ABSTRACT

Title of Dissertation: PERPETUATION OF NON-GENETIC CHANGES AT A TRANSGENE LOCUS IN *C. ELEGANS*

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Development of a multicellular organism from a single cell or from a few cells in every generation relies on the reproducible expression pattern of genes. At the beginning of every generation in every organism, at least a single progenitor cell is necessary to produce the organism. This cell is subjected to reprogramming mechanisms that erase epigenetic information transmitted from the previous generation and as it develops, the organism goes through experiences that affect gene expression. Despite this, developmental processes give rise to nearly the same organism in the next generation, suggesting that the components in the cells are similarly regulated in every generation. How a complex organism can develop and reproduce its gene expression pattern using only the information present within the progenitor cell is not understood. Here, we describe an engineered genetic locus in the nematode worm *C. elegans* that shows

robust transgenerational expression like many loci in the genome but, unlike other tested loci, can be uniquely susceptible to transgenerational silencing by one of two distinct processes. This locus could be silenced by double-stranded RNA (dsRNA) transported from neurons and could also be silenced when inherited solely from the male parent in a genetic cross. Each process could initiate transgenerational silencing within the germline that lasted for >25 generations. The two processes depended on distinct mechanisms to initiate silencing – while neuronal dsRNA required the conserved dsRNA importer SID-1 and the Argonaute RDE-1, mating-induced silencing required the Piwi-interacting Argonaute PRG-1. Both processes engaged the same germline Argonaute HRDE-1 to maintain heritable silencing suggesting that both processes trigger silencing independently but converge on the same pathway for maintenance. No other locus that was tested showed such indefinite silencing by either mechanism, suggesting that most loci are resistant to changes in gene expression. Thus, the discovery of a locus that is susceptible to transgenerational change provides us with the first instance of how a single change at a gene sequence can be used to explain evolution of gene regulation.

PERPETUATION OF NON-GENETIC CHANGES AT A TRANSGENE LOCUS IN *C. ELEGANS*

by

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

To my parents.

Acknowledgements

I have been fortunate enough to work in a lab that made graduate school a rewarding experience while tackling the challenges that the pursuit of Science brings.

I will always be grateful for the generosity and patience with which Dr. Antony Jose nurtured me over the years to become an independent thinker. Antony saw the potential in me as a naïve rotation student and I thank him for his unsparing support in teaching me how to recognize and communicate a scientific problem with clarity. I will look up to Antony as a true scientific mentor because he encouraged me to develop ideas and explanations to scientific observations independent of his own. Not many are endowed with the perspectives of breadth and depth and Antony demonstrated by example how to critically think through both perspectives at the same time.

I thank my lab-mates and colleagues from my years in graduate school for celebrating my successes, for helping me endure through failures and for cheering me on at every step. Among them, I have found what I think will be friends for life, and therefore will be constantly reminded outside of this space for how grateful I am for their friendship.

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Chapter 1: General Introduction

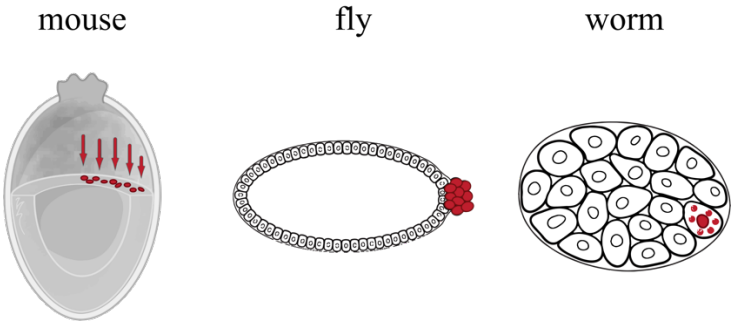
1.1 How is gene expression perpetuated across generations?

Perpetuation of life relies on the reproducible expression pattern of genes across generations. Every species passes through development and reproduction using the bottleneck of at least a single-cell as the progenitor. This indicates that the information required to reproduce the expression of a gene at a given developmental time-point is stored within the single-cell progenitor. This single-cell progenitor is typically the zygote in many organisms. The changes that the zygote of a multicellular organism must go through as a prerequisite for development include repeated rounds of DNA replication, replication of cellular components and cell divisions. In addition to these molecular changes, the primordial germ cells of many organisms undergo epigenetic reprogramming where the extensive erasure of modifications associated with the genome occurs (1). Fascinatingly, despite all these changes, one zygote gives rise to another zygote in the following generation. How the information is transmitted between the two zygotes is a fundamental question that remains to be answered.

The continuity of information between zygotes across generations refers to the continuity of germ plasm – the material within the nuclei of germ cells – from parent to progeny as proposed by August Weismann in *The Continuity of the Germ-Plasm as the Foundation of a Theory of Heredity* (1885) (2). But, Weismann stated that the heritable material from one generation to the next is maintained continuously within

the germplasm and that the material from and changes made in somatic cells are not heritable. This distinction between somatic and germ cells became known as the Weismann barrier and was supported by the observation that many multicellular organisms separate their primordial germ cells early in ontogeny (Fig. 1-1, ref. 3). However, ancestral experiences have been reported to be correlated with changes in the descendants (see below). Thus, either the germline of the parent must have itself experienced the change or alternatively, the somatic cells could have experienced the change, and passed on this information to the germline.

Fig. 1–1. In model organisms, the germline is separated early during development. Whether the **germline** is induced by signals (arrows) from the ectoderm, as in a mouse embryo or whether it is preformed using germline determinants in the oocyte, as in a fly or a worm, the germline forms early in embryogenesis (3).



Studies in many animals raise the possibility of the movement of information from somatic cells of the parent to the progeny. Specifically, environmental stimuli have been correlated with physiological changes across generations. For example, changes in ancestral diet in humans, in mice and in rats were associated with an increased risk of disease (4, 5, 6) and mis-regulation of metabolic genes (6, 7). Exposing mice to an olfactory stimulant under stress conditions resulted in distinct

anatomical, behavioral and gene expression changes in the odor-response pathway of descendants (8). Exposing gestating rats to an endocrine disruptor was correlated with male infertility in descendants (9). These results suggest that the Weismann barrier could be broken but it was unclear how parental changes are transmitted to progeny because many genes are affected at the same time in many tissues in response to a single environmental stimulus.

Organisms across many phyla undergo alterations in the regulatory states of alleles that result in non-Mendelian inheritance of these epigenetic changes. Because of the prevalence of such genes that exhibit susceptibility to non-genetic changes across organisms, a subset of genomic sequences could always be under changeable epigenetic control during development. In most cases, such changes were inherited for more than one successive generation, suggesting that the non-genetic changes may contribute either to the phenotypic diversity or to the evolution of an organism (Table 1-1).

Table 1–1. Non-Mendelian inheritance mechanisms.

Non-mendelian inheritance (ref.)	Description of the epigenetic phenomenon	Is silencing inherited for >1 generation?
Paramutation in plants (10, 11), flies (12, 13), or mice (14)	A process where interaction of homologous alleles results in reversible changes in gene expression at the target allele.	Yes.
RNA induced epigenetic silencing (RNAe) in *C. elegans* (15-20)	Single-copy gene insertions containing *gfp* and *mCherry* expressed in the germline become silenced in a spontaneous manner.	Yes.
Multi-generational RNAe caused by meiotic silencing by unpaired DNA in *C. elegans* (21)	When a transgene is maintained in a meiotically unpaired state for several generations in *C. elegans*, silencing is initiated but is abolished when the transgene is brought back to a homozygous state.	Yes.
RNA-induced epigenetic gene activation (RNAa) (20, 22, 23)	A process where an expressed transgene results in the transactivation of a silenced transgene in the *C. elegans* germline.	Yes.
Meiotic silencing by unpaired DNA (24)	A gene not paired with a homolog during prophase I in meiosis silences expressed homologous genes, independent of their pairing state.	Unknown.
Epigenetic licensing of *fem-1* in *C. elegans* (ref. 25)	Maternal transcript of *fem-1* gene is required to enable zygotic expression of the paternal copy of *fem-1*, which is otherwise silenced.	Yes (could only be tested by repeated backcrossing).
Genomic imprinting and parent of origin effects (26-28)	A gene is silenced within the zygote when it is transmitted through a specific gamete.	Yes.
Transposon silencing in flies (13, 29)	Maternally inherited RNAs silence a paternally inherited gene, usually a transposon, that is absent in the maternal genome.	Yes.
Transvection in flies (30)	Interaction by physical pairing between alleles on homologous chromosomes can result in altered gene expression.	No.
Licensing by DNA sequences in *C. elegans* (31)	Silencing of transgenes in the *C. elegans* germline can be inhibited by stretches of non-coding DNA within the transgene.	Yes.
Transgenerational silencing by neuronal dsRNA (this dissertation)	Transport of dsRNA from neurons to the germline can trigger transgenerational silencing for >30 generations.	Yes.
Mating-induced silencing (this dissertation)	Mating males with a susceptible transgene with wild-type hermaphrodites can trigger transgenerational silencing for >200 generations.	Yes.

Observations made thus far argue that environmental experiences and non-genetic changes accumulated over the course of development of an animal could alter the phenotype and could be communicated as the new phenotype in the zygote of the next generation. Because of the remarkable reproducibility of gene expression in every generation, the components that make up the organism must be regulated in approximately the same way in every generation. Specifically, the arrangement of cellular components such as RNA, DNA, proteins, sugars, and lipids as well as the sequence of DNA, recently defined as the cell code of an animal (32), is therefore nearly the same between parent and progeny but can tolerate small changes. Thus, the reproducibility of the cell code at every successive generation is likely maintained by transgenerational homeostasis mechanisms that are not yet understood (ref. 32, see Fig. 3 therein).

To begin to understand how the cell code is regulated, we could first analyze changes at a single gene across many generations. The nematode worm *C. elegans* is an ideal organism to manipulate a single gene and perform transgenerational studies because its genome has been sequenced and it can transmit epigenetic changes across generations (33).

1.2* C. elegans *is an ideal model system for transgenerational studies

C. elegans is a simple nematode with an organized anatomy that develops stereotypically from the zygote to adulthood. The primordial germ cells are specified

to be distinct from somatic cells early in embryogenesis (34). It is ideal for multigenerational studies without the requirement for mating, even though males can be propagated, because it is a predominantly hermaphroditic worm that produces both oocytes and sperm for self-fertilization and has a short generation time of ~3 days.

1.3 Epigenetic changes can be transmitted across generations in C. elegans

Several observations in *C. elegans* revealed that it has evolved ways to communicate environmental information and changes in physiological traits across generations. Growth of worms under heat stress caused changes in germline gene expression in subsequent generations (35-37). Starvation resulted in increased longevity and resistance to nutritional and temperature stress for more than one generation (38-39). Intriguingly, evidence from these and other transgenerational studies provide support for the activity of chromatin modifying enzymes and small RNAs within the germline in the inheritance of ancestral experiences. For example, removal of germline-specific histone modifiers only in parent animals resulted in increased lifespan in descendants for up to at least two generations (40, 41). Similarly, both temperature-induced stress and starvation-induced longevity was accompanied by transmission of small RNAs across generations (36, 38). It is not yet clear whether these changes are communicated through direct effects on the parental germline or through effects on the parental soma, but it is likely that histone modifications and small RNAs play a key role in inheritance of parental experiences.

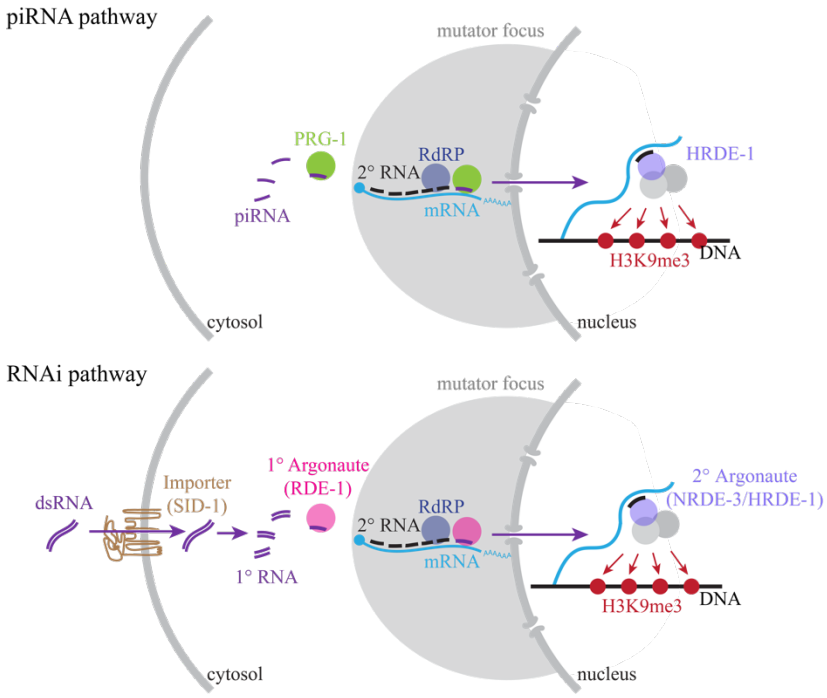
1.4 RNA silencing in C. elegans *can be used to study transgenerational effects*

Extensive studies in *C. elegans* have uncovered that the worm generates thousands of species of non-coding small RNAs, each broadly categorized into a developmental role (42). Of the many classes of non-coding RNAs, the most predominant class that functions in the germline is the Piwi-associated RNAs (piRNAs). This class of small RNAs is conserved across phyla (43-45) and has been described to function in the germline as "sentinels" against foreign sequences that invade the genome, such as transposons and engineered sequences in *C. elegans* (15-17). In the worm, piRNAs are transcribed as individual transcripts that bind to the germline-specific Argonaute PRG-1 and silence invading sequences in the genome across generations using the secondary Argonaute HRDE-1 (Fig. 1-2, *Top*, refs. 15-17, 46). Silencing by piRNAs reflects the robust mechanisms that the germline has in place to prevent instability and maintain continuity of the germline across generations.

C. elegans can also efficiently use double-stranded RNA (dsRNA) introduced exogenously, such as by injection or ingestion, and silence a matching gene in a process called RNA interference (RNAi) (47). Silencing of a gene by exposure of dsRNA to a parent could result in silencing of the same gene in naïve progeny, whether the gene was expressed in the soma (47, 48) or in the germline (15, 18, 49-54). While silencing of a somatic gene was predominantly limited to the progeny of the exposed animal, silencing in the germline has been shown to be multigenerational in several cases. This is either because of engagement of differential silencing machinery or because of differences in the loci expressed in the soma versus the germline. In the germline,

double-stranded RNA can enter the cytosol using the conserved dsRNA-importer, SID-1 (55), gets processed into primary short-interfering RNAs (siRNAs) that bind to a primary Argonaute RDE-1 (56), and one of the siRNA strands can in turn guide RDE-1 to a matching mRNA in perinuclear structures called mutator foci (57). Within these foci, an RNA-dependent RNA polymerase (RdRP) gets recruited to the mRNA resulting in synthesis of templated small RNAs called secondary siRNAs, that bind to the secondary Argonautes NRDE-3 (58) or HRDE-1 (54) within the soma or the germline, respectively. This bound complex can move into the nucleus, bind to the nascent transcript and recruit additional factors resulting in the deposition of the repressive H3K9me3 marks on the gene within the genome (Fig. 1-2, *Bottom*). Therefore, RNAi can be used to manipulate a single gene in a single tissue and to cause heritable changes, making it an excellent tool to understand how changing the epigenetic information at one sequence can affect expression state of that sequence across generations.

Fig. 1–2. Silencing pathways in the *C. elegans* germline.
Silencing of genes in the germline uses different mechanisms depending on the RNA that initiates silencing.

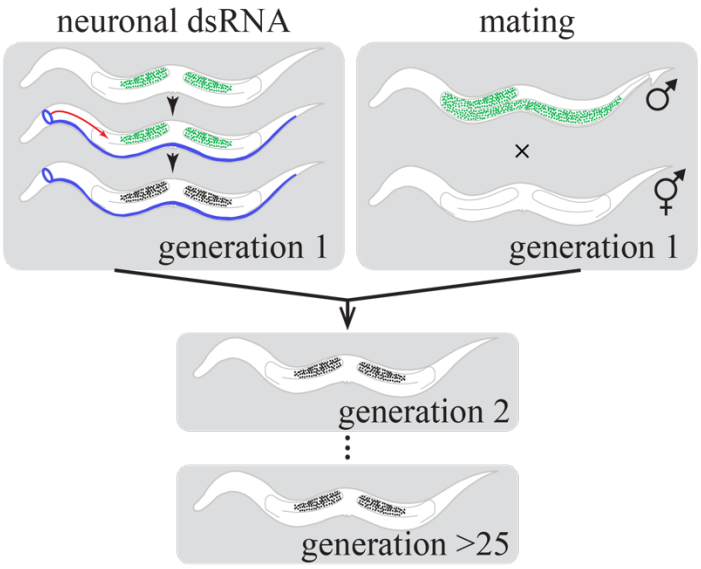


In my dissertation, I report the identification of a single-copy transgene in *C. elegans* as being particularly susceptible to epigenetic changes that can be transmitted across generations, and the analysis of RNA silencing that initiates and maintains these epigenetic changes. My work using this transgene and other loci are relevant for addressing the following questions: (i) Can somatic cells that experience environmental changes transfer information to the germline and across generations in an animal? (ii) How are gene expression states maintained across generations despite changes incurred by parents?

We found that dsRNA made in neurons can be imported into the germline and can cause silencing of a transgene locus for >25 generations (Fig. 1-3, *Left*). Reflecting

the particular susceptibility of the locus, mating males that express the transgene with hermaphrodites that lack the transgene also resulted in silencing that lasted for >150 generations (Fig. 1-3, *Right*). No other loci we tested were susceptible to transgenerational silencing either by neuronal dsRNA or by mating. Genetic analyses suggested that while the mechanisms that initiated silencing in both processes were distinct, the mechanisms that maintained silencing converged on the germline-specific secondary Argonaute HRDE-1. These findings reveal that an indefinite change in the expression state of a gene sequence is likely dictated by components within a cell associated with the sequence. The discovery of this locus provides us with a way to observe both how epigenetic changes first occur at a gene and also how the induced expression state is then maintained in every successive generation.

Fig. 1–3. A locus is particularly susceptible to transgenerational silencing.
A transgene that is expressed in the germline can be **silenced** for >25 generations by dsRNA transported from neurons to the germline (*Left)* or by mating males that express the transgene to hermaphrodites that lack the transgene (*Right*).



Chapter 2: Double-stranded RNA made in neurons can enter the germline and cause transgenerational gene silencing in *C. elegans*

2.1 Preface

All the work presented in this chapter was published with some modifications as: Devanapally S, Ravikumar S, and Jose AM (2015) Double-stranded RNA made in neurons can enter the germline and cause transgenerational gene silencing in *C. elegans*. *Proceedings of the National Academy of Sciences USA*. Feb 17;112(7):2133-8.

Snusha Ravikumar generated the data for Fig. 2-9*E* and Fig. 2-13. The remaining work in this chapter was contributed by me.

Some worm strains were obtained from the *Caenorhabditis elegans* Genetic Stock Center, the Hunter laboratory (Harvard University) and the Seydoux laboratory (Johns Hopkins University). The Hamza laboratory (University of Maryland) provided the bacteria that express *gfp–dsRNA*. Amy Beaven (University of Maryland) trained me on the use of Leica SP5 confocal microscope.

2.2 Introduction

An animal that can transfer gene-regulatory information from somatic cells to germ cells may be able to communicate changes in the soma from one generation to the next. In the worm *C. elegans*, expression of double-stranded RNA (dsRNA) in

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

The germline is separated from the rest of the body, or soma, during early development in most animals, consistent with the suggestion that environmental effects on soma throughout the lifetime of an animal cannot influence inheritance through the germline (59). However, some environmental changes can cause effects that last for three or more generations, even in the apparent absence of changes in the genotype (reviewed in ref. 60). These transgenerational epigenetic effects are presumably initiated either by direct changes within the ancestral germline or by the transfer of information from ancestral somatic cells to the ancestral germline. It is difficult to

distinguish between these possibilities because complex ancestral changes that affect subsequent generations, such as diet (6, 7, 38) or endocrine disruption (9), perturb many genes in many tissues in ways that are as yet unclear. Manipulating the activity of a single gene in specific tissues and across generations can help distinguish between these possibilities. Such specific inactivation of a single gene can be achieved by using dsRNA to trigger RNAi in the worm *Caenorhabditis elegans* (47).

As in most animals, the *C. elegans* germline is set aside early in development—after four cell divisions (34). Gene silencing initiated through RNAi-related mechanisms within the *C. elegans* germline can last for many generations (15-17, 19). Such transgenerational silencing can be triggered by both injected dsRNA (49-51) and ingested dsRNA (48, 50, 53, 54). However, both injection and ingestion can deliver dsRNA directly into the fluid-filled body cavity that surrounds the germline, without entry into the cytosol of any somatic cell (61, 62). Thus, it remains unknown whether somatic cells in *C. elegans* can export signals for delivery into the germline to cause transgenerational gene silencing.

The transfer of gene-specific information from one somatic tissue to another somatic tissue during RNAi has been observed in *C. elegans* (55). Such inter-tissue transfer of gene-regulatory information appears to occur through the transport of forms of dsRNA called mobile RNAs (63). Entry of these mobile RNAs into the cytosol requires the dsRNA-selective importer SID-1 (55, 64, 65). Consequently, when dsRNA is expressed in a variety of somatic tissues such as the gut, muscles, or neurons, SID-1– dependent silencing of genes of matching sequence is observed in other somatic

tissues (61). Because gene silencing by mobile RNAs from neurons appears to be stronger than that by mobile RNAs from other somatic tissues (61), we examined whether neurons export mobile RNAs that can enter the germline to cause transgenerational gene silencing.

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

2.3 Materials and methods

2.3.1 Strains

All strains used are listed in Table 2-1. Strains were generated and maintained by using standard methods (66).

Table 2–1. Strains used.

Strain	**Genotype***
N2	wild type
AMJ2	*eri-1(mg366) nrIs20 (Psur-5::sur-5::gfp) IV; sid-1(qt9) V; qtIs49 (Prgef-1::gfp–dsRNA and pRF4) III*
AMJ154	*eri-1(mg366) nrIs20 IV; qtIs49 III, AMJ265 rrf-1(ok589) I; oxSi487 [Pmex-5::gfp and unc-119(+)] II; unc-119(ed3)? III*

AMJ300 *nrIs20 IV; qtIs49 III, AMJ301 qtIs49 III, AMJ310 eri-1(mg366) nrIs20 IV; mIs10 (Pmyo-2::gfp) V*

AMJ320 *nrIs20 IV; sid-1(qt9) V; qtIs49 III, AMJ324 oxSi487 II; unc-119(ed3)? III; sid-1(qt9) V* (generated by Julia Marré, A.M.J. laboratory, University of Maryland, College Park, MD)

AMJ326 *oxSi487 II; unc-119(ed3)? III; rde-1(ne219) V* (generated by Julia Marré)

AMJ349 *oxSi221 [Peft-3::gfp and unc-119(+)] II; unc-119(ed3)? qtIs49 III*

AMJ361 *oxSi221 II; unc-119(ed3)? qtIs49 III; eri-1(mg366) IV*

AMJ363 *oxSi221 II; unc-119(ed3)? qtIs49 III; eri-1(mg366) IV; sid-1(qt9) V*

AMJ377 *oxSi487 II; unc-119(ed3)? III; eri-1(mg366) IV*

AMJ382 *oxSi221 II; unc-119(ed3)? III; eri-1(mg366) IV*

AMJ463 *oxSi487 II; unc-119(ed3)? III; sid-1(qt9) V; jamEx131 (pHC337 and pHC448)*

AMJ466 *oxSi487 II; unc-119(ed3) III; jamEx132 (pHC337 and pHC448)*

AMJ502 *oxSi487 II; unc-119(ed3) III; jamEx145 (pHC448)*

AMJ471 *jamEx140 (pHC337 and pHC448)*

AMJ533 *rde-1(ne219) V; jamEx140, AMJ542 sid-1(qt9) V; jamEx140*

AMJ577 *hrde-1(tm1200) III [4× outcrossed]*

AMJ581 *oxSi487 dpy-2(e8) II; unc-119(ed3)? III* (generated by Samual Allgood, A.M.J. laboratory, University of Maryland, College Park, MD)

AMJ585 *mut-7(ne4255) III [1× outcrossed]*

AMJ586 *oxSi487 dpy-2(e8) II; unc-119(ed3)? III; rde-1(ne219) V*

AMJ592 *hrde-1(tm1200) III; jamEx140, AMJ593 oxSi487 dpy-2(e8) II; unc-119(ed3)? III; sid-1(qt9) V, AMJ595 oxSi221 II; unc-119(ed3)? qtIs49 III; sid-1(qt9) V*

AMJ598 *oxSi487 dpy-2(e8) II; unc-119(ed3)? III; sid-1(qt9) V; jamEx140, AMJ599 oxSi487 dpy-2(e8) II; unc-119(ed3)? III; rde-1(ne219) V; jamEx140*

AMJ600 *oxSi487 dpy-2(e8) II; unc-119(ed3)? III; jamEx140*

AMJ601 *oxSi487 dpy(e8) II; unc-119(ed3)? mut-7(ne4255) III*

AMJ602 *oxSi487 dpy-2(e8) II; unc-119(ed3)? hrde-1(tm1200) III*

AMJ603 *oxSi487 dpy-2(e8) II; unc-119(ed3)? III; qtEx136 (Prgef-1::unc-22 dsRNA)* (ref. 66)

AMJ620 *oxSi487 dpy-2(e8) II; unc-119(ed3)? hrde-1(tm1200) III; jamEx140 isolate 1*

AMJ621 *oxSi487 dpy-2(e8) II; unc-119(ed3)? hrde-1 (tm1200) III; jamEx140 isolate 2*

AMJ628 *oxSi487 dpy-2(e8) II; unc-119(ed3)? III; jamEx147 (pHC448)*

AMJ639 *mut-7(ne4255) III; jamEx140*

AMJ643 *oxSi487 dpy-2(e8) II; unc-119(ed3)? mut-7(ne4255) III; jamEx140*

AMJ645 *oxSi487 dpy-2(e8) II; unc-119(ed3)? III; eri-1(−) IV; qtEx136*

EG6070 *oxSi221 II; unc-119(ed3) III*

EG6787 *oxSi487 II; unc-119(ed3) III*

GR1373 *eri-1(mg366) IV*

HC195 *nrIs20 IV*

HC196 *sid-1(qt9) V*

HC566 *nrIs20 IV; sid-1(qt9) V*

HC567 *eri-1(mg366) nrIs20 IV*

HC568 *eri-1(mg366) nrIs20 IV; sid-1(qt9) V*

HC780 *rrf-1(ok589) I [2×outcrossed]*

*The term dsRNA is used to refer to any form of base-paired RNA including hairpin RNA and double-stranded RNA for simplicity.

2.3.2 Transgenic animals

Recombinant DNA fragments generated through overlap extension PCR using Expand Long Template polymerase (Roche) were purified by using the QIAquick PCR Purification Kit (Qiagen). Plasmids were purified by using the Plasmid mini kit (Qiagen). PCR products or plasmids were combined with a co-injection marker to transform *C. elegans* by using microinjection (67). The plasmid pHC448 was used as a co-injection marker to express DsRed2 in the pharynx (63); pRF4 was used as a co-injection marker to express *rol-6(su1006)* (67); and pHC337 was used to express an inverted repeat of *gfp* in neurons (61), which is expected to generate a hairpin RNA (designated as *gfp–dsRNA*).

To express *gfp–dsRNA* in the neurons (*Prgef-1::gfp–dsRNA*): A 1:1 mixture of pHC337 (40 ng/μL) and pHC448 (40 ng/μL) in 10 mM Tris·HCl (pH 8.5) was microinjected into the wild-type strain N2 or into strains that express a single copy of *Pmex-5::gfp* in the germline as part of an operon (68) in wild-type [EG6787], *sid-1(−)* [AMJ324], *rde-1(−)* [AMJ326], *rrf-1(−)* [AMJ265], or *eri-1(−)* [AMJ377] backgrounds to generate three independent transgenic lines for each genetic background. In addition, pHC448 (40 ng/μL) in 10 mM Tris·HCl (pH 8.5) was injected into N2, EG6787, or AMJ377 to generate "no dsRNA" control transgenic lines.

2.3.3 Balancing *sid-1*

A transgene integrated on chromosome V [*mIs10 (Pmyo-2::gfp)*] was used to balance *sid-1(qt9)*. In Figs. 2-9*E* and Fig. 2-13, progeny of heterozygous *sid-1(qt9)/mIs10* animals were scored as homozygous mutants if they lacked GFP expression from *mIs10*. Tests using *rde-1* (~4.9 Mb from *sid-1*) suggest a low rate of recombination between *sid-1* and *mIs10*. Specifically, among the progeny of *rde-1(−)/mIs10* heterozygotes that lacked GFP expression from *mIs10*, ~94% (63/67) were found to be homozygous *rde-1(−)* by Sanger sequencing (determined by Edward Traver, A.M.J. laboratory, University of Maryland, College Park, MD).

2.3.4 Genotyping *Prgef-1::gfp–dsRNA*

The integrated transgene *qtIs49* was identified based on the co-segregation of the dominant Rol defect due to the pRF4 co-injection marker that is present along with

Prgef-1::gfp–dsRNA (Figs. 2-1, 2-5*B*, 2-9, 2-2, and 2-10–2-13). The DNA for *Prgef-1::gfp–dsRNA* in transgenes was detected by PCR using the primers GACTCAAGGAGGGAGAAGAG and GAGAGACCACATGGTCCTTC. A fragment of the *rrf-1* gene was amplified as a control by using the primers TGCCATCGCAGATAGTCC, TGGAAGCAGCTAGGAACAG, and CCGTGACAACAGACATTCAATC (Fig. 2*B*).

2.3.5 Feeding RNAi

Worms that were 24 h past the L4 stage were singled onto RNAi plates [NG agar plates supplemented with 1 mMIPTG (Omega) and 25 μg/mL carbenicillin (MP Biochemicals)] with 5 μL of *Escherichia coli* OP50. Twenty-four hours later, once eggs had been laid (typically, all OP50 was consumed by then), the parent worm was picked off the plate, and progeny were fed bacteria with a plasmid expressing *gfp–dsRNA* or with a control plasmid (L4440). For inherited silencing in somatic cells, 3 d later, gravid adults were treated with bleach (0.6% NaOCl and 1.5 M NaOH), and the silencing in progeny, which were protected by their egg shells, was measured when they reached the L4 stage by counting the number of GFP-positive gut nuclei (Fig.2–9*C*) (adapted from ref. 48). For silencing in the germline, 2 d later, the germlines of L4-staged animals were imaged (Fig. 2-8).

2.3.6 Quantification of silencing by imaging

The silencing of GFP expressed from single-copy transgenes *oxSi221* (*Peft-3::gfp*) or *oxSi487* (*Pmex-5::gfp*) in different genetic backgrounds was compared by

imaging L4-staged animals under non-saturating conditions for the brightest strain being compared using a Nikon AZ100 microscope and a Photometrics Cool SNAP HQ2 camera. When the extent of silencing was measured as a single proportion, 95% confidence intervals and P values for comparison of two proportions were calculated as described (61). For Fig. 2-1*B*, a Leica SP5X confocal microscope was used to measure GFP expression. All images being compared were adjusted identically by using Adobe Photoshop for display.

For Fig. 2–9*A*, GFP silencing in gut nuclei was measured by imaging L4-staged animals using a Nikon AZ100 microscope under non-saturating conditions and counting the number of GFP-positive gut nuclei that were above a fixed threshold of brightness. For all other figures, GFP silencing in gut nuclei was measured by counting the number of bright GFP-positive nuclei at a fixed magnification on an Olympus MVX10 fluorescent microscope. Comparison of this counting with measurements of fluorescence intensity (using Nikon AZ100 microscope and NIS Elements software) revealed that false calling of a GFP-positive nucleus as per the conservative criterion described in Fig. 2-10 occurred at most for one nucleus per animal. To measure fluorescence intensity in Fig. 2-10, an L4-staged worm was mounted on a slide after paralyzing the worm by using 3 mM levamisole (Sigma-Aldrich; catalog no. 196142). Fluorescence intensity in each nucleus of the worm was calculated by using the formula $A_n(I_n - I_b)$, where A_n = area of the nucleus; I_n = mean intensity within the nucleus; and I_b = mean intensity in an area of the slide outside the worm.

2.3.7 Semi-quantitative RT-PCR

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

2.3.8 Genetic crosses

Males with an extrachromosomal array were generated for each cross in Fig. 2-7 by mating hermaphrodites that express the extrachromosomal array in wild-type or mutant backgrounds with wild-type males or corresponding mutant males, respectively. For example, to generate *Ex[gfp–dsRNA]; sid-1(−)* males, *Ex[gfp–dsRNA]; sid-1(−)* hermaphrodites were mated with *sid-1(−)* males. For all crosses with *Pmex-5::gfp* animals in Fig. 2-7, *dpy-2(e8)* was used as a linked marker to identify the homozygosity of *Pmex-5::gfp*. Only 3% (6/200) of the Dpy progeny of *Pmex-5::gfp/+ dpy-2(e8)/+* double-heterozygous parents were not homozygous for the *Pmex-5::gfp* transgene (determined by Sam Allgood).

2.4 Results

2.4.1 Neuronal mobile RNAs can enter most somatic tissues and the germline

Genetic analyses suggest that neuronal mobile RNAs are forms of dsRNA (63). Mobile RNAs generated from dsRNA expressed in neurons against the muscle gene *unc-22* can enter muscle cells through the dsRNA importer SID-1 and cause *unc-22* silencing (63). To examine silencing of a gene expressed in multiple tissues by a single source of dsRNA in neurons, we used animals that expressed cytosolic *gfp* (*Peft-3::gfp*) in all somatic tissues and *gfp–dsRNA* in all neurons (*Prgef-1::gfp–dsRNA*) (Fig. 2-1). GFP expression was detectably reduced in most somatic tissues (with the notable exception of the pharynx) in the presence of *Prgef-1::gfp–dsRNA* (Fig. 2-1*A*, *top* vs, *middle*) and this silencing was enhanced in the absence of the exonuclease ERI-1 (Fig.

2-2), consistent with ERI-1 acting to inhibit silencing by imported neuronal mobile RNAs (63). Silencing in all somatic tissues, even in the *eri-1(−)* background, was lost upon removal of the mobile RNA importer SID-1 (Fig. 2-1*A*, *bottom*, and Fig. 2-2), suggesting that all observed silencing was due to mobile RNAs made in neurons.

Fig. 2–1. Neuronal mobile RNAs can cause gene silencing in most somatic tissues and in the germline.
(A) Representative fourth larval (L4)-staged animals that express GFP (**black**) in somatic tissues (*Peft-3::gfp*) in a wild-type (*Top*) background and animals that in addition express dsRNA in neurons against *gfp* (*Prgef-1::gfp–dsRNA*) in wild-type (*Middle*) or *sid-1(−)* (*Bottom*) backgrounds are shown. Silenced tissues and unsilenced pharynx are indicated (*Middle*). Detectable silencing was observed in 100% of wild-type animals (n = 135) and 0% of *sid-1(−)* animals (n = 115). (Scale bars, 50 μm.) Also see Fig. 2–2. (B) Representative L4-staged animals that express GFP (black) in the germline (*Pmex-5::gfp*; outlined) in a wild-type (*Top*) background and animals that in addition express *Prgef-1::gfp–dsRNA* in wild-type (*Middle*) or *sid-1(−)* (*Bottom*) backgrounds are shown. Because of the long exposure time required to acquire these images, variable and irregular autofluorescence due to gut granules was also detected. Detectable silencing was observed in 87% of wild-type animals (n = 54) and 27% of *sid-1(−)* animals (n = 59). (Scale bars, 50 μm)

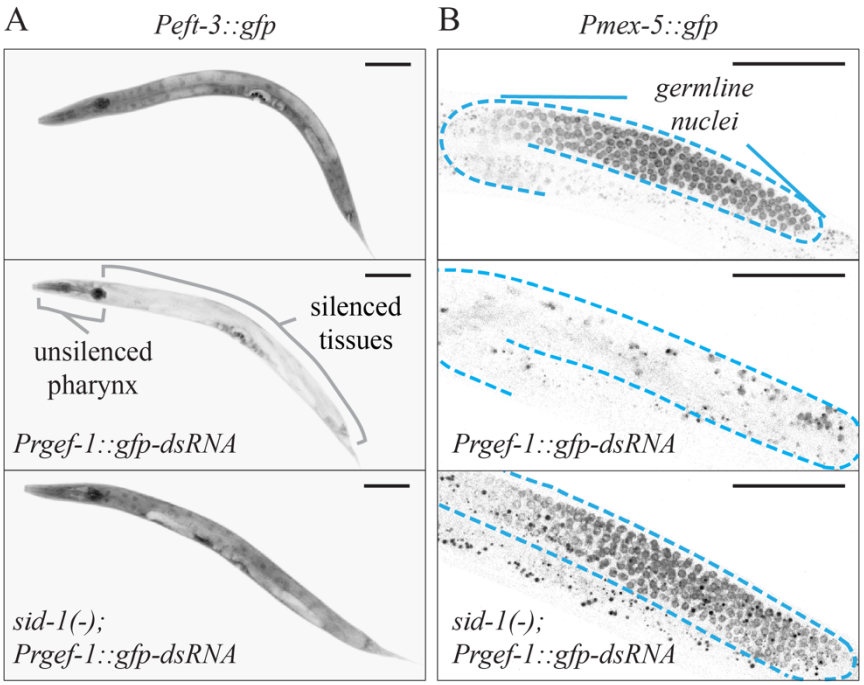
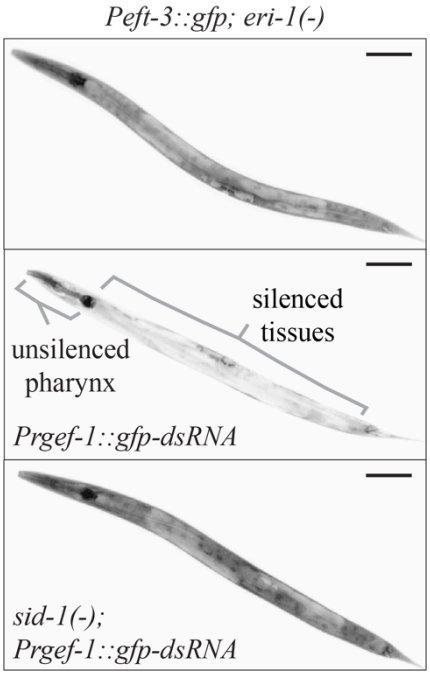


Fig. 2–2. Silencing by neuronal mobile RNAs is dependent on SID-1 even in an enhanced RNAi background.
Representative L4-staged animals that express GFP (**black**) in all tissues (*Peft-3::gfp*) in an *eri-1(−)* (*Top*) background and animals that in addition express dsRNA in neurons against *gfp* (*Prgef-1::gfp–dsRNA*) in *eri-1(−)* (*Middle*) or *eri-1(−); sid-1(−)* (*Bottom*) backgrounds are shown. Detectable silencing was observed in 100% of *eri-1(−)* animals (n = 90) and 0% of *eri-1(−); sid-1(−)* animals (n = 88). Silenced tissues and unsilenced pharynx are indicated (*Middle*). (Scale bars, 50 μm).



To test whether the germline is susceptible to silencing by mobile RNAs, we examined silencing of GFP expression in animals that express *gfp* in the germline (*Pmex-5::gfp*) and neuronal mobile RNAs from a *Prgef-1::gfp–dsRNA* transgene. Like most somatic cells, the germline was susceptible to silencing by neuronal mobile RNAs and the silencing was predominantly dependent on SID-1 (Fig. 2-1*B*). The silencing was sequence-specific and did not occur in animals with transgenic expression of a co-injection marker (Fig. 2-3*A*) or in animals with transgenic expression of *unc-22* dsRNA

in neurons (Fig. 2-3*B*). Furthermore, silencing, as detected by the loss of GFP fluorescence within the germline (Fig. 2-4*A*), was correlated with a reduction in *gfp* mRNA levels (Fig. 2-4*B*). Consistent with mobile RNAs that are imported into the germline being forms of *gfp–dsRNA*, silencing was strongly dependent on the dsRNA importer SID-1 and the primary Argonaute RDE-1 that acts on short dsRNA (56) but independent of the RNA-dependent RNA polymerase RRF-1 that generates single-stranded secondary small RNAs in somatic cells (69) (Fig. 2-4*C*). The residual silencing observed in *sid-1(−)* and *rde-1(−)* animals may reflect additional *sid-1–* and *rde-1*–independent gene silencing mechanisms that can act in the germline (15, 16, 18, 19). Because silencing of a germline target due to dsRNA expression in neurons is greatly reduced in the absence of SID-1 (Fig. 2-1*B*, bottom, and Fig. 2-4*C*), we conclude that SID-1-dependent neuronal mobile RNAs can enter the germline.

Together, our results suggest that neuronal mobile RNAs can enter most somatic tissues as well as the germline to silence genes of matching sequence. Because injection of *in vitro*-synthesized dsRNA can generate signals that are inherited in *C. elegans* (47, 49-51), our observations raise the possibility that neuronal mobile RNAs may also generate such inherited signals upon silencing a gene within the germline or upon silencing a gene in other somatic cells.

Fig. 2–3. Silencing in the germline by neuronal mobile RNAs is sequence-specific. (A) A repetitive transgene that lacks homology to *gfp* sequence does not cause silencing of GFP expression in the germline even in an *eri-1(−)* background. The proportions of animals that showed silencing of GFP expression in the germline were determined for *Pmex-5::gfp* or *Pmex-5::gfp; eri-1(−)* animals that express the co-injection marker alone. (B) Neuronal mobile RNAs against the somatic gene *unc-22* do not cause silencing of GFP expression in the germline. The proportions of animals that showed silencing of GFP expression in the germline were determined for *Pmex-5::gfp* or *Pmex-5::gfp; eri-1(−)* animals that express neuronal *unc-22–dsRNA*. Error bars indicate 95% CI and n > 20 L4-staged animals.

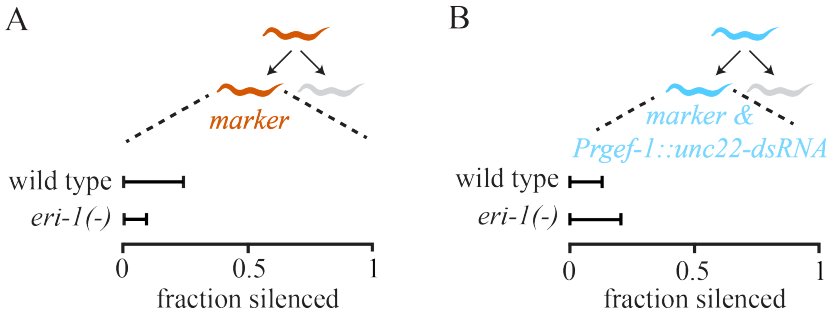
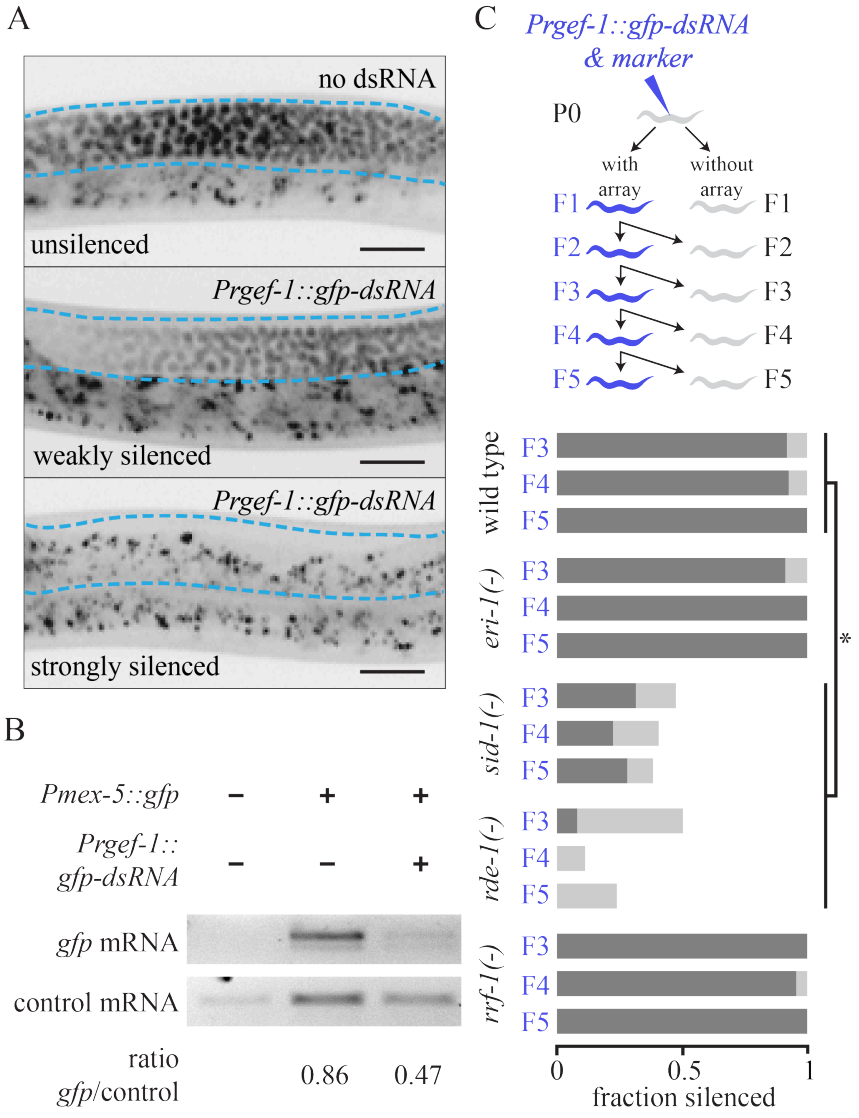


Fig. 2–4. Potent silencing by transgenes that express neuronal dsRNA requires neuronal mobile RNAs even when the transgenes are generated in animals that express the germline target gene.
(A) Extent of germline silencing due to neuronal mobile RNAs can vary. Representative L4-staged animals that express GFP (black) from the *Pmex-5::gfp* transgene in the germline (*Top*) and animals that in addition express dsRNA in neurons against *gfp* (*Prgef-1:: gfp–dsRNA*) but show weak (*Middle*) or strong (*Bottom*) silencing are shown. Because of the long exposure time required for these images, variable and irregular autofluorescence of the gut granules was also detected. (Scale bars, 10 μm.) (B) Loss of GFP fluorescence in the germline is due to reduction in levels of *gfp* mRNA. Semiquantitative RT-PCR was used to detect *gfp* mRNA and *tbb-2* mRNA (control) in wild-type animals, *Pmex-5::gfp* animals, and *Pmex-5::gfp* animals that in addition express *Prgef-1::gfp–dsRNA*. The intensity of the *gfp* band was

normalized to that of the *tbb-2* band in each sample. (C) Silencing of GFP expression in the germline by dsRNA expressed in neurons requires SID-1 and RDE-1, but not ERI-1 or RRF-1. Wild-type, *eri-1(−)*, *sid-1(−)*, *rde-1(−)*, or *rrf-1(−)* animals that express *Pmex-5::gfp* (P0 generation) were injected with constructs to express *Prgef-1::gfp–dsRNA* along with a co-injection marker (*Pmyo-2::DsRed*). For each genetic background, the proportions of worms with fluorescence from the co-injection marker (blue worm) that showed either strong (dark gray bars; as shown in *A*, *Bottom*) or weak (light gray bars; as shown in *A*, *Middle*) silencing of GFP expression in the germline were determined in the F3 (n = 11–24 L4-staged animals), F4 (n = 18–39 L4-staged animals), and F5 (n = 17–29 L4-staged animals) generations. *$P < 0.05$ (Student's *t* test).



2.4.2 Silencing in the germline by neuronal mobile RNAs is inherited for many generations

Injected or ingested dsRNA can cause transgenerational gene silencing of germline genes in *C. elegans* (18, 49-51, 53, 54). However, both forms of dsRNA delivery could result in the direct entry of dsRNA into the germline without entry into the cytoplasm of somatic cells. Ingested dsRNA is transcytosed across the gut into the body cavity that surrounds the germline (61, 62), and it is difficult to avoid spillage of injected dsRNA into the body cavity. These experimental considerations suggest that to test the possibility of somatic tissues initiating transgenerational gene silencing, it is necessary to express silencing triggers within somatic tissues and examine gene silencing within the germline. Although induction using heat-shock of a transgene that encodes a viral genome in somatic tissues caused transgenerational silencing in *C. elegans* (52), such heat-shock induction also led to expression within the germline (figure S5 in ref. 70). Therefore, because of the inherent difficulty in ensuring lack of expression within the germline from transgenes, only germline silencing that is reduced in the absence of the dsRNA importer SID-1 (Fig. 2-1*B*, bottom and Fig. 2-4*C*) can be interpreted as being caused by mobile RNAs.

To determine whether neuronal mobile RNAs that are imported into the germline can cause transgenerational silencing, we examined animals that lack the DNA for *gfp–dsRNA* but whose ancestors expressed neuronal dsRNAs. Because stable transgenic lines of extrachromosomal arrays are generated in *C. elegans* two generations after an animal [parental generation (P0)] is transformed with DNA (i.e. in

the F2 generation) (67), we examined the silencing of GFP expression in wild-type animals of the F3 generation that lacked the *gfp–dsRNA* transgene and in their descendants (Fig. 2-5*A*, *Left*). Animals that lack the *gfp–dsRNA* transgene can be identified by the loss of a red fluorescent co-injection marker, the DNA for which is expected to be incorporated along with the DNA for *gfp–dsRNA* into a single extrachromosomal array upon co-transformation. All F3 animals without the extrachromosomal array showed silencing of GFP expression in the germline (Fig. 2-5*A*, *Right*). Inherited silencing due to the ancestral production of neuronal mobile RNAs persisted for >25 subsequent generations despite unbiased passaging of worms from one generation to the next (Fig. 2-5*A Right* and Fig. 2-6). Consistent with the loss of the *gfp–dsRNA* transgene in animals that lack fluorescence from the co-injection marker, we failed to detect the *gfp–dsRNA* transgene in the DNA of worms that lacked the co-injection marker after 35 cycles of PCR amplification (Fig. 2-5*B*). These results suggest that neuronal mobile RNAs imported into the germline can initiate gene silencing that lasts for many generations in the absence of the ancestral source of neuronal dsRNA.

Fig. 2–5. Neuronal mobile RNAs can cause transgenerational silencing of a germline gene.
(A) Inherited silencing in the germline lasts for >25 generations after the source of neuronal mobile RNAs is lost. (A, *Left*) *Pmex-5:: gfp* animals (P0) were injected with constructs to express neuronal mobile RNAs (*Prgef-1::gfp–dsRNA*) along with a co-injection marker (*Pmyo-2:: DsRed*) to generate F2 transgenic lines. (A, *Right*) The proportions of animals that all lack fluorescence from the co-injection marker (gray worm) but that show either strong (dark gray bars) or weak (light gray bars) silencing in the F3 generation and in successive generations (F4–F30) were determined. Error bars indicate 95% CI and n > 14 L4-staged animals for each generation. Also see Fig. 2-6. Dark gray bars and light gray bars are as in Fig. 2-4*C*. (B) Animals that lack the co-injection marker also lack the *gfp–dsRNA* transgene. Genomic DNA from wild-type animals (no dsRNA), from wild-type animals that express *Prgef-1::gfp–dsRNA*, from *Pmex-5::gfp* animals, and from *Pmex-5::gfp* animals that either have or whose ancestors had extrachromosomal transgenes [i.e., *Pmex-5::gfp* animals that in addition express the co-injection marker alone or along with *Prgef-1::gfp–dsRNA* or apparently lack these extrachromosomal transgenes (gray worm) but that were derived from ancestors that expressed these transgenes] were analyzed. Although the control gene was detected in all cases, a PCR product for the *gfp–dsRNA* transgene was detected only in wild-type animals with *gfp–dsRNA* and in *Pmex5::gfp* animals with *gfp–dsRNA* as evidenced by fluorescence from the co-injection marker.

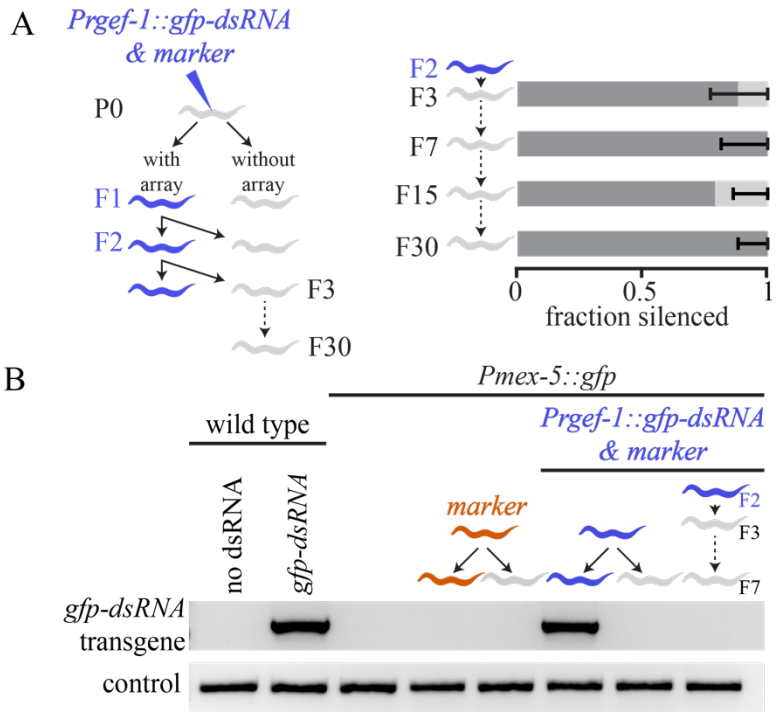
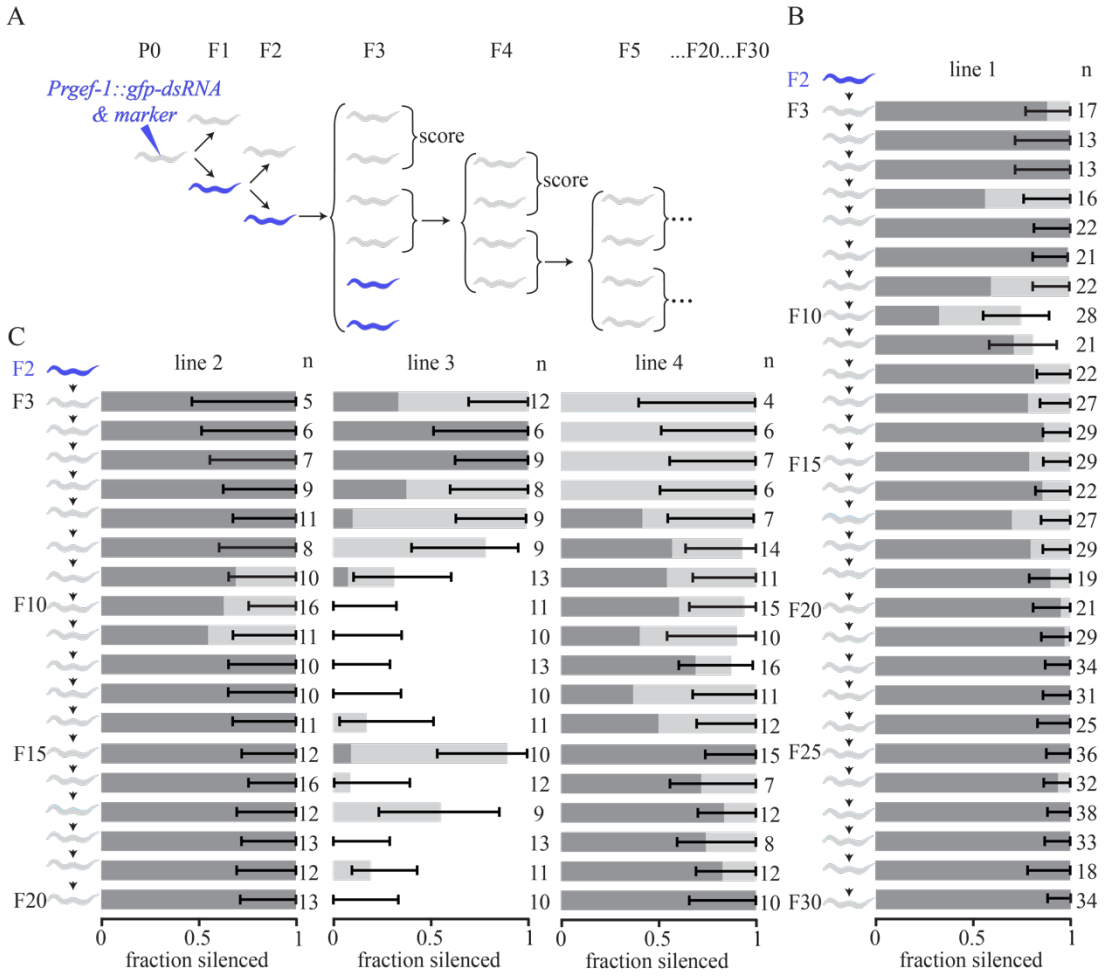


Fig. 2–6. Inherited silencing in the germline can persist for many generations after the source of neuronal mobile RNAs is lost.
(A) Schematic of the assay for transgenerational silencing by neuronal mobile RNAs. *Pmex-5::gfp* animals (P0) were injected with constructs to express neuronal mobile RNA (*Prgef-1::gfp– dsRNA*) along with a co-injection marker (*Pmyo-2::DsRed*) to generate F2 transgenic lines. F3 progeny and their descendants that lost the extrachromosomal array but were derived from F2 transgenic parents were scored for silencing by imaging the germline to detect the silencing of GFP. At each generation, the siblings of the scored animals were propagated to obtain the next generation. (B and C) The persistence of transgenerational silencing varies from one transgenic line to another. The proportions of animals that lack fluorescence from the co-injection marker (gray worm) but that show either strong (dark gray bar) or weak (light gray bar) silencing in the F3 generation and in successive generations (F4–F30 in *B* and F4–F20 in *C*) were determined for four independent transgenic lines (lines 1–4). Error bars indicate 95% CI, and n indicates number of L4-staged animals scored at each generation. Dark gray bars and light gray bars are as in Fig. 2–4*C*.

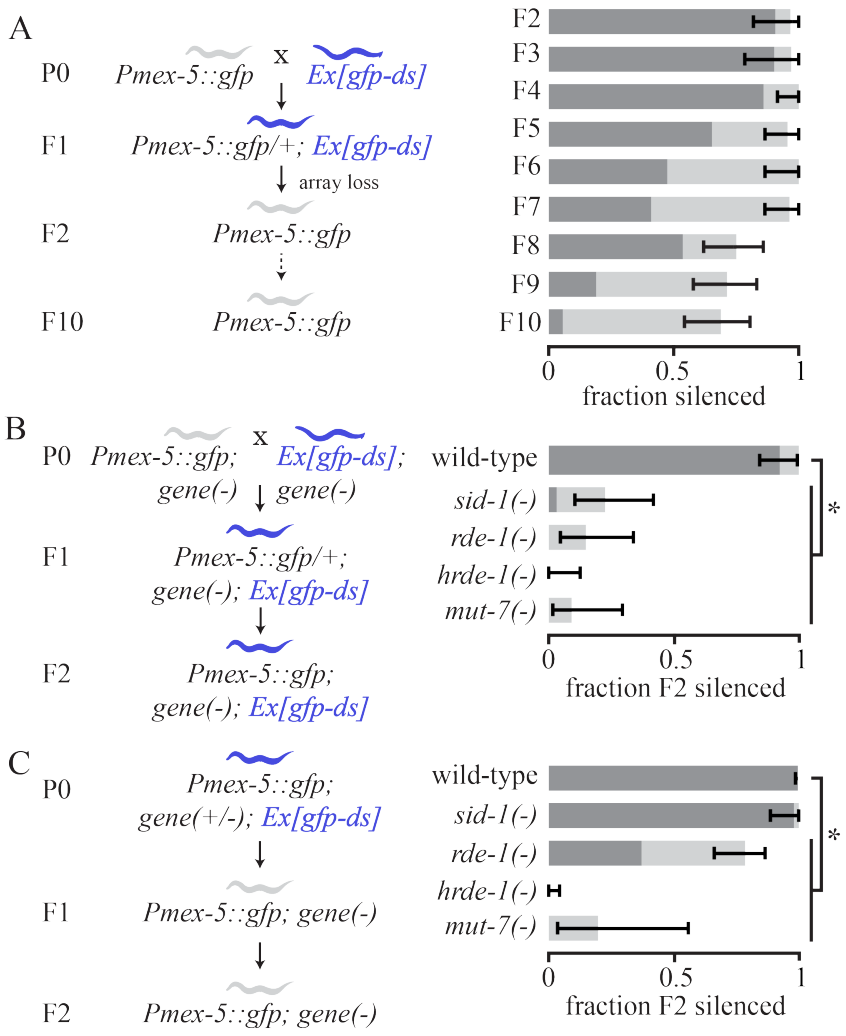


2.4.3 Transgenerational silencing by neuronal mobile RNAs has distinct genetic requirements for initiation and maintenance

Although transgenerational silencing is reliably observed by using multiple transgenic sources of neuronal mobile RNAs (Fig. 2-6), the number of generations that show silencing varied from one transgenic line to another, possibly due to differences in the levels of expression of dsRNA in different transgenic lines. To facilitate comparison of transgenerational silencing across multiple genetic backgrounds and to expose animals to mobile RNAs in defined generations, we chose a single extrachromosomal transgenic line that expresses neuronal mobile RNAs against *gfp* in wild-type animals and crossed it into animals that express *gfp* in the germline. This experimental scheme was then used to determine the genetic requirements for the initiation and maintenance of transgenerational gene silencing.

We found that exposure of a germline target gene to neuronal mobile RNAs for a single generation was sufficient to cause transgenerational silencing (Fig. 2-7*A*). Specifically, when animals with *Pmex-5::gfp* and animals with *Prgef-1::gfp–dsRNA* were mated, the F1 cross progeny that inherited the *Prgef-1::gfp–dsRNA* transgene could initiate transgenerational silencing. This silencing persisted for many generations despite the loss of the source of neuronal mobile RNAs in the F2 generation (Fig. 2-7*A*).

Fig. 2–7. Neuronal mobile RNAs have distinct requirements for the initiation and maintenance of transgenerational silencing.
(A) Expression of neuronal mobile RNAs for one generation is sufficient to initiate multigenerational silencing. *Pmex-5::gfp* animals were crossed with animals that express neuronal dsRNA from an extrachromosomal array (*Ex[gfp–ds]*) and the proportions of animals that lack the extrachromosomal array (gray worm) but that show either strong (dark gray bars) or weak (light gray bars) silencing in the F2 generation and in successive generations (F3–F10) were determined. The loss of *Ex[gfp–ds]* was determined by the loss of the fluorescent co-injection marker. (B) Initiation of silencing by neuronal mobile RNAs requires *sid-1*, *rde-1*, *hrde-1*, and *mut-7*. Wild-type, *sid-1(−)*, *rde-1(−)*, *hrde-1(−)*, or *mut-7(−)* animals that all express *Pmex-5::gfp* were mated with animals of identical genetic backgrounds that all express neuronal dsRNA (*Ex[gfp–ds]*), and the silencing in descendants that had both *Pmex-5::gfp* and *Ex[gfp–ds]* was measured as in *A*. (C) Maintenance of germline gene silencing by neuronal mobile RNAs requires HRDE-1 and MUT-7, but not SID-1 or RDE-1. Wild-type, *sid-1(+/−)*, *rde-1(+/−)*, *hrde-1(+/−)*, or *mut-7(+/−)* animals that all had both *Pmex-5::gfp* and *Ex[gfp–ds]* were allowed to have progeny, and the silencing in wild-type, *sid-1(−)*, *rde-1(−)*, *hrde-1(−)*, or *mut-7(−)* grand progeny animals that all had *Pmex-5::gfp* but that all lacked *Ex[gfp–ds]* was measured as in *A*. The analyzed grand progeny were progeny of animals that also lacked *Ex[gfp–ds]*. Error bars indicate 95% CI. $*P < 0.05$. n > 19 L4-staged animals, except for *mut-7(−)* animals in *C*, where n = 10 L4-staged animals. Dark gray bars and light gray bars are as in Fig. 2–4*C*.



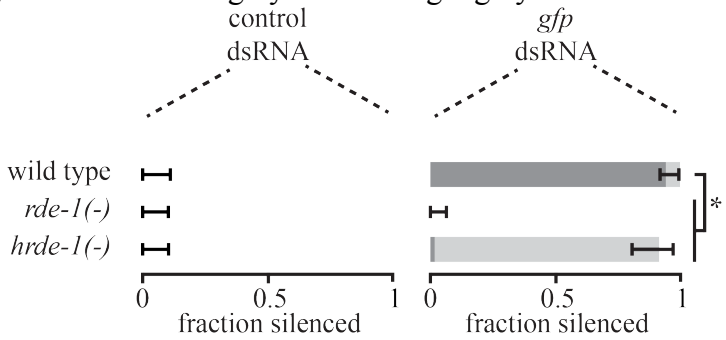
To test whether a gene is required for germline silencing by neuronal mobile RNAs, we used the same experimental scheme as above but with animals that also had a mutation in the gene being tested (Fig. 2-7*B*). For example, to test the requirement for *sid-1*, we mated *sid-1* null mutants [*sid-1(−)*] that express *Pmex-5::gfp* with *sid-1(−)* animals that express *Prgef-1::gfp–dsRNA* and examined silencing in *sid-1(−)* animals of a later generation that express both *Pmex-5::gfp* and *Prgef-1::gfp–dsRNA*

(Fig. 2-7*B*). Germline silencing using this experimental scheme also required SID-1 and RDE-1, in agreement with the results obtained for silencing by transgenic lines that were independently generated in mutant backgrounds (Fig. 2-4*C*). Thus, germline silencing due to neuronal mobile RNAs likely relies on the import of forms of dsRNA through SID-1 and subsequent processing by the primary Argonaute RDE-1 within the germline. Further processing within the germline leads to the production of secondary single-stranded small RNAs. These secondary small RNAs eventually cause gene silencing through mechanisms that require many proteins (reviewed in ref. 42), including the secondary nuclear Argonaute HRDE-1 (54) and the RNase D homolog MUT-7 (71). We found that both HRDE-1 and MUT-7 were required for silencing by neuronal mobile RNAs, suggesting that silencing within the germline is executed by secondary small RNAs and downstream genes. For all genes tested above, the source of neuronal mobile RNAs was present in the animals that were tested. Therefore, the lack of silencing in *sid-1(−)*, *rde-1(−)*, *hrde-1(−)*, and *mut-7(−)* animals reflects a requirement for the corresponding genes in the initiation of germline silencing by neuronal mobile RNAs.

The observed genetic requirements for silencing by neuronal mobile RNAs are distinct from those observed for silencing by ingested or injected dsRNA. Whereas the requirement for SID-1 and RDE-1 is in agreement with the requirement for these genes when silencing is triggered using ingested and injected dsRNA, the requirement for HRDE-1 and MUT-7 is in contrast to the HRDE-1-independent silencing observed in response to ingested dsRNA (ref. 54 and Fig. 2-8) and the MUT-7-independent

silencing observed in response to injected dsRNA (49). These differences might reflect differences in the dosage of dsRNA delivered into the germline using the different methods or the differential engagement of silencing machinery by the different sources of dsRNA used to trigger gene silencing.

Fig. 2–8. Ingested dsRNAs can silence a gene within the germline independent of HRDE-1.
Wild-type, *rde-1(−)*, or *hrde-1(−)* animals that all express *Pmex-5::gfp* were exposed for one generation to bacteria that have either the control L4440 plasmid (control dsRNA) or a plasmid that encodes dsRNA against *gfp* (*gfp dsRNA*) and silencing of GFP expression in the germline was measured. Error bars indicate 95% CI. *****$P < 0.05$. n > 35 L4-staged animals. Dark gray bars and light gray bars are as in Fig. 2–4*C*.



To test whether a gene is required for the maintenance of transgenerational silencing by neuronal mobile RNAs, we examined silencing in animals that had mutations in the gene but were descendants of ancestors that had a wild-type copy of the gene during exposure to neuronal mobile RNAs (Fig. 2-7*C*). For example, to test the requirement for *sid-1* in the maintenance of transgenerational silencing, we examined silencing in *sid-1* null mutants [*sid-1(−)*] that were grand progeny of *sid-1(+/−)* heterozygous animals that were exposed to *Prgef-1::gfp–dsRNA*. Grand progeny were examined for silencing instead of progeny because maternal deposition of mRNA or protein from

heterozygous parents can complicate interpretation of results in *sid-1(−)* progeny. We observed silencing in the *sid-1(−)* grand progeny of *sid-1(+/−)* heterozygous animals that were exposed to *Prgef-1::gfp−dsRNA*, which suggests that SID-1 is not required for the maintenance of transgenerational silencing. Similar experiments with null mutants of *rde-1*, *hrde-1*, and *mut-7* revealed that RDE-1, like SID-1, is dispensable for the maintenance of transgenerational silencing but HRDE-1 and MUT-7 are required for the maintenance of transgenerational silencing.

In summary, our results suggest a model where mobile RNAs exported from neurons enter the germline through SID-1 to cause RDE-1–, MUT-7–, and HRDE-1–dependent silencing in the parent, which is subsequently maintained through a MUT-7− and HRDE-1−dependent but SID-1− and RDE-1−independent mechanism. Because HRDE-1 has been shown to use secondary small RNAs to guide trimethylation of the histone H3 on lysine 9 (H3K9me3) at genes of matching sequence (53, 54), our results suggest that the initiation and maintenance of transgenerational silencing by neuronal mobile RNAs is associated with the deposition of H3K9me3 marks on genes of matching sequence. Although the response to ingested or injected dsRNA strongly suggests that secondary small RNAs are inherited (48, 49, 53), it is possible that in response to neuronal mobile RNAs chromatin marks are inherited across generations. Furthermore, although silencing of somatic genes has been reported to be inherited for a few generations when the silencing is triggered by using ingested dsRNA (48, 53), it is unclear whether silencing of a somatic gene by neuronal mobile RNAs is inherited

and if transgenerational silencing by neuronal mobile RNAs within the germline can spread to somatic cells.

2.4.4 Silencing in somatic cells by neuronal mobile RNAs is not detectably inherited

To measure silencing by neuronal mobile RNAs in somatic cells, we used animals that have two different integrated transgenes—one that expresses nuclear-localized GFP (*sur-5::gfp*) under the control of a promoter that drives expression in all somatic cells (*Psur-5*) and one that expresses *gfp–dsRNA* under the control of a promoter that drives expression in all neurons (*Prgef-1*). Silencing due to neuronal mobile RNAs made from the *Prgef-1::gfp–dsRNA* transgene results in a reduction in fluorescence of nuclear-localized GFP made from the *Psur-5::sur-5::gfp* transgene (Fig. 2-9*A*). This silencing can be most easily observed in the large intestinal cell nuclei and counting the number of GFP-positive gut nuclei provides a reliable measure of silencing that correlates with reduction in *gfp* mRNA levels (Fig. 2-10*A* and *B*). Wild-type animals with neuronal mobile RNAs had, on average, fewer GFP-positive gut nuclei than did animals without neuronal mobile RNAs (Fig. 2-9*B*; 24.2 vs. 29.9 GFP-positive gut nuclei; $P < 0.05$). Consistent with silencing by neuronal mobile RNAs, this silencing was abolished in *sid-1(−)* animals (Fig. 2-9*B*) and not observed in wild-type animals that were merely cocultured with animals that express neuronal mobile RNAs (Fig. 2-10*C*).

Fig. 2–9. Silencing of a somatic gene by neuronal mobile RNAs in parents is not detectably inherited by progeny.
(A) Neuronal mobile RNAs can silence GFP expression in gut cells. Representative L4-staged animals that express GFP (black) in all somatic cells (*Psur-5::sur-5::gfp*) (*Upper*) or that in addition express dsRNA in all neurons (*Prgef-1::gfp–dsRNA*) (*Lower*) are shown. Brackets indicate strongly silenced gut nuclei. (Scale bars, 50 μm.) Also see Fig. 2–10. (B) Double-stranded RNAs expressed in neurons against *gfp* require SID-1 to silence GFP expression in gut cells. The numbers of GFP-expressing gut nuclei were counted in wild-type animals that do not express dsRNA against *gfp* (no dsRNA; gray) and in wild-type, or *sid-1(−)* animals that express *Prgef-1::gfp–dsRNA*. Gray line indicates average number of gut nuclei in L4-staged animals, n > 19 L4-staged animals, and red bar in box plots indicates median. **P* < 0.05 (Student's *t* test). (C–E) An enhanced RNAi background [*eri-1(−)*] was used to maximize the ability to detect inherited silencing. (C) Unlike silencing by ingested dsRNA, silencing by neuronal mobile RNAs is not detectably inherited by progeny. Numbers of GFP-positive gut nuclei in genetically identical progeny of animals that were not exposed to *gfp–dsRNA* (none) or that were exposed to ingested *gfp–dsRNA* or that had one copy of an integrated transgene that expresses *Prgef-1::gfp–dsRNA* (*gfp–ds/+*) were counted. Errors indicate SEM. (D) Unbiased passaging of worms for multiple generations can lead to small differences in gene silencing. Worms that express *Prgef-1::gfp–dsRNA* (P0) were passaged for five generations (F1–F5) by picking a random

worm at each generation, and the numbers of GFP-positive gut nuclei in animals of each generation were determined (see Fig. 2–12 for additional data). Gray line, n, red bar, and asterisks are as in *B*, except for F5, which had n = 8 L4-staged animals. (E) SID-1 is required for silencing by neuronal mobile RNAs even after 17 generations of ancestral silencing by neuronal mobile RNAs. (E, *Left*) Schematic of experimental design to test the requirement for SID-1 in each generation for silencing by neuronal mobile RNAs. At each generation, the numbers of GFP-positive gut nuclei in *sid-1(−/−)* animals were counted, and heterozygous [*sid-1(+/−)]* siblings of any *sid-1(−/−)* animal (F1–F11) or heterozygous siblings of the most silenced *sid-1(−/−)* animal (F12–F18) were passaged. (E, *Right*) The extent of silencing in F1, F11, and F18 are shown (see Fig. 2–13 for additional data). Gray line, red bar, and n are as in *B*.

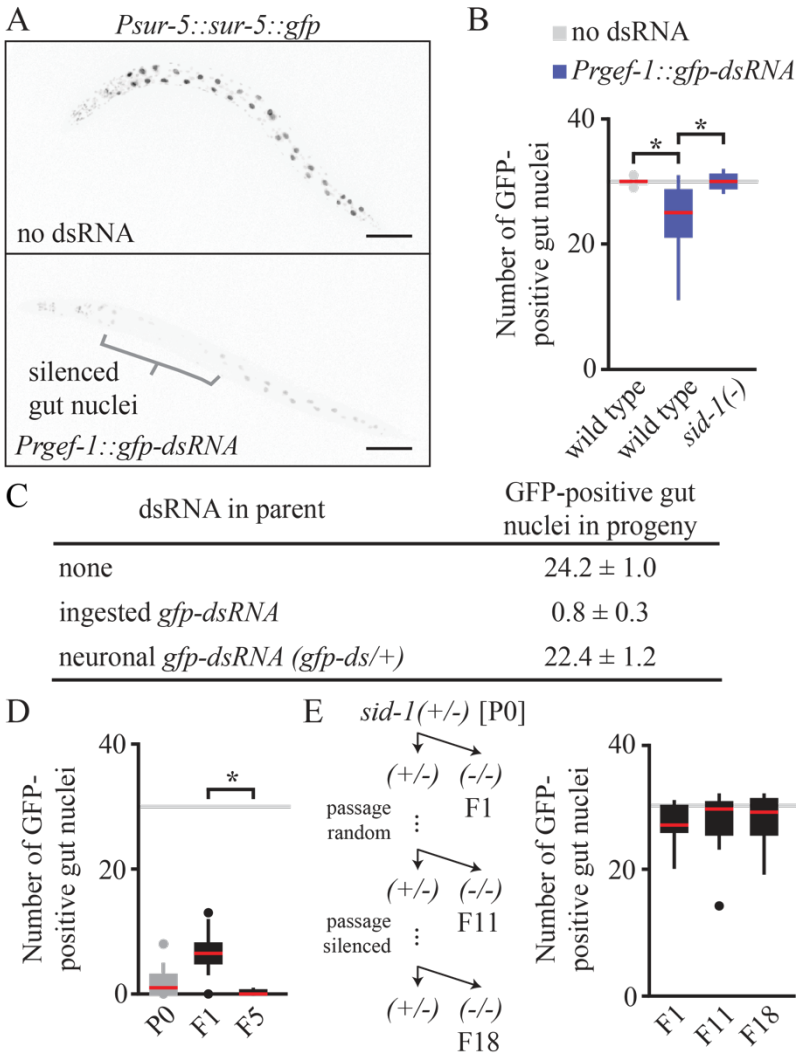
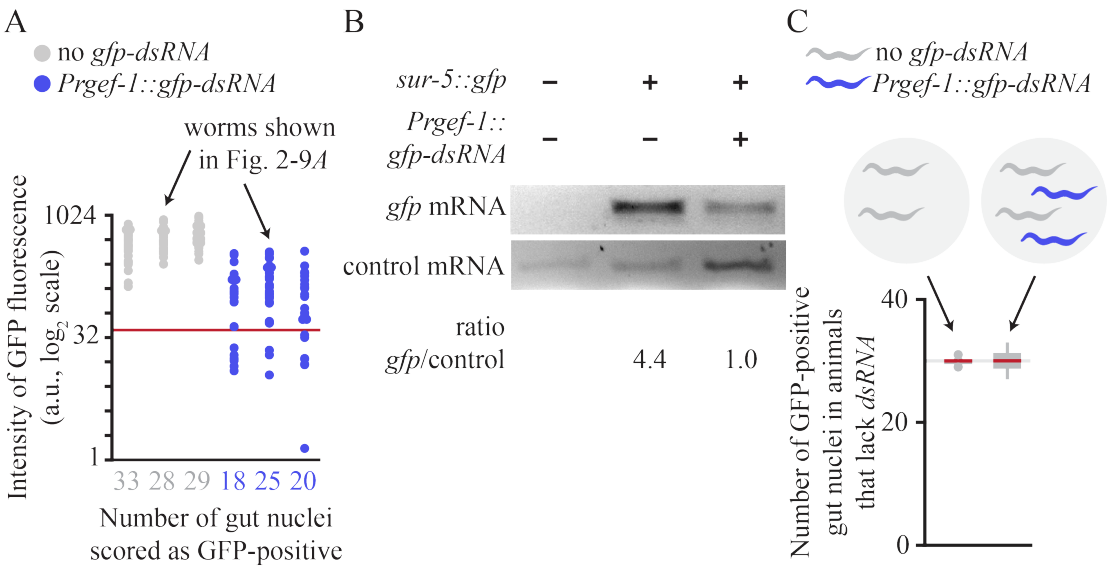


Fig. 2–10. Silencing by neuronal mobile RNAs against *gfp* reduces GFP fluorescence as well as *gfp* mRNA levels and is due to transport of mobile RNAs from neurons to other cells in animals that express dsRNA.
(A) Nuclei counted as showing GFP silencing have several fold lower intensity of GFP fluorescence than even the dimmest nucleus in animals that do not show silencing. Intensity of GFP fluorescence in each gut nucleus of *sur-5::gfp* animals (no *gfp–dsRNA*, gray) or *sur-5::gfp* animals that express neuronal dsRNA (*Prgef-1::gfp–dsRNA*; blue) was measured and compared with the number of nuclei counted as not silenced for each worm (indicated along the x axis). Red line indicates threshold of expression below which a nucleus was scored as silenced in Figs. 2–9. (B) Silencing of somatic GFP by neuronal mobile RNAs is due to reduction in mRNA levels. Semiquantitative RT-PCR was used to detect *gfp* mRNA and *tbb-2* mRNA (control) in wild-type animals, *sur-5::gfp* animals, and *sur-5::gfp* animals that in addition have *Prgef-1::gfp–dsRNA*. The intensity of the *gfp* band was normalized to that of the *tbb-2* band in each sample. (C) Animals that express neuronal mobile RNAs do not cause silencing in animals that lack neuronal mobile RNAs when grown together. The numbers of GFP-positive gut nuclei in animals that express *sur-5::gfp* were determined after growing the strain alone or after growing the strain for 4 d along with animals that contain both *Prgef-1::gfp-dsRNA* (marked with a dominant Rol defect) and *sur-5::gfp*. Gray line and red bar are as in Fig. 2–9*B*, and n > 25 L4-staged animals.



Because the initiation of inherited silencing occurs more frequently in animals that lack the exonuclease ERI-1 (50), we examined the ability of neuronal mobile RNAs to trigger inherited silencing in an *eri-1(−)* background, where trace amounts of

dsRNA (72) and additional mobile RNAs (61) made from the multicopy *Psur-5::sur-5::gfp* transgene could also contribute to silencing. Using this sensitive genetic background, we did not detect inherited silencing by neuronal mobile RNAs (Fig. 2-9*C*) but detected inherited silencing by ingested dsRNAs as reported earlier (Fig. 2-9*C* and ref. 48). We noticed a correlation between an increase in silencing by neuronal mobile RNAs and an increase in parental or ancestral exposure to mobile RNAs (Fig. 2-11). However, the increases in silencing were small and comparable to the small variations in silencing observed in successive generations when worms with *Prgef-1::gfp–dsRNA* of identical genotype were simply passaged (Fig. 2-9*D*). Furthermore, selection of the most silenced or most desilenced animal for four generations introduced marginal differences in silencing between the first and fifth generations (Fig. 2-12). Nevertheless, if marginal increases in inherited silencing accrued over many generations due to the presence of parental neuronal mobile RNAs, such inherited silencing might become independent of neuronal mobile RNAs and thus independent of SID-1 in later generations. However, we did not detect such SID-1–independent silencing even after exposure to 17 generations of silencing by neuronal mobile RNAs (Fig. 2-9*E* and Fig. 2-13). The requirement for *sid-1* in every generation for silencing by neuronal mobile RNAs suggests that transport of neuronal mobile RNAs must occur in every generation to observe silencing in somatic cells.

The absence of robust inherited silencing by neuronal mobile RNAs of genes expressed in somatic cells could be either because somatic silencing does not generate signals for transmission to the next generation or because such signals require a

template of matching sequence in the germline for stability. To test this latter possibility, we examined inherited somatic silencing by neuronal mobile RNAs in animals that express the target gene (*gfp*) in somatic cells as well as in the germline either from a single transgene (germline expression due to *Pmex-5::gfp* and pharyngeal expression due to an additional promoter in the *Pmex-5::gfp* transgene) (Fig. 2-14*A*) or from two separate transgenes (germline expression due to *Pmex-5::gfp* and gut expression due to *sur-5::gfp*) (Fig. 2-14*B*). In both cases, no inherited silencing was detected in somatic cells. These results suggested to us that neuronal dsRNA requires a matching template expressed in the germline for silencing to be inherited. We therefore tested whether another target gene that is expressed in the germline would show inherited silencing. When progeny of animals that express neuronal dsRNA were assayed for silencing of *Pgtbp-1::gftbp-1::gfp* (an endogenous gene tag), the target was silenced in the generation that expressed neuronal dsRNA, but interestingly, not in the progeny animals that mitotically lost the dsRNA array (Fig. 2-14*C*). Thus, not all germline loci can undergo an indefinite change in gene expression upon silencing by neuronal dsRNA, and that the transgene locus *Pmex-5::gfp* is potentially a rare susceptible locus.

Together, our results suggest that neuronal mobile RNAs generate transgenerational silencing signals that have a strong effect on gene expression in the germline and a minimal effect, if any, on gene expression in somatic tissues. Finally, because not all germline target genes are susceptible to silencing by neuronal dsRNA,

the Weismann barrier could be perhaps at the level of the gene and not in the separation between the soma and the germline.

Fig. 2–11. Changes in parental mobile RNA silencing are correlated with small changes in mobile RNA silencing in progeny.
Extent of RNA silencing in parents was varied, and inheritance of silencing was measured by comparing progeny of identical genotype in an *eri-1(−)* background. (*Left*) Dosage of dsRNA transgene in neurons against *gfp* dictates the level of silencing observed. Numbers of GFP-positive gut nuclei were counted in animals that either lack (gray) or that have one (cyan) or two copies (blue) of *Prgef-1::gfp–dsRNA* (*gfp–ds*) transgene. (*Right*) Increased mobile RNA silencing in parents is correlated with a small increase in mobile RNA silencing in progeny. Numbers of GFP-expressing gut nuclei were counted in animals that all expressed *Prgef-1::gfp–dsRNA* (*gfp–ds*) but that were progeny of parents that expressed one copy of *Prgef-1::gfp–dsRNA* (cyan), two copies of *Prgef-1::gfp–dsRNA* for one generation (blue), or two copies of *Prgef-1::gfp–dsRNA* for many generations (black). Gray line, red bar, and asterisks are as in Fig. 2–9*B*. ^*P* = 0.054. n > 15 L4-staged animals. These results are consistent with a small increase in silencing by mobile RNAs due to parental or ancestral silencing signals.

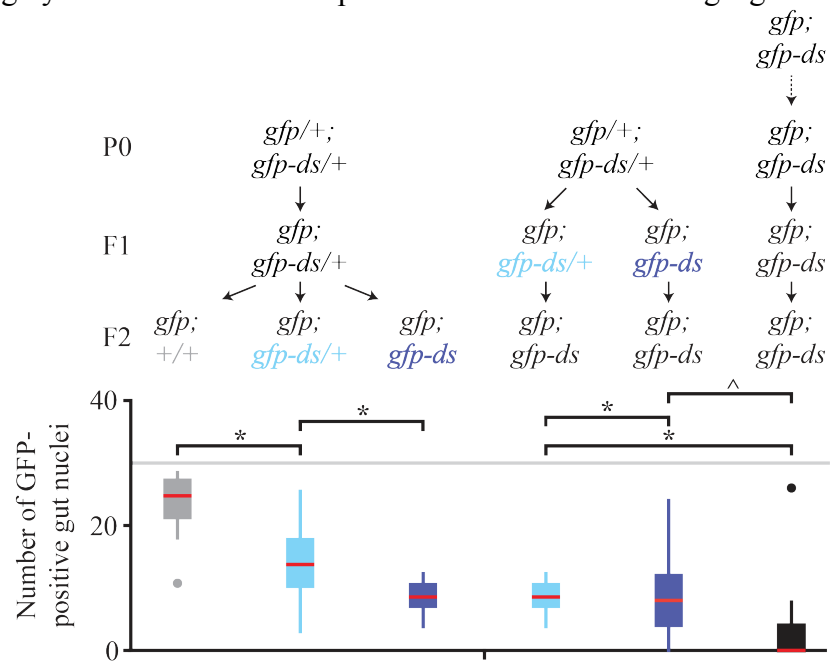


Fig. 2–12. Neuronal mobile RNAs can show small variations in silencing a somatic gene across generations.
Animals that express neuronal mobile RNAs (*Prgef-1:: gfp–dsRNA*) in *Psur-5::sur-5::gfp* (*Left*) or in *eri-1(−) Psur-5::sur-5::gfp* (*Right*) backgrounds were propagated for five generations (F1–F5) in triplicate by selecting at each generation the most silenced animal, the most desilenced animal, or **a random animal** from a starting population of animals (P0) and the numbers of GFP-expressing gut nuclei in L4-staged animals of each generation were counted. Gray line, red bar, and asterisks are as in Fig. 2–9*B*. The number of animals assayed in each generation varied from 1 to 42 and are indicated above each box plot.

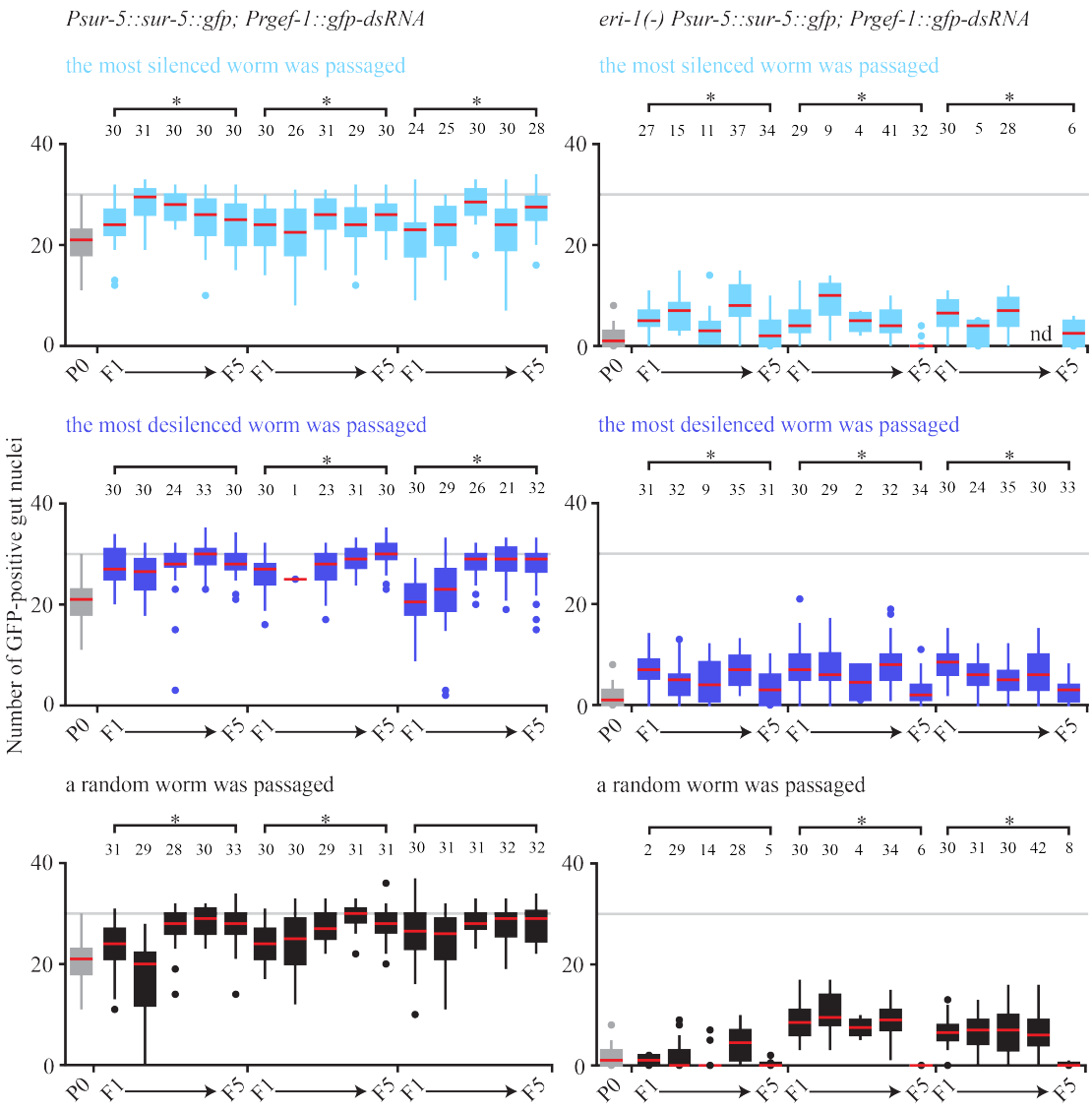


Fig. 2–13. SID-1 is required for silencing by neuronal mobile RNAs even after 17 generations of ancestral silencing.
The numbers of GFP-positive gut nuclei were counted in animals that express neuronal mobile RNAs (*gfp–ds*) and nuclear-localized GFP in all somatic tissues (*gfp*) in an eri-1(−) background (*gfp; gfp–ds*) or in an *eri-1(−); sid-1(−)* background [*gfp; gfp–ds; sid-1(−)*]. By using the schematic described in Fig. 2–9*E*, *sid-1(−/−)* animals were generated after different numbers of generations of *sid-1(+/−)* animals that all had *gfp* and *gfp–ds*. The numbers of GFP-positive gut nuclei were counted in L4-staged *sid-1(−/−)* animals of each generation when *sid-1(+/−)* heterozygous siblings were passaged in triplicate without any selection for 11 generations (A) or when *sid-1(+/−)* siblings of the most silenced *sid-1(−/−)* animal were passaged in triplicate for seven more generations (B). Gray line, n, and red bars are as in Fig. 2–9*B*.

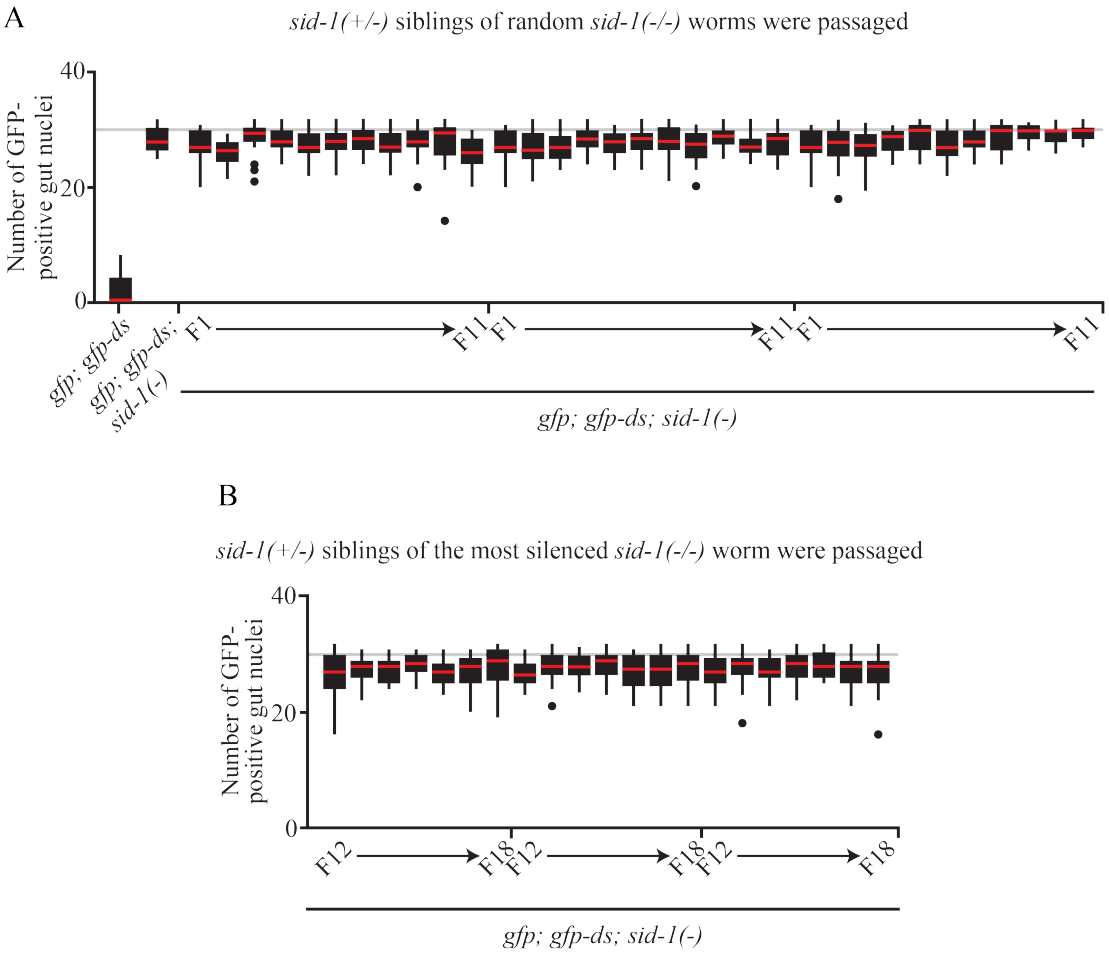
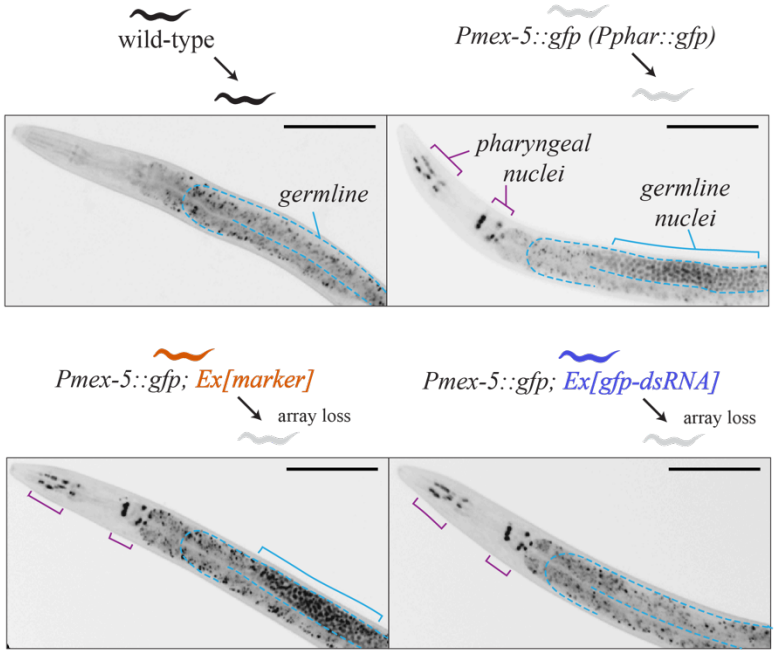


Fig. 2–14. Inherited silencing of a germline gene by neuronal mobile RNAs in the parent does not spread to the soma of the progeny.
(A) Silencing of GFP expression within the germline by neuronal mobile RNAs does not cause detectable inherited silencing of GFP expressed from the same locus in the pharynx of progeny. *Pmex-5::gfp* animals and *Pmex-5::gfp* animals that in addition express an extrachromosomal source of either the co-injection marker (*Ex[marker]*) or neuronal mobile RNAs (*Ex[gfp–dsRNA]*) were passaged and L4-staged progeny that lack the extrachromosomal arrays were imaged under identical conditions. The pharyngeal expression of GFP (black) is from an additional uncharacterized promoter (*Pphar::gfp*) within the *Pmex-5::gfp* transgene and is absent in wild-type worms. Germline (outlined), GFP expression in the germline nuclei (cyan brackets), and GFP expression in pharyngeal nuclei (purple brackets) are indicated. (Scale bars, 50 μm.) (B) Silencing of GFP expression within the germline by neuronal mobile RNAs in the parent does not cause detectable inherited silencing of GFP expressed from a different locus in gut cells of progeny. *Pmex-5::gfp* animals (P0 hermaphrodite) that in addition expressed *Ex[marker]* or *Ex[gfp–dsRNA]* were crossed with *Psur-5::sur-5::gfp* animals (P0 male), and the numbers of GFP-positive gut nuclei were counted in the resulting F1 progeny that lack extrachromosomal arrays. Errors indicate SEM. (C) Not all germline genes can be susceptible to silencing across generations. Silencing in animals that express *Pmex-5::gfp* (*Left*) or *Pgtbp-1::gftbp-1::gfp* (*Right*) as well as neuronal dsRNA (*Ex[gfp–ds]*), in their progeny that lack *Ex[gfp–ds]* was measured as in 3–7*A*. n > 18 L4-staged animals.

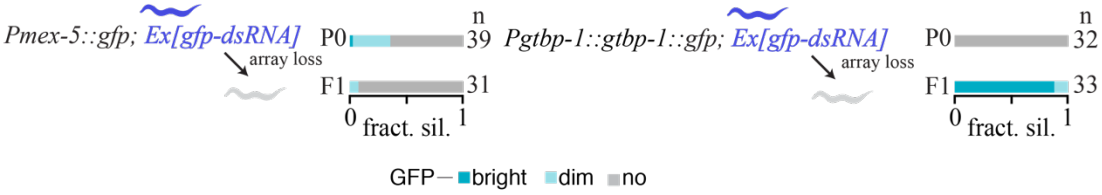
A



B

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

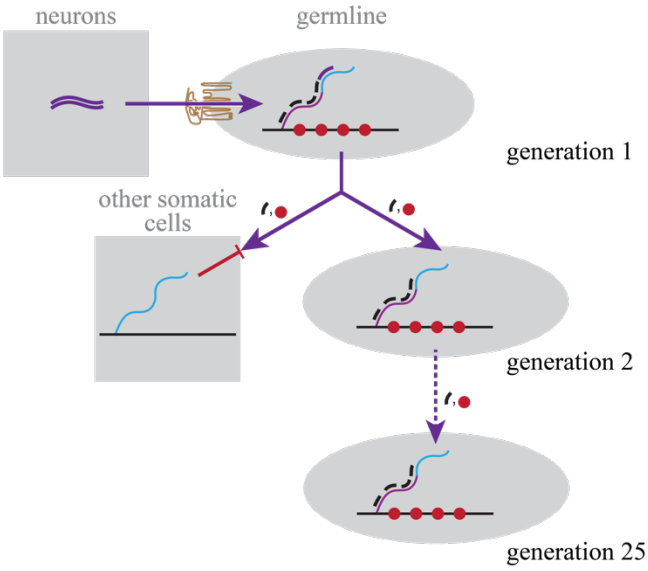
C



2.5 Discussion

We found that neurons can transport forms of dsRNA into the germline to cause silencing that can last for many generations and that such transgenerational silencing is restricted to the germline with distinct genetic requirements for initiation and maintenance (Fig. 2-15).

Fig. 2–15. Model: neuronal mobile RNAs can enter the germline and cause transgenerational silencing.
Double-stranded RNA transcribed in neurons (grey square in generation 1) can be imported into the germline (grey oval) through the **conserved importer SID-1**, and be used for silencing both ***gfp*** and ***mCherry***, likely resulting in deposition of **H3K9me3** (••••) on the **target gene**. This silencing can be inherited in the germline for many generations, by propagation of secondary siRNAs (~) or **H3K9me3**, but not in the soma (grey square in generation 2).



Mobile RNAs that enter the germline can provide an organism with the ability to transfer gene-specific regulatory information from somatic cells across generations and could be one mechanism by which the environment elicits transgenerational effects in animals. Although restricted to the germline, transgenerational silencing by mobile RNAs could underlie effects of the environment across generations in some cases. For example, expression of some genes within the germline can affect longevity (41), and transgenerational silencing of such genes might underlie the longevity that results from ancestral starvation in *C. elegans* (38). Thus, additional experiments are needed to determine the role of mobile RNAs, if any, in the transport of such experience-

dependent information from somatic cells to subsequent generations in *C. elegans*. The presence of a mammalian homolog of the dsRNA importer SID-1 that is also required for the uptake of dsRNAs into cells (73) raises the possibility that dsRNA generated from distant somatic cells—potentially in response to environmental influences—may be imported through SID-1 into the mammalian germline to trigger transgenerational epigenetic changes. Consistent with this possibility, small RNAs have been found in circulation in mammals (74); dsRNAs have been detected in mammalian germ cells (75-77); and injection of RNAs into the early mouse embryo can trigger epigenetic silencing (14). However, even if RNAs from somatic cells are transported to the germline in mammals, they may not always initiate transgenerational inherited effects because they have to escape mechanisms that reprogram epigenetic information in each generation (78). Additional studies are required to determine whether specific mechanisms prevent environmental influences from triggering transmission of information in the form of mobile RNAs from somatic cells to the germline.

Chapter 3: Mating can cause transgenerational gene silencing in *C. elegans*

3.1 Preface

Most of the work presented in this chapter was submitted to the pre-print BioRxiv as: Devanapally S, Allgood S, and Jose AM (2017) Mating can cause transgenerational gene silencing in *Caenorhabditis elegans.*

Sam Allgood, an undergraduate I mentored, worked along with me to generate the data for Figs. 3-1*B*, 3-2, 3-4*A*, 3-4*B*, 3-5, 3-7*A*, 3-7*D*, 3-9*A*, 3-9*D*, 3-10*A*, 3-10*B*, 3-12*B* and 3-12*C*. Maïgane Diop, another undergraduate I mentored, generated the data for Figs. 3-1*A*, 3-12*D*, 3-12*E*. Nate Shugarts performed the Sanger sequencing of *oxSi487*. The remaining work in this chapter was contributed by me.

In addition to the sources mentioned in Chapter 2, some worm strains were obtained from the Cohen-Fix lab (National Institutes of Health).

3.2 Introduction

Gene silencing is a significant obstacle to genome engineering and has been proposed to be a non-self response against foreign DNA (15, 18, 19, 31). Yet, some foreign genes remain expressed for many generations (15, 19, 31) and some native genes remain silenced for many generations (15, 17, 54). How organisms determine whether a sequence is expressed or silenced is unclear. Here we show that a stably expressed foreign DNA sequence in *C. elegans* is converted into a stably silenced

sequence when males with the foreign DNA mate with wild-type hermaphrodites. This conversion does not occur when the hermaphrodite also has exonic sequences from the foreign DNA. Once initiated, silencing persists for many generations independent of mating and is associated with a DNA-independent signal that can silence other homologous loci in every generation. This mating-induced silencing resembles piRNA-mediated silencing because it requires the Argonaute PRG-1 (ref. 46) for initiation and the Argonaute HRDE-1 (refs. 15, 54) for maintenance. Loss of HRDE-1 can revive gene expression even after 150 generations. Thus, our results reveal the existence of a mechanism that maintains gene silencing initiated upon ancestral mating. By allowing retention of potentially detrimental sequences acquired through mating, this mechanism could create a reservoir of sequences that contribute to novelty when activated during evolution.

3.3 Materials and methods

3.3.1 Strains and primers used

Table 3–1. Strains used.

Strain	***Genotype***
N2	wild type
AMJ501	*oxSi487* (*Pmex-5::mCherry::h2b::tbb-2 3'utr::gpd-2 operon::gfp::h2b::cye-1 3' utr + unc-119(+)*) II*; unc-119(ed3)* III?*; sid-1(qt9)* V

AMJ506 *prg-1(tm872)* I*; oxSi487* II*; unc-119(ed3)* III?

AMJ544 *oxSi487* II*; unc-119(ed3)?* III*; nrde-3(tm1116)* X

AMJ545 *oxSi487* II*; unc-119(ed3)?* III*; rde-1(ne219)* V

AMJ577 *hrde-1(tm1200)* III [4x]

AMJ581 *oxSi487 dpy-2(e8)* II

AMJ586 *oxSi487 dpy-2(e8)* II*; unc-119(ed3)?* III*; rde-1(ne219)* V

AMJ591 *jamSi25 [Punc-119deletion *jamSi19]* II [*T*ΔΔ]

AMJ593 *oxSi487 dpy-2(e8)* II*; unc-119(ed3)?* III*; sid-1(qt9)* V

AMJ602 *oxSi487 dpy-2(e8)* II*; unc-119(ed3)? hrde-1(tm1200)* III

AMJ626 *rrf-1(ok589)* I*; oxSi487 dpy-2(e8)* II*; unc-119(ed3)?* III

AMJ646 *dpy-17(e164) unc-32(e189)* III*; rde-1(ne219)* V

AMJ647 *dpy-17(e164) unc-32(e189)* III*; sid-1(qt9)* V

AMJ667 *dpy-20(e1282) ax2053[gtbp-1::gfp]* IV

AMJ673 *rrf-1(ok589)* I*; dpy-2(e8) unc-4(e120)* II

AMJ675 *oxSi487* II*; unc-119(ed3)? hrde-1(tm1200)* III

AMJ683 *oxSi487 dpy-2(e8)* II*; unc-119(ed3)?* III*; nrde-3(tm1116)* X

AMJ689 *rrf-1(ok589)* I*; oxSi487* II*; unc-119(ed3)?* III

AMJ690 *dpy-2(e8) unc-4(e120)* II*; nrde-3(tm1116)* X

AMJ691 *dpy-2(e8) unc-4(e120)* II; *hrde-1(tm1200)* III

AMJ692 *oxSi487 dpy-2(e8)* II [*Ti*]

AMJ693 *dpy-2(e8) unc-4(e120)* II; *Pmex-5::mCherry::mex-5::mex-5 3' utr* IV

AMJ709 *dpy-10(jam21) jamSi25* [*Punc-119deletion *jamSi19*] II [*T*ΔΔ]

AMJ711 *prg-1(tm872)* I [1x]

AMJ712 *dpy-2(e8) unc-4(e120)* II*; Pgtbp-1::gtbp-1::RFP::linker::3xflag::gtbp-1 3'utr* IV

AMJ713 *dpy-2(e8) unc-4(e120)* II*; Ppgl-1::pgl-1::gfp::pgl-1 gfp 3' utr* IV

AMJ714 *oxSi487* II*; unc-119(ed3)? hrde-1(tm1200)* III

AMJ724 *oxSi487* II*; unc-119(ed3)?* III [*Ti*]

AMJ725 *oxSi487* II*; unc-119(ed3)?* III

AMJ727 *dpy-2(e8) unc-4(e120)* II*; Pgtbp-1::gtbp-1::mCherry::gtbp-1 3' utr* IV

AMJ753 *dpy-10(jam38) oxSi487* II*; unc-119(ed3)* III

AMJ763 *dpy-10(jam40) jamSi16* [*Pmex-5::mCherry::h2b::cye-1 3' utr *oxSi487*] II [*T*Δ]

AMJ765 *dpy-10(jam41) jamSi18* [*Pmex-5::mCherry::h2b::cye-1 3' utr *oxSi487*] II [*T*Δ]

AMJ766 *jamSi19* [*Pmex-5::mCherry::h2b::cye-1 3' utr *oxSi487*] II [*T*Δ]

AMJ767 *dpy-10(jam42) jamSi20* [*Pmex-5::mCherry::h2b::cye-1 3' utr *oxSi487*] II [*T*Δ]

AMJ768 *dpy-10(jam43) jamSi21* [*Pmex-5::mCherry::h2b::cye-1 3' utr *oxSi487*] II [*T*Δ]

AMJ769 *dpy-10(jam44) oxSi487* II*; unc-119(ed3)* III

AMJ777	*dpy-10(jam45)* II
AMJ792	*dpy-10(jam46)* II
AMJ844	*oxSi487 dpy-2(e8)* II [*Ti*]
AMJ917	*dpy-10(jam47) jamSi20 [Pmex-5::mCherry::h2b::cye-1 3' utr *oxSi487]* II*; unc-119(ed3)* III [*T*Δ*i*]
AMJ922	*prg-1(tm872)* I [1x]*; dpy-2(e8) oxSi487* II*; unc-119(ed3)?* III
AMJ923	*prg-1(tm872)* I [1x]*; dpy-2(e8) unc-4(e120)* II
AMJ926	*dpy-10(jam39) jamSi27[Pmex-5::mCherry::cye-1 3' utr *jamSi25]* II [*T*ΔΔΔ]
AMJ928	*jamSi27[Pmex-5::mCherry::cye-1 3' utr *jamSi25]* II [*T*ΔΔΔ]
DR439	*unc-8(e49) dpy-20(e1282)* IV
EG6787	*oxSi487* II*; unc-119(ed3)* III
GE1708	*dpy-2(e8) unc-4(e120)* II
HC196	*sid-1(qt9)* V
HC780	*rrf-1(ok589)* I
JH3197	*ax2053* (*gtbp-1::gfp*) IV
JH3270	*Ppgl-1::pgl-1::gfp::pgl-1 gfp 3' utr* IV [gift from Geraldine Seydoux]
JH3296	*Pmex-5::mCherry::mex-5::mex-5 3' utr* IV [gift from Geraldine Seydoux]
JH3323	*Pgtbp-1::gtbp-1::mCherry::gtbp-1 3' utr* IV [gift from Geraldine Seydoux]

JH3337	*Pgtbp-1::gtbp-1::RFP::linker::3xflag::gtbp-1 3'utr* II [gift from Geraldine Seydoux]
OCF62	*jfSi1* [*Psun-1::gfp cb-unc-119(+)*] II*; ltIs38* [*(pAA1) pie-1::GFP::PH(PLC1delta1) + unc-119(+)*] [gift from Orna Cohen-Fix]
OCF69	*ocfSi1* [*Pmex-5::Dendra2::his-58::tbb-2 3' utr + unc-119(+)*] I*; unc-119(ed3)* III [gift from Orna Cohen-Fix]
SP471	*dpy-17(e164) unc-32(e189)* III
WM27	*rde-1(ne219)* V
WM156	*nrde-3(tm1116)* X
WM161	*prg-1(tm872)* I

All strains with fluorescent reporters showed invariable expression of fluorescence, except OCF69 which showed suppression of expression in one of the 34 animals tested.

Table 3–2. Primers used.

P1	ATAAGGAGTTCCACGCCCAG
P2	CTAGTGAGTCGTATTATAAGTG
P3	TGAAGACGACGAGCCACTTG
P4	ATCGTGGACGTGGTGGTTAC
P5	CTCATCAAGCCGCAGAAAGAG
P6	GGTTCTTGACAGTCCGAACG
P7	ACGGTGAGGAAGGAAAGGAG
P8	ACAAGAATTGGGACAACTCCAG

P9 AGTAACAGTTTCAAATGGCCG

P10 TCTTCACTGTACAATGTGACG

P11 CACTATTCACAAGCATTGGC

P12 CGGACAGAGGAAGAAATGC

P13 TGCCATCGCAGATAGTCC

P14 TGGAAGCAGCTAGGAACAG

P15 CCGTGACAACAGACATTCAATC

P16 ACGATCAGCGATGAAGGAG

P17 GGAGATCCATGATTAGTTGTGC

P18 GCAGGCATTGAGCTTGAC

P19 TCATCTCGGTACCTGTCGTTG

P20 AGAGGCGGATACGGAAGAAG

P21 CATAACCGTCGCTTGGCAC

P22 TCGAGTCGTGGTACAGATCG

P23 CATGCTCGTCGTAATGCTCG

P24 CGATCGTGCCAGAACAATCC

P25 ATGAAAGCCGAGCAACAACG

P26 AGAATGATGAGTCGCCACAGG

P27 CATGCACAACAAAGCCGACTAC

P28 TGAGAATACGGTCGCAGTTAGG

P29 ACGGATGCCTAGTTGCATTG

P30 CCTTCCCAGAGGGATTCAAGTG

P31 TCTGTTCCTATTCTGTCTGCAC

P32 CGCGGTTCGCAATAGGTTTC

P33 TCACCTAGTCTGTGCCATTTC

P34 TGCGGGTTTCTGTTAGCTTC

P35 GCACAGACTAGGTGAAAGAGAG

P36 ACCTCCCACAACGAGGATTAC

P37 TGGGCGTGGAACTCCTTATC

P38 GGCGAAGAGCAAAGCAGAG

P39 GGGCCGTTATCCTTTCAAATGC

P40 CATGGGCCACGGATTGTAAC

P41 ACGCATCTGTGCGGTATTTC

P42 ATTTAGGTGACACTATAGGATCAGGTAGTGGCCCACCAGTTTTAGA GCTAGAAATAGCAAG

P43 AAA AGC ACC GAC TCG GT

P44 ATGGTCTCCAAGGGAGAGGAG

P45 GAATCCTATTGCGGGTTATTTTAGCCACTACCTGATCCCTTG

P46 ATTTAGGTGACACTATAGGTGTAATCCTCGTTGTGGGGTTTTAGAG CTAGAAATAGCAAG

P47 CAAGGGATCAGGTAGTGGCTAAAATAACCCGCAATAGGATTC

P48 TAAGGAGTTCCACGCCCAG

P49 TTTCGCTGTCCTGTCACACTC

P50 CGATGATAAAAGAATCCTATTGCGGGTTATTTTTTGAGCCTGCTTTT TTGTACAAACTTG

P51 CAAGTTTGTACAAAAAAGCAGGCTCAAAAAATAACCCGCAATAGG ATTCTTTTATCATCG

P52 AGCTAACAGAAACCCGCATAC

P53 CCTGTCACACTCGCTAAAAACAC

P54 ACAGAAACCCGCATACTCG

P55 ATT TAG GTG ACA CTA TAG ATT CCT TGT TCG GTG CTT GGG TTT TAG AGC TAG AAA TAG CAA G

P56 ATT CCA TGA TGG TAG CAA ACT CAC TTC GTG GGT TTT CAC AAC GGC AAA ATA TCA GTT TTT

P57 ATTTAGGTGACACTATAGCTACCATAGGCACCACGAGGTTTTAGAG CTAGAAATAGCAAG

P58 CAC TTG AAC TTC AAT ACG GCA AGA TGA GAA TGA CTG GAA ACC GTA CCG CAT GCG GTG CCT ATG GTA GCG GAG CTT CAC ATG GCT TCA GAC CAA CAG CCT A

P59 ATTTAGGTGACACTATAGACAAATGCCCGGGGGATCGGGTTTTAGA GCTAGAAATAGCAAG

P60 TGAGGTCAAGACCACCTACAAG

P61 GAATCCTATTGCGGGTTATTTTACTTGCTGGAAGTGTACTTGG

P62 CCAAGTACACTTCCAGCAAGTAAAATAACCCGCAATAGGATTC

P63 GACCACCTACAAGGCTAAGAAG

P64 ATTTAGGTGACACTATAGGGGAGAGGGAAGACCATACGGTTTTAG AGCTAGAAATAGCAAG

P65 GCAAAAATTCCCCGACTTTCCC

P66 GAAAAGTTCTTCTCCTTTACTCATTTTTGAGCCTGCTTTTTTGTAC

P67 GTACAAAAAAGCAGGCTCAAAAATGAGTAAAGGAGAAGAACTTTTC

P68 CCCATGGAACAGGTAGTTTTCC

P69 CGACTTTCCCCAAAATCCTGC

P70 ACAGGTAGTTTTCCAGTAGTGC

P71 AGAGGGATTCAAGTGGGAGAG

P72 TGGGTCTTACCGCGTATACC

P73 TGATCCCTTGTAAAGCTCATCC

P74 GTG TGT GCT GCT CGG TTA AG

P75 AAT TCC ACA GTT GCT CCG AC

P76 TCATCTCGCCCGATTCATTG

P77 CCGTTTCTTCCTGGTAATCC

P78 GGGTGAAGGTGATGCAACATAC

P79 GGGACAACCTGTGTGCATG

P80 AAGGTCCACATGGAGGGATC

P81 AAA GTA ATT CTA CAG TAT TCC TGA GAT G

3.3.2 Nomenclature of transgenes

The letter *T* is used to specify the transgene *oxSi487* in all genetic crosses. The active or expressing allele of *oxSi487* is named as *Tα* and the inactive or the silenced allele of *oxSi487* is named as *Ti* in parents. Genotypes that additionally include a recessive marker (*dpy* or *dpy unc*) are in orange font. See 'Genetic Crosses' for details on recessive mutations used.

3.3.3 Quantification of silencing and measurement of fluorescence intensity

To classify fluorescence intensity, in most cases, animals of the fourth larval (L4) stage or 24 h after the L4 stage were mounted on a slide after paralyzing the worm using 3 mM levamisole (Sigma-Aldrich, Cat# 196142), imaged under non-saturating conditions (Nikon AZ100 microscope and Photometrics Cool SNAP HQ^2 camera), and binned into three groups – bright, dim and not detectable. A C-HGFI Intensilight Hg Illuminator was used to excite GFP or Dendra2 (filter cube: 450 to 490 nm excitation, 495 dichroic, and 500 to 550 nm emission) or mCherry or RFP (filter cube: 530 to 560 nm excitation, 570 dichroic, and 590 to 650 nm emission). Sections of the gonad that are not obscured by autofluorescence from the intestine were examined to classify GFP and mCherry fluorescence from *oxSi487*. Autofluorescence was appreciable when imaging GFP but not when imaging mCherry. For Figs. 3-2*B*, 3-2*E*, 3-5 and 3-10*B*, fluorescence intensity within the germline 24 h after the L4 stage was scored by eye at fixed magnification and zoom using the Olympus MVX10 fluorescent microscope without imaging.

To quantitatively measure fluorescence of mCherry from *T* (Fig. 3-2*D*) and fluorescence from other transgenes (Fig. 3-9*C*), regions of interest (ROI) were marked using either NIS elements or ImageJ (NIH) and the intensity was measured. Background was subtracted from the measured intensity for each image. For Fig. 3-2*D*, intensity was given by (a1.(m1-b)/b + a2.(m2-b)/b)/2, where a1 = area of anterior gonad arm, a2 = area of posterior gonad arm, m1 = mean intensity of anterior gonad arm, m2 = mean intensity of posterior gonad arm, and b = background mean intensity. This measured intensity was then normalized to the least value and plotted on a $\log_2$ scale. The shape of the gonad was traced using the red channel or brightfield image. For Fig. 3-9*C*, intensity was given by x-b, where x = mean intensity of ROI and b = mean intensity of background.

All images being compared were adjusted identically using Adobe Photoshop for display.

3.3.4 Genetic crosses

Three L4 hermaphrodites and 7-13 males were placed on the same plate and allowed to mate for each cross plate. Cross progeny were analyzed three to five days after the cross plate was set up. At least two independent matings were set up for each cross. For crosses in Fig. 3-2 and in Fig. 3-5, the required genotypes were determined by PCR (primers P1, P2, and P3) after scoring all animals and only the data from animals with the correct genotypes were plotted. In Fig. 3-4, 3-6, 3-7*A*, 3-7*C–E*, 3-8, 3-9, 3-10, 3-12, and 3-13, *dpy-2(e8)* (3 cM from *oxSi487*) or *dpy-10(-)* (7 cM from

oxSi487) was used as a linked marker to determine the homozygosity of *T* and *dpy-2(e8) unc-4(e120)* or *dpy-10(-)* was used as a balancer to determine the hemizygosity of *T, T*Δ, and *T*ΔΔ. In Fig. 3-7*D* and 3-10*A Right* (control for *sid-1(-)* and *rde-1(-)*), *unc-8(e49) dpy-20(e1282)* and *dpy-17(e164) unc-32(e189)*, respectively, were used as markers to facilitate identification of cross progeny. Some crosses additionally required identification of cross progeny by genotyping of single worms, including those from Fig. 3-7*A*, 3-7*D–E*, and 3-9*D–E*. Animals from crosses with *prg-1(+/-)* males in Fig. 3-10*A Reft*, and in Fig. 3-10*D* or with *T; prg-1(+/-)* males in Fig. 3-10*A Right were* also genotyped to identify *T; prg-1(-/-)* or *prg-1(-/-)* cross progeny, respectively. In crosses from Fig. 3-7*D* and Fig. 3-9*E*, cross progeny of the required genotype were identified by the absence or presence of pharyngeal mCherry or GFP (refer to Chapter 2), respectively.

3.3.5 Generation and maintenance of *Ti* and *T*Δ*i* strains.

To make hermaphrodites with *Ti* linked to a *dpy* marker, AMJ581 hermaphrodites were mated with N2 males to generate cross progeny males that all show bright mCherry fluorescence from *oxSi487*. These males were then mated with N2 hermaphrodites to give cross progeny (F1) with undetectable mCherry fluorescence. F1 animals were allowed to give progeny (F2) that are homozygous for *oxSi487* as determined by the homozygosity of a linked *dpy-2(e8)* mutation. One such F2 animal was isolated to be propagated as the *Ti* strain (AMJ692).

To make males with *Ti*, *dpy-17(e164) unc-32(e189)* hermaphrodites were mated with EG6787 males to generate cross progeny (F1) hermaphrodites with undetectable mCherry fluorescence. These cross progeny were allowed to give progeny (F2) that are homozygous for *oxSi487*. Two such F2s were isolated to be propagated as two different *Ti* lines. One of these was designated as AMJ724 and used for further experiments. These strains maintained the silencing of *oxSi487* and were heat-shocked to produce males. Genotypes of *Ti* strains were verified using PCR.

To make hermaphrodites with *T*Δ*i* linked to a *dpy* marker, AMJ767 hermaphrodites were mated with N2 males to generate cross progeny males with bright mCherry fluorescence. These males were then mated with GE1708 hermaphrodites to give cross progeny (F1) with undetectable mCherry fluorescence. F1 animals were allowed to give descendants that are homozygous for *T*Δ as determined by genotyping for *jamSi20*. A homozygous descendant was isolated to be propagated as the *T*Δ*i* strain (AMJ917). Genotypes of *T*Δ*i* strains were verified using PCR.

AMJ692 was used to test for recovery of gene expression ~150 generations after it was made. This generation time was estimated as follows: worms were passaged every 3.5 days for 143 generations over a period of 556 days, except for three intervals when they were allowed to starve, and larvae were recovered after starvation. These intervals with recovery from starvation spanned a total of ~6 generations over 49 days. Thus, the total number of generations = 143 + ~6 = ~150 generations. The generation times for AMJ724 and AMJ844 were similarly estimated.

3.3.6 CRISPR-Cas9 mediated editing of *oxSi487*

To generate edits in *oxSi487*, Cas9-based genome editing with a co-conversion strategy (79) was used. Guide RNAs were amplified from pYC13 using primers listed above. The amplified guides were purified (PCR Purification Kit, Qiagen) and tested *in vitro* for cutting efficiency (Cas9, New England Biolabs catalog no. M0386S). For most edits, homology template for repair (repair template) was made from gDNA using Phusion High Fidelity polymerase (New England Biolabs catalog no. M0530S) and gene specific primers to separately amplify regions precisely upstream and downstream of the site to be edited. The two PCR products were used as templates to generate the entire repair template using Phusion High Fidelity Polymerase and the fused product was purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, catalog no. 740609.250). Homology templates to generate *T*ΔΔ and *dpy-10(-)* were single-stranded DNA oligos. Wild-type animals were injected with 1.2 – 12.9 pmol/µl of guide RNAs, 0.08 – 1.53 pmol/µl of homology repair template to make edits in *T* and in *dpy-10* and 1.6 pmol/µl of Cas9 protein (PNA Bio catalog no. CP01). In animals with *T*ΔΔ edit, *Punc-119* deletion resulted in Unc animals due to the *unc-119(ed3)* mutation in the background of EG6787, suggesting that a functional transcript was not made from the remaining part of the rescuing *Punc-119::unc-119::unc-119 3'utr* insertion at *ttTi5605*. Edits were verified using PCR and Sanger sequencing. For additional details on specific reagents, see Table 3-3.

Table 3–3. Reagents used for CRISPR-Cas9 experiments.

Allele name	CRISPR edit	Primers used to make: DNA template for sgRNA transcription	Primers used to make: Homology repair template	Length of homology repair template	Concentration of reagents used (pmol/µl): First sgRNA	Second sgRNA	Homology repair template	*dpy-10* sgRNA	*dpy-10* homology repair template
+/+	*dpy-10(-)* in +/+	P57 (FOR), P43 (REV)	P58 (oligo)	100 b	-	-	-	3.05	0.66
T	*dpy-10(-)* in *oxSi487*		P58 (oligo)	100 b	-	-	-	3.05	0.66
*T*Δ	Deletion of *gfp* & *tbb-2 3'utr* from *oxSi487*	P59 (FOR), P43 (REV)	Left: P60 + P61, Right: P62 + P52, Fusion: P63 + P54	1074 bp	2.96	-	0.08	3.05	0.66
*T*ΔΔ	Deletion of *Punc-119* from *jamSi19 (T*Δ*)*	P55 (FOR), P43 (REV)	P56 (oligo)	60 b	8.4	-	1.53	8.16	1.52
*T*ΔΔΔ	Deletion of *h2b* from *jamSi25 (T*ΔΔ*)*	P42 (FOR), P43 (REV)	Left: P44 + P45, Right: P47 + P48, Fusion: P80 + P81	1604 bp	11.16	12.87	0.31	2.89	0.62

3.3.7 Statistical analyses

For each figure, χ^2 test was used to compare data as indicated in figure legends except in cases where only one category (bright or silenced) was present in both datasets being compared. GFP fluorescence and mCherry fluorescence were each separately compared in all cases. Student's two-tailed *t*-test with unequal variance was used in Fig. 3–9*C*.

3.3.8 Genetic Inferences

Extent of mating-induced silencing is variable in progeny but is initiated in every mating.

The initiation of mating-induced silencing is reliable (observed in >440 animals from 45/45 independent crosses in wild-type and *dpy*- or *unc*-marked genetic backgrounds). In every comparison, precisely the same markers were used in crosses

being compared. Nevertheless, silencing ("dim" + "no" animals) varied from 68% to 100% in cross progeny in these backgrounds. The reason for this variation is unclear. Therefore, we did not strongly infer from small variations observed when testing genetic requirements for initiation (e.g. enhancement of silencing observed in *sid-1(-)* animals and reduction of silencing observed in *hrde-1(-)* animals (Fig. 3–10*A*)).

Lack of silencing when the transgene is inherited only through self-sperm in hermaphrodites could be because of a protective signal transmitted through oocyte.

Hemizygous self-progeny of hemizygous hermaphrodites showed stable expression of *T* for multiple generations (Fig. 3–6*B*). In each generation the transgene is expected to be inherited through self-sperm 50% of the time and a maternal protective signal is required for expression of paternal *T* in genetic crosses (Fig. 3–7). Therefore, this result implies that either a protective signal inherited through oocytes licenses expression of *T* inherited through self-sperm in each generation or that inheritance of *T* through self-sperm does not result in silencing.

The silencing signal can separate from *Ti* in the male germline before meiotic maturation.

While meiosis is completed in sperm before fertilization (80), it is stalled at prophase I in oocytes until fertilization (81). Nevertheless, oocyte meiosis is completed early in the one cell zygote such that only a haploid genome is present in the oocyte pronucleus when it meets the sperm pronucleus. Thus, a DNA-independent signal when transmitted through sperm must have separated from DNA in the male germline but

when transmitted through oocytes can separate from DNA either in the hermaphrodite germline or in the embryo (Fig. 3–9*D*, *E*).

Parental rescue of genes can complicate analysis of newly generated mutants.

Homozygous mutant progeny of heterozygous animals may not show the mutant defect because of rescue by parental gene products – typically maternal rescue. Consistently, only some *hrde-1(-/-)* progeny of *hrde-1(+/-)* animals showed expression but all *hrde-1(-/-)* progeny in the next generation showed expression (Fig. 3–13). All strains analyzed for initiation (Fig. 3–10*A*) and maintenance (Fig. 3–10*D*) requirements had been mutant for at least two generations, except when testing the requirement for *prg-1(-)* in initiation, which was done using *prg-1(-)* animals that were mutant for one generation.

3.4 Results

3.4.1 Inheritance of a transgene through the male causes heritable silencing

Mating is routinely used to introduce genes, including fluorescent reporters, into different genetic backgrounds and it is generally assumed that gene expression is unaffected by this manipulation. While expression from many transgenes is indeed unaffected by mating (Fig. 3-1), we identified a single-copy transgene that violates this rule during the course of our experiments on gene silencing in the hermaphrodite worm *C. elegans* (refer to Chapter 2). This transgene (68) consists of a bicistronic operon that expresses mCherry and GFP in the germline (Fig. 3-2*A*, Fig. 3-3). We observed differences in expression from this transgene depending on the gamete through which

the transgene was inherited (Fig. 3-2*B*). While progeny inheriting the transgene from the oocyte showed uniform fluorescence, progeny inheriting the transgene from the sperm displayed variation in fluorescence that ranged from bright to undetectable – a measurable difference of ~12.5-fold (Fig. 3-2*C*, *D*). Fluorescence of both proteins was similarly affected in each animal (Fig. 3-4), consistent with co-transcriptional or nuclear silencing of the bicistronic pre-mRNA. This silencing was observed in progeny despite stable expression in all male parents (Fig. 3-3*B*), suggesting that silencing is initiated within cross progeny and not in male parents. While not all cross progeny showed silencing, silenced cross progeny tended to have silenced self-progeny in the next generation (Fig. 3-2*E*, Fig. 3-5, also see Genetic Inferences in materials and methods). Thus, gene expression can be affected by the direction of mating and expression in the next generation can depend on the sibling chosen for propagation by selfing. Because this silencing is distinct from previously reported epigenetic silencing phenomena (see Table 3-4 and Discussion), we refer to it as mating-induced silencing.

Fig. 3–1. Expression of many transgenes remains unaffected by mating.
(A–B) An identical sequence (*Pdpy-30::gfp::h2b::tbb-2 3' utr*) inserted at different genomic loci using MosSCI or miniMos (ref. 31) (*A*) or transgenes made using MosSCI (*sun-1::gfp* and *Pmex-5::Dendra2::h2b::tbb-2 3' utr*), CRISPR-Cas9-mediated genome editing (*gtbp-1::gfp*, *mCherry::mex-5*, *gtbp-1::rfp::3xflag*, *pgl-1::gfp*, and *gtbp-1::mCherry*), or bombardment (*Ppie-1::gfp::PH(PLCdelta1)*) were tested for susceptibility to mating-induced silencing as in Fig. 3–2 (*A*). Fluorescence in 100% of F1s is represented in the schematic (blue in *A*). Germlines of representative cross progeny at L4 or adult stage are outlined (*B*). Scale bar = 50 µm. Number of animals assayed, and orange font are as in Fig. 3–7*A*.

A

Insertion by	*Pdpy-30::gfp::h2b::tbb-2 3'utr* at different genomic loci		
MosSCI	*oxSi474*	chr I	♂×♀ → ♀
	oxSi466	chr II	
miniMos	*oxTi138*	chr I	
	oxTi134	chr I	♂×♀ → ♀
	oxTi132	chr V	
	oxTi162	chr III	

B

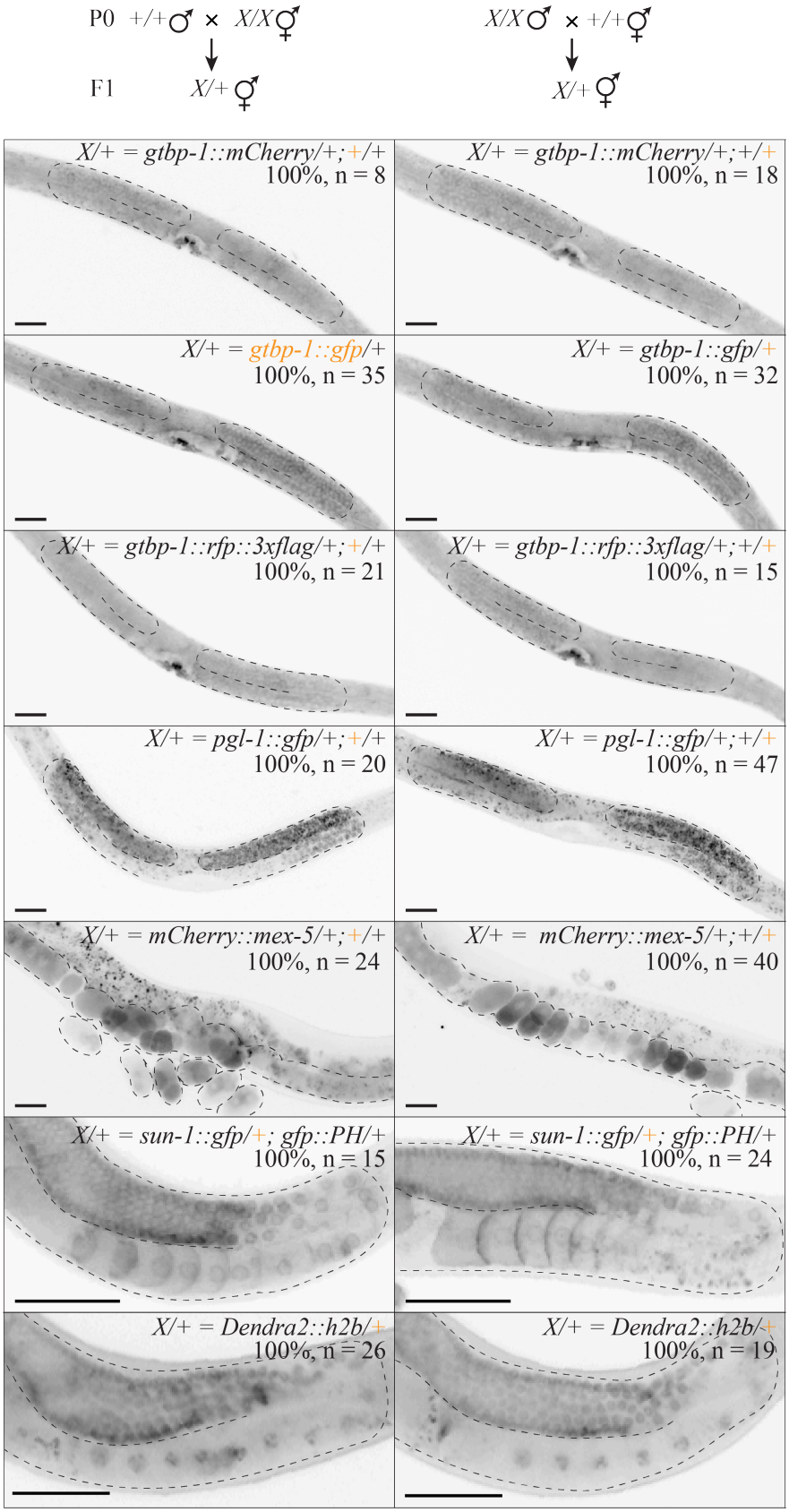

P0 +/+♂ × X/X♀
F1 X/+♂
X/X♂ × +/+♀
X/+♂
X/+ = gtbp-1::mCherry/+;+/+
100%, n = 8
X/+ = gtbp-1::mCherry/+;+/+
100%, n = 18
X/+ = gtbp-1::gfp/+
100%, n = 35
X/+ = gtbp-1::gfp/+
100%, n = 32
X/+ = gtbp-1::rfp::3xflag/+;+/+
100%, n = 21
X/+ = gtbp-1::rfp::3xflag/+;+/+
100%, n = 15
X/+ = pgl-1::gfp/+;+/+
100%, n = 20
X/+ = pgl-1::gfp/+;+/+
100%, n = 47
X/+ = mCherry::mex-5/+;+/+
100%, n = 24
X/+ = mCherry::mex-5/+;+/+
100%, n = 40
X/+ = sun-1::gfp/+; gfp::PH/+
100%, n = 15
X/+ = sun-1::gfp/+; gfp::PH/+
100%, n = 24
X/+ = Dendra2::h2b/+
100%, n = 26
X/+ = Dendra2::h2b/+
100%, n = 19

Fig. 3–2. Mating can cause heritable silencing in progeny.
(A) Schematic of *Pmex-5::mCherry::h2b::tbb-2 3'utr::gpd-2 operon::gfp::h2b::cye-1 3' utr* transgene (called *T* in this study) (also see Fig. 3–3). (B) Hermaphrodites or males that carry *T* were mated with wild-type (+/+) males or hermaphrodites, respectively, and mCherry fluorescence was scored (**bright**, dim, and **not detectable** (no)) in L4-staged hemizygous cross progeny (*T*/+). Number of L4-staged or gravid adult animals scored are indicated (n) for each cross. Bracket indicates relevant comparisons and asterisk indicates $P < 0.01$ (χ^2 test). (C–D) Representative images (*C*) and quantification (*D*) of the germline (outline) of hemizygous animals (*T*/+) scored as having bright (*top*), dim (*middle*), or not detectable (no, *bottom*) levels of mCherry fluorescence. Average (red bar) normalized mCherry fluorescence ($\log_2$ (arbitrary units)) within the germline was calculated for 10 bright (magenta), 5 dim (pink), 5 no (grey), and 5 wild-type (black) L4-staged hermaphrodites. Red arrowheads indicate animals shown in (*C*). Scale bars, 50 µm. (E) mCherry fluorescence intensity was scored in homozygous self-progeny (F2) of some hemizygous cross progeny (F1) shown in (*B*). Each box indicates fluorescence intensity (as in (*D*)) from one adult animal and lines indicate descent. See Fig. 3–5 for additional biological replicates.

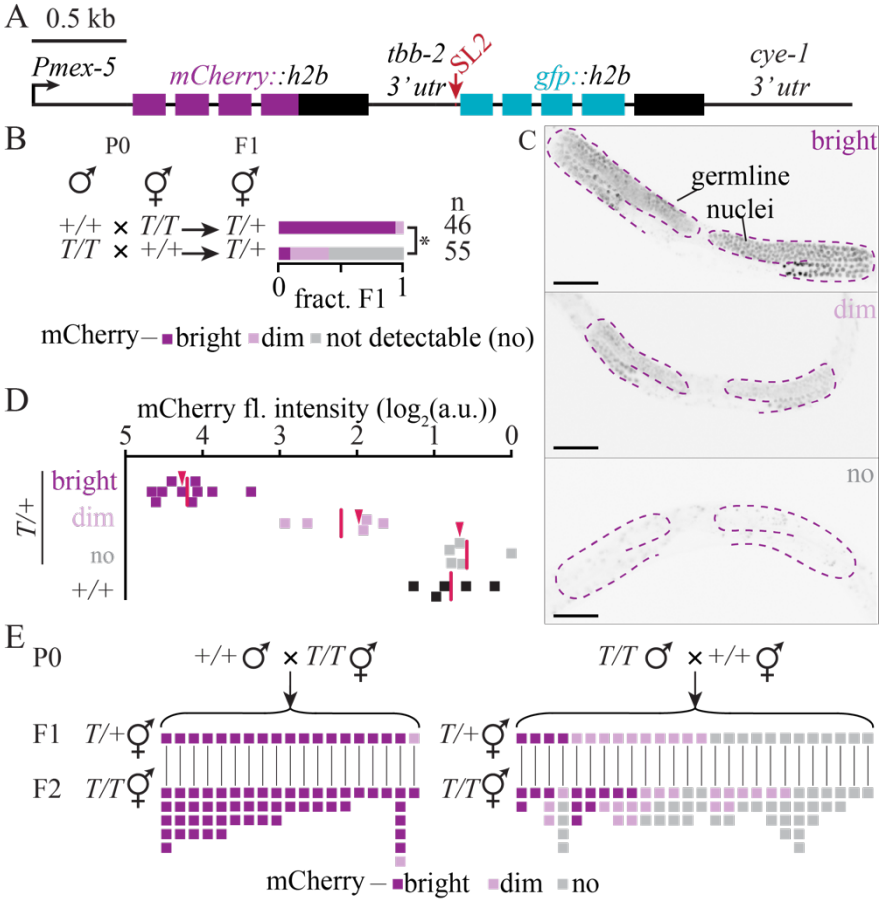


Fig. 3–3. A transgene with foreign DNA coding for two fluorescent proteins shows stable expression within the germline in both hermaphrodites and males.
(A) Schematic of *oxSi487* (*Pmex-5::mCherry::h2b::tbb-2 3' utr::gpd-2 operon::gfp::h2b::cye-1 3' utr*) (ref. 68) within its genomic context where it is present as a single copy transgene as verified by PCR. The transgene consists of *mCherry* and *gfp* genes tagged to *histone 2b* (*his-58/his-66*) arranged in an operon, and is presumably transcribed into a nascent transcript with both *mCherry::h2b* and *gfp::h2b* but present as two separate mature transcripts in the cytosol. Fragments of DNA sequences verified by Sanger sequencing in the strain that expresses *oxSi487* are indicated. The genes surrounding the insertion site of *oxSi487* are shown. (B) Germlines of representative L4-staged hermaphrodites and males showing mCherry::H2B or GFP::H2B expression from *oxSi487* are indicated (dotted outline). Scale bar = 50 µm.

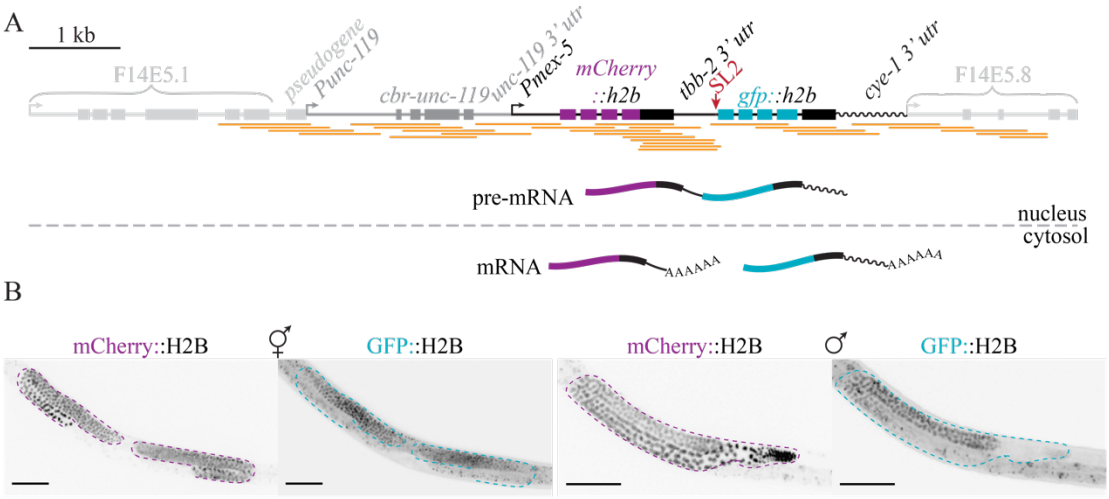


Fig. 3–4. Mating can trigger silencing of both cistrons in an operon.
(A) Cross progeny that inherited *Ta* from one or both parents were analyzed for mCherry and GFP fluorescence. (B) Data from final cross in (*A*) is re-plotted to show mCherry and GFP fluorescence in each individual (outlined box). (C) ***Ta* males** and **non-transgenic hermaphrodites** were mated and cross progeny that were laid in the first 48 h (2 d) or in subsequent ~24 h (1 d) intervals, were collected after moving the P0s at these intervals to fresh plates. Scoring of silencing, number of animals assayed, orange font, brackets and asterisks are as in Fig. 3–7*A*. While silencing triggered by parental ingestion of dsRNA is less effective in later progeny (82), silencing triggered by mating can be equally effective in early and in late progeny.

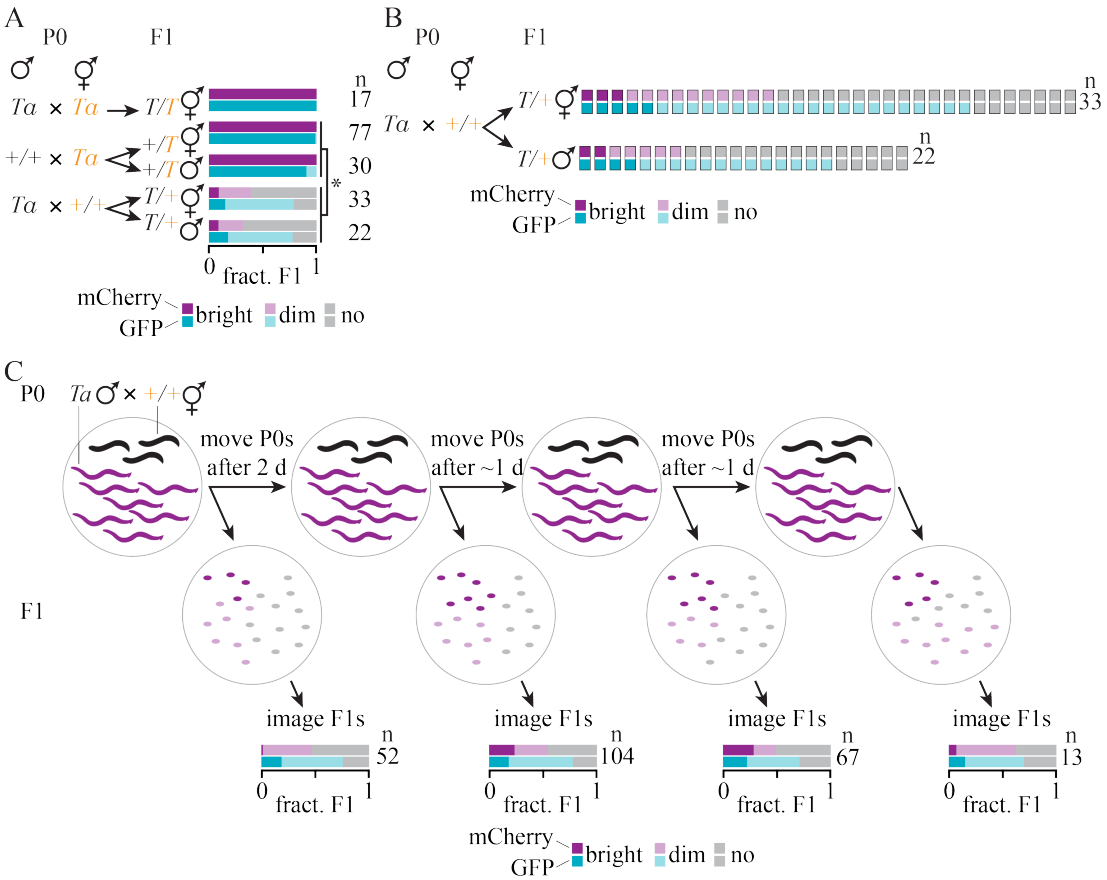


Fig. 3–5. Mating-induced silencing is heritable.
Ta hermaphrodites (*Top*) or males (*Bottom*) were mated with wild-type males or hermaphrodites respectively, in three independent replicates and mCherry fluorescence was scored in hemizygous cross progeny and in homozygous grand-progeny. Each box indicates fluorescence intensity (as in Fig. 3–2*C*, *D*) from a single adult animal and lines indicate descent. See Fig. 3–2*E* for an additional biological replicate. Once initiated by passage through the sperm, mating-induced silencing persists despite passage of *T* through oocytes of hermaphrodites and is therefore unlike genomic imprinting (26, 27), where passage of *T* through oocytes is expected to revive expression.

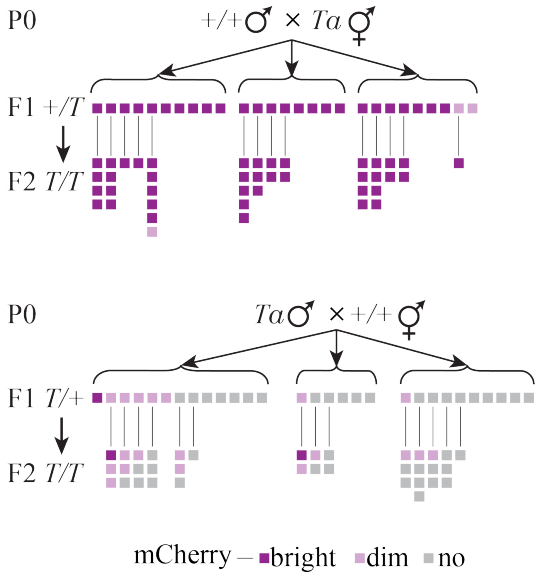


Table 3–4. Similarities and differences between mating-induced silencing and related epigenetic phenomena.

Phenomenon (ref.)	Similarity with mating-induced silencing	Difference from mating-induced silencing
Paramutation in plants (10, 11), flies (12, 13), or mice (14)	Silencing is transgenerational. Silenced allele inherited through either gamete can silence homologous sequences.	Silencing cannot be predictably initiated. When a silenced allele induces meiotically heritable silencing of another allele, this allele also becomes a silencing allele.
RNA induced epigenetic silencing (RNAe) (15-20)	Initiation requires PRG-1; maintenance requires HRDE-1. Silencing is transgenerational.	Silencing cannot be predictably initiated. The same DNA inserted into the same locus can show expression or silencing. Changes upon mating, if any, are unknown.
Multi-generational RNAe caused by meiotic silencing by unpaired DNA (21)	Initiation requires PRG-1. *oxSi487* (*T* in this dissertation) introduced through the male parent showed silencing in cross progeny.	Effect of introducing *oxSi487* through the hermaphrodite parent on silencing in cross progeny or its hemizygous descendants was not tested.
RNA-induced epigenetic gene activation (RNAa) (20, 22, 23)	Extragenic signal can be inherited from male to control gene expression in progeny. Inheritance of an active transgene from hermaphrodite affects expression of paternally inherited transgene.	Extragenic signals inherited from sperm promote expression.
Meiotic silencing by unpaired DNA (24)	Silencing of DNA is epigenetic.	DNA must be upaired during meiosis for silencing.
Epigenetic licensing of *fem-1* (ref. 25)	Maternal transcript of a gene is sufficient to enable expression of the paternal copy in the zygote.	Repeated crossing was required for increased severity of silencing.
Genomic imprinting and parent of origin effects (26-28)	Silencing occurs when a gene is inherited through a specific gamete.	Expression is reset upon passage through the other gamete.
Transposon silencing in flies (13, 29)	Inherited piRNAs silence a paternally inherited gene.	Maternal transcript does not prevent gene silencing.
Transvection in flies (30)	Interaction between alleles on homologous chromosomes can result in changed expression.	Changes in gene expression are not heritable.
Licensing by DNA sequences (31)	Not all transgenes are susceptible to germline silencing.	Initiation of silencing is independent of mating.

3.4.2 Mating-induced silencing does not occur in the presence of a maternally inherited protective signal

Mating-induced silencing was not observed in any descendant of cross progeny that inherited the transgene through both gametes (compare Fig. 3-6*A* with Fig. 3-2*B*). It is possible that the maternal presence of an active, i.e. expressed, transgene (*Tα*) prevents silencing of the paternally inherited transgene. To test if maternal *Tα* in the hermaphrodite parent is sufficient for preventing mating-induced silencing, we mated hemizygous *Tα* hermaphrodites with *Tα* males and examined silencing in progeny that inherited the transgene only from the male (Fig. 3-7*A*). All cross progeny showed stable expression of the paternally inherited transgene (Fig. 3-7*A*), suggesting that the transgene was protected from silencing by an inherited maternal signal. Consistently, no silencing was observed in any self-progeny of hemizygous parents despite the expected inheritance of the transgene through hermaphrodite sperm in 50% of progeny in each generation (Fig. 3-6*B*, also see Genetic Inferences in Methods). Thus, a DNA-independent signal transmitted through oocytes can protect the paternal transgene from mating-induced silencing.

To examine the sequence requirements for the production of the protective signal, we tested whether different homologous sequences could prevent mating-induced silencing. We used genome editing to delete parts of *Tα* (*Pmex-5::mCherry::h2b::tbb-2 3' utr::gpd-2 operon::gfp::h2b::cye-1 3' utr* with *Cbr-unc-119(+)* upstream) (Fig. 3-7*B*, Fig. 3-3*A*). Neither deletion of the *tbb-2 3' utr* and

gfp::h2b sequences (*T*Δ) nor subsequent deletion of upstream sequences (*T*ΔΔ) and *h2b* from *mCherry::h2b* (*T*ΔΔΔ) eliminated the protective signal (Fig. 3-7*B*, *C*). One possible interpretation of these results is that the maternal *mCherry* sequence can protect paternal *gfp::h2b* from silencing, potentially at the level of the bicistronic pre-mRNA. However, because mating-induced silencing occurred despite the presence of two identical *h2b* genes (*his-58* and *his-66*) in the *C. elegans* genome, we infer that not every homologous maternal gene is capable of protecting *Tα* from silencing. Consistently, neither a *Dendra2::h2b* transgene with shared sequences nor *gtbp-1::gfp* could prevent mating-induced silencing of *Tα* (Fig. 3-7*B*, Fig. 3-7*D*). Like maternal *Tα*, maternal *T*ΔΔΔ*α* also retained the property of transmitting a DNA-independent protective signal (Fig. 3-7*E*). Thus, a DNA-independent signal derived from maternal *Pmex-5::mCherry::cye-1 3' utr* is sufficient to protect both *mCherry* and *gfp* of paternal *Tα* from mating-induced silencing (Fig. 3-7*F*).

Fig. 3–6. Inheritance through hermaphrodite sperm does not trigger silencing of the transgene *T*.

(A) *Ta* males were mated with *Ta* hermaphrodites, and mCherry and GFP fluorescence was scored in cross progeny (F1) as well as in self-fertilized grand-progeny (F2) that inherited only the grand-maternal allele or only the grand-paternal allele or both. F1 data shown here is the same as that in Fig. 3–4*A*. (B) *Ta* hermaphrodites were mated with wild-type males and mCherry and GFP fluorescence was scored in hemizygous cross progeny (F1) as well as in descendant hemizygous self-progeny for four generations (F2 through F5). Scoring of silencing, number of animals assayed, and orange font are as in Fig. 3–7*A*. In contrast to previous reports (21), we find that *oxSi487* is not subject to meiotic silencing by unpaired DNA (24).

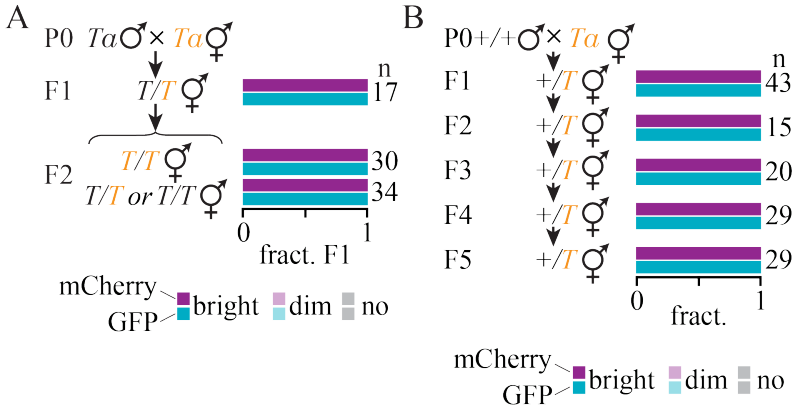
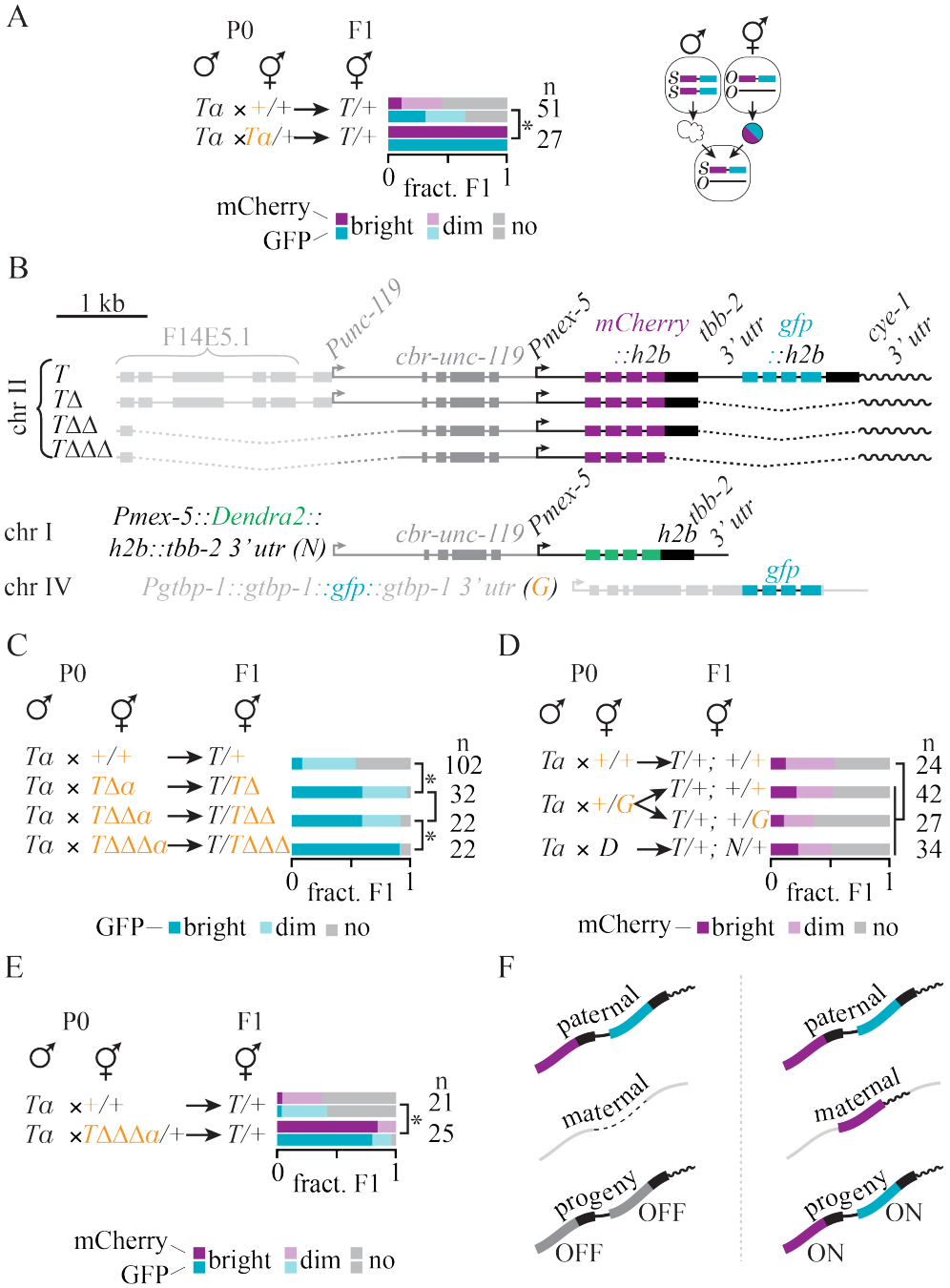


Fig. 3–7. A maternal DNA-independent protective signal can prevent mating-induced silencing.

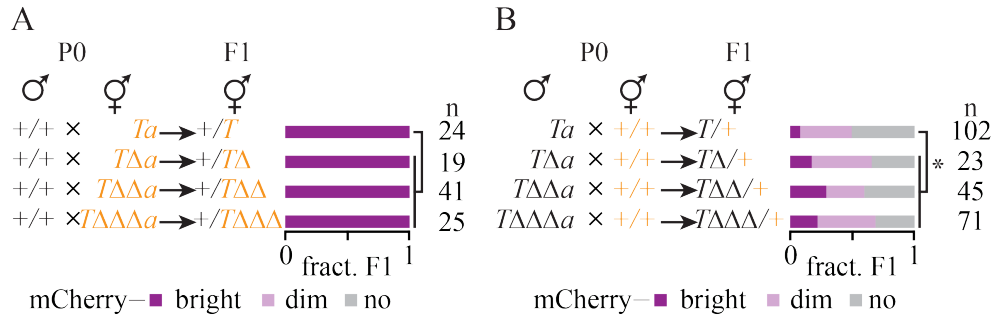
(A) Males that express the active transgene (*Tα*) were mated with non-transgenic (+/+) or hemizygous (*Tα*/+) hermaphrodites, and fluorescence was scored (*top*, mCherry – **bright**, dim, **no**, and *bottom*, GFP – **bright**, dim, **no**) in hemizygous cross progeny that inherited *Tα* through the sperm. Schematic depicts outcome of the test cross: maternally present active transgene (*Tα*, magenta and blue) prevents silencing of *Tα* that is inherited through the sperm (cloud shape) suggesting that the oocyte (circle) carries a DNA-independent protective signal (magenta/blue fill). *s* and *o* label DNA sequences inherited through sperm and oocyte, respectively. Chromosome with (colored boxes) or without (black line) the transgene is as indicated. (B) Schematics of *T*, successive deletions of *T* and other homologous loci. Successive deletions that remove *gfp* and *tbb-2 3' utr* (*T*Δ), a ~3 kb region upstream of the *unc-119(+)* coding region (*T*ΔΔ), and *h2b* (*T*ΔΔΔ) are depicted in their genomic context. Other homologous loci are *Pmex-5::Dendra2::h2b::tbb-2 3' utr* [*N*] and *Pgtbp-1::gtbp-1::gfp::gtbp-1 3' utr* [*G*]. (C) Males that express the active transgene *Tα* were mated with hermaphrodites that lack the transgene or that have a deletion in *T* (as in (*B*)), and GFP fluorescence from paternal *Tα* was scored in cross progeny. (D) Males that express the active transgene *Tα* were mated with hermaphrodites that lack the transgene (+/+) or that express *Pgtbp-1::gtbp-1::gfp::gtbp-1 3' utr* [*G*] or *Pmex-5::Dendra2::h2b::tbb-2 3' utr* [*N*], and mCherry fluorescence from paternally inherited *Tα* was scored in cross progeny. (E) Maternal presence of a single cistron can protect both cistrons of a paternal operon from mating-induced silencing. Males that express the active transgene *Tα* were mated with non-transgenic control (+/+) or hemizygous *Pmex-5::mCherry::cye-1 3' utr* (*T*ΔΔΔ/+) hermaphrodites, and fluorescence from paternal *mCherry* and *gfp* was scored in cross progeny that inherited *Tα* through the sperm. (F) Model depicting maternal expression of *T*ΔΔΔ (magenta) is sufficient to prevent silencing of both *mCherry* and *gfp* from paternal *Tα* in cross progeny. Number of L4-staged or gravid adult animals scored are indicated (n) for each cross. Brackets indicate relevant comparisons and asterisks indicate $P < 0.01$ (χ^2 test in A, C, D, E). Orange font represent chromosomes with a recessive marker (see materials and methods).


A
P0
F1
Ta × +/+ → T/+
Ta × Ta/+ → T/+
n
51
27
0 fract. F1 1
mCherry
GFP
bright
dim
no
B
1 kb
F14E5.1
Punc-119
cbr-unc-119
Pmex-5
mCherry ::h2b
tbb-2 3' utr
gfp ::h2b
cye-1 3' utr
chr II
T
TΔ
TΔΔ
TΔΔΔ
chr I
Pmex-5::Dendra2::
h2b::tbb-2 3'utr (N)
chr IV
Pgtbp-1::gtbp-1::gfp::gtbp-1 3'utr (G)
C
P0
F1
Ta × +/+ → T/+
Ta × TΔa → T/TΔ
Ta × TΔΔa → T/TΔΔ
Ta × TΔΔΔa → T/TΔΔ
n
102
32
22
22
0 fract. F1 1
GFP
bright
dim
no
D
P0
F1
Ta × +/+ → T/+; +/+
Ta × +/G → T/+; +/+
T/+; +/G
Ta × D → T/+; N/+
n
24
42
27
34
0 fract. F1 1
mCherry
bright
dim
no
E
P0
F1
Ta × +/+ → T/+
Ta × TΔΔΔa/+ → T/+
n
21
25
0 fract. F1 1
mCherry
GFP
bright
dim
no
F
paternal
maternal
progeny
OFF
OFF
paternal
maternal
progeny
ON
ON

3.4.3 Sequence requirements for mating-induced silencing

Protection from mating-induced silencing and susceptibility to mating-induced silencing could have different sequence requirements. Therefore, we examined all deletion variants (Fig. 3-7*B*) by crossing males expressing the variant with hermaphrodites without the corresponding transgene. All variants were silenced (Fig. 3-8, also see Genetic Inferences in materials and methods), suggesting that elimination of an operon structure, histone sequences, and upstream *C. briggsae unc-119* sequences did not eliminate the susceptibility to mating-induced silencing. Thus, a minimal gene that has a *mex-5* promoter driving the expression of *mCherry* with *cye-1 3' utr* (*Pmex-5::mCherry::cye-1 3' utr*) is susceptible to mating-induced silencing.

Fig. 3–8. Variants that lack some sequences of the transgene *T* are also susceptible to mating-induced silencing.
(A–B) Hermaphrodites (*A*) or males (*B*) that express *Tα* or that express variants of *Tα* (as in Fig. 3–7*B*) with deletions in *gfp::h2b::tbb-2 3' utr* (*TΔα*), in upstream sequences (*TΔΔα*), and in *h2b* (*TΔΔΔα*) were mated with non-transgenic males or hermaphrodites, respectively, and mCherry fluorescence was scored in cross progeny. Scoring of silencing, number of animals assayed, orange font, brackets and asterisks are as in Fig. 3-7*A*.



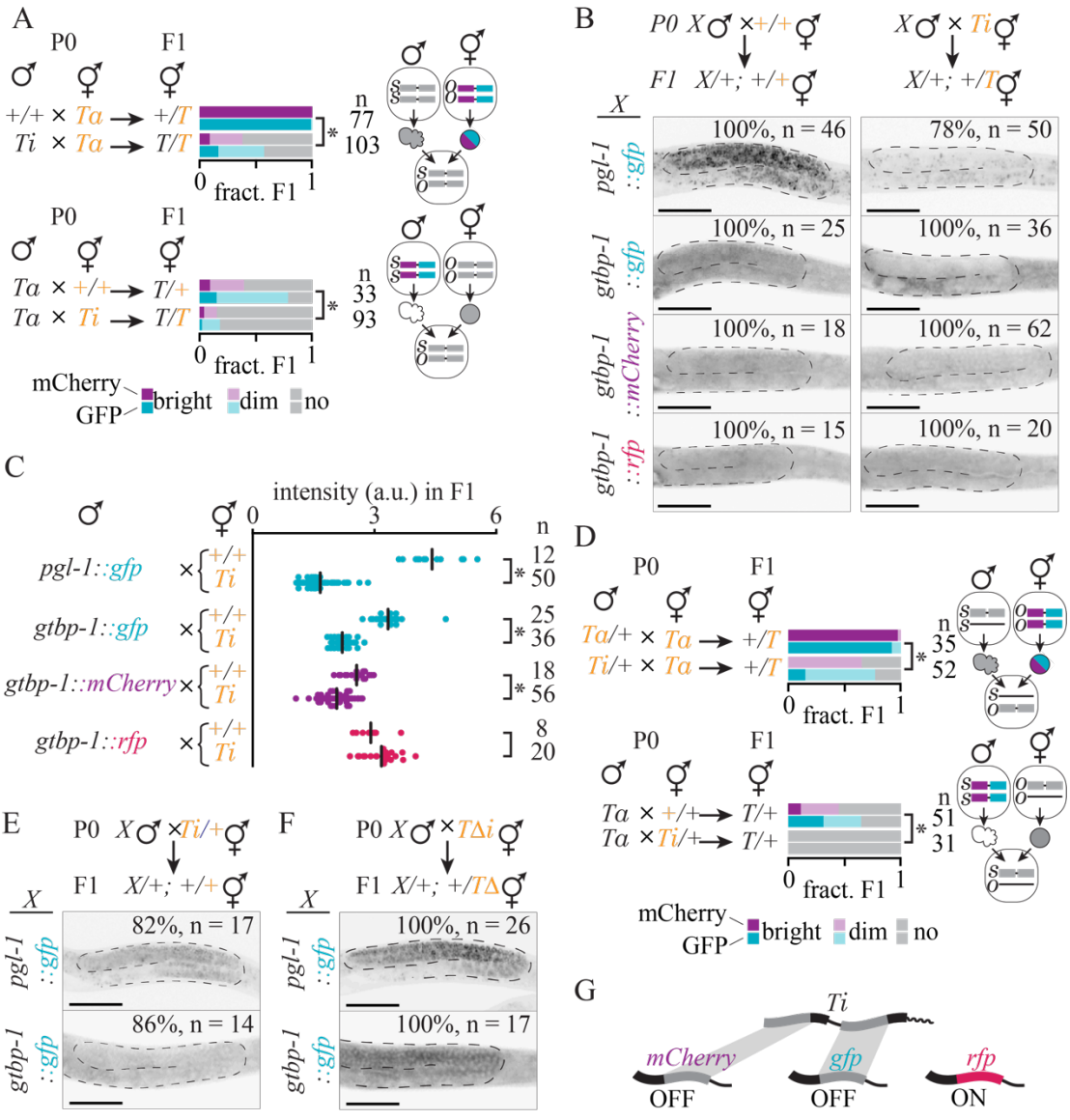
3.4.4 Mating-induced silencing generates a DNA-independent silencing signal

To dissect the properties of mating-induced silencing, we examined the interaction of the inactive, i.e. silenced, transgene (*Ti*) with other homologous sequences. Mating *Ti* males with *Ta* hermaphrodites resulted in cross progeny that showed silencing (Fig. 3-9*A*, *top*) and progeny from the reciprocal cross also showed a small increase in silencing (Fig. 3-9*A*, *bottom*). Thus, *Ti* can silence *Ta in trans*, especially when *Ti* is inherited through the sperm. To examine if *Ti* can silence other homologous loci, we mated *Ta* or *Ti* hermaphrodites with males expressing homologous (*gfp* or *mCherry*) or non-homologous (*rfp*) sequences tagged to endogenous genes present at other genomic loci (Fig. 3-9*B*, *C*). Animals with *Ti* showed silencing of *gfp* and *mCherry*, but not *rfp* (Fig. 3-9*B*, *C*). Interestingly, silencing of the ubiquitously expressed *gtbp-1::gfp* and *gtbp-1::mCherry* was restricted to the germline, and undetectable in somatic tissues (Fig. 3-9*B*). Thus, *Ti* can silence homologous genes expressed from different loci within the germline, suggesting that *Ti* generates a sequence-specific silencing signal that is separable from *Ti*. We therefore tested if parental presence of *Ti* could affect the expression of homologous sequences in progeny. We examined progeny of a hemizygous *Ti* parent that did not inherit *Ti* but did inherit *Ta* or a homologous gene from the other parent. Cross progeny showed silencing in both cases (Fig. 3-9*D*, *E*, also see Genetic Inferences in materials and methods). However, no silencing of the homologous gene was observed in cross progeny when parents that carried a silenced copy of a transgene that lacks the *gfp* (*TΔi*)

were mated with hermaphrodites with the homologous gene (Fig. 3-9*F*). Thus, mating-induced silencing generates a DNA-independent signal that can be inherited through both gametes and can silence homologous sequences in the germline of progeny (Fig. 3-9*G*).

Fig. 3–9. Mating-induced silencing generates a heritable DNA-independent silencing signal.
(A) *Ta* hermaphrodites (*top crosses*) or males (*bottom crosses*) were mated with animals that are non-transgenic (+/+) or with those that carried *Ti*, and fluorescence was scored in cross progeny. Schematics depict outcome of each test cross (as in Fig. 3-7*A*): a silenced transgene (*Ti*, grey) can silence *Ta* when progeny inherit each from different gametes. (B–C) Males that express homologous (***gfp*** or ***mCherry***) or non-homologous (***rfp***) sequences fused to endogenous genes (*X*) expressed in the germline (*pgl-1*) or ubiquitously (*gtbp-1*) were mated with non-transgenic or *Ti* hermaphrodites and fluorescence of GFP (PGL-1::GFP, GTBP-1::GFP), mCherry (GTBP-1::mCherry), or RFP (GTBP-1::RFP) was imaged (*B*) and quantified (*C*) in cross progeny. (D) *Ta* animals (hermaphrodites – *top crosses*; males – *bottom crosses*) were mated with animals that lacked *Ti* (*Ta*/+ in *top* or +/+ in *bottom*) or that carried the inactive transgene (*Ti*/+), and mCherry fluorescence was scored in cross progeny that only inherited *Ta*. Schematics depict outcome of each test cross (as in Fig. 3-7*A*): parental *Ti* can silence *Ta* in progeny, suggesting inheritance of a DNA-independent silencing signal (filled grey) through either gamete. (E) Males that express *pgl-1::gfp* or *gtbp-1::gfp* were mated with hemizygous *Ti* (*Ti*/+) hermaphrodites and GFP fluorescence from the tagged gene was scored in cross progeny that did not inherit *Ti*.

(F) Males that express *pgl-1::**gfp*** or *gtbp-1::**gfp*** were mated with *TΔi* hermaphrodites and GFP fluorescence from the tagged gene was scored in cross progeny. Germlines of representative cross progeny at L4 stage are outlined (*B*, *E*, and *F*). Scoring of silencing, number of animals assayed, and orange font are as in Fig. 3-7*A*. Brackets indicate relevant comparisons and asterisks indicate $P < 0.01$ (χ^2 test in *A*, *D*) or $P < 0.05$ (Student's *t* test in *C*). Percentage of animals with the depicted expression is indicated in each image. (G) Model depicting silencing of homologous (*mCherry* (shaded) in *gtbp-1::**mCherry*** and *gfp* (shaded) in *pgl-1::**gfp*** or *gtbp-1::**gfp***) but lack of silencing of non-homologous (*gtbp-1::**rfp***) genes by *Ti*.



3.4.5 Mating-induced silencing likely relies on the piRNA pathway for initiation and is actively maintained in each generation by a germline Argonaute

The spread of silencing to other loci was not observed in the absence of matching exonic sequences in *Ti* (Fig. 3-9*B*, *C*, *F*). Because this requirement is characteristic of silencing by antisense small RNAs in *C. elegans*, we examined whether genes implicated in RNA-mediated silencing also play a role in mating-induced silencing. Specifically, we tested the requirement of SID-1, RDE-1, RRF-1, NRDE-3, HRDE-1 and PRG-1. To test if each gene is required for initiation, we examined mating-induced silencing in the corresponding mutant backgrounds. Removal of *hrde-1* showed some desilencing and in support of *hrde-1* being required in initiation, independent observations (Pravrutha Raman, A.M.J laboratory) showed that initiation may completely depend on HRDE-1 (data not shown). However, substantial silencing was observed in all other cases except in animals that lack the *prg-1* gene (Fig. 3-10*A*, also see Genetic Inferences in materials and methods). Thus, initiation requires the germline Argonaute PRG-1 and potentially associated germline small RNAs called piRNAs (46). Because the minimal *Pmex-5::mCherry::cye-1 3' utr* is still susceptible to mating-induced silencing (Fig. 3-8), it is likely that piRNAs recognize a part of this sequence. Such piRNA-mediated silencing is expected to be stable for many generations (33). Consistently, we found that mating-induced silencing persisted for >20 generations without selection (Fig. 3-10*B*, Fig. 3-11). The silenced transgene retained the capacity to silence homologous genes in *trans* even after >200 generations (Fig. 3-12*A*) although the DNA-independent silencing signal was not

detectably inherited for more than one generation (Fig. 3-12*B-D*). However, unlike silencing of *Ta* by mating, silencing of *Ta* by *Ti* does not generate a DNA-independent signal (Fig. 3-12*E*). Therefore, the DNA-independent signal made in every generation does not account for the transgenerational stability of mating-induced silencing.

Fig. 3–10. Mating-induced silencing requires the Argonaute PRG-1 for initiation and the Argonaute HRDE-1 for maintenance.
(A) Mating-induced silencing was initiated as in Fig. 3–2 in a wild-type background or in different mutant (*g(-)*) backgrounds (*right*) and compared with control crosses of the same genotypes (*left*). Fluorescence from mCherry and GFP was scored in cross progeny for all tested mutants: *sid-1(-)*, *rde-1(-)*, *rrf-1(-)*, *nrde-3(-)*, *hrde-1(-)* and *prg-1(-)*. Wild-type crosses shown here are the same as in Fig. 3–7*A* and Fig. 3–4*A*. An additional wild-type cross with a different visible marker (mCherry: bright = 5, dim = 6, no = 25 and GFP: bright = 7, dim = 12, no = 17) was performed for comparison with *sid-1(-)* and *rde-1(-)* crosses on the right. (B) Homozygous F2 progeny obtained after initiation of mating-induced silencing were propagated by selfing for 23 generations. mCherry fluorescence intensity was measured in animals (boxes) at F1, F2, F10 and F25 generations. Presence of the transgene was verified by genotyping in F1 and F2 generations and descendants from 3 independent crosses were analyzed. See Fig. 3–11 for passaging scheme. (C–D) *Ti* hermaphrodites that had remained silenced for many generations (*Ti* gen. #) were mated with mutant males (*g(-)*) that lacked *hrde-1*, *nrde-3*, *rde-1*, *rrf-1*, or *sid-1* at the indicated generation and heterozygous cross progeny (*g(-/+)*) were allowed to give homozygous wild-type and homozygous mutant F2 progeny. mCherry and GFP fluorescence was scored in F1 cross progeny and F3 self-progeny of the F2 animals. Use of *prg-1(-/+)* males owing to the poor mating by *prg-1(-)* males in (A) and (D) is indicated (§). Silencing in *prg-1(+/-)* animals is depicted under wild-type F3 animals in the test for *prg-1* requirement. Scoring of silencing, number of animals assayed, orange font, brackets and asterisks are as in Fig. 3–7*A*.

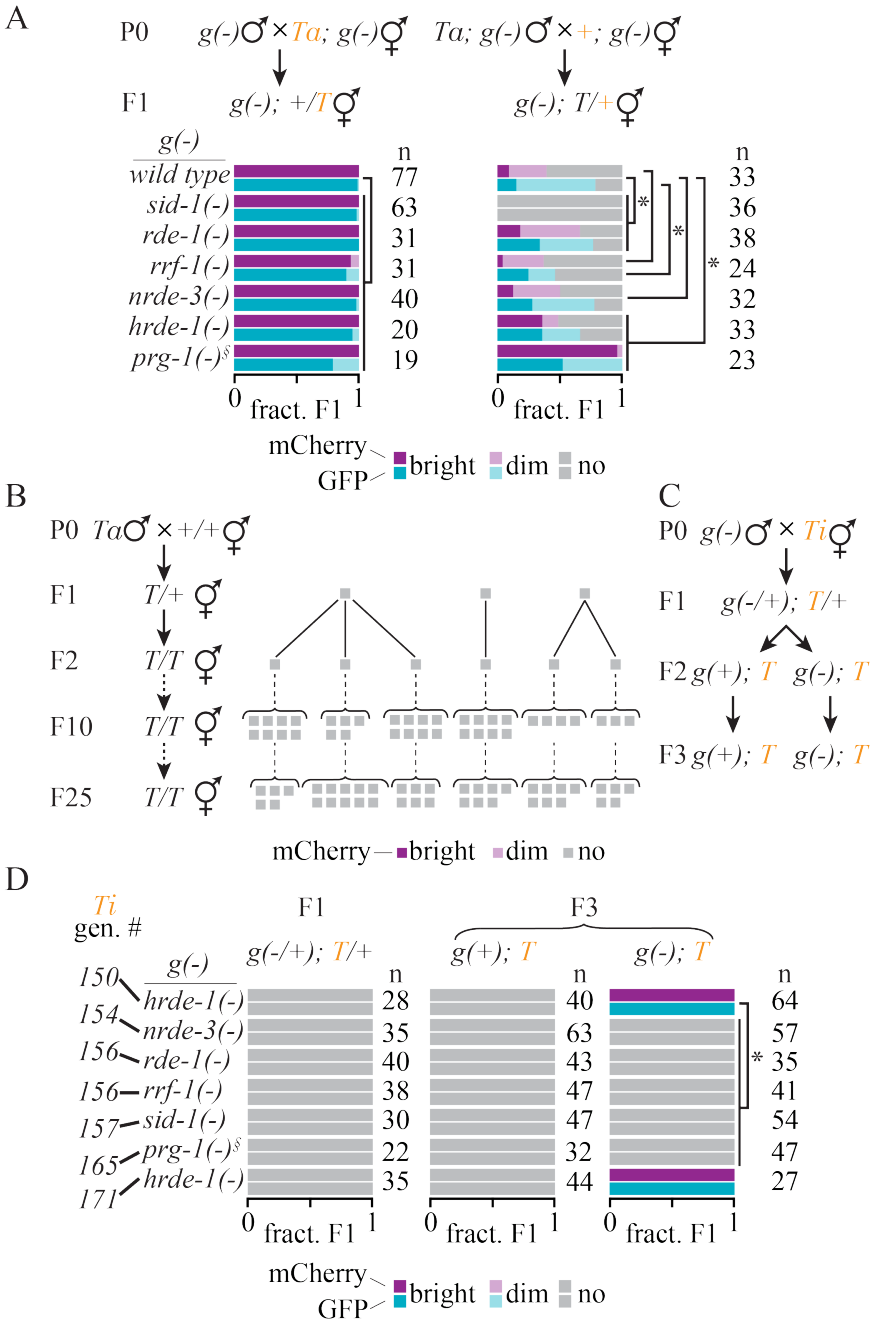

A
P0
g(-)♂ × Ta; g(-)♀
Ta; g(-)♂ × +; g(-)♀
F1
g(-); +/T♀
g(-); T/+♀
g(-)
wild type
sid-1(-)
rde-1(-)
rrf-1(-)
nrde-3(-)
hrde-1(-)
prg-1(-)§
n
77
63
31
31
40
20
19
33
36
38
24
32
33
23
*
0
fract. F1
1
mCherry
GFP
bright
dim
no
B
P0 Ta♂ × +/+♀
F1 T/+ ♀
F2 T/T ♀
F10 T/T ♀
F25 T/T ♀
C
P0 g(-)♂ × Ti♀
F1 g(-/+); T/+
F2 g(+); T g(-); T
F3 g(+); T g(-); T
D
Ti
gen. #
150
154
156
156
157
165
171
g(-)
hrde-1(-)
nrde-3(-)
rde-1(-)
rrf-1(-)
sid-1(-)
prg-1(-)§
hrde-1(-)
F1
g(-/+); T/+
F3
g(+); T
g(-); T
n
28
35
40
38
30
22
35
40
63
43
47
47
32
44
64
57
35
41
54
47
27
*

Fig. 3–11. Passaging scheme to evaluate persistence of transgenerational silencing. *Ta* males were mated with **wild-type hermaphrodites** and **silenced** hemizygous hermaphrodites (grey F1 worms) that lacked mCherry fluorescence were allowed to have homozygous self-progeny. Three silenced F2 progeny (grey F2 worms) were selected to propagate the strain for 23 more generations without additional selection. At each generation indicated, mCherry fluorescence was scored in siblings of the animals that were passaged. See Fig. 3–10*B* for data on fluorescence.

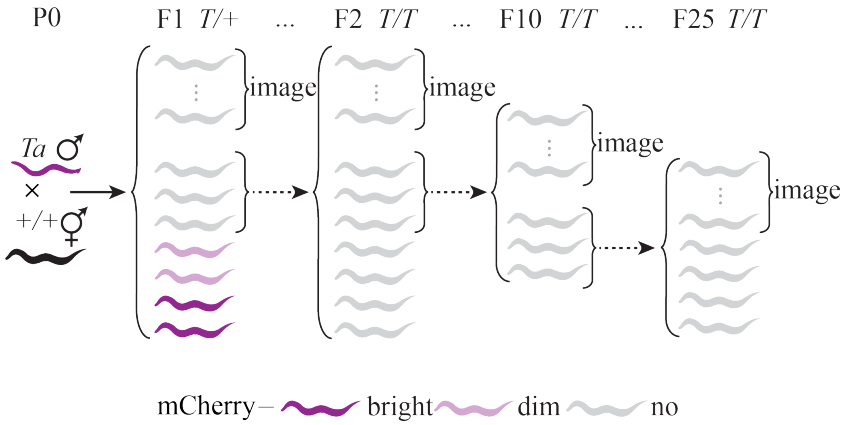
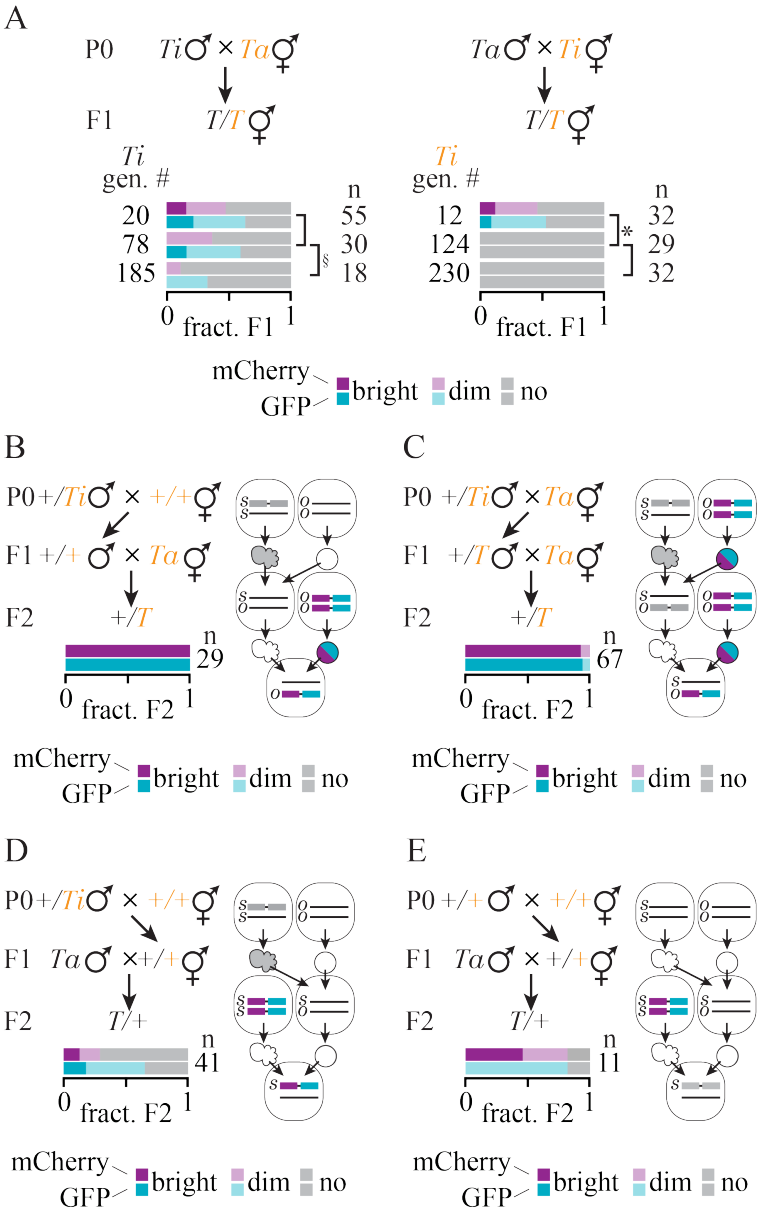


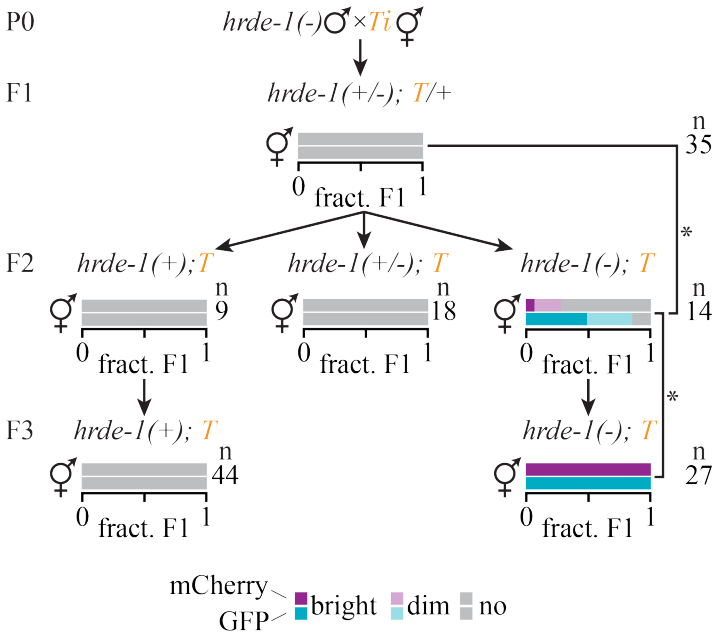
Fig. 3–12. The transgene silenced for >200 generations can silence an active transgene but cannot transmit the silencing signal for more than one generation. (A) *Ta* animals (hermaphrodites – *Left*; males – *Right*) were mated with *Ti* animals that remained silenced for many generations (*Ti* gen. #) after initiation by mating-induced silencing, and mCherry and GFP fluorescence was scored in cross progeny. The combined data from each cross is shown in Fig. 3–9*A*. (B–C) Hemizygous (*B*) *Ti* males (+/*Ti* in *B*) or non-transgenic (+/+ in *C*) males were mated with non-transgenic (+/+) hermaphrodites, resulting in cross progeny hermaphrodites that were then mated with *Ta* males (F1). The subsequent cross progeny (F2) were scored for mCherry and GFP fluorescence. Schematics depict outcome of each test cross (as in Fig. 3–7*A*): paternal inheritance of the DNA-independent silencing signal does not result in further transmission of the signal to descendants (*B*), suggesting that transmission through the sperm and subsequent inheritance of the DNA-independent silencing signal through the oocyte is limited to one generation. (D–E) Males that carry *Ti* (+/*Ti*) were mated

with non-transgenic (*D*) or *Ta* hermaphrodites (*E*), resulting in cross progeny males that were then mated with *Ta* hermaphrodites (F1). The subsequent cross progeny (F2) were scored for mCherry and GFP fluorescence. Schematics depict outcome of each test cross (as in Fig. 3–7*A*): paternal inheritance of the DNA-independent silencing signal does not result in further transmission of the signal to descendants (*D*) despite the presence of *Ta* in the animal (*E*), suggesting that inheritance of the DNA-independent silencing signal is limited to one generation. Scoring of silencing, number of animals assayed, orange font, brackets and asterisks are as in Fig. 3–7*A*. § indicates $P < 0.013$.



If maintenance of silencing for many generations relies on an active process, then loss of genes required for such silencing could result in the recovery of gene expression. Full recovery of gene expression was observed when *hrde-1* was eliminated even after >150 generations (Fig. 3-10*C*, *D*). Silencing persisted in the absence of every other gene (*nrde-3*, *rde-1*, *rrf-1*, *sid-1*, and *prg-1*) that was tested 154 to 165 generations after initiation of mating-induced silencing. Crucially, a subsequent retest of loss of *hrde-1* 171 generations after initiation also resulted in full recovery of gene expression (Fig. 3-10*C*, *D*, Fig. 3-13, also see Genetic Inferences in materials and methods). Current understanding of silencing by HRDE-1 suggests that nascent transcripts are recognized by antisense small RNAs bound to HRDE-1, resulting in the recruitment of histone modifying enzymes that generate H3K9me3 at the locus (54). The recovery of expression upon loss of HRDE-1 suggests that none of these events that depend on this Argonaute are transgenerationally stable, but rather silencing is actively established in every generation.

Fig. 3–13. Maternal rescue of HRDE-1 can maintain transgenerational silencing in some *hrde-1(-)* animals.
hrde-1(-) mutant males were mated with *Ti* hermaphrodites that remained silenced for 171 generations, and mCherry and GFP fluorescence was scored in heterozygous F1 cross progeny (*hrde-1(-/+)*), in F2 descendants that segregated different *hrde-1* genotypes and in F3 descendants that were homozygous wild-type (*hrde-1(+)*) or mutant (*hrde-1(-)*) for *hrde-1*. Refer to Fig. 3–10*C*, *D* for summary of *hrde-1* requirement. Scoring of silencing, number of animals assayed, orange font, brackets and asterisks are as in Fig. 3–7*A*.



3.5 Discussion

Modern genome engineering enables the precise introduction of any sequence into any genome. This study reveals that the fate of such sequences can change during genetic crosses. In progeny of males with a transgene and hermaphrodites without, piRNA-mediated transgenerational silencing is triggered. At genomic loci where this phenomenon can occur, mating of ancestors hundreds of generations ago could have triggered gene silencing that continues to be maintained.

3.5.1 Comparison of mating-induced silencing with related epigenetic phenomena

The hallmarks of mating-induced silencing are: (i) silencing is initiated upon inheritance only through the male sperm; (ii) once initiated, silencing is stable for many generations; (iii) transgenerational silencing is associated with a DNA-independent silencing signal that is made in every generation, can be inherited for one generation,

and can silence homologous sequences; and (iv) maternal exonic sequences can prevent initiation of silencing. While to our knowledge no other known phenomenon shares all of these hallmarks (Table 3-4), phenomena that share some of these features are highlighted below and can inform future mechanistic studies.

Paramutation refers to meiotically heritable changes in gene expression transferred from one allele ("paramutagenic") to another allele ("paramutable") when they interact within a cell (83). In addition to similar heritability, both paramutation (10-12, 14) and mating-induced silencing rely on small RNAs to spread silencing from one locus to another homologous locus. However, there are several aspects of paramutation that were found to be different from mating-induced silencing, when tested. First, a paramutagenic allele often requires associated repetitive sequences (84-86). Second, how a paramutagenic allele first arises remains obscure (83). Third, while some alleles are paramutable, others are not, for reasons that are unknown (12). The reliability of initiating and also protecting from meiotically heritable silencing at a defined single-copy locus described in this study will be useful in discovering possible shared mechanisms that have remained unclear in the ~60 years since the original discovery of paramutation in maize (10).

The unpredictable silencing that occurs at some single-copy reporter transgenes within the *C. elegans* germline has been called RNA-induced epigenetic silencing or RNAe (15). Some studies of RNAe (15, 16), but not others (p.94 in ref. 18) report genetic requirements for initiation and maintenance that are similar to those for mating-induced silencing – *prg-1* only for initiation and *hrde-1* only for maintenance. Although

transgenes silenced through RNAe are associated with more small RNAs than unsilenced transgenes (15), it remains unclear whether this quantitative increase in small RNAs is the cause or consequence of silencing. Nevertheless, a model proposing RNAe as a response to foreign or non-self DNA has emerged (15, 16, 18, 19). This model is inadequate because the same sequence can be either silenced or expressed within the germline (15) and endogenous genes are subjected to transgenerational silencing through similar PRG-1- and HRDE-1-dependent mechanisms (35, 17, 46, 54, 87). Furthermore, the features of a transgene that trigger silencing are unknown. Tethering the Argonaute CSR-1 to the nascent transcript (88) or adding intronic sequences that are found in native germline-expressed genes (31) can increase the frequency of expression of a foreign sequence but does not itself determine whether a sequence is expressed. Thus, despite these efforts, the mechanisms that enable stable expression or silencing of a gene across generations remain unclear.

Unlike RNAe, mating-induced silencing can be predictably initiated and thus provides a reliable assay for evaluating how organisms establish stable expression or silencing of a gene. Our analyses suggest that the decision to express paternal foreign sequences (*mCherry* and *gfp*) is re-evaluated in each generation based upon maternal mRNA (Fig. 3-7). Although mating-induced silencing is not a general property of transgenes (Fig. 3-1), a similar silencing phenomenon with dependence on maternal mRNA has been observed for the endogenous gene *fem-1* (ref. 25). However, it is unknown whether this *fem-1* silencing also shares the *trans* silencing properties and genetic requirements of mating-induced silencing.

Taken together, the paradigm of mating-induced silencing established here provides a reliable model to study epigenetic mechanisms that dictate expression or silencing of a sequence in every generation in otherwise wild-type animals.

3.5.2 Implications for genetic studies

The field of genetics relies heavily on analyses of animals generated by mating. Our study reveals that the direction of a genetic cross could strongly influence the phenotype of cross progeny. Additionally, because not every sibling from a cross has the same phenotype, the choice of the sibling selected for further manipulation can have a profound effect. Subsequent transgenerational persistence of silencing can make phenotype independent of genotype, resulting in erroneous conclusions. Thus, when using genetic crosses to generate strains both the direction of the genetic cross and choice of the individual cross progeny selected for propagation needs to be controlled for – especially when evaluating epigenetic phenomena. For example, we ensured that every cross was performed with the transgene present in the hermaphrodite to avoid initiating mating-induced silencing in our studies examining silencing by dsRNA from neurons (see Chapter 2). Such methodological considerations impelled by this study could impact conclusions drawn from previous studies of epigenetic silencing in *C. elegans*.

3.5.3 Possible impact on evolution

Our results reveal a mechanism that silences genes in descendants in response to ancestral mating. The transgenerational stability of this gene silencing with the

possibility of recovery of expression even after 170 generations (Fig. 3-10) suggests that this mechanism could be important on an evolutionary time scale. Genes subject to such silencing could survive selection against their expression and yet be expressed in descendants as a result of either environmental changes that alter epigenetic silencing or mutations in the silencing machinery (e.g. in *hrde-1*). This mechanism thus buffers detrimental genes from selective pressures akin to how chaperones buffer defective proteins from selective pressures (89). Many endogenous genes in *C. elegans* are silenced by HRDE-1 (refs. 15, 35, 54, 90), some of which could have been acquired when a male with the gene mated with a hermaphrodite without the gene. An interesting direction to explore next is to examine whether this mechanism facilitates adaptation.

Chapter 4: General Discussion

4.1 Introduction

Parental experiences had previously been shown to affect development and gene regulation in progeny but there was no clear mechanistic explanation for it. The work in this dissertation demonstrates that (i) somatic cells that interact with the environment, such as neurons, can send sequence-specific information to the germline in the form of dsRNA, and transmit this information to descendants in the form of gene silencing, and (ii) mating can induce silencing of a susceptible locus without an environmental trigger and this silencing can be robustly inherited for hundreds of generations.

4.2 Comparison of silencing triggered by neuronal dsRNA and mating-induced silencing

We stumbled upon a transgene locus expressed in the germline that, among all the loci tested, was uniquely susceptible to transgenerational silencing by a single generation of exposure to dsRNA transported from neurons or in a single genetic cross of males with the locus to hermaphrodites without the locus. It is interesting to observe that the most significant differences between the two processes are at the level of initiation of silencing (Table 4-1), and the similarities they share are at the level of maintenance of silencing (Table 4-2). Both of the discoveries were only possible because this one locus was particularly susceptible to transgenerational silencing. This

raises the possibility that the capacity to carry information across generations is dictated by the locus and is independent of the mechanisms that initiate silencing. It is imaginable that in evolution, while a parent animal could employ multiple ways to transmit information from somatic cells that experience the environment or information within germline that is induced by the products of parental cytotypes interacting, only some loci in the genome may be responsive to this information to pass it to descendants.

Table 4–1. Differences between silencing by transport of neuronal dsRNA to the germline and mating-induced silencing.

Silencing by neuronal dsRNA	Mating-induced silencing	Fig. reference in this dissertation
Initiation is triggered by dsRNA transported from neurons, and requires SID-1 and RDE-1.	Initiation likely relies on piRNAs because it requires PRG-1 and does not require SID-1.	2-4, 2-7 vs. 3-10
Both genes in the soma and germline are susceptible to silencing for one generation.	Only one gene has been found to be susceptible.	2-1, 2-5 (also see refs. 61, 63) vs. 3-1
Initiation of multigenerational silencing depends on the exposure to dsRNA for at least one generation.	Initiation of multigenerational silencing does not need exposure to an induced trigger.	2-4 – 2-7 vs. 3-2

Table 4–2. Similarities between silencing by transport of neuronal dsRNA to the germline and mating-induced silencing.

Similarity between silencing initiated by neuronal dsRNA and mating-induced silencing	Fig. reference in this dissertation
Transgenerational silencing can be triggered at one specific locus (*oxSi487*) and not at other loci tested.	2-4 – 2-7 vs. 3-1, 3-2
One generation of triggering mechanism is sufficient to cause transgenerational silencing for >25 generations.	2-5, 2-6 vs. 3-10
Both genes in the operon (*gfp* and *mCherry*) are silenced, although less robustly by neuronal dsRNA.	6-1, 6-2 vs. 3-4 – 3-13
The silencing initiated in the germline does not spread to homologous somatic loci or even pharyngeal *gfp* expressed from the same locus.	2-14 vs. 3-9
Initiation of silencing does not require the RdRP RRF-1 and likely depends on at least another RdRP.	2-4, 6-2 vs. 3-10
Maintenance of silencing depends on the germline Argonaute HRDE-1.	2-7, 6-3 vs. 3-10, 3-13
Multigenerational silencing can be disrupted by removing *hrde-1*, but cannot be revived by reintroducing *hrde-1*, suggesting that the signal used in maintenance depends on *hrde-1* to be generated in every generation.	6-3 vs. 6-20

Unlike other loci, the property for the locus *T* to be especially susceptible to parental experience – whether by neuronal dsRNA or by mating – could reflect a difference in the three-dimensional arrangement of molecules that is reproduced in every generation around the locus (32). In support of this view, perturbation of cellular components and processes in *C. elegans* has been shown to affect the susceptibility of loci to transgenerational silencing. For example, removing PRG-1, the Argonaute that

silences transposons using piRNAs and that is required in mating-induced silencing, can make essential developmental genes susceptible to silencing by misrouting the endogenous RNAi proteins (87, 91). However, a complementarity to piRNAs alone does not guarantee susceptibility because not all genes with piRNA target sites can be silenced (Fig. 3-1 and ref. 15). Loss of MET-2, a methyltransferase of H3K9me1/2 marks, caused genes that were previously licensed for expression to become potently susceptible to transgenerational silencing (92). Loci that tend to become particularly susceptible to silencing by small RNAs with a change in cellular components, such as mutations in the endogenous RNAi genes, are targets of the germline Argonaute CSR-1 (87, 91). Therefore, molecularly dissecting all CSR-1 dependent loci, by either examining the sequence characteristics (e.g. if they are repeats in the genome, if they are associated with specific histone modifications, if they are present as operons), by examining the expression pattern of the gene product (e.g. timing of expression, localization and function within a cell) or by examining specific interactions of the loci (e.g. if they bind to components of the piRNA pathway), might distinguish susceptible loci from the resistant ones. Although speculative at this time, a subset of these loci could also be targets of behavioral information transmitted through somatic cells in the form of endogenous dsRNA (93, 95).

4.3 Male-specific inheritance of epigenetic information

DNA methylation is associated with inheritance through the paternal germline in mammals. In mice, retrotransposon-induced DNA methylation state of the *Axin-*

fused gene has been reported to be transmitted from the sperm that results in changes within the soma of the progeny (95). But, any DNA methylation that is inherited must escape two waves of epigenetic reprogramming that the mouse embryo goes through (60), and therefore it is still debated whether DNA methylation is causal in inheritance of parental experiences – especially because DNA methylation does not persist in late-gestation somatic tissues (96) and genetic variation was sufficient to explain changes in the sperm methylome (97). Therefore, if DNA methylation is required for transmission of parental experiences, further work is needed to test how these marks are resistant to epigenetic reprogramming.

There is relatively strong evidence for the transmission of environmental information through the male germline using small RNAs. Because small RNAs can be transmitted without being tightly linked to genomic sequences, they can potentially be used in a more versatile manner (e.g. with respect to timing of production, localization of function) as compared with epigenetic reprogramming as the latter is defined to affect only modifications on the DNA and histones (1). Protein restriction or high fat in paternal diet in male mice could be communicated through small RNAs present in the mature sperm to cause metabolic changes and endogenous gene silencing in offspring (98, 100). Such information could be transmitted by fusion of extracellular vesicles in the epididymis that contain tRNA fragments and immature sperm (98). Injection of sperm RNA from male mice that were subjected to traumatic stress into wild-type fertilized oocytes produced progeny and grand progeny with behavioral patterns of the traumatized male and altered microRNA expression in the stress-

response region of the brain (100). In *C. elegans*, male-specific small RNAs promote thermotolerance in sperm (101) and can be inherited to maintain the memory of gene expression of spermatogenesis and oogenesis in progeny (23). Diffusible silencing signals, potentially secondary siRNAs, that were generated by ancestral exposure to dsRNA were more potent through the sperm than through the oocyte (51). Interestingly, disabling epigenetic reprogramming in *C. elegans* is correlated with spermatogenesis defects (102), changes in methylation at histone H3K4 (18) and an increase in DNA methylation at N^6 adenine (6mA, ref. 103). Because histone modifications are tightly linked to small RNAs, it is likely that the paternal effects are communicated through an interplay between epigenetic marks and small RNAs. Mating-induced silencing provides evidence for specific behavior of paternally inherited sequences and silencing signals (see Fig. 6-19). Therefore, examining whether specific RNAs and epigenetic marks are present in males that express the transgene (*Ta* males) and in males that transmit the silencing signal (*Ti* males) may inform what properties of the locus allow the apparent resistance to transgenerational homeostasis.

4.4 Evolution of genes introduced through the male germline

The phenomenon of mating-induced silencing reveals mechanisms the worm has evolved to recognize a male-inherited sequence, but the fate of the sequence is dictated by whether or not the sequence is present in the hermaphrodite genome (3-7*A*). This is reminiscent of piRNA-mediated repression of paternally inherited transposons that are absent in the maternal genome in *Drosophila* (13), especially because mating-

induced silencing also requires the piRNA Argonaute PRG-1 to silence the paternally inherited transgene (Fig. 3-10*A*). However, unlike in the case of *Drosophila* where mating of such incompatible strains results in dysgenic offspring – possibly due to transposon-mediated gene disruption – mating-induced silencing does not cause developmental defects in the progeny but triggers apparently indefinite silencing of the transgene. It is perhaps conceivable that the descendants whose genomes now contain this transgenic sequence inherited newly from an ancestral male could repurpose the gene, after keeping it transcriptionally inactive for hundreds of generations, which can result in gradual changes over an evolutionary timescale. In support of this view, several genes that originated *de novo* in *D. melanogaster* (104) or during divergence from *D. willistoni* (105) and genes that originated by retrotransposition of non-coding sequences in human lineage (106) evolved functions with roles in the male germline, suggesting that the male germline is where a newly acquired sequence first becomes "domesticated" before developing into a functional gene over millions of years (further discussed in ref. 107).

4.5 Conclusion

This dissertation reveals the existence of certain loci that the worm renders particularly susceptible to indefinite change in gene expression. The barrier likely lies at the level of the locus rather than at the tissue level because even silencing within the germline does not necessarily cause silencing across generations. Because many genes are affected in response to a single environmental stimulus and cause effects across

generations, it could be that the genes that remain affected across many generations are the ones that first underwent a change in some ancestral generation.

Chapter 5: Future Directions

5.1 Preface

The thoughts presented in this chapter were, in addition to discussions with Antony in lab and in meetings, largely influenced by the ideas presented in: Jose AM, 2018. Replicating and Cycling Stores of Information Perpetuate Life. *Bioessays*.

5.2 Introduction

This dissertation describes the discovery of a single engineered locus whose expression can be switched from active to permanently inactive in two ways – using sequence-specific information delivered from neurons or using a genetic cross. In this chapter, I will discuss how this discovery could inspire future work in the field of Biology.

5.3 Implications of neuronal dsRNA transport into the germline resulting in transgenerational gene silencing

Movement of sequence-specific information is a process conserved across the animal kingdom (108). Evidence for RNA movement has been found in many animals such as in insects (109, 110), mice (111), humans (73, 74) and *C. elegans* (108). In this dissertation, we showed that neurons can export dsRNA into the germline and alter gene expression that can last for many generations. While the permanence of switch in gene expression is a property inherent to the target gene (Fig. 2-14 and unpublished

work by F. Ettefa, Jose Lab), this discovery informs some interesting directions to explore in the near future.

A survey of different tissues in *C. elegans* suggests that neurons are the most capable of exporting dsRNA that can silence genes in other tissues (61). Whether other somatic tissues can similarly transport sequence-specific information to the germline and across generations remains unknown. If known, such an observation could elucidate the possibility that non-neuronal tissues that interact with environmental triggers such as diet (intestine, muscles), temperature (hypodermis, muscles), chemicals (hypodermis) etc. may send signals in a dsRNA-dependent form through the germline to descendants. The existence of machinery that transmits somatic information to the germline and across generations could indicate a broader role for RNA transport in the development of an animal. Because SID-1 is required for import of dsRNA and MUT-7 and HRDE-1 for the initiation and propagation of silencing (Fig. 2-7), any endogenously generated dsRNAs in the worm could be identified using animals that lack *sid-1*, *mut-7* and *hrde-1*. As both *sid-1* and *mut-7* genes are conserved in humans (SIDT2 and EXD3), it is conceivable that such mechanisms of transport of somatic RNAs to the germline is a conserved process. Therefore, identification of endogenous dsRNAs as well as additional machinery such as the exporter protein(s) and inter-tissue transporter(s) could be the precursor to understanding dsRNA transport in different phyla.

Once endogenously made dsRNAs have been identified and validated (e.g. by qRT-PCR or northern blotting) in *C. elegans*, *in vivo* labeling of sequence-specific

dsRNA could be used to visually detect movement of the identified dsRNAs from between tissues. As a starting point, endogenously made dsRNAs can be engineered with target RNA sites for molecular beacons (112) or bacteriophage MS2 system (113) to visualize movement in live tissues. In some cases, intercellular movement may be restricted to a subset of dsRNAs and can be visualized using dsRNA-binding fluorescence complementation – a technique that employs fluorescent protein reconstitution when two dsRNA-binding proteins, each fused to one half of a fluorescent protein, are brought together on a target dsRNA (114). For example, this method could be useful when discriminating long dsRNA that binds RDE-4 with a higher affinity than short dsRNA (115). In addition, all endogenously made dsRNA can be visualized by staining cells simultaneously with monoclonal anti-dsRNA antibodies (e.g. J2 and 9D5 (ref. 116). The endogenous population of dsRNA could be measured to see if the levels and co-localization of the antibody signals corresponding to dsRNA can change upon removal of genes necessary for dsRNA stability (*rde-4*) or movement (*sid-1*).

Given that small RNAs have been seen in circulation in disease conditions as well as in response to environmental stimuli, RNAs perhaps play a general role in adaptation of an organism to its surroundings (93). In *C. elegans*, exposure of an odor for up to five generations has been reported to result in a change in the olfactory imprint causing a stark behavioral change inherited for many more generations (117). Such imprints across generations could be communicated from the ancestral generations using dsRNA from neurons. This possibility can be explored by determining whether

SID-1 is required for inheritance of behavioral changes, and if other olfactory imprints can similarly be created by exposure of the worm to other odors, and perhaps even to other stimuli. Evidence for such a possibility could open an exciting field of behavioral engineering that uses the simple system of dsRNA transport. Because neurons form the predominant tissue that senses the environment and have been shown to endogenously produce non-coding RNAs that influence development and memory (45, 118), they are ideal for engineering behavioral changes across generations by expression of dsRNA matching a gene required in development or behavior.

Similar to the process in *C. elegans*, RNAs secreted from cells in other organisms are known to provide viral immunity in mammals. In mice, the homolog of *C. elegans* SID-1, Sidt2, is required to provide immunity by transporting dsRNA associated with viruses from endocytic compartments to the cytosol, resulting in the production of interferons and enhanced immunity in bystander cells (119). Trophoblasts that make up the outer layer of placenta in humans can deliver miRNAs in extracellular vesicles to recipient cells of the fetus resulting in increased resistance to viral infections (120). However, even though mammalian gametes have been shown to carry non-coding RNAs in extracellular vesicles (98), whether such transported RNAs change gene expression across generations is unclear. Therefore, one exciting direction to explore would be to determine the properties of RNAs (e.g. sequence structure, associated proteins, molecules on the extracellular vesicles that provide specificity etc.) inherited in immunity and engineer RNAs that provide antiviral immunity across generations.

5.4 Implications of mating-induced silencing

Findings from mating-induced silencing suggest that mechanisms in the worm can recognize certain sequences and cause silencing when inherited from the male. Because initiation requires the piRNA Argonaute PRG-1, it is possible that there are other genes within the genome that are similarly silenced, but not detected as a defect from a mating. Thus, it would be interesting to identify if certain loci are specifically silenced in cross progeny from a mating by measuring changes in the transcript levels of endogenous genes in cross progeny obtained from reciprocal crosses. Any such loci could be marked as susceptible loci within the genome. Additional evidence suggests that the abundance/stability of the transcript could make the loci susceptible to silencing (Fig. ## in Appendix chapter). Such abundance could be used as an indicator to identify susceptible loci within the genome – perhaps because the transcripts are more available for silencing and subsequently, for the generation of secondary siRNAs.

Whether the non-genetic conflict between hermaphrodite and male genomes that arises during mating-induced silencing can be seen in other wild isolates of *C. elegans* and if it is conserved across other gonochoristic members of the *Caenorhabditis* clade is unknown. To begin with, one could simply test if mating different wild isolates of *C. elegans* such as N2 Bristol hermaphrodites and Hawaiian males or vice-versa results in transgenerational changes in the expression of genes specific one of the parental genomes. This test can then be expanded to other *Caenorhabditis* species that are compatible in a mating. Because *prg-1* is conserved across the *Caenorhabditis* clade and is required to initiate mating-induced silencing,

mutants that lack *prg-1* could also be tested to verify if any detectable transgenerational change in gene expression is now abolished.

5.5 Directions to explore that could explain how life is perpetuated

In each generation of an organism, the information present in minimally a single cell dictates the organism's development. We have seen in this work that a single transgene locus can be amenable to indefinite change whereas other loci tested were not, thus indicating that while the one locus *T* is particularly susceptible, all other loci tested show resistance to changes. These observations lay down the framework for transgenerational homeostasis as has been described (32). To understand the basis for such homeostasis, it is necessary to dissect the features of the susceptible loci and compare them to resistant loci. Any differences (e.g. distribution of small RNAs, repressive chromatin marks like H3K9me3, variation in stability of transcripts – see Fig. ## in Chaper 6) that correlate with resistance vs. susceptibility can be used to switch the properties of different loci using various molecular tools (see Fig. ## in Chapter 6 for preliminary speculation). For example, deactivated CRISPR-Cas9 (121) can be fused to a histone-methylase to modify the chromatin state of a target locus while enzymatic CRISPR-Cas9 can be used for the precise modification of DNA sequences associated with a target locus and susceptibility can be tested in each case. Furthermore, DNA-IP followed by mass spectrometry can be used against the transgene locus to identify the predominant protein complex associated with the transgene locus. This result can be complemented with ChIP-exo sequencing (122) as

well as live-cell imaging of the identified transcription factors (123) along the transgene locus. To determine if there are differences in the 3D-distribution of genetic loci within the nucleus, the structure of the genome in an individual cell could be studied (124). Finally, such examination could be extended to determine whether other DNA sequences within the genome show similar interactions with other proteins or components of the cell to identify an initial list of loci that could be susceptible to modification over generations.

Because it would be incomplete and inaccurate to describe the idea of the cell code from the perspective of a single locus, an understanding of how gene expression patterns are perpetuated for life must consider at once how many different loci interact with other components within the cell such as proteins, organelles, metabolites etc. (32). A biological model that can be used to make such an inquiry is simply the single-cell zygote of an animal. The *C. elegans* zygote provides an ideal system because development in no other organism is so well-defined at the cellular and molecular levels and is also susceptible to robust transgenerational effects. A living *C. elegans* zygote can be subjected to techniques that measure the interaction of genes with proteins in the intermediate future (next ~10 years). To determine molecular interactions using a live worm zygote, gene expression and transcription factor dynamics can be precisely measured at a subset of both resistant and susceptible loci (122, 123, 125) from immediately after the fertilization of the oocyte through the single-cell stage. In addition, multi-spectral microscopy can be adapted to visualize interactions between cell-organelles (126). Such approaches can potentially begin to measure the cell code

of an animal in a rudimentary yet informative manner. To reinforce the results, the observations from live-cell imaging during the development of a zygote can be complemented by measuring biochemical interactions (e.g. genes with transcription factors, enzymes with substrates) and molecular genetics (e.g. if mutations that disrupt the transcription factor or enzyme causes interactions that are subsequently detrimental to the development of the zygote). Any reproducible arrangement of molecules within single-cell zygotes must correspond to the necessary spatio-temporal positions that propagates the cell code. After deduction of a rudimentary cell code, one can determine how the cell code can change under conditions of disease (127), stress, physical forces, environmental stimuli etc. Because components of a cell must need to go through similar interactions each generation to give rise to nearly the same organism, understanding how such information is transmitted between generations using the minimal number of cells is key. In the more distant future, it would be exciting to determine how such spatio-temporal information is encoded and transmitted (32) – whether the information is in the form of a physical reproducible template, perhaps provided by cytoskeleton lattices (128), and/or is in the form of a time-dependent series of molecular interactions that precedes all other interactions resulting in an organism in every generation.

5.6 Conclusion

We found a locus that can be manipulated to discover how and what epigenetic changes can be introduced at a locus that can be reproduced for hundreds of

generations. The identification of only a single locus that can by modified by parental experience across generations reveals to us that most loci within the genome are resistant to indefinite changes. Thus, such resistance provided by transgenerational homeostasis consequently results in the reproducibility of gene expression at the start of every successive generation. This dissertation provides a foundation for understanding mechanisms within the progenitor cell(s) that determine gene expression states in every generation, not only within *C. elegans* but in all cases where transgenerational studies are possible in Biology.

Chapter 6: Appendix

6.1 Preface

Maïgane Diop, an undergraduate I mentored, generated the data for Figs. 6-9, 6-17, and 6-18. Eunice Cho, an undergraduate I mentored, generated the data for Fig. 6-11. The remaining work in this chapter was contributed by me.

Some resources used in this chapter are as mentioned in Chapter 3.

6.2 Introduction

We have shown that dsRNA made in neurons can move to the germline and initiate transgenerational silencing. Further questions that address how transgenerational silencing is maintained, whether the worm makes endogenous dsRNAs to control gene regulation and whether these RNAs move between tissues, including the germline, and cause transgenerational effects remain to be answered. In the first part of this chapter, I present some preliminary results that examine *hrde-1* requirement for the maintenance of silencing, and that test if neuronal mobile RNAs can silence an endogenous germline gene.

The peculiar observation of silencing that is initiated by mating males with a transgene to hermaphrodites that lack the transgene reveals that the worm has evolved unexpected mechanisms to turn off gene expression across generations, independent of the laws of inheritance put forth by Mendel. While there are several other epigenetic phenomena that are also non-Mendelian, mating-induced silencing displays unique

characteristics that have not been observed before (Table 3-4) – such as the existence of maternal protective signal(s) and the *trans* silencing signal that are both separable from the transgene locus. In the second part of this chapter, I expand on the properties of these DNA-independent signals and further probe into what triggers mating-induced silencing.

At the end of each preliminary result, I will briefly discuss future experiments that are informed by each observation.

6.3 Materials and methods

6.3.1 Strains and primers used.

Table 6–1. Strains used.

Strain	**Genotype**
AMJ300	*qtIs49* III; *nrIs20* IV
AMJ364	*jamEx164*
AMJ506	*prg-1(tm872)* I; *oxSi487* II; *unc-119(ed3)*? III
AMJ552	*oxSi487 dpy-2(jam33)* II; *unc-119(ed3)?* III
AMJ577	*hrde-1(tm1200)* III *[4× outcrossed]*
AMJ581	*oxSi487 dpy-2(e8)* II*; unc-119(ed3)?* III
AMJ602	*oxSi487 dpy-2(e8)* II*; unc-119(ed3)? hrde-1(tm1200)* III
AMJ654	*oma-1(zu405)* IV*; sid-1(qt9)* V
AMJ667	*dpy-20(e1282) ax2053* IV

AMJ692 *oxSi487 dpy-2(e8)* II; *unc-119(ed3)?* III

AMJ709 *dpy-10(jam21) jamSi25 [Punc-119deletion *oxSi487]* II

AMJ713 *dpy-2(e8) unc-4(e120)* II*; Ppgl-1::pgl-1::gfp::pgl-1 gfp 3' utr* IV

AMJ715 *oma-1(zu405)* IV*; jamEx163*

AMJ716 *oma-1(zu405)* IV*; jamEx163*

AMJ717 *oma-1(zu405)* IV*; jamEx163*

AMJ723 *oma-1(zu405)* IV*; jamEx163*

AMJ724 *oxSi487* II*; unc-119(ed3)?* III

AMJ725 *oxSi487* II*; unc-119(ed3)?* III

AMJ765 *jamSi18 [dpy-10(cn64) Pmex-5::mCherry::H2B::cye-1 3'UTR *oxSi487]* II*; unc-119(ed3)* III

AMJ767 *jamSi20 [dpy-10(cn64) Pmex-5::mCherry::H2B::cye-1 3'UTR *oxSi487]* II; *unc-119(ed3)* III

AMJ774 *jamSi23* II

AMJ777 *dpy-10(cn64)* II

AMJ844 *oxSi487 dpy-2(e8)* II*; unc-119(ed3)?* III

AMJ918 *jamSi32 [Pmex-5::mCherry(3bpΔ)::H2B::cye-1 3'UTR *jamSi19]; unc-119(ed3)* III

AMJ919 *jamSi33 [Pmex-5::mCherry(2bpΔ)::H2B::cye-1 3'UTR *jamSi25]* II

AMJ920 *dpy-2(e8) unc-4(e120)* II*; ax2053[gtbp-1::gfp]* IV

AMJ922 *prg-1(tm872)* I [1x]*; dpy-2(e8) oxSi487* II*; unc-119(ed3)?* III

AMJ923	*prg-1(tm872)* I [1x]*; dpy-2(e8) unc-4(e120)* II
AMJ926	*jamSi27[Pmex-5::mCherry::cye-1 3'UTR *jamS25] dpy-10(jam39)* II
AMJ928	*jamSi27[Pmex-5::mCherry::cye-1 3' utr *jamSi25]* II [*T*ΔΔΔ]
AMJ930	*dpy-10(jam68)* II
AMJ931	*dpy-10(jam69)* II
AMJ932	*dpy-10(jam70)* II
AMJ1100	*oxSi487 unc-4(e120)* II; *unc-119(ed3)?* III
AMJ1101	*oxSi487 unc-4(e120)* II; *unc-119(ed3)?* III
AMJ1102	*oxSi487 unc-4(e120)* II; *unc-119(ed3)?* III
AMJ1103	*oxSi487 unc-4(e120)* II; *unc-119(ed3)?* III
EG6787	*oxSi487* II; *unc-119(ed3)* III
GE1708	*dpy-2(e8) unc-4(e120)* II
HC195	*nrIs20* IV
OCF62	*jfSi1* [*Psun-1::gfp cb-unc-119(+)*] II*; ltIs38* [*(pAA1) pie-1::GFP::PH(PLC1delta1) + unc-119(+)*] [gift from Orna Cohen-Fix]
TX20	*oma-1(zu405)* IV

Table 6–2. Primers and smFISH probe sequences used.

P82	GAAAGATTTGGAAGAAGAGGCACG
P83	CATTTTTATTGAAACTCCACCATTTTTC
P84	TTCCTTTCTCCGGTAGTAGTGC

P85 GTTTTCACCGTTAACGTTCATCGTCGTCGTCGTCGATGC

P86 GCATCGACGACGACGACGATGAACGTTAACGGTGAAAAC

P87 TGTTCACCACAAATTGAGATGC

P88 AGCCAAAAAACCTCTGTGGCC

P89 CAAGCATCTCAATTTGTGGTGACATTTCCGCCGGTCATTCTG

P90 CAGAATGACCGGCGGAAATGTCACCACAAATTGAGATGCTTG

P91 ATGAACGTTAACGGTGAAAAC

P92 GTCAGTGGAGAGGGTGAAGG

P93 AAGAGTGCCATGCCCGAAG

P94 CCATCGCCAATTGGAGTAGTT

P95 TTTCTGTCAGTGGAGAGGGTG

P96 TGATGATAGCCATGTTATCC

P97 GTGGACCTTGAATCTCATGA

P98 CTCTCCCTCGATCTCGAACTCGTGTC

P99 CTTGGTGACCTTAAGCTTAG

P100 GATATCCCAAGCGAATGGAA

P101 CGTACATGAACTGTGGGGAA

P102 TGCTTGACGTAAGCCTTGGA

P103 GGTAATCTGGGATATCAGCT

P104 GAATCCCTCTGGGAAGGAAA

P105 ATCCTCGAAGTTCATGACTC

P106 GAATCCTGGGTGACGGTGAC

P107 ATGAACTCTCCATCCTGAAG

P108 TCCTCTAAGCTTGACCTTGT

P109 GTCCATCGGATGGGAAGTTG

P110 ATGGTCTTCTTCTGCATGAC

P111 TACATTCTCTCGGAGGAAGC

P112 CTTGATCTCTCCCTTAAGAG

P113 TCCATCCTTAAGCTTAAGTC

P114 TTGACCTCAGCATCGTAGTG

P115 CTTCTTAGCCTTGTAGGTGG

P116 TAAGCTCCTGGAAGCTGGAC

P117 ATCAAGCTTGATGTTGACGT

P118 TGTAATCCTCGTTGTGGGAG

P119 CTCTCGTACTGCTCGACGAT

P120 TTGTAAAGCTCATCCATTCC

P121 AAGTTCTTCTCCTTTACTCA

P122 GAATTGGGACAACTCCAGTG

P123 CCCATTAACATCACCATCTA

P124 CCTCTCCACTGACAGAAAAT

P125 GTAAGTTTTCCGTATGTTGC

P126 TGGAACAGGTAGTTTTCCAG

P127 GGTATCTCGAGAAGCATTGA

P128 TCATGCCGTTTCATATGATC

P129 GGGCATGGCACTCTTGAAAA

P130 TTCTTTCCTGTACATAACCT

P131 GTTCCCGTCATCTTTGAAAA

P132 CCTTCAAACTTGACTTCAGC

P133 ACCTTTTAACTCGATTCTAT

P134 GTGTCCAAGAATGTTTCCAT

P135 GTGAGTTATAGTTGTATTCC

P136 GTCTGCCATGATGTATACAT

P137 CTTTGATTCCATTCTTTTG

P138 CCATCTTCAATGTTGTGTCT

P139 ATGGTCTGCTAGTTGAACGC

P140 CGCC AATTGGAGTA TTTTGT

P141 GTCTGGTAAAAGGACAGGGC

P142 AAGGGCAGATTGTGTGGACA

P143 TCTTTTCGTTGGGATCTTTC

P144 TCAAGAAGGACCATGTGGTC

P145 AATCCCAGCAGCTG TTACAA

P146 TATAGTTCATCCATGCCATG

P147 ATTTAGGTGACACTATAGGGGAGAGGGAAGACCATACGGTTTTAG AGCTAGAAATAGCAAG

P148 ATTTAGGTGACACTATAGGTGTAATCCTCGTTGTGGGGTTTTAGAG CTAGAAATAGCAAG

6.3.2 Transgenic animals

To express *oma-1–dsRNA* in the neurons (*Pneur::oma-1–dsRNA*): A mixture of the following PCR fragments and plasmid was used to inject into wild-type strain N2: *Prgef-1* DNA (made using primers P84 and P85, 10 ng/µl), *oma-1* sense DNA (made using primers P86 and P87, 10 ng/µl), *Punc-119* DNA (made using primers P88 and P89, 10 ng/µl), *oma-1* antisense DNA (made using primers P90 and P91, 10 ng/µl) and pHC448 (40 ng/µL) in 10 mM Tris·HCl (pH 8.5) was microinjected into the wild-type strain N2. *Prgef-1* and *oma-1* sense fragments share an overlapping region at 3' and 5' ends respectively and are expected to form a fused product upon injection *in vivo*. Similar strategy was used with *Punc-119* and *oma-1* antisense fragments. Recombinant DNA fragments generated through PCR on N2 gDNA using Expand Long Template polymerase (Roche) were purified by using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, catalog no. 740609.250).

To express DNA missing in *T*ΔΔ animals (Fig. 6-15): A 4:1 mixture of pRF4 (420 ng/µL) and DNA missing in *T*ΔΔ (38 ng/µL) in 10 mM Tris·HCl (pH 8.5) was microinjected into the wild-type strain N2. DNA missing in *T*ΔΔ was made from AMJ766 lysate using primers P82 and P83 and the PCR product was purified using

NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, catalog no. 740609.250) prior to injecting. Rol transformants were picked at F2 and passaged as AMJ364.

6.3.3 Quantification of silencing

All images were imaged and quantified as described in Section 3.3.3.

6.3.4 Feeding RNAi

Five embryos of either TX20 or AMJ654 were moved to a single RNAi plate in replicates [NG agar plates supplemented with 1 mMIPTG (Omega) and 25 μg/mL carbenicillin (MP Biochemicals)] with 100 μL of bacteria with a plasmid expressing *oma-1–dsRNA* or with a control plasmid (L4440). After two days of growth at a shift between 15°C and 20°C (independent of the temperature shift provided for growth, worms would not be adults by this point), one surviving worm was left, and the other survivors were picked out (typically, some would have died due to the embryonic lethality of the *zu405* allele, which makes this selection process necessary). At the time-point 145 h post embryo, brood size of the fed animals was calculated by counting the number of viable progeny on each plate.

6.3.5 Semi-quantitative RT-PCR

RNA isolation from each strain and conditions used for *tbb-2* RT-PCR are as described in Section 2.3.7. Gene-specific primer designed to reverse-transcribe the antisense strand of *gfp* is P92. The resulting cDNA was used as a template for PCR (30 cycles for *Psur-5::sur-5::gfp*, for *Pmex-5::gfp*, for *tbb-2*) using *Taq* polymerase and

gene-specific primer pairs P93 and P94. P94 has one mismatch with *gfp* but was successful in giving a PCR product in multiple attempts. The antisense RNA from *gfp* was also independently detected by Julia Marré in August 2014 using the gene-specific reverse transcription primer P95.

6.3.6 Single-molecule RNA fluorescence *in situ* hybridization (smFISH)

Custom Stellaris FISH probes were designed against only exons of *mCherry* and *gfp* using the web-based Stellaris FISH Probe Designer from Biosearch Technologies (www.biosearchtech.com/stellarisdesigner). Probes that span the expected exon-exon junctions were avoided to allow for the detection of both mature and nascent transcripts. For Fig. 6-8, extruded gonads of N2, EG6787, and AMJ552 adult hermaphrodites were subjected to the smFISH protocol using *mCherry* and *gfp* probes. For Fig. 6-11, extruded gonads of EG6787, JH3323, JH3296 and AMJ928 adult hermaphrodites were subjected to the smFISH protocol using *mCherry* probes alone. Standard *C. elegans* smFISH protocol followed by DAPI staining was used as described (129, 130). The probe blend to detect *mCherry* includes 25 exon-specific probes (P96 through P120) each tagged with Quasar 570 dye and antisense to *mCherry* RNA. The probe blend to detect *gfp* includes 26 exon-specific probes (P121 through P146) each tagged with Quasar 670 dye and antisense to *gfp* RNA. Images were taken using Leica SP5 confocal microscope with the 63x oil immersion objective at 5x zoom.

6.3.7 Identification of piRNA target sites in the minimal susceptible transgene

The published dataset of 16,003 *C. elegans* piRNA sequences (17) was used to map all piRNA target sites within *oxSi487 cbr-unc119(+)* using Bowtie2 (131, 132) on Galaxy from PSU, the web-based platform for analysis of sequencing data. A 15 nt seed length was provided allowing for at most 2 nt mismatches. Other Bowtie2 parameters used are as follows: Skip first n reads = 0; Only align the first n reads = -1 (off); Trim n bases from high-quality end of each read before alignment = 0; Trim n bases from low-quality end of each read before alignment = 0; Maximum permitted total of quality values at the mismatched read positions = 300; Whether or not to round to the nearest 10 and saturating at 30 = Do not round to the nearest 10; Number of mismatches for SOAP-like alignment policy = 3; Whether or not to try as hard as possible to find valid alignments when they exist = Try hard; Report up to n valid arguments per read = 10; Whether or not to report all valid alignments per read = report all valid alignments; Suppress all alignments for a read if more than n reportable alignments exist = -1 (no limit). This alignment resulted in 20 piRNA target sites in the sense and antisense orientation to the query sequence. Of these 20 sequences, all piRNA target sites that map in an antisense orientation to *mCherry* with *T*ΔΔΔ were selected as the candidate piRNA target sites. The final result was 3 piRNA target sites.

6.3.8 CRISPR-Cas9 mediated genome editing of *mCherry*

To generate missense mutations in *mCherry* in *T**, *T*Δ* and *T*ΔΔ*, CRISPR-Cas9 genome editing was used as described earlier (Section 3.3.6). For

additional details on specific reagents, see Table 5-3. Mutations were generated by non-homologous end-joining, resulting in a 6 bp indel in *T**, a 2 bp indel in *T*Δ*, and a 2 bp deletion in *T*ΔΔ*.

Table 6–3. Reagents used for CRISPR-Cas9 experiments

Allele name	CRISPR edit	Primers used to make DNA template for sgRNA transcription	Concentration of reagents used (pmol/µl)		
			mCherry sgRNA	*dpy-10* sgRNA	*dpy-10* homology repair template
*T**	*mCherry* mutation in *oxSi487*	P147 (FOR), P43 (REV)	1.6	1.2	5.1
*T*Δ*	*mCherry* mutation in *jamSi19* (*T*Δ)	P148 (FOR), P43 (REV)	6.0	3.0	0
*T*ΔΔ*	*mCherry* mutation in *jamSi25* (*T*ΔΔ)	P148 (FOR), P43 (REV)	6.0	3.0	0

6.3.9 Injection of RNA transcribed from the minimal susceptible transgene

To generate *Psp6::mCherry::cye-1 3' utr*, DNA was amplified from AMJ928 gDNA using primers P82 and P83 with Phusion High Fidelity polymerase (New England Biolabs catalog no. M0530S). The PCR product was purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, catalog no. 740609.250) and used for *in vitro* transcription using SP6 RNA polymerase (New England Biolabs catalog no. M0207). The transcribed product was DNase treated (New England Biolabs catalog no. M0303S) and purified using phenol-chloroform extraction. The purified RNA was resolved on a 0.8% agarose gel and was either subjected to size-selected gel purification (to retain only the intact *T*ΔΔ RNA) or was used in its entirety as a heterogenous mixture. N2 hermaphrodites were injected with either size-selected RNA

(25.6 ng/µl) or with the entire heterogenous mixture of transcribed RNA in two replicates (3543 ng/µl – gel shown in Fig. 6-14 or 4985 ng/µl).

6.3.10 Statistical analyses

χ^2 test was used to compare data as indicated in figure legends except in cases where only one category (bright or silenced) was present in both datasets being compared. GFP fluorescence and mCherry fluorescence were each separately compared in all cases. Student's two-tailed *t* test with unequal variance was used in Fig. 6–5.

6.4 Results

6.4.1 Neuronal dsRNA transported to the germline triggers transcriptional silencing that is dependent on the RdRP RRF-1

Because the target *Pmex-5::gfp* in the germline is an operon (*Pmex-5::gfp::mCherry::h2b::tbb-2 3'utr::gfp::h2b::cye-1 3' utr*), we measured silencing of *mCherry* in animals that express neuronal *gfp-dsRNA* and in their progeny that lack *gfp-dsRNA*. Both parent animals with the neuronal dsRNA array and the descendants without the array showed partial silencing of *mCherry*, despite the robust *gfp* silencing (F1 in Fig. 6-1). The partial silencing of *mCherry* could be explained by some silencing of the nascent bicistronic transcript, resulting in some spread of silencing to the *mCherry* sequence. This spread of silencing from *gfp* to *mCherry* was dependent on the import of *gfp-dsRNA* from neurons and on the RdRP RRF-1 (Fig. 6-1). Interestingly,

the partial silencing of *mCherry* was inherited in every generation (F2 through F7 in Fig. 6-1). These results suggest that *gfp* silencing is a pre-requisite to silencing *mCherry* and that a similar mechanism of silencing occurs in every generation as in the parental generation that expressed the dsRNA. Future work could examine whether silencing by neuronal mobile RNAs in the parental generation results in the establishment of chromatin marks (e.g. by histone modifying enzymes) and what genes are required for the inheritance of co-transcriptional silencing of the bicistronic transcript.

Fig. 6–1. Neuronal mobile RNAs can cause transcriptional silencing of the germline target.
Schematic of the transgene *oxSi487* (*Pmex-5::mCherry::h2b::tbb-2 3' utr::gpd-2 operon::gfp::h2b::cye-1 3' utr*) (ref. 49), that is presumably transcribed into a nascent transcript with both *mCherry::h2b* and *gfp::h2b* but present as two separate mature transcripts in the cytosol (*Top*). *Pmex-5::gfp* hermaphrodites were crossed with males that express neuronal *gfp-dsRNA* from an extrachromosomal array (*Ex[gfp–ds]*) and the proportions of animals that lack the extrachromosomal array (gray worm) but that show either strong (dark gray bars) or weak (light gray bars) silencing of *mCherry* (purple error bars) and *gfp* (blue error bars) in the F2 generation and in successive generations (F3–F7) were determined (*Bottom*). The loss of *Ex[gfp–ds]* was determined by the loss of the fluorescent co-injection marker. Error bars indicate 95% CI. **P* < 0.05. n > 17 L4-staged animals. Dark gray bars and light gray bars are as in Fig. 2–4*C*.

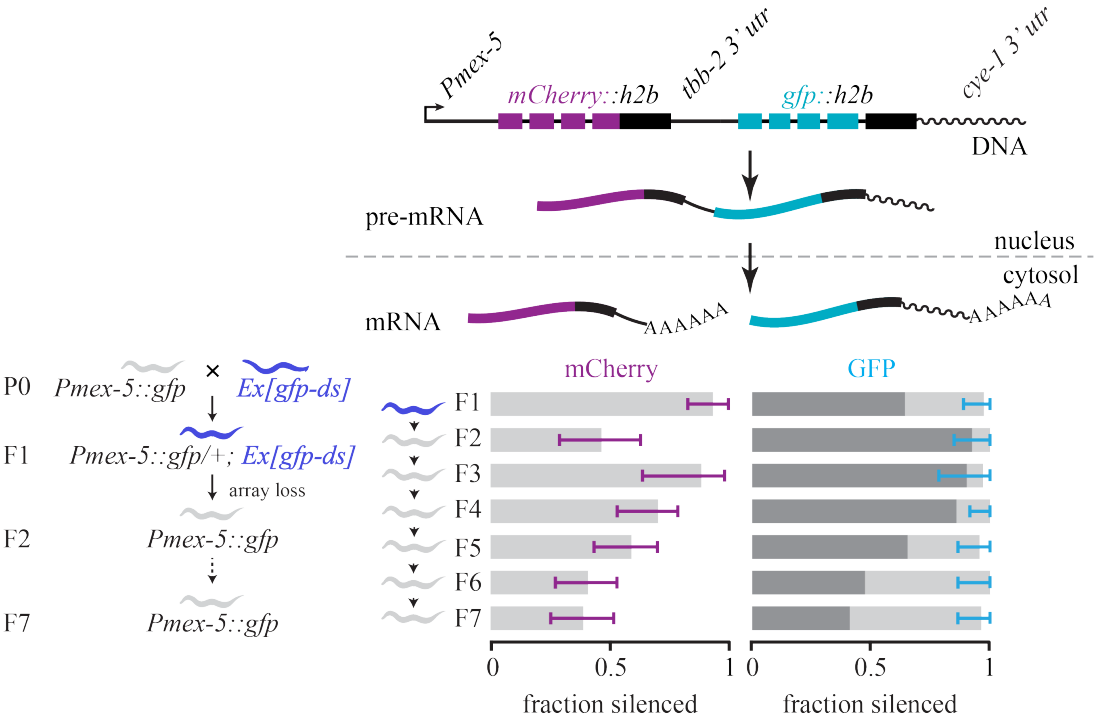
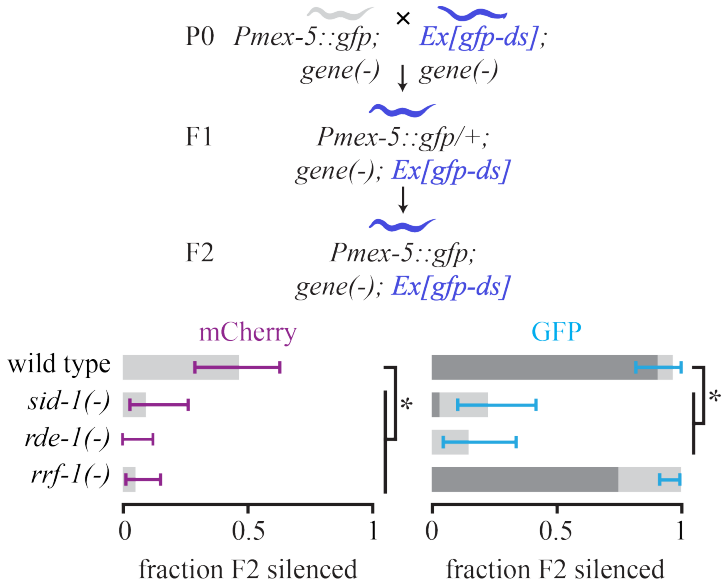


Fig. 6–2. Transcriptional silencing caused by neuronal dsRNA requires the RdRP RRF-1.
Wild-type or *rrf-1(-)* animals that express *Pmex-5::gfp* were mated with animals of identical genetic backgrounds that all express neuronal dsRNA (*Ex[gfp–ds]*), and the silencing in descendants that had both *Pmex-5::gfp* and *Ex[gfp–ds]* was measured as in 2–7*A*. Error bars indicate 95% CI. **P* < 0.05. n > 31 L4-staged animals. Dark gray bars and light gray bars are as in Fig. 2–4*C*.

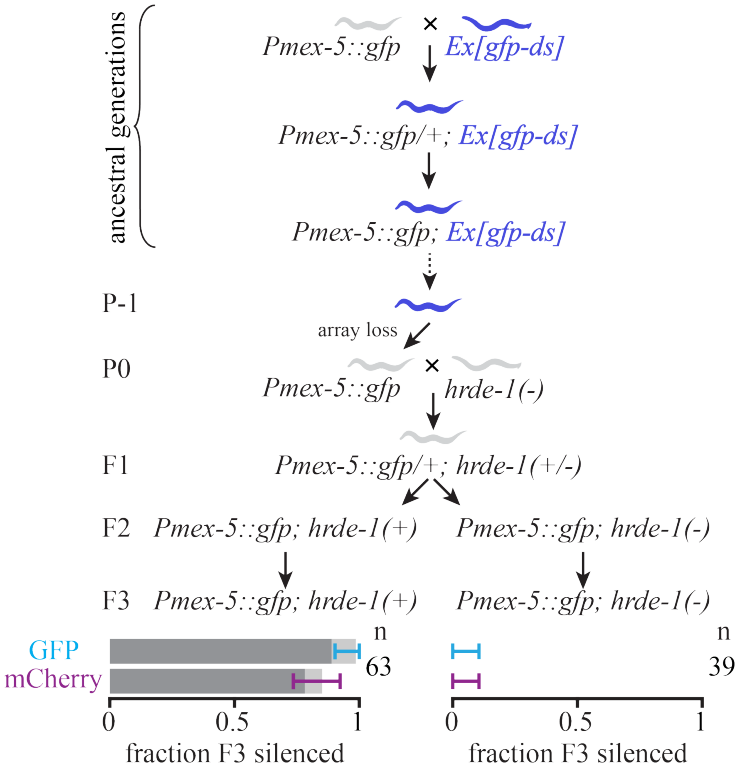


6.4.2 Inherited silencing triggered by transport of neuronal dsRNA to the germline relies on the continued activity of HRDE-1

Neuronal mobile RNAs can trigger transgenerational silencing within the germline (Fig. 2-5, Fig. 2-6, Fig. 2-7*A*) that depends on HRDE-1 for both initiation and maintenance two generations after dsRNA exposure (Fig. 2-7*C*). It is possible that the activity of HRDE-1 in the first generation of inherited silencing (e.g. F2 in Fig. 2-7*C*) is sufficient for transgenerational silencing to continue. We mated progeny of animals that expressed *Prgef-1gfp-dsRNA* that themselves lack the *dsRNA* with *hrde-1(-)* animals and examined silencing in animals two generations later with no *hrde-1* [*hrde-*

1(−)] or in animals with wild-type *hrde-1* [*hrde-1(+)*] (F3 in Fig. 6-3). While animals with wild-type *hrde-1* showed silencing, *hrde-1* mutant animals lacked any silencing. Therefore, one generation of *hrde-1* activity is not sufficient to maintain transgenerational silencing. It is likely that *hrde-1* is continuously required in every generation of silencing to propagate the nuclear silencing of the germline target using secondary siRNAs, as was the case in mating-induced silencing (Fig. 3-10*D*). It will be interesting to test what small RNAs are propagated in the maintenance of silencing, including those originating at endogenous genes, in earlier generations versus in later generations, and compare how the small RNAs change in *sid-1(-); hrde-1(-)* animals.

Fig. 6–3. Maintenance of germline gene silencing triggered by neuronal mobile RNAs requires the continued presence of HRDE-1 in every generation.
Pmex-5::gfp hermaphrodites were mated with males that express neuronal dsRNA (*Ex[gfp–ds]*), and descendants that express both *Pmex-5::gfp* and neuronal dsRNA had progeny that lost the dsRNA (array loss). These animals were mated with *hrde-1(-)* males and silencing was measured in *Pmex-5::gfp* descendants from this cross that were homozygous wild-type or mutant for *hrde-1* for two generations. Error bars indicate 95% CI. **P* < 0.05. n > 38 L4-staged animals. Dark gray bars and light gray bars are as in Fig. 2–4*C*.

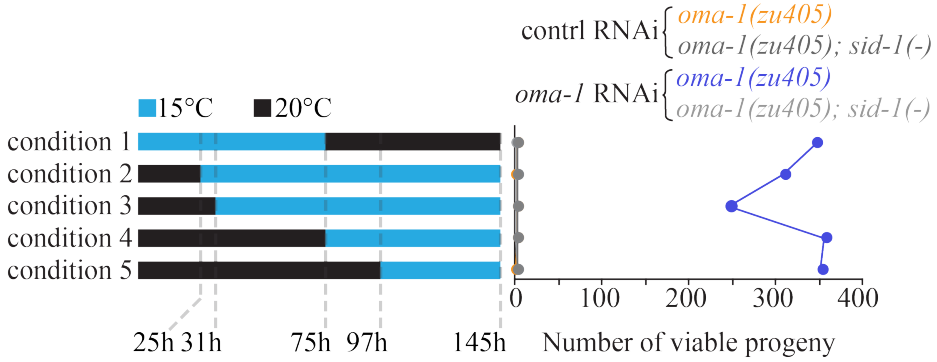


6.4.3 Can neuronal dsRNA silence an endogenous germline gene?

The presence of RNAi inheritance machinery in the germline and dsRNA transport in *C. elegans* suggests that endogenous dsRNA can be made in the soma and transported to the germline. To begin to address this question, we tested whether dsRNA against an endogenous germline gene required SID-1. Specifically, we used a

gain-of-function allele of the oocyte maturation gene *oma-1* as a target for matching dsRNA expressed from neurons. The *oma-1(zu405)* animals produce 30-80% dead embryos at 15°C and 100% dead embryos at the non-permissive temperature of >20°C (51, 133), due to improper degradation of the defective OMA-1 protein (51). RNAi of *oma-1(zu405)* rescues the embryonic lethality (51, 134), resulting in surviving animals, thus facilitating the identification of the silenced phenotype. We performed RNAi of *oma-1(zu405)* animals that express or lack wild-type *sid-1* by growing parent animals from embryo to adulthood on bacteria expressing *oma-1* dsRNA, while they were subjected to either upshift (from 15°C to 20°C) or downshift (from 15°C to 20°C) temperatures during development (conditions 1 through 5 in Fig. 6-4) to identify if there is a critical period during which *oma-1(zu405)* lethality can be rescued. When control RNAi was fed, all conditions of growth resulted in embryonic lethality, suggesting that the restrictive temperature at any stage of parental development can be detrimental to the viability of embryos. However, when *oma-1* dsRNA was fed, the parent animals had viable embryos, although the level of silencing varied with the growth condition. This variation reflects either a temporally changing effect of defective OMA-1 protein and/or the differences in how effective RNAi is at each developmental stage. However, all silencing required *sid-1* because *oma-1(zu405); sid-1(-)* animals produced dead embryos. Thus, consistent with previous observation (54), RNAi against *oma-1* using ingested dsRNA requires the transport of matching dsRNA into the cytoplasm of the tissue that expresses *oma-1*, likely the germline.

Fig. 6–4. Growth of *oma-1(zu405)* animals under restrictive temperature during any period of development results in embryonic lethality that can be rescued by silencing *oma-1(zu405).*
oma-1(zu405) and *oma-1(zu405); sid-1(-)* animals were grown from embryo to adulthood at 15°C or 20°C, under varying temperatures of growth (condition 1 through 5, *Left*). Cultured animals were exposed for one generation on bacteria that have either the control L4440 plasmid (control RNAi) or a plasmid that encodes dsRNA against *oma-1* (*oma-1* RNAi) and silencing of *oma-1(zu405)* by neuronal dsRNA was measured by counting the number of viable progeny of the animals exposed to dsRNA (*Right*). n ≥ 3 parent animals.



We used the convenience of *oma-1(zu405)* silencing to determine whether matching neuronal dsRNA could silence this germline gene in a SID-1-dependent manner. The neuronal dsRNA against *oma-1* was designed such that there is convergent transcription of *oma-1* sense and antisense sequences driven by the pan neuronal promoters *Prgef-1* and *Punc-119*, respectively (see materials and methods). Neuronal *oma-1-dsRNA* could silence *oma-1(zu405)* animals, resulting in viable embryos (Fig. 6-5). Surprisingly, *oma-1(zu405); sid-1(-)* animals also showed a significant amount of silencing, similar to animals with wild-type *sid-1*. Although this result indicates that silencing of *oma-1* by neuronal dsRNA is independent of *sid-1*, it could be explained by how the *oma-1(zu405); sid-1(-)* was made (Fig. 6-6*A*): animals of *Pneur::oma-1-*

dsRNA transgenic line were mated with *sid-1(-)* animals to generate *Pneur::oma-1-dsRNA; sid-1(-)* (P0 in Fig. 6-6*A*). These mutant animals were mated with *oma-1(zu405); sid-1(-)* animals to then generate the final *sid-1(-); oma-1(zu405); Pneur::oma-1-dsRNA* animal (F2 in Fig. 6-6*A*). However, the generation of *Pneur::oma-1-dsRNA* array could have silenced the endogenous wild-type *oma-1*, in which case any possible silencing at the chromatin level could have spread to *oma-1(zu405)* in the mating between *Pneur::oma-1-dsRNA; sid-1(-)* and *oma-1(zu405); sid-1(-)* (P0 in Fig. 6-6*A*). This possibility could explain the apparent *sid-1*-independence of *oma-1(zu405)* silencing by neuronal dsRNA. To rule out this possibility, the *Pneur::oma-1-dsRNA* transgenic line must be generated in an animal with the *oma-1* gene deleted (*oma-1(*Δ*)* in Fig. 6-6*B*). Animals with deleted *oma-1* can still survive because of the redundant *oma-2* gene (134). Using *oma-1(*Δ*)* animals will ensure that the *Pneur::oma-1-dsRNA* does not encounter the target until *oma-1(zu405)* is mated in as a target (F1 in Fig. 6-6*B*) and can answer the question of whether transgenerational silencing can be triggered at an endogenous locus by neuronal mobile RNAs.

Fig. 6–5. Neuronal dsRNA can silence an endogenous gene within the germline.
Wild-type animals and *oma-1(zu405)* or *oma-1(zu405); sid-1(−)* animals that either lack or express neuronal *oma-1-dsRNA* (*[Ex-oma-1-ds]*) from an extrachromosomal array were cultured at 20°C and the number of viable progeny per animal were counted. **P* < 0.05 (Student's *t* test). n ≥ 10 animals per genotype.

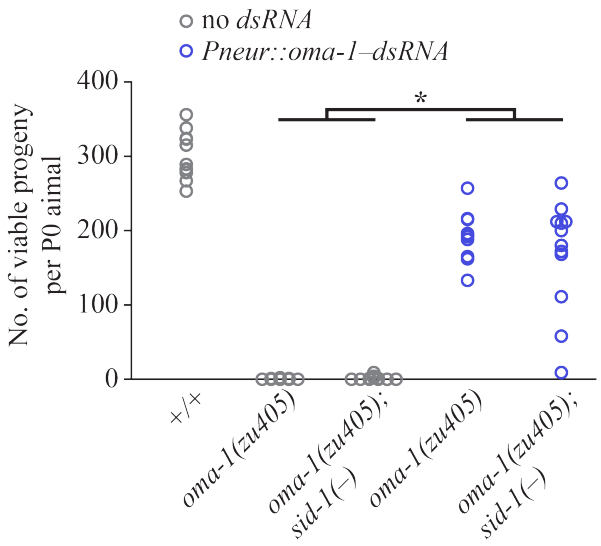
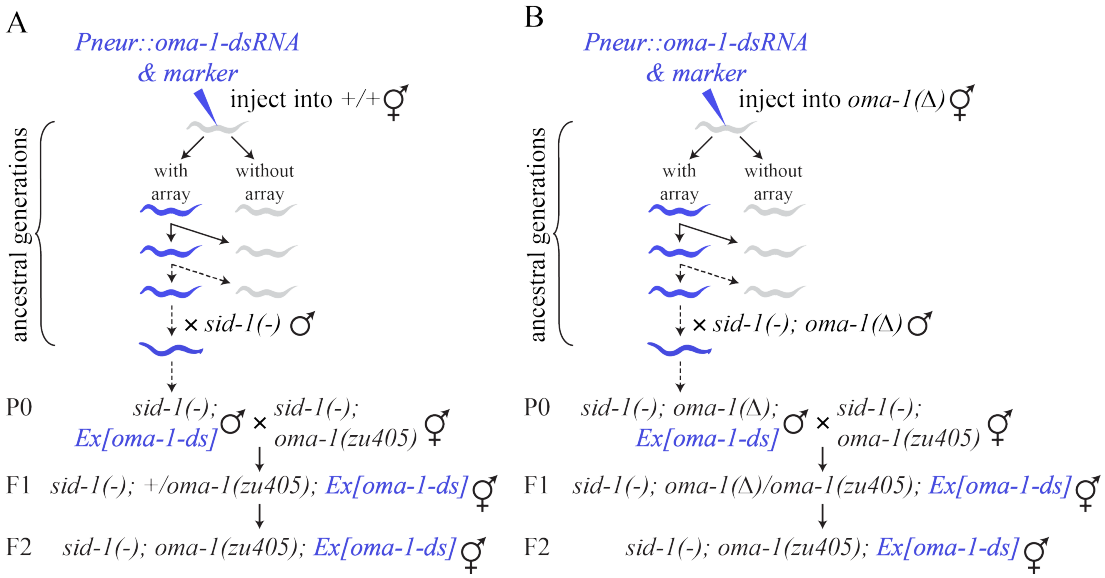


Fig. 6–6. Silencing of wild-type *oma-1* by neuronal dsRNA could explain silencing of *oma-1(zu405)* being independent of SID-1.
(A) Schematic for how *the sid-1(-); oma-1(zu405); oma-1-dsRNA* animals used in Fig. 5-5 was generated: wild-type animals were injected with constructs to express neuronal mobile RNAs (*Pneur::oma-1–dsRNA*) along with a co-injection marker (*Pmyo-2::DsRed*) to generate transgenic lines (blue worm). Animals with the array were mated with *sid-1(-)* males to generate *sid-1(-); oma-1(zu405); Ex[oma-1-ds]* animals. (B) Schematic for how *the sid-1(-); oma-1(zu405); oma-1-dsRNA* animals should be generated, to avoid silencing of any allele of *oma-1* as seen in Fig. 6-5: the cross used here is shown as in (*A*) but the *oma-1-dsRNA* array must be generated by injecting the construct into animals with gene deletion of *oma-1* (*oma-1(Δ)*).

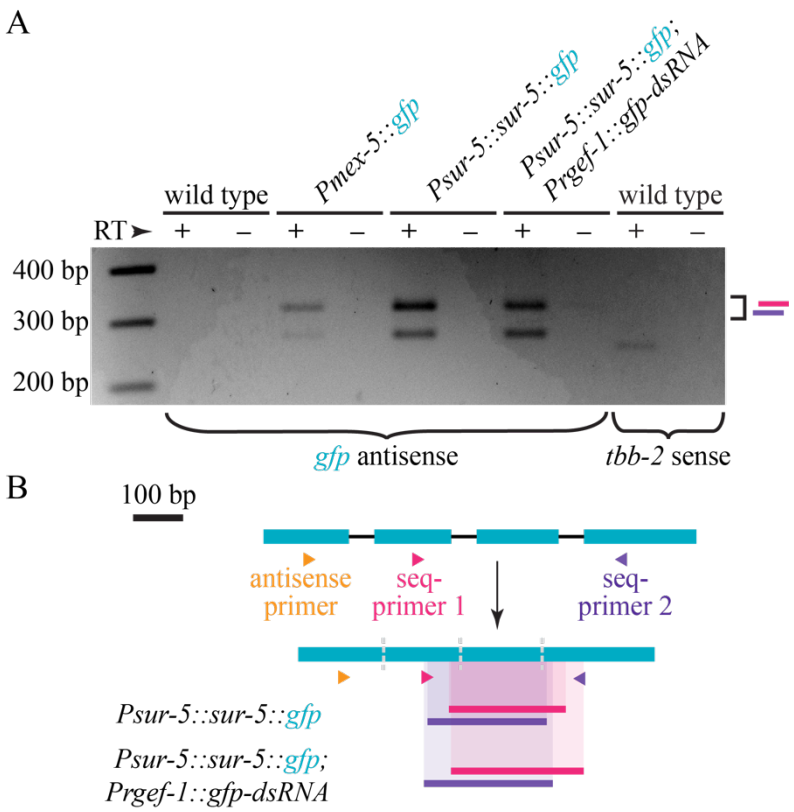


6.4.4 T may generate both sense and anti-sense transcripts that match the sequence of spliced *gfp*

Preliminary results suggest that the locus *T* generates antisense RNA. We discovered that reverse transcription of the antisense strand of *gfp* results in a cDNA product the size of the mature *gfp* mRNA (320 bp band from *T* in *Pmex-5::gfp* strain in Fig. 6-7). The RT-PCR also resulted in a shorter band of ~280 bp. Attempts to sequence each of the two amplicons from *Pmex-5::gfp* were unsuccessful but the 320 bp band sequenced from *Psur-5::sur-5::gfp* and *Psur-5::sur-5::gfp*; *Prgef-1::gfp-dsRNA* animals revealed the presence of intronless antisense *gfp* sequence with intact exon-exon junctions. Although the RT-PCR product from transgene *T* has not yet been sequenced, there is likely antisense *gfp* generated from *T* because: (i) RT-PCR products from *Psur-5::sur-5::gfp* and *Psur-5::sur-5::gfp*; *Prgef-1::gfp-dsRNA* animals tested concomitantly showed DNA bands that correspond to the same size as those from *Pmex-5::gfp* (Fig. 6-7*A*) and were antisense to *gfp* (Fig. 6-7*B*), (ii) the RT-PCR of *gfp* using the same RT primer (as in Fig. 6-7*A*) reproducibly showed bands at 320 bp and ~280 bp from *T* (data not shown), and (iii) RT-PCR of antisense *gfp* using a different RT primer (see materials and methods) showed a similar result of spliced antisense *gfp*. If antisense spliced RNA is indeed produced from *gfp*, it is more likely made by using the sense mRNA as the template rather than by antisense transcription of the gene followed by splicing because the splice acceptor and donor sites in the antisense template are in the antisense orientation. Such generation of dsRNA using reverse transcription of an existing mRNA template occurs using the RdRP RRF-3 in the

endogenous RNAi pathway (135). Existence of dsRNA could either explain the unique susceptibility of *T* to mating-induced silencing or the enhancement of silencing observed in *sid-1* mutants (Fig. 3-10*A*). Future work could examine total RNA-seq to resolve what antisense RNAs are made from *T* and test whether genes required in the endogenous RNAi pathway (e.g. *rrf-3*) are required in the initiation of mating-induced silencing.

Fig. 6-7. The transgene *T* likely generates antisense *gfp* RNA.
(A) Semi-quantitative RT-PCR was used to detect antisense *gfp* transcript levels in wild-type, *Pmex-5::gfp*, *Psur-5::sur-5::gfp* and *Psur-5::sur-5::gfp; Prgef-1::gfp-dsRNA* animals and *tbb-2* mRNA (control) levels in wild-type animals. (B) Schematic represents the coding region of *gfp* before and after introns (black lines) are spliced. The 320 bp RT-PCR product in (black square bracket in *A*) was sequenced using the forward (►) or reverse (◄) primers, after antisense *gfp* was reverse transcribed with the reverse transcription primer (►) and converted to cDNA. Colored lines correspond to fragments of RT-PCR product verified by Sanger sequencing in either *Psur-5::sur-5::gfp* and *Psur-5::sur-5::gfp; Prgef-1::gfp-dsRNA* animals.

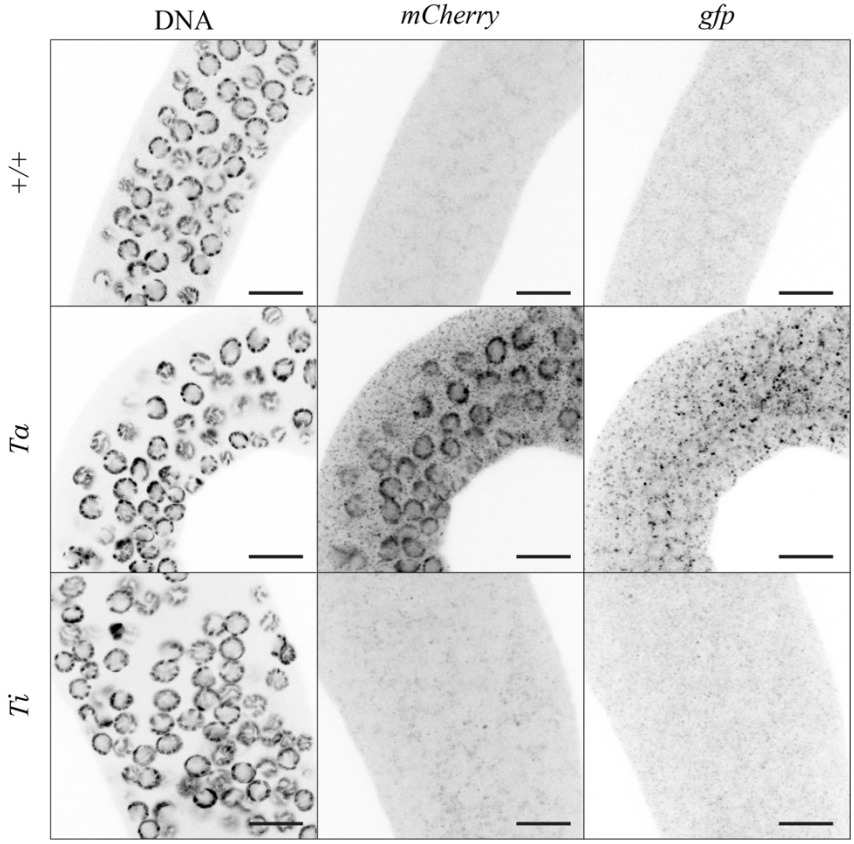


6.4.5 What triggers mating-induced silencing?

It would be interesting to test what the trigger for mating-induced silencing is, although current evidence supports that the silencing mechanism likely affects the transgene at least at the pre-mRNA level. The levels of pre-mRNA and mRNA of both *mCherry* and *gfp* in silenced animals were starkly different from those in active animals, when tested using smFISH probes matching the exonic sequences of each of the transcripts. *Ta* animals showed a strong presence of both *mCherry* and *gfp* RNAs, where *Ti* animals that had been silenced for >200 generations looked similar to animals without the transgene (Fig. 6-8). Future work could determine if a dsRNA triggers the silencing mechanism (for e.g. dsRNA from *T* in Fig. 6-7) using RNAseq and what the

earliest developmental stage is that shows evidence for initiation of silencing in cross progeny that inherit that paternal copy of the transgene.

Fig. 6–8. Mating-induced transgenerational silencing occurs by reduction in transcript levels of both *mCherry* and *gfp*.
Representative pachytene germlines of wild-type hermaphrodites, hermaphrodites that either express the active transgene (*Ta*) or those that have been silenced for >200 generations that were subjected to smFISH against *mCherry* and *gfp* mRNA and pre-mRNA transcripts are shown. DNA was stained using DAPI. Scale bar, 10 µm.



PRG-1 is required in initiation (Fig. 3-10*A* in Chapter 3), but PRG-1-associated piRNAs are known to be maternally deposited, indicating the possibility that maternal PRG-1 plays a key role initiation of mating-induced silencing. We therefore mated

hermaphrodites that lack *prg-1* with *Ta* males that express *prg-1* and examined silencing in homozygous mutant or heterozygous progeny that inherited the transgene only from the male (Fig. 6-9). Similar to cross progeny that lacked zygotic *prg-1* (also see Fig. 3-1*A*), those that expressed zygotic *prg-1* but lacked only maternal *prg-1* showed no silencing (Fig. 6-9), suggesting that zygotic *prg-1* is not sufficient and that maternal *prg-1* is required for the initiation of mating-induced silencing. This requirement for maternal PRG-1 suggests the use of maternally inherited piRNAs in initiation. We therefore determined whether there are potential target sites within the minimal susceptible transgene (*Pmex-5::mCherry::cye-1 3'utr*, see Fig. 6-8*B*) for piRNAs made within *C. elegans* (17). By using the database of 16,003 piRNAs that were recently sequenced (17) to complement the sequence of the minimal transgene (see materials and methods for mapping strategy), we found three piRNA sequences that match perfectly at the seed region with the minimal transgene in an antisense orientation – all mapping to the pre-mRNA sequence of *mCherry* (Fig. 6-10). Whereas these piRNA target sites are not sufficient to trigger mating-induced silencing of any gene with the *mCherry* sequence (Fig. 3-1), initiation could occur using one or more of these piRNAs along with an unknown determinant. Future work that will recode the target sites within *mCherry* and that will delete the three piRNA genes can be used to resolve the possible role of these specific piRNAs in triggering mating-induced silencing.

Fig. 6–9. Mating-induced silencing relies on maternal PRG-1 for initiation.
Mating-induced silencing was initiated by mating male parents that carried at least one wild-type copy of *prg-1* and hermaphrodites that lacked or expressed wild-type *prg-1*. Fluorescence from mCherry and GFP was scored in cross progeny. Scoring of silencing, number of animals assayed, orange font, brackets and asterisks are as in Fig. 3–7*A*.

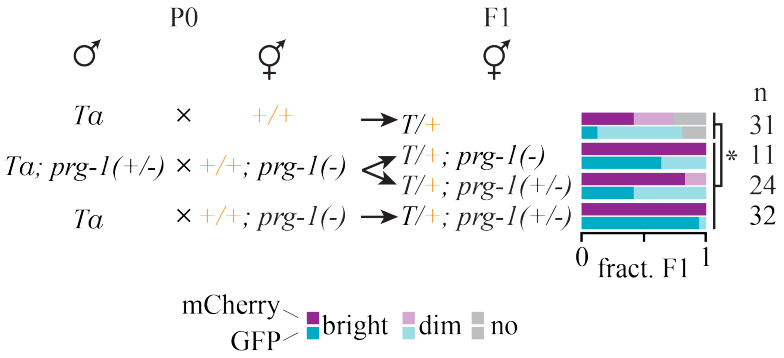
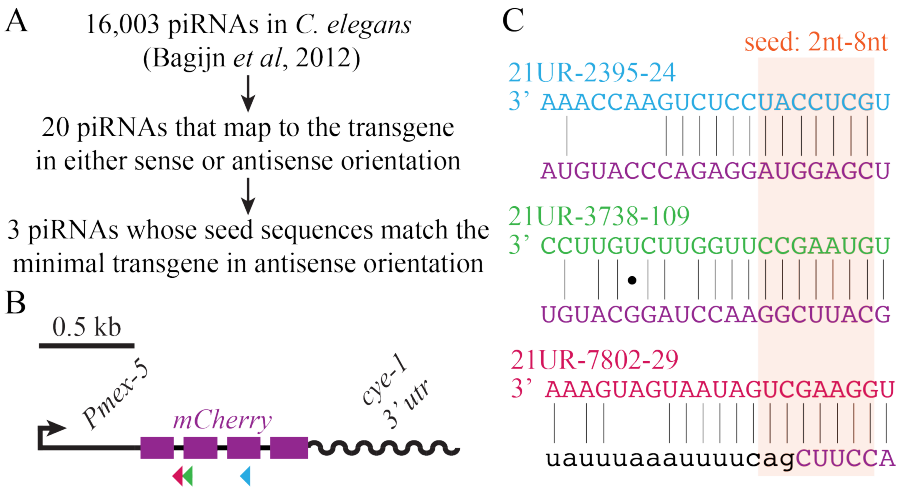


Fig. 6–10. The worm makes piRNAs that match the minimal transgene.
(A) All piRNAs that *C. elegans* makes (17) were used to identify three potential piRNAs targeting the minimal susceptible transgene. See materials and methods for mapping parameters. (B–C) The position where the three piRNAs (colored arrowheads) map in an antisense orientation to the minimal transgene (*Pmex-5::mCherry::cye-1 3' UTR*) (*B*) and the extent to which each piRNA sequence is complementary to different parts of *mCherry* is shown (*C*). Perfect matches (black lines) or wobble base pairs (•) with the *mCherry* exonic (purple lettering) or intronic (**black** lettering) sequences, and the predicted seed regions within the piRNAs (orange box) are depicted.

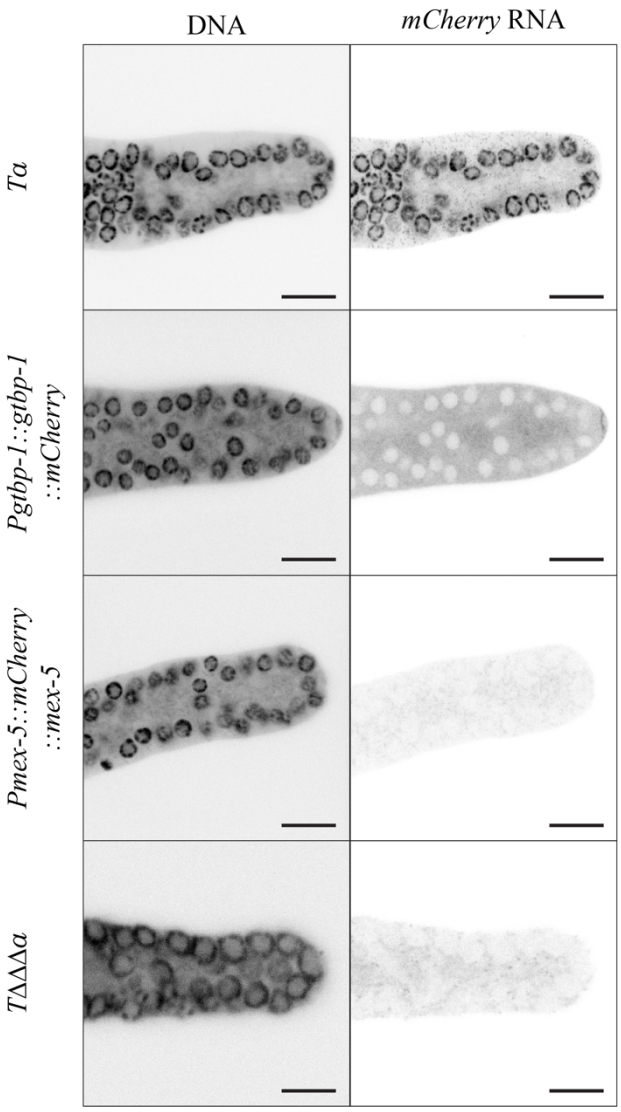


6.4.2 Why are other loci resistant to mating-induced silencing?

Mating-induced silencing was not observed with other loci that contain sequences present in the transgene *T* (e.g. *gfp*, *mCherry*, *h2b*, *Pmex-5*, *tbb-2* 3' UTR) or are present at the same genetic position that *T* is inserted at (i.e. Mos insertion site of *ttTi5605* on chromosome II). It could be that the precise combination of sequences and the genomic location of where *T* is inserted make it susceptible to mating-induced silencing. We therefore examined if such differences could nevertheless result in a similar distribution of transcripts using smFISH of *T* and other loci that express *mCherry* (*Pgtbp-1::gtbp-1::mCherry*, *Pmex-5::mCherry::mex-5*). Surprisingly, we observed that there were qualitative differences between the abundance as well as localization of the transcripts from *T*, *Pgtbp-1::gtbp-1::mCherry*, and *Pmex-5::mCherry::mex-5* (Fig. 6-11). The abundance of *mCherry* transcripts expressed from *T* was far higher than that from the other two loci. Interestingly, *mCherry* was both localized to the nuclear genome and the cytosol. This distribution could indicate that the *mCherry* transcripts from *T* are bound to the chromatin. However, this distribution likely does not contribute to mating-induced silencing because *T*ΔΔΔ animals lack such a nuclear distribution of *mCherry* RNA, and yet show mating-induced silencing (Fig. 6-11). An alternative explanation for the nuclear localization contributing to susceptibility of *T* would be that the gene sequence of *T* was already primed for mating-induced silencing. For example, transcription from a matching piRNA gene could be induced within the genome that depends on the interaction of nuclear *mCherry* RNA with the genomic sequences (136). Such a hypothesis is similar to the piRNA-mediated

nuclear accumulation of retrotransposon transcripts within the female *Drosophila* germline, where the strength of retrotransposons silencing depends on the abundance of the transcripts and on the nuclear presence of transposon transcripts (137). Accordingly, *T*ΔΔΔ, as a consequence, would still remain susceptible to piRNA-mediated silencing despite the deletion of sequences that was required to generate *T*ΔΔΔ. If piRNA production is always a consequence of nuclear accumulation of *mCherry* transcripts as seen with *T*, the prediction is that the male germline might lack the nuclear distribution of *mCherry* because only maternal piRNAs are responsible for mating-induced silencing and that a *de novo* insertion of *T*ΔΔΔ will remain resistant to mating-induced silencing because there would be no history of nuclear localization of the *mCherry* transcript.

Fig. 6–11. smFISH of various loci that express *mCherry* reveals qualitative differences in *mCherry* transcript levels and accumulation.
Representative distal germlines of hermaphrodites that express the active transgene *Tα*, *Pgtbp-1::gtbp-1::mCherry*, *Pmex-5::mCherry::mex-5*, or the minimally susceptible active transgene *T*ΔΔΔ*α*, were subjected to smFISH against *mCherry* mRNA and pre-mRNA transcripts in the same smFISH experiment. A similar accumulation of *mCherry* transcripts was observed with a biological repeat imaged on a different day for *Tα*, *Pgtbp-1::gtbp-1::mCherry* and *Pmex-5::mCherry::mex-5* (data not shown). DNA was stained using DAPI. Scale bar, 10 μm.



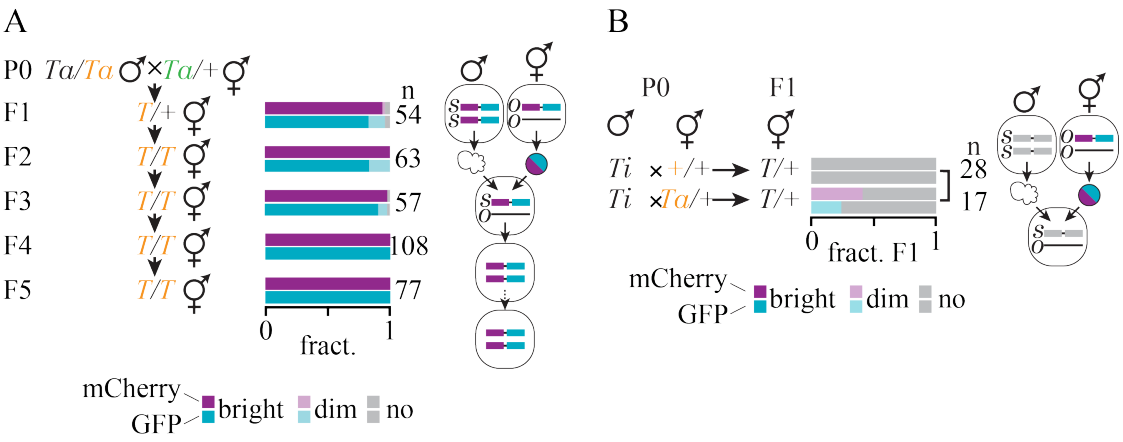
6.4.3 What is the nature of the protective signal?

A DNA-independent protective signal transmitted through oocytes can prevent mating-induced silencing of the paternal transgene (see Fig. 3-7). However, this protective activity on the paternal copy of the transgene may only be effective in the animals that directly inherit the protective signal. It is possible that the paternally inherited transgene could revert to silencing in the next generation, because of the

absence of any protective signal. We therefore tested this possibility by mating hemizygous *Ta* hermaphrodites with *Ta* males and allowed the resulting cross progeny that showed the expected protection from mating-induced silencing (bright F1 in Fig. 6-12) to self-fertilize for several generations. Most descendants that are homozygous for the paternally derived copy of the transgene showed lack of silencing (F2 through F5 in Fig. 6-12), suggesting that the maternal protective signal can prevent the initiation and therefore, can also prevent the transgenerational effects of mating-induced silencing. Despite being able to robustly inhibit initiation of mating-induced silencing, the maternal protective signal could not reactivate a transgene that has been subjected to transgenerational silencing by mating. These results support the idea that transgenerational silencing is tightly dependent on initiation of silencing and that the paternally inherited transgene can escape mating-induced silencing through the activity of a signal separable from the maternal copy of the transgene.

Fig. 6–12. The transgene protected from mating-induced silencing does not undergo transgenerational silencing.
(A) Males that express the active transgene (*Ta*) were mated with hemizygous (*Ta*/+) hermaphrodites, and fluorescence was scored (*top*, mCherry – **bright**, dim, **no**, and *bottom*, GFP – **bright**, dim, **no**) in hemizygous cross progeny (F1) that inherited *Ta* through the sperm and in subsequent homozygous self-progeny for four generations (F2 through F5). Schematic depicts outcome of the test cross: *Ta* that is inherited through the sperm is prevented from silencing by maternally inherited protective signal (magenta/blue fill) and continues to remain expressed across generations, suggesting that the effect of the protective signal within one generation on the transgene inherited through the sperm is sufficient to prevent initiation, and therefore, the maintenance of silencing across following generations. (B) Hemizygous *Ti* males were mated with non-transgenic hermaphrodites or with hemizygous *Ta* hermaphrodites and fluorescence was scored in cross progeny that inherited only the paternal copy of the transgene. *s* and *o* label DNA sequences inherited through male sperm and hermaphrodite oocyte, respectively. Chromosome with (colored boxes) or without (black line) the transgene is as indicated. Scoring of silencing, number of animals assayed, orange font, brackets

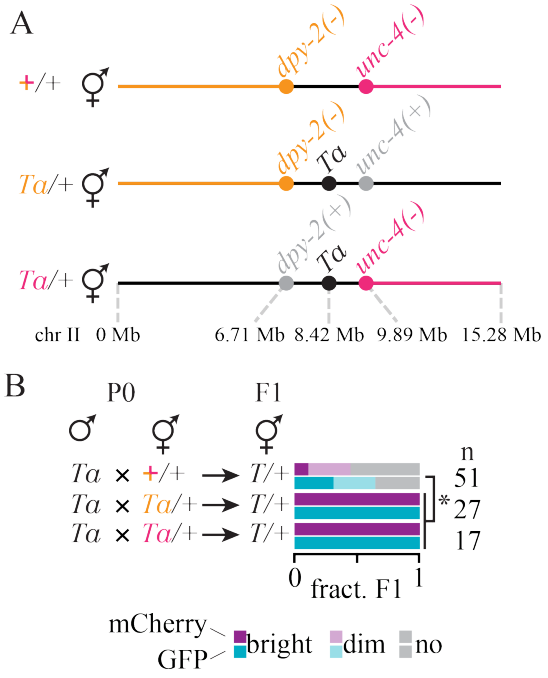
and asterisks are as in Fig. 3–7*A*. Green font represents a recessive mutation in *dpy-10* linked to *Ta*.



The source of the protective signal is likely the maternally present *Ta* but there may instead be another maternal effect suppressor of silencing that is the source of the signal, despite the ability of various outcrosses strains to provide the protective signal (Chapter 3). To further reduce this possibility, we tested the ability of hemizygous *Ta* hermaphrodites after outcrossing the region of the chromosome either upstream (between 0 Mb and 6.71 Mb of chr. II) or downstream (between 9.89 Mb and 15.28 Mb of chr. II) of *Ta* (located at 8.42 Mb) using visible markers (Fig. 6-13*A*). We mated hemizygous *Ta* hermaphrodites with *Ta* males and examined silencing in progeny that inherited the transgene only from the male. As observed before (Fig. 3-7*A*), all cross progeny showed stable expression of the paternally inherited transgene (Fig. 6-13*B*). Thus, the source of the protective signal is, if not the transgene itself, at least closely linked (within approx. ±1.69 Mb) to the transgene and is independent of some sequences within the transgene (see Fig. 3-7*E*). Future work that creates a *de novo* insertion of the minimal transgene can be used to determine whether the transgene

alone is sufficient to provide the maternal signal and if susceptibility of the transgene can be separated from its capacity to protect.

Fig. 6–13. The source of the maternally inherited protective signal is closely linked to the transgene.
(A) Schematics of +/+ [*dpy-2(-)*/+ *unc-4(-)*/+], *dpy-2(-) Tα* [*Tα*/+], and *unc-4(-) Tα* [*Tα*/+] hermaphrodites used in (*B*) to map the position of the maternally inherited protective signal. (B) Males that express the active transgene (*Tα*) were mated with non-transgenic (+/+) or hemizygous (*Tα*/+) hermaphrodites, and fluorescence was scored (*top*, mCherry – **bright**, dim, **no**, and *bottom*, GFP – **bright**, dim, **no**) in hemizygous cross progeny that inherited *Tα* through the sperm. Scoring of silencing, number of animals assayed, brackets and asterisks are as in Fig. 3-7*A*.

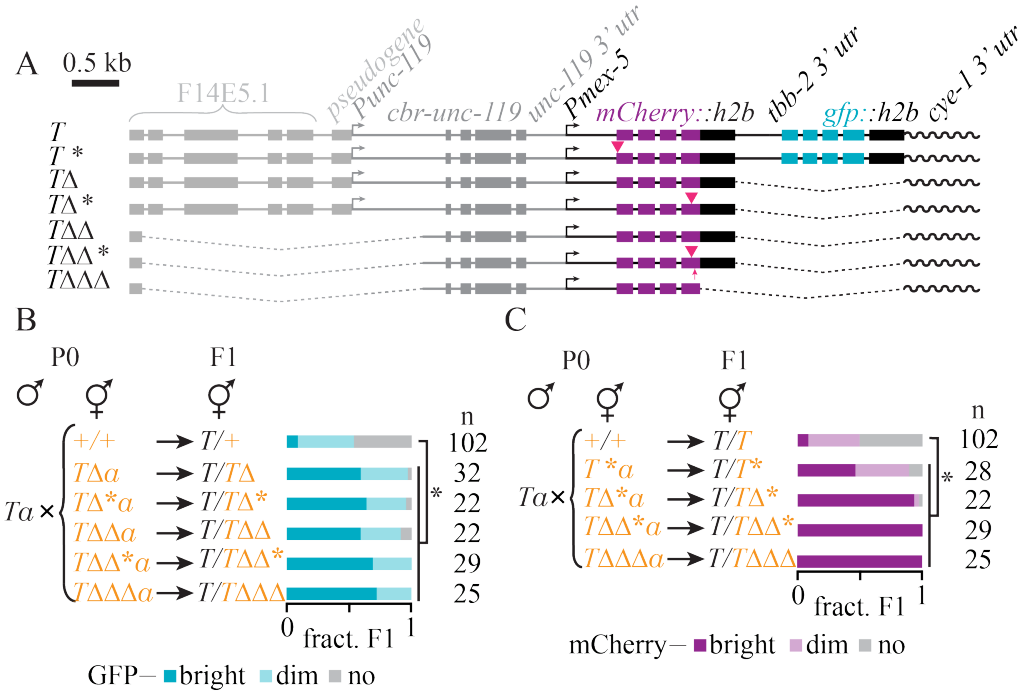


To determine whether any matching maternal transcript is sufficient for protection, we examined if maternal presence of disrupted sequences of the transgene could prevent mating-induced silencing. We used genome editing to make mutations that disrupted the *mCherry* sequence in *Tα* and in its deletion variants that disrupted the *mCherry* sequence, thus creating either missense or frameshift mutations. We mated

hermaphrodites that are hemizygous for one of these variants with *Tα* males and examined silencing in cross progeny that inherited the transgene only from the male. While *gfp* silencing in the cross progeny from all crosses was prevented to a similar extent, there were some differences with *mCherry* silencing in cross progeny from hermaphrodite parents with different *mCherry* variants. Hermaphrodites that carried an in-frame mutation in the first exon of *mCherry* (*T**) prevented mating-induced silencing less often in their progeny (Fig. 6-14) than those with frameshift mutations located in the last exon of *mCherry* (*TΔ** and *TΔΔ**). This small difference in the level of protection could be explained by either the differences in the transcript length of *mCherry* among the variants – with a longer *mCherry* transcript made from *T** while a shorter *mCherry* transcript made from *TΔ** and *TΔΔ**, or the presence of *gfp* in *T** but not *TΔ** and *TΔΔ**. Nevertheless, all variants of *mCherry* significantly prevented mating-induced silencing, with the minimal transgene *TΔΔΔ* still retaining the capacity to protect. Future work to determine the differences in transcript lengths of *mCherry* in *T**, *TΔ**, and *TΔΔ**, introduction of serial stop codons along the length of *mCherry* and *gfp* and a complete deletion of *mCherry::h2b* from *T* could resolve the role of maternal RNA in protection from mating-induced silencing.

Fig. 6–14. Maternal presence of a non-coding copy of the transgene can prevent mating-induced silencing.
(A) Schematics of *T*, successive deletions and/or small indel mutations in *T*. Successive deletions that remove *gfp* and *tbb-2 3' utr* (Δ), a ~3 kb region upstream of the *unc-119(+)* coding region (ΔΔ), and *h2b* (ΔΔΔ) with mutations in *mCherry* that are either in-frame (*T**) or are out of frame, resulting in a stop codon (*TΔ**, *TΔΔ**), are depicted in their genomic context. (B–C) Males that express the active transgene *Ta* were mated with hermaphrodites that lack the transgene or that have a mutated *T* (as in (*A*)), and GFP (*B*) or mCherry (*C*) fluorescence from paternal *Ta* was scored in cross

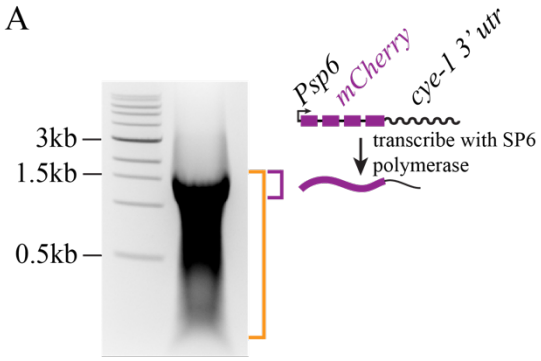
progeny. Number of L4-staged or gravid adult animals scored are indicated (n) for each cross. Brackets indicate relevant comparisons and asterisks indicate $P < 0.01$ (χ^2 test in B, C). Orange font represent chromosomes with a recessive marker (see materials and methods).



To begin to address the possibility that maternal RNA could be the protective signal, we injected RNA transcribed *in vitro* from the minimal protective transgene *T*ΔΔΔ into the germlines of wild-type hermaphrodites, mated them with *Ta* males and examined silencing in cross progeny. None of the cross progeny showed protection from silencing (Fig. 6-15), suggesting that the injected RNA was not stably inherited in all cross progeny, a modified form of transcript is the protective signal or maternal presence of a matching transcript from the transgene is not sufficient for protection. Consistent with the final possibility, we observed that presence of homologous endogenous (*Pmex-5*, *h2b*, *tbb-2 3' utr*, *cye-1 3' utr*) or engineered (*gfp*, *Dendra2::h2b*) sequences in the maternal genome does not detectably provide the protective signal

(Fig. 3-7*D*). Further work is needed to clarify whether the RNAs transcribed from regions at or near the minimal transgene could provide the protective signal when transmitted maternally.

Fig. 6–15. Injected maternal transcript does not detectably prevent mating-induced silencing.
(A) Part of the minimal transgene that is susceptible to mating-induced silencing (*mCherry::cye-1 3' utr*, see Fig. 3–8) was constructed by fusion PCR to be under the control of the SP6 promoter, and used as a template for *in vitro* transcription by SP6 polymerase (schematic). The transcription resulted in the expected single-stranded 1646 bp RNA (purple bracket) along with a heterogeneous mix of smaller RNA products (orange bracket). (B) Germlines of wild-type hermaphrodites were injected with either the size-selected transgene RNA (~) or with the entire heterogeneous mix of transgene RNAs (~~~) and mated with males that express *Ta*, and mCherry and GFP fluorescence was scored in L4 hermaphrodite, L4 male and adult male cross progeny. Bottom row corresponds to a replicate experiment performed using RNA not displayed in *A*. Sizes correspond to base-paired DNA. The size-selected RNA was not sequenced at the time of the experiment.



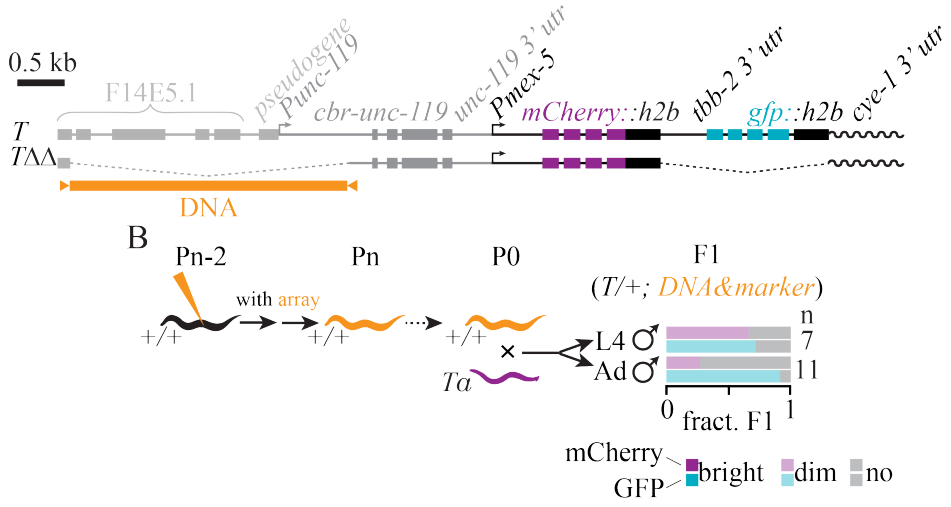
B

P0 ♀	P0 ♂	# of sil. F1/total # of F1s: F1 ♀	F1 ♂
+/+ & ~	*Ta*	n.d.	5/5
+/+ & ~~~	*Ta*	3/3	67/67
+/+ & ~~~	*Ta*	22/22	53/53

Maternal presence of many copies of either the entire transgene or parts of the transgene missing in any of the variants could enhance *gfp* protection. We therefore generated a stable transgenic line carrying an extrachromosomal array generated using

the sequence of the ~3 kb upstream region that was deleted in *T*ΔΔ and the sequence of a dominant visible marker (see materials and methods). Mating hermaphrodites carrying this array with *Ta* males resulted in silencing (Fig. 6-16), suggesting that DNA from the upstream deleted region does not contribute to protective function of the maternal signal. Future work is needed to test if a variant of *Ta* that leaves the minimal *Pmex-5::gfp::cye-1 3'utr* in the genome is sufficient to completely prevent silencing of *gfp* that is inherited from the male parent.

Fig. 6–16. Presence of a region upstream of *T* does not prevent mating-induced silencing.
(A) Schematics of *T* and a mutated copy of *T* with deletions that remove *gfp* and *tbb-2 3' utr* and a ~3 kb region upstream of the *unc-119(+)* coding region (*T*) are depicted in their genomic context (*Top*). The region of DNA amplified using PCR from a strain expressing *T* is depicted (orange). (B) Wild-type animals (Pn-2) were injected with DNA constructs (depicted in *A*) spanning the upstream ~3 kb region deleted in *T*ΔΔ along with a co-injection marker (*rol-6(su1006)*) to generate a transgenic line (orange worm, Pn). The descendant hermaphrodites that express the array (DNA&marker, P0) were mated with males that express *Ta* and GFP or mCherry fluorescence from paternal *Ta* was scored in cross progeny. Scoring of silencing, and number of animals assayed are as in Fig. 3–7*A*.

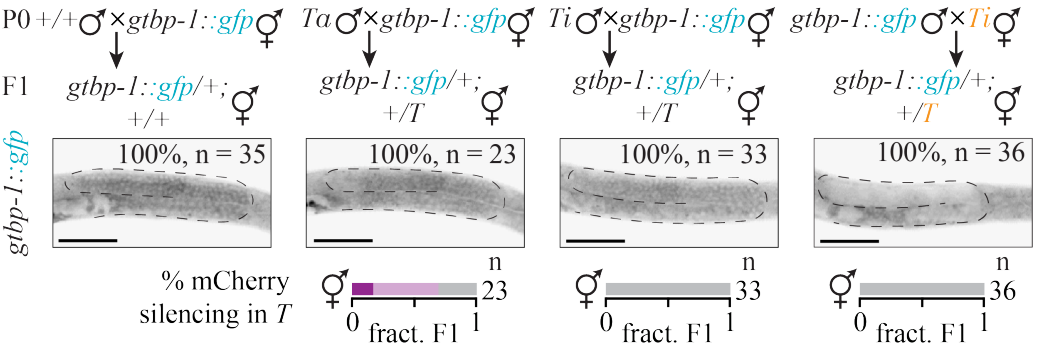


6.4.3 Is the signal that initiates mating-induced silencing separable from the DNA-independent silencing signal?

We found that the signal used to initiate mating-induced silencing of *Tα* is likely different from the silencing signal that acts on homologous genes *in trans*. *Tα* males mated with *gtbp-1::gfp* hermaphrodites had cross progeny that showed mating-induced silencing of *Tα*, as seen from silencing of *mCherry*, but no silencing of *gtbp-1::gfp* (Fig. 6-17). However, the *trans* silencing signal from *Ti* was able to silence *gtbp-1::gfp* (Fig. 6-17). These results separate the role of the two signals (also compare Fig. 3-6*A* v. Fig. 6-18) and strongly indicate that mating-induced silencing is a pre-requisite for the generation of the *trans* silencing signal. The difference between the two classes of signals could reflect a difference in their subcellular localization (for e.g. nuclear vs. cytoplasmic siRNAs), a difference in the timing of when they are generated, or a difference in what part of the transcript they are generated from. Evidence so far makes the last possibility the most likely because *C. elegans* makes two classes of siRNAs – one distal (near 3' end) to the mRNA template, called secondary siRNAs, and one proximal (near 5' end) to it, called tertiary siRNAs – both being chemically identical but different only in their distribution along the matching template (138). Tertiary siRNAs could therefore be used as the signals that could silence other target genes with shared homology. In further support of this hypothesis, secondary siRNAs rely on PRG-1 for their generation while tertiary siRNAs use HRDE-1 for their stability (138), both being observations that also hold true for the initiating signal (Fig. 3-7) and the

trans silencing signal (Fig. 3-9, Fig. 6-17 and Fig. 6-18), respectively. To resolve this hypothesis, future work that tests the genetic requirements in the generation of tertiary siRNAs, such as the role of two RdRPs RRF-1 and EGO-1, and that examines the change in siRNA distribution using RNA-seq in *hrde-1(-)* animals that express both *Ti* and a homologous gene can be informative.

Fig. 6–17. The signal that initiates silencing is different from the signal that silences *in trans*.
Hermaphrodites (*first three crosses*) or males (*last cross*) that express *gfp* ubiquitously (*gtbp-1::gfp*) were mated with non-transgenic, *Ta* or *Ti* males (*first three crosses*) or *Ti* hermaphrodites (*last cross*), respectively, and fluorescence of GFP (from GTBP-1::GFP) and mCherry (from *T*) was imaged in cross progeny. Germlines of representative cross progeny at L4 stage are outlined. Scoring of silencing, number of animals assayed, and orange font are as in Fig. 3–7*A*. *gtbp-1::gfp* in hermaphrodites was linked to the recessive marker *dpy-20*. Percentage of animals with the depicted expression is indicated in each image. Scale bar = 50 µm.

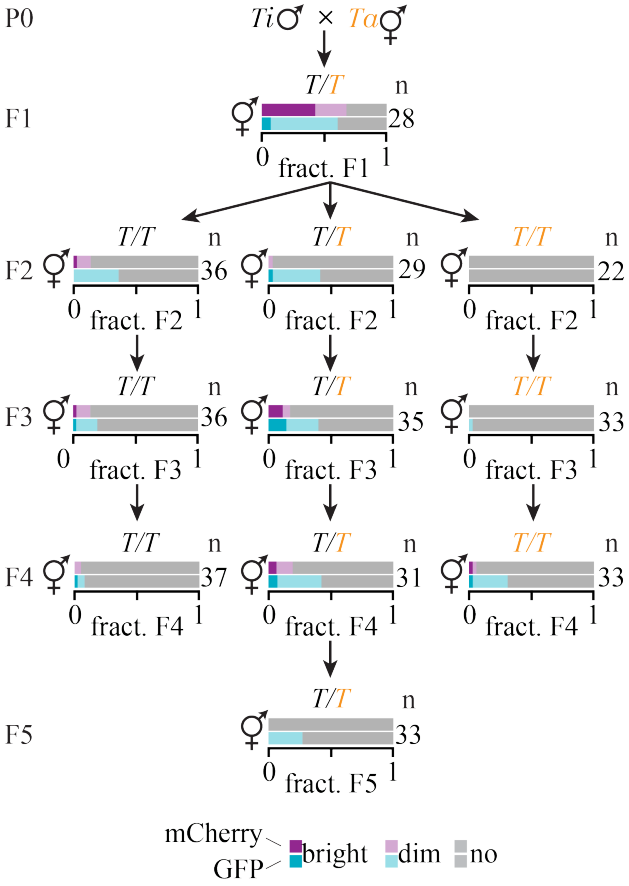


6.4.4 What is the nature of the DNA-independent silencing signal?

Ti can silence *Ta in trans*, perhaps more potently when *Ti* and its associated silencing signal are inherited paternally (Fig. 3-9*A* and *D*). Therefore, we determined whether an active transgene silenced *in trans* by a paternally inherited *Ti* remains silenced across generations. We mated *Ti* males with *Ta* hermaphrodites and examined

silencing in cross progeny (F1 in Fig. 6-18), as well as in homozygous descendants of the cross progeny (F2 in Fig. 6-18) that segregated the parental copies of the transgenes in different combinations. While silencing of the paternally inherited *T* remained stable across generations, silencing of maternally inherited *T* was also stably inherited for several generations, both when transmitted with paternal *T* and when transmitted alone as a homozygote (F3-F5 in Fig. 6-18). Thus, exposure of *Ti* inherited through the sperm to *Ta* for one generation can cause silencing of *Ta* that is transgenerationally stable.

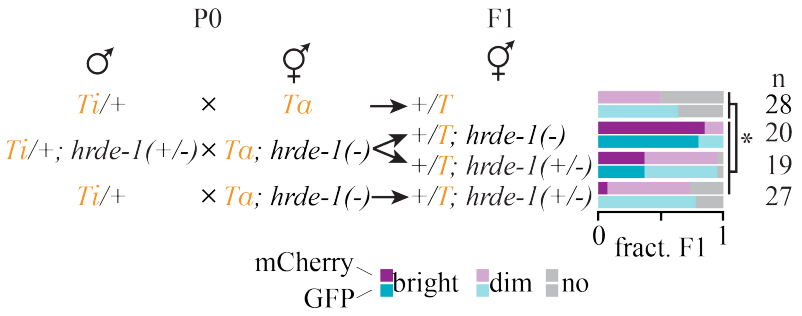
Fig. 6–18. The inactive transgene dominantly silences the active transgene after one generation of interaction.
Ti males were mated with *Ta* hermaphrodites and fluorescence was scored in cross progeny (F1) as well as in descendants (F2 through F5) with that were carried one or both copies of each of either paternally or maternally inherited transgene. Scoring of silencing, number of animals assayed, and orange font are as in Fig. 3–7*A*.



We used the potency of *Ti* inherited through the sperm to dissect the nature of the *trans*-silencing signal. Because the maintenance of silencing of *Ti* requires the germline Argonaute HRDE-1 (Fig. 3-10*D*), and HRDE-1 is associated with small RNAs in the germline, we reasoned that the *trans*-silencing signal may also be HRDE-1-dependent small RNAs. To determine whether HRDE-1 is required for silencing by the DNA-independent silencing signal, we mated *Ti*/+ males wild-type or heterozygous for *hrde-1* mutation with *Ta* hermaphrodites wild-type or homozygous mutant for *hrde-1*, and examined silencing of *Ta* by the DNA-independent silencing in the cross

progeny that inherited only *Tα* through the oocyte (Fig. 6-19). Animals that lacked *hrde-1* showed a robust loss of silencing suggesting that silencing *in trans* is independent of maternal *hrde-1* and uses only zygotic HRDE-1 or paternal HRDE-1 or both (second row in Fig. 6-19). Consistent with the last possibility, animals that were heterozygous for *hrde-1* showed stronger silencing when two copies of wild-type *hrde-1* were present paternally, suggesting that the silencing signal transmitted through the sperm to the progeny is affected by the paternal dosage of HRDE-1 (compare last two rows of Fig. 6-19). Thus, the dependence of the DNA-independent silencing signal on the paternal dosage of *hrde-1* not only provides further evidence to the signal being small RNAs, but also supports the role of the sperm in carrying a potent silencing signal. Whether the silencing signal inherited through oocytes can mediate transgenerational silencing and can depend on maternal dosage of *hrde-1* for silencing *in trans* remains to be seen. Future experiments could examine small RNAs using RNA-seq in embryos generated from the various crosses to identify the DNA-independent silencing signal (Fig. 6-19).

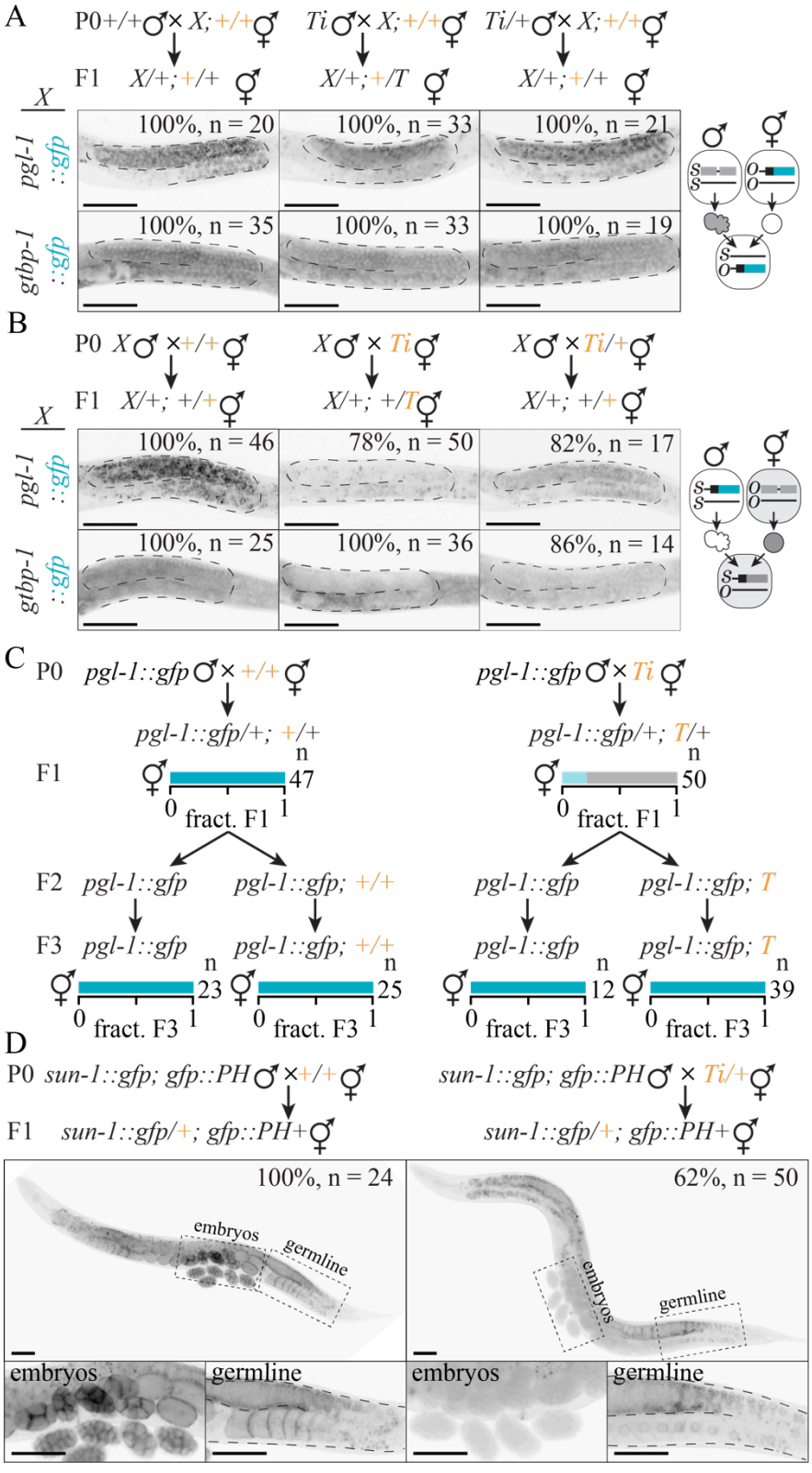
Fig. 6–19. Silencing *in trans* by the DNA-independent silencing signal requires HRDE-1.
Tα hermaphrodites with the wild-type or mutant allele of *hrde-1* were mated with males that carried the inactive transgene (*Ti*/+) in a background wild-type or heterozygous for *hrde-1*, and fluorescence was scored in cross progeny that inherited only *Tα*, and not *Ti*. Scoring of silencing, number of animals assayed, orange font, brackets and asterisks are as in Fig. 3-7*A*.



Given the ability of the silencing signal to be efficiently inherited through the sperm, we tested its ability to silence other homologous loci. Surprisingly, the signal transmitted through the sperm could not detectably silence other homologous loci, while the silencing signal transmitted through the oocyte could (Fig. 6-20*A* v. *B*). The peculiarity of *Ti* inherited through sperm to both silence *Ta* (Fig. 3-9, Fig. 3-12*A* and Fig. 6-18) and cause its transgenerational silencing (Fig. 6-18 F3-F5) but its inability to silence other homologous loci indicates that despite the sperm-inherited silencing signal being equal when mated with hermaphrodites that express *Ta* or that express a homologous locus, the response of the target locus is different. In other words, either (i) *Ta* is simply more susceptible to the silencing signal or, (ii) whatever *Ti* inherited through the sperm needs something provided by a P0 oocyte carrying only *Ta*, but not by a P0 oocyte carrying another homologous locus, for effective transgenerational silencing. Thus, the oocyte could provide an additional unknown factor when transmitting *Ti* to progeny (Fig. 6-20*B*, schematic). Despite this higher potency of *Ti* transmitted through the oocyte, the silencing of the homologous *pgl-1::gfp* (Fig. 6-20*C*) and *gfp-PH* (Fig. 6-20*D*) by *Ti* inherited through the oocyte did not last more than one generation. The lack of *gfp* expression in the embryos of the silenced *gfp::PH/+*

animals is likely a consequence of silencing resulting in absence of maternal deposition of *gfp::PH* (embryos in Fig. 6-20*D*), but future experiments that systematically examine the next generation must be done to resolve this. This result highlights two aspects of silencing that can dictate the efficiency of silencing: the susceptibility of the target locus to the DNA-independent silencing signal and the gamete carrying the silencing signal.

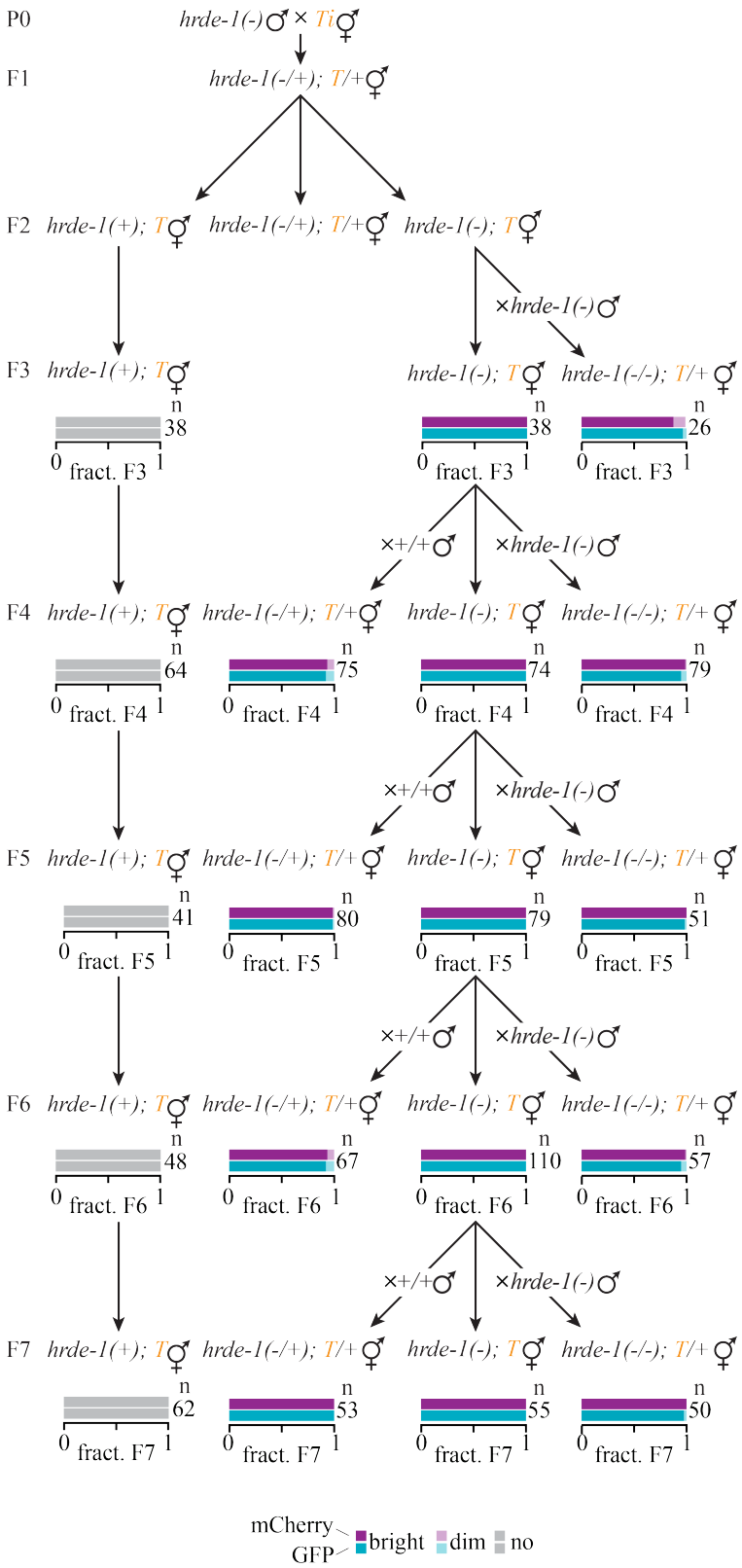
Fig. 6–20. The heritable DNA-independent silencing signal can silence other homologous loci when inherited through the oocyte but not when inherited through the sperm.
(A–B) Hermaphrodites (*A*) or males (*B*) that express the homologous *gfp* sequence fused to endogenous genes (*X*) expressed in the germline (*pgl-1*) or ubiquitously (*gtbp-1*) were mated with non-transgenic, homozygous *Ti* or hemizygous *Ti* (*Ti*/+) male or hermaphrodites, respectively, and fluorescence of GFP (PGL-1::GFP or GTBP-1::GFP) was imaged in cross progeny. Schematics depict outcome of the final test crosses (as in Fig. 3-7*A*): paternal *Ti* cannot silence *X* in progeny, but maternal *Ti* can, suggesting that other homologous loci are susceptible to silencing by the DNA-independent silencing signal (filled grey circle) only when it is inherited through the oocyte. This could imply that there is an additional factor provided by the oocyte (grey filled rounded square) that causes silencing of other homologous loci. (C) Males that express *pgl-1::gfp* or ubiquitously (*gtbp-1*) were mated with non-transgenic (*Left*) or homozygous *Ti* (*Right*) hermaphrodites and GFP fluorescence was scored in heterozygous F1 cross progeny, in F3 descendants of F2 progeny that were homozygous for only *pgl-1::gfp* or homozygous for both *pgl-1::gfp* and the transgene *T*. (D) Males that express sun-*1::gfp*; *gfp::PH* were mated with non-transgenic (*Left*) or hemizygous *Ti* (*Ti*/+, *Right*) hermaphrodites and GFP fluorescence from the tagged genes was imaged in adult cross progeny. Dotted boxes within the top image are expanded below and show the F2 embryos or the posterior gonad arm. Germlines of representative cross progeny at L4 (*A*, and *B*) or adult (*D*) stage are outlined. Scoring of silencing, number of animals assayed, and orange font are as in Fig. 3–7*A*. Percentage of animals with the depicted expression is indicated in each image (*A*, *B*, *D*). Scoring of silencing, number of animals assayed, orange font, brackets and asterisks are as in Fig. 3–7*A*.


A
P0 +/+ ♂ × X;+/+ ♀
Ti ♂ × X;+/+ ♀
Ti/+ ♂ × X;+/+ ♀
F1 X/+;+/+ ♀
X/+;+/T ♀
X/+;+/+ ♀
X
pgl-1 ::gfp
gtbp-1 ::gfp
100%, n = 20
100%, n = 33
100%, n = 21
100%, n = 35
100%, n = 33
100%, n = 19
B
P0 X ♂ × +/+ ♀
X ♂ × Ti ♀
X ♂ × Ti/+ ♀
F1 X/+; +/+ ♀
X/+; +/T ♀
X/+; +/+ ♀
100%, n = 46
78%, n = 50
82%, n = 17
100%, n = 25
100%, n = 36
86%, n = 14
C
P0 pgl-1::gfp ♂ × +/+ ♀
pgl-1::gfp ♂ × Ti ♀
pgl-1::gfp/+; +/+
pgl-1::gfp/+; T/+
F1
n
47
50
0 fract. F1 1
F2 pgl-1::gfp
pgl-1::gfp; +/+
pgl-1::gfp; T
F3 pgl-1::gfp
pgl-1::gfp; +/+
pgl-1::gfp; T
23
25
12
39
0 fract. F3 1
D
P0 sun-1::gfp; gfp::PH ♂ × +/+ ♀
sun-1::gfp; gfp::PH ♂ × Ti/+ ♀
F1 sun-1::gfp/+; gfp::PH+ ♀
sun-1::gfp/+; gfp::PH+ ♀
100%, n = 24
62%, n = 50
embryos
germline

6.4.5 What mechanisms are used in the maintenance of mating-induced silencing?

The silenced transgene remains silenced for >150 generations and requires HRDE-1 at each generation for maintaining (see Fig. 3-10*D*). The continued requirement for the Argonaute suggests that either it is required to generate a silencing signal and cause silencing in progeny that inherit the signal, or that the silencing signal is replicated in each generation independent of HRDE-1 but is inherited every generation as a precursor for silencing that is then used by HRDE-1 for silencing. To distinguish between these possibilities, we disrupted transgenerational silencing by mutating *hrde-1* (as in Fig. 3-10*D*) and re-introduced wild-type *hrde-1* into the subsequent generations (Fig. 6-21). While animals that carried a wild-type copy of *hrde-1* and their progeny were always silenced and those with mutant *hrde-1* showed stable expression (as was seen in Fig. 3-10*D*), the heterozygous *hrde-1* progeny of mutant *hrde-1* animals did not show any silencing (Fig. 6-21). Thus, the silencing signal is tightly linked to the activity of *hrde-1* in every generation and is not independently propagated to be used in silencing by *hrde-1* introduced in a later generation.

Fig. 6–21. The signal used to maintain transgenerational silencing cannot be propagated independent of *hrde-1*.
Ti hermaphrodites that had remained silenced for many generations were mated with *hrde-1* mutant males heterozygous cross progeny were allowed to give homozygous or heterozygous wild-type and homozygous mutant F2 progeny. While homozygous mutant and homozygous wild-type animals were propagated every generation by self-fertilization and their progeny were scored, homozygous mutant animals were crossed every generation with either wild-type or mutant *hrde-1* males and their cross progeny were scored in each generation. Scoring of silencing, number of animals assayed, orange font, brackets and asterisks are as in Fig. 3–7*A*. Note that the reason for why there are no cross progeny of *hrde-1(-/-); T* F2 hermaphrodites mated with N2 males could be because F2 hermaphrodites were genotyped after being mated with male sperm, and therefore any possible *hrde-1(-/-); T* hermaphrodites could only have been detected as heterozygous for *hrde-1* due to the presence of wild-type sperm within the adult hermaphrodites. A repeat of this experiment by Maïgane Diop resulted in a similar distribution of ratios in the same cross at F2 generation.


P0
hrde-1(-)♂ × Ti♀
F1
hrde-1(-/+); T/+♀
F2
hrde-1(+); T♀
hrde-1(-/+); T/+♂
hrde-1(-); T♀
×hrde-1(-)♂
F3
hrde-1(+); T♀
hrde-1(-); T♀
hrde-1(-/-); T/+♀
n 38
0 fract. F3 1
n 38
0 fract. F3 1
n 26
0 fract. F3 1
×+/+♂
×hrde-1(-)♂
F4
hrde-1(+); T♀
hrde-1(-/+); T/+♂
hrde-1(-); T♀
hrde-1(-/-); T/+♀
n 64
0 fract. F4 1
n 75
0 fract. F4 1
n 74
0 fract. F4 1
n 79
0 fract. F4 1
×+/+♂
×hrde-1(-)♂
F5
hrde-1(+); T♀
hrde-1(-/+); T/+♂
hrde-1(-); T♀
hrde-1(-/-); T/+♀
n 41
0 fract. F5 1
n 80
0 fract. F5 1
n 79
0 fract. F5 1
n 51
0 fract. F5 1
×+/+♂
×hrde-1(-)♂
F6
hrde-1(+); T♀
hrde-1(-/+); T/+♂
hrde-1(-); T♀
hrde-1(-/-); T/+♀
n 48
0 fract. F6 1
n 67
0 fract. F6 1
n 110
0 fract. F6 1
n 57
0 fract. F6 1
×+/+♂
×hrde-1(-)♂
F7
hrde-1(+); T♀
hrde-1(-/+); T/+♂
hrde-1(-); T♀
hrde-1(-/-); T/+♀
n 62
0 fract. F7 1
n 53
0 fract. F7 1
n 55
0 fract. F7 1
n 50
0 fract. F7 1
mCherry
GFP
bright
dim
no

6.5 Conclusion

In this dissertation, I describe the discovery of a transgene locus that could be uniquely susceptible to transgenerational gene expression change within a single generation using two distinct trigger mechanisms. Because other loci tested were not susceptible to silencing across generations, despite sharing common regions of homology or genetic positions with the transgene locus *T*, we conclude that most loci within the genome are likely resistant to transgenerational changes when tested under the independent conditions of RNA silencing by neuronal dsRNA or by mating. Thus, the barrier to the communication of any change in information from one generation to the next is likely at the level of the gene, and not necessarily between the soma and the germline. The discovery of a susceptible locus provides us with a precise way to observe how changes first occur at a gene such that they can be maintained for many generations and thus explain how gene regulatory mechanisms may evolve while perpetuating life.

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