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

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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) (Bernstein 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; Guang et al., 2008). 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 nuclease MUT-7 (Ketting et al., 1999), and the WAGO proteins (Yigit et al., 2006), among others

A

Transgene	% (+)	n
gfp::csr-1	6.3%	16
gfp::csr-1[a]	75%	12
gfp::csr-1[b]	0%	60
flag::csr-1	83%	6
gfp::rde-3	17%	18
flag::rde-3	100%	7
rde-3::gfp	93%	14
rde-3::flag	100%	6
gfp::cdk-1	0%	5
gfp::cdk-1[a]	0%	17
gfp::cdk-1[b]	0%	21
cdk-1::gfp	100%	6
gfp::pie-1[a]	96%	23
gfp::pie-1[b]	76%	21

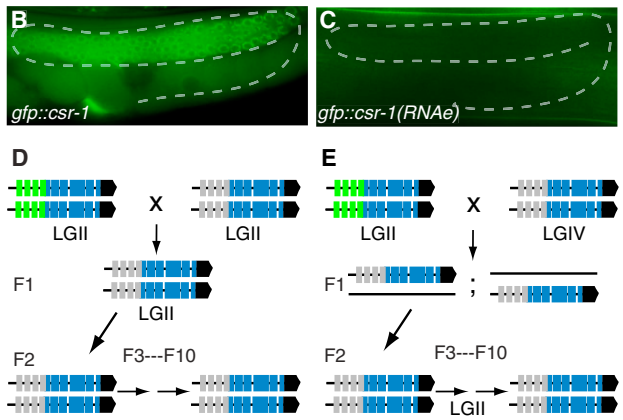

Figure 1. Heritable and Dominant Silencing of Single-Copy Transgenes

(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. GFP::CSR-1 is expressed prominently in the perinuclear P granules in the syncytial germline (dashed outline) and is also visible in the cytoplasm of maturing oocytes.

(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), *neSi8 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.

(Robert et al., 2004). The fact that RDE-1 is not required for transposon and transgene silencing suggests that features unique to transposons and transgenes underlie the initial recruitment of RdRP to these targets and that dsRNA is unlikely to be the trigger.

In the germline, RdRPs not only produce 22G-RNAs that interact with WAGOs, but also produce 22G-RNAs that interact with a distinct AGO, CSR-1, required for fertility and chromosome segregation (Claycomb et al., 2009; Yigit et al., 2006). However, some factors, including RDE-3 and MUT-7, are only required for WAGO 22G-RNA accumulation (Gu et al., 2009), indicating that the CSR-1 and WAGO 22G pathways also involve distinct mechanisms. Indeed, the WAGO and CSR-1 22G pathways together target virtually all germline-expressed mRNAs; however, their targets are largely nonoverlapping (Gu et al., 2009). Furthermore, unlike the WAGO pathway, the CSR-1 22G pathway does not appear to silence its targets (Claycomb et al., 2009). Instead, the CSR-1 pathway may help to define and maintain euchromatic regions along the holocentric chromosomes in order to support the proper assembly of kinetochores.

In most animals, the Piwi family AGOs are required for fertility and transposon silencing (Cox et al., 1998; Juliano et al., 2011). In *C. elegans*, however, the Piwi-related gene product PRG-1 has only been linked to the silencing of one transposon family, Tc3 (Batista et al., 2008; Das et al., 2008). Interestingly, PRG-1 appears to recruit RdRP and the WAGO 22G pathway to maintain Tc3 silencing. Piwi-interacting RNAs (piRNAs) (21U-RNAs in *C. elegans*) are genomically encoded and appear to be expressed as Pol II transcripts whose single-stranded products are processed and loaded onto Piwi (Aravin and Hannon, 2008; Kim et al., 2009). More than 15,000 distinct piRNA species exist in *C. elegans*, and millions of species are expressed in the testes of mammals (Aravin et al., 2006; Batista et al., 2008; Das et al., 2008; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006). The majority of these piRNAs map uniquely to the genome and lack obvious targets. As such, their function remains entirely unknown.

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 *e*pigenetic 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 we analyzed 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).

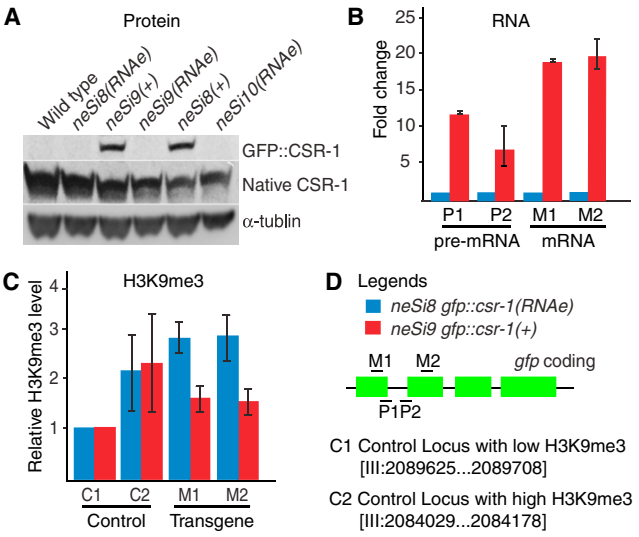

Figure 2. *RNAe* Alleles Exhibit Evidence of Transcriptional Silencing

(A) Analysis of protein expression in wild-type and transgenic strains (as indicated). The blot was probed with anti-GFP (GFP::CSR-1), anti-CSR-1 (Native CSR-1), and anti-α-tubulin (α-tubulin) antibodies (as indicated). The *neSi9 gfp*::*csr-1*(*RNAe*) strain was generated by crossing *neSi9 gfp*::*csr-1*(+) to *neSi10 gfp*::*csr-1*(*RNAe*). The *neSi8 gfp*::*csr-1*(+) strain was generated by crossing *neSi8 gfp*::*csr-1*(*RNAe*) to *rde-3*.

(B and C) qPCR analysis of *gfp::csr-1* mRNA, pre-mRNA, and H3K9me3 levels in silent (blue) and expressed (red) transgenic lines. The strains and probes used are indicated in (D). In (B), *gfp*::*csr-1* expression was normalized to the *clp-3* mRNA. The data are shown as fold change between the expressed and silent *gfp*::*csr-1* alleles. Error bars represent the standard deviation for two experimental replicates. In (C), error bars indicate the standard deviation for three experimental replicates.

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/-6), a Trithorax-related protein (MES-4), and the heterochromatin proteins (HPL-1 and -2) (Couteau et al., 2002; Grishok et al., 2005; Kelly and

Table 1. Genetic Test for Maintenance of Gene Silencing

Gene(Allele)	Gene Function	Transgene Expression	
		gfp::csr-1	*gfp::cdk-1*
rde-1(*ne300*)	Argonaute in RNAi	–	–
prg-1(*tm872*)	Piwi homolog	–	–
rde-3(*ne3370*)	Poly(A) polymerase	+	+
mut-7(*ne4255*)	3′-to-5′ exonuclease	+	+
hpl-1(*tm1624*)	HP1 homolog	–	–
hpl-2(*tm1489*)	HP1 homolog	+[c]	+[b]
hpl-1(*tm1624*) *hpl-2*(*tm1489*)	HP1 homologs	+	+
met-1(n4337) *met-2(n4256)*	methyltransferases	–	NA
mes-3(*bn35*)[a]	Polycomb complex	+[b]	+[b]
mes-4(*bn23*)[a]	Trithorax complex	+[b]	+[b]
wago-1(*tm1414*)	cytoplasmic WAGO	–	+[b]
nrde-3(*tm1116*)	nuclear WAGO	–	NA
wago-9(*tm1200*)	nuclear WAGO	+	+[b]
wago-1(*tm1414*) *wago-9*(*tm1200*)	cytoplasmic and nuclear WAGOs	NA	+
wago-9(*tm1414*) *wago-10*(*tm1186*)	nuclear WAGOs	NA	+
wago-9(*tm1414*) *wago-10*(*tm1186*) *nrde-3*(*tm1116*)	nuclear WAGOs	NA	+
wago-9(*tm1414*) *wago-10*(*tm1186*) *wago-11*(*tm1127*) *nrde-3*(*tm1116*)	nuclear WAGOs	NA	+

NA, not applicable.
[a]Scored in sterile M−Z− mutants.
[b]GFP is partially desilenced (GFP signal is weak in each worm).
[c]GFP is desilenced in fraction of germline in the same worm.

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 strain into strains bearing mutations in RNAi components. Two downstream 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).

We also examined the consequences of crossing strains desilenced in the *rde-3* mutant background back into a wild-type *rde-3*(+) background. We found that, for a *gfp*::*csr-1* transgene desilenced by *rde-3*, 27% of *rde-3*(+) segregants (n = 15) retained expression after outcross (Figure 2A and Figure S1 available online). However, in contrast, strains bearing the *gfp*::*cdk-1* transgene, also desilenced by *rde-3*, were always rapidly and fully resilenced by reintroducing *rde-3*(+) (n > 20).

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 3I). 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 transcriptional 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

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* transgene. 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 *prg-1* and *rde-1* mutants. We chose to inject the *gfp::cdk-1* construct because 100% of MosSCI lines were silent when established in the wild-type background (n = 21) (Figure 1A). In an *rde-1*(*ne300*) mutant strain, we found that the *gfp::cdk-1* transgene was silenced in all three newly isolated lines. Strikingly, however, when we repeated the same experiments with *prg-1*(*tm872*) mutants, the *gfp::cdk-1* transgene was fully active in all five independently generated transgenic lines (Figure 4). Taken together, these findings suggest that PRG-1 and piRNAs are involved in the initiation of transgene silencing, whereas dsRNA (e.g., from bidirectional transcription of the transgene) is not involved.

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, the *gfp*::*cdk-1* transgene was only expressed in 9% (n = 66) of animals heterozygous or homozygous for a wild-type allele of *prg-1*; by the F4 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

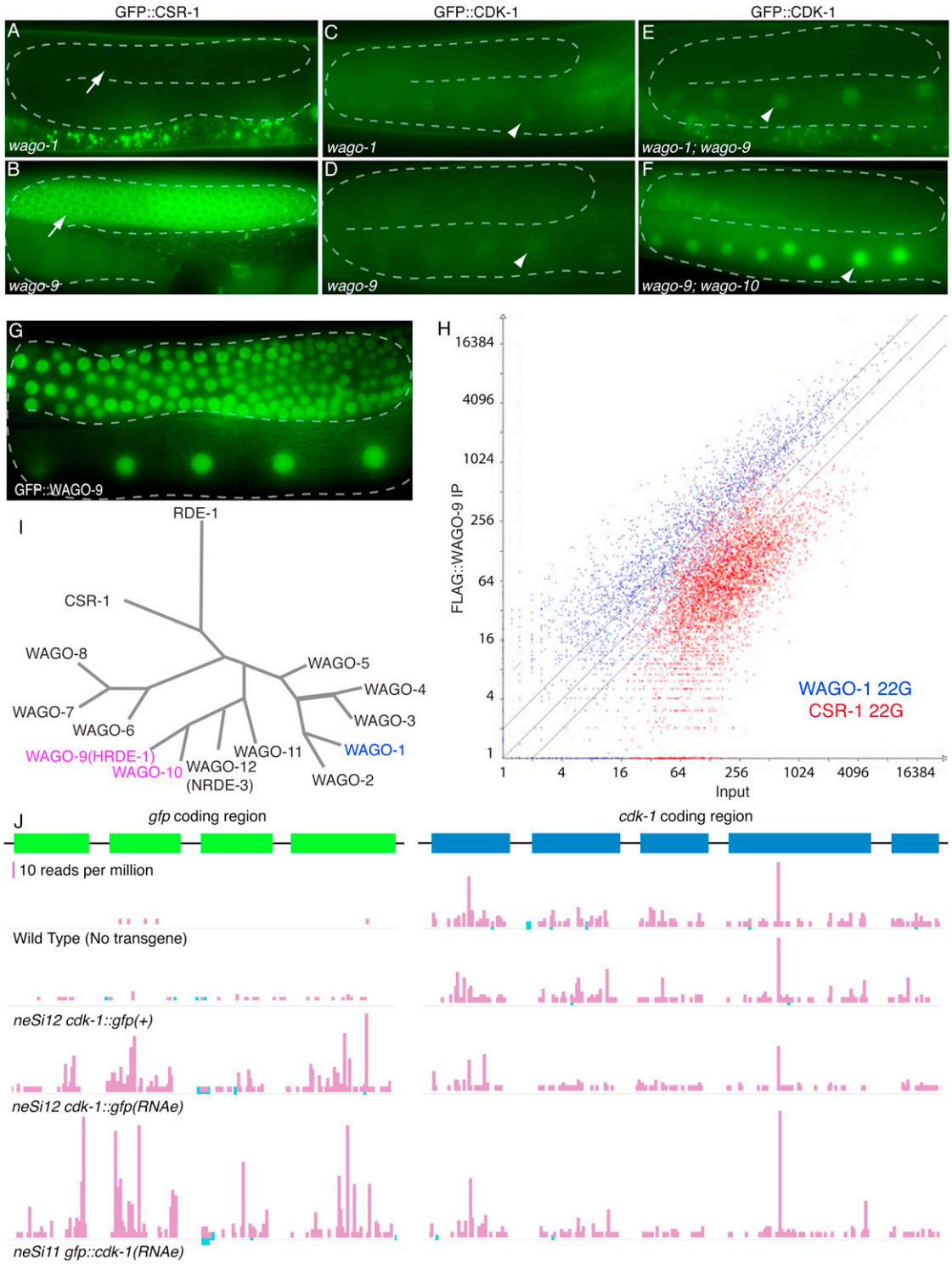

Figure 3. Genetic Requirements for Maintenance of RNAe

(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

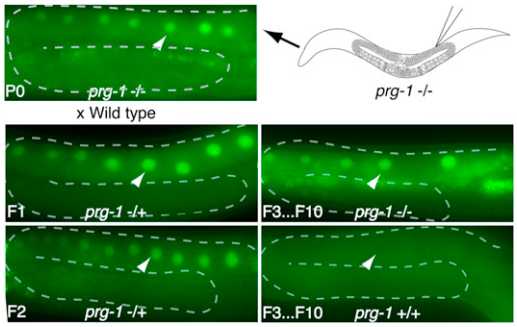

Figure 4. PRG-1 Is Required for the Initiation of RNAe

prg-1(*tm872*) mutant worms injected with the *gfp*::*cdk-1* construct (upper right) give rise to MosSCI lines that express GFP::CDK-1 (P0, upper left). The micrographs show the expression status of GFP::CDK-1 in oocyte nuclei (arrowheads) before (P0) and after outcrossing to wild-type (F1 and F2 panels) and after segregating homozygous *prg-1*(+) and *prg-1*(−) strains for several generations (F3–F10 panels). More than ten worms were examined per generation. Results are detailed in the text.

years ago by different methods. For this analysis, we chose two different transgenes generated by different approaches: *gfp*::*wrm-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*::*wrm-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*::*wrm-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*::*wrm-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*::*wrm-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 (Hornung 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

($\log_2$ 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*).

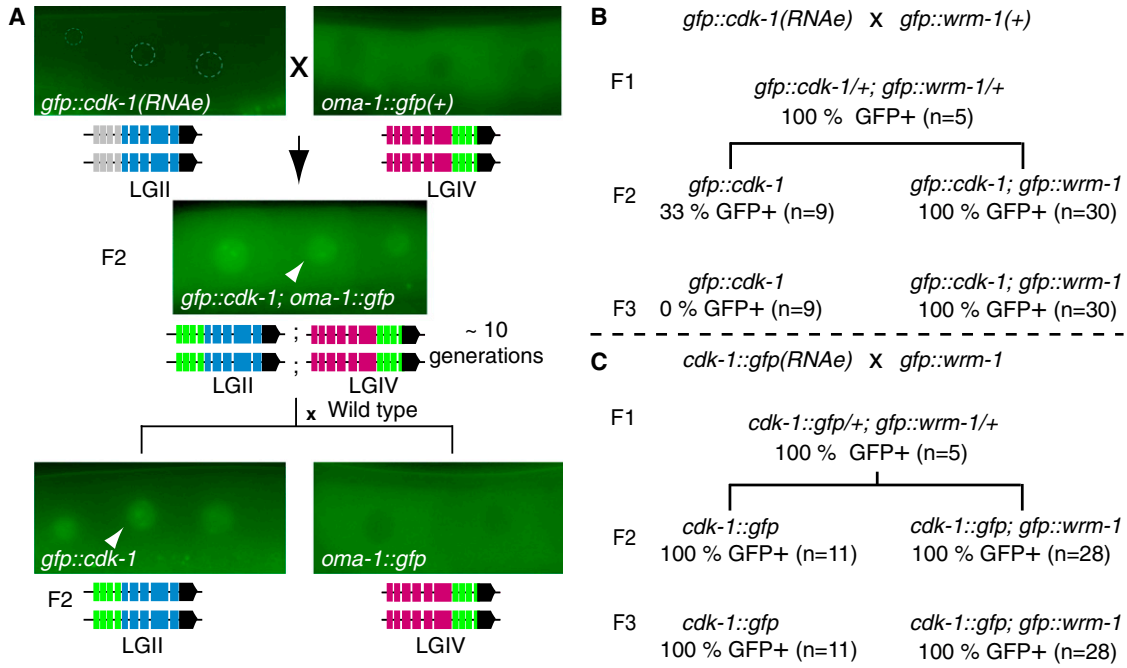

Figure 5. Evidence for a *trans*-Acting Antisilencing Activity

(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 *neIs2 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::WRM-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.

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

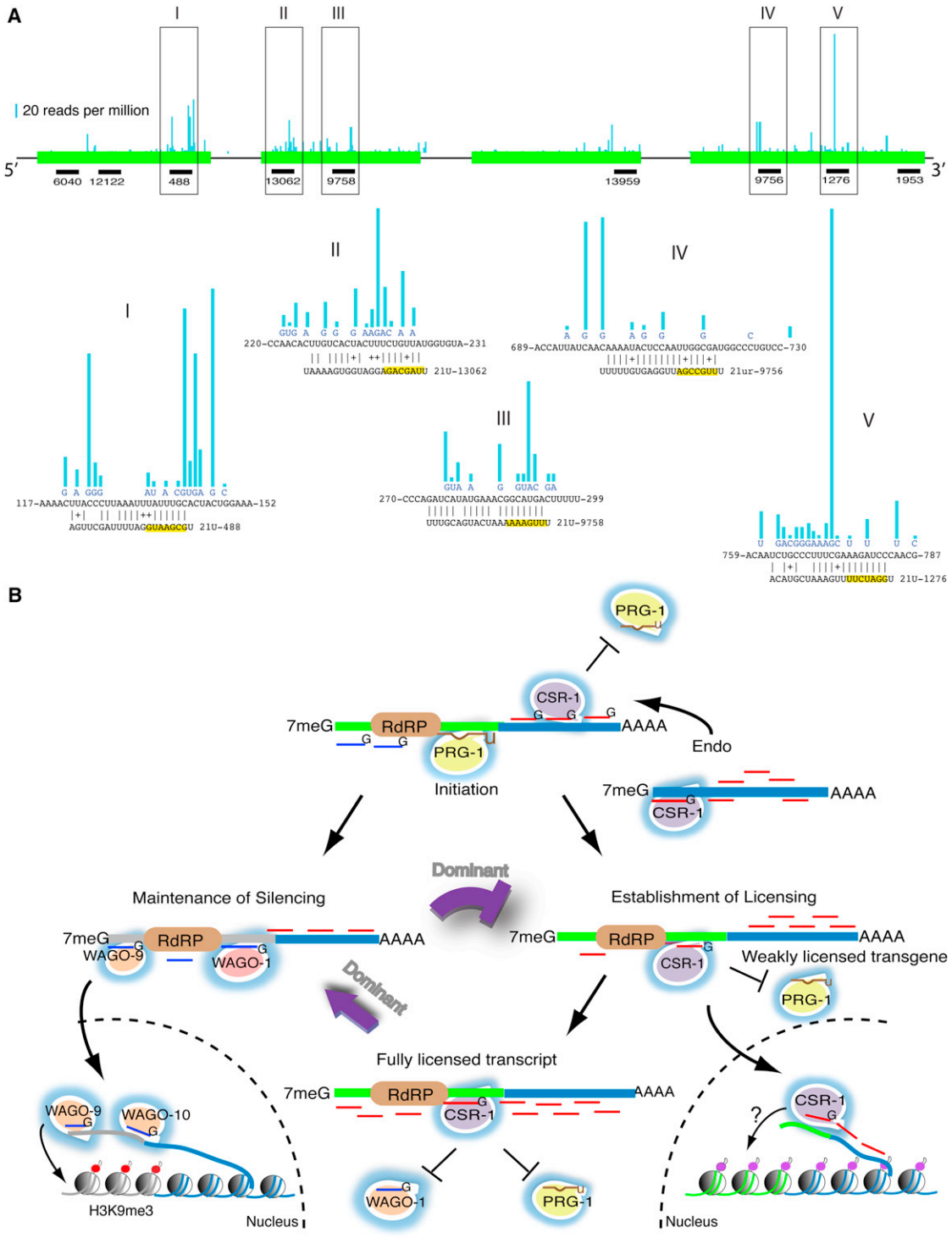

Figure 6. Model: Self-Nonself RNA Recognition in *C. elegans*

(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 ±40 nt 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 germline-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 germline-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 WM186 was injected with a DNA mixture containing 50 ng/μl each of pRF4::*rol-6(su1006)*, pCCM416::*Pmyo-2*::*avr-15*, and pJL44::*Phsp-16.48*::*MosTase*::*glh-2utr* (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 (Frøkjaer-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 × 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 Biotechnologies). *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 Immune Complexes

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 immune complexes 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 in (Gu 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 (30 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 *clp-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 *neSi9 gfp::csr-1*(+), *neSi12 cdk-1::gfp*(+), *tsIs1 oma-1::gfp*(+), or *neIs2 gfp::wrm-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 dH_2O 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.

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Cell

EXTENDED EXPERIMENTAL PROCEDURES

Strain List

Strains used in this study are listed in Table S1.

MosSCI Targeting Constructs

csr-1

A 7.0 kb DNA fragment (*Spe*I – *Bst*Z171) from cosmid (F20D12) containing the entire *csr-1* gene was inserted into a modified version of pCFJ151 (B1496) for LGII or pCFJ178 (B1777) for LGIV (Frøkjaer-Jensen et al., 2008). The *gfp* coding region amplified from pPD95.75 (addgene) or 3x*flag* sequence (GATTACAAAGACCATGATGGTGACTATAAGGATCATGATATTGACTATAAAGACGATG ACGATAAG) was inserted in a *Sma*I site created by site-direct mutagenesis immediately after the initiation codon in the first exon of the *csr-1* gene. The *csr-1* constructs were present at 50 ng/μl in the injection mixture.

rde-3

A 4.4 kb DNA fragment (*Sal*I – *Bam*HI) from cosmid (K04F10) containing the entire *rde-3* coding sequence was cloned into the B1496 vector. A *Bgl*II site was engineered by site-directed mutagenesis immediately after the initiation codon or before the stop codon of *rde-3* to insert *gfp* or 3x*flag* sequence. The *rde-3* constructs were present at 50 ng/μl in the injection mixture.

cdk-1

A 1.8 kb fragment (LGIII: 9747058-9748900) containing the entire *cdk-1* coding region was amplified by PCR and cloned into the B1496 vector. A *Bgl*II site was engineered by site-directed mutagenesis immediately after the initiation codon or before the stop codon of *cdk-1* to insert the *gfp* sequence. The *cdk-1* constructs were present at 10 or 50 ng/μl in the injection mixture.

pie-1

A 2.6 kb fragment (LGI: 12426792-12429422) containing the *pie-1* coding region and a 3.7 kb fragment (pID3.01B, addgene) between *Sca*I and *Not*I sites containing the *pie-1* promoter and *gfp* coding region were combined and inserted into the B1496 vector. The *pie-1* constructs were present at 10 or 50 ng/μl in the injection mixture.

wago-9

A 5.3 kb fragment (LGIII: 4171941-4177280) containing the entire *wago-9* coding region was cloned into the B1496 vector. A *Bam*HI site was created by site direct mutagenesis immediately after the initiation codon of *wago-9* to insert the *gfp* coding region or 3x*flag* sequence. The *wago-9* constructs were present at 10 ng/μl in the injection mixture.

MosSCI by Heat Shock, Ivermection Selection

Several lines that stably transmit the Rol phenotype were used for each MosSCI construct. Starved L1 worms were plated to fifteen 60 mm plates at a density of ~300 worms per plate and grown until the L4 or young-adult stage. Heat-shock induction of Mos Transposase was performed by placing plates in a Ziploc bag and submerging in a 35°C water bath for 1.5 hr. After heat shock, worms were propagated for 2 generations at 25°C. F3 embryos were harvested with a brief hypochlorite treatment, washed with M9 and seeded onto 60 mm NGM plates containing 2 ng/ml ivermection to select against animals carrying the extrachromosomal array. After 3 days at 25°C, wild-type looking worms (non-Unc, non-Rol) were manually picked and transgene insertions were homozygosed for phenotypic analysis.

Primers for qPCR

Primer sequences are listed in Table S2.

Small RNA Cloning and Computational Analysis

Small RNA libraries were prepared using a ligation-dependent method with a 5′ adaptor containing a 4 nt barcode and a 3′ adaptor added to the RNA as described (Gu et al., 2009). The cloning protocol was modified to be suitable for cloning from minute quantities of RNA, such as obtained from a few dissected germlines (W.G. and C.C.M., unpublished data). Libraries were sequenced on an Illumina GAII at the UMass Medical School Deep Sequencing Core. A custom Perl script was used to remove the 5′ barcode and the 3′ adaptor sequences. If the 3′ adaptor was not identified, then incomplete 3′ adapters CTGTA, CTGT, CTG, or CT were removed. Reads of at least 17 nt in length were mapped to the *C. elegans* genome (WormBase release WS215) and miRBase 16 using Bowtie 0.12.7 with the parameter "-v 3 -a --best --strata -m 400". A custom Perl script was used to perform a post-match analysis, only allowing mis-matches with reads ≥ 19 nt: one mismatch for 19 – 21 nt, two for 22–24 nt and three for ≥ 25 nt. The Bowtie parameter '-a --best --strata' was used to return only the best matches. The read count of each sequence was normalized to the number of matches in the genome. To account for differences in sequencing volume between samples, we normalized the total of matched non-structural RNAs to 5 million reads. A custom script and Bioperl was used to draw the scatter plot. The single nt histogram for the start site of matched RNA was obtained using a custom Perl script and the generic genome browser 1.70. All scripts are available upon request.

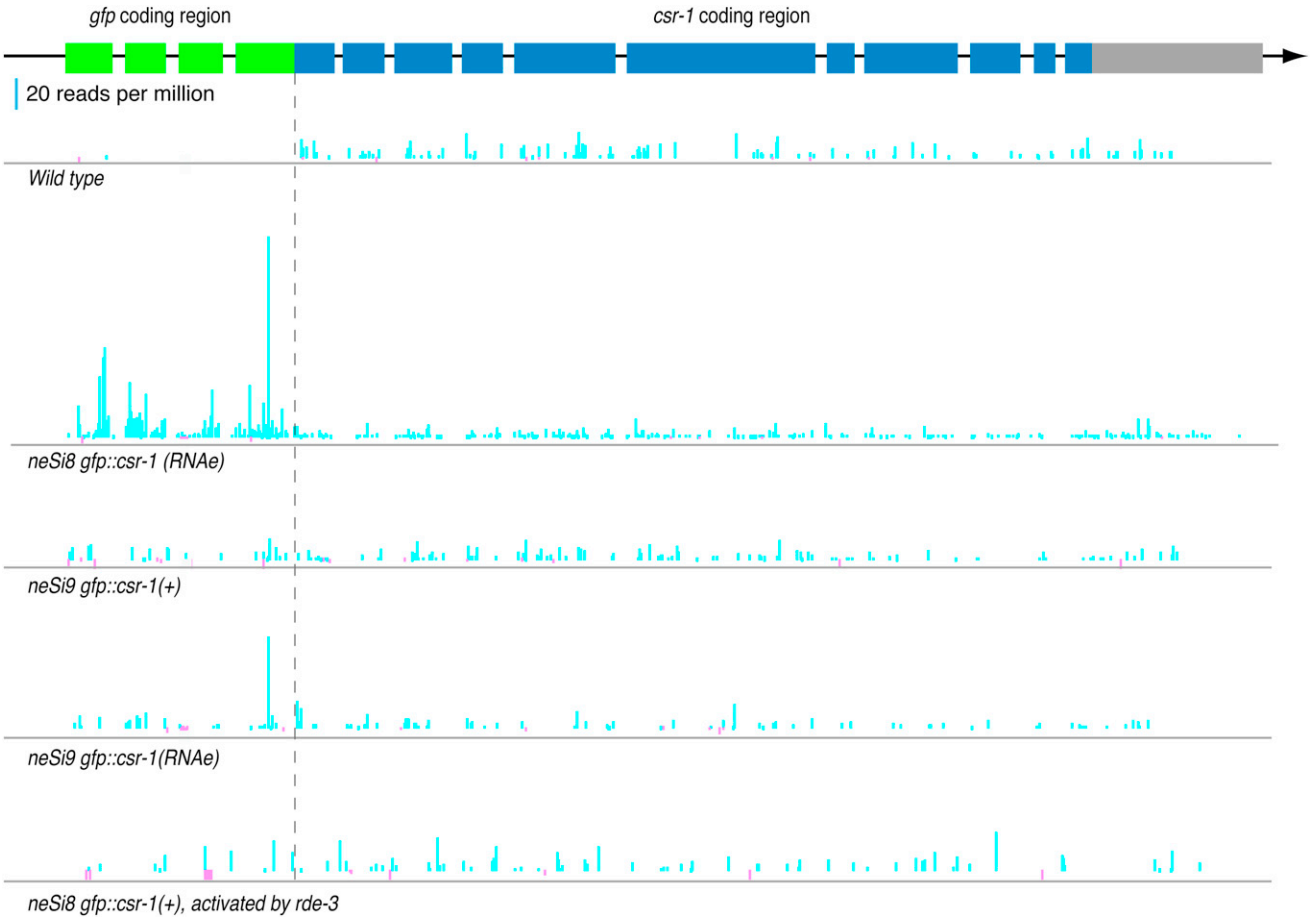

Figure S1. Small RNA Profile of *gfp*::*csr-1* Transgenic Lines, Related to Figure 3

Small RNA density along the *gfp* and *csr-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 (pink) and antisense (light blue). Scale bar indicates 20 reads per million. The strain *neSi9 gfp*::*csr-1(RNAe)* was generated by crossing *neSi9 gfp*::*csr-1*(+) to *neSi10 gfp*::*csr-1(RNAe*). The strain *neSi8 gfp*::*csr-1*(+) was generated by crossing an *rde-3* mutation into the strain *neSi8 gfp::csr-1(RNAe)* and then removing the *rde-3* mutation by crossing *rde-3; neSi8 gfp*::*csr-1* to a wild-type strain.