piRNAs Initiate an Epigenetic Memory of Nonself RNA in the *C. elegans* Germline

Masaki Shirayama,1,2 Meetu Seth,1 Heng-Chi Lee,1 Weifeng Gu,1 Takao Ishidate,1,2 Darryl Conte, Jr.,1 and Craig C. Mello1,2,*
1Program in Molecular Medicine
2Howard Hughes Medical Institute
University of Massachusetts Medical School, 373 Plantation Street, Worcester, MA 01605, USA
*Correspondence: craig.mello@umassmed.edu
http://dx.doi.org/10.1016/j.cell.2012.06.015

**SUMMARY**

Organisms employ a fascinating array of strategies to silence invasive nucleic acids such as transposons and viruses. Although evidence exists for several pathways that detect foreign sequences, including pathways that sense copy number, unpaired DNA, or aberrant RNA (e.g., dsRNA), in many cases, the mechanisms used to distinguish “self” from “nonself” nucleic acids remain mysterious. Here, we describe an RNA-induced epigenetic silencing pathway that permanently silences single-copy transgenes. We show that the Piwi Argonaute PRG-1 and its genomically encoded piRNA cofactors initiate permanent silencing, and maintenance depends on chromatin factors and the WAGO Argonaute pathway. Our findings support a model in which PRG-1 scans for foreign sequences and two other Argonaute pathways serve as epigenetic memories of “self” and “nonself” RNAs. These findings suggest how organisms can utilize RNAi-related mechanisms to detect foreign sequences not by any molecular signature, but by comparing the foreign sequence to a memory of previous gene expression.

**INTRODUCTION**

All organisms balance the need to maintain genetic variation against the danger of accumulating potentially deleterious genes or pathogenic sequences (Antonovics et al., 2011). The experimental introduction of DNA (transgenes) into the germline provides an opportunity to probe an organism’s response to foreign DNA (Rülicke and Hübscher, 2000) and has revealed that organisms use a variety of mechanisms to silence transgenes in the germline (Birchler et al., 2003; Brodersen and Voinnet, 2006). Interestingly, some mutants that disrupt transgene silencing also desilence endogenous genes, including self-replicating elements called transposons (Ketting et al., 1999; Tabara et al., 1999). Thus, the mechanisms involved in transgene silencing protect the genome from invasive DNA elements.

In many organisms, transgene silencing has been linked to factors that are also required for the RNAi pathway (Bosher and Labouesse, 2000). RNAi was first identified as a sequence-specific response triggered by double-stranded RNA (dsRNA) (Fire et al., 1998). During RNAi, dsRNA is processed by the RNase III-related protein, Dicer, into ~21 nucleotide (nt) short-interfering RNAs (siRNAs) (Bermstein et al., 2001; Carmell and Hannon, 2004; Zamore et al., 2000), which are loaded onto Argonaute (AGO) proteins to form the key effectors of RNA-induced silencing complexes (Hammond et al., 2001; Liu et al., 2004; Meister et al., 2004). AGOs are RNase H-related proteins that use the base-pairing potential of small RNA cofactors to guide sequence-specific binding to target sequences (Song et al., 2004). In some cases, AGOs directly cleave their targets; in other cases, AGOs recruit cofactors that direct mRNA destruction or other modes of regulation.

Despite a clear overlap between the mechanisms that mediate RNAi and the silencing of transposons and transgenes, several findings point to distinct triggering mechanisms. For example, the AGO protein RDE-1 is essential for the dsRNA response in *C. elegans* but is not required for transposon or transgene silencing (Tabara et al., 1999). RDE-1 engages siRNAs produced by Dicer and mediates the initial search for target RNAs in the cell (Parrish and Fire, 2001; Yigit et al., 2006). RDE-1 is thought to recruit a cellular RNA-dependent RNA polymerase (RdRP), which then utilizes the target mRNA as a template for the production of secondary siRNAs, termed 22G-RNAs (Gu et al., 2009; Pak and Fire, 2007; Sijen et al., 2001, 2007; Yigit et al., 2006). The 22G-RNAs are loaded onto members of an expanded, partially redundant, group of worm-specific AGOs (WAGOs). WAGOs that localize to the cytoplasm are thought to mediate mRNA turnover, whereas WAGOs that localize to the nucleus mediate transcriptional silencing (Gu et al., 2009; Sijen et al., 2001, 2007; Yigit et al., 2006). Many components of the RNAi pathway that function downstream of RDE-1 are required for transposon and transgene silencing, including the RdRP system (Gu et al., 2009; Smardon et al., 2000), the polynucleotide polymerase RDE-3 (Chen et al., 2005), the nuclelease MUT-7 (Ketting et al., 1999), and the WAGO proteins (Yigit et al., 2006), among others.
Heritable and Dominant Silencing of Single-Copy Transgenes

(A) Transgenic lines created by MosSCI. MosSCI injection mixture made with 1 ng/μl (a) or 50 ng/μl (b) target plasmid for heat-shock method.

(B and C) Fluorescence micrographs of adult hermaphrodite germlines from (B) GFP-positive neSi9 gfp::csr-1(+) and (C) GFP-negative neSi8 gfp::csr-1(RNAe) transgenic lines.

(D and E) Schematic diagrams illustrating the results of genetic crosses between expressed (green) and silenced (gray) gfp::csr-1 transgenic lines (>100 animals scored per generation after F2). In (D), neSi9 gfp::csr-1(RNAe) hermaphrodites were mated with neSi9 gfp::csr-1(+) males. In (E), neSi10 gfp::csr-1(RNAe) hermaphrodites, integrated on chromosome IV (LGIV), were mated to neSi9 gfp::csr-1(+) males, integrated on chromosome II (LGII). In the F2 generation, the neSi9 gfp::csr-1(+) allele was segregated away from neSi10 and propagated for eight more generations.

Here, we use a homologous gene-targeting method called “Mos1-mediated single-copy insertion” (MosSCI; Frøkjaer-Jensen et al., 2008) to show that strains bearing identical single-copy transgenes inserted at the same chromosomal site can exhibit opposite and remarkably stable epigenetic fates, either expressed or silenced. Transgenes consisting of an endogenous germline-expressed gene fused to a relatively long foreign sequence (e.g., gfp) were prone to silencing. By contrast, otherwise identical transgenes fused to a short foreign sequence (e.g., flag) were always expressed. Our genetic and molecular analyses reveal that silencing is dependent on nuclear and cytoplasmic WAGOs and is correlated with the accumulation of 22G-RNAs targeting the foreign portion of the transgene. Importantly, PRG-1 is required to initiate, but not to maintain, silencing. We propose that PRG-1 and its 21U-RNA cofactors scan for foreign RNA sequences and initiate WAGO-maintained gene silencing, and endogenous mRNAs are protected from silencing, perhaps by the CSR-1/22G-RNA pathway.

RESULTS

Heritable and Dominant Silencing of Single-Copy Transgenes

Single-copy insertions can overcome barriers to transgene expression in the germline (Rieckher et al., 2009). Indeed, the single-copy insertion of transgenes at a defined chromosomal locus via the recently developed MosSCI approach reproducibly achieves germline expression (Frøkjaer-Jensen et al., 2008). However, while using MosSCI, we were surprised to find that not all single-copy transgenes were expressed in the germline (Figures 1A–1C). The failure to express was only common for transgene fusions to lengthy foreign sequences, gfp (Figure 1A); transgenes with the flag epitope sequences were nearly always fully expressed (Figure 1A). Furthermore, we observed that transgenes in which gfp was inserted at the 5′ (rather than 3′) end of the construct were much less likely to be expressed (Figure 1A). PCR and sequence analyses indicated that nonexpressed transgenes are structurally identical.
to expressed transgenes, suggesting that the former are actively silenced.

We next crossed a silent line to an expressing line to see which phenotype dominates. Strikingly, we found that 100% of the F1 cross-progeny (n = 12) and F2 self-progeny (n = 24) failed to express gfp in the germline (Figure 1D). Identical results were obtained even when the silent and active alleles were inserted on separate chromosomes (Figure 1E), suggesting that chromosomal pairing is not required for transfer of the silent state. Although transgenes with 3’ gfp insertions were less prone to silencing during transgene formation, they were fully silenced when crossed to a silent line (Figure 3J and data not shown).

We found that either parent could contribute the dominant silencing signal. However, when the silent allele was male derived, it took more than one generation to completely silence the active allele. For example, silencing was observed in 67% (n = 15) of F1 progeny when the silent allele was paternally derived, whereas 100% (n = 12) of F1 progeny were silenced when maternally derived. Nevertheless, regardless of the parent of origin, in the F3 and subsequent generations, 100% of the descendants were GFP negative (n > 100). The silent phenotype was fully penetrant, with no evidence of expression or reversion even after the formerly active allele was resegregated as a homozygote (Figure 1E). These results clearly indicate that the failure to express these single-copy transgenes represents an active silencing process that involves a dominant trans-acting silencing signal. We first observed this dominant silencing activity in crosses with gfp::csr-1, which raised a concern because CSR-1 is an Argonaute that is potentially involved in silencing mechanisms. However, identical results were obtained in crosses with cdk-1 transgenes (data not shown), indicating that there is nothing unusual about the csr-1 transgenic lines.

We refer to this phenomenon as RNA-induced epigenetic silencing (RNAe) because the silent state is stable indefinitely (without evidence of reversion), and (as shown below) maintenance of silencing involves a small RNA silencing signal that is epigenetically programmed (not genomically encoded). We identify transgenes exhibiting this type of silencing by including the term “(RNAe)” after the transgene name (e.g., neSi11 gfp::cdk-1(RNAe)). For clarity, active versions of the same alleles are referred to using (+), e.g., neSi11 gfp::cdk-1 (+).

High-copy transgenes in C. elegans can induce cosuppression of endogenous homologous genes (Dernburg et al., 2000; Ketting and Plasterk, 2000). Several of the transgenes observed are fusion constructs with essential genes (e.g., gfp::cdk-1) and should result in obvious visible phenotypes if the corresponding endogenous locus was cosuppressed. However, no phenotypic evidence of cosuppression was observed in the silent lines analyzed (data not shown), suggesting that, despite the dominant nature of the silencing signal, silencing does not spread to the endogenous locus. To ask whether there is a partial suppression of the endogenous locus, we performed western blot analysis to determine the relative expression of the transgene and endogenous protein products in both active and silent lines. Consistent with the lack of phenotypic evidence for cosuppression, we observed identical levels of endogenous

protein expression in both the active and silent transgenic lines (Figure 2A).

**RNAe Requires Chromatin Factors and Correlates with H3K9me3**

To ask whether silencing is regulated transcriptionally or post-transcriptionally, we isolated total RNA from otherwise identical silent and active gfp::csr-1 strains and measured the abundance of pre-mRNAs and mRNAs by real-time quantitative PCR (qPCR). We found that both the pre-mRNA and mRNA levels were significantly reduced in the silent line compared to the active line (Figures 2B and 2D). Moreover, although a reduction at the pre-mRNA level appeared to account for the majority of silencing, a further reduction was evident at the mRNA level, suggesting that silencing is achieved at both transcriptional and posttranscriptional levels (Figures 2B and 2D).

Previous work has shown that the methylation of lysine 9 on histone H3 (H3K9me), a histone modification associated with silent chromatin, is enriched on high-copy number transgenes in the germline (Bessler et al., 2010; Kelly et al., 2002). Furthermore, germline silencing of high-copy transgenes is dependent on a number of chromatin-associated factors, including the Polycomb group complex (MES-2/3/4), a Trithorax-related protein (MES-4), and the heterochromatin proteins (HPL-1 and 2) (Couteau et al., 2002; Grishok et al., 2005; Kelly and...
The possible involvement of an RNAi-related small RNA pathway.

Factors Maintenance of silencing requires RNAi-related nance of single-copy transgene silencing involves a chromatin hpl-2 and enrichment observed. Finally, we found that chromatin, perhaps accounting for the relatively weak 2-fold enrichment observed. The lysates in chromatin immunoprecipitation (ChIP) experiments using anti-MosSCI allele, but not an active MosSCI allele, were enriched findings, we found that transgene sequences from a silent 68 Cell Fire, 1998; Kelly et al., 1997 ). Consistent with these previous cGFP is desilenced in fraction of germline in the same worm. bGFP is partially desilenced (GFP signal is weak in each worm). aScored in sterile M

table for Maintenance of Gene Silencing

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NA, not applicable.

^aScored in sterile M – Z– mutants.

^bGFP is partially desilenced (GFP signal is weak in each worm).

^cGFP is desilenced in fraction of germline in the same worm.

trans into strains bearing mutations in RNAi components. Two down-stream factors in the exo-RNAi pathway, rde-3 and mut-7, which encode a β-nucleotidyl transferase and a 3’-5’ exonuclease, respectively (Chen et al., 2005; Ketting et al., 1999), are known to be required for the maintenance of transposon silencing and have been implicated in cosuppression (Dernburg et al., 2000; Ketting and Plasterk, 2000) and high-copy number transgene silencing (Tabara et al., 1999). Consistent with the involvement of these factors in the maintenance of RNAe, we found that crossing a silent transgene into these mutant strains resulted in fully restored transgene expression (Table 1).

Nuclear and Cytoplasmic WAGOs Are Required for Silencing Maintenance

Because RDE-3 and MUT-7 are required for the accumulation of RdRP-derived 22G-RNAs that engage WAGOs (Gu et al., 2009; Yigit et al., 2006), we asked whether WAGOs are required for the maintenance of single-copy transgene silencing by crossing silent lines with several different wago mutant strains. We found that a mutation in the predominantly cytoplasmic germline WAGO, wago-1(tm1414) (Gu et al., 2009), partially desilenced a gfp::cdk-1 transgene but did not desilence a gfp::csr-1 transgene (Table 1 and Figures 3A and 3C).

The finding that wago-1 mutants failed to desilence gfp::csr-1 and only partially desilenced gfp::cdk-1 suggested that additional WAGOs contribute to RNAe (Figure 3). Furthermore, because RNAe involves a chromatin component, we suspected that nuclear WAGOs might be important for RNAe. The nuclear WAGO, NRDE-3/WAGO-12, is required for nuclear RNAi and transcripational silencing in somatic tissues (Burton et al., 2011; Guang et al., 2008), and nrde-3 mutants failed to desilence a gfp::csr-1 transgene in the germline (Table 1). However, within the WAGO subclade that includes NRDE-3 (Figure 3I), we identified WAGO-9 (HRDE-1/C16C10.3) as a nuclear WAGO that is restricted to the germline (Figure 3G). Furthermore, we found that wago-9(tm1200) mutants fully desilenced a gfp::csr-1 transgene and partially desilenced a gfp::cdk-1 transgene (Figures 3B and 3D), the converse of the relationship between wago-1(tm1414) and these RNAe lines. The desilencing of gfp::cdk-1 was increased in a wago-1; wago-9 double mutant (Figure 3E).

The wago-9 locus was also identified by two other groups (Ashe et al., 2012 [this issue of Cell]; S.G. Kennedy, personal communication) as a gene required for heritable RNAi (hence its other name, heritable RNAi-defective, hrde-1).

Because gfp::cdk-1 was not completely desilenced by these wago mutant combinations, we asked whether additional members of the nuclear WAGO subclade play a role in gfp::cdk-1 silencing. Indeed, gfp::cdk-1 was strongly desilenced in a wago-9; wago-10 (t22h9.3); wago-11(f49f6a.1); nrde-3 quadruple mutant, as well as in a wago-9; wago-10 double

Fire, 1998; Kelly et al., 1997). Consistent with these previous findings, we found that transgene sequences from a silent MosSCI allele, but not an active MosSCI allele, were enriched in chromatin immunoprecipitation (ChIP) experiments using antibodies specific for H3K9me3 (Figures 2C and 2D). The lysates used were from whole worms; therefore, only a portion of the chromatin present in the total lysate corresponds to germline chromatin, perhaps accounting for the relatively weak 2-fold enrichment observed. Finally, we found that mes-3, mes-4, and hpl-2 mutants all desilenced the gfp::csr-1 and gfp::cdk-1 transgenes (Table 1). These findings suggest that the maintenance of single-copy transgene silencing involves a chromatin component.

Maintenance of Silencing Requires RNAi-Related Factors

The trans-acting nature of the silencing phenomenon suggested the possible involvement of an RNAi-related small RNA pathway. To explore this possibility, we crossed a silent transgenic
mutant (Table 1 and Figure 3F). Taken together, these findings indicate that cytoplasmic and nuclear WAGOs contribute to RNAe in parallel and that the input from cytoplasmic and nuclear WAGOs varies between individual RNAe lines.

The small RNAs that associate with WAGO-1 were previously identified by immunoprecipitation (IP) of FLAG::WAGO-1 followed by deep sequencing of associated small RNAs (Gu et al., 2009). We performed similar studies using a flag::wago-9 construct. We found that the targets of WAGO-9 largely overlap with those of WAGO-1 (Figure 3H). These observations suggest that nuclear and cytoplasmic WAGOs share targets and are likely to share a common 22G biogenesis pathway.

Silencing Correlates with Accumulation of 22Gs

Targeting gfp
To examine the small RNA profile associated with germline silencing, we dissected gonads from different transgenic lines, including active, silent, and converted lines (e.g., active-to-silent and silent-to-active lines) and prepared small RNA libraries for deep sequencing (Figures 3J and S1). Strikingly, each silenced line exhibited a marked accumulation of 22G-RNAs that were restricted to the gfp portion of the transgene sequence (Figures 3J and S1). Consistent with the idea that these 22Gs are WAGO pathway dependent, we found that 22G-RNA levels targeting gfp were significantly reduced in lines converted from silent to active by crossing through an rde-3 mutant background (Figure S1).

Native germline-expressed genes are recognized by low levels of 22G-RNAs that engage CSR-1 (CSR-1-22Gs) (Claycomb et al., 2009). We found that the transgene sequences corresponding to endogenous germline-expressed mRNA sequences always exhibited low 22G-RNA levels similar to those observed for the endogenous sequences in wild-type nontransgenic animals (Figures 3J and S1). These findings suggest that the WAGO-mediated silencing signal only targets the foreign sequences of the transgene.

Initiation of Silencing Requires the Piwi Argonaute PRG-1
Despite interacting with distinct small RNA species, both PRG-1 and RDE-1 function as primary AGOs upstream of WAGO-22G-mediated silencing (Batista et al., 2008; Das et al., 2008; Pak and Fire, 2007; Sijen et al., 2007; Yigit et al., 2006). However, we found that neither prg-1 nor rde-1 mutants could activate an already established silent transgene (Table 1). To explore the possibility that either PRG-1 or RDE-1 is involved in the initiation of RNAe, we generated new transgenic lines by directly injecting into rde-3 mutant animals (Figures 3J and S1). These findings suggest that the WAGO-mediated silencing signal only targets the foreign sequences of the transgene.

When established in the wild-type background, the epigenetic state of a transgene, whether active or silent, is stably maintained over many generations. If PRG-1 is only required for the initiation of silencing, then we expected that active transgenes established in a prg-1 mutant background would remain active even after outcrossing to a wild-type strain. We found that gfp::cdk-1 was expressed in 96% (n = 24) of the heterozygous F1 progeny. However, by the F3 generation, gfp::cdk-1 was silent in all wild-type descendants (Figure 4). Conversely, among the F3 animals that were once again homozygous for the prg-1 mutation, 77% (n = 30) maintained expression of the gfp::cdk-1 transgene (Figure 4). These findings support the idea that PRG-1 is involved in the initiation of gene silencing.

However, the finding that the transgene becomes silent after outcross to wild-type indicates that the active state for this transgene does not become epigenetically stable when propagated in the prg-1 mutant background. This observation raises the possibility that PRG-1 is upstream of competing epigenetic pathways: one that initiates silencing and one that initiates antisilencing (see below and Discussion).

A trans-Acting Antisilencing Signal
The findings described above indicate that extremely stable silencing associated with single-copy transgenes is initiated by piRNAs and requires the same downstream factors that are required for RDE-1-dependent dsRNA-induced silencing. However, unlike the silencing described here, to our knowledge, dsRNA-induced silencing (even when transmitted for numerous generations) has not been observed to become stable. Instead, all previous descriptions of inherited RNAi described reversion frequencies in the range of 80% per generation (Alcazar et al., 2008; Vastenhouw et al., 2006).

We therefore wondered whether PRG-1 somehow initiates a more stable mode of silencing than that initiated through RDE-1. To test this idea, we used gfp dsRNA to initiate silencing of active GFP(+) transgenes and monitored expression for multiple generations after removal of the dsRNA trigger. In each generation, we scored ten animals from each of ten independent lines for a total of 100 worms per generation. For the gfp::csr-1 transgene, we found that, as expected, 100% of the animals were silenced in the F1 generation. Remarkably, however, 100% of gfp::csr-1 worms remained silent in all ten lines for greater than ten generations, with no evidence of reversion. Similar results were obtained for the cdk-1::gfp transgene. This transgene, which was less prone to silencing during initial transgenesis, remained completely silent in six of ten lines, whereas four lines recovered expression. Thus, the susceptibility of these active transgene lines to piRNA-induced silencing mirrors their susceptibility to dsRNA-induced permanent silencing.

The above data suggest that the MosSCI transgenes studied here are more sensitive than endogenous genes to permanent silencing by RNAi. To ask whether this is generally true of transgenes, we asked whether exposure to gfp(RNAi) could permanently silence low-copy transgenes generated several
Figure 3. Genetic Requirements for Maintenance of RNAe

(A–F) Fluorescence microscopy of transgene desilencing in wago mutant backgrounds. The transgenes used were neSi8 gfp::csr-1(RNAe), which localizes to P granules when expressed (indicated by arrows in A and B), and neSi11 gfp::cdk-1(RNAe), which is most prominent in oocyte nuclei (indicated by arrowheads in C–F). (G) WAGO-9 is a germline-expressed nuclear Argonaute. Fluorescence micrograph of GFP::WAGO-9 in the adult hermaphrodite germline. The dashed lines in the micrograph indicate the position of the syncytial germline.

(H) WAGO-9-associated small RNAs overlap extensively with WAGO-1 small RNAs. The plot shows the enrichment of 22G-RNAs in FLAG::WAGO-9 IP relative to input. Each point in the graph corresponds to previously identified WAGO-1 (blue) and CSR-1 (red) target genes. The x and y axes represent the number of 22Gs.
years ago by different methods. For this analysis, we chose two different transgenes generated by different approaches: gfp::wmr-1 (Nakamura et al., 2005), which was produced by injecting an engineered yeast artificial chromosome, and oma-1::gfp (Lin, 2003), which was generated by biolistic gold-particle-mediated transformation (Praitis, 2006). We found that both transgenes were efficiently silenced by RNAi in the F1 (100%, n = 100), but expression always fully recovered after removal of the dsRNA trigger (100% GFP+ by the F3 generation).

Considering the resistance of gfp::wmr-1 and oma-1::gfp to permanent silencing by dsRNA, we wondered whether they might also be resistant to trans-silencing in crosses with silent transgenes. Surprisingly, not only were both gfp::wmr-1 and oma-1::gfp resistant to trans-silencing, but we also found that both transgenes could dominantly activate the expression of a silent transgene in the F1 cross progeny (Figures 5A–5C). Expression was initially low in the F1 and F2, but, when propagated along with gfp::wmr-1 or oma-1::gfp transgenes, the trans-activated transgene alleles became fully expressed by the third generation (Figures 5A–5C). Finally, after propagating the activated transgene lines in the presence of gfp::wmr-1 or oma-1::gfp for a few generations, we segregated the transgenes away from each other. We found that gfp::cdk-1 returned to its silent state (Figure 5B), whereas cdk-1::gfp remained stably expressed after exposure to the active transgene (Figure 5C). Although we need to test more transgenic lines, these findings indicate that a trans-acting dominant mechanism can activate a silent transgene and suggest that activating and silencing signals compete with each other for dominance when transgene alleles interact.

DISCUSSION

Recognition of Self and Nonself Nucleic Acids

Organisms employ an array of mechanisms that afford some control over the expression of foreign sequences (Hormung and Latz, 2010; Murray, 2002). In Drosophila, for example, piRNAs have been shown to mediate transposon silencing in the germline (Malone and Hannon, 2009). In this remarkable system, transposons are thought to move freely at first until a spontaneous insertion into a genomic piRNA-generating locus results in the expression of piRNAs perfectly complementary to the new transposon (Khurana and Theurkauf, 2010). The stable genomic integration of the transposon within the piRNA-generating locus initiates silencing and provides a genetic (rather than epigenetic) memory of the invasive sequence. Maternally inherited piRNAs function to prime production of piRNAs, but this requires a genetic reservoir of transposon sequence in the maternal genome (Brennecke et al., 2008). Even defective transposon remnants embedded in piRNA-producing loci are sufficient to maintain piRNA production in the absence of a functional transposon (Grentzinger et al., 2012). Here, we have shown that C. elegans employs piRNAs in a very different mechanism that recognizes even single-copy foreign sequences and initiates a remarkably stable epigenetic memory of silencing. Rather than depending on the site of integration or on an aberrant feature of the transgene DNA or RNA product, our findings suggest that initiation of silencing involves the comparison of the foreign sequence to an epigenetic memory of previously expressed sequences. Thus, genetically identical individuals in C. elegans can exhibit remarkably stable but opposite patterns of expression.

We propose a model in which three AGO pathways function together in a system that maintains an inventory of expressed mRNAs while constantly scanning for foreign sequences (Figure 6B). In this system, PRG-1 uses genomically encoded piRNA cofactors to scan, via imperfect base-pairing interactions, for foreign RNAs expressed in the germline. Upon targeting, PRG-1 recruits RdRP to produce antisense 22G-RNAs, which are loaded onto WAGO Argonautes. In turn, WAGOs mediate silencing and establish a memory of nonself RNA. A third as yet unidentified pathway provides a memory of self and is capable of acting as an antisilencing signal. Although our studies have not yet identified the antisilencing (self-recognition) mechanism, the CSR-1 22G-RNA pathway provides an attractive candidate for this activity (see further discussion below). We propose that the self-recognition pathway can prevent PRG-1 from recruiting the WAGO pathway, providing a function that helps expressed transgenes to maintain their expression and helps endogenous genes to recover from WAGO-mediated silencing.

(log2 scale) targeting each gene in the input and WAGO-9 IP samples, respectively. The diagonal lines signify 2-fold enrichment (top), identity (middle), and 2-fold depletion (bottom) of 22G-RNAs in the WAGO-9 IP.
(i) Phylogenetic tree of WAGOs, CSR-1, and RDE-1. Adapted from Yigit et al. (2006).
(j) Small RNA density along the gfp- and cdk-1-coding regions of wild-type and indicated transgenic lines. Vertical bars represent the 5' nt of a small RNA, and the height of each bar indicates the number of reads that start at that position. The strand is represented by color; sense (light blue) and antisense (pink). Scale bar indicates ten reads per million. Strain neSi12 cdk-1::gfp(RNAe) was generated by crossing neSi12 cdk-1::gfp(+) to neSi11 gfp::cdk-1(RNAe).
induced by RNAi. The initial decision to silence or express the transgene represents a stochastic outcome of competition between establishment of these epigenetic self- or nonself memories.

Repetitive and Single-Copy Transgenes Exhibit Distinct but Overlapping Silencing Mechanisms

The silencing of high-copy and single-copy transgenes shares several features, including chromatin-related and WAGO 22G pathway requirements. Furthermore, both high-copy (Tabara et al., 1999) and single-copy silencing (the present study) occur independently of RDE-1 and thus are unlikely to be initiated by dsRNA. However, several observations suggest that high-copy transgenes are subject to distinct modes of recognition and silencing. First, high-copy transgenes were at best only partially desilenced in WAGO pathway mutant contexts, such as rde-3 and mut-7 (Tabara et al., 1999 and data not shown), whereas single-copy transgenes were fully desilenced and, in some cases, even maintained their expression after outcrossing to wild-type. Second, high-copy transgenes were fully and rapidly silenced in the germline of prg-1 mutant animals (data not shown), indicating that a distinct initiation step is involved in high-copy number silencing. Third, high-copy number silencing was observed even when only the native germline gene sequences were present in the transgene (data not shown), whereas silencing of the single-copy transgene was correlated with the presence of foreign sequences within the germline-expressed portion of the transgene construct. Finally, unlike the single-copy silencing described here, wherein trans-silencing remains focused on foreign sequences, high-copy transgenes were found to elicit cosuppression of the endogenous gene (Dernburg et al., 2000; Ketting and Plasterk, 2000). Taken together, these observations are consistent with the existence of at least two distinct modes of silencing that act on transgenes: one that depends on high-copy number and can spread throughout the transgene and a second that requires PRG-1 and is restricted to portions of the transgene composed of foreign sequences.

21U-RNAs Complementary to gfp Are Correlated with 22G Biogenesis

Our findings suggest that transgene silencing is initiated by PRG-1 and depends on the presence of foreign gfp sequences in the transgene. In a parallel study, PRG-1 was shown to initiate silencing of synthetic reporters containing sites perfectly complementary to 21U-RNAs (Lee et al., 2012 [this issue of Cell]; Bagijn et al., 2012). Mismatched pairing was also correlated with silencing both on transgenes (Bagijn et al., 2012) and on presumptive endogenous targets (Lee et al., 2012; Bagijn et al., 2012). We have not identified 21U-RNAs that are perfectly complementary to gfp; however, there are dozens of potential high-affinity 21U-RNA-gfp target sites (data not shown). Our

Figure 5. Evidence for a trans-Acting Antisilencing Activity

(A) Schematic illustrating the cross between neSi11 gfp::cdk-1(RNAe) and teIs1 oma-1::gfp(+). The micrographs show the expression status of GFP::CDK-1 in oocyte nuclei (arrowhead) when expressed and OMA-1::GFP in the oocyte cytoplasm. The dashed circles (upper left) show the position of GFP-negative oocyte nuclei in the neSi11 gfp::cdk-1(RNAe) strain. The cartoon below each micrograph indicates whether the transgene is expressed (green) or silent (gray).

(B and C) Schematics illustrating crosses between nels2 gfp::wrm-1(+) males and (B) neSi11 gfp::cdk-1(RNAe) or (C) neSi12 cdk-1::gfp(RNAe) hermaphrodites. After each cross, the two transgenes were either maintained together or were allowed to segregate away from each other. The GFP::WFM-1 signal is very weak and was scored periodically during the analysis. The percentage of GFP+ worms indicates the expression of the CDK-1 fusion proteins.
Figure 6. Model: Self-Nonself RNA Recognition in *C. elegans*

(A) Schematic showing the density of 22G-RNAs targeting gfp in *neSi8 gfp::csr-1/RNAe* worms, as described in the legend of Figure 3J. Scale bar indicates 20 reads per million. The positions of several 21U-RNAs that could base pair with mismatches to the gfp sequence are indicated below the gene diagram. Five major 22G hot spots (numbered boxes) are enlarged to show the base pairing between the candidate 21U-RNA and gfp, as well as the density of 22G-RNAs at single-nucleotide resolution. Each 21U-RNA has, at most, two G:U pairs within the seed region (nt 2–8, yellow highlight) and, at most, three nonseed mismatches (nt 9–21).

(B) Model for the allelic interactions between transgenes observed in this study.
recent studies (Lee et al., 2012) suggest that PRG-1/21U-RNA targeting initiates 22G-RNA biogenesis within a ±40nt window around the site of 21U-RNA complementarity on the target RNA. We found eight regions in gfp in which 22G-RNAs were detected at greater than 75 reads per million in a silent strain (Figure 6A). We identified potential high-affinity 21U-RNA interactions in all eight regions. The potential base-pairing interactions and the proximal 22G-RNAs found in a silent transgenic strain are shown at single-nucleotide resolution in Figure 6A (also see Experimental Procedures). Validation of these candidate 21U-RNA target sites and the general rules that govern piRNA targeting remain to be elucidated.

CSR-1 as an Antisilencing Argonaute
At least three mechanisms must work together to explain the all-or-none nature (expressed or silent) of the epigenetic states observed and the stable heritability of these states once established (Figure 6B). The genetic studies thus far have implicated PRG-1 in the initiation of silencing and the WAGO pathway in the maintenance of silencing. The third pathway required is a “maintenance-of-expression,” or “antisilencing,” pathway. Such a pathway is necessary to explain why, once established, active transgenes are stably transmitted from one generation to the next without undergoing spontaneous silencing. An antisilencing pathway could also explain how certain active transgenes are able to dominantly activate silent transgenes (Figure 6B).

The CSR-1 22G pathway targets endogenous germine-expressed mRNAs (Claycomb et al., 2009) and is an ideal candidate for an antisilencing pathway. In vitro, CSR-1 is catalytically active and capable of cleaving a target (Aoki et al., 2007), whereas the all WAGOs lack key catalytic residues (Yigit et al., 2006). Perhaps CSR-1 can compete by selectively destroying RNAs on which RdRP is bound, thus preventing or attenuating the production of WAGO 22G-RNAs. It is not known how CSR-1 targeting is first established. However, all of the transgenes that we analyzed contain endogenous germine-expressed sequences known to be targeted by CSR-1 22Gs. Perhaps CSR-1 22Gs can spread in trans along a target transcript, as has been shown for the transitive RNAi mediated by WAGOs after dsRNA targeting (Pak and Fire, 2007; Sijen et al., 2007; Yigit et al., 2006). If so, then stable expression of a transgene may reflect the spread of CSR-1 targeting to the foreign portion of the transgene prior to PRG-1 recognition.

Interestingly, although the antisilencing signal initially appears to be sufficient to prevent PRG-1-driven silencing, it is not sufficient to prevent silencing initiated in crosses with a silent transgene or when dsRNA is used to stimulate gene silencing. If CSR-1 22G-RNAs represent the antisilencing signal, then it will be interesting to explore whether the levels of CSR-1 22G-RNAs build up over generations. If so, then the older transgenes, which were able to activate a silent transgene, may show relatively high levels of CSR-1 22G-RNAs targeting gfp when compared to newly established lines. However, it is also possible that as yet unknown features of the chromatin environments of the different transgenes drive their different sensitivity to trans-silencing and their differing abilities to trans-activate or to recover from silencing spontaneously.

Finally, it is worth noting that PRG-1 may function upstream of RdRP recruitment for both the CSR-1 and WAGO pathways. If so, then the decision to express or silence a new transgene may represent the result of a competition between the CSR-1 and WAGO pathways for RdRP loading, downstream of this initial recruitment. An expectation for such a model would be that both the maintenance of silencing (nonself) and maintenance-of-expression (self) pathways should fail to initiate when PRG-1 is absent. To further explore this question, it will be important to analyze the behavior of additional transgenes established in the prg-1 mutant background.

RNA-Induced Epigenetic Inheritance
Here, we have described a remarkably stable form of epigenetic inheritance (RNAe) that is initiated by C. elegans piRNAs. Though RNAe likely serves as a defense against transposons and other invasive sequences, it is also possible that it could have a more general role with significant potential impact on evolution. For example, RNAe could accelerate evolutionary change by heritably modulating the expression of unpaired parental alleles to allow the phenotypic expression of recessive traits among F1 progeny. Consistent with this idea, a recent report has shown that a paternally derived allele with no homolog in the hermaphrodite genome is subject to dominant silencing and that silencing was prevented by injecting single-stranded RNAs matching the coding region of the absent gene into hermaphrodite gonads prior to the cross (Johnson and Spence, 2011). These observations are consistent with a mechanism for the licensing of gene expression by maternal RNA and, along with the present study, support the existence of an epigenetic switch that is sensitive to prior expression of a gene. These phenomena are also similar to a form of allelic interaction known as paramutation that has been described in organisms ranging from mice to corn (Erhard and Hollick, 2011). Thus, it appears likely that diverse organisms can both track and respond epigenetically to the history of gene expression. In C. elegans, this process overlaps mechanistically with RNAi but involves a distinct triggering mechanism that requires the genomically encoded piRNAs. Mammalian genomes encode abundant piRNA species that are analogous to C. elegans 21U-RNAs. Our findings raise the intriguing possibility that piRNAs of mammals and other animals function in epigenetic programming.

EXPERIMENTAL PROCEDURES
Genetics
All C. elegans strains were derived from the Bristol N2 strain and cultured as described (Brenner, 1974). The strains used in this study are listed in Table S1.

MosSCI by Direct Injection
MosSCI lines were generated by the direct insertion method using strains EG4322 and EG5003, as described (Frøkjaer-Jensen et al., 2008). Targeting vectors are described in the Supplemental Information.

MosSCI by Heat Shock and Ivermectin Selection
Strain WM1186 was injected with a DNA mixture containing 50 ng/µl each of pRF4::rol-6(su1006), pCCM416::Jmyo-2::avr-15, and pJL44::Phsp16.48::MosTase::gh-2ub (Frøkjaer-Jensen et al., 2008) and either 1 ng/µl or 50 ng/µl of targeting vector. MosSCI was performed using the heat-shock
method (Frokjaer-Jensen et al., 2008), and single-copy insertion lines were selected on ivermectin to select against animals carrying the extrachromosomal array. Additional details are provided in the Supplemental Information.

**Small RNA Cloning from Isolated Germlines**

Ten gonads from each strain were dissected in 1 x PBS containing 0.1 mM EDTA, 1 mM Aurin tricarboxylate, 0.1% Tween 20, and 0.2 mM levamisole (Wang et al., 2009). Total RNAs were extracted with five volumes of TRI Reagent (MRC). Small RNAs were gel purified and cloned as described (Gu et al., 2009). gfp::csr-1 small RNAs were pretreated with Tobacco Acid Phosphatase (TAP, Epicenter Biotecologies), gfp::cdk-1 and cdk-1::gfp small RNAs were pretreated with CIP/PNK (NEB). Libraries were sequenced in the UMass Deep Sequencing Core using an Illumina GAII instrument.

**Small RNA Cloning from FLAG::WAGO-9 Immunocomplexes**

Synchronous adult flag::wago-9 worms were dounced in a stainless steel homogenizer. FLAG::WAGO-9 was immunoprecipitated from 20 mg of lysate essentially as described (Gu et al., 2009). Small RNAs were extracted from WAGO-9 immunocomplexes as well as a portion of the input lysate gel purified, pretreated with TAP, cloned, and sequenced as above.

**Computational Analysis of Small RNAs**

Deep sequencing data were processed and analyzed using custom Perl scripts (Gu et al., 2009). Definition of WAGO and CSR-1 22Gs are described (Claycomb et al., 2009). Candidate 21U-RNAs that target gfp were identified by searching for seed sequences (nt 2–8) that base pair with, at most, two G:U wobbles and allowing, at most, three unpaired nonseed residues (nt 9–21). Additional details are provided in the Supplemental Information. Perl scripts are available on request.

**Chromatin Immunoprecipitation**

ChIP was performed essentially as described (Claycomb et al., 2009) except that synchronized adult neSi8 gfp::csr-1 (RNAe) and neSi9 gfp::csr-1(+) worms were dounced in a stainless steel homogenizer (50 strokes) prior to crosslinking with 2.6% formaldehyde. Immunoprecipitations were performed in a total volume of 1 ml (5 mg) with 10 µg of anti-histone H3 (ab1791, Abcam) or anti-H3K9me3 (ab8898, Abcam) antibodies. Immune complexes were recovered with 50 µl of Protein A Dynabeads (Invitrogen). Three independent ChIP experiments were performed and analyzed by quantitative PCR.

**Quantitative PCR**

Quantitative PCR was performed as described (Claycomb et al., 2009) using an ABI 7500 Fast Real-Time PCR instrument. For RNA analysis, cDNA was generated from 1 µg of total RNA using random hexamers and Superscript III Reverse Transcriptase (Invitrogen). gfp::csr-1 expression was measured relative to cep-3 mRNA levels. H3K9me3 ChIP was first normalized to histone H3 ChIP, and fold enrichment was then determined relative to an H3K9me3-negative control locus. Primer sequences are provided in the Supplemental Information.

**Transgenerational RNAi Phenotype**

A single neSi8 gfp::csr-1(+), neSi12 cdk-1::gfp(+), tala1 oma-1::gfp(+), or neIs2 gfp::wmr-1(+) adult worm was placed onto each of ten plates seeded with gfp(RNAi) food. A single F1 worm from each plate was transferred to OP50 (control) or gfp(RNAi) food, and each line was maintained for ten generations by transferring a single worm from each plate to the corresponding food source, OP50 or gfp(RNAi). In each generation, ten progeny from each plate were scored for gfp expression (100 total for each condition).

**Western Blot Analysis**

Antibodies used for western blotting are anti-CSR-1 (Claycomb et al., 2009), anti-GFP (A01704, Genscript), and anti-α-Tubulin (MCA78A, Serotec) antibodies.

**Microscopy**

Transgenic worms were mounted in diH2O on RITE-ON glass slides (Beckton Dickinson). Epi-fluorescence and differential interference contrast (DIC) microscopy were performed using an Axioplan2 Microscope (Zeiss). Images were captured with an ORCA-ER digital camera (Hamamatsu) and AxioVision (Zeiss) software.

**ACCESSION NUMBERS**

Illumina data are available from GEO under the accession number GSE38724.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, one figure, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.06.015.

**ACKNOWLEDGMENTS**

We thank Kirsten Hagstrom and Anna Zinovyeva for primer information, technical advice on ChIP experiments, and helpful discussion. We are grateful to the members of the Mello lab for input and discussion, Sandra Vergara for help with genetic studies, William Stanney III for technical help, and Elaine Youngman and Colin Conine for advice on figure presentation. Some strains used in this study were obtained from the Caenorhabditis Genetic Center. H.-C.L. is supported by a Ruth L. Kirschstein National Research Service Award (GM099372). C.C.M. is a Howard Hughes Medical Institute Investigator and is supported by NIH grant GM058800.

Received: June 5, 2012
Revised: June 15, 2012
Accepted: June 15, 2012
Published online: June 25, 2012

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