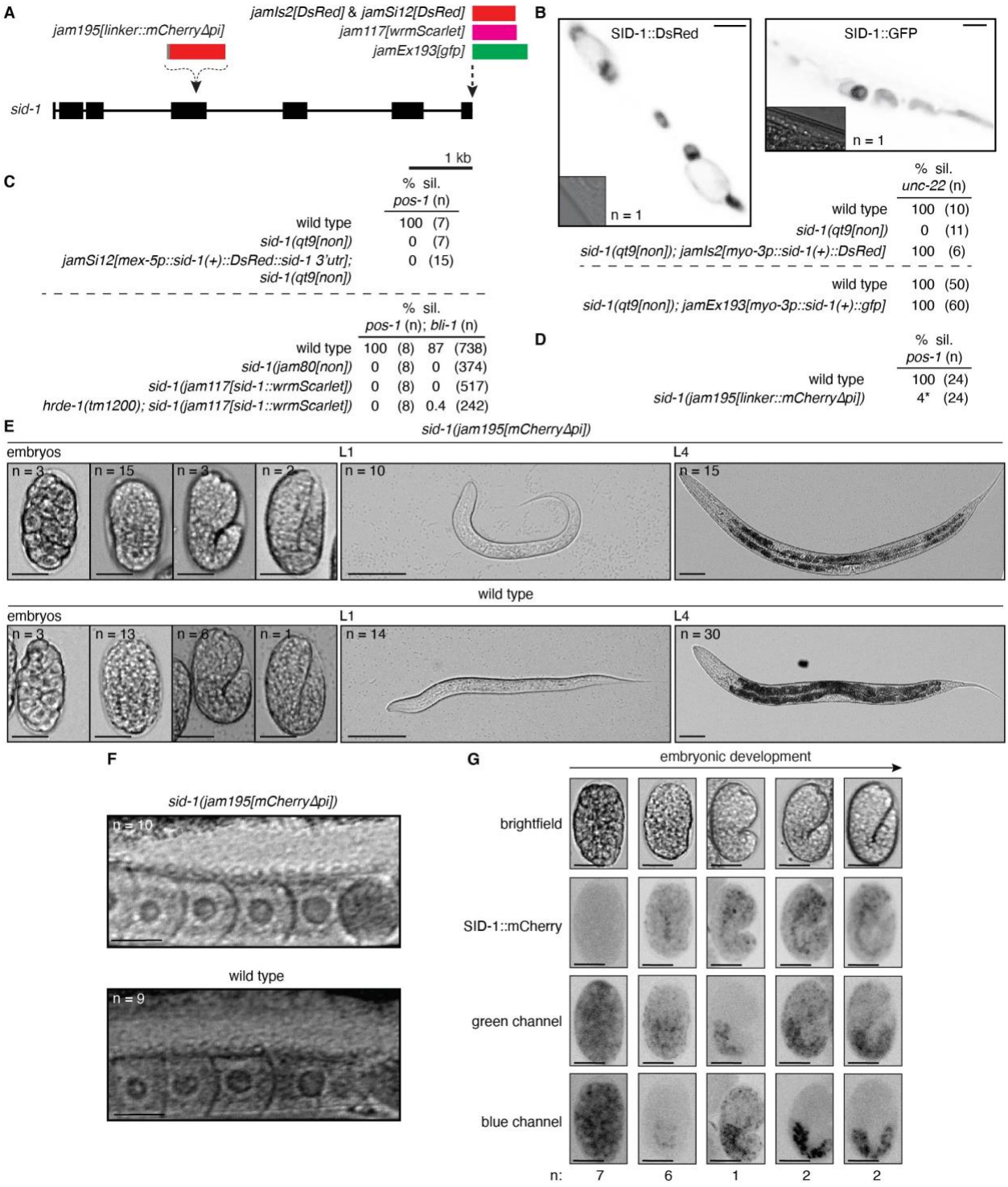

Figure S5. An internally tagged and partially functional SID-1 fusion protein shows dynamic changes in SID-1 expression across development. (A) Schematic of transgenic *sid-1* reporters or modifications at the *sid-1* gene generated using Cas9-mediated genome editing. An integrated *sid-1::DsRed* array (*jamIs2[DsRed]*), a single-copy *sid-1::DsRed* transgene (*jamSi12[DsRed]*) inserted using Mos-mediated single copy insertion[90], insertion of *wrmScarlet* at the *sid-1* locus (*jam117[wrmScarlet]*), an extrachromosomal array of *sid-1::gfp*

(*jamEx193[gfp]*), and an insertion of *mCherry* sequence that lacks piRNA binding sites[64-65] along with a linker at the *sid-1* locus (*jam195[linker::mCherryΔpi]*, also referred to as *jam195[mCherryΔpi]*) are depicted. (B) C-terminal SID-1 fusion proteins expressed from multicopy transgenes apparently rescue function and show intracellular localization. (*top*) Fluorescence images of SID-1::GFP (*left*) and SID-1::DsRed (*right*) fusion proteins expressed from multicopy arrays in the muscle (*Pmyo-3*). Insets, brightfield images; scale bars, 5 μm. (*bottom*) Percentages of *unc-22* silencing (% sil.) upon ingestion of bacterial *unc-22*-dsRNA in *sid-1(qt9[non])* animals with and without these transgenes. Numbers of animals scored (n) are indicated. (C) C-terminal SID-1 fusion proteins expressed from a single copy transgene or the endogenous *sid-1* locus appear non-functional. (*top*) Percentage of *pos-1* silencing after ingestion of bacterial *pos-1*-dsRNA in *sid-1(qt9[non])* animals with or without a single-copy transgene designed to express SID-1::DsRed in the germline (*jamSi12[mex-5p::sid-1(+)::DsRed::sid-1 3'utr]*). (*bottom*) Percentages of *pos-1* (*left*) or *bli-1* (*right*) silencing after ingestion of bacterial *pos-1* or *bli-1*-dsRNA in animals with the endogenous *sid-1* gene tagged at the 3'end with *wrmScarlet* sequence (*sid-1(jam117[sid-1::wrmScarlet])*) with or without the secondary Argonaute HRDE-1. Numbers of animals scored (n) are indicated. Wild-type and *sid-1(-)* animals (*sid-1(qt9[non])*, *top*; *sid-1(jam80[non])*, *bottom*) were used as controls. When expressed in single copy, animals expected to have C-terminal fusions of SID-1 (SID-1::DsRed and SID-1::wrmScarlet) remained RNAi defective, suggesting that the SID-1 fusion proteins are either not expressed because of silencing at the *sid-1* locus or that the tagging disrupts SID-1 protein function or stability. Consistent with loss of protein function and/or stability upon C-terminal tagging, RNAi was not restored upon loss of HRDE-1 (*hrde-1(tm1200)*), which is expected to disrupt silencing (if any), and an internal tag of SID-1 showed substantial function (Figure 3B). Given the diversity of gene products that can be made from multicopy transgenes[42], we reason that the apparent functionality of C-terminal fusions of SID-1 expressed from multicopy arrays in (B) reflects the activity of variants that could be untagged. Therefore, the observed subcellular localization in (B) cannot be attributed to functional SID-1. (D) Percentage of *pos-1* silencing after ingestion of bacterial *pos-1*-dsRNA in wild-type and *sid-1(jam195[linker::mCherryΔpi])* animals. Numbers of animals scored (n) are indicated. Asterisk indicates weak silencing in one animal (partially viable F1 brood). (E and F) Representative brightfield images of *sid-1(jam195[mCherryΔpi])* (*top*) and wild-type (*bottom*) animals corresponding to mCherry images in Figures 3C and 3D. Images of (E) embryos (*left*), L1 animals (*middle*), L4 animals (*right*), and (F) adult gonad arms are shown. Numbers of images for each stage (n) are depicted. For adult gonad arms imaged in (F), only the proximal germline was visible in 1/10 *sid-1(jam195[mCherryΔpi])* and 5/9 wild-type animals. Scale bars for embryos (E) and adult gonad arms (F), 20 μm. Scale bars for larvae (E), 50 μm. (G) Representative brightfield (*first row*), SID-1::mCherry (*second row*), green channel (*third row*) and blue channel (*fourth row*) images of *sid-1(jam195[mCherryΔpi])* embryos throughout embryogenesis. Green and blue channel images are depicted to highlight potential sources of autofluorescence, if any, in the mCherry channel. The numbers of embryos represented (n) are depicted (*bottom*) and 100% of embryos exhibited the represented patterns. Scale bar, 20 μm.

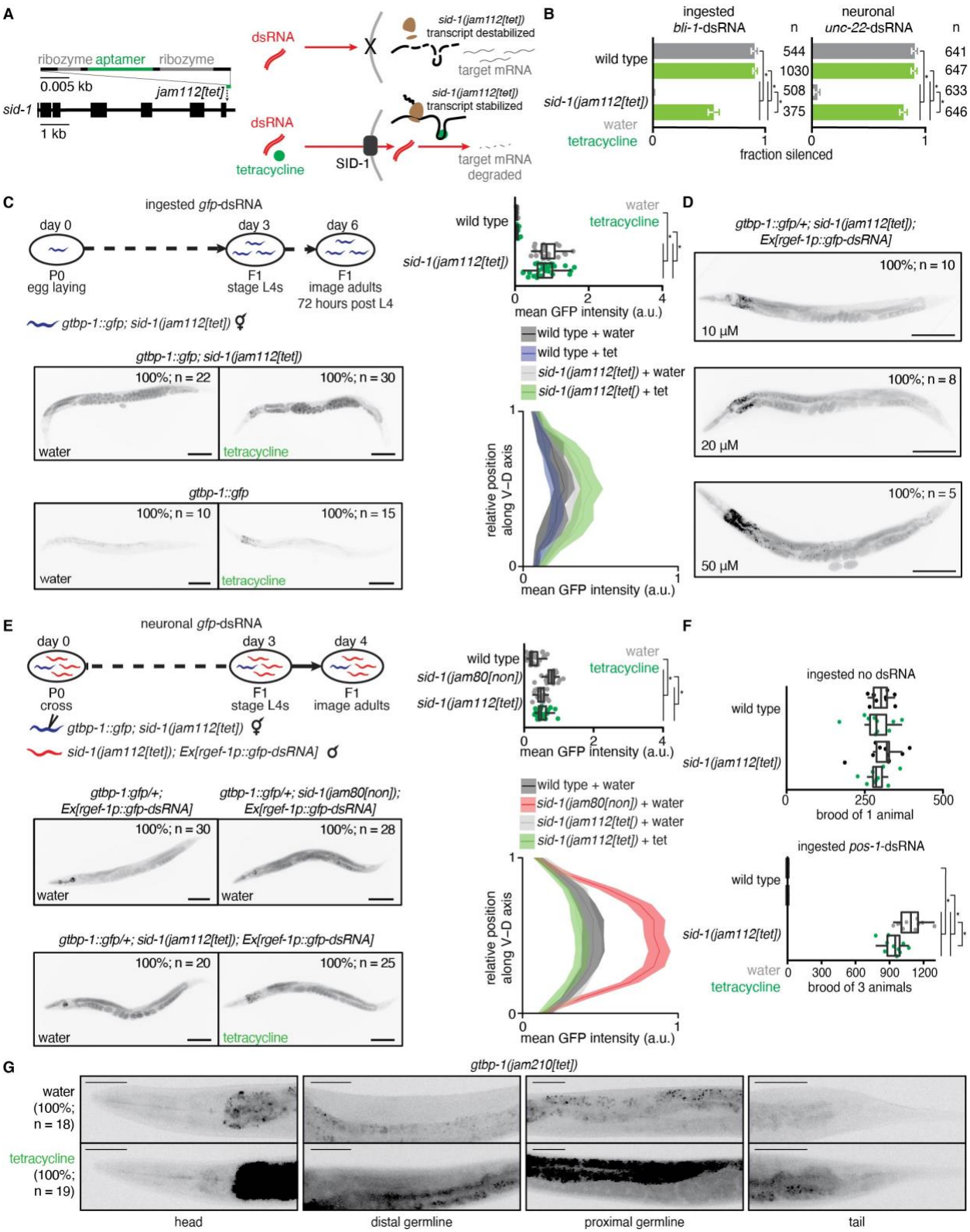

Figure S6. Tetracycline-induced functional rescue of *sid-1* expression is evident in somatic tissues but not within the germline. (A) Schematic illustrating a cell expressing *sid-1* transcript with a tetracycline aptazyme[45] inserted into the *sid-1* 3'UTR (*left*) in the presence (*bottom right*)

or absence (*top right*) of tetracycline. Tetracycline stabilizes *sid-1* transcripts by inhibiting ribozyme-based cleavage in the 3'UTR and thereby allows for the expression of SID-1 protein and dsRNA import. (B) Fraction of wild-type or *sid-1(jam112[tet])* animals silenced after ingestion of *bli-1*-dsRNA (*left*) or expression of neuronal *unc-22*-dsRNA (*right*) in the presence of water (grey bars) or 10 μM tetracycline (green bars). Numbers of animals scored for silencing (n) are depicted. (C) The extent of *gfp* silencing in *gtbp-1::gfp; sid-1(jam112[tet])* day 3 adult animals after ingestion of *gfp*-dsRNA in the presence of water or 10 μM tetracycline. A schematic illustrating the experimental design (*top left*), representative images of animals from each condition with numbers of animals imaged (n) and percentages of animals represented (*bottom left*), and quantification of representative germline (*top right*) and somatic (*bottom right*) GTBP-1::GFP intensity (a.u.) are depicted. Mean germline GFP intensity was measured in representative regions of the posterior germline and somatic GFP intensity was measured along a dorsal to ventral axis in the tail region (shaded region represents 95% CI) to avoid increased autofluorescence in the intestines of animals exposed to tetracycline. Scale bars, 100 μm. (D) Representative images of *gtbp-1::gfp; sid-1[jam112[tet])* F1 day 1 adult animals after P0 and F1 ingestion of *gfp*-dsRNA until day 1 of F1 adulthood in the presence of different concentrations of tetracycline (10 μM, 20 μM, 50 μM). Higher concentrations of tetracycline did not enhance silencing in *gtbp-1::gfp; sid-1(jam112[tet])* animals. Scale bars, 100 μm. (E) The extent of *gfp* silencing in cross progeny of *gtbp-1::gfp; sid-1(jam112[tet])* hermaphrodites injected with water or 10 μM tetracycline and *sid-1(jam112[tet]); Ex[rgef-1p::gfp-dsRNA]* males in the presence of water or 10 μM tetracycline. A schematic illustrating the experimental design including injection of *gtbp-1::gfp; sid-1(jam112[tet])* hermaphrodites with water or 10 μM tetracycline (*top left*), representative images of animals with the *Ex[rgef-1p::gfp-dsRNA]* array from each condition with numbers of animals imaged (n) and percentages of animals represented (*bottom left*), and quantification of representative germline (*top right*) and somatic (*bottom right*) GFP intensity (a.u.) as in (C) are depicted. Scale bars, 100 μm. (F) Total brood of wild-type or *sid-1(jam112[tet])* animals after culturing on OP50 *E. coli* or *pos-1*-dsRNA bacteria in the presence of water or 10 μM tetracycline. Silencing by *pos-1*-dsRNA typically results in inviable embryos (wild type, *bottom*), but culturing *sid-1(jam112[tet])* with 10 μM tetracycline and *pos-1*-dsRNA only resulted in a minor decrease in brood size (*sid-1(jam112[tet]*), *bottom*). This decrease was not observed when *sid-1(jam112[tet])* animals were cultured on 10 μM tetracycline plates in the absence of *pos-1*-dsRNA (*top*, brood of 1 animal; *bottom*, brood of 3 animals). (G) Representative fluorescence images of GTBP-1::GFP (black) in the heads, distal germlines, proximal germlines, and tails of *gtbp-1::gfp* animals with a tetracycline-aptazyme sequence inserted into the *gtbp-1::gfp* 3'UTR (*gtbp-1(jam210[tet])*) after culturing with water or 10 μM tetracycline. The numbers of animals imaged (n) and the percentages of animals with the represented expression patterns are depicted. An increase in GTBP-1::GFP intensity was observed in the soma and germline, but increased fluorescence in the intestine cannot be distinguished from increased autofluorescence caused by culturing with 10 μM tetracycline. Scale bars, 50 μm.

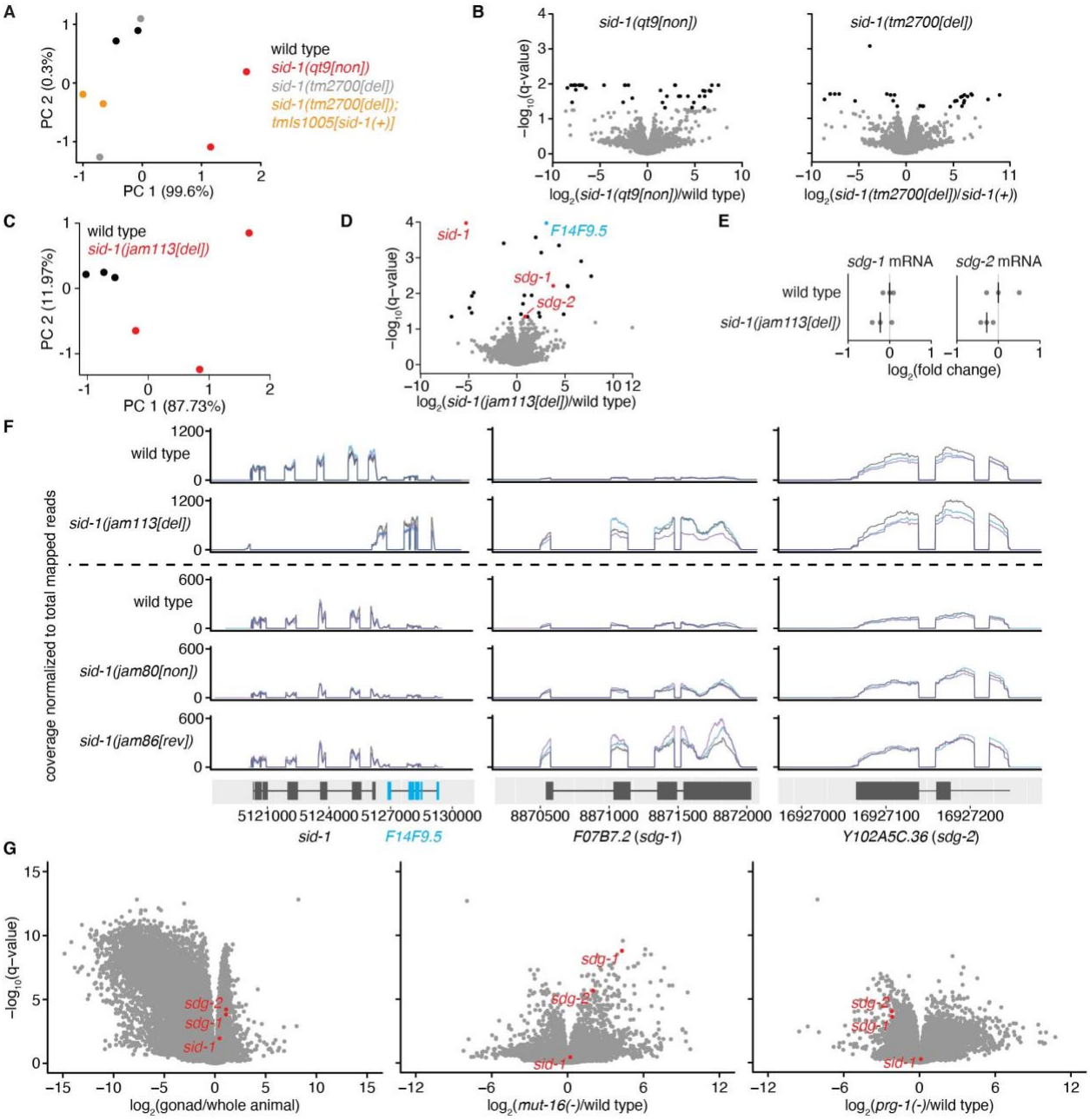

Figure S7. Selective disruption of *sid-1* followed by restoration to wild type reveals two *sid-1*-dependent transcripts expressed in the germline that show heritable change. (A) Principal components explaining the variance between wild type (black), *sid-1(qt9[non])* (red), *sid-1(tm2700[del])* (grey) and *sid-1(tm2700[del]); tmIs1005[sid-1(+)]* (orange) animals. (B) Volcano plots of changes in the abundance of polyA+ RNA in *sid-1(qt9[non])* vs. wild-type animals (*left*) and *sid-1(tm2700[del])* vs. *sid-1(tm2700[del]); tmIs1005[sid-1(+)]* animals (*right*) (black, $q < 0.05$; grey, $q > 0.05$). No significantly misregulated genes were detected in both comparisons. (C) Principal components explaining the variance between wild type (black) and *sid-1(jam113[del])* (red) animals. (D) Volcano plots of changes in the abundance of polyA+ RNA in *sid-1(jam113[del]*) animals compared with wild-type animals (black, $q < 0.05$; red, $q <$

0.05 and with change in the same direction in *sid-1(jam80[non])*; see Figure 3B, *top*). (E) Levels of spliced *sdg-1* and *sdg-2* transcripts in animals of the indicated genotypes measured using RT-qPCR. The median (line) of three technical replicates is plotted for each of three biological replicates. $P > 0.05$ with Bonferroni correction using two-tailed Student's t-test for wild type to mutant comparisons. Levels of *sid-1* transcripts were not detectable in *sid-1(jam113[del])* animals due to absence of *sid-1* coding sequence (data not shown). (F) Read coverage in biological triplicate (black, blue and purple) at *sid-1* and *F14F9.5* (*left*), *W09B7.2/F07B7.2* (*sdg-1*) (represented by *F07B7.2* locus, *middle*) and *Y102A5C.36* (*sdg-2*) (*right*) of polyA+ RNA in wild-type and *sid-1(jam113[del])* animals (*top*), and in wild-type, *sid-1(jam80[non])*, and *sid-1(jam86[rev])* animals (*bottom*) normalized to total mapped reads per sample. Deletion of *sid-1* coding sequence caused accumulation of transcripts from *F14F9.5* (blue), requiring point mutation (*jam80[non]*) for selective disruption of *sid-1* (see Figure 3). (G) Volcano plots of changes in the abundance of RNA in wild-type gonads vs. whole animals (*left*), *mut-16(-)* vs. wild-type animals (*middle*), and *prg-1(-)* vs. wild-type animals (*right*) using data from ref.[49]. *sdg-1, sdg-2* and *sid-1* transcripts are highlighted (red).

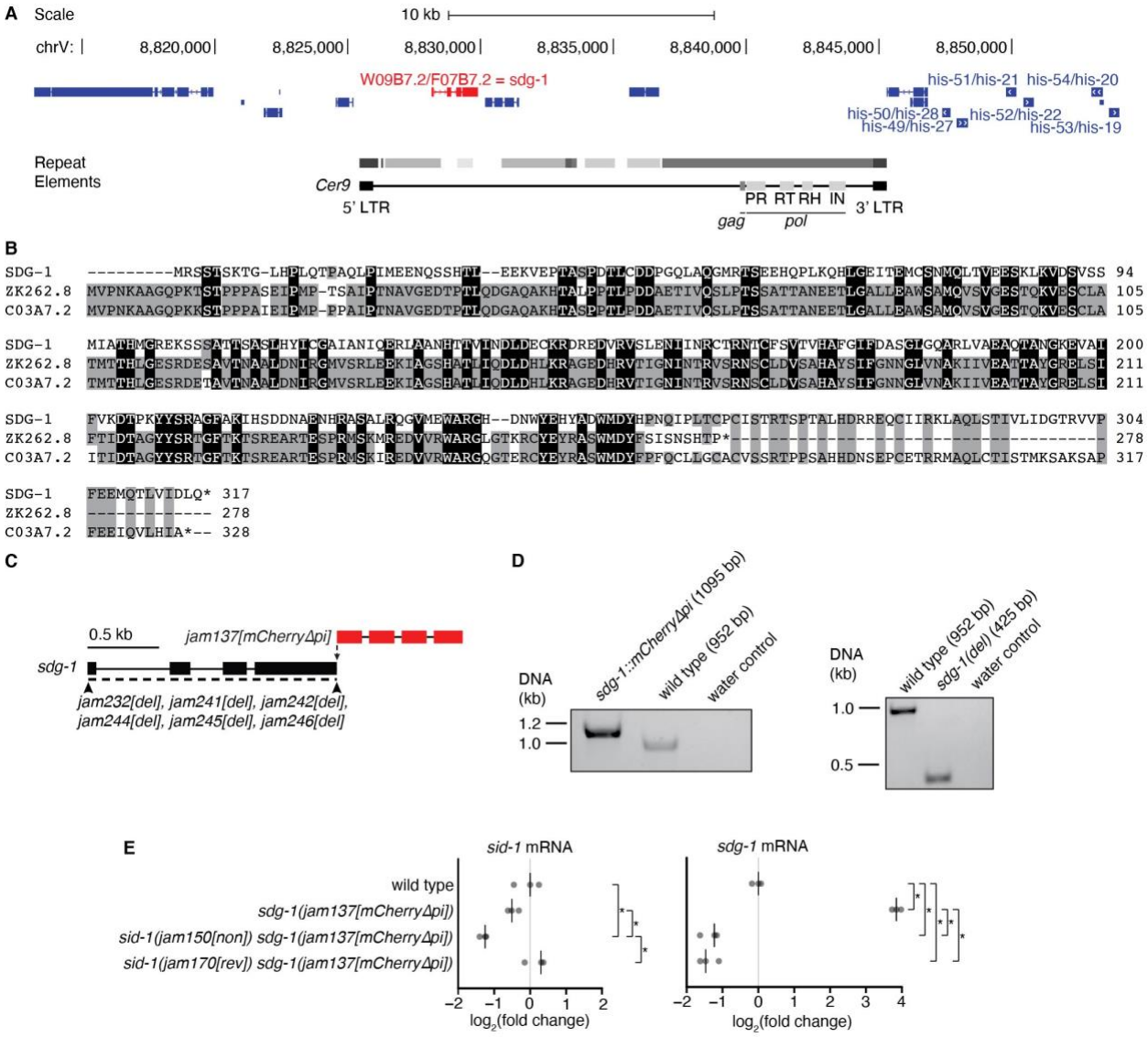

Figure S8. The *sid-1*-dependent gene *sdg-1* is expressed from two identical loci (*W09B7.2/F07B7.2*) and loss of its expression in *sid-1(non)* animals fails to recover in *sid-1(rev)* animals. (A) Schematic adapted from UCSC Genome Browser depicting *W09B7.2/F07B7.2* (red) located within a repeated ~40-kb locus on chromosome V (8813207-8896495 depicted; duplicate locus at 8855302-8896495) that includes many histone genes (dark blue; duplicate genes also depicted). *W09B7.2/F07B7.2* are located within full-length *Cer9* retrotransposons with repeated regions in grey (darker color indicates fewer repeat element-associated mismatches/insertions/deletions). Loci encoding *gag* and *pol* elements (PR: protease, RT: reverse transcriptase, RH: RNaseH, IN: integrase) within *Cer9* are depicted. (B) Alignment of the SDG-1 protein sequence encoded by *W09B7.2/F07B7.2* to the paralogs ZK262.8 and C03A7.2 with conserved residues between two (grey) or three (black) proteins highlighted. (C) Schematic depicting insertion of *mCherry* sequence that lacks piRNA binding sites[64-65] at the 3' end of *sdg-1*(*jam137[mCherryΔpi]*), as well as deletion of the *sdg-1* coding sequence (*jam232*, *jam241*, *jam242, jam244, jam245,* and *jam246*). (D) Genotyping gel showing insertion of *mCherryΔpi* sequences (1095 bp) (*left*) or deletion of *sdg-1* coding sequences (425 bp) (*right*) at both loci of *sdg-1*. Absence of wild-type bands in either case confirm genome editing of both

copies. (E) Levels of spliced *sid-1* (*left*) and *sdg-1* (*right*) transcripts in wild-type animals and *sdg-1(jam137[mCherryΔpi])* animals with a wild-type (+), *sid-1(jam150[non])* or *sid-1(jam169[rev])* background measured using RT-qPCR. The median of three technical replicates is plotted for each of three biological replicates (bar indicates median). Asterisks indicate $P < 0.05$ with Bonferroni correction using two-tailed Student's t-test.

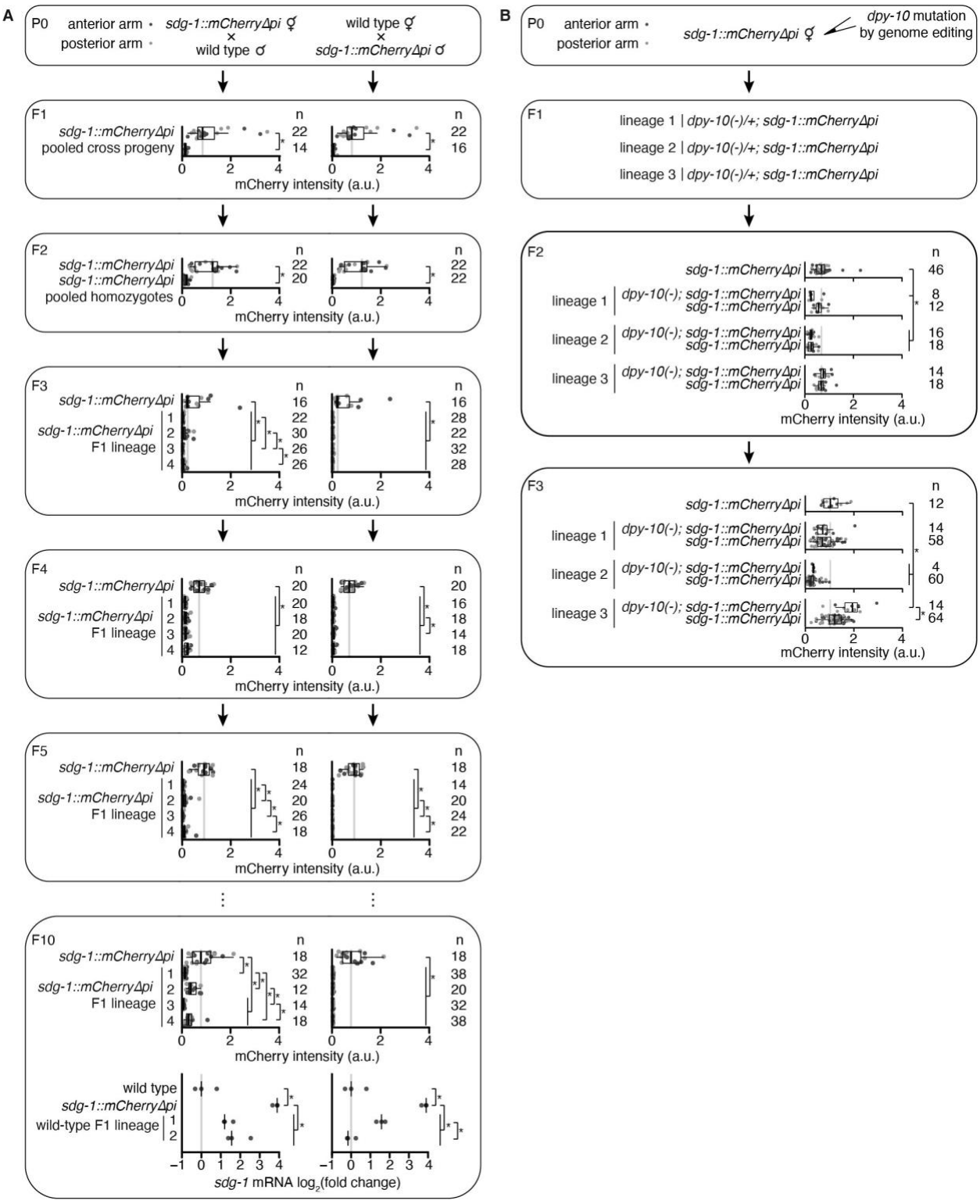

Figure S9. Mating but not genome editing can initiate distinct heritable changes in *sdg-1* expression. (A, P0 to F10, *top*) Quantification of SDG-1::mCherry fluorescence intensity (a.u.) in adult gonad arms (anterior arm, dark grey; posterior arm, light grey) across generations after mating hermaphrodite and male *sdg-1(jam137[mCherryΔpi])* animals with male and

hermaphrodite wild-type animals, respectively. The generations assayed and numbers of gonad arms quantified (n) are indicated. In F1 and F2, fluorescence intensity values of animals with lineages that were not propagated to F10 but were heterozygous or homozygous *sdg-1(jam137[mCherryΔpi])*, respectively, were pooled with values of animals with lineages that were propagated to F10. In F3 to F10, *top*, animals from four different F1 lineages were scored. Fluorescence intensity of animals descending from the self-progeny of P0 *sdg-1(jam137[mCherryΔpi])* animals was measured in each generation and is depicted, with the same data plotted for each mating direction for comparison. Asterisk indicates $P < 0.05$ with Bonferroni correction using Mann-Whitney U test for two-sided comparisons. (A, F10, *bottom*) Levels of spliced *sdg-1* mRNA transcripts in wild-type animals, *sdg-1(jam137[mCherryΔpi])* animals and two lineages of wild-type F10 progeny from each cross direction, measured using RT-qPCR. The median of three technical replicates is plotted for each of three biological replicates (bar indicates median). Asterisks indicate $P < 0.05$ with Bonferroni correction using two-tailed Student's t-test. (B, P0 and F1) Schematic illustrating mutation of *dpy-10* in three P0 lineages of *sdg-1(jam137[mCherryΔpi]* animals and subsequent segregation of the *dpy-10* mutation. (B, F2 and F3) Both *dpy-10(-)* and *dpy-10(+)* F2 and F3 animals from each of the three P0 lineages were imaged and SDG-1::mCherry intensity was quantified (a.u.) in adult gonad arms (anterior arm, dark grey; posterior arm, light grey). Minor differences in SDG-1::mCherry expression were observed between mutants and nonmutants in some cases, as well as between lineages. The numbers of gonad arms quantified (n) are depicted. Asterisks indicates $P < 0.05$ with Bonferroni correction using Mann-Whitney U test for two-sided comparisons.

Table S1. Strains.

Strains	Genotype
AMJ3	*sid-1(qt9) V; jamIs2[myo-3p::sid-1::gfp]*
AMJ308	*ccIs4251[myo-3p::gfp::lacZ::nls & myo-3p::mito-gfp & dpy-20(+)] I; sid-1(qt9) V*
AMJ327	*ccIs4251[myo-3p::gfp::lacZ::nls & myo-3p::mito-gfp & dpy-20(+)] I; sid-1(qt9) V; jamIs2[myo-3p::sid-1 cDNA::DsRed]*
AMJ471	*jamEx140[rgef-1p::gfp-dsRNA & myo-2p::DsRed]*
AMJ477	*qtEx136[rgef-1p::unc-22-dsRNA & rgef-1p::DsRed]*
AMJ576	*jamSi12[mex-5p::sid-1::DsRed::sid-1 3'UTR]; unc-119(ed3) III; sid-1(qt9) V*
AMJ577	*hrde-1(tm1200[4X outcrossed]) III*
AMJ581	*oxSi487[mex-5p::mCherry::h2b::gfp::h2b & Cbr-unc-119(+)] dpy-2(e8) II; unc-119(ed3) III*
AMJ706	*sid-1(qt9) V; jamEx193[myo-3p::sid-1::gfp]*
AMJ819	*eri-1(mg366) gtbp-1(ax2053[gtbp-1::gfp]) IV*
AMJ837	*jamEx209[rgef-1p::PH::miniSOG & myo-2p::DsRed]*
AMJ936	*jamEx210[rgef-1p::PH::miniSOG & rgef-1p::DsRed]*
AMJ1007	*eri-1(mg366) IV; jamEx213[rgef-1p::PH::miniSOG & rgef-1p::bli-1-dsRNA & myo-2p::DsRed]*
AMJ1009	*eri-1(mg366) gtbp-1(ax2053[gtbp-1::gfp]) IV; jamEx214[rgef-1p::PH::miniSOG & rgef-1p::gfp-dsRNA & myo-2p::DsRed]*
AMJ1019	*jamSi36[rgef-1p::PH::miniSOG & Cbr-unc-119(+)] II; unc-119(ed3) III*
AMJ1108	*eri-1(mg366) IV; sid-1(qt9) V; jamEx213[rgef-1p::PH::miniSOG & rgef-1p::bli-1-dsRNA & myo-2p::DsRed]*
AMJ1114	*sid-1(qt9) V; jamEx213[rgef-1p::PH::miniSOG & rgef-1p::bli-1-dsRNA & myo-2p::DsRed]*
AMJ1120	*rme-2(jam71[deletion]) IV; sid-1(qt9) V*
AMJ1123	*jamEx213[rgef-1p::PH::miniSOG & rgef-1p::bli-1-dsRNA & myo-2p::DsRed]*
AMJ1131	*rme-2(jam71[deletion]) IV*
AMJ1134	*jamEx214[rgef-1p::PH::miniSOG & rgef-1p::gfp-dsRNA & myo-2p::DsRed]*
AMJ1151	*sid-1(tm2700) V; jamEx213[rgef-1p::PH::miniSOG & rgef-1p::bli-1-dsRNA & myo-2p::DsRed]*
AMJ1153	*sid-1(tm2700)[3X outcrossed] V*
AMJ1159	*sid-1(jam80[nonsense]) V*
AMJ1173	*eri-1(mg366) IV; sid-1(tm2700) V; jamEx213[rgef-1p::PH::miniSOG & rgef-1p::bli-1-dsRNA & myo-2p::DsRed]*
AMJ1217	*sid-1(jam86[revertant]) V*
AMJ1220	*hrde-1(tm1200) III; gtbp-1(ax2053[gtbp-1::gfp]) IV*
AMJ1280	*sid-1(jam115[sid-1::wrmScarlet13]) V*
AMJ1281	*rme-2(jam116[rme-2::wrmScarlet13]) IV*
AMJ1282	*sid-1(jam117[sid-1::wrmScarlet]) V*
AMJ1284	*rme-2(jam119[rme-2::wrmScarlet]) IV*
AMJ1312	*sid-1(jam80[nonsense]) V; jamEx214[rgef-1p::PH::miniSOG & rgef-1p::gfp-dsRNA & myo-2p::DsRed]*

AMJ1323 *sid-1(jam112[sid-1::tetracycline-K4-aptazyme::3'UTR]) V*
AMJ1324 *sid-1(jam113[deletion]) V*
AMJ1330 *sid-1(jam112[sid-1::tetracycline-K4-aptazyme::3'UTR]) V; qtEx136[rgef-1p::unc-22-dsRNA & rgef-1p::DsRed]*
AMJ1332 *sid-5(jam122[deletion]) X*
AMJ1350 *sid-1(jam112[sid-1::tetracycline-K4-aptazyme::3'UTR]) V; jamEx140[rgef-1p::gfp-dsRNA & myo-2p::DsRed]*
AMJ1355 *gtbp-1(ax2053[gtbp-1::gfp]) IV; sid-1(jam112[sid-1::tetracycline-K4-aptazyme::3'UTR]) V*
AMJ1365 *hrde-1(tm1200) III; sid-1(jam117[sid-1::wrmScarlet]) V*
AMJ1366 *rme-2(jam71[deletion]) IV; sid-1(jam113[deletion]) V*
AMJ1367 *sid-1(jam113[deletion]) V; sid-5(jam122[deletion]) X*
AMJ1368 *sid-2(jam134[deletion]) III*
AMJ1372 *W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1380 *sid-2(jam134[deletion]) III; sid-1(jam113[deletion]) V*
AMJ1383 *gtbp-1(ax2053[gtbp-1::gfp]) IV; nrde-3(tm1116) X*
AMJ1389 *sid-1(jam150[nonsense]) W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1399 *sid-1(jam157[nonsense]) V*
AMJ1405 *sid-1(jam163[revertant]) V*
AMJ1406 *sid-1(jam164[revertant]) V*
AMJ1407 *sid-1(jam165[revertant]) V*
AMJ1408 *sid-1(jam166[revertant]) V*
AMJ1409 *sid-1(jam167[revertant]) V*
AMJ1410 *sid-1(jam168[revertant]) V*
AMJ1412 *sid-1(jam170[revertant]) W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1413 *sid-1(jam171[revertant]) W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1438 *sid-1(jam172[sid-1 N-term::mCherryΔpi::sid-1 C-term]) V*
AMJ1442 *sid-1(jam173[nonsense]) W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1443 *sid-1(jam174[nonsense]) W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1444 *sid-1(jam175[nonsense]) W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1445 *sid-1(jam176[nonsense]) W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1446 *sid-1(jam177[nonsense]) W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1447 *W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) rde-1(jam178[nonsense]) V*
AMJ1448 *W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) rde-1(jam179[nonsense]) V*
AMJ1449 *W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V; sid-3(jam180[nonsense]) X*

AMJ1450	*W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V; sid-3(jam181[nonsense]) X*
AMJ1451	*deps-1(jam182[nonsense]) I; W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1452	*deps-1(jam183[nonsense]) I; W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1479	*sid-1(jam189[deletion]) W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1480	*sid-1(jam190[deletion]) W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1481	*sid-1(jam191[deletion]) W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1482	*sid-1(jam192[deletion]) W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1485	*sid-1(jam195[sid-1 N-term::linker::mCherryΔpi::sid-1 C-term]) V*
AMJ1504	*oxSi487[mex-5p::mCherry::h2b::gfp::h2b & Cbr-unc-119(+)] dpy-2(e8) II; unc-119(ed3) III; sid-1(jam80[nonsense]) V*
AMJ1542	*gtbp-1(jam210[gtbp-1::gfp::tetracycline-K4-aptazyme::3'UTR]) IV*
AMJ1574	*deps-1(jam229[nonsense]) I; sid-1(jam170[revertant]) W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1575	*deps-1(jam230[nonsense]) I; sid-1(jam170[revertant]) W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
AMJ1577	*W09B7.2/F07B7.2(jam232[del]) V*
AMJ1612	*W09B7.2/F07B7.2(jam241[del]) V*
AMJ1613	*W09B7.2/F07B7.2(jam242[del]) V*
AMJ1615	*W09B7.2/F07B7.2(jam244 [sdg-1 ORF deleted from jam137]) V*
AMJ1616	*W09B7.2/F07B7.2(jam245 [sdg-1 ORF deleted from jam137]) V*
AMJ1617	*W09B7.2/F07B7.2(jam246 [sdg-1 ORF deleted from jam137]) V*
AMJ1662	*znfx-1(gg544[3xflag::gfp::znfx-1]) II; W09B7.2/F07B7.2(jam137[W09B7.2/F07B7.2::mCherryΔpi]) V*
DH1390	*rme-2(b1008) IV*
EG4322	*ttTi5605 II; unc-119(ed9) III*
EG6787	*oxSi487[mex-5p::mCherry::h2b::gfp::h2b & Cbr-unc-119(+)] II; unc-119(ed3) III*
FX02700	*sid-1(tm2700) V*
FX15992	*sid-1(tm2700) V; tmIs1005[sid-1(+) & vps-45 mini]*
GR1373	*eri-1(mg366) IV*
HC196	*sid-1(qt9) V*
HC731	*eri-1(mg366) IV; sid-1(qt9) V*
JH3197	*gtbp-1(ax2053[gtbp-1::gfp]) IV*
N2	wild type
YY916	*znfx-1(gg544[3xflag::gfp::znfx-1]) II*

Table S2. Oligonucleotides.

Name	Sequence
P1	caccttcgccaattatcacctc
P2	cgtcagcttctgattcgacaac
P3	ataaggagttccacgcccag
P4	ctagtgagtcgtattataagtg
P5	tgaagacgacgagccacttg
P6	ggaacatatggggcattcg
P7	cagacctcacgatatgtggaaa
P8	gcttcacctgtcttatcactgc
P9	cgcggcgactttggttaaatc
P10	ggcttgacaaacgtcagcttc
P11	tcatctcggtacctgtcgttg
P12	agaggcggatacggaagaag
P13	cataaccgtcgcttggcac
P14	aatgggtgagatgggcttaag
P15	gcacttcgatatttcgcgccaa
P16	gaaccaatgtggcacgaaac
P17	gcaaaacttcgattaacattttcatggcctcctccgagaacg
P18	cgttctcggaggaggccatgaaaatgttaatcgaagttttgc
P19	ggtaccctctagtcaaggcctatagaaaagttgaaatatcagtttttaaaaa
P20	cacgaatcattctctgtctgaaacattcaattg
P21	cagacagagaatgattcgtgtttatttgataattttaatg
P22	cggaggaggccatgaaaatgttaatcgaagttttgc
P23	taacattttcatggcctcctccgagaac
P24	aattactctactacaggaacaggtggtgg
P25	gttcctgtagtagagtaattttgttttccctatc
P26	ggctacgtaatacgactcacagtggctgaaaatttatgc
P27	gagcagcagaatacgagctc
P28	gaaaagttcttctcctttactcatgaaaatgttaatcgaagttttgc
P29	gcaaaacttcgattaacattttcatgagtaaaggagaagaacttttc
P30	ctctcagtacaatctgctctg
P31	gaatacgagctcagaactcg
P32	atgccgcatagttaagccag
P33	atcgacgacgacgacgatcagcagtaaagaagcttgcatgcctgcag
P34	atgttgaagagtaattggacgtcatccatccagcagcac
P35	gtccaattactcttcaacatcccta
P36	ctttactgctgatcgtcg
P37	tctctccctaggcacaacgatggatacgctaac
P38	gagagacctaggcacgatgagcatgatttgacg
P39	atttaggtgacactatagctaccataggcaccacgaggttttagagctagaaatagcaag
P40	gcaccgactcggtgcca
P41	cacttgaacttcaatacggcaagatgagaatgactggaaaccgtaccgcatgcggtgcctatggtagcggagct tcacatggcttcagaccaacagccta
P42	atttaggtgacactatagcaaggcgcatggttctcagttttagagctagaaatagcaag

P43	atttaggtgacactatagcaactttcatgcaataaatgttttagagctagaaatagcaag
P44	ttctttcattcttttcataatctcactcaccatgatattgcatgaaagttgataatgtctactagtactg
P45	aaacaccaacaacgcaatcc
P46	tgacctcatcatctcctccag
P47	tccgaatctgaaccacgaatg
P48	atttaggtgacactatagcattcaatcgagactgcagttttagagctagaaatagcaag
P49	agcctataatctatatcagcattcaatcaaggctacacggttacgatcaggttttgatggaaatgagggt
P50	atttaggtgacactatagcattcaatcaaggctacagttttagagctagaaatagcaag
P51	aagcctataatctatatcagcattcaatcgagactgcacggttacgatcaggttttgatggaaatgaggg
P52	tgaaatatgaaaaaccggat
P53	tcattaatacacgcaaaacttcgattaacattttcatggtcagcaagggagaggcagttatcaaggagttcatgcgt ttcaaggtccaacgagcgttccgagggacgtcactccaccggaggaatggacgagctctacaagtagagtaatt ttgttttccctattcgtttcttcatatttcaacttttctcctgcctta
P54	actcggcttcttcggttcc
P55	aacaccagatcactgcgtagag
P56	aaggtccaacgagcgttccg
P57	atggtcagcaagggagagg
P58	cttgtagagctcgtccattcct
P59	attgtgaacctggaaaaatg
P60	tttcactatcagtggcttcacctgtcttatcactgcttcttgtatactgaacgacgttaaacacatctcactttaacattt agaaattaaaactcctcatcggtttttcatatttcaacttttctcctgccttaatacgtagcccatctctcatttcttcatg ttttaagaactttctgaatctatgtaattagttgg
P61	tttttggcacagtttttgct
P62	ggaattagagactagagctt
P63	cgtgtctctcacaacagccgtttctctaacagaaaaaccttctttttgttgatgtttgtctaaaatcgatttttttcagcaag aaatcgagaaactggaacgagctttggtaagtttttgttcctcgaagtgtaaataattgagtaaaagctttcttattga aaaaaaaaacgaatgttcaaattatgaagattgaaaaatg
P64	tttcccgcgtactcctctc
P65	ctaagaccaacatccaagctcg
P66	tcacatttggcgaggagcca
P67	aatcgaatgactccagcgaa
P68	cagacgtttggctatacgcc
P69	caactggtttcgtcagatcggcttccgcaccatttgccggtgtgatccgtttcgaaaatgatagtttattaatggtca gcaagggagaggcagttatcaaggagttcatgcgtttcaagttccgagggacgtcactccaccggaggaatgg acgagctctacaagtgaattctactacaaaattactaaatcagatgtct
P70	ctgctttgatggccgaatactg
P71	aaacaaaaatatacaaatcg
P72	ccttcgctacattggaaagc
P73	catatgaaatttttaaataaagttgttttctaactgttcccaatattcttaaatcccattgaacagaatttcattttcaaaac cctgatattttcaggaattttattccaataatatgattttgaaaaactattaatcttacctgtgcatcaataaagatcttgt gagtatatcatcgatcacagtctccgatttgtctg
P74	ggtcttacccattccaacatcg
P75	ttcgctacattggaaagctgg
P76	cacgcctatgttcccttgtc
P77	ttcatgcgtttcaagttccg

P78	tcgattaacattttctagagtaattttgttttcccaaacaaacaaaggcgcgtcctggattcgtacaaaacataccag atttcgatctggagaggtgaagaatacgaccacctgtacatccagctgatgagtcccaaataggacgaaacgcg ctcaaacaaacaaactatccggtttttcatatttcaacttttctcct
P79	tctcccacttgaatccctctg
P80	ccaaatgttgagccagtcac
P81	ttgaggaaatgcagacgctcgttatcgacctccagatggtctccaagggagagga
P82	tgttattttgagggagccaaatgttgagccagtcagccactacctgatcccttgt
P83	gctgaaggtggatagtgtctc
P84	gagttcggaagtaaaccgtgg
P85	tcaccatctgggaggtgttcacatttggcgaggagccataggtcggctgtcgagccatcgatgtgctcaa
P86	agacgaaagggtgagaactttg
P87	cgcgaggatatgcagttcac
P88	agcattcaatcgagactgca
P89	acaagaaggaaaaaggagaa
P90	aatgcgggacaaaattagaagctttccgttctcccaaacaaacaaaggcgcgtcctggattcgtacaaaacatac cagatttcgatctggagaggtgaagaatacgaccacctgtacatccagctgatgagtcccaaataggacgaaac gcgctcaaacaaacaaatttttccttcttgtaagaattgcacatccattag
P91	cacatggtccttcttgagtttg
P92	acggtgaggaaggaaaggag
P93	agcattcaatcaaggctaca
P94	cgaagtaaaacaattcatgt
P95	gcttcgatctttaaaaagcgaagtaaaataatttatgtcagaacgggatggagaagatccagagccgaag
P96	tggctcatggacgggaaag
P97	ggaacaggcaacgagatgg
P98	cgtggcacatactttccgttgttg
P99	gtcatctccgacgagcac
P100	ttccgttgttggcttcgttg
P101	tgcacggcgtatcaaactg
P102	ggccattgggagaacttcg
P103	tgacggcctcttctacatatcg
P104	ccgcaagtctctcctgtatg
P105	gctgaaggtggatagtgtctc
P106	attgctccgcaaatgtagtgg
P107	gctgctcaagcaaatcgaatg
P108	ttatcacggtggagaacagc
P109	ttggtagggaatcggctgg
P110	tcaaattgttgaagagatca
P111	cagcagaaaatcaaattgttgaagagatcacagctatggtctccaagggagagga
P112	cggtttccctcttctacgctcgtttcttgattttcgccactacctgatcccttgt
P113	caacgggacatggatttgag
P114	ttgaatttcccggtttccctc
P115	tgttgaagagatcacagcta
P116	cagcagaaaatcaaattgttgaagagatcacagctggtggcggtggatcgggaggaggaggttcgggtggcg gaggcagtatggtctccaagggagaggaagataacatggctat
P117	taatacgactcactatagg
P118	cccacactaccatcggcgctac

P119	cactcttactgctaccaacgcttctggaagcgacaaacat
P120	atgtttgtcgcttccagaagcgttggtagcagtaagagtg
P121	tcgttgttccaggagatcagaaaacagcaactgttccaaa
P122	tttggaacagttgctgttttctgatctcctggaacaacga
P123	acccacttcacagtcgattcactcaacaagggagatcatt
P124	aatgatctcccttgttgagtgaatcgactgtgaagtgggt
P125	tagaaaaaatgagtaaaggagaagaacttttcactggagt
P126	actccagtgaaaagttcttctcctttactcatttttcta
P127	agtttgaaggtgataccttgttaatagaatcgagttaaa
P128	tttaactcgattctattaacaagggtatcaccttcaaact
P129	ggattacacatggcatggatgaactatacaaatgcccggg
P130	cccgggcatttgtatagttcatccatgccatgtgtaatcc
P131	acauuccagucaguggugaaccaacuccaacaauuacuuggacuuucgaa
P132	uucgaaaguccaaguaauuguuggaguugguucaccacugacuggaaugu
P133	ugguccuucuugaguuuguaacagcugcugggauuacacauggcauggau
P134	auccaugccauguguaaucccagcagcuguuacaaacucaagaaggacca
P135	5’Atto 565-auccaugccauguguaaucccagcagcuguuacaaacucaagaaggacca
P136	5’Atto 488-ugguccuucuugaguuuguaacagcugcugggauuacacauggcauggau
P137	aggcgacccgtgcggagccagacgtttggctatacgcctgaattcgattcgaaactaccatgaagagtgg
P138	cgtttggctatacgccggg
P139	tccgttgacagaggttacatgc
P140	agcgtcttccagcagaaatg
P141	cttcatggtagtttcgaatcgactt
P142	gctaccataggcaccgcatg
P143	ctggttgagcttctcattct
P144	ccaaatgttgagccagtcac
P145	tccgtttttttcgaaacttttcgtaatatttttgtttcttcaattgatctcttgaatattcatcgtgaatta
P146	gagttcggaagtaaaccgtgg
P147	gctgaaggtggatagtgtctc
P148	cgcagtacgcagagtgaac
P149	gatggtctccaagggagagg
P150	ttacagtaaaacagccggatcccaccgagaatggtctccaagggagaggaagataacatg
P151	tctcccacttgaatccctctg
P152	atcgtcttgatcgacggaacac
P153	ttgaggtggtttatctctggac
P154	cttgtagttcccgtcatctttg
P155	atttcgttctgattccgtgagg