2018-02-27

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The Coding Regions of Germline mRNAs Confer Sensitivity to Argonaute Regulation in *C. elegans*

**Highlights**

- *C. elegans* germline mRNAs differ in sensitivity to piRNA targeting

- piRNA targeting of coding regions provides incremental control of gene expression

- Piwi Argonaute surveillance occurs upstream of nonsense-mediated decay

- Model, piRNAs scan mRNAs within perinuclear nuage prior to translation initiation

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**In Brief**

Some *C. elegans* transgenes resist piRNA silencing. Seth et al. map resistance to endogenous sequences within transgenes and show that artificially increasing piRNA targeting can incrementally reduce expression without silencing. Their findings identify coding regions as part of a rich piRNA regulatory landscape within perinuclear nuage.
The Coding Regions of Germline mRNAs Confer Sensitivity to Argonaute Regulation in *C. elegans*

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https://doi.org/10.1016/j.celrep.2018.02.009

SUMMARY

Protein-coding genes undergo a wide array of regulatory interactions with factors that engage non-coding regions. Open reading frames (ORFs), in contrast, are thought to be constrained by coding function, precluding a major role in gene regulation. Here, we explore Piwi-interacting (pi)RNA-mediated transgene silencing in *C. elegans* and show that marked differences in the sensitivity to piRNA silencing map to the endogenous sequences within transgene ORFs. Artificially increasing piRNA targeting within the ORF of a resistant transgene can lead to a partial yet stable reduction in expression, revealing that piRNAs not only silence but can also “tune” gene expression. Our findings support a model that involves a temporal element to mRNA regulation by germline Argonautes, likely prior to translation, and suggest that piRNAs afford incremental control of germline mRNA expression by targeting the body of the mRNA, including the coding region.

INTRODUCTION

Cells utilize RNA-guided search mechanisms to find and regulate genetic information. Mechanisms of this type include the Argonaute-mediated response termed RNA interference (RNAi) (Fire et al., 1998) and the independently evolved bacterial antiviral CRISPR/CAS system (Bhaya et al., 2011; Marraffini and Sontheimer, 2010). In addition to cellular defense, organisms employ RNA-guided mechanisms to regulate endogenous gene expression. For example, the microRNA (miRNA) Argonaute-mediated pathway employs cellular transcription to produce RNA guides that carry out mRNA regulation (Ghildiyal and Zamore, 2009; Lee et al., 1993; Wightman et al., 1993). The miRNA Argonaute system tolerates mismatched pairing between miRNA and target mRNA, allowing the few hundred miRNAs typically present in most animal genomes to regulate a substantial fraction of mRNAs (Grimson et al., 2007; Helwak et al., 2013; Lewis et al., 2005).

Among the most enigmatic of small RNA pathways is the Piwi-interacting (pi)RNA pathway (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Ruby et al., 2006). piRNAs engage Argonaute proteins related to the *Drosophila* Piwi (P element-induced, wimpy testes) protein (Cox et al., 1998; Lin and Spradling, 1997). piRNAs derive from precursors that are transcribed by RNA polymerase II, and their production requires nucleolytic processing at their 5’ and 3’ ends (Ipsaro et al., 2012; Izumi et al., 2016; Nishimasu et al., 2012; Tang et al., 2016). While some piRNAs target transposons, many have no perfectly matched mRNA targets (Bagijn et al., 2012; Lee et al., 2012; Vourekas et al., 2012).

Studies on mouse Piwi proteins suggest that they may regulate endogenous genes. For example, Goh et al. (2015) provide evidence for piRNA-directed targeting of meiotically expressed protein-coding genes in the mouse testes. Another study suggests that piRNAs may direct massive mRNA elimination in elongating spermatids (Gou et al., 2015). In *C. elegans*, piRNAs can induce stable transgenerational silencing of foreign genes and are thought to do so while allowing mRNA-like, partially mismatched base-pairing (Ashe et al., 2012; Bagijn et al., 2012; Lee et al., 2012; Shirayama et al., 2012). Upon target binding, the Piwi-related protein (PRG-1) recruits RNA-dependent RNA polymerase (RdRP) to produce secondary small RNAs (22G-RNAs) that load onto members of an expanded group of worm-specific Argonautes (WAGO). WAGO pathways in turn, maintain and propagate a form of epigenetic silencing termed RNA-induced epigenetic silencing (RNAe) (Shirayama et al., 2012).

A curious feature of RNAe is that WAGO targets the foreign portions of a silenced transgene (e.g., the gfp open reading frame [ORF]) but not the endogenous sequences fused to gfp within the same transgene (Shirayama et al., 2012). How these endogenous sequences are protected from WAGO targeting remains unclear, but a recent study suggests that it is not simply because piRNAs fail to target sequences that resist RNAe (Shen et al., 2018). Indeed, Shen et al. (2018) revealed that piRNAs bind with miRNA-like seed and supplementary pairing
but do so within the ORFs as well as the UTRs of essentially all germline mRNAs.

Interestingly, a third germline Argonaute system, the CSR-1 pathway, engages small RNAs produced by RdRP that are antisense to most germline mRNAs (Claycomb et al., 2009). CSR-1 targeting correlates with resistance to WAGO silencing. Several lines of evidence suggest that CSR-1 provides a protective memory of self-gene expression and that this protection is necessary for germline mRNAs to avoid piRNA silencing. First, as noted above, the diversity and relaxed-targeting rules of piRNAs mean that germline mRNAs cannot entirely avoid piRNA targeting. Second, essentially all expressed germline mRNAs are targeted by CSR-1, and with very few exceptions (Gerson-Gurwitz et al., 2016), their expression is not increased in csr-1 mutants. Thus, CSR-1 does not silence the vast majority of its targets. Third, when transgenes are introduced at single copy, in defined chromosomal locations, only those transgenes containing foreign sequences (e.g., gfp) undergo silencing (Shirayama et al., 2012). Fourth, some gfp transgenes escape piRNA-induced silencing and also resist WAGO silencing. This resistance correlates with targeting of the gfp sequences by CSR-1 22G-RNAs (Seth et al., 2013). Fifth, when CSR-1 targets gfp sequences, the transgene can transactivate silenced gfp transgenes (Shirayama et al., 2012), and its ability to transactivate depends on CSR-1 activity (Seth et al., 2013). Moreover, artificially tethering CSR-1 to a target mRNA can drive the activation of a normally silent transgene (Wedele et al., 2013). Finally, when CSR-1 activity is depleted, piRNA targeting increases on germline mRNAs transcriptome wide (Shen et al., 2018).

Thus, numerous lines of evidence suggest that CSR-1 targeting provides a memory of self-gene expression that is necessary to protect mRNAs from piRNA-mediated silencing in C. elegans. The term RNA activation, RNAa, describes the process by which a CSR-1-targeted transgene can activate a silent transgene that shares sequence identity (Seth et al., 2013). Interestingly, both CSR-1 and WAGO Argonautes and their associated small RNAs are transmitted to offspring in both the sperm and the egg, providing a mechanism for the inheritance of these “memories” of parental gene-expression states (Conine et al., 2010, 2013).

In C. elegans, both CSR-1 and PRG-1, as well as members of the WAGO Argonaute family, reside within perinuclear germline nuage structures termed P granules (Batista et al., 2008; Claycomb et al., 2009; Gu et al., 2009). CSR-1 is required for the perinuclear localization of the P granules, suggesting that the targeting of nascent mRNAs by CSR-1 may induce the recruitment (or condensation) of P granules at the nuclear periphery (Claycomb et al., 2009; Updike and Strome, 2009). In wild-type animals, P granules surround nuclei in close apposition to nuclear pores, and mRNAs are thought to transit through P granules after nuclear exit (Schisa et al., 2001; Sheth et al., 2010). These observations have prompted the hypothesis that P granules represent a ribosome-free zone where nascent mRNAs may undergo regulation in germ cells prior to the onset of translation (Sheth et al., 2010; Updike and Strome, 2010; Updike et al., 2011).

Here, we explore marked differences in the sensitivity of two transgenes to piRNA-induced silencing. We map the sequences that confer these differences to the ORFs of the endogenous sequences in these transgenes. We show that resistance to silencing does not depend (solely) on CSR-1 targeting. We show that piRNA surveillance occurs even on transcripts that undergo nonsense-mediated decay, a co-translational surveillance mechanism that destroys transcripts containing premature stop codons (Baker and Parker, 2004; Chang et al., 2007). Our findings support a model that involves regulation by germline Argonautes at a step prior to mRNA translation and suggest that regulation occurs within a context of other, as yet unknown regulators that also engage the coding region of the mRNA to afford incremental control of germline mRNA expression.

RESULTS

In the course of our investigation of transgene interactions, we identified numerous gfp fusions that were able to transactivate silent gfp transgenes. These activating transgenes included oma-1::gfp, wrm-1::gfp, oma-2::gfp, and pie-1::gfp (Shirayama et al., 2012). A search for features of these transgenes or their corresponding endogenous genes that might explain their properties did not reveal any obvious correlations. The transgenes themselves were not more abundantly expressed nor were the corresponding endogenous genes expressed at higher levels. Moreover, as compared to the endogenous regions in transgenes prone to silencing, the endogenous sequences within RNAe-competent transgenes were neither targeted by more CSR-1 22G-RNAs nor by fewer piRNAs (Figure S1; Shen et al., 2018). These observations suggest that the anti-silencing features of RNAs transgenes do not result from bypassing or overwhelming the RNAe system. Instead, we speculated that they somehow promote the spread of CSR-1 targeting from the endogenous sequences to the gfp sequences in the transgene (Seth et al., 2013; Shirayama et al., 2012).

Balanced Silencing and Activating Signals

To explore the question of why transgenes differ in sensitivity to silencing, we decided to make a detailed investigation of the RNAe transgene oma-1::gfp. During this analysis, we found that multiple independently isolated oma-1::gfp transgenes could reproducibly transactivate the silent, RNAe, transgenes gfp::cdk-1 and gfp::csr-1 (green arrows, Figure 1A). Surprisingly, however, we identified occasional pairings in which RNAa failed to occur (red arrows, Figure 1A), and the transgenes instead adopted a stable balanced state, with the oma-1::gfp transgene expressed and the RNAe transgenes silent (Figure 1B). Consistent with the idea that an RNAe-dependent mechanism maintains silencing in this balanced state, we found that the introduction of a mutation in rde-3(ne3370), a gene required for RNAe (Shirayama et al., 2012), robustly reactivated GFP::CSR-1 expression.

PRG-1 activity was previously shown to be required to initiate RNAe or to re-silence a transgene activated by RNAa but not to maintain RNAe once established (Seth et al., 2013; Shirayama et al., 2012). We therefore asked whether prg-1 activity is required to maintain silencing in the balanced oma-1::gfp gfp::csr-1 double transgenic strain. We found that while, as expected, the gfp::csr-1 single transgenic strain remained silenced in prg-1 mutants (n > 20), the GFP::CSR-1 protein became robustly expressed in 100% of the prg-1(tm872) homozygous strains that also contained oma-1::gfp (n = 60; Figure 1C). Thus, oma-1::gfp...
can transactivate gfp::csr-1 inserted at cxTi10882 on LGIV but only when prg-1 activity is absent. These findings indicate that, although oma-1::gfp exerts a positive influence on the gfp::csr-1 transgene located at cxTi10882, it is not sufficient to overcome the combined activities of the WAGO-silencing machinery and continued piRNA/PRG-1 targeting. Thus, in addition to initiating RNAes as previously shown (Shirayama et al., 2012), these findings show that PRG-1 can also function in some instances to maintain and reinforce silencing.

**Transgenes Differ in Their Responses to piRNA Targeting**

In the above experiments, differences in the chromosomal insertion sites were correlated with differences in the interactions between transgenes. While it would be very interesting to understand these chromosomal influences in more detail, we decided to first attempt to address the more tractable question of why transgenes inserted at the same location respond differently to piRNA targeting. To address this question, we chose to compare the silencing-resistant oma-1::gfp and the silencing-prone gfp::cdk-1 transgenes. We asked how these two transgenes responded to increased piRNA targeting. Using CRISPR-mediated homologous recombination (Dickinson and Goldstein, 2016; Friedland et al., 2013; Jinek et al., 2013; Kim et al., 2014; see Supplemental Experimental Procedures), we replaced the most abundantly expressed piRNA, 21ux-1 (Gu et al., 2012), with a sequence antisense to gfp (Figure 2A) and then crossed the 21ux-1(anti-gfp) worms to animals expressing cdk-1::gfp. As expected, the cdk-1::gfp transgene was rapidly silenced after crossing to 21ux-1(anti-gfp) worms (n > 20) (Figure S2A), indicating that the engineered piRNA is expressed and functional. Small RNA sequencing also confirmed robust expression of the 21ux-1(anti-gfp) piRNA. Nevertheless, the 21ux-1(anti-gfp) piRNA failed to silence the oma-1::gfp transgene, even when 21ux-1(anti-gfp) was homozygous in the strain (n > 20) (Figure S2B). Strikingly, however, when we crossed these strains together to generate a double transgenic cdk-1::gfp; oma-1::gfp strain homozygous for 21ux-1(anti-gfp), rather than observing transactivation of cdk-1::gfp, we observed silencing of oma-1::gfp (n > 20) (Figure S2C). Thus, the addition of the 21ux-1(anti-gfp) piRNA abolishes the ability of oma-1::gfp to transactivate a silent transgene and renders oma-1::gfp sensitive to transitive silencing.

We next used high-throughput sequencing to ask how the addition of 21ux-1(anti-gfp) influenced 22G-RNA induction on the silencing-resistant oma-1::gfp and on the expressed but more silencing-prone cdk-1::gfp transgenes. Strikingly, on cdk-1::gfp we found that 22G-RNA induction occurred both locally, i.e., near the 21ux-1(anti-gfp) target site, and at numerous regions distributed along the gfp portion of the transgene. The 21ux-1(anti-gfp) target site was correlated with three major peaks of 22G-RNA biogenesis initiating at C residues at both ends and the middle of the target mRNA region (Figures 2D and 2E). By contrast, in the resistant oma-1::gfp transgenics, we observed a dramatically muted 22G-RNA response with only a single abundant 22G-RNA species positioned near the 3′ end of the 21ux-1(anti-gfp) piRNA target region (Figures 2B and 2C). This finding suggests that some property of the oma-1::gfp mRNA prevents the accumulation and spread of 22G-RNA production along the length of the transgene.

Although immunofluorescence analysis indicated that oma-1::gfp remained expressed, we also monitored the levels of the corresponding mRNA and protein products. As expected, the cdk-1::gfp transgene was strongly silenced both at the mRNA and protein levels (Figures 2F and 2G). However, though still visible by microscopy, the mRNA and protein levels of oma-1::gfp signal were clearly reduced (Figures 2F and 2G), suggesting that the additional piRNA can partially reduce oma-1::gfp expression without inducing RNAes.

**Increasing piRNA Targeting Can Drive PRG-1-Dependent Silencing**

The above findings prompted us to ask whether engineering additional piRNAs that target oma-1::gfp could ultimately render the transgene sensitive to piRNA-induced silencing. Using
CRISPR, we replaced two adjacent abundantly expressed piRNAs (21ur-11498 and 21ur-2675) (Figure 3A) with sequences antisense to the oma-1 mRNA. This double piRNA mutant strain also failed to silence oma-1::gfp (Figure S3A). However, when all three engineered piRNAs (both anti-oma-1 piRNAs and the 21ux-1(anti-gfp) piRNA) were present together in the same strain, we finally observed oma-1::gfp silencing (Figure S3B).

As expected, we found that the addition of the new piRNAs was correlated with the accumulation of 22G-RNAs near the target sites, especially the 21ux-1(anti-gfp) target site (Figures 3B–3D). As expected, the oma-1::gfp transgene was strongly silenced both at the mRNA and protein levels (Figures 3E and 3F). Interestingly, crossing the prg-1 (tm872) mutation into this silenced strain reactivated oma-1::gfp (n > 20). Thus, silencing of oma-1::gfp via these engineered piRNAs requires continuous PRG-1 targeting.

**Properties Intrinsic to the oma-1 Coding Sequences Confer Resistance to Silencing**

We next wished to explore which features render the oma-1::gfp transgene more resistant to silencing than the 21ux-1::gfp transgene. Therefore, we performed a number of swaps of sequence domains from these genes and monitored how these changes affected expression when re-inserted within exactly the same chromosomal location. We found that neither the promoter, 3'UTR, nor introns of oma-1::gfp were required for its RNAa properties (Figures 4A and 4B). An oma-1::gfp fusion driven by the cdk-1 promoter and 3' UTR was resistant to
Figure 3. Increasing piRNA Targeting Induces *oma-1::gfp* to Silence

(A) Schematic representing the replacement of 21U-2675 IV and 21U-11498 IV with anti-oma-1 sequence.

(B–D) Schematics showing plots of small RNA species induced along the entire *oma-1::gfp* transgene in (B) wildtype, (C) 21uIV-11498(anti-oma1) and 21uIV-2675(anti-oma1) animals, and (D) 21uIV-11498(anti-oma1), 21uIV-2675(anti-oma1), and 21ux-1(anti-gfp) animals. In the browser schematic, *oma-1* sequences are blue, fused to green gfp sequences. The positions of each artificial piRNA are indicated by red dash marks beneath the small RNA graphs. The 5' ends of small RNA reads are plotted, and the height indicates abundance in reads per million.

(E) qRT-PCR analysis of wild-type (WT) and *oma-1::gfp* RNA from total RNA prepared from different transgenic strains (as indicated). Error bars represent the standard deviation for three replicates in one experiment.

(F) Western blot analysis of GFP protein expression in wild-type and transgenic strains (as indicated). As a loading control, the blot was stripped and re-probed for the germline-specific GLH-4 protein.

See also Figure S3.
Figure 4. Properties Intrinsic to the oma-1 Coding Sequences Confer Resistance to Silencing

(A and B) Promoters, UTRs, and introns do not determine transgene sensitivity/resistance to silencing. Schematics indicating the exon-intron structure of fusion genes analyzed are displayed alongside tabulations of their respective RNAe and RNAa activities. Transgenes were scored as (+) if they could act in trans to

(legend continued on next page)
silencing and retained the ability to transactivate a silent gfp transgene (Figure 4A). Conversely, cdk-1::gfp controlled by the oma-1 promoter and 3’ UTR remained prone to silencing (Figure 4A).

The above findings suggest that the sequences comprising the coding region of oma-1 confer resistance to silencing. To more directly test this idea we decided to re-code the OMA-1 protein to maximize nucleotide differences while maintaining the amino acid sequence of OMA-1. Upon introduction, we found that all of the (n > 20) independently generated codon-altered oma-1::gfp transgenes analyzed were silent, with no detectable GFP fluorescence or mRNA expression (Figures 4B and 4D). Moreover, we found that a silent codon-altered oma-1::gfp transgene was able to act in trans to silence an expressed allele of cdk-1::gfp (Figure 4C). Consistent with an RNAe mechanism, we found that introducing a mutation in rde-3 resulted in the activation of the silent re-coded oma-1::gfp allele (n > 20) and, as expected, also caused the loss of 22G-RNAs targeting the transgene (Figures 4F and 4G). Instead of exhibiting 22G-RNA accumulation only within the gfp portion of the transgene, as is normally seen in RNAe-silenced transgenes (e.g., gfp::cdk-1) (Shirayama et al., 2012), we observed RDE-3-dependent 22G-RNA accumulation throughout the codon-altered region of oma-1 in this strain (Figures 4F and 4G). Thus, the RNAe machinery appears to target both the oma-1 and gfp sequences equally to drive silencing of this transgene.

A possible explanation for the above findings was that altering the codons of oma-1 rendered the mRNA less optimal for translation (Presnyak et al., 2015), perhaps predisposing it to silencing. We therefore generated a second transgene in which we shifted the oma-1 ORF by removing one nucleotide after the ATG, replacing 11 stop codons with sense codons, and adding a nucleotide just before the gfp sequence to maintain the gfp reading frame. This +1-frame transgene encodes a novel protein with non-optimal codons but with a nucleotide sequence nearly identical to oma-1. Although the +1-frame transgene did not produce a visible GFP signal, the level of mRNA was similar to that produced by the unaltered oma-1::cDNA::gfp transgene (Figure 4D). Moreover, we found that this +1-frame transgene was able to transactivate a silent gfp transgene (Figure 4E). Taken together, these findings suggest that the nucleotide sequence of the oma-1 coding region, and not, for example, the additional production of OMA-1 protein, confers resistance to RNAe-mediated silencing.

A plausible model for how oma-1::gfp acquires RNAe activity is that pre-existing CSR-1 22G-RNAs (templated from the endogenous oma-1 mRNA) spread to the nearby gfp sequences via local recruitment of RdRP to the oma-1 region of the oma-1::gfp mRNA. Altering the codons of oma-1 could thus abolish CSR-1-dependent protection of the oma-1 region, essentially making the entire gene into a “foreign” transgene, and thus prone to piRNA targeting. To test this idea, we made a strain that completely lacks all genomic sequences complementary to the oma-1::gfp transgene. We used genome editing to remove the entire oma-1 gene including its 3’ UTR. Although small regions of oma-2 have nucleotide sequences similar to oma-1, none of these regions produces CSR-1 22G-RNAs that match oma-1. Deletion of endogenous oma-1 should thus completely remove all portions of the transcriptome that could provide transitive CSR-1 22G-RNAs complementary to the oma-1::gfp transgene. Surprisingly, upon de novo introduction into this strain, we found that the oma-1::gfp transgene (n = 2) continued to exhibit robust RNAa activity in crosses with a silent gfp::cdk-1 strain that also contains the complete genomic deletion of oma-1 (Figure 4H). These findings suggest that the resistance of oma-1::gfp to RNAs reflects intrinsic properties of the transgene coding sequences.

Premature Nonsense Mutations Do Not Prevent RNAa and RNAe

In many eukaryotes, mRNAs containing a premature stop codon undergo degradation via a conserved pathway known as nonsense-mediated decay (NMD) (Baker and Parker, 2004; Chang et al., 2007). Since both RNAs and RNAe utilize mRNAs as templates for secondary sRNA production, we reasoned that NMD silencing could suppress template levels necessary for these Argonaute-mediated pathways. Alternatively, since many endogenous RNAe targets are pseudo genes that contain numerous stop codons (Gu et al., 2009), NMD might act to predispose mRNAs to silencing.

To explore the relationship between NMD and Argonaute pathways, we generated transgenes containing premature stop codons and assayed their ability to respond to RNAa and RNAe surveillance mechanisms. To explore the consequences of premature stop codons on an RNAe-inducing transgene, we constructed the silencing-prone gfp::cdk-1 transgene with a stop codon in the second exon of gfp (n > 3) (Figure 5A). We first confirmed that this transgene was sensitive to NMD. As expected, we found that expression of gfp (Y74stop)::cdk-1 was not detected when introduced into wild-type animals, and expression was not restored in rde-3 mutants (data not shown). When crossed to an expressed cdk-1::gfp transgene, we found that all of the
pression by NMD (Lin, 2003). We therefore introduced this potently induce silencing (n > 20, Figure 5A).

(R106stop) that was previously shown to be subject to sup-

(ayama et al., 2012)( Shen et al., 2018), further expanding their
tolerate mismatched pairing (Ashe et al., 2012; Bagijn et al.,

animals (Aravin et al., 2006; Girard et al., 2006; Grivna et al.,

through the body of the mRNA to promote mRNA expression.

of silencing depends on opposing pathways that also act

inclusive of the ORF can modulate gene expression. The degree

We have shown that piRNA targeting within the body of an mRNA

sensitive to the inclusion of nonsense mutations within the

ORFs of the inducing alleles, suggesting that these pathways

scan mRNAs independently and likely upstream of the NMD pathway.

DISCUSSION

Regulation of mRNA Expression through the Entire Transcrip

We have shown that piRNA targeting within the body of an mRNA

inclusive of the ORF can modulate gene expression. The degree

of silencing depends on opposing pathways that also act

through the body of the mRNA to promote mRNA expression.

piRNAs are abundantly expressed in the germlines of diverse an-

imals (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Ruby et al., 2006), and like miRNAs, piRNAs tolerate mismatched pairing (Ashe et al., 2012; Bagijn et al.,

2012; Goh et al., 2015; Lee et al., 2012; Reuter et al., 2011; Shir-

ayama et al., 2012) (Shen et al., 2018), further expanding their

potential target space. Thus, the vast repertoire of piRNAs in

metazoan germlines provides a wealth of potential post-tran-

scriptional regulatory capacity. Our findings suggest that piRNAs

can access and regulate mRNAs throughout the mature tran-

script, including the ORF, and that even the coding regions of

mRNAs are free to sample regulatory inputs from piRNAs over
evolutionary time. Moreover, because piRNAs are expressed

as independent genes, they are also free to evolve independen-
dantly, unconstrained by the coding requirements of their target

How might the cell achieve mRNA regulation of the type

observed here? One attractive model is suggested by the prom-

inent localization of the Argonaute machinery (including PRG-1,

CSR-1, and WAGO-1) within nuage (P granules) in the perinu-
clear zone (Batista et al., 2008; Claycomb et al., 2009; Gu et al., 2009). If nascent mRNAs are subject to scanning as they

emerge from the nucleus prior to ribosome access, then the

entire transcript including the ORF should be accessible to

piRNA targeting. Increased piRNA targeting could reduce escape of the transcript and promote retention within a zone

where WAGO 22G-RNA amplification occurs. Conversely, fac-
tors that promote escape from this regulatory zone will promote

mRNA expression in cis (see model, Figure 6).

A paradox of the RNAa and RNAe systems is that RNAa, an

activating mechanism, involves a cleavage-competent Argona-

ute, CSR-1, while RNAe, a silencing mechanism, involves

several cleavage-incompetent Argonautes. WAGO Argonautes,

unlike CSR-1, lack the conserved residues that coordinate Mg

within their RNase H domains (Yigit et al., 2006). The key to this

paradox is likely explained by the importance of amplification in

the RNAe silencing mechanism. WAGO targeting leads to massive

amplification of 22G-RNAs on WAGO targets, and only after 22G-

RNA levels rise does silencing occur. 22G-RNA amplification is

thought to occur within a subdomain of the P granule called the

mutator focus (Phillips et al., 2012). By not cleaving their targets,

WAGO Argonautes may ensure the preservation of the template

RNAs needed to amplify and propagate the silencing signal. The

paradox of CSR-1 as a protective, and yet cleavage-competent,

Argonaute could in turn be explained if CSR-1 preferentially rec-

ognizes and cleaves the RdRP templates involved in WAGO-

22G-RNA amplification, while avoiding the cleavage of the intact

mRNAs corresponding to CSR-1 targets. This could explain

how CSR-1 actively, and rapidly, disarms the silencing machinery

when WAGOs become directed toward CSR-1 protected mRNA.

Balance and Tuning of Argonaute-Mediated Regulation

The above discussion suggests that Argonaute pathways are

amplified and strongly transitive. But can they be balanced and

tuned? In this study, we have explored transgene interactions

in which the expression states of two complementary genes

remain balanced, one OFF and one ON. This bimodal behavior

suggests that the ultimate expression state of an mRNA is
determined by mechanisms that sum activating and silencing

signals, in cis, along the mRNA. For a gene that inherently resists

piRNA silencing, we found that artificially increasing piRNA tar-

geting led to reductions in expression that were stably inherited,
demonstrating that piRNA can lower germline gene expression without completely silencing their targets. Importantly, the levels of WAGO or Piwi targeting are not the sole determinant of a gene’s expression state. Rather, our findings also suggest that positive influences from unknown factors that act in cis on the mRNA sequences can override or partially counteract these silencing signals to preserve mRNA expression.

Our search for regions of a gene that confer resistance to silencing identified the ORF as necessary and sufficient. Analysis of these regions reveals no obvious features—e.g., absence of piRNA targeting or high levels of CSR-1 targeting—that could explain the reproducible ability of transgenes containing these sequences to activate silent transgenes. These findings suggest that, while CSR-1 mediates the transitive aspect of RNAa, other, unknown features also underlie the ability of such transgenes to acquire or recruit CSR-1 targeting to their mRNA products.

Indeed, recent studies demonstrate that the entire germline transcriptome is under piRNA surveillance and that C. elegans piRNAs have physiologically important effects on the expression of at least one endogenous mRNA, xol-1 (Shen et al., 2018; Tang et al., 2018). The xol-1 mRNA behaves like the transgenes we describe here. We have shown that adding one new piRNA that targets oma-1::gfp reduced its expression but that adding three was necessary to drive complete silencing. Similarly, multiple piRNAs target xol-1 cooperatively, inducing a large accumulation of WAGO 22G-RNAs (Shen et al., 2018). Moreover, xol-1 silencing, like that of oma-1::gfp, requires continuous PRG-1 activity. Hundreds of other worm genes appear to be actively silenced by the piRNA pathway. Thus, future studies may identify additional physiologically important functions to the regulatory mechanisms revealed in these transgene studies. In summary, our findings suggest a temporal aspect to Argonaute-mediated regulation (likely prior to mRNA translation) and support the notion that C. elegans germline mRNAs, inclusive of their coding regions, undergo a period of comprehensive piRNA scanning during mRNA transit through perinuclear nuage.

Figure 6. Model. piRNAs Scan mRNAs within Perinuclear Nuage prior to Translation Initiation
Schematic showing mRNPs exiting the nucleus through P granules. Binding factors and possibly covalent modification put in place during mRNA transcription and processing influence sensitivity to piRNA scanning. Three Argonaute systems within P granules are shown engaging the entire transcript including the ORF. The balance of positive and negative signals along an mRNA determines the fraction of molecules that escape destruction and gain access to the translation machinery.

EXPERIMENTAL PROCEDURES

Strains and Genetics
The C. elegans strains used in this study (Table S1) were derived from the Bristol N2 strain and cultured as described (Brenner, 1974). Transgenic strains were made using the MosSCI heat shock protocol combined with ivermectin selection as described (Frokjær-Jensen et al., 2014; Shirayama et al., 2012).

Generation of Transgenic Strains by CRISPR/CAS9
21ux-1(anti-gfp), 21uv-11498(anti-oma1), and 21uv-2675(anti-oma1) were generated by a co-CRISPR strategy using unc-22 sgRNA as a co-injection marker to enrich CRISPR/CAS9-mediated genome editing events (Kim et al., 2014). The vector expressing rol-6 (su1098), a dominant allele conferring a roller phenotype, was used as a co-injection marker.

Small RNA Cloning and Deep Sequencing
Total RNA was extracted from adult worms using Trizol (Molecular Research Center). Small RNAs were further enriched using MirVana Kit (Thermo Fisher Scientific). Samples from wild-type and mutant were pretreated with a homemade 5′ polyphosphatase and were ligated to the 3′ adaptor linker 1 (5′-rAppAGATCGGAAGAGCACACGTCTGAACTCCAGTCA/3′ddC/3′0) us-

mg T4 RNA ligase 2 (M0351S, NEB). Subsequently, the 5′ adaptor (rArCrUrUrCrUrUrCrUrUrCrArCrGrArCrGrArCrUrUrCrUrUrCrUrUrCrUrUrCrGrArUrCrU) was ligated using T4 RNA ligase 2 (M0351S, NEB). Subsequently, the 5′ adaptor (rArCrUrUrCrUrUrCrUrUrCrUrUrCrArCrGrArCrGrArCrUrUrCrUrUrCrUrUrCrUrUrCrGrArUrCrU) was ligated using T4 RNA ligase 1 (M0204S, NEB). The ligated products were con- verbalized to cDNA using SuperScript III (Thermo Fisher Scientific). Libraries were amplified by PCR and sequenced using the HiSeq or Miseq systems (Illumina) at the UMass Medical School Deep Sequencing facility.

Data Analysis
Small RNA sequencing results were analyzed using an established pipeline (Gu et al., 2012). Briefly, sequencing reads were sorted according to barcode sequences, and both 5′ and 3′ adaptor sequences were removed using a custom Perl script. Reads starting with G and 21 to 23 nt in length were mapped to WormBase WS215 allowing at most two mismatches and normalized to non-structural RNA reads. To account for differences in sequencing depth among samples, each read was normalized to total number of reads. Normalized counts were visualized in the UCSC genome browser. All scripts are available upon request.

Quantitative RT-PCR
The reverse transcription (RT) was performed using randomized primers by SuperScript III (Thermo Fisher Scientific). RT reaction was conducted in triplicate reactions. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Gene-specific primers were used to amplify gfp transcript. These transcripts were normalized to primers specific to csr-1 transcripts. All statistical analysis was performed in Microsoft Excel. Error bars in the graph represent the standard deviation (SD).
Western Blotting Analysis
Cell lysate was prepared from synchronized population of L4 larvae and gravid adults. 50 µg lysate was loaded onto the precast polyacrylamide gel (Thermo Fisher Scientific), subjected to electrophoresis, and transferred onto the polyvinylidene difluoride membrane (Bio-Rad) with Trans-Blot Turbo Transfer System (Bio-Rad). The primary antibodies used were polyclonal anti-GLH-4 and polyclonal rabbit anti-GFP (GenScript, A01704). The secondary antibody is goat-anti-rabbit HRP (Abcam).

Imaging and Microscopy
Transgenic worms expressing GFP were mounted on RITE-ON glass slides (Bekton Dickinson) in the presence of 0.2 mM levamisole. Epi-fluorescence and differential interference contrast (DIC) microscopy were performed using an Axioplan2 Microscope (Zeiss). Images were processed using Axiovision software (Zeiss).

DATA AND SOFTWARE AVAILABILITY
The accession number for the small RNA data generated in this paper is NCBI: DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures, Supplemental Experimental Procedures, and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.02.009.

ACKNOWLEDGMENTS
We thank members of the Mello and Ambros labs for discussions and suggestions; D. Conte Jr. for critical review of the manuscript; W. Gu for providing the reagents for library preparation and the technical support; S. Vergara for technical support on northern blot; and E. Kittler and the UMass Deep Sequencing Core for Illumina sequencing. Some of the strains were provided by the Caenorhabditis Genetics Center supported by the NIH (P40 OD010440). W.T. is supported by the Hope Funds for Cancer Research Postdoctoral Fellowship (HFCR-15-06-03) and the NIH Pathway to Independence Award (GM124460). H.-C.L. is supported by the NIH Pathway to Independence Award (GM108866). The work was supported by a Hughes Medical Institute International Student Research Fellowship (G9107986) to M. Seth and NIH grants HD078253 to Z.W. and GM058800 and HD078253 to C.C.M. C.C.M. is a Howard Hughes Medical Institute Investigator.

AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
The authors declare no competing interests.

Received: September 11, 2017
Revised: January 26, 2018
Accepted: February 1, 2018
Published: February 15, 2018

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