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Short Article

Protection of Germline Gene Expression by the *C. elegans* Argonaute CSR-1

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SUMMARY

In *Caenorhabditis elegans*, the Piwi-interacting small RNA (piRNA)-mediated germline surveillance system encodes more than 30,000 unique 21-nucleotide piRNAs, which silence a variety of foreign nucleic acids. What mechanisms allow endogenous germline-expressed transcripts to evade silencing by the piRNA pathway? One likely candidate in a protective mechanism is the Argonaute CSR-1, which interacts with 22G-small RNAs that are antisense to nearly all germline-expressed genes. Here, we use an in vivo RNA tethering assay to demonstrate that the recruitment of CSR-1 to a transcript licenses expression of the transcript, protecting it from piRNA-mediated silencing. Licensing occurs mainly at the level of transcription, as we observe changes in pre-mRNA levels consistent with transcriptional activation when CSR-1 is tethered. Furthermore, the recruitment of CSR-1 to a previously silenced locus transcriptionally activates its expression. Together, these results demonstrate a rare positive role for an endogenous Argonaute pathway in heritably licensing and protecting germline transcripts.

INTRODUCTION

Several small RNA-mediated gene-silencing pathways that defend their endogenous genomes against potentially deleterious foreign nucleic acids such as viruses, transposable elements, and transgenes have been well-characterized (Ketting, 2011). However, while less understood, small RNA pathways are also strong candidates for maintaining a balance between the silencing of exogenous (nonself) sequences and the appropriate expression of endogenous (self) sequences. In the *Caenorhabditis elegans* germline, recognition of foreign nucleic acid, such as that of a *green fluorescent protein* (*gfp*) transgene, by the Piwi-interacting small RNA (piRNA) pathway initiates a cascade of cytoplasmic and nuclear epigenetic gene-silencing events that efficiently and heritably halt the expression of the foreign nucleic acid (Lee et al., 2012; Shirayama et al., 2012; Luteijn et al., 2012; Ashe et al., 2012; Bagijn et al., 2012; Buckley et al., 2012). In this pathway, the Piwi, PRG-1 coupled with over 30,000 different piRNA sequences (also called 21U-RNAs in *C. elegans*) identifies foreign RNA sequences by incomplete complementarity and then initiates the production of a secondary type of small RNAs, called 22G-RNAs (named for their average size and 5′ nucleotide) (Lee et al., 2012; Shirayama et al., 2012; Gu et al., 2012; Batista et al., 2008; Ruby et al., 2006; Das et al., 2008; Bagijn et al., 2012; Ashe et al., 2012). The 22G-RNAs are synthesized by RNA-dependent RNA polymerases (RdRPs) and are loaded onto the worm Argonautes WAGO-1, WAGO-9, and WAGO-10 (Shirayama et al., 2012; Ashe et al., 2012; Buckley et al., 2012). These WAGO complexes in turn silence the invading nucleic acid both at the transcriptional and posttranscriptional levels (Shirayama et al., 2012; Ashe et al., 2012; Buckley et al., 2012; Luteijn et al., 2012). With this vast silencing potential of piRNAs, one key question is: how are endogenous germline sequences exempted or protected from silencing by the piRNA pathway?

The essential Argonaute CSR-1 (chromosome segregation and RNAi deficient) associates with small RNAs (22G-RNAs) that are complementary to nearly all germline-expressed genes (4,200 out of about 5,000 total germline expressed genes) and is recruited to chromatin at its target gene genomic loci in a small-RNA-dependent manner (Claycomb et al., 2009; Wedeles et al., 2013). Despite possessing ''slicer'' activity in vitro, loss of *csr-1* has little effect on the steady-state levels of target transcripts in vivo (Aoki et al., 2007; Claycomb et al., 2009). Thus, CSR-1 does not appear to silence its targets in vivo and is a strong candidate for an Argonaute that could oppose piRNA silencing to protect germline transcription.

Several *gfp*-possessing transgenes were shown to be efficiently silenced by the piRNA-mediated germline surveillance pathway in a process named RNA epigenetics or RNAe (Shirayama et al., 2012). Silent transgenes (*RNAe* alleles) are capable of inducing the silencing of nearly all other active (expressed) *gfp* transgenes (*gfp+*) in a dominant manner when introduced into the same worm strain. This transitive silencing effect is thought to be mediated by WAGO Argonaute complexes that are loaded with small RNAs sharing complementarity to *gfp* sequences, and they are thus capable of acting on any *gfp*-containing transgenes (Shirayama et al., 2012).

We explored RNAe in conjunction with a transcript tethering assay that we recently developed to test whether CSR-1 has the capacity to protect germline transcripts from piRNA-mediated silencing. In the tethering assay, a *gfp* transcript containing Phage Lambda *box b* RNA hairpins (*gfp::boxb*) is expressed under the control of a germline promoter, and CSR-1 possessing a Phage Lambda N antitermination protein fragment (CSR-1::λN) is recruited to the *box b* RNA hairpins.

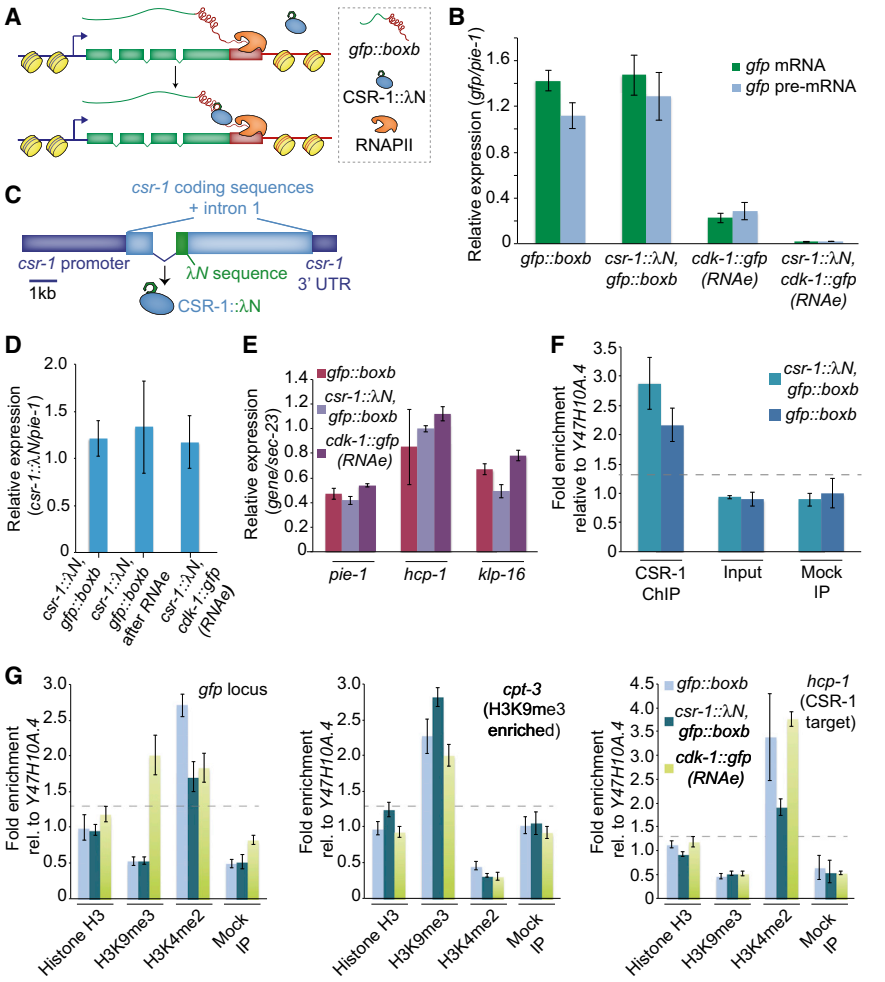

Figure 1. Characterization of Strains Used in the Tethering Assay

(A) A diagram of the *gfp::boxb* tethering locus on chromosome (LG) II is shown. The expression of *gfp* is driven by the germline promoter for *pie-1*. Five *box b* hairpin sites are present at the 3′ end of the *gfp* transcript, followed by the *pie-1* 3′ UTR. CSR-1::λN recognizes the *box b* hairpin sequences.

(B) The *gfp* mRNA (green) and pre-mRNA (blue) levels for the *gfp::boxb, csr-1::λN, gfp::boxb*, *cdk-1::gfp(RNAe)* (WM243), and *csr-1::λN, cdk-1::gfp (RNAe)* strains were measured by qRT-PCR and are expressed relative to the germline expressed gene *pie-1*. For all parts of this figure (unless noted), error bars represent SEM and the samples are the average of three to five biological replicates (of 50–100 adult hermaphrodites per sample).

(C) The structure of the *csr-1::λN* transgene is depicted. The scale bar represents 1 kb.

(D) The *csr-1::λN* mRNA levels for the *csr-1::λN, gfp::boxb, csr-1::λN, gfp::boxb* (after *RNAe*), and *csr-1::λN, cdk-1::gfp (RNAe)* strains were measured by qRT-PCR and are relative to *pie-1*.

(E) The mRNA levels for three representative CSR-1/22G-RNA germline gene targets (*pie-1*, *hcp-1*, and *klp-16*) were measured in the *gfp::boxb* (red), *csr-1::λN, gfp::boxb* (lilac), and *cdk-1::gfp(RNAe)* (WM243) (purple) strains using qRT-PCR. The values are expressed relative to the nontarget germline gene, *sec-23*.

(F) CSR-1 chromatin immunoprecipitation (ChIP) followed by quantitative PCR (qPCR) demonstrates that CSR-1 associates with the *gfp::boxb* locus. Endogenous CSR-1 is present in both strains and can associate with *gfp::boxb*. When CSR-1::λN is present, the levels of CSR-1 at the *gfp::boxb* transgene locus are consistently higher. This result is consistent with weak licensing of the *gfp::boxb* transgene in an otherwise wild-type strain background, which enables this transgene to be expressed initially and silenced by the *cdk-1::gfp(RNAe)* allele. Values at the *gfp::boxb* tethering locus are relative to the *Y47H10A.4* control locus, which is not a target of CSR-1. Input samples and mock ChIPs (performed with beads only) are shown. Error bars are the SD of triplicate experiments. The dashed gray line at 1.33-fold enrichment marks the lower boundary for enrichment by a successful CSR-1 ChIP.

(G) ChIP for histone H3, histone H3 lysine 4 dimethylation (H3K4me2; associated with transcription), histone H3 lysine 9 trimethylation (H3K9me3; associated with transcriptionally silent states) was performed in *gfp::boxb* (blue), *csr-1::λN, gfp::boxb* (teal), and *cdk-1::gfp(RNAe)* (WM243) (green) strains. qPCR was used to examine the *gfp* locus, *cpt*-3 (H3K9me3 enriched locus), and *hcp-1* (CSR-1 target and H3K4me2 enriched locus) relative to the *Y47H10A.4* control locus. Error bars are the SD of triplicate experiments. The dashed gray line at 1.33-fold marks the lower boundary of enrichment. See also Figure S1.

Using this system, we show that tethering CSR-1 to the germline *gfp::boxb* transcript protects this transcript from silencing via the piRNA pathway. We go on to demonstrate that introduction of CSR-1::λN into a previously silenced *gfp::boxb(RNAe)* strain can reactivate expression and that in the continued presence of CSR-1::λN and *gfp::boxb*, a previously silenced *cdk-1::gfp(RNAe)* allele (Shirayama et al., 2012) can be activated in *trans*. Together, our findings reveal a positive and heritable role for CSR-1 in licensing germline gene expression that counteracts the silencing activities of the piRNA surveillance pathway.

RESULTS AND DISCUSSION

An AGO/Transcript Tethering Assay Enables Interrogation of AGO Function In Vivo

We observed an RNA-dependent interaction between CSR-1 and RNA Polymerase II (RNAPII) by coimmunoprecipitation (coIP) experiments, supporting the notion that CSR-1 is recruited in a sequence-specific manner to its target gene loci via an association with nascent transcripts (Figure S1A available online). Thus, to assess the role of CSR-1 in regulating transcription of its gene targets, we developed an in vivo RNA tethering assay to recruit CSR-1 to nascent transcripts (Baron-Benhamou et al., 2004; Bühler et al., 2006).

In this tethering system, we used the germline *pie-1* promoter, intron, and 3′ UTR (Reese et al., 2000) to drive the expression of a *green fluorescent protein* (*gfp*) transcript, followed directly by five Phage Lambda *box b* hairpin RNA sequences (Figure 1A). The *gfp::boxb* transgene was inserted in a single copy onto chromosome II using the Mos1-mediated single-copy insertion (MosSCI) method (Frøkjaer-Jensen et al., 2008, 2012) and confirmed to be expressed by quantitative RT-PCR (qRT-PCR) (Figure 1B).

A transgene encoding CSR-1 with the addition of the 22-amino-acid Phage Lambda N (λN) antitermination peptide at

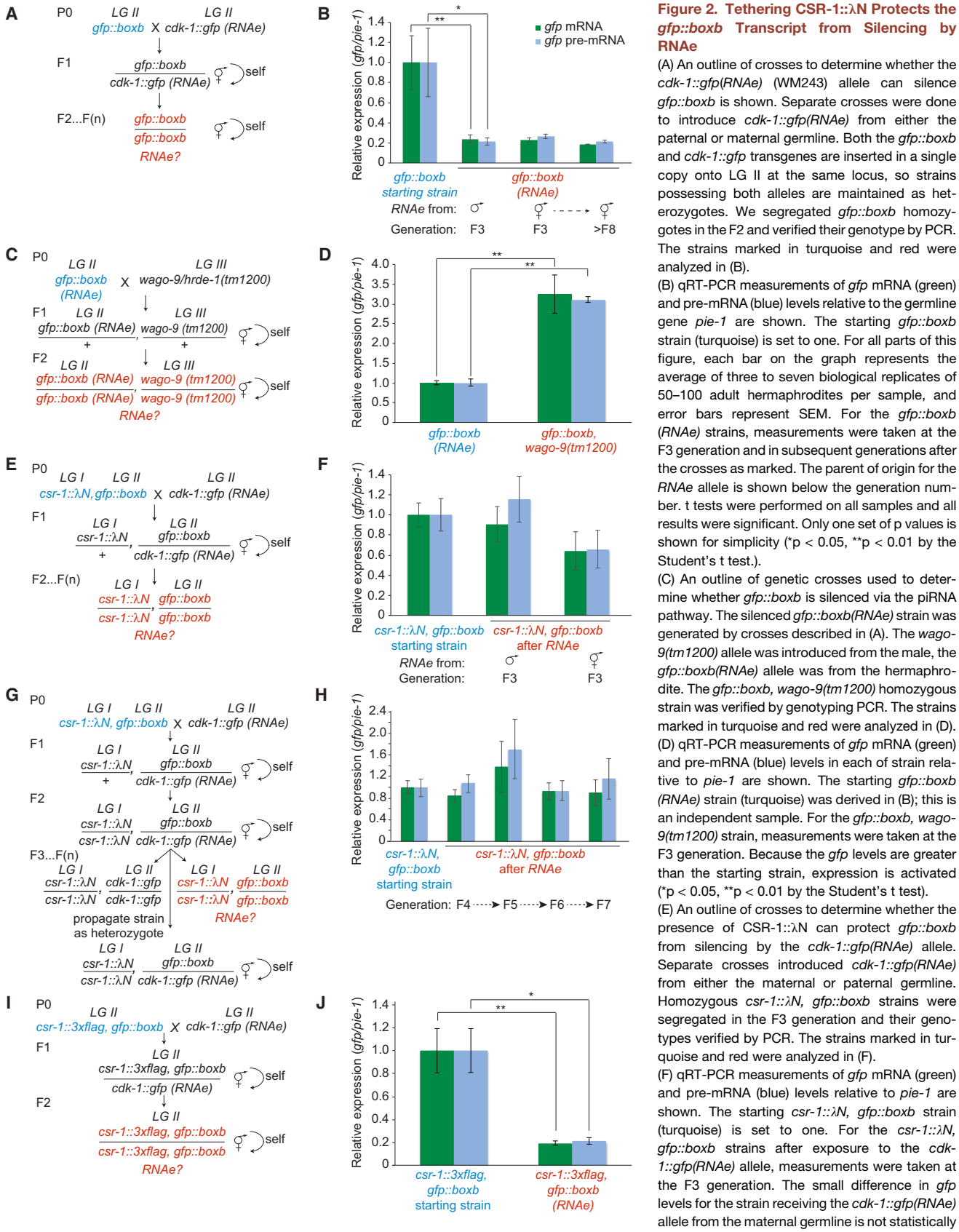

Figure 2. Tethering CSR-1::λN Protects the *gfp::boxb* Transcript from Silencing by RNAe

(A) An outline of crosses to determine whether the *cdk-1::gfp(RNAe)* (WM243) allele can silence *gfp::boxb* is shown. Separate crosses were done to introduce *cdk-1::gfp(RNAe)* from either the paternal or maternal germline. Both the *gfp::boxb* and *cdk-1::gfp* transgenes are inserted in a single copy onto LG II at the same locus, so strains possessing both alleles are maintained as heterozygotes. We segregated *gfp::boxb* homozygotes in the F2 and verified their genotype by PCR. The strains marked in turquoise and red were analyzed in (B).

(B) qRT-PCR measurements of *gfp* mRNA (green) and pre-mRNA (blue) levels relative to the germline gene *pie-1* are shown. The starting *gfp::boxb* strain (turquoise) is set to one. For all parts of this figure, each bar on the graph represents the average of three to seven biological replicates of 50–100 adult hermaphrodites per sample, and error bars represent SEM. For the *gfp::boxb (RNAe)* strains, measurements were taken at the F3 generation and in subsequent generations after the crosses as marked. The parent of origin for the *RNAe* allele is shown below the generation number. t tests were performed on all samples and all results were significant. Only one set of p values is shown for simplicity (*$p < 0.05$, **$p < 0.01$ by the Student's t test.).

(C) An outline of genetic crosses used to determine whether *gfp::boxb* is silenced via the piRNA pathway. The silenced *gfp::boxb(RNAe)* strain was generated by crosses described in (A). The *wago-9(tm1200)* allele was introduced from the male, the *gfp::boxb(RNAe)* allele was from the hermaphrodite. The *gfp::boxb, wago-9(tm1200)* homozygous strain was verified by genotyping PCR. The strains marked in turquoise and red were analyzed in (D).

(D) qRT-PCR measurements of *gfp* mRNA (green) and pre-mRNA (blue) levels in each of strain relative to *pie-1* are shown. The starting *gfp::boxb (RNAe)* strain (turquoise) was derived in (B); this is an independent sample. For the *gfp::boxb, wago-9(tm1200)* strain, measurements were taken at the F3 generation. Because the *gfp* levels are greater than the starting strain, expression is activated (*$p < 0.05$, **$p < 0.01$ by the Student's t test).

(E) An outline of crosses to determine whether the presence of CSR-1::λN can protect *gfp::boxb* from silencing by the *cdk-1::gfp(RNAe)* allele. Separate crosses introduced *cdk-1::gfp(RNAe)* from either the maternal or paternal germline. Homozygous *csr-1::λN, gfp::boxb* strains were segregated in the F3 generation and their genotypes verified by PCR. The strains marked in turquoise and red were analyzed in (F).

(F) qRT-PCR measurements of *gfp* mRNA (green) and pre-mRNA (blue) levels relative to *pie-1* are shown. The starting *csr-1::λN, gfp::boxb* strain (turquoise) is set to one. For the *csr-1::λN, gfp::boxb* strains after exposure to the *cdk-1::gfp(RNAe)* allele, measurements were taken at the F3 generation. The small difference in *gfp* levels for the strain receiving the *cdk-1::gfp(RNAe)* allele from the maternal germline is not statistically significant by the Student's t test.

(legend continued on next page)

amino acid 165 was inserted on chromosome I by MosSCI (Figure 1C) (Baron-Benhamou et al., 2004). The expression of the *csr-1::λN* transgene was driven by the native *csr-1* promoter and predicted 3′ UTR (Figure 1C), and expression was confirmed by qRT-PCR (Figure 1D). We observed that the transgene could rescue the sterility of a *csr-1(tm892)* mutant in a manner comparable to a previously described *pie-1::csr-1::3xflag* transgene (brood size averages: *csr-1::λN*, *csr-1(tm892)* = 31 ± 13 progeny; *csr-1* mutants = no progeny; Bristol N2 wild-type worms = 214 ± 9 progeny), indicating the ability of the CSR-1::λN protein to function in a wild-type manner and thus presumably bind endogenous 22G-RNAs (Claycomb et al., 2009). Furthermore, endogenous CSR-1 targets are expressed at consistent levels in the *csr-1::λN* strain (along with other strains used herein), demonstrating that they are unaffected by the tethering assay or its components (Figure 1E).

Because the Lambda N peptide recognizes *box b* hairpin RNA sequences (Baron-Benhamou et al., 2004; Keryer-Bibens et al., 2008), CSR-1::λN can be recruited to the *gfp::boxb* transcript without the requirement of small RNAs (Figure 1F). Such a system has been employed successfully in *Schizosaccharomyces pombe* to interrogate the influence of Ago1 small RNA pathway components on chromatin (Bühler et al., 2006) and in tissue culture models to assess the influence of microRNA pathway components on target transcripts (Eckhardt et al., 2011; Pillai et al., 2004, 2005).

In our tethering system, we observed that the *gfp::boxb* allele was initially expressed, or weakly licensed, even without CSR-1::λN present in the strain (Figures 1B and 1F). This observation is consistent with a previous model and with the fact that not every single-copy transgene is automatically silenced by the piRNA pathway in the germline (Shirayama et al., 2012). The weak licensing by endogenous CSR-1 is likely due to the fact that the transgene possesses *pie-1* regulatory sequences, which are an endogenous target of CSR-1/22G-RNA complexes (Claycomb et al., 2009). Thus, the recruitment of endogenous CSR-1 complexes is likely to enable weak licensing of *gfp::boxb* and allows the transgene to initially evade silencing by the piRNA surveillance pathway. Consistent with expression of the transgene and targeting by the CSR-1 pathway, we observed enrichment of histone H3 lysine 4 dimethylation (H3K4me2) at the *gfp::boxb* locus, as is the case for CSR-1 target genes such as *hcp-1* (Figure 1G). A repressive histone mark, histone H3 lysine 9 trimethylation (H3K9me3), was depleted at the *gfp::boxb* and *hcp-1* loci and enriched at the silent *cdk-1::gfp(RNAe)* locus as well as a locus known to be enriched for H3K9me3, *cpt-3* (Figure 1G) (Shirayama et al., 2012). Note that the slight enrichment of H3K4me2 at the *cdk-1::gfp(RNAe)* locus was not expected and may be intriguing for future studies.

This tethering system provides a versatile and simplified in vivo context to interrogate the role of CSR-1 in regulating chromatin and transcription and enables studies of CSR-1 function that would otherwise be difficult given the essential nature of *csr-1*. After our initial characterization of the tethering system, we set out to use this assay to test the following model (initially proposed, but not tested, by Shirayama et al., 2012). Because of its capacity to interact with the majority of germline transcripts, CSR-1 could protect germline transcripts against the vast silencing potential of the piRNA germline surveillance pathway.

The piRNA Genome Surveillance Pathway Silences the *gfp::boxb* Transcript

First, we tested whether the *gfp::boxb* transgene (*gfp+*) could be silenced by an *RNAe* allele by crossing a silent *cdk-1::gfp(RNAe)* allele (Shirayama et al., 2012) into the *gfp::boxb(gfp+)* strain (Figure 2A). After the introduction of the *cdk-1::gfp(RNAe)* allele, we then segregated it away from the *gfp::boxb* allele, verified genotypes by PCR, and assayed the homozygous *gfp::boxb* strains for *gfp* expression by qRT-PCR. In all crosses, *gfp::boxb* mRNA expression was significantly reduced after exposure to RNAe relative to the starting *gfp::boxb* strain (Figure 2B). This effect appeared to be mediated at the transcriptional or pre-mRNA stability level, as *gfp* pre-mRNA levels were also significantly reduced relative to the starting *gfp::boxb* strain (Figure 2B). We propagated the *gfp::boxb* silenced strains (*gfp::boxb(RNAe)*) for a number of generations, and for each subsequent generation, we assayed *gfp* mRNA and pre-mRNA levels. The *gfp::boxb(RNAe)* strains remained silenced for *gfp* expression relative to the starting *gfp::boxb* strain for more than eight generations, indicating that they were permanently and heritably silenced (Figure 2B).

To verify that the silencing of the *gfp::boxb* transgene occurred through the piRNA germline surveillance pathway, we tested whether loss of a key factor in the pathway, WAGO-9/HRDE-1, would abrogate the silencing of *gfp::boxb(RNAe)*. WAGO-9 was previously shown to be one of three WAGOs required for the maintenance of RNAe, as loss of *wago-9* alleviated the silencing of *RNAe* alleles (Shirayama et al., 2012). We crossed a *wago-9(tm1200)* allele into the *gfp::boxb(RNAe)* strain and segregated doubly homozygous animals, as verified by genotyping PCR (Figure 2C). Indeed, we observed a resumption of *gfp* mRNA and pre-mRNA expression in *gfp::boxb, wago-9* animals,

(G) An outline of the genetic crosses to demonstrate that the continued presence of the *cdk-1::gfp(RNAe)* allele within a *csr-1::λN, gfp::boxb* strain does not alter the expression of *gfp::boxb*. The starting strain for these experiments possessed a *cdk-1::gfp(RNAe)* allele from the maternal germline (Figure 2F, hermaphrodite symbol). The strain was propagated as homozygous for *csr-1::λN* on LG I and heterozygous for *gfp::boxb* and *cdk-1::gfp(RNAe)* on LG II. In each generation (F3….F(n)) *csr-1::λN, gfp::boxb* homozygotes were segregated and verified by genotyping PCR. The strains marked in turquoise and red were analyzed in (H).
(H) qRT-PCR measurements of *gfp* mRNA (green) and pre-mRNA (blue) levels relative to *pie-1* over several generations are shown. The starting *csr-1::λN, gfp::boxb* strain (turquoise) is set to one.
(I) An outline of genetic crosses to show that *csr-1::λN* tethering is important for the protection of *gfp::boxb* expression. *csr-1::3xflag* (WM193) was previously shown to rescue the *csr-1(tm892)* allele (Claycomb et al., 2009). A recombinant chromosome possessing both *csr-1::3xflag* and *gfp::boxb* was generated by standard genetic approaches and verified by genotyping PCR. The *csr-1::3xflag*, *gfp::boxb* males were crossed to the *cdk-1::gfp(RNAe)* strain, and then *csr-1::3xflag*, *gfp::boxb* alleles were segregated from *cdk-1::gfp(RNAe)* and maintained as homozygotes. The strains marked in turquoise and red were analyzed in (J).
(J) qRT-PCR measurements of *gfp* mRNA (green) and pre-mRNA (blue) levels relative to *pie-1* are shown. The starting *csr-1::3xflag, gfp::boxb* strain (turquoise) is set to 1. The F3 generation for the *csr-1::3xflag, gfp::boxb (RNAe)* strain is shown (*$p < 0.05$, **$p < 0.01$ by the Student's t test). See also Figure S2.

compared to the starting *gfp::boxb(RNAe)* strain (Figure 2D), thus indicating that the silencing of *gfp::boxb* is executed through the piRNA surveillance pathway.

CSR-1::λN Tethering Stably Protects the *gfp::boxb* Transcript from piRNA Silencing

We next tested whether the presence of CSR-1::λN in the same strain as *gfp::boxb* could protect against the silencing effect incurred from introducing an *RNAe* allele. In a series of crosses, we introduced the *RNAe* allele into a *csr-1::λN, gfp::boxb(gfp+)* strain (Figure 2E). Again, we segregated and verified the genotypes of *csr-1::λN, gfp::boxb* homozygotes and assayed *gfp* levels. In all crosses, the presence of CSR-1::λN protected the *gfp::boxb* allele from silencing and maintained the expression of *gfp* mRNA and pre-mRNA at levels comparable to that of the starting strain, *csr-1::λN, gfp::boxb* (Figure 2F, compare to Figure 2B). In support of a transcriptional mode of action for *csr-1::λN*, CSR-1 was not enriched at the *gfp::boxb* locus in the *gfp::boxb(RNAe)* strain, but it was enriched in the *csr-1::λN, gfp::boxb(gfp+)* starting strain and in the *csr-1::λN, gfp::boxb(gfp+)* strain after exposure to RNAe (Figure S1B).

To determine whether *gfp::boxb* expression was heritably protected over multiple generations in the presence of *csr-1::λN* and *cdk-1::gfp(RNAe)*, we maintained a strain possessing *csr-1::λN, gfp::boxb/cdk-1::gfp(RNAe)* for many generations and assayed *gfp* mRNA and pre-mRNA levels at each generation throughout its propagation. We segregated *csr-1::λN, gfp::boxb* homozygotes in each generation to ask whether *gfp::boxb* expression was maintained despite the continued presence of the *cdk-1::gfp(RNAe)* allele (Figure 2G). In each generation tested, *gfp::boxb* mRNA and pre-mRNA levels remained comparable to the levels of the starting *csr-1::λN, gfp::boxb* strain (Figure 2H). These results indicate that CSR-1::λN acts to stably and continuously protect the *gfp::boxb* transgene from RNAe-mediated germline silencing.

To assure that our observations of the protective capacity of *csr-1::λN* were attributable to tethering CSR-1::λN to the *gfp::boxb* transcript, we asked whether *csr-1::3xflag* (Claycomb et al., 2009) (which is not capable of being tethered to the *gfp::boxb* transcript) was able to protect *gfp::boxb* from silencing by RNAe (Figure 2I). After exposure to the *cdk-1::gfp(RNAe)* allele, *gfp* mRNA and pre-mRNA levels in the *csr-1::3xflag, gfp::boxb* strain were significantly reduced relative to the starting strain, comparable to levels observed in the *gfp::boxb(RNAe)* strain (Figure 2J; compare to Figure 2B). Furthermore, to ensure that the protective capacity of CSR-1::λN was not simply due to increased levels of CSR-1 expression, we examined the mRNA levels of *csr-1* in all strains used in this study as well as the protein levels of CSR-1 for several key strains (Figure S2). We observed no significant differences in mRNA or protein levels in our strains. Together, these results indicate that the protective capacity of CSR-1::λN is achieved by its tethering to the target transcript.

Tethering CSR-1::λN to a Previously Silenced *gfp::boxb* Transcript Activates Its Expression

Having established that CSR-1::λN can protect *gfp::boxb* from silencing by the piRNA pathway, we asked whether the introduction of *csr-1::λN* into a previously silenced *gfp::boxb(RNAe)* strain (Figure 2B) is sufficient to reactivate *gfp::boxb* expression (Figure 3A). Here, we found that the *gfp::boxb* was not initially reactivated in the F1 or F2 generations (Figure 3B). Ultimately, by the F4 generation, the strain resumed *gfp* mRNA expression comparable to the initial *csr-1::λN, gfp::boxb* strain (Figure 3B). Furthermore, we observed the same result using multiple *gfp::boxb(RNAe)* strains that differed only in the initial parent from which the *RNAe* allele was introduced (Figure 3B). These results suggest that CSR-1::λN recruitment to a target gene is not simply protective but can also activate gene expression over several generations, perhaps after a sufficient accumulation of CSR-1::λN at the target locus and/or sufficient production of *gfp* small RNAs has been achieved.

CSR-1 Activates an *RNAe* Allele over Multiple Generations in the Absence of Tethering

Related to our observation that CSR-1::λN can activate *gfp::boxb* expression, it was previously shown that, although an *RNAe* allele acts in a dominant manner to silence most transgenes possessing sequences in common, the *RNAe* alleles can be activated in some very specific instances, particularly by the introduction of either *oma-1::gfp* or *gfp::wrm-1* transgenes (which are *gfp+*) into the silenced strain (Shirayama et al., 2012). In this study, the authors proposed that this dominant activation capacity was due to the long-term propagation of the *oma-1::gfp* or *gfp::wrm-1* strains, leading to the ultimate recognition and conversion of these transgenes as "self" (Shirayama et al., 2012). The conversion to "self" was hypothesized to involve the loading of *gfp* small RNAs into CSR-1 complexes over a number of generations (Shirayama et al., 2012), suggesting that active transgenes must be recognized by CSR-1 to be expressed. With the tools in hand to experimentally test this model, we asked whether the *cdk-1::gfp(RNAe)* allele could be activated in our system by the continued presence of *csr-1::λN, gfp::boxb* in the same strain (Figure 3C). We observed that when the *cdk-1::gfp(RNAe)* allele initially came from the paternal germline, the allele was activated in as little as three generations and remained expressed (Figure 3D). When the initial *cdk-1::gfp(RNAe)* allele originated from the maternal germline, we did not observe its activation until generation F7 in the presence of *gfp::boxb* and CSR-1::λN (Figure 3D). Our observation of differences in the potency of activation is consistent with previously reported differences in silencing by the maternal and paternal germline. It is also worth noting that *gfp::boxb(RNAe)*, in which CSR-1::λN tethering occurs, is activated at an earlier generation than *cdk-1::gfp(RNAe)*. Importantly, we never observed activation of *cdk-1::gfp(RNAe)* expression when cultured for many generations in the presence of *csr-1::λN* alone (Figure 1B). These results are striking and demonstrate that silent transgenes can be converted to active transgenes over successive generations in the presence of a fully licensed *gfp+* transgene (see Figure 4). Because the *cdk-1::gfp* transgene does not possess *box b* sites, we hypothesize that *gfp* small RNAs produced from the *gfp::boxb* allele are loaded into CSR-1 complexes to target and activate the expression of *cdk-1::gfp.*

Our data suggest that *gfp* small RNAs from active genes are loaded into CSR-1 complexes and are important for activating or maintaining gene expression. To test this model, we asked whether the introduction of CSR-1::λN into the *gfp::boxb* strain at the same time as *cdk-1::gfp(RNAe)* would provide the same

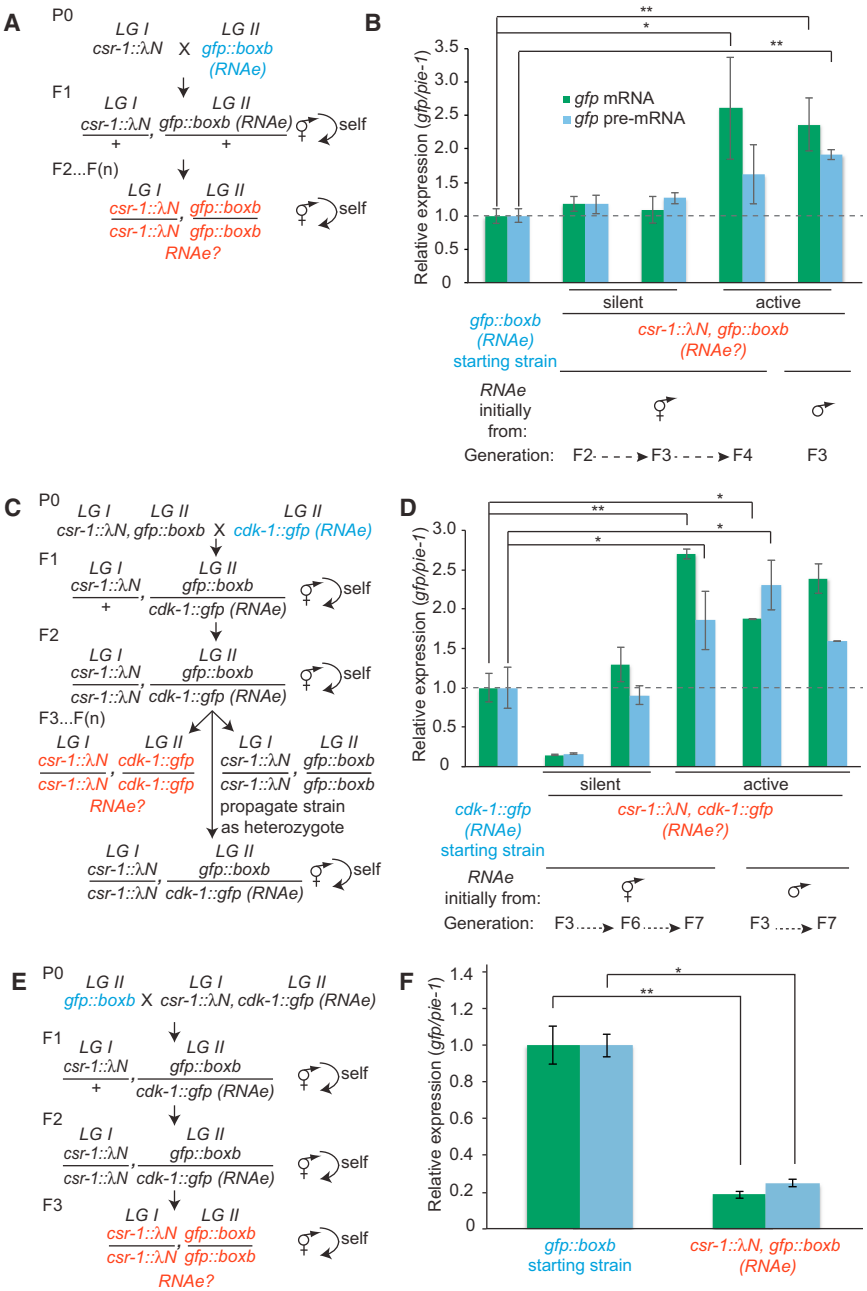

Figure 3. The Introduction of CSR-1::λN into Silent *(RNAe)* Strains Activates *gfp* Expression

(A) An outline of crosses to determine whether the introduction of *csr-1::λN* can activate the expression of a silenced *gfp::boxb(RNAe)* strain (generated in Figures 2A and 2B). The *csr-1::λN* allele was introduced from the paternal germline and the silenced *gfp::boxb(RNAe)* allele was from the hermaphrodite. The strains marked in turquoise and red were analyzed in (B).

(B) qRT-PCR measurements of *gfp* mRNA (green) and pre-mRNA (blue) levels relative to the germline gene *pie-1* are shown. The starting *gfp::boxb (RNAe)* strain (turquoise) is set to one. For all parts of this figure, each bar on the graph represents the average of three to seven biological replicates from samples of 50–100 adult hermaphrodite worms and error bars represent SEM. After introduction of *csr-1::λN* into *gfp::boxb(RNAe)* strains, measurements were taken from each generation indicated. Two *gfp::boxb(RNAe)* strains were used in these experiments and only differ in which parent introduced the initial *cdk-1::gfp(RNAe)* silencing signal, as designated. Strains with *gfp* levels comparable to the starting *gfp::boxb(RNAe)* strain are silenced, while strains with *gfp* levels significantly greater than the starting strain are activated (*$p < 0.05$, **$p < 0.01$ by the Student's t test).

(C) A diagram of genetic crosses used to show that the continued presence of *csr-1::λN, gfp::boxb* within the *cdk-1::gfp(RNAe)* strain activates *cdk-1::gfp(RNAe)* over several generations is shown. These crosses are the same as those in Figures 2G and 2H, except that we focused on the homozygous *csr-1::λN, cdk-1::gfp(RNAe)* animals. In each generation (F3….F(n)), *csr-1::λN, cdk-1::gfp(RNAe)* homozygotes were segregated and verified by genotyping PCR. The strains marked in turquoise and red were analyzed in (D).

(D) qRT-PCR measurements of *gfp* mRNA (green) and pre-mRNA (blue) levels relative to the *pie-1*. The starting *cdk-1::gfp(RNAe)* strain (turquoise) is set to one. Generations are as indicated. Strains with *gfp* levels comparable to the starting *cdk-1::gfp(RNAe)* strain are considered to remain silenced, while strains with *gfp* levels significantly greater than the starting strain are considered to be activated (*$p < 0.05$, **$p < 0.01$ by the Student's t test). The parent of origin for the original silencing *cdk-1::gfp(RNAe)* allele is noted, and had an impact on how quickly activation occurred.

(E) An outline of crosses to determine whether the introduction of *csr-1::λN* at the same time as *cdk-1::gfp(RNAe)* can protect *gfp::boxb* expression is shown. The *csr-1::λN*, *cdk-1::gfp(RNAe)* alleles were introduced from the paternal germline and the silenced *gfp::boxb(RNAe)* allele was from the hermaphrodite. The strains marked in turquoise and red were analyzed in (F).

(F) qRT-PCR measurements of *gfp* mRNA (green) and pre-mRNA (blue) levels relative to *pie-1*. The starting *gfp::boxb* strain (turquoise) is set to one. The F3 generation for the *csr-1::λN, gfp::boxb(RNAe)* strain is shown (*$p < 0.05$, **$p < 0.01$ by the Student's t test).

protective capacity as when CSR-1::λN was already present in the *gfp::boxb* strain prior to exposure to *cdk-1::gfp(RNAe)* (Figure 3E). In this situation, CSR-1::λN would not be tethered to the *cdk-1::gfp* transcript and CSR-1 complexes would likely possess few *gfp* small RNAs. After crossing *csr-1::λN, cdk-1::gfp(RNAe)* into the *gfp::boxb* strain, we segregated *csr-1::λN, gfp::boxb* homozygous animals (Figure 3E) and verified genotypes by PCR. Notably, we observed that *gfp* mRNA and pre-mRNA levels were dramatically reduced in this strain relative to the *gfp::boxb* starting strain and comparable to when no CSR-1::λN was present (Figure 3F; compare to Figure 2B). This indicates that CSR-1::λN must be present at the target transcript and/or loaded with *gfp* small RNAs prior to exposure to RNAe to have a protective effect on *gfp::boxb* transcription.

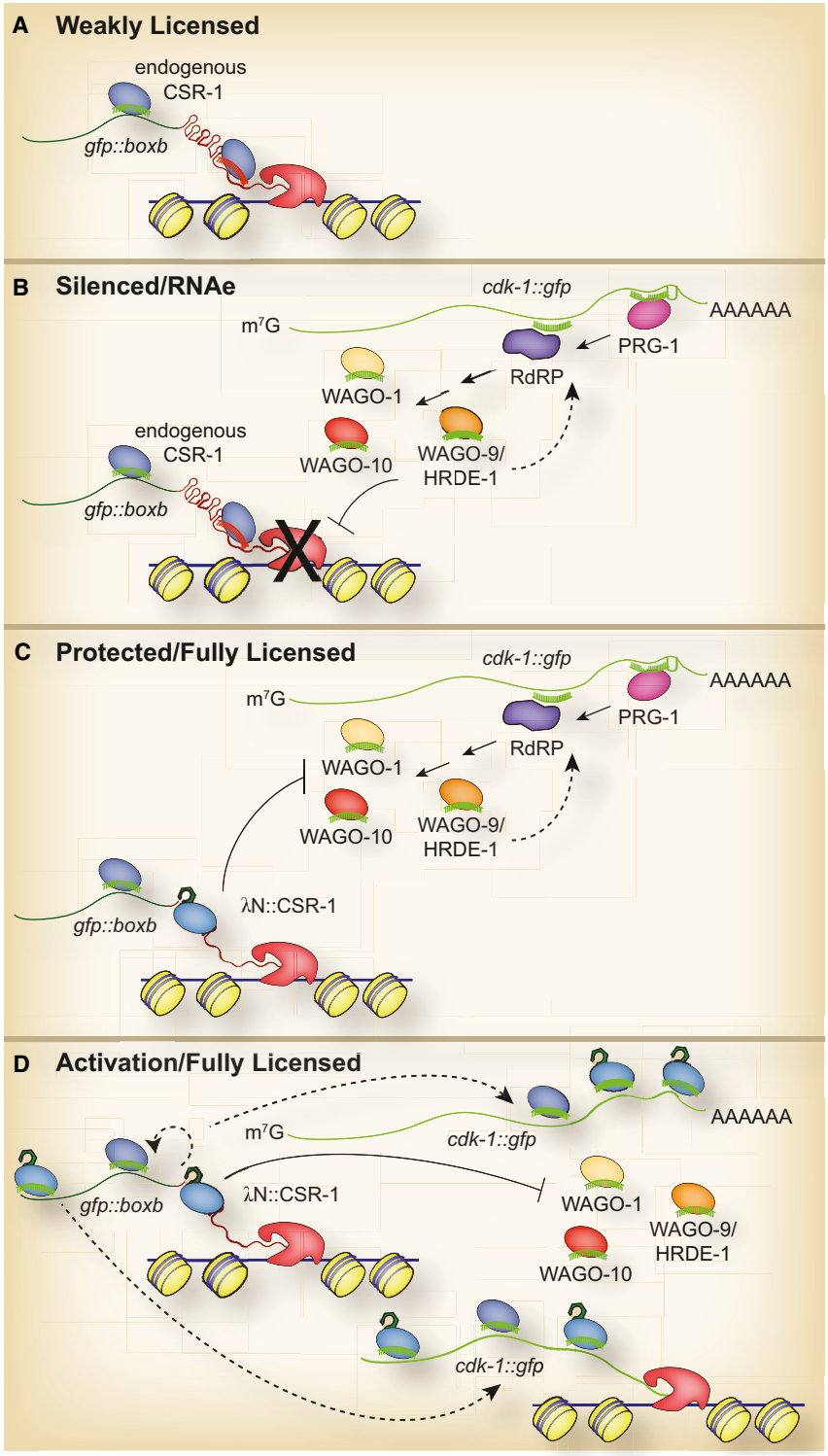

Figure 4. A Model to Summarize How CSR-1 Tethering Can License and Activate Gene Expression

(A) The *gfp::boxb* transcript possesses *pie-1* regulatory elements that can recruit endogenous CSR-1 via 22G-RNAs and allow for the weak licensing of *gfp*.

(B) When exposed to RNAe, expression of the *gfp::boxb* transcript is halted by the piRNA pathway. Endogenous CSR-1 in not sufficient to protect the *gfp::boxb* transcript from the piRNA pathway silencing. (Green small RNAs target *gfp*, and red small RNAs target *box b*.)

(C) The tethering of CSR-1::λN to *gfp::boxb* enables a more stable, or fully licensed, expression state and renders *gfp::boxb* impervious to silencing.

(D) Over several generations, *gfp* small RNAs loaded into CSR-1 complexes are capable of acting in *trans* to license expression of *cdk-1::gfp*. Tethering CSR-1::λN to *gfp::boxb* may improve *gfp* small RNA production and/or loading into CSR-1 complexes, making the activity of the CSR-1 pathway more robust and capable of overcoming silencing by the piRNA pathway.

CONCLUSIONS

Our results demonstrate a positive gene regulatory role for a *C. elegans* Argonaute as a protector of the germline transcriptome against the silencing activities of the piRNA genome surveillance pathway and highlight the stable and heritable nature of small RNA pathway outcomes (Figure 4). These results were obtained using a powerful in vivo tethering assay that enabled us to test the effects of CSR-1 on its targets in an otherwise wild-type background, thus allowing us to avoid any experimental complications due to the essential nature of *csr-1*. Despite the potential developmental complications in *csr-1* mutants, it will ultimately be important to determine whether our observations of the protective nature of CSR-1 can be extended to the 4,200 endogenous CSR-1 target genes and to unravel the molecular mechanisms by which this AGO licenses germline genes and transmits patterns of gene expression across generations.

EXPERIMENTAL PROCEDURES

Genetics and Generation of Transgenic *C. elegans* Strains

Crosses were carried out by standard genetic approaches (Brenner, 1974). Transgenic strains were generated using the MosSCI method (Frøkjaer-Jensen et al., 2008, 2012). Strains are detailed in Supplemental Experimental Procedures.

qRT-PCR

qRT-PCR was performed as described previously (Claycomb et al., 2009), with the exception that SuperScript VILO reverse transcriptase and a StepOnePlus

Real Time-PCR machine were used (Life Technologies). All primers are detailed in Supplemental Experimental Procedures.

Chromatin Immunoprecipitation

ChIP was performed as described previously (Claycomb et al., 2009), except that young adult worms were used. All primers are detailed in Supplemental Experimental Procedures.

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.2013.11.016.

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