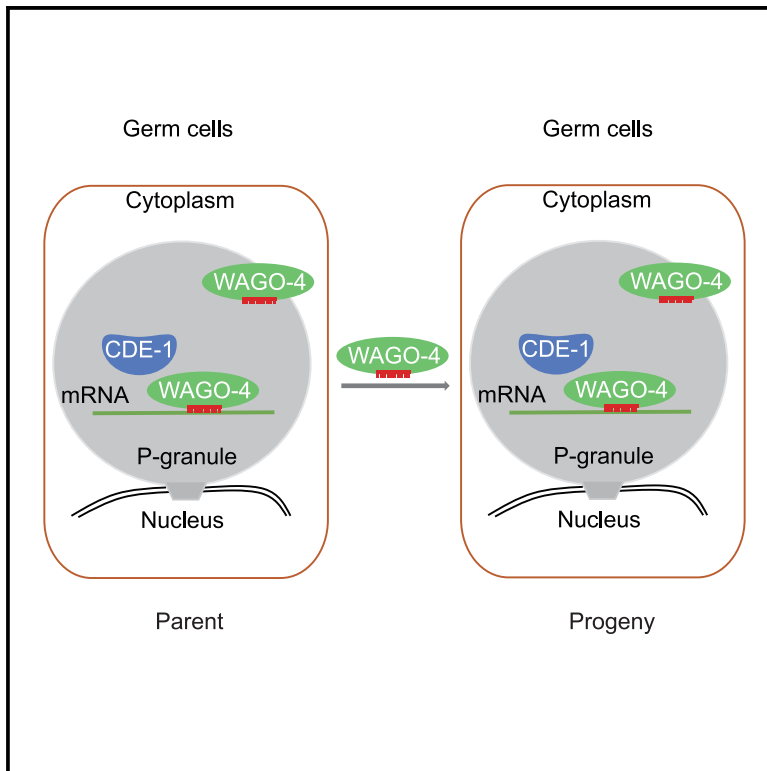


Cell Reports

A Cytoplasmic Argonaute Protein Promotes the Inheritance of RNAi

Graphical Abstract



Authors

Fei Xu, Xuezhu Feng, Xiangyang Chen, ..., Ting Xu, Minjie Hong, Shouhong Guang

Correspondence

sguang@ustc.edu.cn

In Brief

Xu et al. explore the function of a cytoplasmic Argonaute protein WAGO-4 in mediating the multigenerational inheritance of RNAi in *C. elegans*. This work reports that WAGO-4 binds to 22G-RNAs that target germline-expressed protein-coding genes. A polyU polymerase CDE-1 may promote RNAi inheritance by uridylating WAGO-4-associated 22G-RNAs.

Highlights

- WAGO-4 promotes multigenerational inheritance of RNAi
- WAGO-4 is a germline-expressed cytoplasmic Argonaute protein
- WAGO-4 binds 22G-RNAs, which contain untemplated 3' end uridylation
- WAGO-4 promotes the transmission of siRNAs for RNAi inheritance

Data and Software Availability

GSE17787
GSM455394
GSE112475



A Cytoplasmic Argonaute Protein Promotes the Inheritance of RNAi

Fei Xu,^{1,3} Xuezhu Feng,^{1,3} Xiangyang Chen,¹ Chenchun Weng,¹ Qi Yan,¹ Ting Xu,¹ Minjie Hong,¹ and Shouhong Guang^{1,2,4,*}

¹Hefei National Laboratory for Physical Sciences at Microscale, School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, China

²CAS Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences, Beijing, China

³These authors contributed equally

⁴Lead Contact

*Correspondence: sguang@ustc.edu.cn

<https://doi.org/10.1016/j.celrep.2018.04.072>

SUMMARY

RNAi-elicited gene silencing is heritable and can persist for multiple generations after its initial induction in *C. elegans*. However, the mechanism by which parental-acquired trait-specific information from RNAi is inherited by the progenies is not fully understood. Here, we identified a cytoplasmic Argonaute protein, WAGO-4, necessary for the inheritance of RNAi. WAGO-4 exhibits asymmetrical translocation to the germline during early embryogenesis, accumulates at the perinuclear foci in the germline, and is required for the inheritance of exogenous RNAi targeting both germline- and soma-expressed genes. WAGO-4 binds to 22G-RNAs and their mRNA targets. Interestingly, WAGO-4-associated endogenous 22G-RNAs target the same cohort of germline genes as CSR-1 and contain untemplated addition of uracil at the 3' ends. The poly(U) polymerase CDE-1 is required for the untemplated uridylation of 22G-RNAs and inheritance of RNAi. Therefore, we conclude that, in addition to the nuclear RNAi pathway, the cytoplasmic RNAi machinery also promotes RNAi inheritance.

INTRODUCTION

RNAi-elicited gene silencing is heritable and can perpetuate for a number of generations in *C. elegans* (reviewed in Heard and Martienssen, 2014; Miska and Ferguson-Smith, 2016; Rechavi and Lev, 2017). Both exogenously derived small interfering RNAs (exo-siRNAs) and endogenous small RNAs, such as endo-siRNAs and PIWI-interacting small RNAs (piRNAs), can trigger heritable RNAi. Transgenerational inheritance of RNAi allows organisms to remember their exposure to genome parasites, such as viruses and transposons, transmit the experience to descendants, and promote evolutionary advantages to enable selection for physiologically important traits. Although many lines of evidence have demonstrated that the RNAi machinery and small RNAs are involved in the initial establishment and ultimate maintenance of silencing, the precise nature of the trait-

specific information that is transmitted from the parents to their progeny remains largely unclear (Ashe et al., 2012; Buckley et al., 2012; Burton et al., 2017; Kalinava et al., 2017; Klosin et al., 2017; Lev et al., 2017; Luteijn et al., 2012; Marré et al., 2016; Shirayama et al., 2012; Spracklin et al., 2017; Weiser et al., 2017).

The mechanisms of transgenerational inheritance of RNAi are widely being investigated in *C. elegans*. The RNAi-mediated silencing effect can be transmitted via parental gametes (Alcazar et al., 2008; Grishok et al., 2000) and its maintenance depends on the expression of the targeted genes in germline and post-transcriptional mechanisms (Minkina and Hunter, 2017). Chromatin-modifying enzymes and their associated factors, including HPL-2, SET-25, SET-32, MES-2, and HDA-4 are engaged in the re-establishment and maintenance of transgenerational gene silencing (Ashe et al., 2012; Luteijn et al., 2012; Mao et al., 2015; Shirayama et al., 2012; Vastenhouw et al., 2006). The RNAi spreading defective factors RSD-2 and RSD-6 also promote genome silencing by maintaining siRNA populations (Sakaguchi et al., 2014). Especially, the nuclear RNAi defective (Nrde) pathway plays essential roles in the inheritance of RNAi silencing (Ashe et al., 2012; Buckley et al., 2012; Burton et al., 2011; Gu et al., 2012; Mao et al., 2015; Marré et al., 2016; Shirayama et al., 2012; Weiser et al., 2017). The germline-expressed nuclear Argonaute protein HRDE-1 may carry heritable small RNAs and engage in RNAi inheritance (Buckley et al., 2012). It was shown that the nuclear RNAi pathway maintains siRNA expression in the progeny of double-stranded RNA (dsRNA)-treated animals (Burton et al., 2011).

Although the precise nature of the trait-specific information that is directly inherited remains unknown, both small RNAs and dsRNAs have been reported to transmit from the parents to progeny in *C. elegans* (Rechavi and Lev, 2017). In addition, it is unclear whether and to what extent the cytoplasmic RNAi machinery contributes to the inheritance of RNAi (Spracklin et al., 2017). The cytoplasmic Argonaute protein WAGO-1 has been implicated in the maintenance of silencing (Shirayama et al., 2012). Yet another study showed that WAGO-1 is not required for RNAi inheritance of a *pie-1_p::gfp::h2b* transgene (Buckley et al., 2012).

To further understand the mechanisms of RNAi inheritance, we searched for factors required for silencing a germline-expressed *h2b::gfp* transgene in the progenies of animals



exposed to exogenous dsRNA. We identified the cytoplasmic Argonaute protein WAGO-4. WAGO-4 is specifically required for the exogenous dsRNA-induced silencing of germline-expressed genes. WAGO-4 binds to germline-enriched 22G-RNAs containing untemplated addition of uracil at 3' ends, which depends on the poly(U) polymerase CDE-1. After fertilization, in the zygotes and early embryos, WAGO-4 exhibits asymmetrical translocation to the germline. Therefore, we conclude that cytoplasmic RNAi machineries also contribute to the inheritance of RNAi, likely through the Argonaute protein WAGO-4.

RESULTS

WAGO-4 Is Required for Inheritance of RNAi

DsRNA targeting *lin-15b* elicits a multivulva (Muv) phenotype in enhanced RNAi (*eri*) animals through the nuclear RNAi defective (*Nrde*) pathway (Guang et al., 2008). However, the animals exhibit the Muv phenotype only in the F1 progeny but not in the parent animals, suggesting that the progenies have inherited a silencing signal. We have previously tested a number of chromatin modification factors and Argonaute proteins by examining *lin-15*-RNAi induced Muv phenotype and *lir-1*-RNAi-induced larval arrest. However, we failed to identify any except for *nrde* genes and *mes-2* that were required for both RNAi-induced phenotypes (Mao et al., 2015). Interestingly, we found that an Argonaute gene, *wago-4*, was required for *lin-15*-RNAi-induced Muv phenotype in the F1 progeny but not for *lir-1*-RNAi-induced larval arrest in the parental generation, suggesting that WAGO-4 may act through the germline to mediate RNAi inheritance.

WAGO-4 belongs to the worm-specific clade Argonaute proteins (Yigit et al., 2006). We acquired two deletion alleles *tm1019* and *tm2401*, from the National BioResource Project (NBRP) (Figure S1A). In addition, we used dual single guide RNA (sgRNA)-mediated CRISPR/Cas9 technology to generate an allele, *ust42* (Figure S1A) (Chen et al., 2014). *tm1019* lacks the MID domain of Argonaute proteins; however, the remainder of the protein is translated in frame. The alleles, *tm2401* and *ust42*, result in stop codons and are likely to be null alleles. *wago-4(ust42)* has been outcrossed twice and is used as the reference allele.

We confirmed that WAGO-4 is involved in dsRNA-induced gene silencing by feeding RNAi targeting a number of soma- and germline-expressed genes. RDE-4 is a dsRNA-binding protein that is indispensable for feeding RNAi. *rde-4* mutants were resistant to the RNAi targeting *pos-1*, *lir-1*, and *lin-15b* (Figure S1B). The nuclear Argonaute protein NRDE-3 was required for dsRNA targeting both *lir-1* and *lin-15b*, the two examples used in nuclear RNAi analysis (Guang et al., 2008). *wago-4* was required for exogenous RNAi targeting *pos-1* or *lin-15b*, but not for RNAi targeting *lir-1* gene. These data suggest that WAGO-4 is involved in dsRNA-induced gene silencing. Because *pos-1* is mainly silenced by RNAi in cytoplasm (Guang et al., 2008), these data also suggest that WAGO-4 could conduct gene silencing in the cytoplasm.

We further examined whether *wago-4* is required for the inheritance of RNAi (Figure 1A). We first used a germline-expressed *pie-1_{pr}::gfp::h2b* (abbreviated as *h2b::gfp*) transgene as a re-

porter, which can inherit RNAi-induced gene silencing for multiple generations (Buckley et al., 2012). The nuclear Argonaute *hrde-1* was not required for exogenous *gfp* dsRNA to silence the *h2b::gfp* transgene in the parental generation but essential for the silencing in F1 generation (Figure 1B). Similarly, *wago-4* was not required for exogenous *gfp* dsRNA to silence the *h2b::gfp* transgene in the parental (P0) generation, but was necessary for silencing in F1 progeny. Next, we used a soma-expressed *sur-5::gfp* transgene as another reporter of RNAi inheritance. *sur-5::gfp* was silenced by exogenous *gfp* dsRNA in both P0 and F1 generations in wild-type animals (Figure 1C). In *wago-4* mutant animals, *sur-5::gfp* was silenced in P0 generation but desilenced in F1 generation, suggesting that the progeny failed to inherit the silencing effect. Interestingly, the fluorescence intensity of both *h2b::gfp* and *sur-5::gfp* transgenes did not reach the levels of untreated animals in *wago-4* mutants, suggesting the presence of a weak RNAi inheritance even in the absence of *wago-4* (Figures 1B and 1C). The *C. elegans*' genome encodes 27 Argonaute proteins, 12 of which belong to the WAGO clade (Yigit et al., 2006). We speculate that other WAGO proteins may function redundantly with WAGO-4 in mediating the inheritance of RNAi.

To further demonstrate that WAGO-4 promotes RNAi inheritance, we generated a single-copy WAGO-4 transgene *wago-4_{pr}::3xFLAG::GFP::WAGO-4* (abbreviated as *GFP::WAGO-4*) using MosSCI technology (Frøkjær-Jensen et al., 2014). This transgene rescued *pos-1* and *mex-5* dsRNA-elicited embryonic lethality and partially rescued *lin-15* dsRNA-induced Muv phenotype in *wago-4(ust42)* animals (Figures S1C–S1E), suggesting that the *GFP::WAGO-4* represents the function and activity of endogenous WAGO-4 protein. *dpy-11* is an ortholog of human TMX1 and TMX4 genes and is required for body morphogenesis (Minkina and Hunter, 2017). dsRNA targeting *dpy-11* in *eri-1* parental animals induced a dumpy phenotype in the F1 progeny in the absence of further RNAi, suggesting that the RNAi signal has been inherited (Figure 1D). While *rde-4* animals did not respond to feeding RNAi at all, the depletion of NRDE-3 or WAGO-4 both caused the loss of dumpy phenotype in F1 animals, suggesting that NRDE-3 and WAGO-4 were required for the inheritance of RNAi-targeting somatic genes. The introduction of *GFP::WAGO-4* transgene rescued the inheritance defects in *wago-4* mutants (Figure 1D), which confirmed the role of WAGO-4 in RNAi inheritance.

WAGO-4 Is a Germline-Expressed Argonaute Protein and Required for the Silencing of Germline-Expressed Genes

We downloaded the expression data of *wago-4* from Wormbase (version WS260). *wago-4* was exclusively detected in the hermaphrodite germline but not in the soma (Figure 2A). Consistently, *GFP::WAGO-4* was exclusively expressed in the germline and all oocyte cells in gravid adults in hermaphrodites but not significantly expressed in the male germline (Figures 2B and S2A). In early embryos, WAGO-4 is expressed in the P1 and EMS cells (Figure S2B). In late embryos, WAGO-4 was exclusively expressed in Z2/Z3 cells. Interestingly, we observed that WAGO-4 accumulated at some distinct perinuclear foci (Figure 2B). Many RNA processing and regulatory factors, including

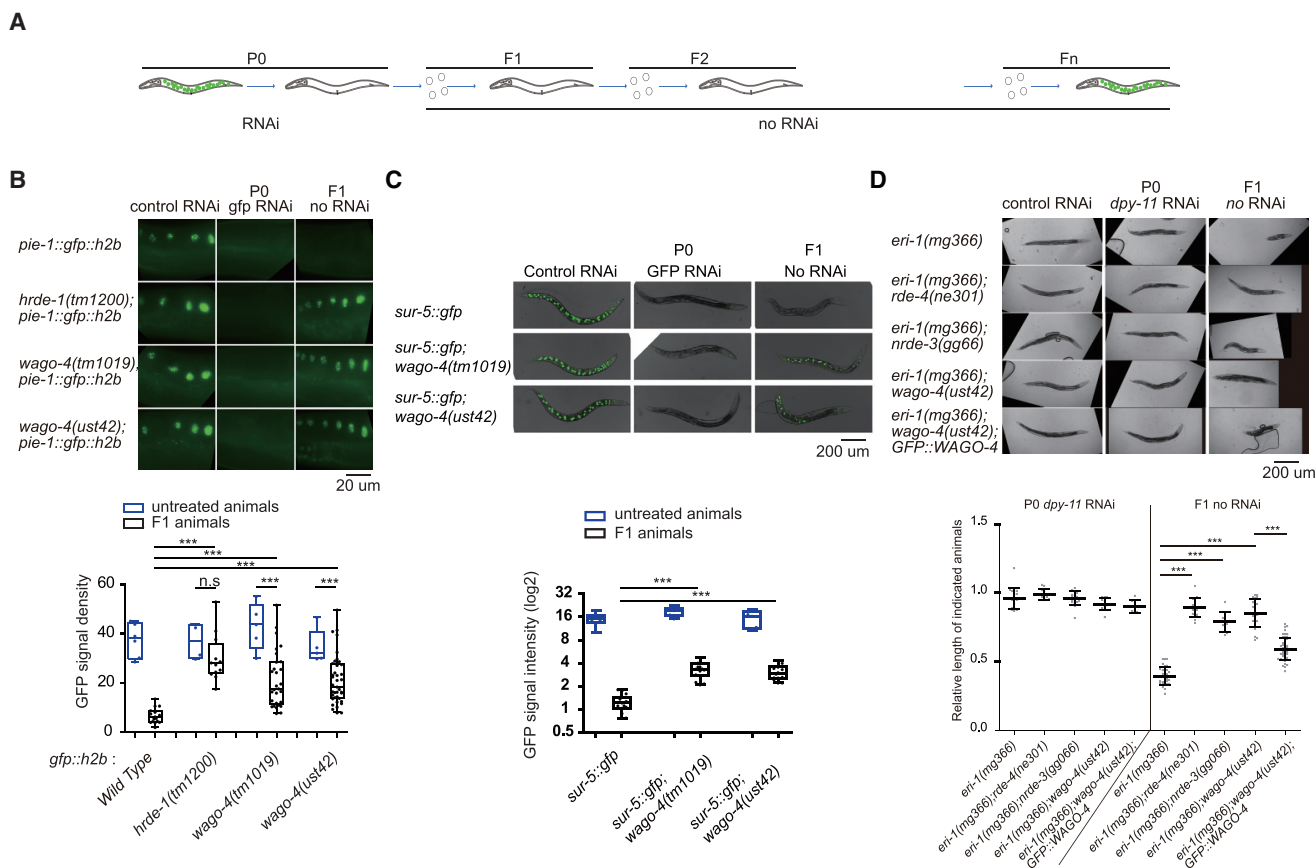


Figure 1. WAGO-4 Is Required for the Inheritance of RNAi

(A) A scheme of RNAi procedures.

(B and C) *pie-1_P::gfp::h2b* (B) and *sur-5::gfp* (C) transgenic animals were exposed to bacteria expressing *gfp* dsRNA, respectively. F1 embryos were isolated and grown on control bacteria in the absence of further *gfp* dsRNA treatment. GFP expression was imaged (top) and the levels were scored (bottom).

(D) Rescue assay with the GFP::WAGO-4 transgene. The indicated animals were treated with *dpy-11* dsRNA and F1 animals were transferred to control bacteria (top). The levels of dumpy were scored (bottom). Mean $\pm$ SD. *** p < 0.001.

the RNAi machinery, are enriched in the perinuclear region and exhibit distinct foci, which are termed P-granules (Chen et al., 2016; Claycomb et al., 2009; Gu et al., 2009; Tu et al., 2015; Weides et al., 2013). We crossed *GFP::WAGO-4* with a chromatin marker strain *mCherry::H2B* and the P-granule marker strain *mRuby::PGL-1*, respectively, and found that WAGO-4 partially co-localized with the P-granule marker PGL-1 but not with the chromatin marker (Figure 2C).

WAGO-4 lacks the DDH catalytic triad of amino acids considered necessary for Argonaute-based slicer activity (Yigit et al., 2006). We tested whether WAGO-4 was required for RNAi targeting germline-expressed mRNAs. We fed L1 animals on dsRNA, harvested L4 animals of the same generation 48 hr later, and quantified mRNA levels by real-time PCR. We found that WAGO-4 was required for the decrease of the targeted mRNAs (Figure 2D), suggesting that either WAGO-4 contains some slicer activity, or siRNA/WAGO-4 complex silences targeted mRNAs through other mechanisms. Interestingly, *mex-5* and *pos-1* mRNAs were not fully desilenced in *wago-4* mutants, suggesting that other germline Argonaute proteins may function redundantly with WAGO-4 to silence germline genes.

WAGO-4 and HRDE-1 Act Differently in Promoting RNAi Inheritance

Both HRDE-1 and WAGO-4 are required for the inheritance of RNAi, yet they exhibit distinct subcellular locations. We hypothesized that they function via different mechanisms or at distinct steps in the process of transmitting siRNAs and/or re-establishing the silencing effects in the progeny.

To test this hypothesis, we fed *sur-5::gfp* parental animals with *gfp* dsRNA and examined *gfp* expression in F1 embryos and L4 larva without further feeding RNAi. *sur-5::gfp* was silenced in wild-type animals but not in *rde-1* mutants in the parental and F1 animals (Figures 3A and S3). In *hrde-1* and *nrde-1/2/4* mutants, *sur-5::gfp* was silenced in both parent animals and F1 embryos, indicating that the animals defective for nuclear RNAi are able to inherit *gfp* silencing signals. During larval development, the heritable *gfp* silencing was relieved in *nrde-1/2/4* mutants (Figure S3), which was also shown in *nrde-3* mutants previously (Burton et al., 2011). However, *hrde-1*(−) mutants still exhibited gene silencing in L4 larva in the soma, further supporting that *hrde-1*(−) animals inherited silencing signals (Figures 3A and S3). Strikingly, when *wago-4*(−);*sur-5::gfp* animals were

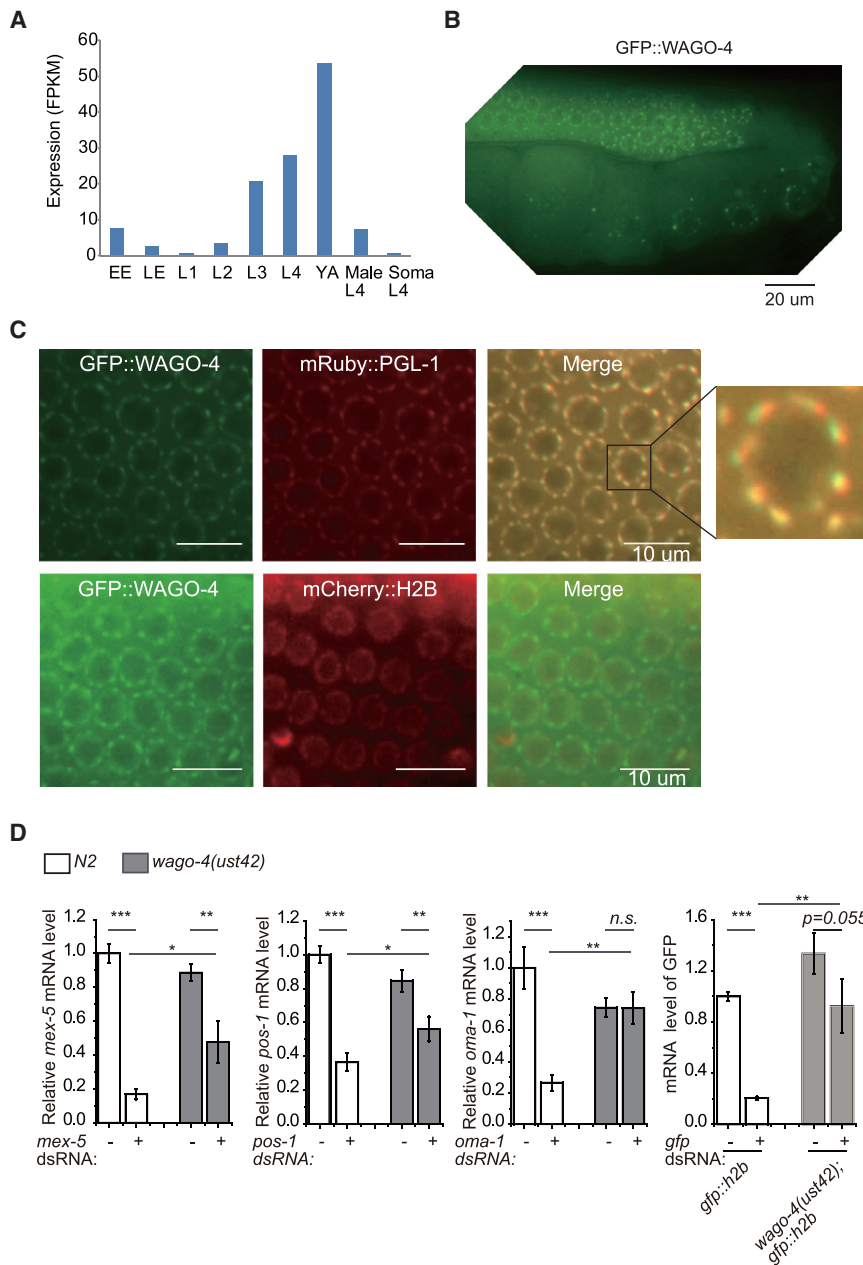


Figure 2. WAGO-4 Is a Germline-Expressed Argonaute Protein

(A) The expression levels of *wago-4* mRNA at different developmental stages. Data were downloaded from Wormbase (version WS260). EE, early embryos; LE, late embryos; YA, young adults. (B) Images of the germline of the GFP::WAGO-4 strain. (C) Pachytene germ cells of GFP::WAGO-4 and the chromatin marker H2B::mCherry and P-granule marker mRuby::PGL-1 were imaged. (D) Wild-type and *wago-4(-)* animals were treated with indicated dsRNAs. Total RNAs were isolated at L4 stage of P0 generation and the indicated mRNAs were quantified by real-time PCR. mean $\pm$ SD. $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant.

is closest to CSR-1, WAGO-10 is closest to HRDE-1, and PPW-2 is closest to WAGO-4 based on sequence comparison (Gu et al., 2009). However, none of these three proteins was required for the inheritance of RNAi targeting the *h2b::gfp* transgene (Figure 3B). These data suggest that either WAGO-4 and the nuclear Argonaute HRDE-1 and NRDE-3 played special roles in RNAi inheritance, or C04F12.1, WAGO-10, and PPW-2 exhibit functional redundancy (Shirayama et al., 2012).

WAGO-4 Acts in Multigenerational Inheritance of RNAi

To test whether *wago-4* is required for multigenerational inheritance of RNAi, we fed germline-expressed *h2b::gfp* animals with *gfp* dsRNA and scored *gfp* levels in later generations. The *h2b::gfp* transgene was silenced in the parental generation and the silencing was maintained to F1 and F2 generations, although at reduced levels (Figure 4A). Both *hrde-1* and *wago-4* were required for the inheritance of *gfp* silencing to F1 and F2 generations.

fed with *gfp* dsRNA, the *sur-5::gfp* was expressed in both F1 embryos and L4 larva (Figure 3A), although not to the levels of the *rde-1* mutant. These results suggest that WAGO-4 is likely engaged in transmitting small RNAs from the parents to zygotes or at least at an earlier step than HRDE-1 to re-establish the silencing state in the progeny.

Previous genetic screens failed to identify WAGO proteins other than HRDE-1 that was required to mediate the inheritance of RNAi targeting *h2b::gfp* transgene (Buckley et al., 2012). The identification of WAGO-4 in RNAi inheritance suggested that these WAGO proteins should be re-investigated individually. We selected three other Argonaute proteins, in which C04F12.1

Then we fed GFP::WAGO-4 animals with dsRNA targeting *oma-1* only in P0 generation and isolated WAGO-4-bound siRNAs in later generations, followed by small RNA deep sequencing. WAGO-4 bound to *oma-1* siRNAs not only in the P0 and F1 generations, but also in the F3 generation (Figures 4B and 4C), suggesting that WAGO-4 act in multigenerational inheritance of RNAi.

Many RNAi inheritance mutants, including *hrde-1*, exhibit a Mrt phenotype that gradually loses the fertility along generations (Buckley et al., 2012; Spracklin et al., 2017). We compared the Mrt phenotype of *hrde-1* and *wago-4* animals by examining their brood size at 25°C (Figure 4D). While *hrde-1* animals gradually

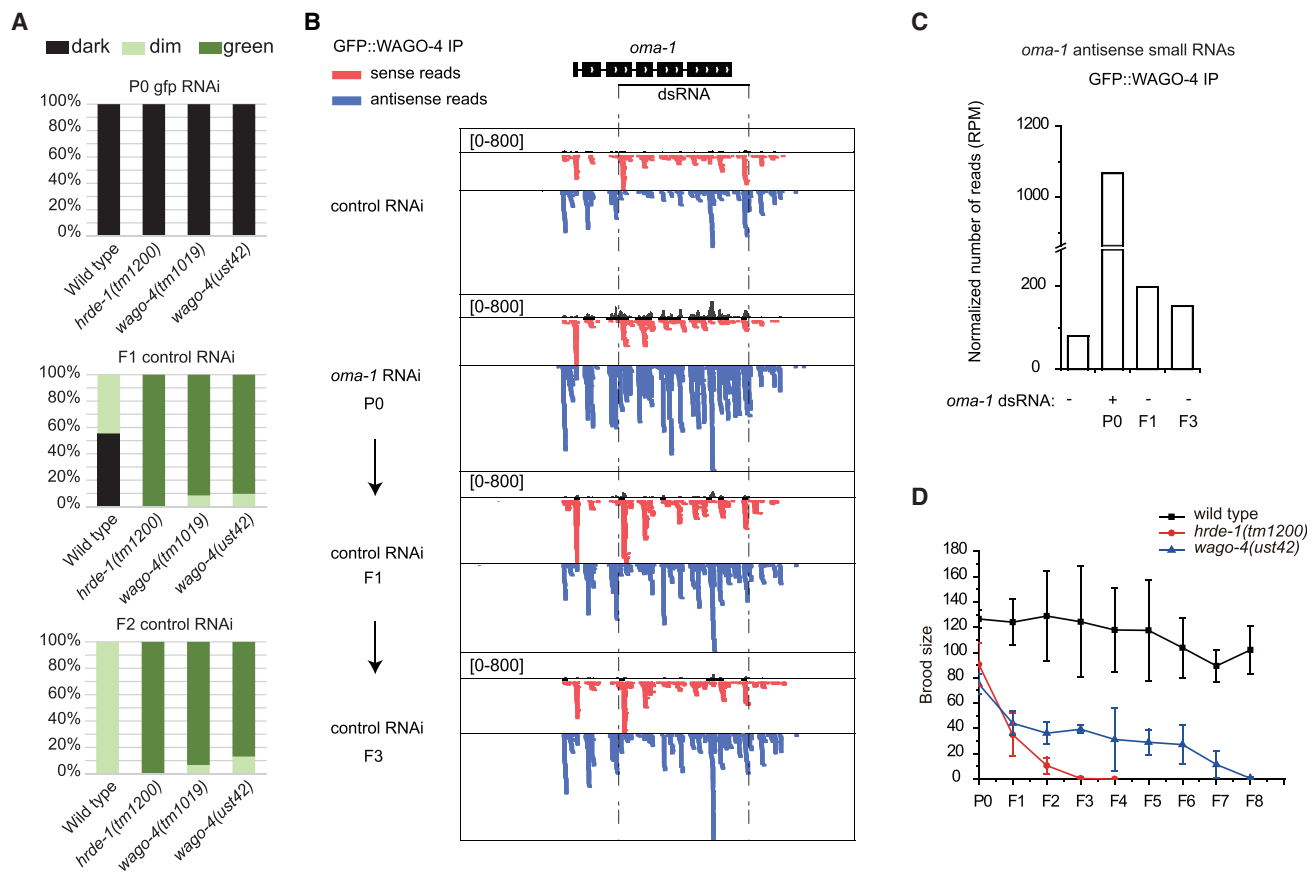


Figure 4. WAGO-4 Acts in Multigenerational Inheritance of RNAi

(A) *pie-1_{pr}::gfp::h2b* transgenic animals were exposed to bacteria expressing *gfp* dsRNA. F1 and F2 embryos were isolated and grown on control bacteria in the absence of further *gfp* dsRNA treatment. Percentage of F1 and F2 animals expressing GFP was counted ($n > 50$).
(B) WAGO-4-associated small RNAs targeting *oma-1* locus is shown. Red ticks are sense reads to *oma-1* sequence, blue ticks are antisense reads. GFP::WAGO-4 animals were treated with *oma-1* dsRNA at P0 generation and then fed on control bacteria in subsequent generations. GFP::WAGO-4 was immunoprecipitated from young adult animals and the associated small RNAs were deep sequenced. Animals fed on control RNAi bacteria were set as control.
(C) Normalized number of antisense reads of indicated animals.
(D) Brood size of indicated animals by Mrt assay at 25°C. $n = 20$, mean $\pm$ SD.

secondary siRNAs, and suggests that WAGO-4 bound to secondary siRNAs (Pak and Fire, 2007).

We further characterized WAGO-4-associated endogenous siRNAs. WAGO-4-associated endogenous siRNAs were 22 nt in length (Figure 5D), and the vast majority of the siRNAs started with G or GA at their 5' ends (Figures 5D and S4B), which are consistent with the properties of 22G-RNA. Approximately 93% of WAGO-4-bound 22G-RNAs target protein-coding genes (Figure 5E). We selected potential WAGO-4 targets that had greater than 25 reads per million and identified 4,774 genes (Table S1).

We then compared WAGO-4-bound 22G-RNAs to other siRNA categories (Gu et al., 2009; Tu et al., 2015; van Wolfswinkel et al., 2009). Consistent with its germline expression, WAGO-4 targets exhibited a pronounced overlap with *gfp-4*-dependent genes (Figure 5F). Although WAGO-1, WAGO-4, and CSR-1 are all expressed in germline, WAGO-4 targets dramatically overlapped with those of CSR-1 but not WAGO-1, suggesting that WAGO-4 and CSR-1 may regulate the same

cohorts of protein-coding genes in the germline. However, although *csr-1* mutant is homozygous lethal and exhibits chromosomal segregation defects (Claycomb et al., 2009; Gerson-Gurwitz et al., 2016), *wago-4* animals are largely normal and have similar brood size to that of wild-type animals at 20°C (Figure S4C). We quantified the mRNA levels of WAGO-4 targets, but failed to detect pronounced desilencing of the mRNA levels in *wago-4* mutants compared to wild-type animals (Figure S4D). These data suggest that CSR-1 and WAGO-4 may function differently in gene regulation and animal development, although they bind to the same cohorts of germline-expressed 22G-RNAs.

WAGO-4-Associated 22G-RNAs Contain Untemplated 3' End Uridylation

We analyzed the 3' ends of WAGO-4-associated 22G-RNAs and found that ~7.6% of them contained untemplated addition of a uracil (Figure 6A). For reads longer than 23 nt, ~31% of them contained an extra U and 24.5% contained extra UU

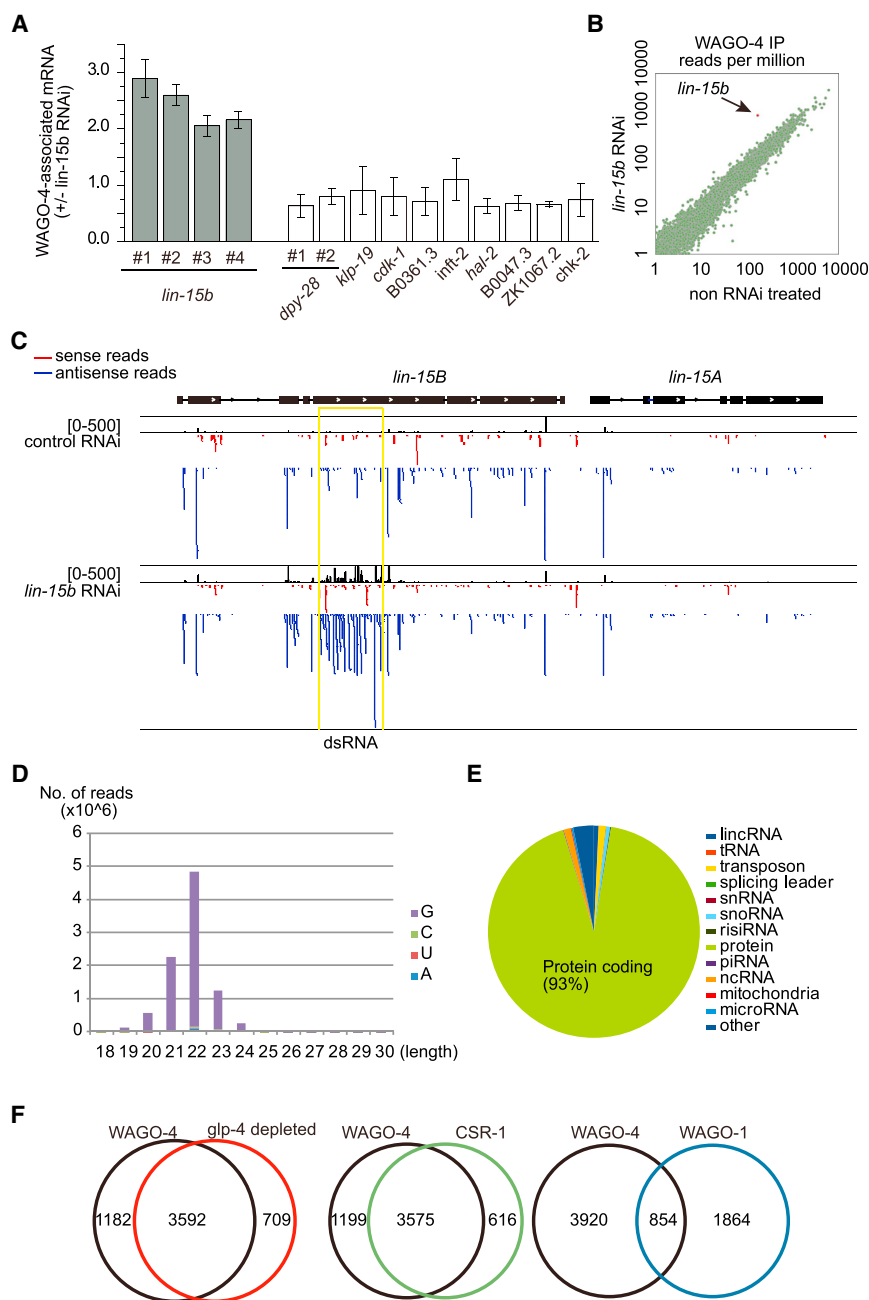


Figure 5. WAGO-4 Binds to 22G-RNAs that Target Germline-Expressed Protein Coding Genes

(A) WAGO-4 was immunoprecipitated at young gravid stage after *lin-15b* RNAi, and the associated RNAs were quantified by real-time PCR. Mean $\pm$ SD. $n = 3$.
(B) WAGO-4 was immunoprecipitated at young gravid stage, and the associated siRNAs were deep sequenced. The number of reads were normalized and then compared.
(C) Reads targeting *lin-15b/a* genomic loci were plotted. Yellow box indicates the dsRNA-targeted region.
(D) The length distribution and the first nucleotides of WAGO-4-associated siRNAs were analyzed.
(E) WAGO-4-associated 22G-RNAs were grouped into different categories.
(F) WAGO-4-associated 22G-RNAs were compared with other known siRNA categories.

ated 22G-RNAs by deep sequencing WAGO-4-associated small RNAs from *cde-1(tm1021);3xFLAG::GFP::WAGO-4* animals. The vast majority of WAGO-4-associated 22G-RNAs still targeted protein-coding genes (Figure S5B) and exhibited a tendency toward GA at 5' end (Figure S5A), suggesting that CDE-1 is not essential for the biogenesis of WAGO-4-associated siRNAs. Yet the untemplated addition of uracil was decreased in *cde-1* mutants. The percentage of an extra U was 7.6% in wild-type animals and 2.5% in *cde-1* animals (Figure 6B). For reads longer than 23 nt, the percentage of an extra U was 31% in wild-type animals and 19% in *cde-1* animals, respectively (Figure 6C).

Noticeably, in *cde-1* mutants, WAGO-4 bound ~24-fold more antisense ribosomal siRNAs (risiRNA) (31,409 reads per million in *cde-1* versus 1,322 reads per million in wild-type animals) and 22-fold more small RNAs related to splicing leaders (Figure S5B). We re-analyzed the published deep sequencing

datasets and identified similar increase of risiRNAs in *cde-1* mutants (data not shown) (van Wolfswinkel et al., 2009). How CDE-1 is involved in risiRNA biogenesis remains unclear (Zhou et al., 2017).

CDE-1-mediated uridylation is thought to destabilize CSR-1-bound 22G-RNAs (van Wolfswinkel et al., 2009). We re-analyzed published data of small RNAs associated with CSR-1 in *cde-1* and wild-type animals (van Wolfswinkel et al., 2009). CSR-1 targets with >25 reads per million were selected. As reported, CSR-1 bound more 22G-RNAs of CSR-1 targets in the *cde-1* mutant (Figure 6D). In contrast, WAGO-4 bound

more 22G-RNAs of WAGO-4 targets in the *cde-1* mutant (Figure 6E). In contrast, WAGO-4 bound

more 22G-RNAs of WAGO-4 targets in the *cde-1* mutant (Figure 6E). In contrast, WAGO-4 bound

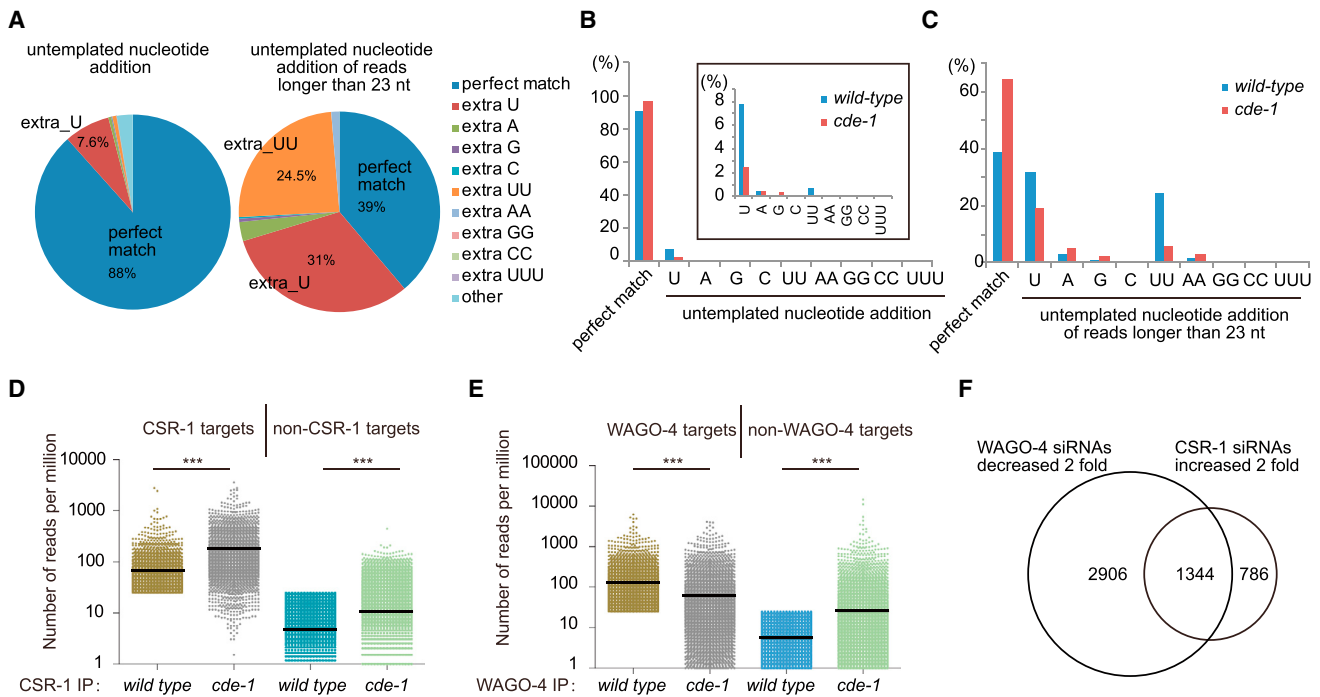


Figure 6. CDE-1 Uridylates WAGO-4-Associated 22G-RNAs

(A) The untemplated 3' end addition of nucleotides in WAGO-4-bound 22G-RNAs was analyzed. Left: total reads. Right: reads longer than 23 nt. (B) Comparison of the untemplated addition of nucleotides in WAGO-4-associated 22G-RNAs in wild-type animals and *cde-1* mutants. The inset plot is a zoom-in of the main figure. (C) Comparison of the untemplated addition of nucleotides in WAGO-4-bound 22G-RNAs for reads longer than 23 nt. (D) Analysis of CSR-1 22G-RNAs in *cde-1* mutants. (E) Analysis of WAGO-4 22G-RNAs in *cde-1* mutants. (F) Comparison of WAGO-4 and CSR-1 22G-RNAs in *cde-1* mutants. *** $p < 0.001$.

fewer 22G-RNAs of WAGO-4 targets in the *cde-1* mutant (Figure 6E). Previous studies suggested that there exists a loading balance between WAGO pathway and CSR-1 pathway, which can be regulated by CDE-1 mediated uridylation (de Albuquerque et al., 2015; Phillips et al., 2015; van Wolfswinkel et al., 2009). We selected WAGO-4-associated 22G-RNAs that were decreased 2-fold and CSR-1-associated 22G-RNAs that were increased 2-fold in the *cde-1* mutant and found that 1,344 genes were decreased in WAGO-4 binding but simultaneously increased in CSR-1 binding (Figure 6F). These data suggest that CDE-1-engaged uridylation likely stabilizes the binding of 22G-RNAs to WAGO-4 but destabilizes the binding to CSR-1.

We then asked whether *cde-1* is required for the inheritance of RNAi. Consistent with a recent report, *cde-1* was not required for exogenous dsRNA to silence the germline-expressed *h2b::gfp* transgene in the parental generation but was imperative for silencing in F1 progeny (Figure S5C) (Spracklin et al., 2017). Taken together, we hypothesize that CDE-1 mediates RNAi inheritance by uridylating WAGO-4-bound 22G-RNAs.

WAGO-4 Promotes the Transmission of siRNAs for RNAi Inheritance

We hypothesized that WAGO-4 is able to directly transmit siRNAs from parents to progeny. Three lines of evidence cumulatively supported this idea. First, WAGO-4 was ex-

pressed in both germline cells and oocytes (Figures 2B and 7A). In -3 to -1 oocytes, WAGO-4 still exhibited distinct foci, but changed localization from peri-nuclear region to cytoplasm. Second, WAGO-4 is expressed in one-cell embryos (Figure 7B). Early in the one-cell stage before chromosomal alignment, WAGO-4 is evenly distributed in the zygote without pronounced aggregation. After chromosomal alignment, WAGO-4 began to accumulate in P-granule foci at the posterior ends. The expression of WAGO-4 in -1 oocyte and the one-cell embryos suggest that WAGO-4 is able to carry siRNAs from parents to progeny.

Third, we used the subcellular localization of GFP::NRDE-3 as a reporter of siRNA abundance to test whether the germline-expressed WAGO-4 is required for the siRNA accumulation in the soma in the progeny. NRDE-3 is a somatic Argonaute protein that localizes to the nucleus when it binds to siRNAs. In *eri-1* animals, NRDE-3 accumulates in the cytoplasm in the absence of siRNA binding (Guang et al., 2008). In *eri-1;GFP::NRDE-3*, but not *eri-1;wago-4;GFP::NRDE-3*, animals, feeding RNAi targeting soma-expressed *dpy-11* and *lin-15b* genes elicited a cytoplasm-to-nucleus translocation of GFP::NRDE-3 in the F1 embryos (Figure 7C). Because the presence of mRNA templates were required for the amplification of secondary siRNAs, the siRNAs targeting soma-expressed genes (*dpy-11* and *lin-15b*) were unable to be amplified in the

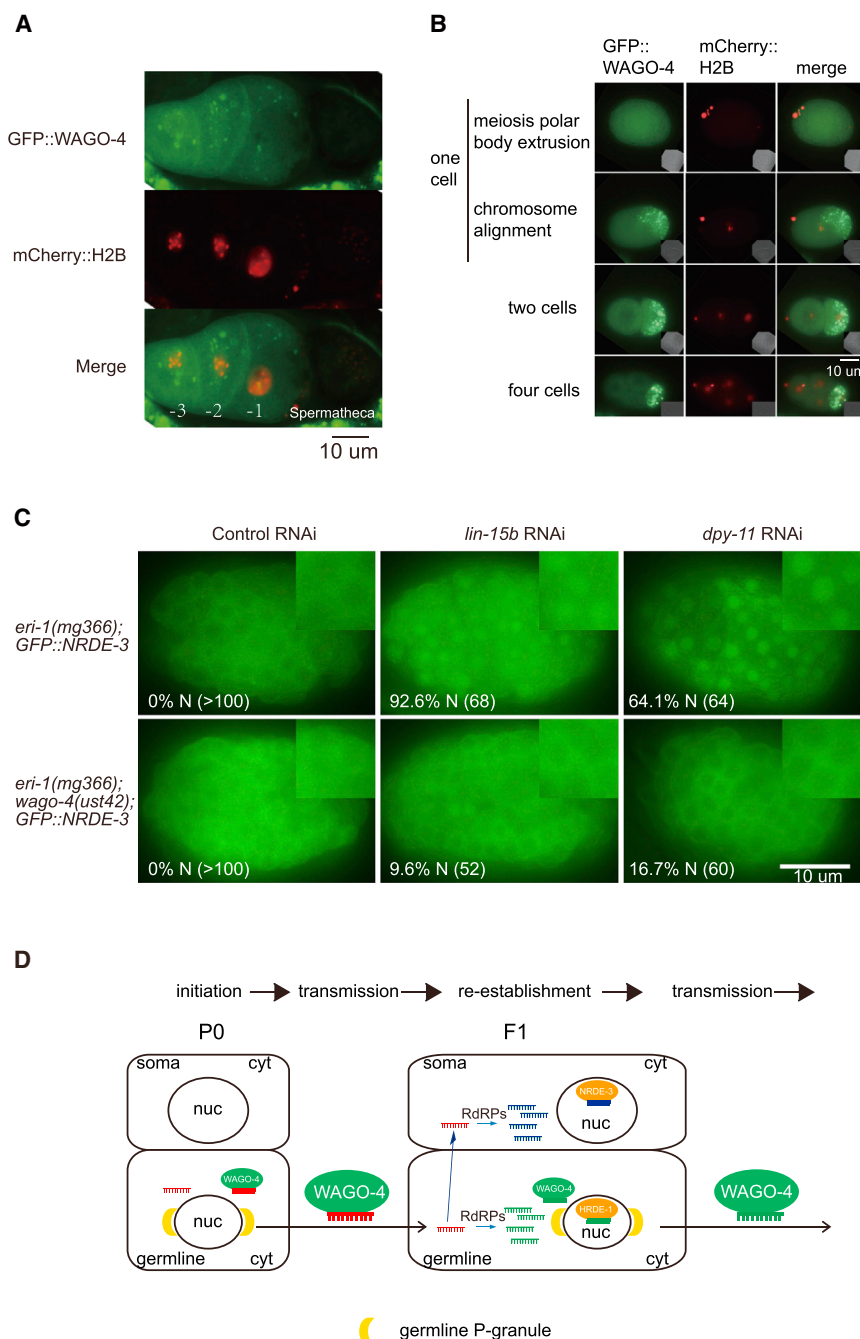


Figure 7. WAGO-4 Promotes Transmission of siRNAs from Parents to Progeny

(A and B) Images of oocytes (A) and early embryos (B) of the *GFP::WAGO-4;mCherry::H2B* strain.

(C) Images of F1 embryos of the indicated animals after feeding RNAi targeting L4 animals for 24 hr. The percentage of nuclear localized GFP::NRDE-3 in F1 embryos was indicated. The number of scored animals were indicated in the parentheses. N, nucleus.

(D) A working model of WAGO-4-mediated inheritance of RNAi. WAGO-4 is involved in transmitting siRNAs along generations as well as conducting gene silencing in the germline. Inherited small RNAs targeting soma-expressed genes are translocated to the soma, amplified, and subsequently silence somatic gene expression. Inherited siRNAs targeting germline-expressed genes are amplified and silence gene expression in the germline of the progeny. WAGO-4 transmits germline siRNAs to the next generation and maintains the silencing status for multiple generations.

DISCUSSION

The inheritance of RNAi in *C. elegans* results from multiple steps, including initiation, transmission, and re-establishment of silencing. Here, we demonstrated that the cytoplasmic Argonaute protein WAGO-4 and the poly(U) polymerase CDE-1 play important roles in promoting inheritance of RNAi.

WAGO-4 Promotes RNAi Inheritance

The nuclear RNAi pathway has been shown to play essential roles in transgenerational inheritance of RNAi (reviewed by Heard and Martienssen, 2014; Rechavi and Lev, 2017). Here, we demonstrated that WAGO-4 promotes the inheritance of RNAi. WAGO-4 partially localized to the perinuclear P-granule foci, suggesting that cytoplasmic RNAi machinery is also involved in the inheritance of RNAi. P-granules contain numerous RNA processing and regulatory factors, including a few WAGOs (Chen et al., 2016). In a

recent genetic screen, Spracklin et al. (2017) identified two cytoplasmic proteins GLH-1/VASA and HRDE-2 in transgenerational inheritance of RNAi, further supporting that the cytoplasmic factors are indispensable for the inheritance of RNAi. Previously, the cytoplasmic Argonaute protein WAGO-1 has been implicated in the maintenance of silencing (Shirayama et al., 2012). Yet another study showed that WAGO-1 is not required for RNAi inheritance of a *pie-1_p::gfp::h2b* transgene (Buckley et al., 2012). Therefore, a systematic investigation of the function of WAGO-1 in RNAi inheritance is needed.

Taken together, these data suggest that WAGO-4 may directly promote the transmission of siRNAs from the parents to the progeny.

Our data suggest that WAGO-4 is involved in the transmission of siRNAs from the parents to progeny. First, WAGO-4 is expressed in both –1 oocyte and the one-cell embryos, suggesting that WAGO-4 is able to carry 22G-RNAs from the parental oocyte to the progeny. Second, using the subcellular localization of GFP::NRDE-3 as a siRNA reporter, we showed that the germline expressed Argonaute protein WAGO-4 is required for the production of siRNAs in the soma. A working model of RNAi inheritance was shown in Figure 7D.

Epigenetic changes can arise in the F1 embryos that are present inside the parents when the parental animals are exposed to environmental stimuli. These phenomena can be called as parental effects or intergenerational epigenetic inheritance (Anava et al., 2015; Heard and Martienssen, 2014; Houri-Zeevi and Rechavi, 2017). The epigenetic changes that are inherited and maintained for more than two or three generations are then called transgenerational inheritance. Stable maintenance of silencing requires an additional class of siRNAs that must be amplified in each generation. These siRNAs can be classified as secondary or tertiary siRNAs (Sapetschnig et al., 2015). WAGO-4 binds to siRNAs in F3 generation after initial RNAi, suggesting that WAGO-4 is engaged in transgenerational inheritance of RNAi and binds to secondary and tertiary siRNAs.

WAGO-4- and CSR-1-associated 22G-RNAs target similar cohorts of germline-expressed protein-coding genes. However, *csr-1* mutant is homozygous lethal, while *wago-4* mutants are largely normal when grew at 20°C, suggesting that these two Argonaute proteins may use different gene regulatory mechanisms (Shirayama et al., 2012). Interestingly, although WAGO-4 lacks the DDH catalytic triad of amino acids considered necessary for Argonaute-based slicer activity (Yigit et al., 2006), it was required for the silencing of the targeted mRNAs.

Untemplated Uridylation by CDE-1 Is Required for RNAi Inheritance

An important question is how Argonaute proteins selectively identify and bind to their siRNA ligands from a pool of short nuclear acids in the cell. In *Arabidopsis*, the 5' terminal nucleotide directs the sorting of small RNAs into different Argonaute complexes (Mi et al., 2008; Montgomery et al., 2008; Takeda et al., 2008). In *C. elegans*, 22G-RNAs may be sorted to distinct Argonaute proteins upon target recognition. Here, our data suggested that untemplated uridylation at the 3' ends may function as an additional sorting signal by modulating the binding affinity of 22G-RNAs to different Argonaute proteins (de Albuquerque et al., 2015; Phillips et al., 2015; van Wolfswinkel et al., 2009). Alternatively, uridylation of 22G-RNAs may change their binding affinity to only one Argonaute, while more or less of the rest 22G-RNAs bind to the other Argonaute proteins.

The poly(U) polymerase CDE-1 (also known as PUP-1 and CID-1) adds untemplated uracil to the 3' termini of RNAs in *C. elegans* (Kwak and Wickens, 2007; van Wolfswinkel et al., 2009). Previous studies suggested that a small RNA loading balance between WAGO pathway and CSR-1 pathway may exist, which is regulated by CDE-1 mediated uridylation (de Albuquerque et al., 2015; Phillips et al., 2015; van Wolfswinkel et al., 2009). Because CDE-1 interacts with a RNA-dependent RNA polymerase EGO-1, but not with the Argonaute protein CSR-1 (van Wolf-

swinkel et al., 2009), and EGO-1 is required for the production of 22G-RNAs and acts upstream of the loading of 22G-RNAs to Argonaute proteins, we speculated that uridylation happens upstream of small RNA loading to Argonaute proteins in *C. elegans*. Interestingly, in fission yeast, the uridyl transferase Cid16 can add non-templated nucleotides to Argonaute-bound small RNAs *in vitro* (Pisacane and Halic, 2017). Thus, we could not exclude the possibility that CDE-1 might be able to uridylate Argonaute-bound siRNAs after loading, because we were only measuring the steady-state levels of Argonaute-bound siRNAs by immunoprecipitation from worm extracts. Further *in vitro* biochemical experiments are required to examine the binding affinities of uridylated 22G-RNAs to different Argonaute proteins and how 3' uridylation affects the stability and target recognition of small RNAs.

Noticeably, Spracklin et al. (2017) identified that CDE-1 is required for the inheritance of RNAi, for which the mechanism is not fully understood. Our data suggest that CDE-1 may mediate RNAi inheritance by regulating the binding of 22G-RNAs to WAGO-4. Yet *cde-1* mutants exhibited a weaker inheritance defects than *wago-4* animals. Therefore, the function of CDE-1 in RNAi inheritance needs further investigation.

EXPERIMENTAL PROCEDURES

Strains

Bristol strain N2 was used as the standard wild-type strain. All strains were grown at 20°C. The strains used in this study are listed in the Supplemental Experimental Procedures.

RNAi

RNAi experiments were conducted as described previously (Timmons et al., 2001). HT115 bacteria expressing the empty vector L4440 (a gift from A. Fire) were used as controls. Bacterial clones expressing dsRNA were obtained from the Ahringer RNAi library (Kamath et al., 2003) and were sequenced to verify their identities. *lin-15b* RNAi clones were described previously (Guang et al., 2008).

RNAi Inheritance Assay

Synchronized L1 animals of the indicated genotypes were exposed to bacteria expressing *gfp* dsRNA. F1 embryos were collected by hypochlorite/NaOH treatment and grown on HT115 control bacteria. The expression levels of GFP in both parental generation and progeny were visualized and scored.

Images were collected on Leica DM2500 microscope. For the quantitation of GFP intensity, the average fluorescence intensities of L4 worms (0.5-s exposure) and embryos (1-s exposure) were analyzed using ImageJ (Borges and Martienssen, 2015). The background of each individual animal was subtracted using “the rolling ball radius” model of 3 × 3 pixels with the smoothing feature disabled. For subcellular localization analysis, ~10 germlines were imaged and analyzed by ImageJ.

Images of co-localization analysis were collected on Leica DM4 B microscope system.

Mrt Assay

wago-4(ust42)(2x) was successively outcrossed twice with N2 animals to get *wago-4(ust42)(4x)* before conducting Mrt experiments. Twenty larval stage L3 animals were singled onto OP50 plates at each generation and cultured at 25°C. The average brood size at each generation was calculated.

Construction of Plasmids and Transgenic Strains

For 3xFLAG::GFP::WAGO-4, the 3xFLAG::GFP coding region was PCR amplified from plasmid pSG085 with the primers 5'-AGCTCTTCCTATGGACTA CAAAGACCATGAC-3' and 5'-ATAGCTCCACCTCCACCTCTTTGTATAGTT CATCCATGCC-3'. The predicted *wago-4* promoter was amplified from N2 genomic DNA with primers 5'-CGTGGATCCAGATATCTGCAGGTTCCGT

TACGCTCTCTCTCCAG-3' and 5'-TGTAAGTCCATAGGAAGAGCTGGCATCCTTCTC-3'. The *wago-4* coding region and predicted 3' UTR were then amplified by PCR from N2 genomic DNA with primers 5'-GGAGGTGGAGGAGGTG GAGCTATGCCAGCTCTTCTCCAGTC-3' and 5'-GACTCACTAGTGGGCA GATCTCATCCCTGATGCCAAGCCGAC-3'. The plasmid pCFJ151 was digested with *Sbf*I and *Bgl*II. The three above PCR fragments were cloned into linearized pCFJ151 with the ClonExpress MultiS One Step Cloning kit (Vazyme C113-02) to generate a 3xFLAG::GFP::WAGO-4 fusion gene. The transgene was then integrated into the *C. elegans* genome using the *mos1*-mediated single copy insertion (*MosSCI*) method (Frøkjær-Jensen et al., 2014).

Dual sgRNA-Directed *wago-4* Deletion

Dual sgRNA-guided chromosome deletion was conducted as previously described (Chen et al., 2014). We manually searched for target sequences consisting of G(N)19NGG near the desired mutation sites in N2 genomic region. We replaced the *unc-119* target sequence in the pU6::unc-119 sgRNA vector with the desired target sequence using overlapped extension PCR with primer pairs sgRNA-1F: 5'-GAAGATCCGCTCATGGTCAAGTTTGTAGAG CTAGAAATAGCAAGTTA-3' and sgRNA-1R: 5'-CATGAGCGGATCTTCAAA CATTTAGATTGCAATTCAATTATATAG-3'; sgRNA-2F: 5'-GAATATTGCACC CGGTATTTGTTTTAGAGCTAGAAATAGCAAGTTA-3' and sgRNA-2R: 5'-CCG GGTGCAATATTCAACATTAGATTGCAATTCAATTATATAG-3'; sgRNA-3F: 5'-GTTGAGAGAAGGCGCAGTAAGTTTGTAGAGCTAGAAATAGCAAGTTA-3' and sgRNA-3R: 5'-GCGCCTTCTCTGAACAAACATTTAGATTGCAATTCAAT TATATAG-3'. Plasmid mixtures containing 50 ng/μL each of pU6::wago4 sgRNA1-3, 50 ng/μL Cas9 expression plasmid, 5 ng/μL pCFJ90, and 5 ng/μL pCFJ104 were co-injected into N2 animals. The deletion mutants were screened by PCR.

qRT-PCR

RNAs were isolated from indicated animals using a dounce homogenizer (pestle B) in TRIzol solution followed by DNase I digestion (QIAGEN), as described previously (Guang et al., 2008). cDNAs were generated from RNAs using the iScript cDNA Synthesis Kit (Bio-Rad) according to the vendor's protocol. qPCR was performed using a MyIQ2 real-time PCR system (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad). The primers that were used in qRT-PCR are provided in the Supplemental Experimental Procedures. *eft-3* mRNA was used as an internal control for sample normalization. Data analysis was performed using a $\Delta\Delta$ CT approach.

RNA Immunoprecipitation

RNA immunoprecipitation (RIP) experiments were performed as previously described with adult animals (Guang et al., 2008). Briefly, gravid adults were sonicated in sonication buffer (20 mM Tris-HCl [pH 7.5], 200 mM NaCl, 2.5 mM MgCl₂, and 0.5% NP-40). The lysate was pre-cleared with protein G-agarose beads (Roche) and incubated with anti-FLAG M2 agarose beads (Sigma #A2220). The beads were washed extensively and 3xFLAG::GFP::WAGO-4 and its associated RNAs were eluted with 100 μg/mL 3xFLAG peptide (Sigma). The eluates were incubated with TRIzol reagent followed by isopropanol precipitation. WAGO-4-bound RNAs were quantified by real-time PCR.

Isolation and Sequencing of WAGO-4-Associated Small RNAs

WAGO-4-associated siRNAs were isolated from gravid adults as described above. The precipitated RNAs were treated with calf intestinal alkaline phosphatase (CIAP, Invitrogen), re-extracted with TRIzol, and treated with T4 polynucleotide kinase (T4 PNK, New England Biolabs) in the presence of 1 mM ATP (Zhou et al., 2014).

WAGO-4-bound siRNAs were subjected to deep sequencing using an Illumina platform, according to the manufacturer's instructions by the Beijing Genomics Institute (BGI Shenzhen). Small RNAs ranging from 18 to 30 nt were gel-purified and ligated to a 3' adaptor (5'-pUCGUAUGCCGUCUUCUGCUU GdT-3'; p, phosphate; idT, inverted deoxythymidine) and a 5' adaptor (5'-GU UCAGAGUUCUACAGUCCGACGAUC-3'). The ligation products were gel-purified, reverse transcribed, and amplified using Illumina's sRNA primer set (5'-CAAGCAGAAGACGGCATACGA-3'; 5'-AATGATACGCGACCAACCGA-3'). The samples were then sequenced using an Illumina HiSeq platform.

The Illumina-generated raw reads were first filtered to remove adaptors, low-quality tags, and contaminants to obtain clean reads at BGI Shenzhen. Clean reads ranging from 18 to 30 nt were mapped to the unmasked *C. elegans* genome and the transcriptome assembly WS243, respectively, using Bowtie2 with default parameters (Langmead and Salzberg, 2012). The number of reads targeting each transcript was counted using custom Perl scripts and displayed by IGV (Thorvaldsdóttir et al., 2013). The number of total reads mapped to the genome minus the number of total reads corresponding to sense rRNA transcripts (5S, 5.8S, 18S, and 26S) was used as the normalization number to exclude the possible degradation fragments of sense rRNAs.

Statistics

Bar graphs with error bars were presented with mean and SD. All the experiments were conducted with independent *C. elegans* animals for indicated times. Statistical analysis was performed with two-tailed Student's *t* test.

DATA AND SOFTWARE AVAILABILITY

Argonaute-associated small RNAs from published data were re-analyzed. CSR-1 IP in wild-type animals and in *cde-1* mutants, NCBI: GSE17787 (van Wolfswinkel et al., 2009); WAGO-1 IP, NCBI: SRR030711 (Gu et al., 2009); *glp-4(bn2)*, NCBI: GSM455394 (Gu et al., 2009); WAGO-4 IP, NCBI: GSE112475.

All other data and materials are available upon request.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.04.072>.

ACKNOWLEDGMENTS

We are grateful to Dr. Scott Kennedy and members of the Guang lab for their comments. We are grateful to Dr. Ho Yi Mak, the International *C. elegans* Gene Knockout Consortium, and the National Bioresource Project for providing the strains. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). This work was supported by grants from the Chinese Ministry of Science and Technology (2017YFA0102903), the National Natural Science Foundation of China (81501329, 31671346, and 91640110), Anhui Natural Science Foundation (1608085MC50), and the Major/Innovative Program of Development Foundation of Hefei Center for Physical Science and Technology (2017FXZY005).

AUTHOR CONTRIBUTIONS

F.X., X.F., and S.G. designed the experiments. F.X., X.F., X.C., C.W., Q.Y., T.X., and M.H. performed experiments. F.X., X.F., and S.G. analyzed the data. F.X., X.F., and S.G. wrote the manuscript. All authors have discussed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: December 17, 2017

Revised: March 24, 2018

Accepted: April 16, 2018

Published: May 22, 2018

REFERENCES

Alcazar, R.M., Lin, R., and Fire, A.Z. (2008). Transmission dynamics of heritable silencing induced by double-stranded RNA in *Caenorhabditis elegans*. *Genetics* 180, 1275–1288.

- Anava, S., Posner, R., and Rechavi, O. (2015). The soft genome. *Worm* 3, e989798.
- Ashe, A., Sapetschnig, A., Weick, E.M., Mitchell, J., Bagijn, M.P., Cording, A.C., Doebley, A.L., Goldstein, L.D., Lehrbach, N.J., Le Pen, J., et al. (2012). piRNAs can trigger a multigenerational epigenetic memory in the germline of *C. elegans*. *Cell* 150, 88–99.
- Borges, F., and Martienssen, R.A. (2015). The expanding world of small RNAs in plants. *Nat. Rev. Mol. Cell Biol.* 16, 727–741.
- Buckley, B.A., Burkhart, K.B., Gu, S.G., Spracklin, G., Kershner, A., Fritz, H., Kimble, J., Fire, A., and Kennedy, S. (2012). A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. *Nature* 489, 447–451.
- Burton, N.O., Burkhart, K.B., and Kennedy, S. (2011). Nuclear RNAi maintains heritable gene silencing in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* 108, 19683–19688.
- Burton, N.O., Furuta, T., Webster, A.K., Kaplan, R.E., Baugh, L.R., Arur, S., and Horvitz, H.R. (2017). Insulin-like signalling to the maternal germline controls progeny response to osmotic stress. *Nat. Cell Biol.* 19, 252–257.
- Chen, X., Xu, F., Zhu, C., Ji, J., Zhou, X., Feng, X., and Guang, S. (2014). Dual sgRNA-directed gene knockout using CRISPR/Cas9 technology in *Caenorhabditis elegans*. *Sci. Rep.* 4, 7581.
- Chen, J.X., Cipriani, P.G., Mecnas, D., Polanowska, J., Piano, F., Gunsalus, K.C., and Selbach, M. (2016). In vivo interaction proteomics in *Caenorhabditis elegans* embryos provides new insights into P granule dynamics. *Mol. Cell. Proteomics* 15, 1642–1657.
- 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.
- de Albuquerque, B.F., Placentino, M., and Ketting, R.F. (2015). Maternal piRNAs are essential for germline development following de novo establishment of endo-siRNAs in *Caenorhabditis elegans*. *Dev. Cell* 34, 448–456.
- Frokjær-Jensen, C., Davis, M.W., Sarov, M., Taylor, J., Flibotte, S., LaBella, M., Pozniakovskiy, A., Moerman, D.G., and Jorgensen, E.M. (2014). Random and targeted transgene insertion in *Caenorhabditis elegans* using a modified Mos1 transposon. *Nat. Methods* 11, 529–534.
- Gerson-Gurwitz, A., Wang, S., Sathe, S., Green, R., Yeo, G.W., Oegema, K., and Desai, A. (2016). A small RNA-catalytic argonaute pathway tunes germline transcript levels to ensure embryonic divisions. *Cell* 165, 396–409.
- Grishok, A., Tabara, H., and Mello, C.C. (2000). Genetic requirements for inheritance of RNAi in *C. elegans*. *Science* 287, 2494–2497.
- 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.
- Gu, S.G., Pak, J., Guang, S., Maniar, J.M., Kennedy, S., and Fire, A. (2012). Amplification of siRNA in *Caenorhabditis elegans* generates a transgenerational sequence-targeted histone H3 lysine 9 methylation footprint. *Nat. Genet.* 44, 157–164.
- Guang, S., Bochner, A.F., Pavelec, D.M., Burkhart, K.B., Harding, S., Lachowicz, J., and Kennedy, S. (2008). An Argonaute transports siRNAs from the cytoplasm to the nucleus. *Science* 321, 537–541.
- Heard, E., and Martienssen, R.A. (2014). Transgenerational epigenetic inheritance: myths and mechanisms. *Cell* 157, 95–109.
- Houri-Zeevi, L., and Rechavi, O. (2017). A matter of time: small RNAs regulate the duration of epigenetic inheritance. *Trends Genet.* 33, 46–57.
- Kalinava, N., Ni, J.Z., Peterman, K., Chen, E., and Gu, S.G. (2017). Decoupling the downstream effects of germline nuclear RNAi reveals that H3K9me3 is dispensable for heritable RNAi and the maintenance of endogenous siRNA-mediated transcriptional silencing in *Caenorhabditis elegans*. *Epigenetics Chromatin* 10, 6.
- Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., et al. (2003). Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* 421, 231–237.
- Klosin, A., Casas, E., Hidalgo-Carcedo, C., Vavouri, T., and Lehner, B. (2017). Transgenerational transmission of environmental information in *C. elegans*. *Science* 356, 320–323.
- Kwak, J.E., and Wickens, M. (2007). A family of poly(U) polymerases. *RNA* 13, 860–867.
- Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359.
- Lev, I., Seroussi, U., Gingold, H., Bril, R., Anava, S., and Rechavi, O. (2017). MET-2-dependent H3K9 methylation suppresses transgenerational small RNA inheritance. *Curr. Biol.* 27, 1138–1147.
- Luteijn, M.J., van Bergeijk, P., Kaaij, L.J., Almeida, M.V., Roovers, E.F., Berezhikov, E., and Ketting, R.F. (2012). Extremely stable Piwi-induced gene silencing in *Caenorhabditis elegans*. *EMBO J.* 31, 3422–3430.
- Mao, H., Zhu, C., Zong, D., Weng, C., Yang, X., Huang, H., Liu, D., Feng, X., and Guang, S. (2015). The Nrde pathway mediates small-RNA-directed histone H3 lysine 27 trimethylation in *Caenorhabditis elegans*. *Curr. Biol.* 25, 2398–2403.
- Marré, J., Traver, E.C., and Jose, A.M. (2016). Extracellular RNA is transported from one generation to the next in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* 113, 12496–12501.
- Mi, S., Cai, T., Hu, Y., Chen, Y., Hodges, E., Ni, F., Wu, L., Li, S., Zhou, H., Long, C., et al. (2008). Sorting of small RNAs into Arabidopsis argonaute complexes is directed by the 5' terminal nucleotide. *Cell* 133, 116–127.
- Minkina, O., and Hunter, C.P. (2017). Stable heritable germline silencing directs somatic silencing at an endogenous locus. *Mol. Cell* 65, 659–670.
- Miska, E.A., and Ferguson-Smith, A.C. (2016). Transgenerational inheritance: models and mechanisms of non-DNA sequence-based inheritance. *Science* 354, 59–63.
- Montgomery, T.A., Howell, M.D., Cuperus, J.T., Li, D., Hansen, J.E., Alexander, A.L., Chapman, E.J., Fahlgren, N., Allen, E., and Carrington, J.C. (2008). Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. *Cell* 133, 128–141.
- Pak, J., and Fire, A. (2007). Distinct populations of primary and secondary effectors during RNAi in *C. elegans*. *Science* 315, 241–244.
- Phillips, C.M., Brown, K.C., Montgomery, B.E., Ruvkun, G., and Montgomery, T.A. (2015). piRNAs and piRNA-dependent siRNAs protect conserved and essential *C. elegans* genes from misrouting into the RNAi pathway. *Dev. Cell* 34, 457–465.
- Pisacane, P., and Halic, M. (2017). Tailing and degradation of Argonaute-bound small RNAs protect the genome from uncontrolled RNAi. *Nat. Commun.* 8, 15332.
- Rechavi, O., and Lev, I. (2017). Principles of transgenerational small RNA inheritance in *Caenorhabditis elegans*. *Curr. Biol.* 27, R720–R730.
- Sakaguchi, A., Sarkies, P., Simon, M., Doebley, A.L., Goldstein, L.D., Hedges, A., Ikegami, K., Alvares, S.M., Yang, L., LaRocque, J.R., et al. (2014). *Caenorhabditis elegans* RSD-2 and RSD-6 promote germ cell immortality by maintaining small interfering RNA populations. *Proc. Natl. Acad. Sci. USA* 111, E4323–E4331.
- Sapetschnig, A., Sarkies, P., Lehrbach, N.J., and Miska, E.A. (2015). Tertiary siRNAs mediate paramutation in *C. elegans*. *PLoS Genet.* 11, e1005078.
- Shirayama, M., Seth, M., Lee, H.C., Gu, W., Ishidate, T., Conte, D., Jr., and Mello, C.C. (2012). piRNAs initiate an epigenetic memory of nonself RNA in the *C. elegans* germline. *Cell* 150, 65–77.
- Spracklin, G., Fields, B., Wan, G., Vijayendran, D., Wallig, A., Shukla, A., and Kennedy, S. (2017). Identification and characterization of *Caenorhabditis elegans* RNAi inheritance machinery. *Genetics* 206, Published online May 22, 2017. <https://doi.org/10.1534/genetics.116.198812>.
- Takeda, A., Iwasaki, S., Watanabe, T., Utsumi, M., and Watanabe, Y. (2008). The mechanism selecting the guide strand from small RNA duplexes is different among argonaute proteins. *Plant Cell Physiol.* 49, 493–500.

- Thorvaldsdóttir, H., Robinson, J.T., and Mesirov, J.P. (2013). Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief. Bioinform.* *14*, 178–192.
- Timmons, L., Court, D.L., and Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* *263*, 103–112.
- Tu, S., Wu, M.Z., Wang, J., Cutter, A.D., Weng, Z., and Claycomb, J.M. (2015). Comparative functional characterization of the CSR-1 22G-RNA pathway in *Caenorhabditis nematodes*. *Nucleic Acids Res.* *43*, 208–224.
- van Wolfswinkel, J.C., Claycomb, J.M., Batista, P.J., Mello, C.C., Berezikov, E., and Ketting, R.F. (2009). CDE-1 affects chromosome segregation through uridylation of CSR-1-bound siRNAs. *Cell* *139*, 135–148.
- Vastenhouw, N.L., Brunschwig, K., Okihara, K.L., Müller, F., Tijsterman, M., and Plasterk, R.H. (2006). Gene expression: long-term gene silencing by RNAi. *Nature* *442*, 882.
- Wedeles, C.J., Wu, M.Z., and Claycomb, J.M. (2013). Protection of germline gene expression by the *C. elegans* Argonaute CSR-1. *Dev. Cell* *27*, 664–671.
- Weiser, N.E., Yang, D.X., Feng, S., Kalinava, N., Brown, K.C., Khanikar, J., Freeberg, M.A., Snyder, M.J., Csankovszki, G., Chan, R.C., et al. (2017). MORC-1 integrates nuclear RNAi and transgenerational chromatin architecture to promote germline immortality. *Dev. Cell* *41*, 408–423.
- 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.
- Zhou, X., Xu, F., Mao, H., Ji, J., Yin, M., Feng, X., and Guang, S. (2014). Nuclear RNAi contributes to the silencing of off-target genes and repetitive sequences in *Caenorhabditis elegans*. *Genetics* *197*, 121–132.
- Zhou, X., Feng, X., Mao, H., Li, M., Xu, F., Hu, K., and Guang, S. (2017). RdRP-synthesized antisense ribosomal siRNAs silence pre-rRNA via the nuclear RNAi pathway. *Nat. Struct. Mol. Biol.* *24*, 258–269.