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Analysis of the *C. elegans* Argonaute Family Reveals that Distinct Argonautes Act Sequentially during RNAi

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SUMMARY

Argonaute (AGO) proteins interact with small RNAs to mediate gene silencing. *C. elegans* contains 27 AGO genes, raising the question of what roles these genes play in RNAi and related gene-silencing pathways. Here we describe 31 deletion alleles representing all of the previously uncharacterized AGO genes. Analysis of single- and multiple-AGO mutant strains reveals functions in several pathways, including (1) chromosome segregation, (2) fertility, and (3) at least two separate steps in the RNAi pathway. We show that RDE-1 interacts with trigger-derived sense and antisense RNAs to initiate RNAi, while several other AGO proteins interact with amplified siRNAs to mediate downstream silencing. Overexpression of downstream AGOs enhances silencing, suggesting that these proteins are limiting for RNAi. Interestingly, these AGO proteins lack key residues required for mRNA cleavage. Our findings support a two-step model for RNAi, in which functionally and structurally distinct AGOs act sequentially to direct gene silencing.

INTRODUCTION

The term RNA interference (RNAi) was initially coined to describe a gene-silencing mechanism induced by the experimental introduction of RNA into the nematode *C. elegans* (Rocheleau et al., 1997; Fire et al., 1998). Subsequent work in numerous organisms revealed that key steps in the RNAi pathway are shared by a diverse and truly remarkable set of endogenous gene regulatory mechanisms (for review see Zamore and Haley, 2005). Among others, these include mechanisms that downregulate endogenous genes and restrain the expression of selfish or exogenous genetic material, mechanisms that direct transcriptional gene silencing and alter chromatin to promote kinetochore function and chromosome segregation, and, perhaps most remarkable of all, a mechanism in *Tetrahymena*, in which the genomic content of nuclei are compared within a shared cytoplasm prior to chromatin modification and targeted DNA elimination. The term RNAi is often used now to refer to the shared portion of all of these diverse pathways.

During RNAi, members of the Dicer family of proteins process dsRNA to initiate gene silencing (reviewed in Carmell and Hannon, 2004). Dicer can process dsRNAs derived from either exogenous or endogenous sources, generating small interfering (si)RNAs of approximately 21 nucleotides that guide sequence-specific silencing (for review see Simard and Hutvagner, 2005). In addition to processing dsRNA substrates, Dicer copurifies with a large complex that loads the siRNAs into the RNA-induced silencing complex (RISC) (Liu et al., 2003; Pham et al., 2004; Tomari et al., 2004; Chendrimada et al., 2005;).

Several studies, including recent elegant structural and functional studies, suggest that members of the AGO protein family are key components of RISC (Liu et al., 2004; Meister et al., 2004; Song et al., 2004). In *C. elegans,* the AGO protein RDE-1 is required for silencing in response to experimentally introduced dsRNA (Tabara et al., 1999). AGO proteins have also been implicated in gene silencing in fungi, plants, protozoans, and metazoans including humans (reviewed in Carmell et al., 2002). Most

organisms have multiple members of the AGO protein family, and several studies suggest that these proteins are specialized to perform distinct functions. For example, two closely related *C. elegans* AGO proteins, ALG-1 and ALG-2, are not required for silencing in response to exogenous (exo) or transgene-derived dsRNA but are essential for the processing and function of the Dicer-derived, developmentally important small RNA species termed microRNAs (miRNAs) (Grishok et al., 2001).

Biochemical studies indicate that AGO proteins interact with Dicer (Hammond et al., 2001; Chendrimada et al., 2005; Tabara et al., 2002) and that small RNAs generated by Dicer are loaded directly onto AGO proteins to form active RISC (reviewed in Filipowicz, 2005). Once charged with a small RNA, AGO proteins are thought to mediate the target-sensing and effector steps in all RNAi-related mechanisms. Two distinct RNA binding domains in AGO proteins, the PAZ (Piwi/Argonaute/Zwille) and PIWI domains, appear to facilitate interactions with the 3′ and 5′ termini (respectively) of the small single-stranded RNA guides, leaving internal nucleotides available for base pairing (reviewed in Song and Joshua-Tor, 2006). Upon target recognition, base-pairing interactions and helix formation are predicted to place the phosphodiester backbone of the target RNA in proximity to the catalytic center of the RNase H-related PIWI domain. In the case of siRNA RISC (siRISC), this interaction is thought to lead directly to target mRNA cleavage. In other RISC complexes, such as the majority of miRISC complexes in animals, helix formation is interrupted by imperfect base pairing, preventing direct cleavage of the target RNA and allowing other forms of regulation, such as inhibition of mRNA translation.

Here we show that AGO proteins not only function in several different pathways in *C. elegans* but that, surprisingly, distinct AGOs function sequentially during RNAi. Our findings support a model in which the RDE-1 protein engages siRNAs derived from Dicing of the trigger dsRNA (primary siRNAs), while a set of several other AGO proteins interact with siRNAs that are amplified during the silencing process (secondary siRNAs). Overexpression of the downstream (or secondary) AGO proteins causes the accumulation of high levels of siRNAs and results in animals that are hypersensitive to RNAi. These findings suggest that secondary AGO protein levels are limiting for RNAi in *C. elegans*. The secondary AGO proteins lack key metal-coordinating residues in their RNase H-related PIWI domains, perhaps explaining why siRISC-mediated cleavage activity has not been detected to date in *C. elegans*. Finally, we provide evidence that endogenous (endo)-RNAi pathways also utilize AGO proteins at two steps and appear to converge on the same secondary AGOs that function in the exogenous dsRNA-induced, or exo-RNAi, pathway. In summary, our findings point to diverse roles for AGO proteins in *C. elegans* and support an AGO-relay mechanism involving structurally and functionally distinct AGOs that act sequentially during the initiation and effector steps of RNAi.

RESULTS

RDE-1 Interacts with Trigger-Derived Single-Stranded RNA

Genetic and biochemical studies place the *C. elegans* AGO protein RDE-1 at an upstream step in the RNAi pathway (Grishok et al., 2000; Tabara et al., 2002). To ask if RDE-1 interacts with siRNAs derived directly from the processing of the exo-trigger dsRNA, which are present at very low levels (Parrish et al., 2000), we utilized a sensitive assay that employs a 2′-*O*-methylated RNA affinity matrix to trap sequence-specific AGO/siRNA-mediated RNA binding events (Hutvagner et al., 2004). When whole animal lysates are exposed to this matrix, siRNA protein complexes are able to interact with the 2′-*O*-methylated RNA through sequence-specific base pairing but are unable to cleave the modified RNA backbone and are therefore retained on the affinity matrix (see Figure 1A).

We found that, after exposure of animals to dsRNA, the RDE-1 protein exhibits sequence-specific interactions with both the sense and antisense 2′-*O*-methylated RNA matrices. These interactions were specific for the trigger dsRNA sequence to which the animals were exposed (Figure 1B). This interaction was not detected when animals were exposed to the bacterially expressed dsRNA trigger for 1 hr or less (Figure 1C), suggesting that internalization and processing of the trigger dsRNA in the animal is required to form an RDE-1 complex capable of sequence-specific binding to the affinity matrix.

Consistent with processing of the original dsRNA trigger into single-stranded guide RNAs, we found that pretreatment of the extracts with the single-stranded ribonucleases RNase A/T1, but not with the dsRNA-specific nuclease RNase V1, dramatically reduced the interaction of RDE-1 with the 2′-*O*-methyl target RNA matrices (Figure 1D). The sequence-specific retention of RDE-1 on the 2′-*O*-methylated matrices occurred with similar efficiency regardless of whether a target mRNA was expressed in the strain (Figure 1E, compare lanes 1 and 2).

To further analyze the step at which RDE-1 functions in RNAi, we tested the binding of the RDE-1 protein to the 2′-*O*-methyl matrices in various RNAi-deficient mutant backgrounds. In the strong loss-of-function *sid-1(ne328)* mutant, which has defects in dsRNA uptake and systemic transport to tissues in the body (Winston et al., 2002), RDE-1 exhibited a markedly reduced interaction with the 2′-*O*-methyl target sequences (Figure 1E, lane 4). In contrast, in an RNAi-deficient, *m*ultiple-*AGO* mutant (MAGO) strain (described below) and in a strain deficient in *rrf-1* that encodes an *R*NA-*d*ependent *R*NA *p*olymerase (RdRP)-related protein that is thought to amplify the silencing signal (Smardon et al., 2000; Sijen et al., 2001; Conte and Mello unpublished), the RDE-1 protein was still recruited to the 2′-*O*-methyl matrices (Figure 1E, lanes 5 and 6). These findings support the placement of RDE-1 downstream of the systemic transport of dsRNA into tissues and upstream of the amplification of the silencing signal.

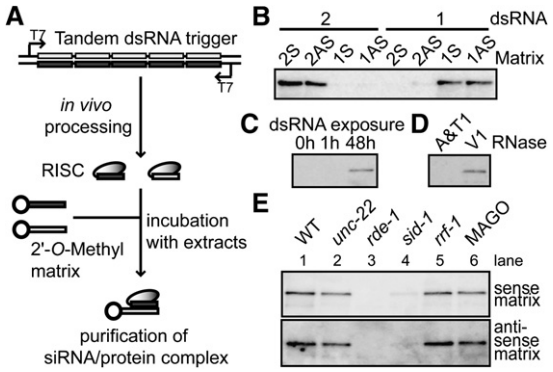

Figure 1. Sequence Specificity and Genetics of RDE-1/RNA Affinity Matrix Binding

(A) Schematic representation of the strategy used to recover proteins interacting with low-abundance (primary) siRNAs.

(B)–(E) Western blot analysis to detect HA::RDE-1 (B–D) or endogenous RDE-1 protein (E) in lysates prepared from worms treated as diagrammed in (A), using nonoverlapping 40 nt segments of GFP as dsRNA triggers. In (B) RDE-1 exhibits sequence-specific interactions with the 2′-*O*-methyl matrices. In (C) the association of RDE-1 with trigger-derived RNA requires prolonged exposure of worms to the dsRNA-expressing *E. coli*. Animals were either not exposed to *E. coli*-expressing dsRNA (0 hr) or were allowed to feed on the *E. coli* for 1 hr or 48 hr as indicated. In (D) the RDE-1 interaction with the 2′-*O*-methyl matrix depends on single-stranded RNA. Prior to exposure to the affinity matrix, worm lysates were pretreated with either the dsRNA-specific nuclease RNase V1 (V1) or with the single-stranded RNA-specific nucleases RNase A and RNase T1 (A&T1). Under these conditions, unmodified control RNAs were totally degraded, while the 2′-*O*-methyl modified oligonucleotides were unaffected (data not shown, Tabara et al., 2002; Sproat et al., 1989). In (E) genetic analysis of RDE-1 affinity-matrix binding is shown. dsRNA triggers and 2′-*O*-methyl affinity matrices were prepared using a 40 nt region of the *unc-22* gene that is deleted in *unc-22*(*st528*), a functionally wild-type allele that harbors an in-frame deletion. The RNAi-deficient mutant strains analyzed are *unc-22*(*st528*), *rde-1*(*ne300*), *sid-1*(*ne328*), and *rrf-1*(*pk1417*).

RDE-1 Does Not Interact with Secondary siRNAs

During RNAi in *C. elegans* the target mRNA appears to serve as a template for the RdRP-dependent amplification of the silencing signal (Sijen et al., 2001). The secondary siRNAs produced through this amplification process are abundant enough to detect by northern blot analysis and consist of the antisense polarity only (Grishok and Mello Unpublished; Sijen et al., 2001).

To ask whether RDE-1 interacts with these amplified secondary siRNAs we exposed animals to dsRNA and examined RDE-1 immune complexes for associated small RNAs by northern blot analysis. For this analysis we targeted a green fluorescent protein (GFP)-transgenic strain that produces abundant and easily detected secondary siRNAs after exposure to GFP dsRNA. In these studies, neither sense nor antisense siRNAs were detected in RDE-1 immunoprecipitates (data not shown). To ask if low levels of the siRNAs corresponding to the amplified region interact with RDE-1, we used sense and antisense 2′-*O*-methyl matrices complementary to GFP sequences

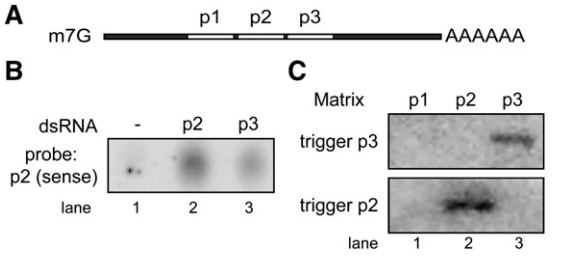

Figure 2. RDE-1 Does Not Interact with Secondary siRNAs

(A)–(C) Schematic representation of the GFP transcript, showing the relative positions of targeted regions. The dsRNA triggers and 2′-*O*-methyl affinity matrices were prepared as described in Figure 1A, using sequences corresponding to the three 40 nt regions of GFP indicated in the diagram. Lysates prepared from GFP-transgenic animals exposed to the dsRNA triggers (p2 and p3) were used for (B) northern blot analysis of small RNA species, and (C) Western blot analysis for RDE-1 protein after exposure to affinity matrices (as indicated). In (B) the RNA probe used was derived from region p2. Note that small RNAs corresponding to region p2 are detected even when region p3 is used as the trigger.

located 5′ of the region targeted by the dsRNA trigger (regions p2 and p1 in Figure 2A). After triggering RNAi with dsRNA targeting region p3, we confirmed by northern blot analysis that secondary siRNAs could be detected with a probe derived from region p2 (Figure 2B). Although RDE-1 was readily recovered on the 2′-*O*-methyl matrix corresponding to the trigger, RDE-1 was not recovered on the 2′-*O*-methyl matrix corresponding to the upstream region, region p2 (Figure 2C, top panel). When RNAi was initiated using a trigger dsRNA targeting region p2, we found that RDE-1 was readily recovered on the region-p2-specific affinity matrix (Figure 2C, bottom panel), demonstrating that the p2 matrix is functional. These data suggest that the RDE-1 protein only interacts with the very low abundance primary siRNAs and not with the much more abundant secondary siRNAs derived from the amplification process.

Genetic Analysis of AGO Mutants in *C. elegans*

Since RDE-1 does not appear to interact with secondary siRNAs, we reasoned that one or more of the numerous RDE-1 homologs in the *C. elegans* genome might play this downstream role in the RNAi pathway. The *C. elegans* genome contains a set of 27 annotated AGO-related genes (Figure 3A). To begin to assign functions to these genes we first used RNAi to target each gene for silencing. In addition, we generated deletion alleles for all of these genes except for *rde-1* and *alg-2*, for which alleles were already available (see Figure S1).

The two most highly conserved members of the *C. elegans* AGO family, *alg-1* and *alg-2*, have overlapping functions in the miRNA pathway and are essential for development (Grishok et al., 2001). Our analysis revealed that two additional AGOs, F20D12.1, which we have renamed *csr-1,* and *prg-1* are also essential for development. Depletion of *csr-1* by RNAi resulted in penetrant

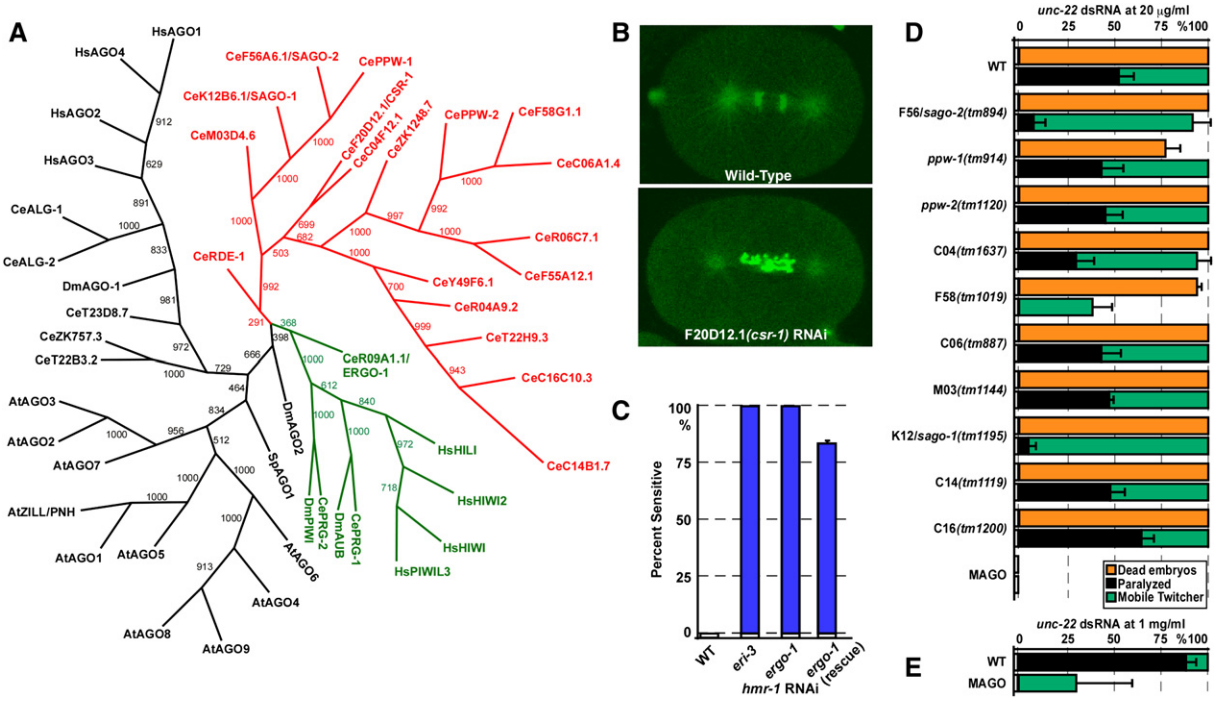

Figure 3. AGO Genes Are Required for RNAi and Development

(A) Phylogenetic tree of representative AGO proteins from plants, animals, and fungi. The AGO group with representatives in all three kingdoms is indicated in black and the PIWI group common to all metazoans is indicated in green. An expanded group of *C. elegans* proteins about equally distant from the PIWI and AGO subgroups is shown in red. ClustalW was used for the alignment, and the tree was created by bootstrapping and neighbor-joining methods using Phylip® software. Ce *(Caenorhabditis elegans)*, At *(Arabidopsis thaliana)*, Hs *(Homo sapiens)*, Sp *(Schizosaccharomyces pombe)*.

(B) *csr-1*/F20D12.1 is required for chromosome segregation. Histone- and tubulin-GFP fluorescence images of wild-type and *csr-1*/F20D12.1(RNAi) embryos at anaphase of the first cell division.

(C) *ergo-1*(*tm1860*) exhibits enhanced RNAi. The broods of between seven and ten animals (~80 embryos per animal) were scored per genotype, and the percent of embryos sensitive to RNAi targeting the *hmr-1* E-cadherin gene is shown. Expression of wild-type ERGO-1 from a transgene (*ergo-1* rescue) partially restores resistance to RNAi. Failure to see a more robust rescue may reflect the poor expression of the *ergo-1*(+) high-copy number transgene in the germline.

(D and E) Multiple red-clade AGOs contribute to RNAi. For germline RNAi, nine to ten animals were exposed to *pos-1*(*RNAi*) by feeding, and the percent *pos-1* embryonic lethal embryos produced is shown (orange bars). For somatic RNAi, between four and ten animals were injected with 20 µg/ml *unc-22* dsRNA (D) or with 1 mg/ml *unc-22* dsRNA (E), and the percent paralyzed progeny (black bars) or twitching but motile progeny (green bars) are shown. The error bars (C–E) represent the 95% confidence interval.

embryonic lethality with defects in the organization of chromosomes at metaphase of each early embryonic cell cycle and the formation of anaphase DNA bridges (Figure 3B and data not shown). Most *csr-1* deletion homozygotes are sterile, but some hermaphrodites produce a few embryos with chromosome segregation defects identical to those observed in *csr-1(RNAi)* embryos. The *csr-1* mutant is also partially deficient in germline RNAi (see Figures S2A and S2B). Thus *csr-1* defines a new gene class, *csr* (pronounced "caesar"), whose members exhibit loss-of-function phenotypes with defects in both chromosome segregation and *R*NAi. A mutation in *prg-1(tm872)*, a member of the metazoan-specific Piwi subfamily of AGO genes, exhibited a reduced brood size and a temperature-sensitive sterile phenotype (Figure S2C), consistent with previous findings linking *prg-1* to germline maintenance (Cox et al., 1998).

A single mutant, R09A1.1, which we have renamed *ergo-1* for endogenous RNAi-deficient *Argo*naute mutant, exhibited an enhanced sensitivity to RNAi (Figure 3C). This enhanced RNAi phenotype was partially rescued by the introduction of an *ergo-1* wild-type transgene, supporting the idea that the enhanced RNAi phenotype is due to a loss of *ergo-1* activity (Figure 3C). As implied by its name, *ergo-1* activity is required for an endo-RNAi pathway (see below).

Multiple AGOs Contribute Incrementally to RNAi

We assayed each viable AGO mutant allele for sensitivity to RNAi. We also used a sequential RNAi assay to search for potential involvement of each AGO in RNAi (see Figure S2D). These assays defined *ppw-1* (also observed in Tijsterman et al., 2002) and F58G1.1 as partially deficient in RNAi. These two genes represent divergent

members of an expanded clade of AGOs present in *C. elegans* (Figure 3A, red branches). To more carefully examine the activities of the other members of this clade, we analyzed mutant alleles of these genes using a more sensitive microinjection assay optimized for detecting deficiencies in RNAi.

In this more sensitive assay we targeted the muscle-specific *unc-22* gene and set the dose of dsRNA for microinjection at 20 μg/ml, which is sufficient to induce approximately 50% paralyzed and 50% motile twitching animals after injection into wild-type animals. These assays revealed that while two mutants, *ppw-1* and F58G1.1, were partially deficient in germline RNAi (Figure 3D, orange bars), four mutants, K12B6.1, F56A6.1, C04F12.1, and F58G1.1, were partially deficient in RNAi targeting the somatic gene *unc-22* (Figure 3D, green and black bars). For reasons described below, we have renamed K12B6.1 and F56A6.1 *sago-1* and *sago-2,* respectively.

We next examined the consequences of creating a multiple mutant including alleles of four genes implicated in RNAi by their single mutant phenotypes (*ppw-1*, *sago-1*, *sago-2*, and F58G1.1). In this MAGO strain we also included alleles of two additional genes, C06A1.4, a close homolog of F58G1.1, and M03D4.6, a close homolog of *sago-2* and *ppw-1*. Both C06A1.4 and M03D4.6 are now predicted to be pseudogenes and, perhaps consistent with this designation, their inclusion in multiple mutant strains did not appear to result in any enhancement of the RNAi defect in our assays (see Figure S3A). The MAGO strain, comprised of the *ppw-1*(*tm914*), *sago-1*(*tm1195*), *sago-2*(*tm894*), F58G1.1(*tm1019*), C06A1.4(*tm887*), and M03D4.6(*tm1144*) alleles, was resistant to both germline and somatic RNAi (Figure 3D). This strain was still weakly sensitive to RNAi in response to injected dsRNA at concentrations of 1 mg/ml (Figure 3E). Nevertheless, this strain was strongly deficient in RNAi by feeding and was suitable for the functional studies described below. The MAGO strain also exhibits a temperature-dependent reduction in fertility when cultured at 25°C but has no other easily discernable phenotypes (data not shown).

AGOs Required for RNAi Exhibit Qualitatively Distinct Activities

To compare the activities of AGO genes, we performed rescue assays in which we used the potent muscle-specific *myo-3* promoter to overexpress individual AGOs in the muscles of the *rde-1* and MAGO strains. Consistent with the idea that RDE-1 and the MAGO components are not interchangeable, we found that overexpression of RDE-1 rescued the *rde-1* mutant but failed to rescue RNAi in the MAGO strain (Figure 4A). Conversely, overexpression of wild-type or GFP-tagged alleles of the MAGO components, *sago-1*, *sago-2*, and *ppw-1*, strongly rescued the MAGO strain but failed to rescue the RNAi defect of the *rde-1* mutant strain (Figure 4A). These findings suggest that *sago-1, sago-2*, and *ppw-1* encode functionally interchangeable proteins whose overexpression can compensate for the collective RNAi defect of the MAGO

A Rescue assays distinguish rde-1 and the MAGO activities:

Transgene	Mutant Strain *rde-1*(*ne300*)	(n)	MAGO	(n)
none	-	(72)	-	(82)
myo-3::GFP	-	(66)	-	(80)
myo-3::RDE-1::HA	+	(58)	-	(78)
myo-3::GFP::SAGO-1	-	(61)	+	(49)
myo-3::GFP::SAGO-2	-	(76)	+	(75)
myo-3::PPW-1	-	(46)	+	(73)
myo-3::GFP::ALG-1	-	(63)	-	(69)
myo-3::PRG-1	-	(50)	-	(63)
myo-3::CSR-1	-	(77)	-	(47)

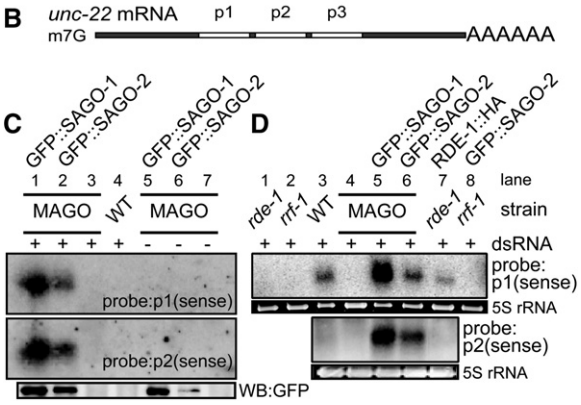

Figure 4. GFP::SAGO-1 and GFP::SAGO-2 Rescue the MAGO Strain and Interact with Secondary siRNAs

(A) Rescue of the RNAi-deficient phenotypes of the *rde-1* and MAGO strains via *myo-3*-promoter-driven expression of AGO genes (as indicated). Transgenic animals were cultured on *unc-22* dsRNA-expressing bacteria. Animals were scored for the *unc-22(RNAi)* phenotype. The (+) indicates Unc (RNAi-responsive) animals while (–) indicates NonUnc (RNAi-deficient) animals. One hundred percent of the animals scored (n) showed the indicated phenotype.

(B) Schematic diagram indicating the regions within the *unc-22* gene used to prepare RNA probes.

(C and D) Northern blot analysis of small RNAs in (C) GFP::AGO immune complexes and (D) total lysates. The strains and probes are as indicated; the dsRNA trigger was derived from region p2. The lower panel in (C) is a western blot probed with a GFP-specific monoclonal antibody. In (D) the RNAi-deficient alleles analyzed are *rde-1*(*ne300*) and *rrf-1*(*pk1417*); the 5S ribosomal RNA is shown as a loading control. In the upper panel of (D) the p1-specific probe is a Starfire probe comprised of a 40 nt segment of region p1.

strain. RDE-1, on the other hand, appears to have a qualitatively distinct activity. We also attempted to rescue the *rde-1* and MAGO strains using other AGO family members. The microRNA-AGO *alg-1*, as well as *prg-1* and *csr-1*, failed to rescue either *rde-1* or the MAGO strain (Figure 4A).

SAGO-1 and SAGO-2 Interact with Secondary siRNAs

The findings that at least three AGOs, SAGO-1, SAGO-2, and PPW-1, appear to differ functionally from RDE-1 in our muscle-specific rescue assays prompted us to ask whether these AGOs might interact with secondary siRNAs. To address this question, northern blot analysis

was performed to detect small RNAs associated with GFP-tagged SAGO-1 and SAGO-2. Indeed, secondary siRNAs derived both from within the trigger region (Figure 4B, probe p2) and from the region upstream of the trigger dsRNA (Figure 4B, probe p1) were detected in GFP-immune complexes recovered from the corresponding MAGO-rescued strains (Figure 4C, lanes 1 and 2). We did not detect siRNAs using a probe located just downstream (3′) of the trigger dsRNA (probe p3 in Figure 4B and data not shown), and we did not detect sense siRNAs associated with these immune complexes using probes from any of the three regions (p1, p2, or p3, data not shown).

Interestingly, we noticed that strains overexpressing GFP::SAGO-1 exhibited an enhanced level of RNAi overall. For example, 100% (n = 49), of the myo-3p::GFP::SAGO-1 transgenic animals exhibited a paralyzed *unc-22* RNAi phenotype, whereas wild-type animals failed to exhibit paralyzed twitchers and were instead strong, but still motile, twitchers after 36 hr of exposure to *unc-22* RNAi (n = 54).

Consistent with the increased level of silencing in these strains, we found that the levels of secondary siRNAs were substantially increased relative to wild-type levels in strains overexpressing SAGO-1 (Figure 4D, compare lanes 3 and 5). The overaccumulation of siRNAs was less evident in the GFP::SAGO-2 transgenic strain (Figure 4D, compare lanes 3 and 6). This appears to reflect relatively weaker expression from the GFP::SAGO-2 transgene (see western blot, lower panel in Figure 4C). As expected from previous studies (Grishok and Mello unpublished, Sijen et al., 2001), only siRNAs of the antisense polarity were detected in these assays (data not shown).

Taken together the findings (1) that mutations in *sago-1* and *sago-2* lead to reduced RNAi activity, (2) that these mutations appear to disrupt RNAi downstream of the interaction of RDE-1 with primary siRNAs, (3) that overexpression leads to increased RNAi activity and to the rescue of secondary siRNA levels, and (4) that the rescuing proteins coimmunoprecipitate with secondary siRNAs, strongly support the notion that at least these two AGOs (and likely others) interact with and stabilize secondary siRNAs to direct silencing during RNAi.

Consistent with the idea that RDE-1 is functionally distinct from these AGOs, we found that although HA::RDE-1 fully rescues the RNAi defect of *rde-1(ne300)*, its overexpression does not lead to any observable increase in secondary siRNA levels (Figure 4D, lane 7) and does not result in any detectable interaction between HA::RDE-1 and secondary siRNAs (data not shown). Finally, consistent with the placement of SAGO-1 and SAGO-2, either at the same step or downstream of RdRP-dependent secondary-siRNA production, we found that overexpression of SAGO-2 failed to rescue the RNAi-deficient phenotype of an *rrf-1*/RdRP mutant strain (data not shown) and, as expected, also failed to rescue secondary siRNA accumulation in the *rrf-1* mutant background (Figure 4D, top panel, lane 8).

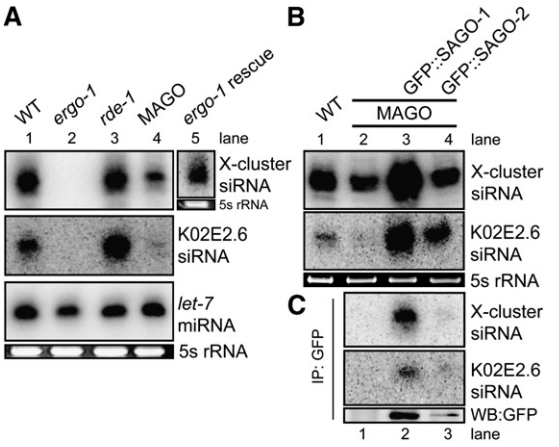

Figure 5. *ergo-1*(*tm1860*) and the MAGO Strain Are Deficient in Endo-siRNA Expression

(A and B) Northern blot analysis of endogenous small RNAs in wild-type and various mutant and transgenic rescued strains, as indicated. The 5S ribosomal RNA blots are provided as loading controls. In (A) the RNAi-deficient alleles analyzed are *rde-1*(*ne300*) and *ergo-1(tm1860)*. (C) Immunoprecipitation (IP)-northern blot analysis (top two panels) and IP-western blot analysis (bottom panel) of GFP-immune complexes recovered from rescuing GFP::SAGO-1 and GFP::SAGO-2 transgenic strains. Probes for the K02E2.6 and the X-cluster endo-siRNAs and for the *let-7* miRNA are described in Duchaine et al. (2006).

Based on the strong genetic and physical criteria linking *sago-1* and *sago-2* to secondary siRNAs, we propose to define this gene class as *sago* (pronounced "say-go"), for synthetic secondary-siRNA defective *AGO* mutants. This class of AGOs is likely to include *ppw-1,* a close homolog of *sago-1* and *sago-2,* as well as other members of the expanded clade of AGO genes in *C. elegans* (see Figure 3A and Discussion).

An Endogenous Small RNA Pathway Requires ERGO-1 and the SAGO Proteins

The finding that increasing the levels of the SAGO proteins increases RNAi activity suggests that these AGOs are present in limited supply. In *C. elegans,* silencing in response to exogenous, experimentally delivered dsRNA (exo-RNAi) is increased when certain endo-RNAi pathways are compromised by mutation (Duchaine et al., 2006; Lee et al., 2006). These findings suggest that the exo-RNAi and endo-RNAi pathways may converge on, and compete for, an unknown limiting factor shared by both pathways. Because the SAGO proteins are limiting for exo-RNAi we wondered if they might encode components of this shared limiting activity. Consistent with this idea, we found that siRNAs derived from an endogenous *C. elegans* gene, K02E2.6, and from an apparently noncoding X chromosome cluster are both reduced in the MAGO strain (Figures 5A and 5B).

Expression of GFP::SAGO-1 and GFP::SAGO-2 in the muscles of MAGO animals rescued the accumulation of the X-cluster and K02E2.6 endo-siRNA species (Figure 5B). As with the secondary exo-siRNAs (see

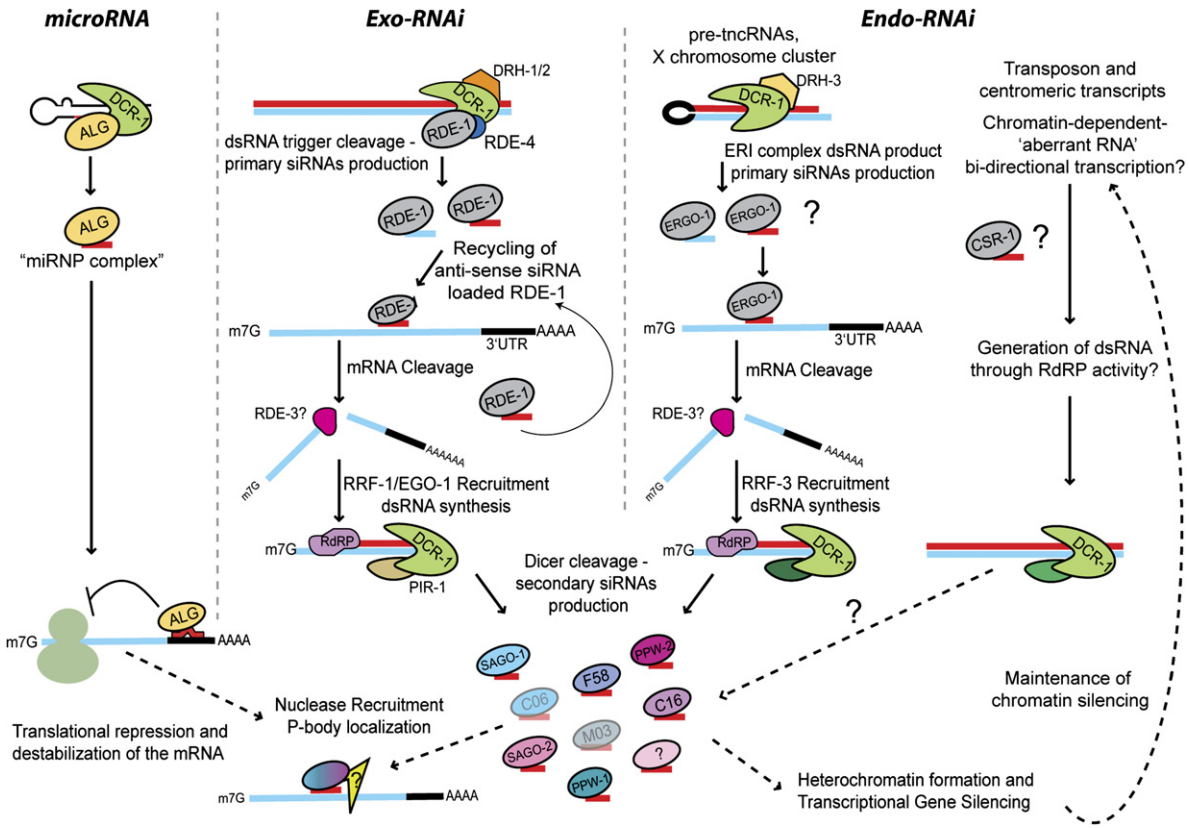

Figure 6. Model

Schematic representations of RNAi-related pathways in *C. elegans*. Exo- and endo-RNAi pathways are proposed to involve sequential rounds of AGO action involving primary siRNA containing AGO complexes (gray ovals), and secondary siRNA containing AGO complexes (colored ovals). The miRNA pathway is proposed to involve a single AGO-mediated step. Distinct DCR-1 complexes are proposed to recognize the dsRNA substrates illustrated in the diagram. Evidence exists for several of these complexes, including the ALG, RDE-1, ERI, and PIR-1 containing DCR complexes (Tabara et al., 2002; Duchaine et al., 2006). After primary-siRNA-directed cleavage, a protein complex potentially containing RDE-3 (Chen et al., 2005, pink object) is proposed to mark the 3′ end of the 5′ cleavage product and to recruit RdRP. The question marks and dashed lines indicate speculative elements in the model.

Figure 4C), these endo-siRNA species accumulate to levels that are higher than wild-type levels in strains overexpressing these AGOs (Figure 5B). Note that the level of endo-siRNA accumulation correlates with the level of SAGO-protein expression as measured in the western blot (Figure 5B, lower panel). Like the secondary exo-siRNAs, we found that the endo-siRNAs also coimmunoprecipitate with GFP::SAGO-1 and GFP::SAGO-2 (Figure 5C).

Interestingly, endo-siRNA levels were even more dramatically reduced in *ergo-1*(*tm1860*) AGO mutant animals (Figure 5A, lane 2), in which exo-RNAi is enhanced (see Figure 3C and Discussion). Furthermore, consistent with competition between the ERGO-1 and RDE-1 pathways, the levels of K02E2.5 endo-siRNAs were increased in animals deficient for *rde-1* (see Figure 5A and Discussion). There were no significant changes in the level of *let-7* miRNA expression in these strains (Figure 5A). Expression of a partially rescuing *ergo-1*(+) transgene in the *ergo-1*(*tm1860*) mutant strain partially restored the expression of the X-cluster-derived endo-siRNA species (Figure 5A, right panel).

DISCUSSION

Through a combination of forward genetics, reverse genetics, and proteomics, we have arrived at a model for RNAi (Figure 6) that explains how multiple, small RNA-mediated silencing pathways interact with each other and converge on shared components of the RNAi machinery. This model explains how RNA-silencing pathways can achieve both specificity and amplification. According to this model, upon exposure to *E. coli* expressing dsRNA, intestinal cells take up and disseminate small quantities of dsRNA to other tissues via a systemic mechanism that depends in part on the SID-1 channel protein (Feinberg and Hunter, 2003; Winston et al., 2002). The dsRNA is then processed by a Dicer complex that includes the dsRNA binding protein RDE-4 and the AGO

protein RDE-1 (Tabara et al., 2002). A scanning phase of RNAi follows, in which RDE-1/primary-siRNA complexes search for target mRNA sequences. RDE-1 then recruits RdRP, perhaps indirectly through an initial round of target mRNA cleavage. This initial targeting by RDE-1 is sufficient to initiate amplification but insufficient, by itself, to cause silencing (due to the low levels of the primary siRNAs).

The target mRNA is proposed to act as a template for the primer-independent synthesis of new dsRNA (see also Duchaine et al., 2006). RdRPs related to those involved in RNAi have been shown to catalyze primer-independent RNA synthesis (Makeyev and Bamford, 2002). Recruitment of RdRP directly to the target mRNA, without the need for priming, would permit new dsRNA synthesis without consuming the original trigger-derived siRNAs. This process would allow each of the rare RDE-1/siRNA complexes to be recycled to target multiple transcripts and would thus permit multiple rounds of RdRP-dependent amplification. According to this model, a second Dicer complex would then act to process the RdRP products and to load the amplified secondary siRNAs onto members of a group of partially redundant secondary AGOs that include SAGO-1, SAGO-2, and likely other related proteins.

The RDE-1 and the SAGO proteins exhibit structural differences that may help explain their distinct biological activities. An alignment of members of the AGO protein family reveals that most members of this family, including RDE-1 and ERGO-1, exhibit conservation of key metal-coordinating residues in the RNase H-related PIWI domain (D,D, and H residues in Figure 7). SAGO-1, SAGO-2, and several other members of the expanded *C. elegans* AGO clade (red branches in Figure 3A), including the other components of the RNAi-deficient MAGO strain (Figure 7, blue shaded sequences), conspicuously lack these residues. Thus, while RDE-1 might be expected to retain catalytic activity, the SAGO proteins would very likely require accessory factors to mediate target mRNA turnover (model, Figure 6).

The model for RNAi proposed above provides two opportunities for amplification. First the RDE-1/siRNA complex, although low in abundance, is proposed to work repeatedly to generate multiple templates for RdRP. Second, Dicer is proposed to process each RdRP-derived dsRNA product into several secondary siRNAs. Acting together, these two steps ([1] repeated mRNA targeting by the RDE-1/primary-siRNA complex, followed by [2] RdRP-dependent dsRNA synthesis and Dicer processing) could generate potentially thousands of secondary siRNA for each original primary siRNA.

While amplification of the silencing signal would have obvious benefits for suppressing viral gene expression, this is balanced against a danger of amplifying off-target silencing. Conceivably, any off-target cleavage events mediated by the primary-siRNA/RDE-1 complex could lead to a chain reaction of silencing with obvious deleterious consequences. The model for silencing proposed

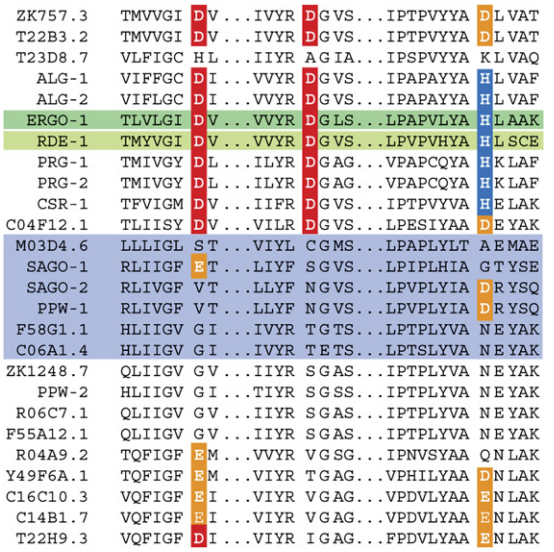

Figure 7. Secondary AGOs Lack Key Catalytic Residues

Alignment of *C. elegans* AGO proteins in three regions with similarity to the catalytic center of RNase H. Within these regions two key aspartic acid residues (highlighted in red) and a histidine residue (highlighted in dark blue) coordinate a magnesium ion at the catalytic center of the RNase H enzyme. Substitutions compatible with metal binding are indicated in orange. The RDE-1 and ERGO-1 amino-acid sequences are highlighted in shades of green, while those of the MAGO strain components are highlighted in blue.

here could safeguard against off-target amplification in three ways. First, since RDE-1 does not need to silence the target mRNA by itself, the target-scanning step mediated by RDE-1 can afford to incorporate a very high degree of selectivity. Second, since the downstream AGOs lack catalytic residues required for mRNA cleavage, they may be unable to generate cleaved substrates for further amplification. And finally, the downstream AGO proteins are present in limited supply and thus provide limited capacity to support multiple simultaneous silencing reactions.

Perhaps consistent with the idea that safeguards exist to prevent the initiation of off-target silencing, the injection of concentrated dsRNA, or even the promoter-driven expression of dsRNA, cannot bypass the requirement for *rrf-1,* the RdRP required for amplification. Furthermore, although we have shown that RDE-1 still appears to interact with primary siRNAs in *rrf-1* mutants, neither the primary nor the secondary siRNAs are detectable in *rrf-1* mutants, even in the presence of abundant promoter-driven dsRNA (Sijen et al., 2001; Conte and Mello, unpublished). These results suggest that the processing of trigger dsRNA and loading into the RDE-1 complex may be inherently inefficient. Alternatively, mechanisms may exist that function to limit the formation of the RDE-1/primary-siRNA complex, even in the presence of large quantities of trigger dsRNA. Such mechanisms could be important to limit the pioneering round of target recognition by RDE-1 and

thus to minimize the risk of amplifying off-target silencing reactions.

Intersecting RNAi Pathways in *C. elegans*

Several of our findings suggest that ERGO-1 may function in the endo-RNAi pathway in a manner analogous to the role of RDE-1 in the exo-RNAi pathway. Furthermore, our findings support the hypothesis that the ERGO-1 and RDE-1 pathways converge on the SAGO proteins (Figure 6). Consistent with this model, the MAGO strain, which includes lesions in *sago-1* and *sago-2,* exhibits defects in both secondary siRNA accumulation and in the accumulation of endo-siRNA species. The convergence of several pathways on members of the secondary group of AGOs may provide selective pressure for the maintenance of this amplified gene family.

ERGO-1 is required for endo-siRNA accumulation, and lesions in *ergo-1* enhance exo-RNAi. These findings support the placement of ERGO-1 upstream of the convergence between the endo- and exo-RNAi pathways in the model (Figure 6). Accordingly, while mutations in *ergo-1* prevent the accumulation of endo-siRNAs, they do not interfere with exo-siRNA production. Instead, by eliminating an abundant endo-siRNA species that would otherwise compete with exo-siRNAs for loading onto the limiting SAGO proteins, lesions in *ergo-1* enhance the exo-RNAi pathway (Figure 6).

The ERI proteins and the RdRP RRF-3 may function along with ERGO-1 in the production of endo-siRNAs (Figure 6 and Duchaine et al., 2006). ERGO-1 has a potentially intact catalytic domain and in this respect is structurally similar to RDE-1 (Figure 7). Conceivably, low levels of dsRNA synthesis from endogenous loci could provide precursors for the production of primary endo-siRNAs that are loaded onto ERGO-1. ERGO-1, through RNA-scanning, target cleavage, and RRF-3-recruitment, may then direct the accumulation of abundant secondary endo-siRNA species that interact with, and compete for, the SAGO proteins.

AGOs and Transcriptional Gene Silencing

Transcriptional silencing appears to be an important mode of RNAi-directed silencing in *C. elegans*. While this has been best studied in fungi (reviewed in Grewal and Rice, 2004), elements of a transcriptional silencing pathway exist in a variety of organisms (Reviewed in Wassenegger, 2005). In *C. elegans*, transgene silencing and cosuppression, which are maintained in part by chromatin-related silencing pathways (Tabara et al., 1999; Ketting et al., 1999; Grishok et al., 2005; Robert et al., 2005), require a subset of the genes implicated in exo-dsRNA-induced RNAi.

Here we have shown that CSR-1, an essential AGO protein, is required, directly or indirectly, for chromosome segregation in *C. elegans*. In addition CSR-1 appears to contribute to germline RNAi. Expression of CSR-1 in the muscle failed to rescue the secondary-AGO defect in our assays, raising the possibility that CSR-1 functions at yet another step in the RNAi pathway or requires specific cofactors that are not present in muscle cells. One interesting possibility is that germline RNAi has a strong transcriptional silencing component and that CSR-1 plays a role in mediating chromatin effects important for both germline RNAi and chromosome segregation (model, Figure 6).

An emerging theme from this and several other recent studies is the remarkable importance of AGO proteins for germline maintenance and function. In *C. elegans* at least four distinct groups of AGO genes are required for fertility. These include *csr-1*, *prg-1/prg-2, alg-1/alg-2*, and the multiple AGO mutant strain (MAGO) that includes *sago-1* and *sago-2*. In the mouse, all three members of the Piwi/*prg* AGO family, Miwi (Deng and Lin, 2002), Mili (Kuramochi-Miyagawa et al., 2004), and Miwi2 (G.J. Hannon, personal communication) are required for male fertility. Two recent reports have shown that an abundant species of ~30 nucleotide siRNAs (named piRNAs) interacts with Mili in meiotic spermatocytes (Aravin et al., 2006; Girard et al., 2006). Interestingly, piRNAs accumulate asymmetrically in a manner analogous to the secondary and X-cluster-derived siRNAs found in *C. elegans*. Clearly, there is still much to learn about the production and function of small RNAs. The paradigms of sequential AGO action and of intersection between AGO-mediated silencing pathways are likely to be important for understanding the diversity and complexity of RNAi-related mechanisms in numerous organisms.

EXPERIMENTAL PROCEDURES

Worm Strains

The Bristol strain N2 was used as the standard wild-type strain. The AGO alleles and strains used in this study are described in the text and are listed in Table S1. Additional alleles used in this study are *rrf-1(pk1417)* I, *alg-2(ok304)* II, *sid-1(ne328)* V, and *unc-22(st528)* IV. Deletions mutations were obtained as previously reported (Gengyo-Ando and Mitani, 2000). *C. elegans* culture and genetics were as described in Brenner (1974).

Rescue Experiments

For *myo-3* promoter-driven expression in muscle, AGO ORFs were cloned into pPD96.52 (from Andrew Fire). Transgenic animals were generated by coinjection of the plasmid constructs at 10 μg/ml with the marker plasmid pRF4 (Mello et al., 1991) at 100 μg/ml. Extrachromosomal arrays were integrated by UV treatment (Evans, 2006). *ergo-1* rescued lines were generated by coinjecting a genomic PCR fragment produced using forward primer ATGTTTCAAAAAAAGTTATGG CC and reverse primer GAAAAAGAATGAATGAACTGC at a 5 μg/ml concentration, along with the marker plasmid pTG96 (Yochem et al., 1998) at 100 μg/ml.

RNAi Experiments

RNAi was carried out as previously reported (Fire et al., 1998; Timmons et al., 2001). Worms were grown on NGM plates containing 1 mM IPTG unless otherwise stated. The sequences used to generate short tandem RNAi triggers, as well as the complementary 2′-*O*-methyl affinity matrices were the following: 5′-AAG GTA TTG ATT TTA AAG AAG ATG GAA ACA TTC TTG GAC A-3′ and 5′-TGT CCA AGA ATG TTT CCA TCT TCT TTA AAA TCA ATA CCT T-3′ (GFP food region 1), 5′-AAG TTT GAA GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA A-3′ and 5′-TTA ACT CGA TTC TAT TAA CAA GGG TAT CAC CTT CAA ACT T-3′ (GFP food

region 2), 5′-TTT CAA AGA TGA CGG GAA CTA CAA GAC ACG TGC TGA AGT C-3′ and 5′-GAC TTC AGC ACG TGT CTT GTA GTT CCC GTC ATC TTT GAA A;3′ (GFP food region 3), 5′-GGA TAT GTC GTT GAA CGT TTT GAG AAG AGA GGT GGC GGT G-3′ and 5′-CAC CGC CAC CTC TCT TCT CAA AAC GTT CAA CGA CAT ATC C-3′ (for unc-22 RNAi trigger). The nonspecific 2′-*O*-methyl oligonucleotide had the following sequence: 5′-CAU CAC GUA CGC GGA AUA CUU CGA AAU GUC-3′. The 2′-*O*-methyl-modified RNA oligonucleotides were obtained from Integrated DNA Technologies. Biotin was attached to the 5′ end of the modified oligonucleotides via a six-carbon spacer arm.

Biochemistry and Molecular Biology

Protein and RNA purifications were performed as previously described (Duchaine et al., 2006). Western blot analysis, immunoprecipitation of GFP-tagged protein complexes, as well as 2′-*O*-methyl oligonucleotide affinity matrix studies were performed as reported in Hutvagner et al. (2004). To remove nonspecific 2′-*O*-methyl oligonucleotide interactors, the clarified worm lysate was preincubated for 45 min with an unrelated 2′-*O*-methyl oligonucleotide.

Antibodies used in this study are as follows: (1) monoclonal HRP conjugated anti-HA (Roche), (2) an affinity-purified polyclonal anti-RDE-1 antibody, or (3) full-length A.v. Polyclonal Antibody (BD Bioscience). Images were collected on a LAS-3000 Intelligent Dark-Box (Fujifilm). Northern blot analysis was performed as described in Duchaine et al. (2006).

Imaging and Video Microscopy

DIC and fluorescence images were collected as reported in Duchaine et al. (2006).

Supplemental Data

Supplemental Data include one table and three figures and can be found with this article online at http://www.cell.com/cgi/content/full/127/4/747/DC1/.

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