Mating can cause transgenerational gene silencing in *Caenorhabditis elegans*

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Abstract

Gene silencing is a significant obstacle to genome engineering and has been proposed to be a non-self response against foreign DNA[1,2,3,4]. Yet, some foreign genes remain expressed for many generations[1,3,4] and some native genes remain silenced for many generations[1,5,6]. 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. 7) for initiation and the Argonaute HRDE-1 (ref. 1,5) 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.

Results

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 (Extended Data Fig. 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*[8]. This transgene[9] consists of a bicistronic operon that expresses mCherry and GFP in the germline (Fig. 1a, Extended Data Fig. 2). We observed differences in expression from this transgene depending on the gamete through which the transgene was inherited (Fig. 1b). 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. 1c, d). Fluorescence of both

proteins was similarly affected in each animal (Extended Data Fig. 3), 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 (Extended Data Fig. 2b), 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. 1e, Extended Data Fig. 4, also see Genetic Inferences in 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 Extended Table 1 and Supplementary Discussion), we refer to it as mating-induced silencing.

Mating-induced silencing was not observed in any descendant of cross progeny that inherited the transgene through both gametes (compare Extended Data Fig. 5a with Fig. 1b). It is possible that the maternal presence of an active, i.e. expressed, transgene ($T\alpha$) prevents silencing of the paternally inherited transgene. To test if maternal $T\alpha$ in the hermaphrodite parent is sufficient for preventing mating-induced silencing, we mated hemizygous $T\alpha$ hermaphrodites with $T\alpha$ males and examined silencing in progeny that inherited the transgene only from the male (Fig. 2a). All cross progeny showed stable expression of the paternally inherited transgene (Fig. 2a), 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 (Extended Data Fig. 5b, 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 $T\alpha$ (*Pmex-5::mCherry::h2b::tbb-2 3' utr::gpd-2 operon::gfp::h2b::cye-1 3' utr* with *Cbr-unc-119(+)* upstream) (Fig. 2b, Extended Data Fig. 2a). Neither deletion of the *tbb-2 3' utr* and *gfp::h2b* sequences ($T\Delta$) nor subsequent deletion of upstream sequences ($T\Delta\Delta$) and *h2b* from *mCherry::h2b* ($T\Delta\Delta\Delta$) eliminated the protective signal (Fig. 2b, 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 $T\alpha$ from silencing. Consistently, neither a *Dendra2::h2b* transgene with shared sequences nor *gtbp-1::gfp* could prevent mating-induced silencing of $T\alpha$ (Fig. 2b, Fig. 2d). Like maternal $T\alpha$, maternal $T\Delta\Delta\Delta\alpha$ also retained the property of transmitting a DNA-independent protective signal (Fig. 2e). 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 $T\alpha$ from mating-induced silencing (Fig. 2f).

Protection from mating-induced silencing and susceptibility to mating-induced silencing could have different sequence requirements. Therefore, we examined all deletion variants (Fig. 2b) by crossing males expressing the variant with hermaphrodites without the corresponding transgene. All variants were silenced (Extended Data Fig. 6, also see Genetic Inferences in 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.

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 $T\alpha$ hermaphrodites resulted in cross progeny that showed silencing (Fig. 3a, *top*) and progeny from the reciprocal cross also showed a small increase in silencing (Fig. 3a, *bottom*). Thus, Ti can silence $T\alpha$ *in trans*, especially when Ti is inherited through the sperm. To examine if Ti can silence other homologous loci, we mated $T\alpha$ 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. 3b, c). Animals with Ti showed silencing of *gfp* and *mCherry*, but not *rfp* (Fig. 3b, 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. 3b). 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. 3d, e, also see Genetic Inferences in Methods). 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. 3f).

The spread of silencing to other loci was not observed in the absence of matching exonic sequences in *Ti* (Fig. 3c, e). 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 the double-stranded RNA (dsRNA) importer SID-1 (ref. 10), the primary Argonaute RDE-1 (ref. 11), the RNA-dependent RNA polymerase RRF-1 (ref. 12), the somatic secondary Argonaute NRDE-3 (ref. 13), and two germline Argonautes, HRDE-1 (ref. 5) and PRG-1 (ref. 7). To test if each gene is required for initiation, we examined mating-induced silencing in the corresponding mutant backgrounds. Substantial silencing was observed in all cases except in animals that lack the *prg-1* gene (Fig. 4a, also see Genetic Inferences in Methods). Thus, initiation requires the germline Argonaute PRG-1 and potentially associated germline small RNAs called piRNAs[7]. Because the minimal *Pmex-5::mCherry::cye-1 3' utr* is still susceptible to mating-induced silencing (Extended Data Fig. 6), it is likely that piRNAs recognize a part of this sequence. Such piRNA-mediated silencing is expected to be stable for many generations[14]. Consistently, we found that mating-induced silencing persisted for >20 generations without selection (Fig. 4b, Extended Data Fig. 7). The silenced transgene retained the capacity to silence homologous genes in *trans* even after >200 generations (Extended Data Fig. 8a) although the DNA-independent silencing signal was not detectably inherited for more than one generation (Extended Data Fig. 8b). However, unlike silencing of *Ta* by mating, silencing of *Ta* by *Ti* does not generate a DNA-independent signal (Extended Data Fig. 8c). Therefore, the DNA-independent signal made in every generation does not account for the transgenerational stability of mating-induced silencing.

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. 4c, 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. 4c, d, Extended Data Fig. 9, also see Genetic Inferences in Methods). Current understanding of silencing by HRDE-1 suggests that nascent transcripts are recoginized by antisense small RNAs bound to HRDE-1, resulting in the recruitment of histone modifying enzymes that generate H3K9me3 at the locus[5]. 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.

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 (also see Supplemental Discussion). 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.

Methods Summary

All *C. elegans* strains were generated and maintained by using standard methods[15]. Animals with the transgene *T* (*oxSi487*) were introduced into mutant genetic backgrounds through genetic crosses using transgenic hermaphrodites and mutant males to avoid initiation of mating-induced silencing. Cross progeny from genetic crosses were identified by balancing or marking *oxSi487* with recessive mutations *dpy-2(e8) unc-4(e120)* or *dpy-2(e8)*, respectively. In some crosses, cross progeny were identified by genotyping for *oxSi487* transgene using PCR. Genome editing was performed using Cas9 protein and sgRNA[16]. Silencing of all transgenic strains was measured by imaging under identical nonsaturating

conditions using a Nikon AZ100 microscope. Quantification of images was performed using NIS Elements (Nikon) and ImageJ (NIH). Detailed procedures are provided in Supplementary Material.

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Author contributions S.D., S.A., and A.M.J. designed and analyzed experiments. S.D. and S.A. performed experiments. S.D. and A.M.J. wrote the manuscript. All authors edited the manuscript.

Author Information The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to A.M.J. (amjose@umd.edu)

Figures and Legends

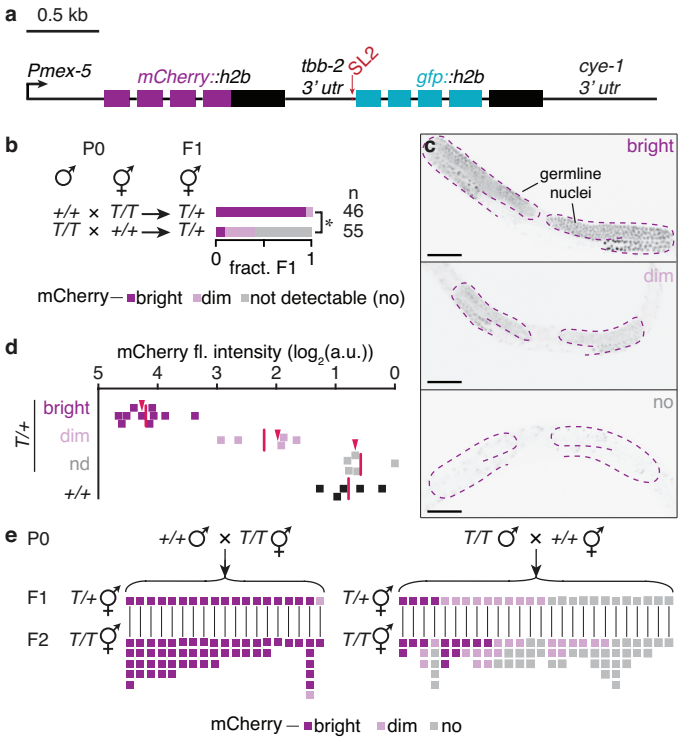

Figure 1. Mating can cause heritable silencing in progeny.

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 Extended Data Fig. 2). **b**, Hermaphrodites or males that carry *T* were mated with wild-type (+/+) males or hermaphrodites, respectively, and mCherry fluorescence was scored (magenta, bright; pink, dim; and grey, 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 (magenta 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 Extended Data Fig. **4** for additional biological replicates.

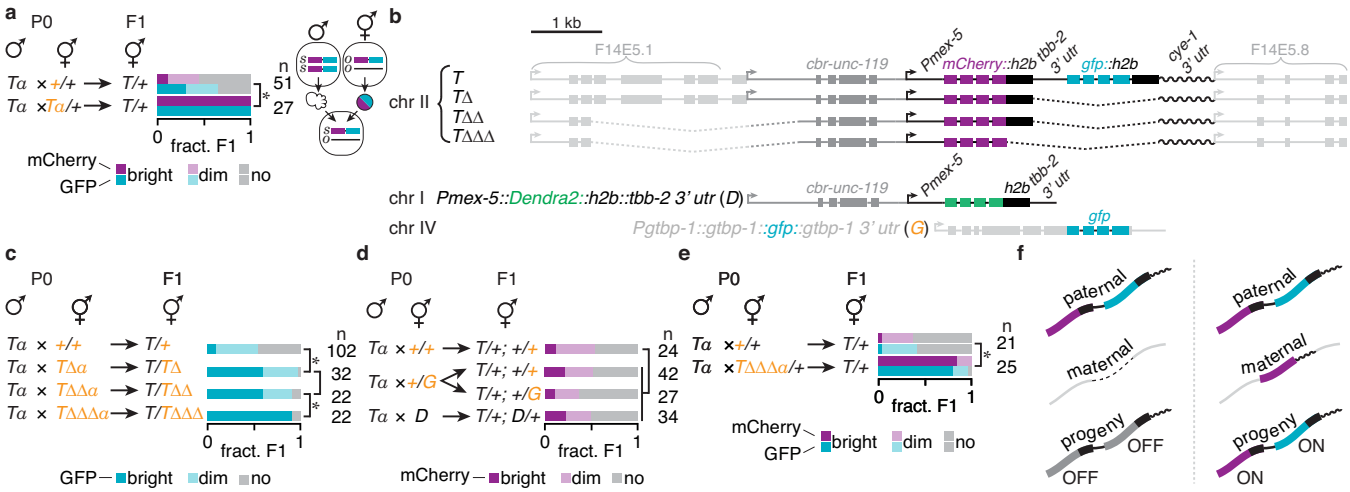

Figure 2. A maternal DNA-independent protective signal can prevent mating-induced silencing. **a**, Males that express the active transgene (*Tα*) were mated with non-transgenic (+/+) or hemizygous (*Tα*/+) hermaphrodites, and fluorescence was scored (*top*, mCherry – magenta, bright; pink, dim; grey, no, and *bottom*, GFP – blue, bright; cyan, dim; grey, 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* [*D*] 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* [*D*], 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 *T*α through the sperm. **f**, Model depicting maternal expression of *T*ΔΔΔ (magenta) is sufficient to prevent silencing of both *mCherry* and *gfp* from paternal *T*α 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 Methods).

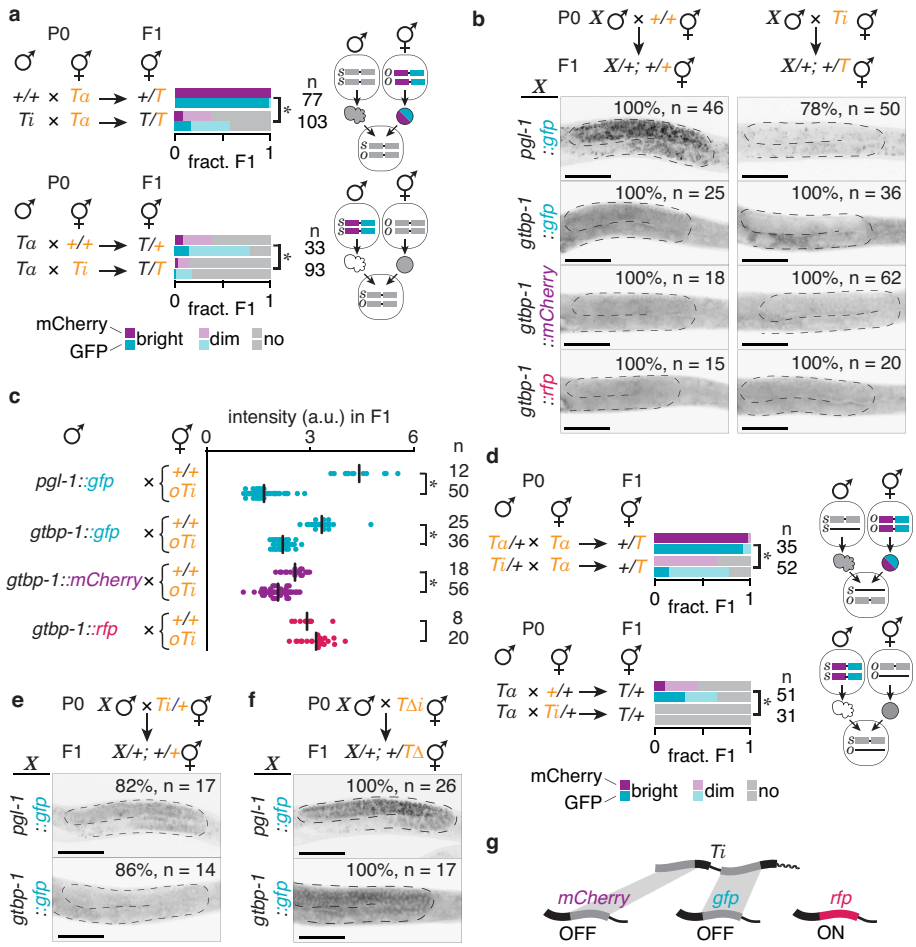

Figure 3. Mating-induced silencing generates a heritable DNA-independent silencing signal.

a, *Tα* 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. 2a): a silenced transgene (*Ti*, grey) can silence *Tα* 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**, *Tα* animals (hermaphrodites – *top crosses*; males – *bottom crosses*) were mated with animals that lacked *Ti* (*Tα*/+ in *top* or +/+ in *bottom*) or that carried the inactive transgene (*Ti*/+), and mCherry fluorescence was scored in cross progeny that only inherited *Tα*. Schematics depict outcome of each test cross (as in Fig. 2a): 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. 2a. 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*.

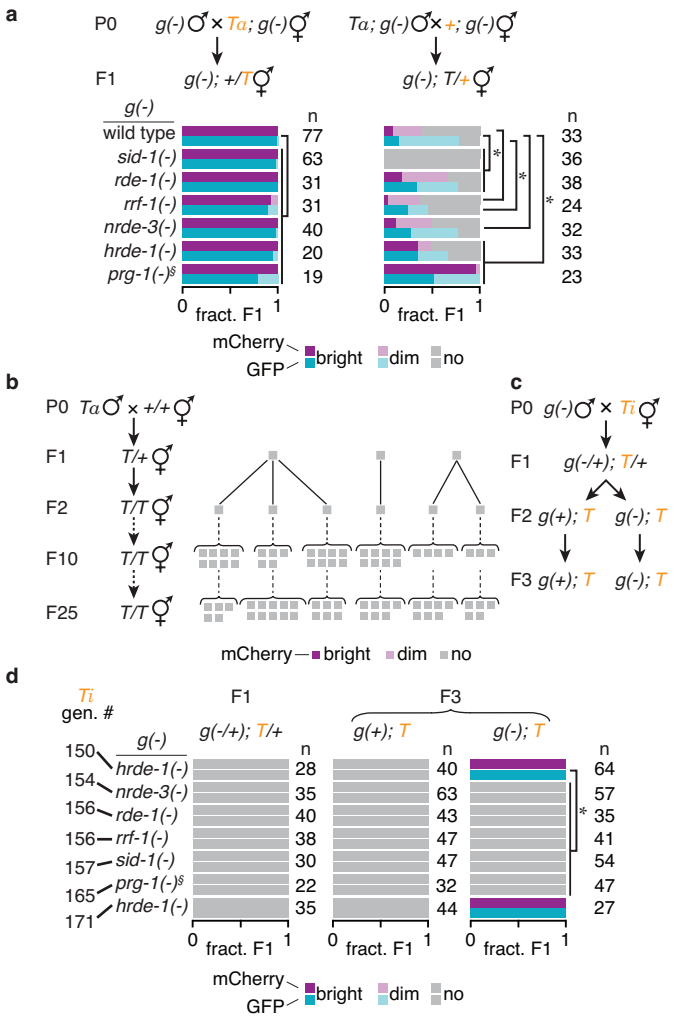

Figure 4. Mating-induced silencing requires the Argonaute PRG-1 for initiation and the Argonaute HRDE-1 for maintenance.

a, Mating-induced silencing was initiated as in Fig. 1 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. 2a and Extended Data Fig. 3a. 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 Extended Data Fig. 7 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. 2a.

SUPPLEMENTARY MATERIAL

Materials and Methods

Strains used

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*; mCherry at cut (sens5) for gene K08F4.2*
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.

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 ATTTAGGTGACACTATAGGATCAGGTAGTGGCCCACCAGTTTTAGAGCTAGAAATAGCAAG

P43 AAA AGC ACC GAC TCG GT

P44 ATGGTCTCCAAGGGAGAGGAG

P45 GAATCCTATTGCGGGTTATTTTAGCCACTACCTGATCCCTTG

P46 ATTTAGGTGACACTATAGGTGTAATCCTCGTTGTGGGGTTTTAGAGCTAGAAATAGCAAG

P47 CAAGGGATCAGGTAGTGGCTAAAATAACCCGCAATAGGATTC

P48 TAAGGAGTTCCACGCCCAG

P49 TTTCGCTGTCCTGTCACACTC

P50 CGATGATAAAAGAATCCTATTGCGGGTTATTTTTTGAGCCTGCTTTTTTGTACAAACTTG

P51 CAAGTTTGTACAAAAAAGCAGGCTCAAAAAATAACCCGCAATAGGATTCTTTTATCATCG

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 ATTTAGGTGACACTATAGCTACCATAGGCACCACGAGGTTTTAGAGCTAGAAATAGCAAG

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 ATTTAGGTGACACTATAGACAAATGCCCGGGGGATCGGGTTTTAGAGCTAGAAATAGCAAG

P60 TGAGGTCAAGACCACCTACAAG

P61 GAATCCTATTGCGGGTTATTTTACTTGCTGGAAGTGTACTTGG

P62 CCAAGTACACTTCCAGCAAGTAAAATAACCCGCAATAGGATTC

P63 GACCACCTACAAGGCTAAGAAG

P64 ATTTAGGTGACACTATAGGGGAGAGGGAAGACCATACGGTTTTAGAGCTAGAAATAGCAAG

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

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 $T\alpha$ 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.

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 Fig. 1b, 1e, 4b, and Extended Data Fig. 4, 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. 1d) and fluorescence from other transgenes (Fig. 3c), 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. 1d, 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. 3c, 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.

Genetic crosses. Three L4 hermaphrodites and 7-13 males were placed on the same plate and allowed to mate for each cross plates. 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. 1 and in Extended Data Fig. 4, 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. 2a, 2c-e, 3, 4,and Extended Data Fig. 3, 5, 6, 8, and 9, *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. 2d and 4a *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. 2a, 2d, e, and 3d, e. Animals from crosses with *prg-1(+/-)* males in Fig. 4a *left*, and in Fig. 4d or with *T; prg-1(+/-)* males in Fig. 4a *right were* also genotyped to identify *T; prg-1(-/-)* or *prg-1(-/-)* cross progeny, respectively. In crosses from Fig. 2d and Fig. 3e, cross progeny of the required genotype were identified by the absence or presence of pharyngeal mCherry or GFP[8], respectively.

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.

CRISPR-Cas9 mediated editing of *oxSi487*. To generate edits in *oxSi487*, Cas9-based genome editing with a co-conversion strategy[16] 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 Extended Data Table 2.

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. 3c.

Data availability. The data that support the findings of this study are available from the corresponding author upon request.

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 + nd 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. 4a)).

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 (Extended Data Fig. 5b). 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. 2). 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[17], it is stalled at prophase I in oocytes until fertilization[18]. 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. 3d, 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 (Extended Data Fig. 9). All strains analyzed for initiation (Fig. 4a) and maintenance (Fig. 4d) 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.

Supplemental Discussion

Comparison of mating-induced silencing with related epigenetic phenomena

The hallmarks of mating-induced silencing are: (1) silencing is initiated upon inheritance only through the male sperm; (2) once initiated, silencing is stable for many generations; (3) 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 (4) maternal exonic sequences can prevent initiation of silencing. While to our knowledge no other known phenomenon shares all of these hallmarks (Extended Data Table 1), 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 (reviewed in 19). In addition to similar heritability, both paramutation[20,24,37,38,43] 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[21,22,23]. Second, how a paramutagenic allele first arises remains obscure[19]. Third, while some alleles are paramutable, others are not, for reasons that are unknown[20]. 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[24].

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[1]. Some studies of RNAe[1,25], but not others (p.94 in (ref. 2)) 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[1], 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[1,2,3,25]. This model is inadequate because the same sequence can be either silenced or expressed within the germline[1] and endogenous genes are subjected to transgenerational silencing through similar PRG-1- and HRDE-1-dependent mechanisms[5,6,7,26,27]. Furthermore, the features of a transgene that trigger silencing are unknown. Tethering the Argonaute CSR-1 to the nascent transcript[28] or adding intronic sequences that are found in native germline-expressed genes[4] 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. 2). Although mating-induced silencing is not a general property of transgenes (Extended Data Fig. 1), a similar silencing phenomenon with dependence on maternal mRNA has been observed for the endogenous gene *fem-1* (ref. 29). 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 mechansims that dictate expression or silencing of a sequence in every generation in otherwise wild-type animals.

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 intiating mating-induced silencing in our studies examining silencing by dsRNA from neurons[8]. Such methodological considerations impelled by this study could impact conclusions drawn from previous studies of epigenetic silencing in *C. elegans*.

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. 4) 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[30]. Many endogenous genes in *C. elegans* are silenced by HRDE-1 (ref. 1, 5, 27, 31), 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.

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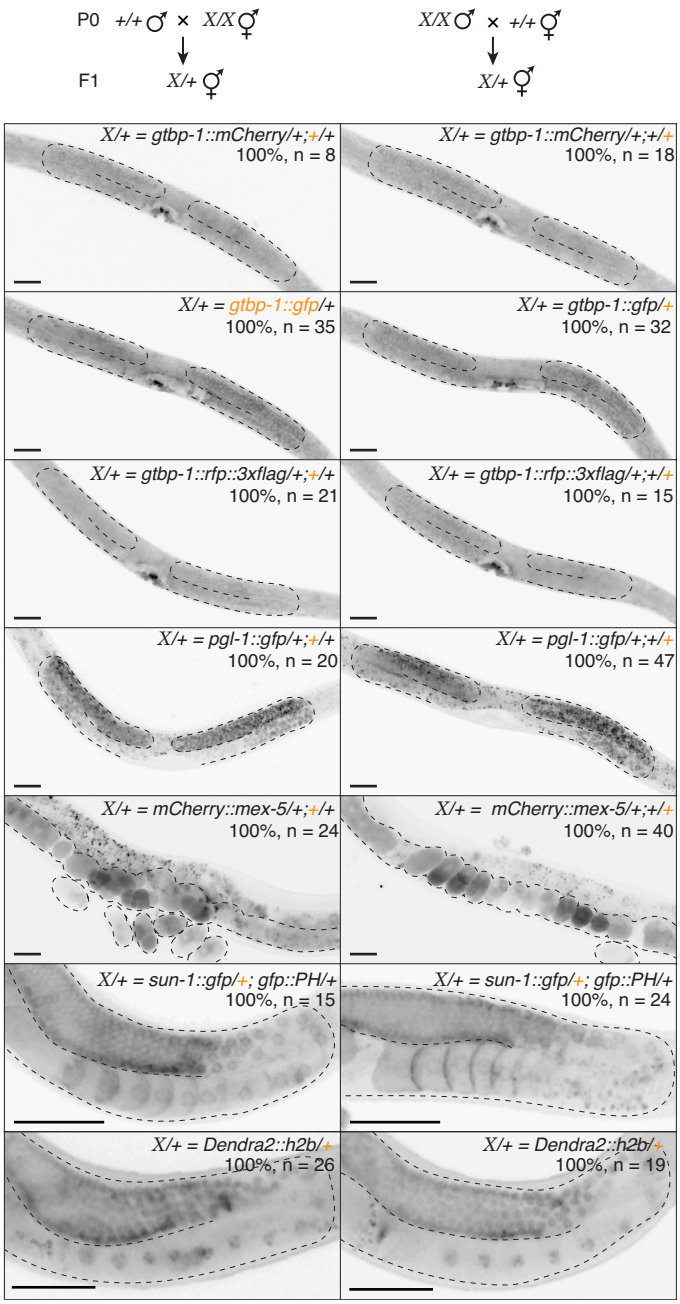
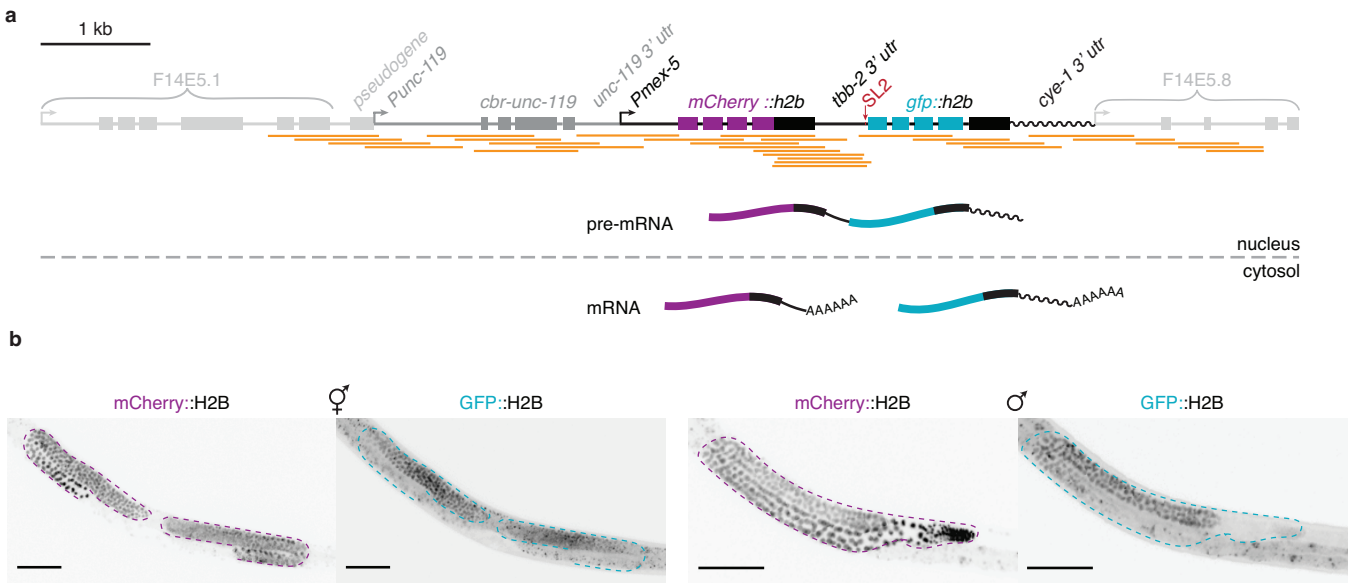
Extended Data Figures and Legends



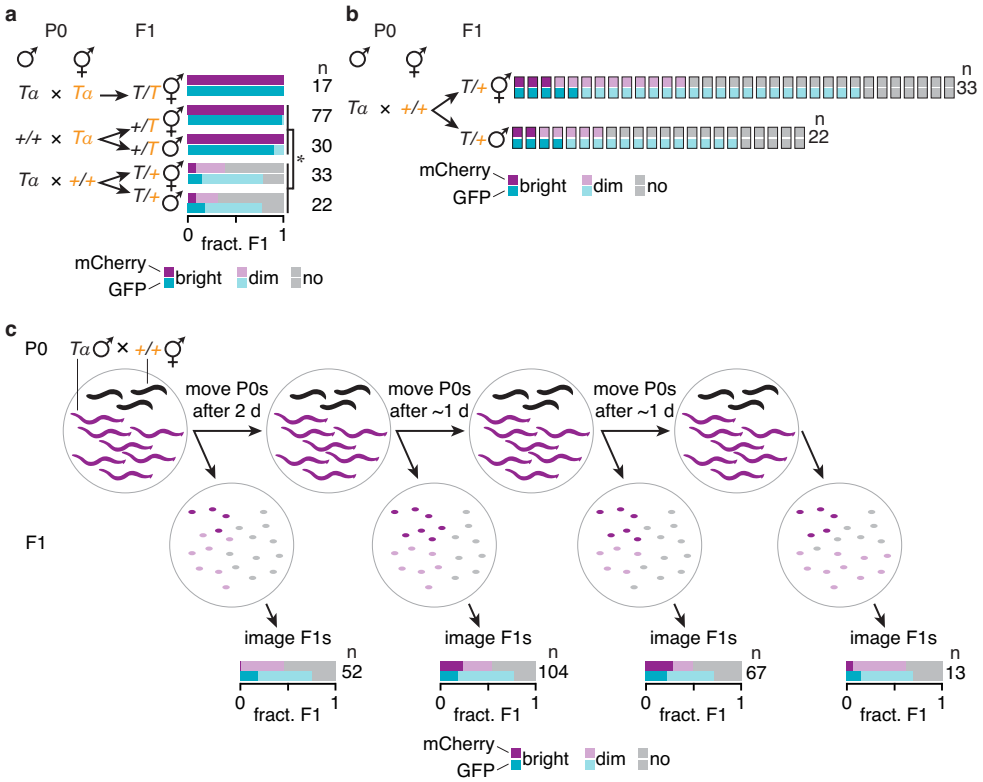
Extended Data Fig. 1. Expression of many transgenes remains unaffected by mating.

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. 1. Germlines of representative cross progeny at L4 or adult stage are outlined. Scale bar = 50 µm. Number of animals assayed and orange font are as in Fig. 2a.



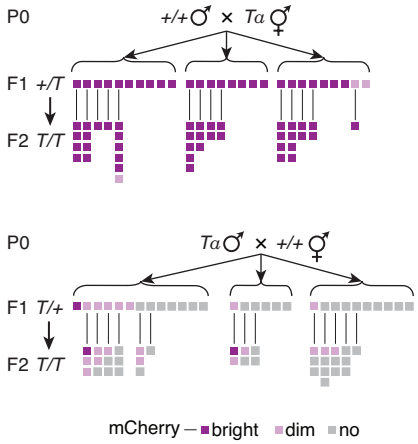
Extended Data Fig. 2. A transgene with foreign DNA coding for two fluorescent proteins shows stable expression within the germline in both hermaphrodites and males.

a, Schematic of *oxSi487* (*Pmex-5::mCherry::h2b::tbb-2 3' utr::gpd-2 operon::gfp::h2b::cye-1 3' utr*) (ref. 9) 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-58/66) 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. Orange lines correspond to fragments of DNA verified by Sanger sequencing in the strain that expresses *oxSi487*. 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.



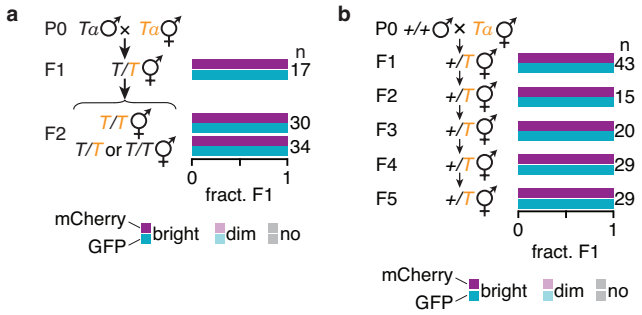
Extended Data Fig. 3. Mating can trigger silencing of both cistrons in an operon.

a, Cross progeny that inherited *Tα* 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**, *Tα* males (magenta) and non-transgenic hermaphrodites (black) 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. 2a. While silencing triggered by parental ingestion of dsRNA is less effective in later progeny[32], silencing triggered by mating can be equally effective in early and in late progeny.



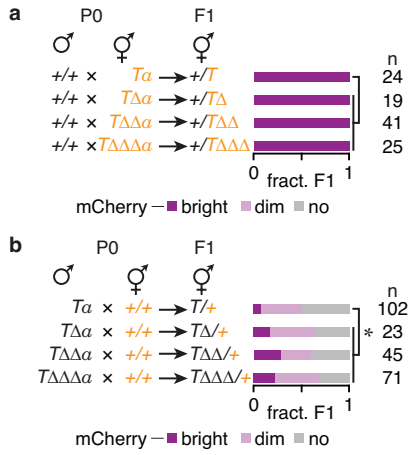
Extended Data Fig. 4. Mating-induced silencing is heritable.

Tα 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. 1c, d) from a single adult animal and lines indicate descent. See Fig. 1e 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[33,34], where passage of *T* through oocytes is expected to revive expression.



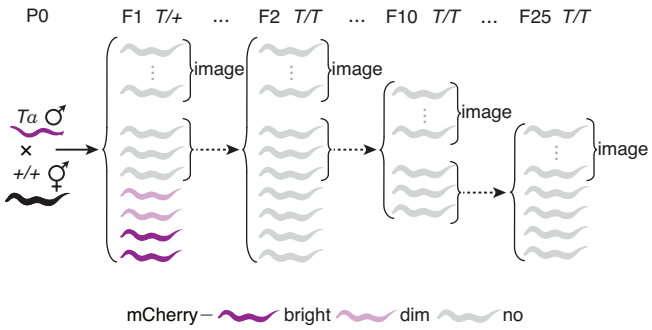
Extended Data Figure 5. Inheritance through hermaphrodite sperm does not trigger silencing of the transgene *T*.

a, *Tα* males were mated with *Tα* 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 Extended Data Fig. 3a. **b**, *Tα* 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. 2a. In contrast to previous reports[35], we find that *oxSi487* is not subject to meiotic silencing by unpaired DNA[36].



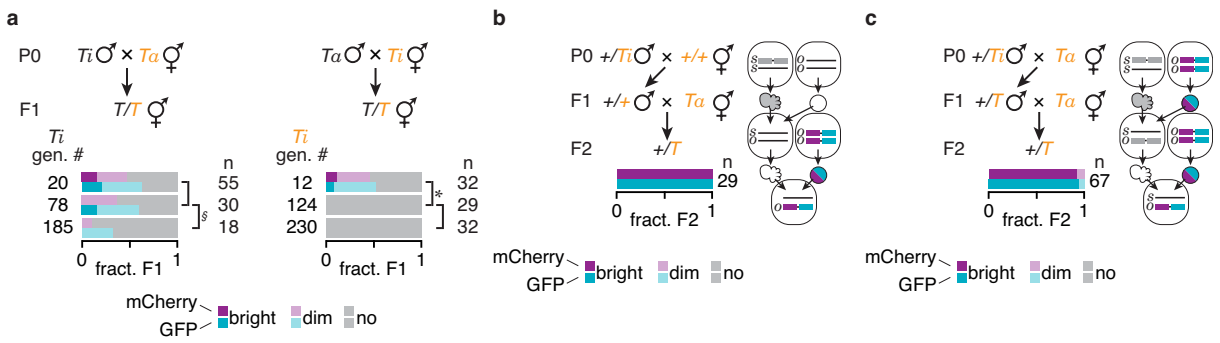
Extended Data Figure 6. Variants that lack some sequences of the transgene *T* are also susceptible to mating-induced silencing.

a, **b**, Hermaphrodites (**a**) or males (**b**) that express *T*α or that express variants of *T*α (as in Fig. 3b) with deletions in *gfp::h2b::tbb-2 3' utr* (*T*$\Delta\alpha$), in upstream sequences (*T*$\Delta\Delta\alpha$), and in *h2b* (*T*$\Delta\Delta\Delta\alpha$) 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. 2a.



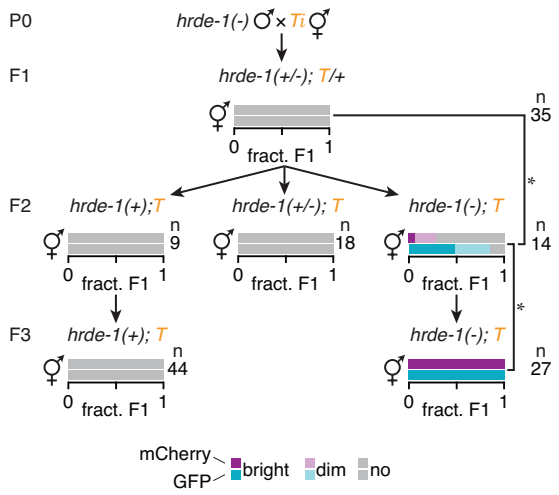
Extended Data Figure 7. Passaging scheme to evaluate persistence of transgenerational silencing.

Tα males (magenta) were mated with wild-type hermaphrodites (black) 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. 4b for data on fluorescence.



Extended Data Figure 8. 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* strains 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. 2a. **b**, **c**, Males that carry *Ti* (*Ti*/+) were mated with non-transgenic (**b**) or *Ta* hermaphrodites (**c**), 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. 2a): paternal inheritance of the DNA-independent silencing signal does not result in further transmission of the signal to descendants (*left*) despite the presence of *Ta* in the animal (*right*), 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. 2a. § indicates $P < 0.013$.



Extended Data Figure 9. 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. 4c, d for summary of *hrde-1* requirement. Scoring of silencing, number of animals assayed, orange font, brackets and asterisks are as in Fig. 2a.

Extended Data Tables

Extended Data Table 1. Comparison of mating-induced silencing with related epigenetic phenomena.

Phenomenon	Similarity with mating-induced silencing	Difference from mating-induced silencing
Paramutation in plants [24,37], flies[20,43], or mice[38]	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)[1,2,3,6,25]	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[35]	Initiation requires PRG-1. *oxSi487* (*T* in our study) 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)[39,40]	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[36]	Silencing of DNA is epigenetic.	DNA must be upaired during meiosis for silencing.
Epigenetic licensing of *fem-1* (ref. 29)	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[33,34,41]	Silencing occurs when a gene is inherited through a specific gamete.	Expression is reset upon passage through the other gamete.
Transposon silencing in flies[42,43]	Inherited piRNAs silence a paternally inherited gene.	Maternal transcript does not prevent gene silencing.
Transvection in flies[44]	Interaction between alleles on homologous chromosomes can result in changed expression.	Changes in gene expression are not heritable.
Licensing by DNA sequences[4]	Not all transgenes are susceptible to germline silencing.	Initiation of silencing is independent of mating.

Extended Data Table 2. Details of reagents used for Cas9-mediated genome editing.

Allele name	CRISPR edit	Primers used to make:		Length of homology repair template	Concentration of reagents used (pmol/μl)				
		DNA template for sgRNA transcription	Homology repair template		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* and *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