Article

EGO-1, a *C. elegans* RdRP, Modulates Gene Expression via Production of mRNA-Templated Short Antisense RNAs

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Summary

Background: The development of the germline in *Caenorhabditis elegans* is a complex process involving the regulation of thousands of genes in a coordinated manner. Several genes required for small RNA biogenesis and function are among those required for the proper organization of the germline. EGO-1 is a putative RNA-directed RNA polymerase (RdRP) that is required for multiple aspects of *C. elegans* germline development and efficient RNA interference (RNAi) of germline-expressed genes. RdRPs have been proposed to act through a variety of mechanisms, including the posttranscriptional targeting of specific mRNAs, as well as through a direct interaction with chromatin. Despite extensive investigation, the molecular role of EGO-1 has remained enigmatic.
Results: Here we use high-throughput small RNA and messenger RNA sequencing to investigate EGO-1 function. We found that EGO-1 is required to produce a distinct pool of small RNAs antisense to a number of germline-expressed mRNAs through several developmental stages. These potential mRNA targets fall into distinct classes, including genes required for kinetochore and nuclear pore assembly, histone-modifying activities, and centromeric proteins. We also found several RNAi-related genes to be targets of EGO-1. Finally, we show a strong association between the loss of small RNAs and the rise of mRNA levels in *ego-1(−)* animals.
Conclusions: Our data support the conclusion that EGO-1 produces triphosphorylated small RNAs derived from mRNA templates and that these small RNAs modulate gene expression through the targeting of their cognate mRNAs.

Introduction

Several processes, including RNA interference (RNAi) in *C. elegans*, quelling in *Neurospora*, and posttranscriptional gene silencing in plants, have been shown to be related in both their method of action and their required components [1–3]. A fundamental link between these processes is their use of double-stranded RNA (dsRNA) and/or short interfering RNAs (21–25 nt) as key effector molecules and reaction intermediates [4–7].

In addition to these exogenous silencing mechanisms, there is growing evidence for endogenous small RNAs playing a critical role in development. In *C. elegans*, one well-defined small RNA-based regulatory pathway involves a two-step process for endogenous small RNA production. The primary step in this pathway involves the RNA-directed RNA polymerase (RdRP) RRF-3, the dsRNA-binding protein RDE-4, and the

RNase-III-like enzyme DCR-1 in producing a small initial pool of 26 nt, 5′-monophosphorylated small RNAs [8–11]. The secondary step involves the activity of the RdRP RRF-1 in the soma and involves the production of a much larger pool of approximately 22 nt, 5′-triphosphorylated small RNAs, which are essential for effective gene silencing [8–11].

The properties of small RNAs produced during exogenously triggered RNAi in *C. elegans* provide further evidence for an amplification step in small RNA production and function. Initial RNAi experiments with exogenous long dsRNA (>500 nt) showed that as little as a few molecules per cell led to silencing [5]. Additionally, small RNAs have been found to map both upstream and downstream of the initial dsRNA trigger [12, 13]. Finally, the majority of small RNAs present during an RNAi response is triphosphorylated at their 5′ ends and maps antisense to exonic sequences [13]. Small RNAs that carry triphosphorylated 5′ termini are likely the direct products of RdRP initiation (unlike DCR-1 cleavage products that have been shown to carry 5′ monophosphates).

EGO-1: A Germline RdRP
The RdRP EGO-1 is related to RRF-1 [14] and is a candidate to perform the secondary step in small RNA production in the germline. *ego-1(−)* animals are inefficient in exogenous RNAi against germline-expressed genes [14]. Moreover, EGO-1 is important in multiple aspects of germline development [14–17].

ego-1 appears to belong to a functional group of at least four loci with germline roles. Mutations in *ego-1*, *csr-1*, *drh-3*, and *ekl-1* all exhibit defects in heterochromatin assembly on unpaired DNA [17, 18] and are enhancers of lethality in *ksr-1*, an important component of the Ras-ERK signaling pathway [19]. CSR-1 is a member of the large *C. elegans* Argonaute family and has been shown to bind small RNAs [20–22]. CSR-1 is also required for transgene-mediated cosuppression [23] and efficient RNAi of germline-expressed genes [24]. DRH-3 is a DEAH/D-box helicase that associates with the Dicer protein DCR-1 and is also required for efficient RNAi in the germline and for the production of endogenous small RNAs [25]. EKL-1 is a Tudor domain-containing protein that has also been shown to be required for efficient germline RNAi, transgene silencing, and cosuppression [19]. Antibodies to these four proteins have been reported to stain structures associated with DAPI-stained chromosomes undergoing mitosis in fixed *C. elegans* embryos. However, in adult germline tissue, in which EGO-1 function is essential, EGO-1 staining is not evident on chromosomes. Rather, antibody staining of adult germline tissue suggests that EGO-1 associates with perinuclear RNA-containing granules [21].

EGO-1 Function
Although much is known about the physical morphology of *ego-1(−)* mutant animals, little is known about the molecular phenotype in these animals. This has left several questions unanswered: What is EGO-1 doing to promote the proper development of the germline, and, specifically, what genes are being misregulated in the absence of EGO-1?

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A

L3 ego−1(om84) sRNA

L3 ego−1(+) sRNA

B

L4 ego−1(om84) sRNA

L4 ego−1(+) sRNA

C

AD ego−1(om84) sRNA

AD ego−1(+) sRNA

ALL GENES
sRNA DOWN in ego−1(om84)
sRNA UP in ego−1(om84)
ratio ego−1(om84)/ego−1(+) mRNA
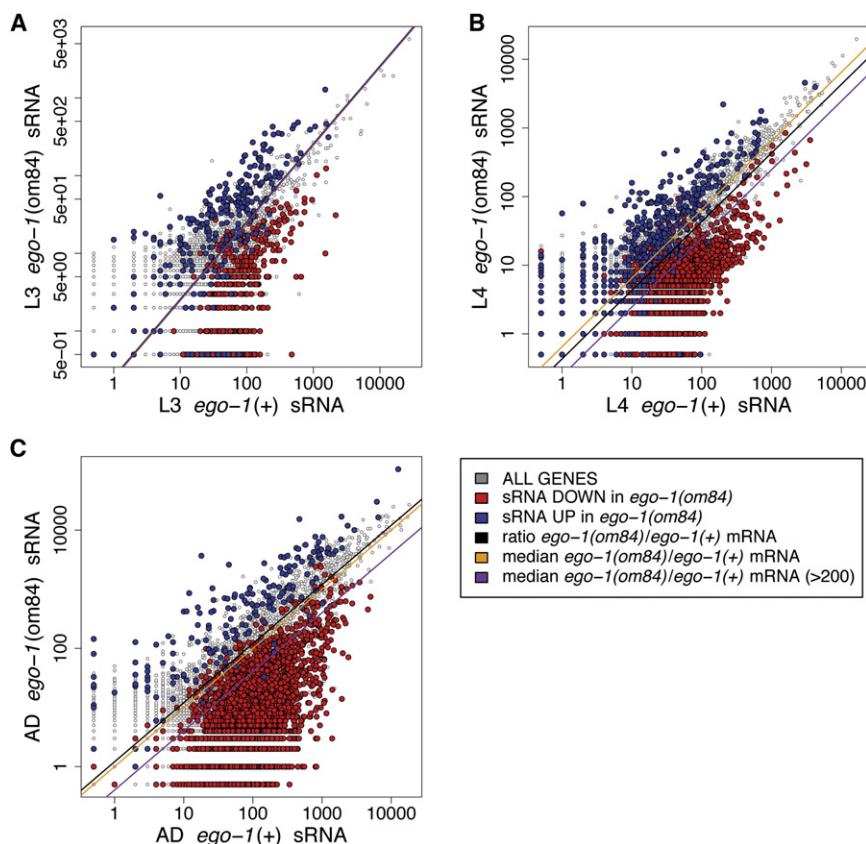median ego−1(om84)/ego−1(+) mRNA
median ego−1(om84)/ego−1(+) mRNA (>200)

Figure 1. Stage-by-Stage Small RNA Replicates

Scatter plots depict a gene-by-gene comparison of small RNA abundance. Each plot compares data from *ego-1*(+) and *ego-1*(om84) animals at a single developmental stage. Each point represents a single gene, with an (x, y) coordinate defined by raw (nonnormalized) counts of corresponding antisense small RNAs from the indicated libraries. A black line shows the ratio of total counts in each pair of samples giving the expected parity between samples. Two median lines are shown for each data set: (1) median of the ratio of *ego-1*(om84)/*ego-1*(+) on a gene-by-gene basis, and (2) median of the ratio of *ego-1* (om84)/*ego-1*(+) on a gene-by-gene basis using only those genes for which the sum of *ego-1* (om84) and *ego-1*(+) counts is greater than 200. Shown are genes whose small RNA abundance increases (blue) or decreases (red) 3-fold (2-fold in L3) in *ego-1*(om84) (posterior probability ratio [PPR] < 0.005) in an independent replicate of the specified stage.
(A) L3.
(B) L4.
(C) Adult.

Determination of Putative EGO-1 Targets

Comparing count numbers on a gene-by-gene basis for libraries from different stages, we found hundreds of genes that show a significant difference in small RNA abundance at each stage. These differences are highly reproducible in comparing distinct independent data sets (Figure 1). In addition, we observed a large number of genes that show a constant difference in multiple stages (Figure 2). Based on previous data showing that the majority of EGO-1 expression occurs in L4 and adult animals [14, 16], we initially focused our analysis on the genes affected in these two developmental stages.

We found that 437 gene loci had at least 3-fold fewer small RNAs (posterior probability ratio [PPR] < 0.005) in both L4 and adult *ego-1*(om84) samples. We also found that 20 genes showed at least 3-fold more small RNAs (PPR < 0.005) in both L4 and adult *ego-1*(om84) samples (Figure 2D1; Table S1).

As a working hypothesis, we consider small RNAs that are lost in *ego-1*(om84) animals to potentially represent direct products of EGO-1. RNAs that are enriched in *ego-1*(om84) would be unlikely to derive from synthesis by EGO-1 (these could show enrichment due to the loss of true EGO-1 products). We also note the alternative hypothesis that some or all of both sets of loci could be indirect targets or could be affected through downstream consequences of developmental defects in *ego-1* mutant animals.

Despite the low levels of EGO-1 expression in L3 animals [16], we did find overlap between our candidate targets in L4 and adult samples with our L3 samples. We found that 60 genes that showed significantly fewer small RNAs in L4 and adult *ego-1*(om84) samples also showed at least 2-fold fewer small RNAs in L3 samples (p = ~1.30×10^{-14}). We found that eight genes that showed significantly more small RNAs in L4 and adult *ego-1*(om84) samples also showed at least 2-fold more small RNAs in L3 samples (p = ~1.64×10^{-19}) (Table S2; Figure 2D2; null hypothesis: no relationship between L3 targets and L4/adult targets).

Results

To better understand the role of EGO-1 in germline development and RNAi, we used an RNA-seq approach to track small RNA and messenger RNA levels from *ego-1*(om84) and control animals. To avoid complications of variable embryonic development, we used strains in which the temperature-sensitive allele *fem-1(hc17ts)* had been introduced into both mutant and control animals. Using *fem-1(hc17ts)*, all animals were feminized via growth at the restrictive temperature of 25°C (all library data are summarized in Table S1 available online).

EGO-1-Dependent Small RNA Production

To determine what populations of small RNAs are dependent on EGO-1 activity, we sequenced small RNA libraries from staged L3, L4, and adult animals. Previous work has indicated that a majority of RdRP-produced small RNAs are triphosphorylated at their 5′ ends [9, 13]. In order to capture all putative EGO-1-produced small RNAs, we first applied a 5′-phosphate-independent protocol for sequencing [8].

We sequenced multiple independent libraries of 5′-phosphate-independent small RNAs from L3 (two independent libraries), L4 (two independent libraries), and adult (three independent libraries)-staged experimental and control animals. Data from replicates at each stage were examined independently. To test the reproducibility of library preparation and sequencing, we compared count numbers between replicate samples on a gene-by-gene basis (Figure 1; Figure S1). These comparisons show a strong reproducibility of differential expression results from our library preparation and sequencing.

To further characterize the temporal characteristics of EGO-1 action, we examined individual stage targets on each of the other stages. In Figure 3, we have plotted the aggregate small RNA counts from *ego-1(om84) fem-1(hc17)* and *fem-1 (hc17)* for L3, L4, and adult small RNA libraries. For each stage (L3, L4, adult), we have highlighted putative EGO-1 targets from each of the three stages individually. For example, when we examine aggregate L3 small RNA abundance, we have highlighted those genes that were found to be significantly different in L3 (Figure 2A1), L4 (Figure 2B1), and adult (Figure 2C1). Similar comparisons were performed for aggregate L4 (Figure 2B) and adult (Figure 2C) samples. In analysis of these results, EGO-1 targets as a whole from any individual stage exhibit a strong parallel trend in their small RNA abundance through the other stages examined.

EGO-1-Dependent Small RNAs Derive from Germline-Expressed mRNAs

In our analysis of EGO-1-dependent small RNAs, we found that those specific RNAs that are missing in *ego-1(−)* animals are significantly more likely to be antisense to an mRNA molecule (p = ~1.05×10^{-12}). We also found that EGO-1 produces small RNAs that span the length of the target gene (Figure 3; Figure S3). This suggests two features of the EGO-1-dependent small RNA populations. First, EGO-1 activity yields small RNAs antisense to a set of transcribed loci, and second, these small RNAs generally span each targeted transcript.

EGO-1 could conceivably copy genomic DNA, initial (unprocessed) transcripts, or processed mRNA. Our data are most consistent with the use of processed mRNA. First, we observe very few small RNAs that map to introns. Second, we found a substantial number of EGO-1-dependent small RNAs that span exon-exon junctions (Figure 4). Finally, we found small RNAs that span the 3′ untranslated region and polyA junctions [26, 27] for several L4/adult targets (Figure S4); no such small RNAs were found in *ego-1(−)* mutant populations.

Small RNA regulation can be used to extinguish residual expression of transcripts that are normally absent in a tissue [28] or to modulate expression of genes following their intended time of action [29]. Examination of putative EGO-1 targets inferred from our sequencing supports a focus for EGO-1 action on genes that have been active in the oogonial germline. First, examining a list of strongly oogonial-enriched mRNAs [30] shows a significant overrepresentation of these genes among putative EGO-1 targets. We found that both putative L3/L4/adult (p = ~2.16×10^{-8}) and putative L4/adult (p = ~9.99×10^{-16}) targets were significantly enriched for germline-expressed genes [30]. Second, we observe a relative underrepresentation of EGO-1 targets on the X chromosome (Figure 5; Figure S5). The paucity of EGO-1 targets on the X chromosome would be consistent with a set of germline targets, given that the X chromosome is known to be substantially de-enriched for germline-expressed genes [30].

Analysis of Putative EGO-1 Targets

We found several functionally related sets of genes to be prominent among EGO-1 targets, including genes involved in (1) RNAi-related processes [24, 31–35] (L4/AD: p = ~2.19×10^{-8}), (2) Ras-ERK signaling [19] (L4/AD: p = ~4.07×10^{-7}), (3) nuclear pore assembly/function [16] (L4/AD: p = ~9.62×10^{-9}), (4) histone methyltransferases [17, 18] (L4/AD: p = ~1.01×10^{-6}), and (5) chromosome segregation [21] (L4/AD: p = ~3.26×10^{-9}). These results are of particular interest given the phenotypic effects of EGO-1 on each of these cellular and/ or biochemical processes.

Additionally, we found several unexpected groups of genes to be overrepresented among EGO-1 targets. Genes required for nonsense-mediated mRNA decay (L4/AD: p = ~1.98×10^{-5}), as well as a number of predicted ubiquitin ligases (L4/AD: p = ~5.08×10^{-4}) and serine/threonine phosphatases (L4/AD: p = ~8.69×10^{-4}), are highly enriched in our data sets. Groups of putative EGO-1 targets are summarized in Table 1.

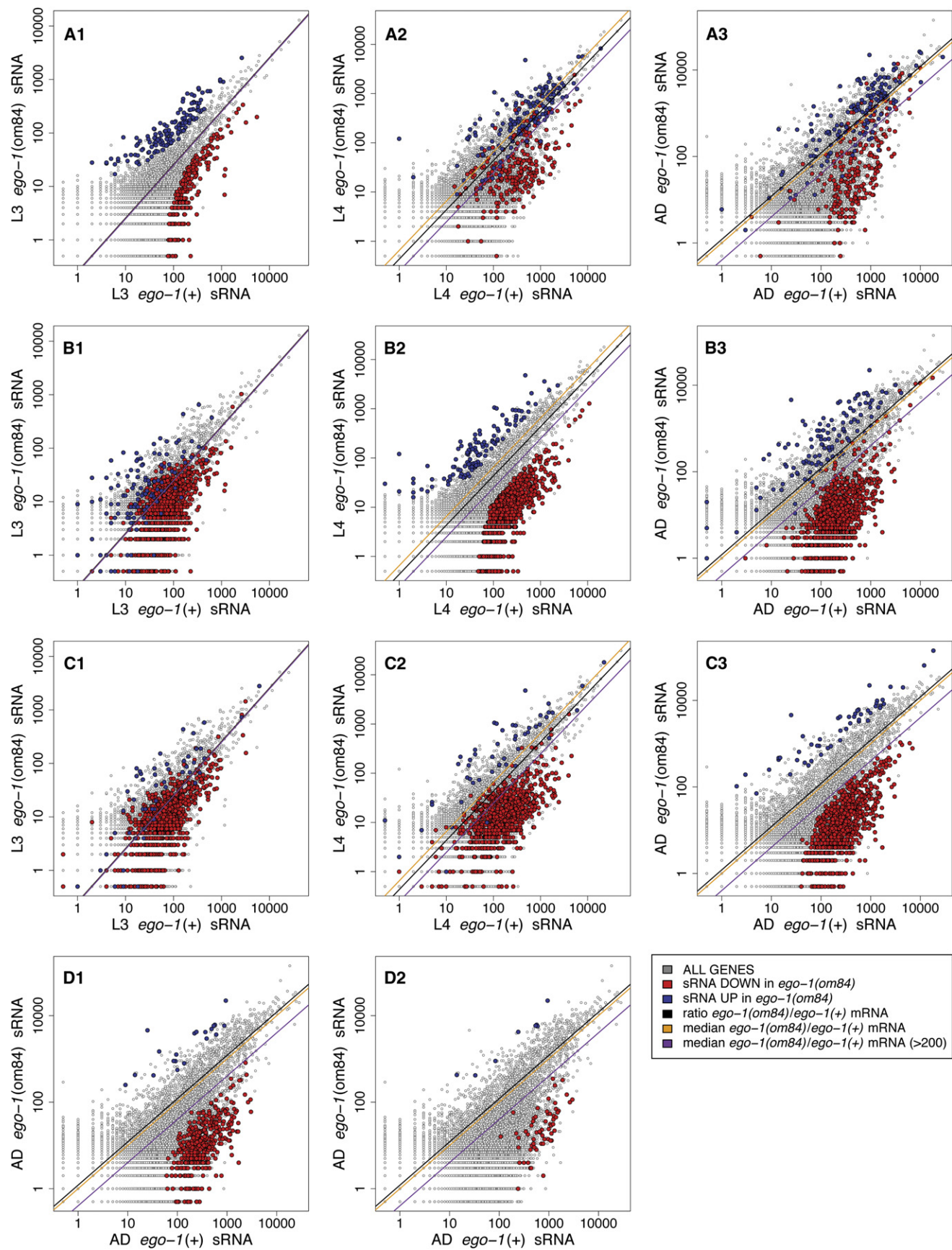### Genetic Requirements for EGO-1-Dependent Small RNA Production

To determine what other RNAi factors might be required for the production of EGO-1-dependent small RNAs, we analyzed a series of other small RNA libraries from several sources. We analyzed 5′-phosphate-independent libraries from four RNAi-related mutants (*rde-1(ne300)*, *rde-4(ne299)*, *rrf-1 (pk1417) glp-4(bn2)*, and MAGO [WM126]; Figures S2A–S2H). In addition, we also analyzed data from CSR-1 protein complexes and several other RNAi-related mutants (*csr-1 (tm892)*, *ego-1(om97)*, *drh-3(ne4253)*, *ekl-1(tm1599)*, *rrf-3 (pk1426)*, *rrf-1(pk1417)*, *eri-1(mg366)*, *dcr-1(mg375)*, and *ergo-1(gg098)*; Figure S2IAB). Some libraries were from previously published work (including *ego-1(om97)* adults) [9, 21, 34], whereas others were from ongoing studies of the exogenous RNAi response in this laboratory (Julia Pak, personal communication). Although different conditions and staging were used in some of these cases (L4 versus adult and RNAi versus standard growth media), we have been able in each case to compare each mutant to an isogenic control under identical conditions.

As validation of the analysis, we found that our putative direct EGO-1 targets are also lost in a single *ego-1(om97)* library reported by Claycomb et al. when matched to a comparable *ego-1(+)* library from the same investigators [21] (p = ~5.13×10^{-28}; Figure S2K). *ego-1(om84)* and *ego-1(om97)* both contain stop codons early in the coding region, and EGO-1 protein is not detected in extracts from either mutant [16].

The critical RNAi Argonaute factor RDE-1 has been shown to function in the response to foreign dsRNA and may recruit RdRPs to small RNA/mRNA complexes [2, 36, 37]. We saw no requirement for RDE-1 in the production of small RNAs derived from putative EGO-1 target loci (Figures S2A and S2E).

Interestingly, we did see a requirement for the dsRNA binding protein RDE-4 [36, 37] for maximal accumulation of putative EGO-1 products from both L4/adult and L3/L4/adult targets (p = ~0.044 and p = ~6.22×10^{-3}, respectively; Figures S2B and S2F).

Additionally, we found putative products from EGO-1 targets to be decreased in *rrf-1(pk1417) glp-4(bn2)* animals (Figures S2C and S2G). Although putative products from EGO-1 targets are decreased in *rrf-1(pk1417) glp-4(bn2)*, they do not appear to be decreased in *rrf-1(pk1417)* (Figures S2U and S2Z). This result is not surprising; given that RRF-1 expression is highest in somatic tissues [12, 14], whereas EGO-1 expression is highest in germline tissues [14], we might predict that these two proteins would have nonoverlapping targets (with limited effects in *rrf-1* mutants). The loss of EGO-1 target small RNAs in *rrf-1(pk1417) glp-4(bn2)* animals would be consistent with the loss of germline tissue in these animals.

Legend:
- ALL GENES
- sRNA DOWN in *ego-1(om84)*
- sRNA UP in *ego-1(om84)*
- ratio *ego-1(om84)/ego-1(+)* mRNA
- median *ego-1(om84)/ego-1(+)* mRNA
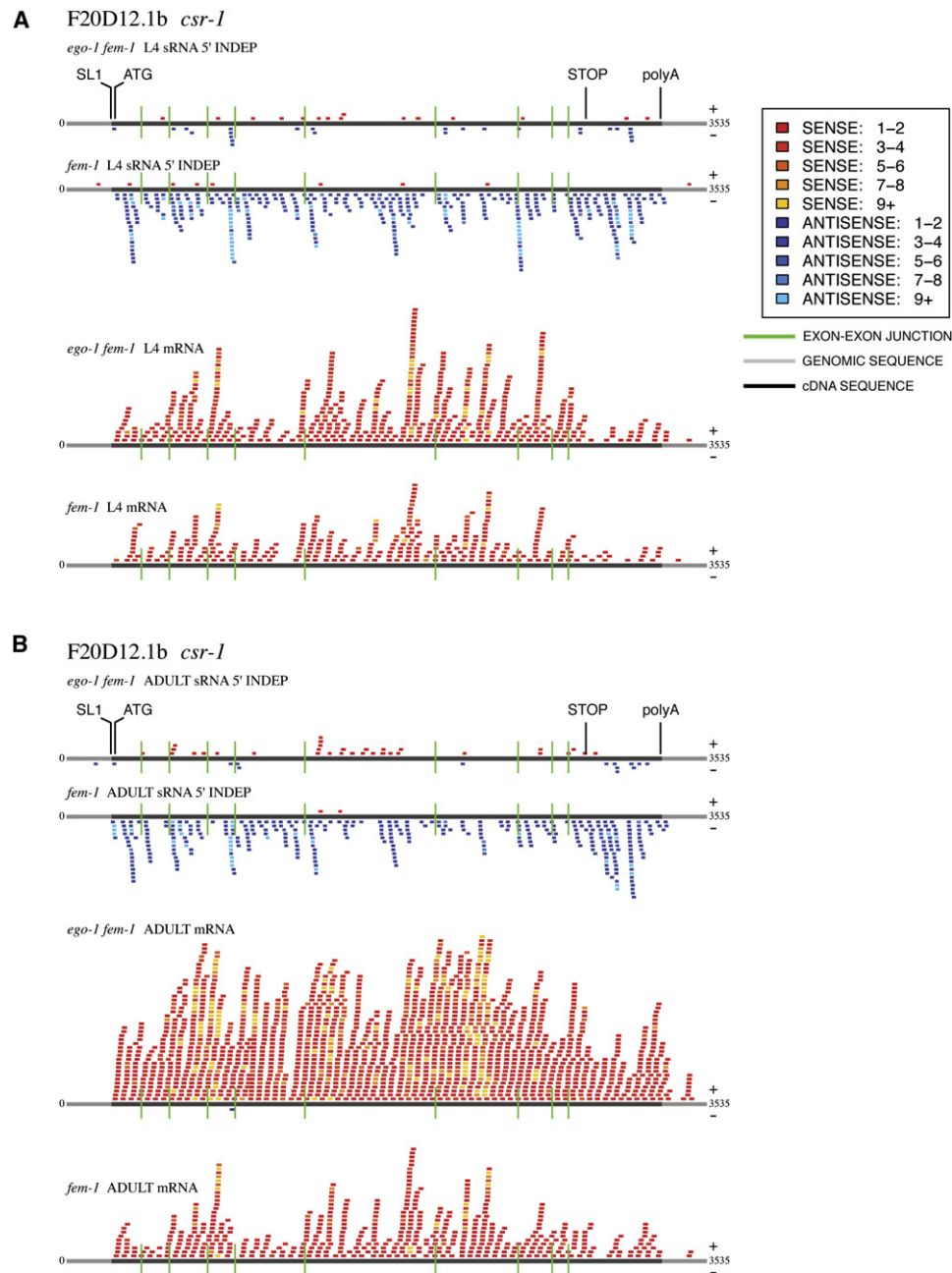- median *ego-1(om84)/ego-1(+)* mRNA (>200)

Figure 3. Small RNA and mRNA Sequencing Reads of *csr-1*

Sense (shades of red) and antisense (shades of blue) reads of 5′-independent small RNAs and mRNA (experimental: *ego-1(om84) fem-1(hc17)*; control: *fem-1(hc17)*) mapped to *csr-1* (spliced). Green lines represent exon-exon junctions.

(A) L4-staged animals.

(B) Adult-staged animals.

We did not find a significant shift in the multiple Argonaute mutant WM126 (Figures S2D and S2H) or in mutants lacking the RNAi components ERI-1, ERGO-1, or RRF-3 (Figures S2S, S2T, S2W–S2Y, and S2AB). We did, however, find that EGO-1 targets became a significantly larger part of the small RNA pool in the helicase domain-specific allele *dcr-1(mg375)*

Figure 2. Stage-by-Stage Comparison of EGO-1 Targets

(A1–D2) Scatter plots depict a gene-by-gene comparison of small RNA abundance. Plotted: aggregated small RNA abundance for multiple replicates of L3, L4, or adult animals (gray). Highlighted: potential EGO-1 targets in L3 (A1–A3), L4 (B1–B3), adult (C1–C3), L4/AD (D1), L3/L4/AD (D2). (Potential EGO-1 targets are defined as those genes whose antisense small RNA abundance changes at least 2-fold [L3] or 3-fold [L4 and adult] and who have a PPR < 0.005 in multiple replicates.) Two median lines are shown for each data set: (1) median of the ratio of *ego-1(om84)/ego-1(+)* on a gene-by-gene basis, and (2) median of the ratio of *ego-1(om84)/ego-1(+)* on a gene-by-gene basis using only those genes for which the sum of *ego-1(om84)* and *ego-1(+)* counts is greater than 200. A black line shows the total ratio of total counts in each pair of samples giving the expected parity between samples.
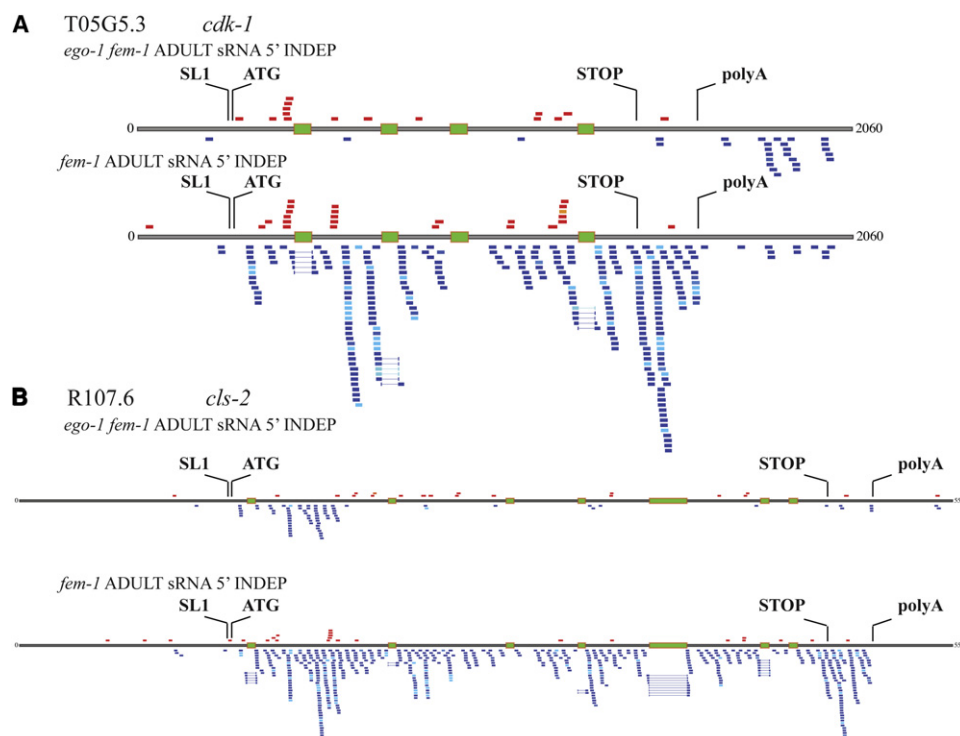
Figure 4. Small RNA and mRNA Sequencing Reads of *cdk-1* and *cls-2*

Sense (shades of red) and antisense (shades of blue) reads of 5′-independent small RNAs (experimental: adult *ego-1(om84) fem-1(hc17)*; control: adult *fem-1(hc17)*). Green bars represent introns.

(A) *cdk-1*.

(B) *cls-2*.

(Figures S2V and S2AA). The latter (modest) effect may represent the loss of helicase-dependent small RNAs from other pathways.

As we described previously, EGO-1 has been functionally linked to CSR-1, DRH-3, and EKL-1 in a number of cellular processes. CSR-1 is a *C. elegans* Argonaute that physically interacts with small RNAs and is required for proper chromosome segregation [20, 21, 24]. DRH-3 interacts with DCR-1 and is required for germline development and efficient germline RNAi [25]. EKL-1 is also required for efficient RNAi, as well as transgene silencing and cosuppression in the germline [19]. Using our statistical criteria, it appears that none of these three factors is required in EGO-1-dependent small RNA accumulation (Figures S2L, S2M, S2Q, and S2R). However, in examining the data, the number of antisense small RNA counts in these *drh-3(ne4253)* and *ekl-1(tm1599)* data sets is quite low. These low counts create more extreme median values and may cause a skewed perspective of the data.

Although CSR-1 may not be involved in EGO-1 target small RNA production, we see a dramatic shift in EGO-1 targets in RNAs selected by CSR-1 immunoprecipitation [21]. We found that 295 of 437 L4/adult EGO-1 targets are enriched in CSR-1 complexes (p = ~2.05×10^{-13}; Figure S2I). Included among these 295 genes are several RNAi-related genes (*csr-1*, *tsn-1*, *mut-14*, *mut-16*), centromere and kinetochore components (*hcp-1*, *hcp-3*, *hcp-4*, *klp-19*), and Ras-related genes (*cdc-42*, *lin-9*, *rho-1*, *trr-1*). The strong overlap between CSR-1-bound small RNAs and EGO-1 targets suggests that although CSR-1 may not be involved in the production of EGO-1-dependent

small RNAs, it may be important in the function of these small RNAs.

Analysis of 5′-Monophosphate-Enriched Small RNA Populations

To further probe the physical structure of EGO-1-dependent small RNAs, we sequenced a pool of small RNAs using an alternative procedure that relies on a 5′ monophosphate (thus capturing primarily 5′-P small RNAs). This analysis was performed on two biological replicates of adult experimental (*ego-1(om84) fem-1(hc17)*) and adult control (*fem-1(hc17)*) animals. Each library was amplified in two independent reactions and sequenced (for a total of four sequenced groups each from experimental and control animals). These libraries, as well as adult 5′-P-independent libraries, were aligned to the *C. elegans* genome and transcriptome, and small RNAs that matched perfectly antisense to genes were counted. In addition to alignment of these small RNAs, all libraries were also aligned to *C. elegans* microRNAs and 21U RNAs.

When we compared 5′-P-dependent and 5′-P-independent libraries, we found that small RNAs antisense to genes are >100-fold enriched relative to microRNAs in 5′-P-independent sequencing. We observed a similar fold enrichment in small RNAs antisense to genes in 21U RNA data sets. We found that small RNAs antisense to genes are almost 74-fold enriched relative to 21U RNAs in 5′-P-independent sequencing. EGO-1 targets show a similar level of fold enrichment.

This dramatic fold enrichment strongly suggests that the vast majority of small RNAs captured in our 5′-P-independent capture procedure are not 5′ monophosphorylated. Based
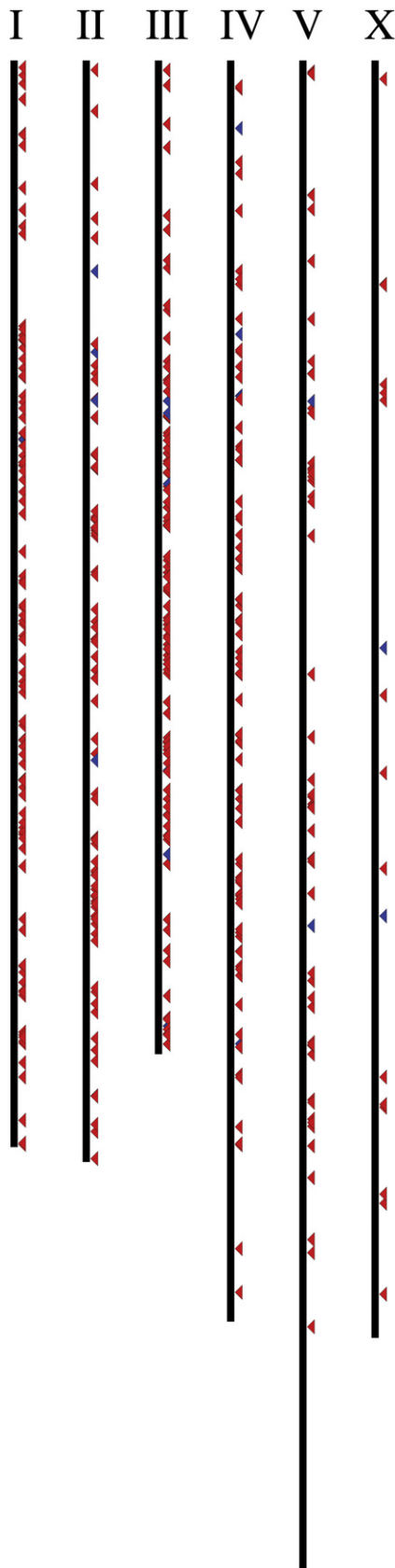
Figure 5. Chromosomal Positions of EGO-1 Targets

L4 and adult. Genes whose small RNA abundance is decreased 3-fold (red, down) or increased 3-fold (blue, up) in *ego-1[om84]* relative to *ego-1[+]* with

Table 1. Putative EGO-1 Target Groups

Gene Group	L3/L4/AD	L4/AD	Examples
RNAi	33.4×	10.1×	CSR-1, TSN-1, C16C10.3, F58G1.1, T23D8.7
Methyltransferases	21.9×	9.0×	SET-21(H3K9), SET-25 (H3K9), SET-33(H3K9), SET-2(H3K4), SET-4 (H3K20)
Nuclear pore complex	–	15.2×	LMN-1, NPP-7(Nup98), NPP-11(Nup98), NPP-5 (Nup107), NPP-8(Nup155), NPP-3(Nup205)
Centromere/kinetochore	61.1×	18.9×	HCP-1(CENP-F), HCP-3 (CENP-A), HCP-4(CENP-C), HCP-6, KNL-1, KNL-3, ROD-1
Kinesins	32.1×	13.2×	KLC-1, KLP-7, KLP-15, KLP-16, KLP-18, ZEN-4
NMD	–	15.7×	SMG-2, SMG-3, PAA-1, RUVB-1, RUVB-2
Ser/Thr Phosphatases	–	5.4×	GSP-1, PPH-6, SUR-6, PPTR-1
Ubiquitin ligases	–	3.1×	BRC-1, CUL-3, NCL-1, UBC-9, UBC-13, WWP-1
RAS-related	–	4.8×	CCR-4, LET-60, LIN-15b, RAB-8, RHO-1, TRR-1
P granule	5.0×	7.3×	CAR-1, GLD-3, GLH-4, PRG-1, SNR-1, XYG-9
Transposon silencing	51.7×	14.2×	MUT-14, MUT-16, PRG-1, K07C5.4

Gene groups and fold enrichment (for the group) in L3/L4/AD and L4/AD *ego-1* small RNA data sets. For fold enrichment: RNAi: L3/L4/AD, $p = 5.40 \times 10^{-7}$; L4/AD, $p = 2.19 \times 10^{-8}$. Methyltransferases: L3/L4/AD, $p = 3.76 \times 10^{-4}$; L4/AD, $p = 1.01 \times 10^{-6}$. Nuclear pore complex: L4/AD, $p = 9.62 \times 10^{-9}$. Centromere/kinetochore: L3/L4/AD, $p = 8.64 \times 10^{-7}$; L4/AD, $p = 3.26 \times 10^{-9}$. Kinesins: L3/L4/AD, $p = 1.24 \times 10^{-4}$; L4/AD, $p = 4.43 \times 10^{-8}$. Nonsense-mediated mRNA decay (NMD): L4/AD, $p = 1.98 \times 10^{-5}$. Ser/Thr phosphatases: L4/AD, $p = 8.69 \times 10^{-4}$. Ubiquitin ligases: L4/AD, $p = 5.08 \times 10^{-4}$. Ras-related: L4/AD, $p = 4.07 \times 10^{-7}$. P granule: L3/L4/AD, $p = 0.020$; L4/AD, $p = 3.87 \times 10^{-14}$. Transposon silencing: L3/L4/AD, $p = 6.40 \times 10^{-8}$; L4/AD, $p = 4.10 \times 10^{-9}$.

upon the two 5′-end capture procedures and several pieces of published data [9, 13, 38], it seems likely that the majority of these non-5′-monophosphorylated RNAs are in fact triphosphorylated and are products of RdRPs. We found that small RNAs antisense to L4/adult EGO-1 target loci are approximately 64-fold enriched to microRNAs and 43-fold enriched to 21U RNAs in 5′-P-independent sequencing and are therefore likely triphosphorylated products of EGO-1 RdRP function.

Analysis of Messenger RNA Levels

To determine whether changes in small RNA abundance correlate with mRNA levels, we performed mRNA tag sequencing on L3, L4, and adult animals. We isolated poly(A)⁺ RNA and fragmented this RNA to the 100–200 nucleotide range. Using

PPR < 0.005. Chromosome size and gene positions are drawn to scale. Chr. I: 104 genes down, 1 gene up. Chr. II: 82 genes down, 5 genes up. Chr. III: 101 genes down, 6 genes up. Chr. IV: 79 genes down, 4 genes up. Chr. V: 56 genes down, 2 genes up. Chr. X: 15 genes down, 2 genes up. We found only 15 putative targets (437 total) to be on the X chromosome ($p = \sim 1.49 \times 10^{-13}$). Correcting for multiple hypotheses, we found that EGO-1 targets are significantly underrepresented on the X chromosome.
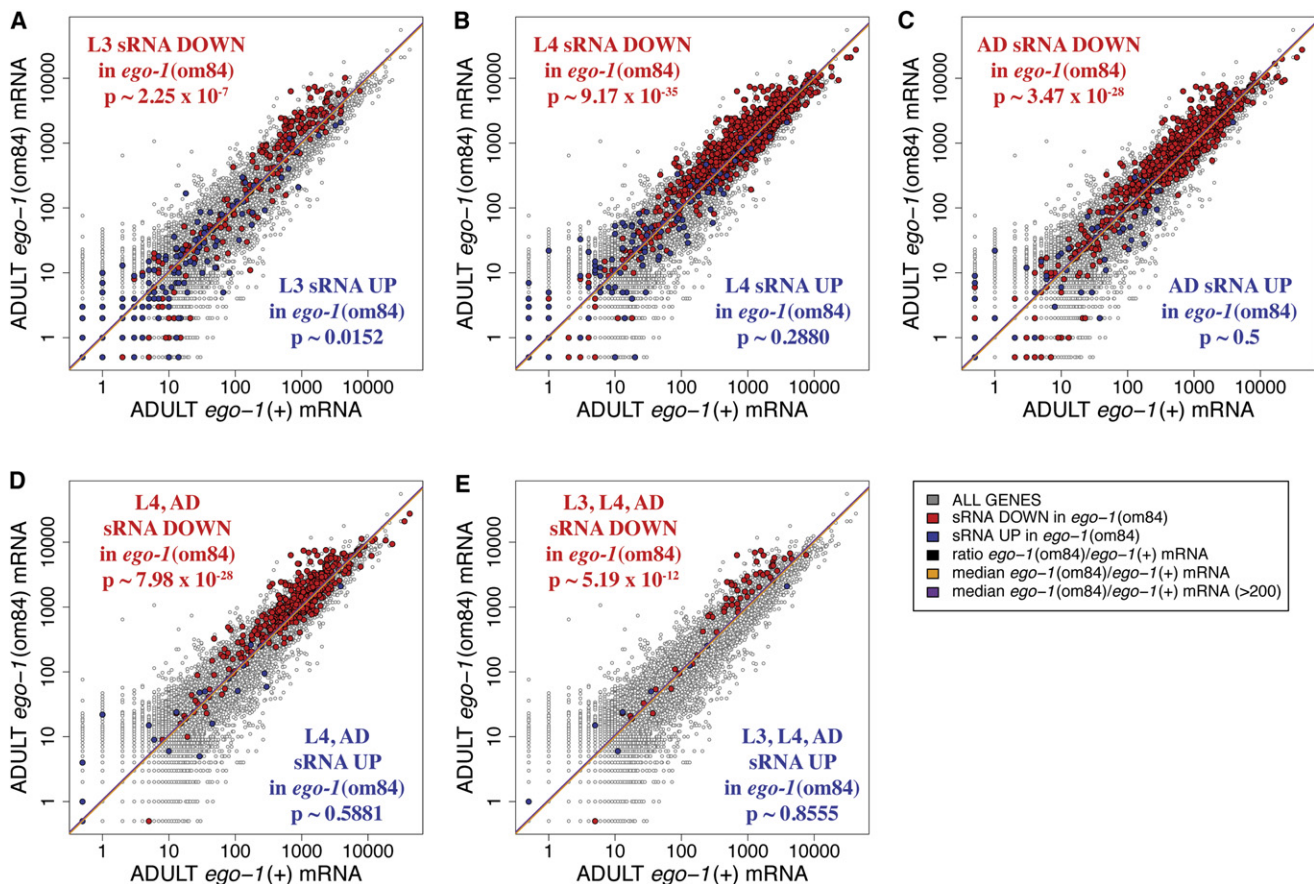
Figure 6. Summary of Adult mRNA Abundance

Scatter plots depict a gene-by-gene comparison of mRNA abundance in staged adult animals (gray), and highlighted are genes whose small RNA (sRNA) abundance is down in *ego-1(om84)* (red) and genes whose sRNA abundance is up in *ego-1(om84)* (blue). Because the key question for these data was the existence of an inverse relationship between sRNA and mRNA abundance, a central aspect of the data is the median values of the ratio of mRNA levels in *ego-1(om84)*/mRNA levels in *ego-1(+)*. Two median lines are shown for each data set: (1) median of the ratio of *ego-1(om84)*/*ego-1(+)* on a gene-by-gene basis, and (2) median of the ratio of *ego-1(om84)*/*ego-1(+)* on a gene-by-gene basis using only those genes for which the sum of *ego-1(om84)* and *ego-1(+)* counts is greater than 200. A black line shows the total ratio of total counts in each pair of samples giving the expected parity between samples. Gene counts summaries are as follows.

(A) Changes in L3 sRNA abundance: 243 genes down 2-fold (red, p = ~2.25 × 10^{-7}) and 145 genes up 2-fold (blue, p = ~0.0152).
(B) Changes in L4 sRNA abundance: 1066 genes down 3-fold (red, p = ~9.17 × 10^{-35}) and 115 genes up 3-fold (blue, p = ~0.2880).
(C) Changes in adult sRNA abundance: 880 genes down 3-fold (red, p = ~3.47 × 10^{-28}) and 51 genes up 3-fold (blue, p = ~0.5).
(D) Changes in L4 and adult sRNA abundance: 437 genes down 3-fold (red, p = ~7.98 × 10^{-28}) and 20 genes up 3-fold (blue, p = ~0.5881).
(E) Changes in L3, L4, and adult sRNA abundance: 60 genes down 2-fold in L3 and 3-fold in L4 and adult (red, p = ~5.19 × 10^{-12}) and 8 genes up 2-fold in L3 and 3-fold in L4 and adult (blue, p = ~0.8555).

sense reads, we calculated fold changes and posterior probability ratios for these mRNA libraries.

Using a 2-fold change with PPR < 0.005, we found that 132 of 19,231 distinct gene models tested differ in abundance between experimental (*ego-1(om84) fem-1(hc17)*) and control (*fem-1(hc17)*) in the L3 stage (47 up in *ego-1(om84)*, 85 down in *ego-1(om84)*). We found that 119 genes differ in abundance in L4 (25 up, 94 down) and 113 genes differ in abundance in adult (56 up, 57 down).

Comparison of EGO-1 Small RNA Targets with mRNA Abundance

We performed a sensitive evaluation of reciprocity in mRNA and small RNA changes in *ego-1* mutant animals through a quantitative comparison of all genes. A sensitive comparison is important because it might be expected that some targets would show only modest differences in mRNA levels. Of the

437 genes whose 5′-triphosphorylated small RNA levels decrease in an *ego-1(om84)* background in L4 and adult, we found that 288 show increased mRNA levels in L4 samples (p = ~1.40 × 10^{-11}; Figure S6D) and 330 show increased mRNA levels in adult samples (p = ~7.98 × 10^{-28}; Figure 6D). Of the 60 genes whose 5′-triphosphorylated small RNA levels decrease in an *ego-1(om84)* background in L3, L4, and adult, we found that 49 show increased mRNA levels in L4 (p = ~3.78 × 10^{-7}; Figure S6E) and 55 show increased mRNA levels in adult (p = ~5.19 × 10^{-12}; Figure 6E). Of the 20 genes whose 5′-triphosphorylated small RNA levels increase in an *ego-1(om84)* background in L4 and adult animals, we saw no significant shift in mRNA levels at any stage (Figure 6; Figure S6).

By further focusing on mRNA abundance in L4 and adult stages, we found 16 genes (ten up, six down) that have significant abundance differences in both stages. Of these 16 genes,

Table 2. Summary of Genes with Significant Change in Small RNA and mRNA Abundance

		L4				Adult			
		sRNA		mRNA		sRNA		mRNA	
		FC	P	FC	P	FC	P	FC	P
mes-6	polycomb-like	0.15–0.17	$<10^{-15}$	2.18–2.46	$<10^{-15}$–10^{-3}	0.01–0.04	$<10^{-15}$–10^{-6}	2.14–4.16	$<10^{-15}$
F01G4.4	unknown function	0.03–0.14	$<10^{-15}$	3.27–5.31	$<10^{-15}$	0.01–0.06	$<10^{-15}$	2.81–3.15	$<10^{-15}$
csr-1	Argonaute	0.02–0.04	$<10^{-15}$	2.09–2.73	$<10^{-15}$	0.002–0.02	$<10^{-15}$	2.56–5.61	$<10^{-15}$
klp-7	kinesin	0.03–0.14	$<10^{-15}$	3.11–4.35	$<10^{-15}$	0.01–0.09	$<10^{-15}$	6.12–8.27	$<10^{-15}$
T12E12.2	heterchromatin-associated protein HP1	0.07–0.08	$<10^{-15}$	2.26–4.33	$<10^{-15}$–10^{-3}	0.02–0.17	$<10^{-15}$	2.64–2.97	$<10^{-15}$
T12E12.3	unknown function	0.12–0.15	$<10^{-15}$	2.94–6.29	$<10^{-15}$	0.01–0.10	$<10^{-15}$	5.38–9.90	$<10^{-15}$

Fold-change (FC) and posterior probability (P) ratios for six genes whose small RNA abundance differs by at least 3-fold and whose mRNA abundance differs by at least 2-fold in L4 and adult animals (*ego-1(om84) fem-1(hc17)* versus *fem-1(hc17)*). Calculations for fold-change and P ratios are found in the Experimental Procedures.

six also show significant change in 5′-P-independent small RNA levels at both the L4 and adult stages (Table 2). For each of these six genes, we see an inverse relationship between 5′-P-independent small RNAs (up in *ego-1(om84)*) and mRNA abundance (down in *ego-1(om84)*). These six genes represent several classes that may be important in understanding the *ego-1(om84)* phenotype, including chromosome segregation and RNAi (*csr-1*, Figure 4), cell division (*klp-7*, Figures S2A and S2B), germline development (*mes-6*, Figures S2C and S2D), and heterochromatin formation (T12E12.2, Figures S2E and S2F).

These data support a reciprocal relationship between small RNA populations and consequences at the mRNA level. In particular, loci that yield large numbers of EGO-1-dependent small RNAs show substantially greater expression (relief of inhibition) in *ego-1* mutant animals.

Discussion

We have described a large class of genes that behave as targets of EGO-1 in both L4 and adult animals, with our results showing that small RNAs antisense to these genes are substantially reduced in *ego-1(−)* animals. These EGO-1 targets include histone methyltransferases, kinetochore and centromeric components, P granule components, RNAi-related factors, and Ras-related genes. Additional classes of EGO-1 targets include a number of regulatory factors, including Ser/Thr phosphatases and ubiquitin ligases, that are not currently associated with specific aspects of the observed *ego-1* mutant phenotype.

In addition to these 437 targets, it appears very likely that there are also additional EGO-1 targets. We found a number of interesting genes that fall just outside of our rather strict fold-change and/or statistical criteria. Included among this group are *ego-1* itself, *drh-3*, and *ekl-1*, as well as critical RNAi components RRF-3, ERI-1, and DCR-1 (Table S3). The number and function of putative EGO-1 targets point to EGO-1 having a central role in the modulation of a number of cellular processes, including endogenous RNAi.

One possible mode of action of EGO-1 and EGO-1 products would be in the downregulation of target mRNAs. Although the mechanisms by which triphosphorylated small RNAs lead to mRNA turnover remain unclear, these RNAs are associated with downregulation of target mRNAs during RNAi and in the endogenous RRF-3 pathway [9–11, 13]. We found that EGO-1 target mRNAs as a whole show a significant shift in their expression in *ego-1(om84)* animals. The regulatory shifts that are observed in mRNA-seq data are not all or nothing. Rather, it appears that the RdRP system is used to achieve a globally modest regulation of a large class of mRNA targets.

Does the EGO-1 target family comprise the genes in which expression is valuable in specific stages of germline development but that are not needed or damaging at subsequent stages? This idea would closely correspond to another *C. elegans* RdRP, RRF-3, which downregulates a set of target mRNAs during spermatogenesis [8, 9]. In both spermatogenesis and oogenesis, a defined and intricate developmental pathway requires a carefully choreographed engagement of specific protein factors at specific times [39–41].

Many mRNAs expressed in oogenesis may encode factors that are only needed in transient stages, with much of the regulation occurring posttranscriptionally [42]. One mechanism to effect a temporal "bump" in expression is to have a specific negative regulator turn on precisely at the time that expression of the target needs to be extinguished. Because making new specific regulatory machinery for each stage of oogenesis may be evolutionarily expensive, RdRPs may provide a modular mechanism that allows any mRNA to acquire a signal that will serve as a negative regulator that only activates after the mRNA has accumulated.

The identification of large numbers of target loci highlights the questions of both identity and number of physiologically critical targets. The dramatic germline defects and unconditional sterility of *ego-1* mutants could be caused by a specific misregulated target locus or a combined misregulation of many such loci, with the complexity of both the target pool and the observed phenotype certainly consistent with a contribution of many targets to aspects of the phenotype. Critical analysis of individual functional contributions of specific EGO-1 targets to organized germline development should emerge as biochemical and/or genetic analysis of individual target loci proceeds, as a more detailed view of the subcellular *ego-1(−)* mutant phenotype emerges, and as tools for examining multigenic influences on the critical subcellular phenotypes are developed.

RdRP-based modulation of gene expression has been characterized in fungi, plants, and animals [8, 9, 14, 43–45]. Although not present in vertebrates, the eukaryotic RdRP protein superfamily that includes EGO-1 is present in vertebrate ancestors [46]. Observed RdRP activities in higher systems reserve the possibility of similar mechanisms modulating development in these systems [47, 48].

References

1. Cogoni, C., and Macino, G. (1999). Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. Nature *399*, 166–169.

2. Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., and Mello, C.C. (1999). The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. Cell *99*, 123–132.

3. Vaucheret, H., Béclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Mourrain, P., Palauqui, J.C., and Vernhettes, S. (1998). Transgene-induced gene silencing in plants. Plant J. *16*, 651–659.

4. Ratcliff, F., Harrison, B.D., and Baulcombe, D.C. (1997). A similarity between viral defense and gene silencing in plants. Science *276*, 1558–1560.

5. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature *391*, 806–811.

6. Elbashir, S.M., Lendeckel, W., and Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. *15*, 188–200.

7. Hamilton, A.J., and Baulcombe, D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science *286*, 950–952.

8. Gent, J.I., Schvarzstein, M., Villeneuve, A.M., Gu, S.G., Jantsch, V., Fire, A.Z., and Baudrimont, A. (2009). A *Caenorhabditis elegans* RNA-directed RNA polymerase in sperm development and endogenous RNA interference. Genetics *183*, 1297–1314.

9. Gent, J.I., Lamm, A.T., Pavelec, D.M., Maniar, J.M., Parameswaran, P., Tao, L., Kennedy, S., and Fire, A.Z. (2010). Distinct phases of siRNA synthesis in an endogenous RNAi pathway in *C. elegans* soma. Mol. Cell *37*, 679–689.

10. Han, T., Manoharan, A.P., Harkins, T.T., Bouffard, P., Fitzpatrick, C., Chu, D.S., Thierry-Mieg, D., Thierry-Mieg, J., and Kim, J.K. (2009). 26G endo-siRNAs regulate spermatogenic and zygotic gene expression in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA *106*, 18674–18679.

11. Vasale, J.J., Gu, W., Thivierge, C., Batista, P.J., Claycomb, J.M., Youngman, E.M., Duchaine, T.F., Mello, C.C., and Conte, D., Jr. (2010). Sequential rounds of RNA-dependent RNA transcription drive endogenous small-RNA biogenesis in the ERGO-1/Argonaute pathway. Proc. Natl. Acad. Sci. USA *107*, 3582–3587.

12. Sijen, T., Fleenor, J., Simmer, F., Thijssen, K.L., Parrish, S., Timmons, L., Plasterk, R.H., and Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. Cell *107*, 465–476.

13. Pak, J., and Fire, A. (2007). Distinct populations of primary and secondary effectors during RNAi in *C. elegans*. Science *315*, 241–244.

14. Smardon, A., Spoerke, J.M., Stacey, S.C., Klein, M.E., Mackin, N., and Maine, E.M. (2000). EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. Curr. Biol. *10*, 169–178.

15. Qiao, L., Lissemore, J.L., Shu, P., Smardon, A., Gelber, M.B., and Maine, E.M. (1995). Enhancers of *glp-1*, a gene required for cell-signaling in

Caenorhabditis elegans, define a set of genes required for germline development. Genetics *141*, 551–569.

16. Vought, V.E., Ohmachi, M., Lee, M.H., and Maine, E.M. (2005). EGO-1, a putative RNA-directed RNA polymerase, promotes germline proliferation in parallel with GLP-1/notch signaling and regulates the spatial organization of nuclear pore complexes and germline P granules in *Caenorhabditis elegans*. Genetics *170*, 1121–1132.

17. Maine, E.M., Hauth, J., Ratliff, T., Vought, V.E., She, X., and Kelly, W.G. (2005). EGO-1, a putative RNA-dependent RNA polymerase, is required for heterochromatin assembly on unpaired dna during *C. elegans* meiosis. Curr. Biol. *15*, 1972–1978.

18. She, X., Xu, X., Fedotov, A., Kelly, W.G., and Maine, E.M. (2009). Regulation of heterochromatin assembly on unpaired chromosomes during *Caenorhabditis elegans* meiosis by components of a small RNA-mediated pathway. PLoS Genet. *5*, e1000624.

19. Rocheleau, C.E., Cullison, K., Huang, K., Bernstein, Y., Spilker, A.C., and Sundaram, M.V. (2008). The *Caenorhabditis elegans ekl* (enhancer of *ksr-1* lethality) genes include putative components of a germline small RNA pathway. Genetics *178*, 1431–1443.

20. Aoki, K., Moriguchi, H., Yoshioka, T., Okawa, K., and Tabara, H. (2007). *In vitro* analyses of the production and activity of secondary small interfering RNAs in *C. elegans*. EMBO J. *26*, 5007–5019.

21. Claycomb, J.M., Batista, P.J., Pang, K.M., Gu, W., Vasale, J.J., van Wolfswinkel, J.C., Chaves, D.A., Shirayama, M., Mitani, S., Ketting, R.F., et al. (2009). The Argonaute CSR-1 and its 22G-RNA cofactors are required for holocentric chromosome segregation. Cell *139*, 123–134.

22. Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G., and Mello, C.C. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. Cell *106*, 23–34.

23. Robert, V.J.P., Sijen, T., van Wolfswinkel, J., and Plasterk, R.H.A. (2005). Chromatin and RNAi factors protect the *C. elegans* germline against repetitive sequences. Genes Dev. *19*, 782–787.

24. Yigit, E., Batista, P.J., Bei, Y., Pang, K.M., Chen, C.C., Tolia, N.H., Joshua-Tor, L., Mitani, S., Simard, M.J., and Mello, C.C. (2006). Analysis of the *C. elegans* Argonaute family reveals that distinct Argonautes act sequentially during RNAi. Cell *127*, 747–757.

25. Duchaine, T.F., Wohlschlegel, J.A., Kennedy, S., Bei, Y., Conte, D., Jr., Pang, K., Brownell, D.R., Harding, S., Mitani, S., Ruvkun, G., et al. (2006). Functional proteomics reveals the biochemical niche of *C. elegans* DCR-1 in multiple small-RNA-mediated pathways. Cell *124*, 343–354.

26. Mangone, M., Manoharan, A.P., Thierry-Mieg, D., Thierry-Mieg, J., Han, T., Mackowiak, S.D., Mis, E., Zegar, C., Gutwein, M.R., Khivansara, V., et al. (2010). The landscape of C. elegans 3′UTRs. Science *329*, 432–435.

27. Lamm, A.T., Stadler, M.R., Zhang, H., Gent, J.I., and Fire, A.Z. (2011). Multimodal RNA-seq using single-strand, double-strand, and CircLigase-based capture yields a refined and extended description of the C. elegans transcriptome. Genome Res. *21*, 265–275.

28. Stark, A., Brennecke, J., Bushati, N., Russell, R.B., and Cohen, S.M. (2005). Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3′UTR evolution. Cell *123*, 1133–1146.

29. Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. Cell *75*, 855–862.

30. Reinke, V., Gil, I.S., Ward, S., and Kazmer, K. (2004). Genome-wide germline-enriched and sex-biased expression profiles in *Caenorhabditis elegans*. Development *131*, 311–323.

31. Tijsterman, M., Ketting, R.F., Okihara, K.L., Sijen, T., and Plasterk, R.H.A. (2002). RNA helicase MUT-14-dependent gene silencing triggered in *C. elegans* by short antisense RNAs. Science *295*, 694–697.

32. Sijen, T., and Plasterk, R.H.A. (2003). Transposon silencing in the *Caenorhabditis elegans* germ line by natural RNAi. Nature *426*, 310–314.

33. Wang, G., and Reinke, V. (2008). A *C. elegans* Piwi, PRG-1, regulates 21U-RNAs during spermatogenesis. Curr. Biol. *18*, 861–867.

34. Gu, W., Shirayama, M., Conte, D., Jr., Vasale, J., Batista, P.J., Claycomb, J.M., Moresco, J.J., Youngman, E.M., Keys, J., Stoltz, M.J., et al. (2009). Distinct argonaute-mediated 22G-RNA pathways direct genome surveillance in the *C. elegans* germline. Mol. Cell *36*, 231–244.

35. Steiner, F.A., Okihara, K.L., Hoogstrate, S.W., Sijen, T., and Ketting, R.F. (2009). RDE-1 slicer activity is required only for passenger-strand

cleavage during RNAi in *Caenorhabditis elegans*. Nat. Struct. Mol. Biol. *16*, 207–211.

36. Parrish, S., and Fire, A. (2001). Distinct roles for RDE-1 and RDE-4 during RNA interference in *Caenorhabditis elegans*. RNA *7*, 1397–1402.

37. Tabara, H., Yigit, E., Siomi, H., and Mello, C.C. (2002). The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in *C. elegans*. Cell *109*, 861–871.

38. Lau, N.C., Lim, L.P., Weinstein, E.G., and Bartel, D.P. (2001). An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. Science *294*, 858–862.

39. Kimble, J., and Crittenden, S.L. (2005). Germline proliferation and its control. In WormBook, The *C. elegans* Research Community, ed. doi/10.1895/wormbook.1.13.1. http://www.wormbook.org.

40. Greenstein, D. (2005). Control of oocyte meiotic maturation and fertilization. In WormBook, The *C. elegans* Research Community, ed. doi/10.1895/wormbook.1.53.1. http://www.wormbook.org.

41. Strome, S. (2005). Specification of the germ line. In WormBook, The *C. elegans* Research Community, ed. doi/10.1895/wormbook.1.9.1. http://www.wormbook.org.

42. Racher, H., and Hansen, D. (2010). Translational control in the *C. elegans* hermaphrodite germ line. Genome *53*, 83–102.

43. Dougherty, W.G., and Parks, T.D. (1995). Transgenes and gene suppression: telling us something new? Curr. Opin. Cell Biol. *7*, 399–405.

44. Schiebel, W., Pélissier, T., Riedel, L., Thalmeir, S., Schiebel, R., Kempe, D., Lottspeich, F., Sänger, H.L., and Wassenegger, M. (1998). Isolation of an RNA-directed RNA polymerase-specific cDNA clone from tomato. Plant Cell *10*, 2087–2101.

45. Shiu, P.K.T., and Metzenberg, R.L. (2002). Meiotic silencing by unpaired DNA: properties, regulation and suppression. Genetics *161*, 1483–1495.

46. Zong, J., Yao, X., Yin, J., Zhang, D., and Ma, H. (2009). Evolution of the RNA-dependent RNA polymerase (RdRP) genes: duplications and possible losses before and after the divergence of major eukaryotic groups. Gene *447*, 29–39.

47. Chao, M. (2007). RNA recombination in hepatitis delta virus: implications regarding the abilities of mammalian RNA polymerases. Virus Res. *127*, 208–215.

48. Lehmann, E., Brueckner, F., and Cramer, P. (2007). Molecular basis of RNA-dependent RNA polymerase II activity. Nature *450*, 445–449.