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Short Article

The *C. elegans* CSR-1 Argonaute Pathway Counteracts Epigenetic Silencing to Promote Germline Gene Expression

Meetu Seth,[1,2,3,4] Masaki Shirayama,[1,2,3,4] Weifeng Gu,[1,2] Takao Ishidate,[1,2,3] Darryl Conte, Jr.,[1,2] and Craig C. Mello[1,2,3,*]
[1]Program in Molecular Medicine
[2]RNA Therapeutics Institute
[3]Howard Hughes Medical Institute
University of Massachusetts Medical School, 368 Plantation Street, Worcester, MA 01605, USA
[4]These authors contributed equally to this work
*Correspondence: craig.mello@umassmed.edu
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SUMMARY

Organisms can develop adaptive sequence-specific immunity by reexpressing pathogen-specific small RNAs that guide gene silencing. For example, the *C. elegans* PIWI-Argonaute/piwi-interacting RNA (piRNA) pathway recruits RNA-dependent RNA polymerase (RdRP) to foreign sequences to amplify a transgenerational small-RNA-induced epigenetic silencing signal (termed RNAe). Here, we provide evidence that, in addition to an adaptive memory of silenced sequences, *C. elegans* can also develop an opposing adaptive memory of expressed/self-mRNAs. We refer to this mechanism, which can prevent or reverse RNAe, as RNA-induced epigenetic gene activation (RNAa). We show that CSR-1, which engages RdRP-amplified small RNAs complementary to germline-expressed mRNAs, is required for RNAa. We show that a transgene with RNAa activity also exhibits accumulation of cognate CSR-1 small RNAs. Our findings suggest that *C. elegans* adaptively acquires and maintains a transgenerational CSR-1 memory that recognizes and protects self-mRNAs, allowing piRNAs to recognize foreign sequences innately, without the need for prior exposure.

INTRODUCTION

Epigenetics is often defined as the stable transmission of gene-expression programs through mitotic or meiotic cell division without alteration in the DNA sequence (Bird, 2007). In eukaryotic cells, epigenetic inheritance can be driven by covalent modifications to chromatin, often referred to as chromatin marks or simply as epigenetic marks (Grewal and Elgin, 2007; Henderson and Jacobsen, 2007; Lippman and Martienssen, 2004; Strome and Lehmann, 2007).

An emerging theme in epigenetic regulation is the frequent involvement of noncoding RNAs (Daxinger and Whitelaw, 2012; Grewal and Elgin, 2007; Henderson and Jacobsen, 2007; Lessing et al., 2013; Lim and Brunet, 2013). In many organisms, epigenetic silencing has been linked to RNAi-related mechanisms, which involve small noncoding RNAs termed short interfering RNAs (siRNAs; see Ghildiyal and Zamore, 2009). Interestingly, the best-studied examples of RNAi-related epigenetic silencing also involve chromatin marks and their associated enzymatic mediators (Grewal and Elgin, 2007; Lippman and Martienssen, 2004), suggesting that RNAi and chromatin-modifying mechanisms reinforce and synergize with each other. Whereas the propagation of chromatin marks occurs in *cis*, RNAi can propagate in *trans*, allowing coordinate regulation of alleles on sister chromatids or of whole gene families such as transposons dispersed throughout the genome.

The core effectors of all RNAi-related pathways are Argonaute proteins. Argonautes present their guide RNAs for base pairing with target sequences and, upon binding, can cleave the target RNA and/or recruit cofactors that mediate posttranscriptional or transcriptional silencing (Ghildiyal and Zamore, 2009; Kuhn and Joshua-Tor, 2013). Although they are much less common, there are several examples of small-RNA pathways that appear to activate gene expression. For example, studies in human cultured cells have implicated small RNAs and/or Argonautes in gene activation, a phenomenon referred to as RNAa (Janowski et al., 2007; Li et al., 2006; Place et al., 2008). In these examples, targeting is thought to occur within the promoter region of the gene, perhaps acting on nascent promoter-derived transcripts, and is correlated with the induction of chromatin marks characteristic of gene activation. In plants, small double-stranded RNAs (dsRNAs) have been implicated in the activation of the Petunia pMADS3 homeotic gene and are thought to act by promoting DNA-methylation at a CpG site within an intronic *cis*-promoter element (Shibuya et al., 2009).

Two major groups of Argonaute proteins, the AGO proteins and the PIWI proteins, are encoded by animal genomes. PIWI Argonautes are expressed abundantly in the germline, where they engage small-RNA species termed piwi-interacting RNAs (piRNAs; for review, see Juliano et al., 2011). In *C. elegans*, the PIWI Argonaute PRG-1 engages over 30,000 distinct genomically encoded piRNA species (Batista et al., 2008; Das et al., 2008; Gu et al., 2012). Recent studies have shown that PRG-1 initiates silencing of transgenes containing foreign, non-*C. elegans* sequences (Shirayama et al., 2012), and suggested

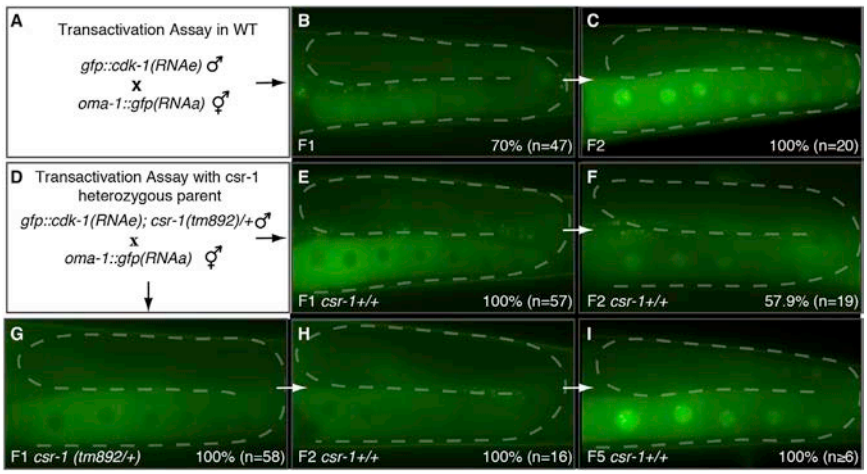

Figure 1. CSR-1 Is Required for RNAa

(A and D) Schematic diagrams of crosses between silenced (RNAe) and licensed (RNAa) GFP transgenic strains as indicated.

(B, C, and E–I) Epifluorescence images of representative germlines (outlined with dashes) in first (F1) and subsequent (F2, F3, and F5) generations. The cytoplasmic fluorescence signal is OMA-1::GFP; the nuclear signal is GFP::CDK-1. The percentages indicate the number of animals that exhibited the phenotype shown in this and subsequent figures.

See also Figure S1.

that it does so while allowing imperfect base pairing with target sequences (Bagijn et al., 2012; Lee et al., 2012; Shirayama et al., 2012). Upon recognition of foreign sequences, PRG-1 is thought to recruit a cellular RNA-dependent RNA polymerase (RdRP), which in turn amplifies the silencing signal by producing antisense siRNAs that are perfectly complementary to the foreign sequences. These amplified siRNAs are loaded onto members of an expanded clade of worm-specific Argonautes (termed WAGO Argonautes), which are implicated in both cytoplasmic and nuclear gene silencing (Buckley et al., 2012; Gu et al., 2009; Guang et al., 2008; Yigit et al., 2006). The result is a remarkably stable mode of epigenetic silencing, termed RNA-induced epigenetic silencing (RNAe) (Shirayama et al., 2012). Alleles that are silenced by RNAe send *trans*-acting Argonaute-small-RNA signals that act in a sequence-specific manner to induce the permanent transgenerational silencing of their targets (Shirayama et al., 2012). The maintenance of RNAe requires chromatin factors, including heterochromatin protein 1 (HP1) and multiple histone methyltransferases (Ashe et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). Given the high numbers and sequence diversity of *C. elegans* piRNAs, the allowance of two or three mismatches during target recognition should suffice, in principle, for piRNAs to bind virtually any foreign RNA sequence. However, piRNAs should also recognize endogenous RNAs, and therefore the piRNA surveillance model requires that "self"-RNA be protected from RNAe (Shirayama et al., 2012).

The CSR-1 Argonaute engages antisense siRNAs that are complementary to the majority (or perhaps all) endogenous germline-expressed genes (Claycomb et al., 2009; Gu et al., 2009). This finding and the fact that CSR-1 targets do not appear to exhibit CSR-1-dependent silencing make this Argonaute a candidate for a self-RNA recognition factor. Paradoxically, however, CSR-1 protein has been shown to exhibit slicer activity in vitro (Aoki et al., 2007), and *csr-1* mutants are partially deficient in dsRNA-induced silencing (Claycomb et al., 2009; Yigit et al., 2006). The siRNAs that engage CSR-1, like those that engage WAGO Argonautes, are RdRP products. *C. elegans* RdRP products are often referred to as 22G-RNAs because they exhibit a predominant length of 22 nt and a strong bias for a 5′ guanosine.

Evidence for a transactivating signal that can counteract RNAe was discovered in crosses between an RNAe transgene and homologous actively expressed transgenes (Shirayama et al., 2012). Because this process involves the epigenetically transmitted, RNA-induced transactivation of a silent allele (see below), we refer to the phenomenon as "RNAa" (for RNA-induced epigenetic gene activation). Transgene alleles that are capable of sending the activating signal, such as *oma-1::gfp(RNAa)*, are designated as RNAa alleles.

Here, we show that CSR-1 is required for RNAa, and that the ability of a foreign sequence to direct transactivation is correlated with acquisition of CSR-1-associated small RNAs antisense to the foreign sequence. In contrast to previously studied RNAa phenomena, the CSR-1-associated activating small RNAs target sequences that are present in the mature mRNA, rather than promoter or intron sequences. We show that propagation of an RNAe and an RNAa allele together for multiple generations results in a gradual transfer of a stable, expressed state to the formerly silent transgene. Finally, consistent with the idea that RNAa counteracts PRG-1 recognition, we show that resilencing of a transactivated RNAe allele depends on PRG-1 activity. Our findings suggest that CSR-1 small RNAs constitute a memory of previous germline-gene expression that protects endogenous genes from piRNA recognition. This self-memory system allows foreign sequences to be recognized innately without the need for prior exposure. Taken together, these findings and previous work on RNAe suggest that the *C. elegans* germline employs Argonaute-small-RNA complexes as transgenerational binary signals that program and reinforce the ON/OFF expression state for thousands of germline genes.

RESULTS

CSR-1 Is Required for RNAa

As a first test of whether transactivation depends on CSR-1 activity, we crossed *oma-1::gfp(RNAa)* to *gfp::cdk-1(RNAe)* and exposed newly hatched F1 cross progeny to either *csr-1(RNAi)* by feeding or to a control RNAi. Since OMA-1::GFP is expressed uniformly in oocyte cytoplasm (Lin, 2003), transactivation in this assay is evidenced by accumulation of the nuclear GFP::CDK-1 gene product (as shown in Figures 1A–1C). When cross progeny were exposed to a control RNAi directed against *sel-1*, an abundant germline gene with a function unrelated to small-RNA pathways, we found that 100% (n = 66) of the F1s exhibited transactivation of *gfp::cdk-1(RNAe)* (Figure S1A available online).

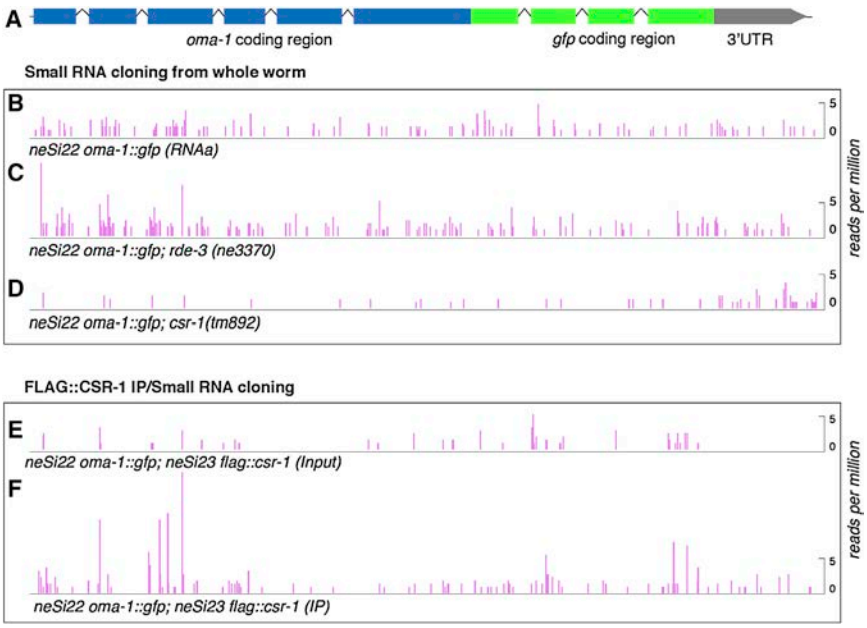

Figure 2. CSR-1-Associated Small RNAs Targeting GFP in *neSi22 oma-1::gfp(RNAa)*

(A) Schematic of the *oma-1::gfp* transgene. The exon-intron structure is indicated with boxes and lines, respectively.

(B–F) Plots showing the density of antisense small RNAs mapping along *oma-1::gfp* in wild-type (B) and mutant strains *rde-3* (C) and *csr-1* (D). In (E and F), the histograms show the read densities of small RNAs obtained from the same lysate before (Input) and after FLAG::CSR-1 IP. The height of each peak corresponds to the number of RNA reads that begin at that position per million total reads.

See also Figure S2.

In contrast, we found that 0% (n = 80) of F1s exposed to *csr-1(RNAi)* exhibited GFP::CDK-1 nuclear expression (Figure S1A). These findings suggest that CSR-1 activity is required in the zygote for transactivation of an RNAe allele.

We next wished to explore the consequences of reducing the dose of *csr-1* activity. To do this, we conducted the transactivation assay using heterozygous *csr-1(tm892)* null mutant animals (Figure 1D), which exhibit wild-type fertility. Interestingly, we found that transactivation failed to occur when either transgenic parent was heterozygous for *csr-1(tm892)* (Figures 1E, 1G, S1A, and S1B). We found that 100% of the F1 cross progeny failed to activate *gfp::cdk-1(RNAe)* when the *csr-1(tm892)* mutant was introduced from the father (n = 115) or the mother (n = 15). This parental effect indicates that zygotic expression of CSR-1, although necessary (as suggested by the RNAi studies above), is not sufficient for transactivation. Even F1 progeny homozygous for wild-type *csr-1(+)* activity failed to exhibit transactivation if either parent was heterozygous for *csr-1(tm892)* (Figure 1E). As expected, when F1 wild-type *csr-1(+)* hermaphrodites were allowed to self-cross, we observed transactivation in the germlines of their F2 progeny (57.9%, n = 19; Figure 1F). In contrast, heterozygous *csr-1(tm892)* hermaphrodites produced self-progeny that failed to exhibit transactivation (0%, n = 16; Figure 1H), and transactivation was only restored among their wild-type progeny in subsequent generations (100%, n ≥ 6; Figure 1I).

RNAa Activity Correlates with the Accumulation of CSR-1 22G-RNAs

A previous study indicated that 22G-RNAs targeting *cdk-1::gfp*, a neutral transgene that is expressed but sensitive to silencing via RNAe, are present at very low levels—much lower, for example, than the level of CSR-1 22G-RNAs targeting the endogenous *cdk-1* portion of the transgene (Shirayama et al., 2012). The genetic analysis of RNAa described above suggests that transactivation of an RNAe allele is acutely sensitive to the dose of CSR-1 activity. We therefore wondered whether small RNAs targeting *gfp* in the *oma-1::gfp(RNAa)* strain might be enriched to levels similar to those observed in an endogenous germline-expressed gene, and whether they depend on CSR-1 activity. To explore this possibility, we first analyzed total small-RNA levels targeting *oma-1::gfp* in wild-type animals and in mutants defective in RNAa (*csr-1(tm892)*) or RNAe (*rde-3(ne3370)*). In wild-type and *rde-3* mutant animals, we found that 22G-RNAs targeting *gfp* exhibited levels similar to those found for 22G-RNAs targeting *oma-1* itself (Figures 2B and 2C). Conversely, and consistent with the idea that these *gfp*-targeted 22G-RNAs are in the CSR-1 pathway, we found that small RNAs targeting *gfp* were reduced by 73% in *csr-1(tm892)* mutants, which is similar to the reduction observed for small RNAs targeting *oma-1* and other germline-expressed RNAs (Figure 2D and data not shown).

We next examined the physical association of *gfp*-directed 22G-RNAs by sequencing RNAs recovered in Argonaute protein immunoprecipitation (IP) complexes. To do this, we conducted IP assays using the epitope-tagged Argonaute proteins FLAG::CSR-1 and FLAG::WAGO-9/HRDE-1 (Ashe et al., 2012; Buckley et al., 2012; Shirayama et al., 2012). Consistent with their genetic dependence on *csr-1*, we found that 22G-RNAs antisense to *gfp* were enriched (3.14-fold) in the FLAG::CSR-1 IP from *oma-1::gfp* transgenic animals (Figures 2E and 2F), and were not enriched in the FLAG::WAGO-9/HRDE-1 IP (Figures S2A and S2B). For comparison, we also performed IP studies in a *gfp::cdk-1(RNAe)* strain. As expected, we found a reciprocal relationship in this silent strain: 22G-RNAs targeting *gfp* were depleted (3.35-fold) in the FLAG::CSR-1 IP relative to input (Figures S2C and S2E), and were enriched (1.75-fold) in the FLAG::WAGO-9/HRDE-1 IP (Figure S2D).

Thus, we showed that in three small-RNA sequencing libraries independently prepared from *csr-1(+)* animals, the level of 22G-RNAs targeting *gfp* was similar to that of CSR-1 22G-RNAs targeting the *oma-1*-derived portion of the RNAa transgene. Furthermore, we showed that these *gfp* 22G-RNAs were depleted in *csr-1* mutants and enriched in the CSR-1 IP. In contrast, an RNAe transgenic strain exhibited *gfp* 22G-RNAs that were enriched in the WAGO-9 IP and depleted in the CSR-1 IP. Finally, a strain with a neutral transgene (sensitive to

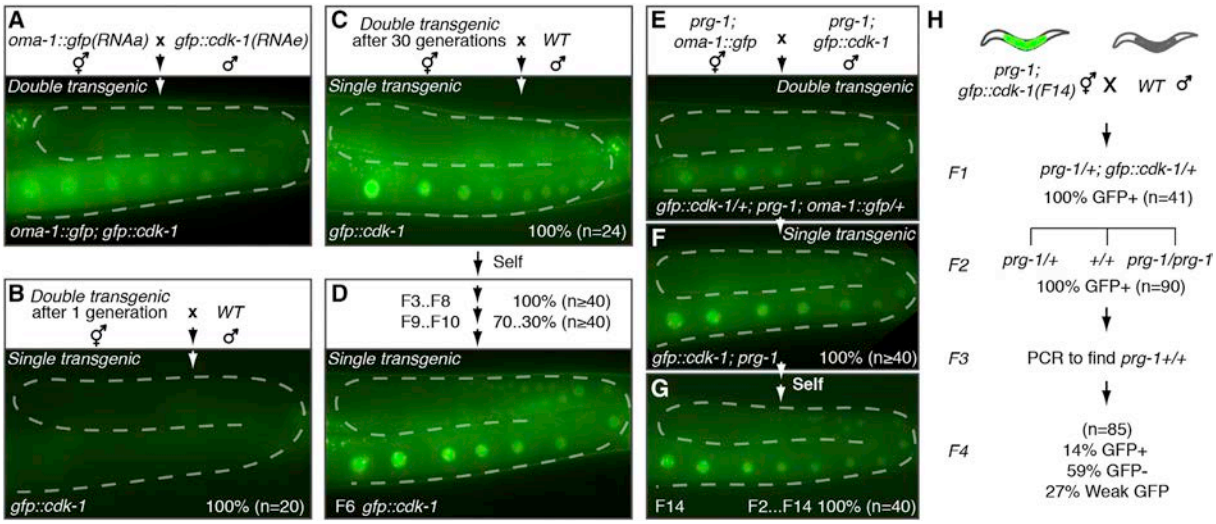

Figure 3. RNAa Counteracts Piwi-Dependent Silencing and Acts over Multiple Generations to Establish an Active Epigenetic Gene-Expression State

(A–H) Genetic crosses with corresponding epifluorescence images showing representative germlines of the resulting progeny. The percentage of animals that expressed *gfp::cdk-1* (nuclear GFP signal) at each generation and the number of animals scored (n) are indicated.

(A–D) Analysis of the effect of RNAa exposure on the durability of gene activation in wild-type animals. Newly transactivated F2 double-transgenic animals (A) were outcrossed to wild-type (WT), either immediately or after propagation as a double-transgenic strain for 30 generations, to obtain the *gfp::cdk-1* "single-transgenic" animals shown in (B) and (C), respectively. Siblings of the animals shown in (C) were allowed to produce self-progeny (D) for multiple generations, and GFP fluorescence was scored in each generation as indicated.

(E–H) Analysis of the genetic influence of Piwi (*prg-1*) on transactivation. RNAa and RNAe transgenes that were established in a wild-type background were crossed into *prg-1* before the transactivation assay shown in (E) was conducted. After one generation, *oma-1::gfp* was segregated away to yield the *gfp::cdk-1* single-transgenic animals assayed in (F). Siblings of the animals shown in (F) were allowed to produce self-progeny for multiple generations, and GFP fluorescence was scored in each generation (G) as indicated. In (H), *gfp::cdk-1*was outcrossed from the *prg-1(tm872)* mutant background and the animals were scored for GFP in subsequent generations as indicated.

RNAe) exhibited very low levels of *gfp* 22G-RNAs relative to the levels of CSR-1 22G-RNA targeting the endogenously derived portion of the transgene (Shirayama et al., 2012). Taken together, these findings indicate that the RNAa activity of *oma-1::gfp* correlates with the accumulation of CSR-1 22G-RNAs targeting the foreign, *gfp* sequences of the transgene.

Multigenerational Exposure to RNAa Can Gradually License an RNAe Allele

The above findings indicate that *C. elegans* transgenes can adopt at least three different states: (1) a dominant-acting *trans*-silencing state (RNAe); (2) a neutral, expressed state that is sensitive to *trans*-silencing; and (3) a dominant transactivating state (RNAa). Previous studies have shown that an RNAe allele can transfer the silent state to a neutral allele. We therefore wished to know whether transient exposure to an RNAa allele could stably activate (or license) the expression of an RNAe allele. To explore this possibility, we set up a series of crosses between an RNAa transgene and a number of distinct RNAe transgenes. After establishing the double-transgenic lines, we outcrossed the strains to wild-type to separate the two transgenes again, and then monitored expression and RNAa or RNAe status. We found that different transgenes behaved differently in these crosses. For example, *gfp::cdk-1(RNAe)* was activated in the presence of *oma-1::gfp(RNAa)* (Figure 3A), but was immediately silenced after the RNAa transgene was crossed away (Figure 3B; Shirayama et al., 2012). In contrast, a *cdk-1::gfp(RNAe)* allele remained stably expressed after transient exposure to the RNAa transgene (Shirayama et al., 2012). Finally, a *gfp::csr-1(RNAe)* transgene was never activated upon exposure to *oma-1::gfp(RNAa)*. Instead, each allele maintained its expression status in the double homozygote (silent *gfp::csr-1(RNAe)* and active *oma-1::gfp(RNAa)*; data not shown).

We next wanted to explore whether prolonged exposure to RNAa could influence the tendency of *gfp::cdk-1* to revert back to an RNAe status. Consistent with this idea, after propagating the *oma::gfp; gfp::cdk-1* double-transgenic strain for ten generations and then outcrossing to wild-type to separate the two transgenes, we found that the *gfp::cdk-1(+)* transgene remained expressed for one full generation after separation before resilencing. Interestingly, the period of sustained expression increased to nearly ten generations when *gfp::cdk-1* and *oma-1::gfp(RNAa)* were separated after 30 generations of copropagation (Figures 3C and 3D). However, we found that, even though expression of the formerly RNAe transgene was stabilized by long-term exposure to RNAa, the RNAa status was not transferred. Instead, the activated transgene remained sensitive to silencing when exposed through a genetic cross to *gfp::csr-1(RNAe)* (100%, n = 24). Taken together, these findings suggest that an RNAa transgene can, over time, influence the epigenetic stability of an RNAe allele. However, the transfer of RNAa status is either very slow or dependent on other factors that remain to be identified.

RNAa Counteracts PRG-1-Dependent Silencing

The PIWI Argonaute PRG-1 is required for the initiation of RNAe, but not for the maintenance of silencing (Shirayama et al., 2012). We therefore wondered whether PRG-1 activity is required to reinitiate silencing of an RNAe transgene after transactivation. To test this possibility, we first crossed the *gfp::cdk-1(RNAe)* and *oma-1:gfp(RNAa)* transgenes into the *prg-1(tm872)* mutant background. As expected, we found that singly, each transgene maintained its silent or active expression state in the *prg-1* mutant background. We then repeated the transactivation crosses by mating these *prg-1* mutant strains (Figure 3E). As observed in the wild-type *prg-1(+)* background, the *gfp::cdk-1(RNAe)* transgene was activated in the F1 cross progeny (Figure 3E). We then allowed the two transgenes to segregate from one another. Strikingly, we found that 100% of the F2–F14 *gfp::cdk-1* transgenic animals examined maintained expression in the absence of the *oma-1::gfp(RNAa)* transgene (Figures 3F and 3G). Thus in the absence of *prg-1* activity, the RNAa allele is not required to maintain the activated status of the formerly RNAe transgene. We next crossed these actively expressing *prg-1* mutant transgenic animals to wild-type to restore *prg-1* activity. We found that once they were homozygous for *prg-1(+)* activity, 85% of the animals examined (n = 85) exhibited resilencing of the transgene by the F4 generation (Figure 3H). These findings indicate that *prg-1* is required to reinitiate silencing on an RNAe transgene, and that RNAa opposes this PRG-1-dependent silencing activity.

DISCUSSION

A Genome-wide Mechanism for the Epigenetic Adaptation of Gene Expression

The term "epigenetics" is used to describe many diverse types of biological events, ranging from the activity of prions (Halfmann and Lindquist, 2010) to the transmission of heritable membrane structures (Harold, 2005), and extending even to cellular differentiation events (Goldberg et al., 2007). In a recent review, Adrian Bird (2007) suggested a compelling definition for chromatin-focused epigenetic events as "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states." A key element of this definition is that epigenetic chromatin marks are seen as responsive and adaptive; they help to canalize and buffer gene-expression programs that may have more direct upstream triggers. Our findings are consistent with this adaptive view of epigenetic programming. They suggest how Argonaute-small-RNA pathways can work in concert with chromatin pathways to create heritable binary signals that communicate a memory of germline gene expression from one generation to the next. In this system, small RNAs can both perpetuate expression states in *cis* and signal adapted gene-expression states to dispersed alleles of a gene.

In this work we focused on the role of Argonaute-small-RNA pathways in the control of transgene expression states. Yet these Argonaute pathways also act globally in the germline to target expressed (CSR-1-targeted) and silenced (WAGO-targeted) genes genome wide. A parallel paper by Conine et al. (2013) shows that CSR-1 is required to promote the expression of many male-specific germline genes. In the absence of paternal CSR-1 activity, males are initially fertile but progressively become sterile over a period of five to six generations. This "germline-mortal" phenotype is consistent with previous work on the loss of specific Argonaute-silencing pathways (Buckley et al., 2012) and may reflect a gradual loss of the "adapted" epigenetic state reinforced by these small-RNA pathways.

Studies on *prg-1* mutants suggest that the default state for transgene expression is "ON." Therefore, a simple model for the CSR-1 pathway is that it prevents the incursion of silencing signals within its targeted sequence domain (see model in Figure 4). It is possible that CSR-1 prevents PRG-1 and WAGO silencing by using its slicer activity to destroy template RNAs engaged in RdRP transcription and WAGO loading. Understanding the mechanistic details of RNAa will require further exploration of how chromatin and small-RNA pathways change as alleles switch from a silenced to an expressed status, and will also require new tools for directly intervening in the feed-forward Argonaute and chromatin pathways. A recent study describes one such tool, a tethering system that recruits CSR-1 to target sequences through direct RNA binding, thus activating an RNAe allele without the need for a transactivating allele and its cognate small RNAs (Wedeles et al., 2013, this issue).

An Innate Sequence-Specific Genome-Defense Mechanism

The findings described here support a model for genome defense that employs a truly surprising strategy—one that permits a rapid "innate" and yet sequence-specific response without the need for prior exposure to a pathogenic sequence or for structural triggers of pathogen-specific activity such as the expression of long dsRNA. Instead, our findings suggest that the recognition of foreign sequences in *C. elegans* depends directly on the Piwi pathway, which scans for foreign sequences (Ashe et al., 2012; Lee et al., 2012; Shirayama et al., 2012), and indirectly on the CSR-1 pathway, which protects endogenous germline-expressed genes from piRNA-mediated recognition. Thus, sequence specificity is achieved not by capturing and remembering foreign sequences, as in some systems (Khurana et al., 2011; Sorek et al., 2008), but rather by remembering all self-sequences, thereby permitting the innate recognition of foreign sequences (see model in Figure 4).

Under some circumstances, foreign sequences appear to be adopted as self. One possible model for this adoption process is that CSR-1 recognition can spread in *cis* from fused endogenous sequences within a transgene (model, Figure 4). Targeting by CSR-1 within the endogenous sequences could promote the local recruitment of RdRP, leading to the de novo synthesis of CSR-1 22G-RNAs within the adjacent foreign sequences. Molecular spreading of this type has been observed in gene silencing in both plants and animals (Axtell et al., 2006; Pak and Fire, 2007; Sijen et al., 2001, 2007). The decision to silence or license a newly introduced transgene would then be determined through a competition between *cis* spreading of CSR-1 recognition and initial recognition by the PRG-1/21U-RNA pathway (model, Figure 4). For some transgenes, such as *oma-1::gfp(RNAa)*, this process leads to the "adoption" of the foreign sequences (through acquisition of CSR-1 targeting), permitting

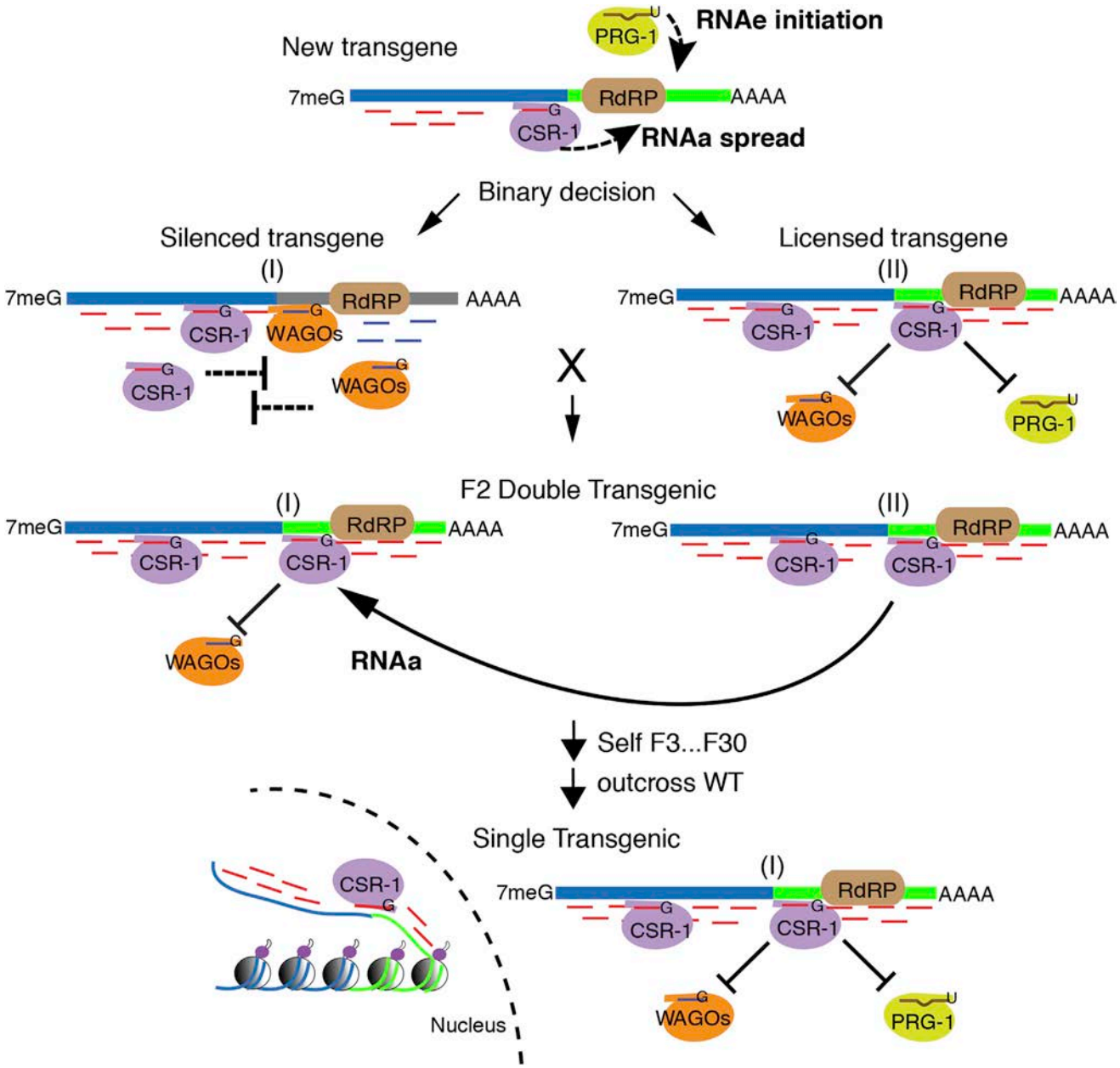

Figure 4. Model for Transactivation by CSR-1
See Discussion for details.

these *gfp* sequences to transactivate homologous transgenes (model, Figure 4).

CONCLUSIONS

Epigenetic pathways are diverse and can differ widely from organism to organism. This is particularly true for Argonaute pathways, which exhibit evidence of extensive gene duplication and pathway diversification in both plants and animals (Cerutti and Casas-Mollano, 2006; Ghildiyal and Zamore, 2009). The rapid evolution of these pathways could reflect selective pressure exerted in response to their targets, which in most organisms include a striking genomic load of transposons. Although the details may differ from one system to another, the concepts revealed in one organism will likely be relevant in other systems. For example, it is now clear that a dynamic interplay between Argonaute/small-RNA pathways and chromatin modifiers is involved both in the silencing of repetitive gene families and in essential chromosome functions, such as kinetochore assembly and chromosome segregation, in organisms as diverse as fungi, plants, and animals (Grewal and Elgin, 2007).

Here, we have shown that *C. elegans* employs Argonautes to protect expressed genes from silencing. Interestingly, although

the interaction between an RNAa allele and an RNAe allele resulted in a rapid reversal of the silenced state, conversion of the formerly silent allele to a state permissive of independent, sustained gene expression required dozens of generations of continuous exposure to RNAa. This slow conversion of the RNAe allele is consistent with the adaptive definition of an epigenetic process (Bird, 2007) and could reflect a gradual elimination of either small RNAs or chromatin marks that can stimulate resilencing (or possibly a slow accumulation of chromatin marks that enforce expression). CSR-1 localizes on chromatin and immunoprecipitates with target DNA sequences (Claycomb et al., 2009). Thus, CSR-1 could influence chromatin directly, perhaps by engaging nascent transcripts at target genes. It will be interesting in the future to determine whether CSR-1 actively recruits chromatin modifiers to promote gene expression. Furthermore, CSR-1 and members of the WAGO family are abundantly expressed in both oocytes and mature sperm (Claycomb et al., 2009; Conine et al., 2010, 2013; Gu et al., 2009; Shirayama et al., 2012). Germline transmission of these Argonautes and their associated small RNAs may thus have genome-wide effects on epigenetic inheritance, with potentially significant evolutionary implications.

EXPERIMENTAL PROCEDURES

Genetics

The *C. elegans* strains used in this study (see Supplemental Experimental Procedures) were derived from the Bristol N2 strain and cultured as described previously (Brenner, 1974). Strain WM288 contains a single-copy *oma-1::gfp* transgene that was created using the MosSCI heat-shock protocol combined with ivermectin selection as described previously (Shirayama et al., 2012).

Small-RNA Cloning and Deep Sequencing

Total RNA was extracted from ten young adult worms (Shirayama et al., 2012). Small RNAs (18–40 nt) were gel purified, treated with tobacco acid pyrophosphatase to generate monophosphate 5′ ends, ligated to 5′ and 3′ linkers, and converted to cDNA (Gu et al., 2009; Shirayama et al., 2012). Illumina adapters were added by PCR (Gu et al., 2009; Shirayama et al., 2012). To clone CSR-1-associated small RNAs, M2 FLAG antibody (Sigma) was used to immunoprecipitate FLAG::CSR-1 from 20 mg of lysate from synchronous adult worms homogenized in a stainless-steel dounce (Gu et al., 2009). Small RNAs were extracted from FLAG::CSR-1 immune complexes and processed for deep sequencing as described above. Libraries were sequenced in the UMass Medical School Deep Sequencing Core using an Illumina GAII instrument.

For AGO IP studies, the relative enrichment was measured by calculating the (number of antisense GFP reads)/(total number of genome-matching antisense reads) in the Input and the IP, and then dividing the two numbers.

Computational Analysis

Deep-sequencing data were processed and analyzed using Bowtie (version 0.12.7) (Langmead et al., 2009) and custom Perl scripts (Gu et al., 2009; Shirayama et al., 2012). Small-RNA reads were mapped to WormBase WS215 and normalized to nonstructural RNA reads or to the total number of small RNAs that map antisense to protein-coding genes. CSR-1 small-RNA targets were defined previously (Claycomb et al., 2009; Gu et al., 2009). All scripts are available upon request.

Microscopy

Transgenic worms expressing GFP were mounted on RITE-ON glass slides (Becton Dickinson) in the presence of 0.2 mM levamisole. Epifluorescence and differential interference contrast 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 the Gene Expression Omnibus under the accession number GSE49532.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.11.014.

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