Diversification of small RNA pathways underlies germline RNAi incompetence in wild *C. elegans* strains

Han Ting Chou¹, Francisco Valencia¹, Jacqueline C. Alexander¹, Avery Davis Bell¹, Diptodip Deb¹, Daniel A. Pollard², Annalise B. Paaby^{1,3}

1. School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA, USA

2. Biology Department, Western Washington University, Bellingham, WA, USA

3. Correspondence: paaby@gatech.edu

ABSTRACT

The discovery that experimental delivery of dsRNA can induce gene silencing at target genes revolutionized genetics research, by both uncovering essential biological processes and creating new tools for developmental geneticists. However, wild-type *C. elegans* strains vary dramatically in their response to exogenous RNAi, challenging our characterization of RNAi in the lab relative to its activity and significance in nature. Here, we investigate why some strains fail to mount a robust RNAi response to germline targets. We observe diversity in mechanism: in some strains, the response is stochastic, either on or off among individuals, while in others the response is consistent but delayed. Increased activity of the Argonaute PPW-1, which is required for germline RNAi in the laboratory strain N2, rescues the response in some strains, but dampens it further in others. Across strains, we observe variability in expression of known RNAi genes and strain-specific instances of pseudogenization and allelic divergence. Our results support the conclusions that Argonautes share overlapping functions, that germline RNAi incompetence is strain-specific but likely caused by genetic variants at common genes, and that RNAi pathways are evolving rapidly and dynamically. This work expands our understanding of RNAi by identifying conserved and variable pathway components, and it offers new access into characterizing gene function, identifying pathway interactions, and elucidating the biological significance of RNAi.

INTRODUCTION

The landscape of molecular and developmental genetics in *C. elegans* has been transformed by the ability to silence genes by feeding worms *E. coli* bacteria engineered to express RNA matching worm gene targets (Kamath *et al.*, 2003; Rual *et al.*, 2004; Timmons & Fire, 1998). This tool emerged from the seminal discovery that double stranded RNA induces gene suppression (Fire *et al.*, 1998). The discovery, awarded the Nobel prize in 2006, launched research into new mechanisms of gene regulation and led to the eventual characterization of the microRNA (miRNA), short interfering RNA (siRNA), and PIWI-interacting (piRNA) pathways. These pathways overlap in gene set and molecular mechanisms, but differ in origination of the RNA trigger molecules and mediate processes as diverse as gene regulation in cell growth and tissue differentiation, adaptive immunity against pathogens, transgenerational epigenetic inheritance, and germline defense against transposons (Grishok, 2013; Wilson & Doudna, 2013). Many of the genes that encode this machinery are shared across plants, animals, and fungi, and appear deeply conserved within the eukaryotic lineage (Shabalina & Koonin, 2008; Wynant *et al.*, 2017).

The umbrella term “RNA interference” (RNAi) refers to the general mechanism of gene silencing via dsRNA, and those shared pathways that mediate it across diverse processes (Yigit *et al.*, 2006). The complexity of RNAi is reflected in the many overlapping ways its activity can be classified: by endogenous biogenesis of the small RNA trigger versus exogenous or environmental delivery; as nuclear RNAi, which induces transcriptional gene silencing, versus post-transcriptional cytoplasmic RNAi; as a phenomenon localized to the cell in which the dsRNA trigger occurs, versus systemic RNAi, which involves transport mechanisms; by silencing genes expressed in the soma versus targets in the germline. These classifications emerged as experiments revealed distinct genetic mechanisms among them. However, all RNAi processes silence complementary mRNA targets via the association of small RNAs with Argonaute effector proteins (Wilson & Doudna, 2013).

The Argonaute superfamily includes the ancient AGO proteins; the PIWI Argonautes, which are conserved in animals; and in *C. elegans*, the WAGO proteins, an expanded clade of Argonautes specific to nematodes (Youngman & Claycomb, 2014). The expansion of Argonautes in nematodes may underlie the extraordinary diversification of worms across environments, perhaps having

enabled adaptations associated with environmental sensing, parasitism, and immunity (*Buck & Blaxter, 2013*). Several viruses that infect *C. elegans* and other *Caenorhabditis* species have been discovered, notably in isolates with defective RNAi, and antiviral immunity shows a clear association with an active RNAi response (*Félix et al., 2011; Sarkies et al., 2013; Schott et al., 2005; Wilkins et al., 2005; Yigit et al., 2006*). Yet, experimental RNAi appears partially distinct from the antiviral response, as variation in RNAi sensitivity does not completely correlate with immunity, and the systemic and transgenerational properties of RNAi are not observed in viral infection (*Ashe et al., 2013, 2015; Félix et al., 2011*). The adaptive functionalization of RNAi genes and pathways across processes emphasizes the essentiality of RNAi, but our understanding of its biological significance remains incomplete.

Although *C. elegans* sits at the epicenter of expansive research programs into gene silencing by small RNAs, worms vary significantly in capacity for RNAi. Across the *Caenorhabditis* genus, some species, like *C. elegans*, will mount an RNAi response upon environmental exposure to dsRNA, but some require injection of the trigger, indicating a deficiency in spreading the response systemically, and some show no response (*Nuez & Félix, 2012; Winston et al., 2007*). Within *C. elegans*, wild-type strains also vary. The universal laboratory strain N2 is robustly sensitive to RNAi, but RNAi response varies quantitatively among other isolates, for targets in both the germline and soma, and some strains show incompetence for RNAi by feeding or injection (*Félix et al., 2011; Paaby et al., 2015; Tijsterman et al., 2002*). For example, the Hawaiian isolate CB4856 appears largely insensitive to RNAi in the germline. Its insensitivity has been mapped to the *ppw-1* locus, which encodes a WAGO Argonaute, and is presumably explained by a single nucleotide deletion producing a frameshift and early stop in the first half of the transcript (*Tijsterman et al., 2002*). To date, this is the only causal variant identified for natural differences in RNAi, though genetic mapping crosses indicate that the weak germline RNAi in CB4856 is genetically complex and likely involves additional factors (*Elvin et al., 2011; Pollard & Rockman, 2013; Tijsterman et al., 2002*).

In this study, we investigate the genetic basis of germline RNAi deficiency in wild *C. elegans* strains. Earlier work employing a high throughput RNAi assay targeting maternal-effect embryonic genes in wild isolates revealed a spectrum of germline RNAi competence, from strains with negligible response on most targets to several that were more sensitive than the

laboratory strain N2 (*Paaby et al., 2015*). Here, we evaluate strains with negligible germline RNAi, with the goals of, firstly, accurately measuring RNAi activity, and secondly, characterizing mechanisms of failure. We evaluate the response at the organismal, cellular, and gene expression levels, and observe distinct variations in how and why germline RNAi fails. We also observe meaningful allelic variation at known RNAi genes in these strains and in the *C. elegans* population at large, suggesting that selection has diversified RNAi activity at both the sequence and functional levels.

RESULTS

Germline RNAi varies in expressivity and penetrance over reproductive age and among genotypes

Previous work has shown that the WAGO Argonaute PPW-1 is essential for germline RNAi. In the RNAi-responsive laboratory strain N2, a 1.5kb intragenic deletion spanning the PAZ and PIWI domains in *ppw-1* (*pk1425*) eliminates germline RNAi by feeding (*Tijsterman et al., 2002; Yigit et al., 2006*). Germline RNAi deficiency in the Hawaiian isolate CB4856, which is one of the most insensitive wild-type strains so far observed (*Paaby et al., 2015*), has been mapped to the *ppw-1* locus, which in CB4856 includes a frameshift and early stop upstream of the critical PAZ and PIWI domains (*Elvin et al., 2011; Pollard & Rockman, 2013; Tijsterman et al., 2002*).

We measured the germline RNAi responses of N2, N2 carrying the 1.5kb deletion allele (N2^{*ppw-1(del)*}), CB4856, and QX1211, a wild isolate that exhibits germline RNAi incompetence similar to CB4856 (*Paaby et al., 2015*). We targeted the maternal-effect, embryonic-required genes *par-1* and *pos-1*, which have commonly been used to measure germline RNAi (*Elvin et al., 2011; Pollard & Rockman, 2013; Tijsterman et al., 2002*), by feeding hermaphrodites *E. coli* expressing target dsRNA; we then counted dead embryos in the next generation. Using a conventional approach, in which hermaphrodites are pooled on plates and offspring are scored in a window of relatively early egg-laying (*Kamath et al., 2001; Pollard & Rockman, 2013*), we observed high lethality for N2 and very low or negligible lethality in the three low-response strains (Figure 1A).

However, following an initial trigger, amplification within the RNAi response can induce strong effects later (Billi *et al.*, 2014), and continual exposure to RNAi by feeding also means that individuals ingest increasing numbers of trigger molecules as they age. To evaluate whether and how the RNAi response changes over time within individual animals, we scored the penetrance of embryonic lethality over the entire reproductive lifespan of egg-laying hermaphrodites. In this assay we used *par-1*, which provides the more sensitive readout since it is not as lethal. Here, N2 exhibited complete lethality in all but the earliest offspring, while the low-response strains showed unique patterns of a slow or incomplete response (Figure 1B). Nearly all N2^{*ppw-1(del)*} embryos hatched, including late-age embryos, indicating that the loss of *ppw-1* is not compensated by any other genes in the N2 background. However, embryonic lethality increased over time in CB4856. This suggests that the frameshift lesion in CB4856 may not be a null allele, permitting some PPW-1 activity; or that other genes in the CB4856 background may partially compensate for the loss of PPW-1, promoting a delayed RNAi response.

To distinguish between these alternatives, we tested a strain carrying the CB4856-derived allele of *ppw-1* (*pk2505*) introgressed into the N2 background (N2^{*ppw-1(CB4856)*}). This strain produced an intermediate response: more responsive than CB4856, especially in the earliest time interval, but never as responsive as N2, even in late age (Figure 1B). This suggests that both explanations matter. N2^{*ppw-1(CB4856)*} is likely more responsive than CB4856 due to its N2 background, consistent with previous reports that the *ppw-1* allele does not fully explain insensitivity in CB4856 (Pollard & Rockman, 2013; Tijsterman *et al.*, 2002). However, the stronger response of N2^{*ppw-1(CB4856)*} relative to N2^{*ppw-1(del)*} suggests that the naturally-occurring frameshift mutation in CB4856 is not as deficient as the 1.5kb deletion, or that additional polymorphisms within the introgressed CB4856 interval modify the frameshift allele. This outcome would be consistent with an earlier investigation of germline RNAi in CB4856, which implicates genetic complexity in the *ppw-1* genome region (Pollard & Rockman, 2013).

Figure 1. Embryonic lethality following RNAi against germline-expressed targets. (A) Hatched larvae and dead embryos laid in a 4-6hr window within the first 8hrs of egg-laying were scored for pooled hermaphrodites across replicate plates. (B) To assess the germline RNAi response over reproductive lifespan, embryonic lethality was scored for individuals. Each point represents the proportion of embryos laid on a plate by a single hermaphrodite that died in the given time interval. The data include all offspring of all hermaphrodite mothers; time intervals were chosen to space out the number of offspring per plate (~30-100); x-axis labels indicate the approximate midpoint of the time intervals. Connecting lines indicate the sequence of offspring plates for each individual. Embryonic lethality for all strains on the negative control empty vector was negligible (data not shown).

QX1211 exhibited low lethality in the earliest laid embryos, but subsequently produced a bimodal response, suggesting that RNAi in this strain is either “on” or “off” in individual animals. Notably, the response does not appear to increase in the “off” individuals over time; instead, they exhibit negligible embryonic lethality over the entire reproductive period. These findings show that wild isolates CB4856 and QX1211 are capable of germline RNAi, though their responses are limited. Further, they show distinct patterns of activity: in CB4856, the response is delayed and incomplete; in QX1211, it is partially delayed, with higher expressivity and variable penetrance.

Target transcript knockdown confirms distinct RNAi responses across strains

The use of an end-point phenotype to read out the RNAi response, in this case embryonic lethality, cannot capture activity at the molecular or cellular level. So, to assess the RNAi response with high spatial and temporal resolution within embryos, we used single-molecule fluorescence *in situ* hybridization (smFISH) to visualize transcripts of germline RNAi targets. Since smFISH visualizes individual molecules via hybridization of dozens of oligonucleotide probes, which in aggregate produce a detectable fluorescent spot (*Raj et al., 2008*), it captures signals from intact RNAs, not those degraded by RNAi.

We examined *par-1* transcript levels in *par-1* RNAi-treated and -untreated embryos of N2, CB4856 and QX1211. We collected embryos from gravid hermaphrodites in early reproductive maturity, in a narrow two-hour window, to maximize precision in estimating the RNAi response. At this timepoint, many *par-1* transcripts are degraded in RNAi-treated N2 embryos (Figure 2A-B). Treated N2 embryos of this timepoint go on to show complete lethality, but in CB4856 and QX1211, lethality is not yet penetrant (Figure 2C).

For all three strains, we observed robust expression of the target gene in untreated embryos. This outcome diminishes the likelihood that the variable embryonic lethality we had observed was driven by differences in native expression of the target gene, rather than differences in RNAi. In RNAi-treated embryos, the transcript levels further confirm the distinct RNAi responses we previously observed: N2 showed a steep drop in abundance, CB4856 showed no change, and

Figure 2. Transcript abundance in individual RNAi-treated and untreated embryos, visualized via single-molecule fluorescence in situ hybridization (smFISH). (A) - (B) Representative embryos are shown from strain N2, from a mother reared in the control condition or with RNAi against *par-1*; red spots indicate *par-1* transcripts and blue DAPI staining shows nuclei, which were used to identify embryo stage. (C) Embryonic lethality was simultaneously measured in matched samples. To limit variation due to reproductive age of the mothers, we collected embryos from a tightly controlled time window in early reproduction. (D) Transcript abundance for early stage embryos (up to four cells). (E) Transcript abundance for *par-1* for the same experiment, but now including later stage embryos with up to 30 nuclei. Green (negative control) and blue (RNAi treatment) lines indicate the linear regression of transcript counts onto embryonic stage; gray shading indicates the 90% confidence interval. For (D) and (E), each point represents one embryo. Significance levels (*t*-tests): $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*).

QX1211 showed N2-like levels for some, but not most, embryos (Figure 2D). Specifically, in early stage embryos (up to four cells), N2 shows a significant reduction in *par-1* transcript abundance after *par-1* RNAi ($t=-16.34$, $df=32$, $p<0.001$), while CB4856 and QX1211 do not ($t=0.41$, $df=56.31$, $p=0.684$ and $t=1.15$, $df=95.03$, $p=0.255$, respectively) (Figure 2D). However, for CB4856, the variance in abundance is slightly higher for treated embryos than for control embryos ($F=1.94$, $df=32,40$, $p=0.048$), which may represent the onset of an RNAi response. In QX1211, the variance of treated embryos is substantially higher ($F=2.94$, $df=100,32$, $p<0.001$) and is bimodally distributed, with low-abundance embryos at levels similar to N2. We observed a similar pattern in QX1211 for a second germline target, *par-4*, in which treated embryos showed a slight reduction in average abundance but substantial increase in variance ($t=-2.64$, $df=24.20$, $p=0.014$ and $F=5.50$, $df=20,36$, $p<0.001$, respectively) (Figure 2D). These results, which mirror the observation of on/off embryonic lethality in QX1211 (Figure 1B), are further evidence that induction of RNAi can occur early but is stochastic within this strain.

To examine how transcript abundance changes with embryonic development across strains, we evaluated embryos with up to 30 nuclei. In the control condition, we observed that *par-1* transcripts decrease with embryonic stage (Figure 2E) (*Charles et al., 2021*), and that the rate of decrease was consistent across strains (ANCOVA model comparison, $p=0.299$). However, overall N2 exhibited significantly fewer *par-1* transcripts than either CB4856 or QX1211 ($p<0.001$). Thus, while we conclude that changes in transcript abundance between control and treatment conditions (or lack thereof) are good indicators of an RNAi response, we cannot rule out the possibility that differences in native gene expression between strains might also mediate differences in apparent response for specific targets.

The strain-specific patterns of RNAi we observed in early-stage embryos (Figure 2D) persisted across development (Figure 2E). We considered how transcript abundance changed between treated and untreated embryos within each strain, after adjusting for embryo stage. For N2, most of the variance in transcript abundance can be explained by treatment ($\omega^2=0.586$), then embryo stage ($\omega^2=0.248$), then by the interaction between them ($\omega^2=0.059$) (ANCOVA model comparison, $p<0.001$ for all). This makes sense, as transcripts are dramatically depleted in RNAi-treated N2 embryos. For CB4856 and QX1211, however, the treatment condition explains very little of the variance in transcript abundance ($\omega^2=0.010$, $p=0.043$ and $\omega^2=0.014$, $p=0.037$,

respectively). Instead, the variance observed for CB4856 is well explained by embryo stage ($\omega^2=0.581$, $p<0.001$) and minimally by the interaction ($\omega^2=0.017$, $p=0.012$), consistent with the conclusion that the RNAi response is negligible in CB4856 at this stage of germline development. In QX1211, variance in transcript abundance is less well explained overall, since incompleteness of the RNAi response adds noise to the transcript counts in treated QX1211 embryos ($\omega^2=0.208$, $p<0.001$ for embryo stage and $p=NS$ for the interaction).

Reduced PPW-1 function explains weak germline RNAi response for some, but not all, low-response strains

We surveyed the several hundred wild isolates in the *Caenorhabditis elegans* Natural Diversity Resource (CeNDR) database (Cook *et al.*, 2017) and found that the naturally-occurring stop-gain/frameshift mutation in *ppw-1* (Tijsterman *et al.*, 2002) is unique to CB4856. Nevertheless, we hypothesized that variation in PPW-1 activity might account for the observed differences in germline RNAi among wild strains. To test whether reduction of PPW-1 function might be a universal aspect of reduced germline RNAi, we performed complementation tests by crossing N2 wild-type and null alleles of *ppw-1* to seven wild isolates. We evaluated CB4856, QX1211, and five additional strains, selected based on prior observations of weak germline RNAi (data not shown) (Paaby *et al.*, 2015) and representation of nucleotide diversity and divergence across the global population (Cook *et al.*, 2017).

We crossed each wild isolate to N2, which carries its native, wild-type copy of *ppw-1*, and also to N2 carrying the *ppw-1* deletion allele (*pk1425*). But because N2 carries a sperm-delivered toxin allele at *peel-1*, which causes genetic incompatibility with strains that do not carry the linked zygotic antidote at *zeel-1* (Seidel *et al.*, 2008, 2011), we generated a strain derived from N2 with both the *ppw-1* deletion and an allele of *peel-1* (*kah126*) that disables the toxin by a frameshift insertion in the second exon (*N2^{ppw-1(del);peel-1}*). Use of this allele eliminates embryonic lethality that would otherwise arise in the F2 generation, from F1 heterozygotes derived from incompatible strains. The presence of this *peel-1* allele had no effect on our measured RNAi response in either sensitive or resistant backgrounds (Figure 3A, Figure S1), so we used it in all comparisons requiring the N2 background.

Figure 3. Complementation tests between seven wild isolates with low RNAi response and the RNAi-sensitive laboratory strain N2, with and without the *ppw-1* deletion allele. Response was measured by embryonic lethality following RNAi by feeding against the embryonic target *par-1*. (A) To circumvent embryonic lethality arising from the *peel-1/zeel-1* genetic incompatibility, we used a null allele of the sperm-delivered toxin *peel-1* in N2, which has no effect on RNAi in either the responsive (N2) or the resistant (N2^{ppw-1(del)}) backgrounds. A single copy of *ppw-1* is sufficient to fully restore the germline RNAi response in N2. (B) - (E) Complementation tests for seven wild isolates with weak germline RNAi, representing a diversity of genetic backgrounds. For DL238 (C), rather than the average across pooled hermaphrodites, each point represents the proportion of dead embryos from one individual; the overall pattern qualitatively replicates that which we observed in pooled hermaphrodites (Figure S2). For (B) - (E), embryonic lethality in the negative control condition is shown only when it was observed to be greater than zero. Significance levels (Tukey's contrasts): p<0.001 (***), p<0.01 (**), p<0.05 (*).

For each cross, we compared the response of the individual wild isolate to the two heterozygote genotypes in the F1 generation, with and without the deletion at *ppw-1* inherited from the N2 chromosome. As previously, we induced *par-1* RNAi in the (F1) hermaphrodite germline and measured embryonic lethality in the following generation. To avoid confounding differences in developmental timing with variation in RNAi response, we scored all progeny from the first 15hrs of egg-laying from a small pool of hermaphrodites (~100-200 embryos) on each replicate plate. Though N2 homozygous for the *ppw-1* deletion fails to exhibit an RNAi response, one copy of wild-type *ppw-1* fully rescues it, indicating that *ppw-1* is haplo-sufficient, at least in the N2 background (Figure 3A). Therefore, we reasoned that if weak RNAi response in the wild isolates was a consequence of reduced PPW-1 activity, any restoration of RNAi response in the F1 genotypes would be greater in the genotype with the functional N2 *ppw-1* allele.

Across the seven *C. elegans* isolates, we observed four distinct response patterns: (i) no rescue, (ii) *ppw-1*-dependent rescue, (iii) *ppw-1*-dependent suppression, and (iv) *ppw-1*-independent rescue. These results indicate that among wild-type strains, PPW-1 activity varies, PPW-1 activity differentially affects germline RNAi due to interaction with other varying factors, or both. The results suggest that weak germline RNAi is multigenic within each strain, since in each instance of rescue, the response was still lower than N2 levels, indicating the presence of factors other than *ppw-1*.

(i) Strain JU1522 showed no rescue, i.e., no improved RNAi response in either F1 genotype (Figure 3B). This outcome does not necessarily mean that weak RNAi in JU1522 is independent of *ppw-1*, but it does indicate that none of the alleles that promote RNAi in N2, including *ppw-1*, are haplo-sufficient to increase the response in the JU1522 background.

(ii) As expected, CB4856 exhibited *ppw-1*-dependent rescue: we observed a significantly increased RNAi response when crossed to N2, but only in the background with the wild-type *ppw-1* allele (Figure 3C). This is consistent with prior reports implicating *ppw-1* insufficiency as a major cause of weak germline RNAi in this strain (Pollard & Rockman, 2013; Tijsterman et al., 2002). Strain DL238 exhibited a similar pattern. However, replicate to replicate variation in embryonic lethality was high in DL238 F1s, so we investigated whether this could be explained by potentially stochastic induction of the RNAi response between individual worms. This

appears to be the case: tested individually, we observed that some hermaphrodites produced no dead embryos and others over 30% (Figure 3C). Although DL238 does not carry the *pha-1* zygotic antidote to a second genetic incompatibility, arising from the *sup-35* toxin carried by N2 (Ben-David *et al.*, 2017) and for which we did not control, we saw only very weak penetrance of this effect (Figure 3C).

(iii) Unexpectedly, QX1211 demonstrated *ppw-1*-dependent suppression: the heterozygote with the *ppw-1* deletion allele exhibited a significant increase in embryonic lethality, implying that reduction of *ppw-1* in this strain promotes germline RNAi (Figure 3D). Like DL238, QX1211 lacks the *pha-1* antidote (Ben-David *et al.*, 2017). Here, the heterozygote genotypes on the negative control and the wild-type heterozygote on *par-1* showed embryonic lethality arising from the incompatibility.

(iv) Strains CB4852, KR314, and ECA369 exhibited *ppw-1*-independent rescue, in which the two heterozygote genotypes exhibited levels of embryonic lethality that were equivalent to each other and significantly higher than the wild isolate on its own. This suggests that N2 alleles other than *ppw-1* promote the RNAi response in these genetic backgrounds (Figure 3E).

RNAi against GFP confirms strain-specific variation

Wild isolates exhibit heritable variation in embryonic pathways, as evidenced by differences in lethality following single-gene perturbations; these mutations may confound our ability to measure RNAi using those targets (Paaby *et al.*, 2015). To verify that the responses we measured here were due to deficiencies in germline RNAi, and not to differences in developmental pathway function (e.g. for *par-1*), we introgressed a germline-expressed GFP construct (*stIs10024*, in which GFP fused to histone H2B is driven by *pie-1*) into four of our low-response strains and measured fluorescence following RNAi against GFP. The strains represented the four distinct RNAi response patterns observed in the *ppw-1* complementation tests (Figure 3).

As expected, strains CB4856, QX1211 and JU1522 showed negligible RNAi response in young hermaphrodite germlines, with no initial difference in GFP fluorescence intensity between

Figure 4. RNAi against germline-expressed GFP in wild-type strains. (A) - (F) Representative images of control and treated worms, all at 24hrs post L4 stage, are shown for N2, CB4856 and QX121. Green indicates GFP fluorescence; the red channel is overlaid on these images to show autofluorescence. (G) Whole-worm fluorescence intensity for worms imaged at 6, 24, and 48hrs post L4 stage; each point represents fluorescence measured for a single individual. (H) The difference in fluorescence intensity between treated and untreated samples; error bars represent standard error. Significance levels (Tukey's contrasts): $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*).

treated and untreated worms (Figure 4). Also consistent with measurements of RNAi using *par-1*, CB4856 showed diminished fluorescence in older hermaphrodites, confirming a delayed response, and JU1522 showed no evidence of RNAi. Overall, QX1211 exhibited a very weak RNAi response, though we did not observe any on/off variation in fluorescence across individual worms. Strain ECA369, however, showed a significant reduction in GFP fluorescence even at the earliest timepoint, suggesting that apparent resistance to *par-1* RNAi may be mediated by alleles modifying PAR-1 function rather than germline RNAi.

Genetic complementation between wild isolates implicates diverse and polygenic basis for germline RNAi incompetence

To examine whether the alleles that limit germline RNAi are unique or shared across wild isolates, we performed complementation tests by crossing low-response strains to each other. As above, we measured embryonic lethality following *par-1* RNAi exposure in the F1 generation. We performed a total of six tests, with each pairwise cross for strains CB4856, DL238, KR314 and QX1211 (Figure 5). We chose these strains based on their apparent diversity in PPW-1 function (Figure 3) and compatibility at the *zeel-1;peel-1* locus (Andersen *et al.*, 2012).

The CB4856 × QX1211 heterozygote produced significantly more dead embryos, indicating an increased RNAi response, than either strain on its own (Figure 4A). This implies that alleles that dampen the RNAi response are not shared between these two strains, as their function is at least partially rescued by the alternate genetic background. Given that CB4856 and QX1211 exhibit responses that are dependent on *ppw-1* but opposite to each other (Figure 3), their complementation may be occurring at *ppw-1* itself.

DL238 had exhibited a *ppw-1*-dependent response (Figure 3), suggesting that, like CB4856, it may be deficient in PPW-1 activity. However, CB4856 × DL238 hermaphrodites also showed a rescued RNAi response (Figure 5B), again implicating distinct mechanisms in these strains. Interestingly, we observed that the direction of the CB4856 × DL238 cross affected the magnitude of the RNAi rescue. For all pairwise crosses, we performed matings in both directions except those with KR314, as males in this strain are infertile (Hodgkin & Doniach, 1997). The direction of the cross had no effect, except for CB4856 × DL238, in which heterozygotes sired

Figure 5. Pairwise complementation tests between four wild isolates with low RNAi response. (A) - (F) Response was measured by embryonic lethality following RNAi by feeding against the embryonic target *par-1*. The heterozygote genotypes were generated by crossing males and hermaphrodites in both directions (A) - (C), except for crosses with strain KR314, which does not produce fertile males (D) - (F). With the exception of CB4856 × DL238 (B), in which hermaphrodites sired by DL238 exhibited a significantly stronger response than those sired by CB4856, cross direction had no effect on embryonic lethality in the next generation and plots show pooled data. Significance levels (Tukey's contrasts): p<0.001 (***), p<0.01 (**), p<0.05 (*).

by DL238 exhibited a stronger response than those sired by CB4856 (Figure 5B). This bias is not observed in the negative control and is not explained by a *pha-1;sup-35* incompatibility between CB4856 and DL238, which is not expected to drive F2 lethality (Ben-David *et al.*, 2017).

DL238 failed to complement QX1211 (Figure 5C), but did complement KR314 (Figure 5D), indicating shared and distinct mechanisms, respectively. These outcomes for DL238 are the opposite of those observed for CB4856 crossed to the same strains (Figure 5A, 5E), reinforcing the conclusion that CB4856 and DL238 harbor distinct genetic mechanisms for weak germline RNAi, despite similar responses under *ppw-1* manipulation (Figure 3). Further evidence for distinct genetic mechanisms is seen in the complementation of KR314 × QX1211 (Figure 5F).

These observations of complementation between wild strains indicate that natural populations harbor unique alleles mediating germline RNAi, and that variation in RNAi response is polygenic. Coupled with the results of the *ppw-1* complementation tests (Figure 3), which suggest that weak RNAi is multigenic within strains, we conclude that variation in germline RNAi is a highly complex trait.

Patterns of gene expression implicate differential regulation of RNAi factors

To evaluate whether additional causal factors for weak germline RNAi include known RNAi genes, including other Argonautes, we examined expression variation at 62 loci (listed in Table 1). We performed RNA-seq on low-response strains CB4856 and QX1211, on responsive strains N2 and JU1088, and on strain EG4348, which shows an intermediate response (Paaby *et al.*, 2015). RNA samples were prepared from young, reproductively mature hermaphrodites reared on *par-1* RNAi, *pos-1* RNAi, and the empty feeding vector as a negative control.

For each gene, we looked for evidence of differential expression between each wild isolate and N2 without inducing RNAi (the control condition), or between *par-1* or *pos-1* RNAi and the control within each strain. In both analyses, we observed significant differences in relative expression at multiple genes, in both directions. In other words, the directionality of expression was not associated with a putatively active RNAi response (Figure 6A-B).

Figure 6. Differential gene expression for RNAi factors across strain and RNAi conditions. (A) - (B) We examined 62 genes for differential expression via RNA-seq; only those with significant results (FDR<0.1) are displayed. (A) Up or down arrows indicate differential expression between N2 and low-response strains CB4856 and QX1211, moderately responsive strain EG4348, and high-response strain JU1088, tested without induction of RNAi. The (*) at *ZK218.8* indicates differential expression beyond the y-axis scale; this gene is not expressed in the N2 reference strain and has been classified as a pseudogene. (B) Arrows indicate differential expression within each strain, between the control condition (no RNAi) and RNAi against *par-1* and *pos-1*. (C) - (D) Expression differences via droplet digital PCR for *ppw-1* and *sago-2*. Ten strains were evaluated (Figure S4); a subset are shown here. Across strains, significant differences (Tukey's contrasts, $p < 0.05$) are indicated by letter groupings; for example, QX1211, JU1088, and N2 have equivalent concentrations of *ppw-1*, while CB4856 and JU1522 concentrations are significantly different from all others. Within each strain, significant differences between developmental stages (pairwise contrasts with Bonferroni correction) are indicated by: $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*).

Relative to N2, strains with weakest germline RNAi exhibited the greatest degree of differential expression. That is: QX1211, then CB4856 showed the most differences across the gene set; the moderately responsive strain EG4348 showed a handful of modest differences; and the strongly responsive strain JU1088 showed a modest difference in expression for only one gene (Figure 6A). Of the genes differentially expressed (FDR<0.1) by both CB4856 and QX1211, the direction of expression was concordant but for one exception (*rde-11*). Notable include a strong reduction in expression by QX1211 at *prg-2*; reduction by both QX1211 and CB4856 at *eri-5*, *ppw-1*, *sid-1*, and *wago-2*; and increased expression by both strains at *sago-2* and *ZK218.8*. The unnamed gene *ZK218.8*, previously identified as a potential Argonaute (Yigit, 2007), is classified as a pseudogene and remains unexplored in the literature. We observed no expression of *ZK218.8* in N2, JU1088 or EG4348, but it is actively transcribed in CB4856 and QX1211, suggesting pseudogenization may have occurred in some strain lineages but not in others.

Relative to N2, strains with the greatest degree of differential expression were also the most genetically diverged. However, RNAi incompetence does not appear to be a function of genetic distance from the reference strain. Highly diverged isolates ECA701, JU561, and XZ1516 (Crombie et al., 2019) were responsive to *par-1* RNAi (data not shown), and RNAi sensitivity for 55 wild isolates in (Paaby et al., 2015) showed no relationship with divergence from N2 (Figure S3).

Within each strain, we observed more instances of differential expression in the *par-1* vs control comparison than in the *pos-1* comparison. Only JU1088, which exhibits the strongest germline RNAi response of these five strains (Paaby et al., 2015), showed significant differences on *pos-1*, all of which were in the same direction as the *par-1* responses (Figure 6B). All of the genes that were differentially expressed (FDR<0.1) for multiple strains showed consistent directionality as well. None of the strains exhibited differential expression at *ppw-1*, but CB4856, N2, and JU1088 showed strong up-regulation of *sago-2* on *par-1* RNAi.

The WAGO Argonautes *ppw-1* and *sago-2*, which reside ~17cM apart on chromosome I, share high sequence identity. In some strains, including QX1211, poor mapping of short reads to the reference genome (Cook et al., 2017) suggests instances of gene divergence or duplication. We resolved sequence ambiguities via de novo assembly of paired-end reads and long read

sequencing, and observed that QX1211 carries *ppw-1*-like alleles at both loci (File S1). To validate our RNA-seq observations of *ppw-1* and *sago-2* expression, and to evaluate additional strains, we designed a droplet digital PCR (ddPCR) experiment to measure both transcripts simultaneously and discriminate between them using transcript-specific labels. We tested all strains thus far discussed: the seven low-response strains for which we tested *ppw-1* function (Figure 3), as well as responsive strains N2 and JU1088 and moderately responsive strain EG4348. We measured native expression; worms were not treated with RNAi. To evaluate whether *ppw-1* or *sago-2* expression changed as worms aged, we also collected RNA from two developmental timepoints, young adult and mid adult.

Consistent with the RNA-seq results, CB4586 exhibited very low *ppw-1* expression (Figure 6C, Figure S4), and CB4856 and QX1211 exhibited high *sago-2* expression (Figure 6D, Figure S4). Overall, *ppw-1* expression was about an order of magnitude higher than that of *sago-2*, and the expression levels of both genes differed significantly across strains (Table S1). Changes in expression between developmental timepoints also differed significantly across strains, for both genes (Figure 6C-D).

Expression levels of *ppw-1* and *sago-2* showed no clear correlation with RNAi responsiveness. Across all ten strains, the combined expression of *ppw-1* and *sago-2* was both highest and lowest in two strongly resistant strains: QX1211 and JU1522, respectively (Figure 6C-D). We have consistently observed negligible germline RNAi in JU1522, including no rescue when crossed with N2 (Figure 3), and no response even at later age (Figure 4); this strain exhibits the weakest response we have observed. One possibility is that both high and low expression of *ppw-1* and *sago-2* can limit germline RNAi. This hypothesis is consistent with our observation that a haploid dose of *ppw-1* increases the RNAi response in QX1211 (Figure 3D), and with the prior finding that *ppw-1* and *sago-2* encode functionally interchangeable proteins that can compensate each other (Yigit *et al.*, 2006), but not with the observation that overexpression these factors increases RNAi sensitivity in N2 (Yigit *et al.*, 2006). N2 and JU1088, the two strains with the most robust germline RNAi response in our analysis, exhibit intermediate baseline levels of *ppw-1* and *sago-2* combined (Figure S4), and both exhibit significant up-regulation of *sago-2* on *par-1* RNAi (Figure 6B). Notably, so does CB4856; it shows the highest baseline level of *sago-2* (Figure S4), and over time increases *sago-2* expression at a higher rate than N2 (Figure 6B). This

may explain why CB4856, which carries a putatively deficient *ppw-1* allele (Tijsterman *et al.*, 2002), is able to mount a delayed germline RNAi response while the N2 *ppw-1* null mutant is not (Figure 1B).

***C. elegans* RNAi genes show lineage-specific diversification and pseudogenization**

To identify candidate genes for the weak RNAi responses we observed, we examined genotypes at known RNAi genes (listed in Table 1) among the seven low-response strains. Of 62 genes, five harbored at least one instance of a high impact mutation predicted to disrupt the encoded protein. Occurrences were mostly strain-specific: in addition to the frameshift mutation at *ppw-1* in CB4856 (Tijsterman *et al.*, 2002), we observed high impact variants at *ergo-1* in ECA369, at *sago-2* in DL238, at *lin-15b* in QX1211, and at *eri-7* in CB4856, DL238 and ECA369 (Table S2). Given that the mutation at *ppw-1* does not fully explain weak germline RNAi in CB4856 (Tijsterman *et al.*, 2002) and that additional causal factors appear to be linked nearby (Figure 1B, (Pollard & Rockman, 2013), we propose *eri-7*, which is 0.34cM from *ppw-1*, as a candidate; in addition to the high impact mutation, which putatively affects splicing, CB4856 exhibits reduced *eri-7* expression (Figure 6).

We also observed alleles that were diverged from the N2 reference genotype but did not carry disruptive high impact variants, suggesting that selection has driven potentially divergent gene function. We classified such “functional diverged alleles” as those with at least five amino acid substitutions, at least 1% divergence across sites, and no high impact mutations. QX1211 harbored 14 functional diverged alleles (Table S2), a subset of which are also differentially expressed: *eri-1*, *eri-6*, *eri-9*, *nyn-2* and *rde-12* (Figure 6). Notably, we observed elevated variation at *hsf-1*. This transcription factor is a master regulator of many RNAi genes, and HSF-1 activity explains stochastic differences in heritable RNAi responses among individuals with an isogenic N2 background (Houri-Zeevi *et al.*, 2020). Two of the seven strains carry functional diverged alleles at *hsf-1*, and another four show poor read mapping, suggesting further allelic divergence (Table S2). Relative to N2, *hsf-1* expression is reduced in QX1211 (Figure 6).

To better characterize patterns of molecular diversity at RNAi genes, we expanded our 62-gene analysis to the population level by querying 403 strain isotypes in CeNDR (Cook *et al.*, 2017).

Table 1. Molecular diversity at 62 RNAi genes, from 403 strain isotypes. “Functional diverged alleles” are those with at least 1% nucleotide divergence from the reference genome, including at least five moderate mutations, such as amino acid substitutions, and no disruptive high impact mutations, such as frameshifts or stop-gains. “Pseudogenized alleles” are those with at least one high impact mutation called with high confidence and at which 1% or more of the sites are diverged or missing, or those with at least 50% of the sites with missing calls. Potential pseudogenization was assessed with respect to the reference genome and considered even for those Argonautes already classified as putative pseudogenes on Wormbase.

Class	Gene	Nucleotide diversity (π)	No. strains with functional diverged alleles	No. strains with pseudo-genized alleles
Argonaute (AGO)	<i>alg-1</i>	7.20E-04	0	0
	<i>alg-2</i>	8.53E-04	0	19
	<i>alg-3</i>	6.86E-04	0	1
	<i>alg-4</i>	6.08E-04	10	5
	<i>alg-5</i>	3.75E-04	0	0
	<i>rde-1</i>	4.98E-04	2	0
Argonaute (PIWI)	<i>ergo-1</i>	1.18E-03	0	8
	<i>prg-1</i>	1.77E-03	0	0
Argonaute (WAGO)	<i>C04F12.1</i>	2.50E-04	0	34
	<i>csr-1</i>	6.59E-04	0	0
	<i>hrde-1</i>	1.84E-04	4	3
	<i>nrde-3</i>	3.39E-04	22	0
	<i>ppw-1</i>	2.77E-04	0	2
	<i>ppw-2</i>	5.35E-04	0	0
	<i>sago-1</i>	1.23E-03	0	4
	<i>sago-2</i>	1.26E-03	1	25
	<i>wago-1</i>	9.37E-04	0	0
	<i>wago-10</i>	9.64E-06	58	2
	<i>wago-4</i>	7.70E-06	0	0
	<i>wago-5</i>	9.17E-06	0	48
	<i>C06A1.4</i>	1.07E-03	0	0
Argonaute (pseudo)	<i>C14B1.7</i>	6.47E-04	0	0
	<i>H10D12.2</i>	3.00E-04	0	25
	<i>prg-2</i>	6.59E-04	0	3
	<i>wago-11</i>	7.58E-05	0	64
	<i>wago-2</i>	7.78E-04	0	0
	<i>ZK218.8</i>	4.08E-04	0	11
	<i>dcr-1</i>	6.37E-04	49	0
Other RNAi factor	<i>drh-1</i>	2.35E-04	40	0
	<i>drsh-1</i>	1.11E-03	0	0
	<i>ego-1</i>	1.07E-04	0	0

Class	Gene	Nucleotide diversity (π)	No. strains with functional diverged alleles	No. strains with pseudo-genized alleles
Other RNAi factor	<i>eri-1</i>	2.37E-04	23	0
	<i>eri-12</i>	5.00E-04	23	0
	<i>eri-3</i>	4.37E-04	9	0
	<i>eri-5</i>	7.15E-04	0	1
	<i>eri-6</i>	6.04E-04	36	20
	<i>eri-7</i>	1.74E-04	0	12
	<i>eri-9</i>	7.00E-04	26	0
	<i>hrde-2</i>	9.92E-04	0	0
	<i>hsf-1</i>	5.54E-03	119	0
	<i>lin-15b</i>	2.61E-04	9	53
	<i>mut-7</i>	8.90E-04	0	0
	<i>nck-1</i>	5.01E-04	0	0
	<i>nhl-2</i>	2.22E-04	0	0
	<i>nyn-2</i>	4.47E-04	2	1
	<i>rde-10</i>	1.00E-03	21	1
	<i>rde-11</i>	5.45E-05	0	1
	<i>rde-12</i>	2.46E-03	54	0
	<i>rde-2</i>	1.48E-04	0	0
	<i>rde-4</i>	7.21E-04	0	0
	<i>rde-8</i>	5.03E-06	0	0
	<i>rme-2</i>	2.09E-04	0	0
	<i>rrf-1</i>	1.09E-03	9	0
	<i>rrf-3</i>	1.19E-03	14	0
	<i>rsd-2</i>	2.67E-03	151	4
	<i>rsd-3</i>	2.00E-04	35	0
	<i>rsd-6</i>	2.68E-04	28	0
	<i>sid-1</i>	9.03E-04	0	1
	<i>sid-2</i>	1.44E-03	54	3
	<i>sid-3</i>	2.09E-04	56	1
	<i>sid-5</i>	1.23E-03	0	0
	<i>tofu-5</i>	6.59E-04	0	0

Estimates of nucleotide diversity (π) per gene were generally high (Table 1) relative to genome-wide averages (Lee et al., 2021), with $\pi > 0.001$ for 14/62 genes (13/55 genes not characterized as pseudogenes). Elevated variation was often, but not always, associated with high-impact mutations in one or more strains (Figure S5). Therefore, to distinguish between instances of pseudogenization and gene loss versus allelic diversification arising from directional or balancing selection acting on functional sequences, we classified alleles as possible pseudogenes

if they harbored at least one high impact mutation called with high confidence and if at least 1% of the sites were diverged or missing relative to the reference genome, or if over 50% of sites were missed calls; we classified alleles as functionally diverged as defined above.

Given the expansion and diversification of Argonautes in nematodes (*Buck & Blaxter, 2013*), we hypothesized that the WAGO genes might be relatively unconstrained and therefore especially susceptible to pseudogenization. Indeed, we saw multiple instances of putative gene loss in one or more strains at WAGOs, but we also saw putative loss across all classifications, including the PIWIs, AGOs, and other RNAi factors (Table 1). Excluding seven genes classified as pseudogenes on Wormbase (*Harris et al., 2020*), 40% (22/55) of the RNAi genes indicated pseudogenization in one or more strains (Table 1). Consistent with active transcription of putative pseudogene *ZK218.8* in some non-reference strains (Figure 6A), we conclude that RNAi genes exhibit an unusually high level of lineage-specific gene loss in *C. elegans*.

Across the population, we found that 25 genes harbor functional diverged alleles (Table 1). These alleles most often occurred in multiple strains, unlike the pseudogenized alleles, which often occurred singly. A comprehensive analysis is beyond the scope of this paper, but these results are consistent with positive selection driving or maintaining divergent gene function. For example, at *hsf-1* we observed very high polymorphism, including amino acid changes at intermediate frequencies, but no instances of disruptive high-impact mutations (Figure 7A). (We also observed poor read mapping across the locus, which indicates further divergence but may also obscure deleterious mutations.) The apparent absence of loss-of-function alleles at *hsf-1* suggests purifying selection, and its diversification suggests evolutionary lability, potentially as a keystone regulator of small RNA pathways (*Houri-Zeevi et al., 2020*). At other genes, high polymorphism may have accumulated under relaxed selection. The PIWI Argonaute *ergo-1*, for example, exhibits lineage-specific pseudogenization (Figure 7B). Although some of the RNAi genes are near invariant (Figure 7C, Figure S5), the overall level of very high allelic divergence at Argonautes and other known RNAi factors indicates that RNAi pathways are likely evolving dynamically within the *C. elegans* population. These findings suggest that variation at known RNAi genes likely contributes to the observed variability in RNAi response, and that functional characterizations of these genes, which have been universally achieved in N2, may be strain specific.

Figure 7. Population-level allelic diversity in RNAi genes across 403 strains. (A) The transcription factor *hsf-1* shows elevated polymorphism indicative of functional divergence, including: multiple changes to the amino acid sequence, mutations segregating at intermediate frequency, and no observed high impact mutations that disrupt the protein. Each observed mutation is represented as a single point; the up- and down-triangles representing indels are scaled by indel length (range = 1-99, mean = 6.8). (B) The Argonaute *ergo-1* also exhibits elevated polymorphism, but accumulation of high impact mutations in some alleles suggests gene loss by pseudogenization. In this haplotype network, each circle represents a unique haplotype (of one or more strains) and hatch marks indicate mutations. (C) In contrast, *rde-2* exhibits conservation. Most strains belong to the common haplotype; haplotype circles are scaled by frequency.

DISCUSSION

In this study, we demonstrate that the failure of some wild-type *C. elegans* strains to mount a robust germline RNAi response occurs by a diversity of mechanisms. Rather than identifying one or more common, shared factors that explain RNAi incompetence, our results indicate that RNAi fails for different reasons in different strains and that the same genes can produce opposite responses. Coupled with high levels of divergence and lineage-specific pseudogenization at known RNAi genes, these findings reposition our understanding of RNAi pathways and processes.

Sydney Brenner's selection of *C. elegans* as a model species, and N2 as the strain of study, was fortuitous for the future discovery of RNAi (Félix, 2008). This system remains the most fertile area for elucidating gene regulation by small RNAs (Youngman & Claycomb, 2014), but our understanding is incomplete without incorporating how RNAi phenomena vary at the population level. The N2 reference genome is both independent of (for purposes of experimental control) and inextricably linked to (via evolution) the genomes of other *C. elegans* strains. Thus, RNAi genes and the interactions between them reside in an expanded context, shaped by the evolutionary history of the population. Our finding that germline RNAi incompetence is highly strain-specific offers direct access into identifying conserved and labile components of RNAi pathways. For example, the apparent dispensability of some Argonautes for target cleavage, or gene silencing generally (Billi *et al.*, 2014), is probably best understood in the context of a pool of interchangeable factors (Yigit *et al.*, 2006), which our data on *ppw-1* and *sago-2* support.

We show that many RNAi genes have been pseudogenized along specific lineages within the population (Table 1). However, evidence of directional or diversifying selection along other lineages, and at other genes (Table 1), suggests that intraspecific differences in germline RNAi incompetence have not evolved by a straightforward accumulation of loss-of-function variants. Instead, we conclude that RNAi pathways are evolving dynamically in the contemporaneous population. A more complete sequence analysis, on wild-type genomes without ambiguities at unmapped loci, will be valuable in refining microevolutionary dynamics in RNAi pathways and identifying testable hypotheses with respect to allele function and interaction. For example, the

non-additivity we observed for *ppw-1* (Figure 3) indicates strain-specific differences that may well occur for other Argonautes, and which will be best interpreted in aggregate.

The variation in *ppw-1* phenotypes and diversification of other RNAi factors (Figure 3, Table 1) indicate that the activity of RNAi genes is highly context-dependent. Background effects have always plagued genetic analysis (Chandler *et al.*, 2013), but the retention of hyperdiverse regions across the *C. elegans* genome (Lee *et al.*, 2021), the potential for strong epistasis in the species (Campbell *et al.*, 2018), and high evolvability of RNAi (Nuez & Félix, 2012) suggest that this system is especially susceptible to functional variability. As such, genetic analysis of RNAi phenotypes in non-reference strains has the potential to yield orthogonal insights into RNAi biology and complement our existing body of knowledge derived from experiments in the lab strain N2 (Chandler *et al.*, 2013).

Given the essentiality of RNAi to numerous biological processes—and the centrality of *C. elegans* in RNAi research—is the variation in this system surprising? Perhaps not: the variation we describe here mirrors patterns of RNAi incompetence observed over longer evolutionary timescales. The ability to silence genes by dsRNA appears intermittently, and shows evidence of rapid evolution within the *Caenorhabditis* genus (Nuez & Félix, 2012; Winston *et al.*, 2007), across nematodes generally (Buck & Blaxter, 2013; Dalzell *et al.*, 2011), and in other systems (Obbard *et al.*, 2009). Argonautes and associated RNAi factors also exhibit taxon-specific patterns of gene duplication, loss, and diversification, likely representative of diversification of biological functions (Buck & Blaxter, 2013; Dalzell *et al.*, 2011; Obbard *et al.*, 2009). RNAi exhibits great lability over long timescales (Nuez & Félix, 2012), which we now observe over very short timescales as well.

The variability we observe suggests that RNAi experiments in the lab do not necessarily target the phenomena most relevant to the animal itself. Exogenous RNAi likely induces responses that evolved for antiviral immunity, but our understanding of nematode immunity is still young (Ashe *et al.*, 2013, 2015; Félix *et al.*, 2011; Sarkies *et al.*, 2013; Wilkins *et al.*, 2005; Yigit *et al.*, 2006). Orsay virus, the only natural-occurring virus known to infect *C. elegans*, invades intestinal cells and is horizontally, but not vertically, transmitted (Félix *et al.*, 2011; Franz *et al.*, 2014). However, vertically transmissible viral-like RNAs have been detected in the germlines of wild-caught

Caenorhabditis isolates (Richaud *et al.*, 2019), suggesting undiscovered host-pathogen dynamics. The piRNA pathway is active in the germline and presumed critical for maintaining genome integrity (Wilson & Doudna, 2013; Youngman & Claycomb, 2014). We found that germline RNAi increases in strain QX1211 after consecutive generations at $\geq 18^{\circ}\text{C}$ (unpublished data), which we hypothesize may be associated with changes in piRNA-like pools of small RNAs that co-occur with onset of the mortal germline phenotype (Frézal *et al.*, 2018). One possibility is that competition between exogenous and endogenous RNAi forces induction of one pathway over the other (Yigit *et al.*, 2006; Youngman & Claycomb, 2014), which may explain the stochasticity in QX1211 phenotypes. Since *ergo-1* laboratory mutants show enhanced exo-RNAi but reduced endo-RNAi (Yigit *et al.*, 2006), this gene, which exhibits extreme allelic diversification in nature (Table 1, Figure 6), may gatekeep RNAi pathway activity differently in different isolates. Another possibility is that in the wild, RNAi in the germline matters most to future generations. Strains resistant to RNAi upon exposure can show transgenerational sensitivity (Tijsterman *et al.*, 2002), and a growing body of research emphasizes the outsized role of RNAi in transgenerational inheritance (Houri-Zeevi *et al.*, 2020, 2021). Thus, germline RNAi incompetence as we observe it in the lab may only be part of the story in nature.

RNAi is a complex and intricate collection of phenomena. RNAi responses are sensitive to environmental conditions (Houri-Zeevi *et al.*, 2021) and susceptible to unexpected outcomes (DeSouza *et al.*, 2019), which in turn could be mediated differently in different genetic backgrounds. Much of our effort here went to developing protocols that could discriminate between distinct RNAi responses in wild-type strains while avoiding confounding effects. For example, to prevent native differences in developmental timing or gene expression from confounding RNAi phenotypes (Shiu & Hunter, 2017), we exposed the parental generation to dsRNA as unfed L1 larvae instead of as L4s (Kamath *et al.*, 2001). However, the extent of condition-dependent interactions in RNAi may preclude true experimental control. For example, temperature likely plays an unseen hand in RNAi experimentation, given the exquisite sensitivity of *C. elegans* to temperature (Testa *et al.*, 2020) and the intimate relationship between temperature and other stresses and RNAi (Frézal *et al.*, 2018; Houry-Zeevi *et al.*, 2021; Pagliuso *et al.*, 2021). We controlled environmental temperature by conducting all RNAi assays at 20°C , but RNAi competence may be maximized at different temperatures in different strains. On the other hand, temperature may be used to uncover plastic and adaptive responses (Paaby & Testa, 2018). In this system, which

increasingly appears to be dominated by transgenerationally inherited small RNA programs (*Houri-Zeevi et al., 2021*), temperature may prove a valuable tool in exposing the variability and function of *C. elegans* inheritance systems in the wild (*Frézal et al., 2018*).

MATERIALS AND METHODS

Strains used in this study

Table S3 contains a complete list of strains used in this study. To introduce germline-expressed GFP into wild isolates, we introgressed *zuIs178 [his-72(1kb 5' UTR)::his-72::SRPVAT::GFP::his-72 (1KB 3' UTR) + 5.7 kb XbaI - HindIII unc-119(+)]*; *stIs10024 [pie-1::H2B::GFP::pie-1 3' UTR + unc-119(+)]* into strains CB4856, ECA369, JU1522 and QX1211 by crossing to RW10029 and backcrossing to the wild strain for 10-18 generations.

Worm husbandry

Worms were cultured following standard protocol (*Stiernagle, 2006*), though we added 1.25% agarose to plates used to maintain non-N2 wild isolates, to avoid burrowing. Worms were maintained at 20°C without starving for at least three generations before initiating an experiment, with the exception of QX1211, which was maintained at 18°C to avoid induction of the mortal germline phenotype (*Frézal et al., 2018*).

RNA interference

General culture conditions

RNAi was induced by feeding and experiments were carried out on plates, at 20°C, based on methods previously described (*Ahringer, 2006; Kamath et al., 2001*). In brief: to target endogenous germline-expressed genes, we fed worms HT115 *E. coli* bacteria that had been transformed with the pL4440-derived *par-1* (H39E23.1), *par-4* (Y59A8B.14), *pos-1* (F52E1.1), or GFP feeding vector (*Timmons et al., 2001*). The *par-1* and *pos-1* vectors were obtained from the Ahringer feeding library (*Kamath & Ahringer, 2003*); *par-4* was a gift from M. Mana. To target GFP, we

transformed HT115 with pL4417, which carries 0.7 kb of GFP coding sequence (*Timmons et al., 2001*). We used *E. coli* carrying the empty pL4440 vector as a negative control. Bacteria were streaked from frozen stocks onto LB agar plates with carbenicillin (25 ug/mL) and tetracycline (12.5 mg/mL); liquid cultures were inoculated with 5-10 colonies from <1 week old plates, into LB broth with carbenicillin (50 ug/mL) and tetracycline (12.5 mg/mL) and incubated for 16-18hrs shaking at 37°C, then amplified in a 1:200 dilution with carbenicillin (50 ug/mL) for 6hrs. Seeded plates were incubated in the dark at room temperature and used no earlier than 44hrs and no later than 78hrs. Experimental worms were exposed to RNAi bacteria as L1s by hatching on RNAi plates, synchronized either by bleaching (*Stiernagle, 2006*) or by timed egg-laying by the hermaphrodite mothers.

Embryonic lethality assays

To measure RNAi response by phenotypic penetrance, we targeted *par-1* or *pos-1* transcripts in the hermaphrodite germline and measured embryonic lethality in the next generation. Experimental worms were reared on RNAi plates and transferred as L4s to fresh RNAi plates for the egg-laying assay, remaining continuously exposed to RNAi bacteria since their hatching. For all experiments except those explicitly testing variation in penetrance between individual worms (Figure 1B, Figure 3C), the L4 hermaphrodites were pooled in small groups of 4-6 on 6-10 replicate assay plates. For the complementation tests (Figure 3, Figure 4, Figure S2), all embryos within the first ~15hrs of egg-laying were scored for hatching, typically 100-200 embryos per plate. For assays testing RNAi within a defined window of reproductive maturity, we scored the embryos laid in a 4-6hr window within the first 8hrs of egg-laying (Figure 1A), or a 2hr window 4hrs after egg-laying began (Figure 2C). For the experiment measuring RNAi in individual worms over their reproductive lifespan (Figure 1B, Figure S1), the L4 hermaphrodites were singled to RNAi plates, permitted to lay embryos, and continually transferred to fresh plates until they ceased to lay, or laid only unfertilized eggs. To score embryos as dead or alive, we removed the egg-laying adult(s), incubated the plates at 20°C for 24hrs, and counted (dead) embryos and hatched larvae using a stereoscope. Experiments included 6-10 (RNAi treatment) or 4-6 (negative control) replicate plates.

To test the effect of genotype on embryonic lethality following exposure to RNAi, the counts of dead embryos and hatched larvae from each replicate plate were bound together as a single response variable and modeled with a generalized linear model with a quasibinomial error structure, implemented by the *glm* function in R. The model included a single linear predictor for genotype and took the form $E(Y)=g^{-1}(\beta_0+\beta_{\text{genotype}})$. Within each experiment, differences between specific genotypes were assessed by pairwise contrasts using the "Tukey" specification in the function *glht* in the R package *multcomp* (Hothorn *et al.*, 2008).

RNAi against GFP

To measure germline RNAi by GFP knockdown, worms carrying a histone-linked GFP driven by a *pie-1* promoter were fed RNAi bacteria targeting GFP. Synchronized animals were grown on RNAi plates, then individually selected for imaging at the following stages: young adults (6±2hrs after exiting L4 stage), day one adults (24±2hrs), and day 2 adults (48±2hrs). For whole worm fluorescence imaging, animals were anesthetized with 10mM NaN₃ and mounted on 2% agarose pads, then imaged using a 10× objective with the PerkinElmer UltraVIEW VoX spinning disk confocal microscope equipped with an EM-CCD camera. Raw images were exported as OME.TIFF files. We used Fiji (Schindelin *et al.*, 2012) to acquire the sums of intensity in the Z projection, then quantitated the GFP fluorescence by subtracting the integrated intensity of the background, over the area of the worm, from the integrated intensity of the whole animal. To test whether RNAi-treated worms exhibited reduced fluorescence relative to control worms, we analyzed the six samples (three treatment timepoints, three control timepoints) for each strain using a one-way ANOVA, then performed treatment-control contrasts within each timepoint using the R function *TukeyHSD()*.

Single-molecule fluorescence *in situ* hybridization

Sample preparation and imaging

Custom Stellaris FISH probes were designed with the Stellaris Probe Designer (LGC Biosearch Technologies). We excluded polymorphic sites during probe design. Worms were synchronized on tryptone-free NGM agar plates at the L1 stage and reared on RNAi bacteria as described

above. Embryos were extracted by standard bleaching/washing, fixed using 3.7% formaldehyde in RNase-free phosphate buffered saline, and hybridized (100nM at 37°C for 4hrs) with a Quasar 570 labeled probe set targeting either *par-1* or *par-4*, following the manufacturer's instructions. Samples were mounted using VECTASHIELD antifade mounting medium with DAPI (Vector Labs #H-1200) on no. 1 cover slides. Images were captured with a 100X oil immersion objective on a PerkinElmer UltraVIEW VoX spinning disk confocal microscope equipped with an EM-CCD camera and piezoelectric motorized stage. Three-dimensional image stacks were collected using Volocity 3D visualization software (PerkinElmer) and exported as TIFF files.

Quantitative Analysis

We created image segmentation masks and counted chromosome clusters manually using ImageJ (*Schneider et al., 2012*). Single molecule FISH spot quantification was performed using Aro, a MATLAB-based, machine learning pipeline designed for single-molecule visualization in worm embryos (*Wu & Rifkin, 2015*). We used multiple samples from each of the genetic backgrounds and treatments to create the training sets for the random forest classifier. To test whether means or variances in transcript counts differed for RNAi-treated versus untreated samples within a strain, we applied two-sample *t*-tests and *F*-tests, respectively. For these tests, we only considered early stage embryos (up to four cells). To evaluate changes in transcript abundance over a wider range of embryonic development, we considered embryos with up to 30 nuclei and used ANCOVA to ask whether, adjusted for embryo stage, transcript levels varied across strains within the negative control condition; and whether, adjusted for embryo stage, transcript levels varied between control and treatment conditions within a strain. We used minimal model selection to test for changes in the way transcript level depended upon embryo stage (i.e. changes in slope). We estimated ANCOVA effect sizes as ω^2 using the R package *sjstats* (*Lüdtke, 2018*).

RNA-seq

Library preparation and sequencing

Healthy cultures of strains N2, CB4856, QX1211, JU1088 and EG4348, reared for several generations without starving or bleaching, were bleached to retrieve large numbers of embryos.

Synchronized L1 larvae were reared on plates with *par-1*, *pos-1*, or empty RNAi feeding vector, details as described above. Young, reproductively mature hermaphrodites were washed off plates and rinsed twice with M9, then RNA was extracted with TRIzol (Invitrogen #15596026) and RNeasy columns (Qiagen #74104), following (He, 2011). All samples were collected and processed simultaneously and in triplicate, starting with replicate plates of worms. cDNA was generated from fresh RNA samples, using 500ng of RNA and 10 cycles of PCR. Libraries were prepared with the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #7760) and quality checked using an Agilent 2100 Bioanalyzer. BluePippon (Sage Science) was used to size-select fragments in the pooled library, and sequencing was performed using Illumina NextSeq for single-end 75bp reads. Sequencing was performed by the Molecular Evolution Core facility at the Georgia Institute of Technology.

Alignment and gene expression quantification

Before alignment, Illumina TruSeq adapters were trimmed from RNA-seq reads using Trimmomatic (v0.3.9) (Bolger et al., 2014) with the following parameters: *ILLUMINACLIP:TruSeq3-SE.fa:1:30:12*. Trimmed reads were aligned to the *C. elegans* genome (cel1 release ws263) using STAR (v2.6.1a) (Dobin et al., 2013) with the following parameters (all others default): *--outSAMunmapped Within --outMultimapperOrder Random --outFilterMismatchNmax 33 --seedSearchStartLmax 33 --alignSJoverhangMin 8 --outFilterScoreMinOverLread 0.3 --alignIntronMin 40 --alignIntronMax 2200 --varVCFfile --outSAMattributes NH HI AS nM vG vA vW --waspOutputMode SAMtag*. The *--varVCFfile* and *--waspOutputMode* parameters (as well as output attributes *vG*, *vA*, and *vW*) run the WASP (van de Geijn et al., 2015) algorithm during alignment, identifying reads that cover single nucleotide variants occurring in any of the four non-reference strains and determining whether the read would map uniquely when containing both the reference and alternate alleles. This process enables exclusion of reads that would be discarded when containing non-reference alleles but included when containing reference alleles, allowing better comparison across strains. Single nucleotide variants in any of the four non-N2 strains in the CeNDR (Cook et al. 2017) VCF (WI.20180527.hard-filter.vcf) were masked by WASP by including the input *--varVCFfile* in STAR.

Reads were assigned to genes and counted using an extension of the ‘HTSeq-count’ method (Anders *et al.*, 2015), wherein totals of all reads, all unique reads, and all reads passing WASP’s filter (aligning uniquely when containing either the reference allele or the alternate allele, whichever was actually observed) mapping to each gene were returned. This was implemented with a custom Python script using the *htseq* module (v0.11.3).

Differential expression analysis

Differential gene expression was analyzed in R (v4.0.3) (R Core Team, 2020) using the *DESeq2* package (v1.28.1) (Love *et al.*, 2014); effect sizes and p-values were corrected using the ‘ashr’ method from the *ashr* package (v2.2-47) (Stephens, 2017). The counts for each gene were modeled using the *DESeq2* negative binomial generalized linear model as follows:

$$\log_2(q_{ij}) = \beta_i x_j + \beta_i y_j,$$

where for gene *i*, sample *j*, *q* is proportional to the true concentration of RNA fragments for a gene, with *q* derived from observed WASP-passing read counts per gene via negative binomial gene-specific dispersion modeling including sample-specific size factors (Love *et al.*, 2014). β_i gives the log2 fold changes for gene *i*. *x* represents the biological replicate and *y* represents the strain-treatment combination for the sample, with a total of 15 combinations: five strains by three RNAi treatments, including the control.

Results were extracted as contrasts between the strain-treatment combinations of interest, e.g. strain vs strain on the control condition, or treatment vs control for a single strain. Genes were considered differentially expressed if their ashhr-adjusted p-values were < 0.1. These adjusted p-values represent FDRs that account for variance, initial effect size and the number of tests performed. The effect sizes reported are ashhr-adjusted log2 fold changes.

Droplet digital PCR

To design an unbiased primer set, we identified regions of identical sequence between *ppw-1* and *sago-2* and across the ten strains of interest. Following (Kamitaki *et al.*, 2018), we chose primers to target both genes, and probes to discriminate between *ppw-1* (FAM) and *sago-2* (HEX). Sequences are as follows: forward (CTTGGTACCGCTCCGCTC), reverse

(GCTGATTCGGTTTGATCGTC), *ppw-1* probe (AGACGAGAAATGTGGAGAGGGGAA), *sago-2* probe (AGACGAGAAATGAGGAGTGGGGAA). Both probes anneal in the same location, ensuring competition between them.

Worms from strains N2, CB4856, CB4852, DL238, ECA369, EG4348, JU1088, JU1581, KR314, and QX1211 were reared under standard conditions (as above), bleached to isolate embryos, and grown to reproductive maturity. RNA was extracted with TRIzol (Invitrogen #15596026) and RNeasy columns (Qiagen #74104), following (He, 2011). RNA was collected at two timepoints, early and middle reproductive maturity (68±2hrs and 90±2hrs after bleaching, respectively). RNA sample concentrations were quantified and standardized using a Nanodrop (Thermo Scientific), and cDNA was synthesized using the ProtoScript II First Stand cDNA Synthesis Kit (NEB #E6560S). The experiment was replicated as follows: from each experimental condition, we collected two RNA samples, for two biological replicates; within the plate, each reaction was duplicated, for two technical replicates; and we conducted the entire experiment twice.

Droplet digital PCR was carried out with the Bio-Rad QX200 system following the manufacturer's protocol, and results were obtained using the QuantaSoft software (Bio-Rad), via both automatic thresholding and manual droplet selection. All samples produced >8000 droplets and results from all samples were retained. Concentration, given by number of copies per µL, was modeled with a quasipoisson error structure using the *glm()* function in R. As *ppw-1* was detected at an order of magnitude higher than *sago-2*, we analyzed the two genes separately. By model selection, we identified the minimal model that best described the observed differences in concentration. For the *ppw-1* analysis, we dropped run date from the model, as it was not significant; for *sago-2*, run date contributed <1% to the total observed deviance (Table S1), but was nevertheless significant, so it was retained. The final models were: Concentration ~ Strain*DevStage/BiolRep for *ppw-1*, and Concentration ~ RunDate + Strain*DevStage/BiolRep for *sago-2*. To determine which strains differed in *ppw-1* or *sago-2* levels, we performed pairwise contrasts among strains using the *TukeyHSD()* function and a family-wise confidence level of 95% (only a subset of comparisons are reported in the text). To determine which strains showed differences in concentration according to developmental stage, we performed pairwise

contrasts using the *lsmeans()* function in the package *lsmeans*, using a confidence level of 95% following a Bonferroni correction for multiple tests.

Genotype and sequence analysis

To evaluate population-level allelic variation at known RNAi genes, we queried the *C. elegans* Natural Diversity Resource (<https://elegansvariation.org>), which provides genotype data for 403 wild isotypes from short read sequence data mapped to the N2 reference genome (*Cook et al., 2017*). Specifically, we downloaded the VCF (WI.20200815.hard-filter.isotype.vcf) and used the R package *VariantAnnotation* (*Obenchain et al., 2014*) to extract information about mutations and mapping coverage, and the package *PopGenome* (*Pfeifer et al., 2014*) to estimate nucleotide diversity, at each gene. Haplotype networks for individual genes were determined using the R package *pegas* (*Paradis, 2010*).

For a subset of strains, we verified and/or supplemented the genotype data with de novo-assembled genome data and long read data. Genomic DNA of strains AB2, EG4347, EG4348, JU1088, JU1171, PB306, PX174, QX1211 and QX1216 was prepared using standard phenol/chloroform extraction and ethanol precipitation. Samples were cleaned with DNA Clean & Concentrator columns (Zymo Research #D4004) and libraries were prepared using NEBNext Ultra II FS DNA Library Prep Kit for Illumina (New England Biolabs #E7805) and Multiplex Oligos for Illumina (NEB #E7500), with customized fragmentation and purification steps to enrich for desired sizes. A final DNA size selection targeting 650bp±50bp was performed using BluePippin (Sage Science). The libraries were sequenced on a HiSeq 2500 (Illumina) on Rapid Run Mode (paired-end 2x250bp) in the Molecular Evolution Core at the Georgia Institute of Technology. Raw data were trimmed using Cutadapt (v1.18) (*Martin, 2011*) and quality control was performed with FastQC (*Andrews, 2021*). Reads were then assembled into contigs with DISCOVAR *de novo* (v52488) (Broad Institute) using default parameters. Separately, QX1211 and JU1088 genomic DNA samples were snap frozen with liquid nitrogen and sent to the Georgia Genomics and Bioinformatics Core (GGBC) at the University of Georgia. Quality was assessed by Qubit (Invitrogen) and NanoDrop (Thermo Scientific), molecular weight distribution was assessed by fragment analysis, and sizes >15kb were selected by BluePippin (Sage Science). Each sample was sequenced on a single SMRT Cell on the PacBio Sequel I platform (Pacific

Biosciences). Genome assembly was performed by the GGBC using Canu (v1.7) (*Koren et al., 2017*).

Computing

Unless otherwise specified, all analyses were performed in R (*R Core Team, 2020*) and figures were generated with the packages *ggplot2* (*Wickham, 2016*) and *ggpubr* (*Kassambara, 2020*). Computationally intensive jobs, including read mapping and genome assembly, were performed on PACE, the high performance computing platform at the Georgia Institute of Technology.

ACKNOWLEDGEMENTS

We wish to acknowledge the core facilities at the Parker H. Petit Institute for Bioengineering and Bioscience at the Georgia Institute of Technology for the use of their shared equipment, services and expertise. Specifically, we are grateful to Aaron Lifland at the Optimal Microscopy Core for training in confocal imaging, and Anton Bryksin, Naima Djeddar, and Shweta Biliya at the Molecular Evolution Core for collaboration on the sequencing projects. We also thank the Georgia Genomics and Bioinformatics Core at the University of Georgia for PacBio sequencing and genome assembly. We thank Miyeko Mana for the gift of the *par-4* RNAi clone, and Lijiang Long and Patrick McGrath for strain PTM377. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). This research was supported in part through research cyberinfrastructure resources and services provided by the Partnership for an Advanced Computing Environment (PACE) at the Georgia Institute of Technology. This research was funded by NIH grant R35 GM119744 to A.B.P. and NSF grant MCB1518314 to D.A.P.

LITERATURE CITED

- Ahringer, J. (2006). Reverse genetics. In *WormBook: The Online Review of C. elegans Biology [Internet]*. WormBook. <https://www.ncbi.nlm.nih.gov/books/NBK19711/>
- Anders, S., Pyl, P. T., & Huber, W. (2015). HTSeq—A Python framework to work with high-throughput sequencing data. *Bioinformatics (Oxford, England)*, 31(2), 166–169. <https://doi.org/10.1093/bioinformatics/btu638>

984 Andersen, E. C., Gerke, J. P., Shapiro, J. A., Crissman, J. R., Ghosh, R., Bloom, J. S., Félix, M.-A., &
985 Kruglyak, L. (2012). Chromosome-scale selective sweeps shape *Caenorhabditis elegans* genomic
986 diversity. *Nature Genetics*, 44(3), 285–290. <https://doi.org/10.1038/ng.1050>

987 Andrews, S. (2021). *FastQC: A quality control tool for high throughput sequence data*.
988 <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>

989 Ashe, A., Bélicard, T., Le Pen, J., Sarkies, P., Frézal, L., Lehrbach, N. J., Félix, M.-A., & Miska, E. A.
990 (2013). A deletion polymorphism in the *Caenorhabditis elegans* RIG-I homolog disables viral RNA
991 dicing and antiviral immunity. *ELife*, 2, e00994. <https://doi.org/10.7554/eLife.00994>

992 Ashe, A., Sarkies, P., Le Pen, J., Tanguy, M., & Miska, E. A. (2015). Antiviral RNA Interference against
993 Orsay Virus Is neither Systemic nor Transgenerational in *Caenorhabditis elegans*. *Journal of*
994 *Virology*, 89(23), 12035–12046. <https://doi.org/10.1128/JVI.03664-14>

995 Ben-David, E., Burga, A., & Kruglyak, L. (2017). A maternal-effect selfish genetic element in
996 *Caenorhabditis elegans*. *Science (New York, N.Y.)*, 356(6342), 1051–1055.
997 <https://doi.org/10.1126/science.aan0621>

998 Billi, A. C., Fischer, S. E. J., & Kim, J. K. (2014). Endogenous RNAi pathways in *C. elegans*.
999 *WormBook: The Online Review of C. Elegans Biology*, 1–49.
1000 <https://doi.org/10.1895/wormbook.1.170.1>

1001 Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence
1002 data. *Bioinformatics (Oxford, England)*, 30(15), 2114–2120.
1003 <https://doi.org/10.1093/bioinformatics/btu170>

1004 Buck, A. H., & Blaxter, M. (2013). Functional diversification of Argonautes in nematodes: An expanding
1005 universe. *Biochemical Society Transactions*, 41(4), 881–886. <https://doi.org/10.1042/BST20130086>

1006 Campbell, R. F., McGrath, P. T., & Paaby, A. B. (2018). Analysis of Epistasis in Natural Traits Using
1007 Model Organisms. *Trends in Genetics: TIG*, 34(11), 883–898.
1008 <https://doi.org/10.1016/j.tig.2018.08.002>

1009 Chandler, C. H., Chari, S., & Dworkin, I. (2013). Does your gene need a background check? How genetic
1010 background impacts the analysis of mutations, genes, and evolution. *Trends in Genetics: TIG*, 29(6),
1011 358–366. <https://doi.org/10.1016/j.tig.2013.01.009>

1012 Charles, S., Aubry, G., Chou, H.-T., Paaby, A. B., & Lu, H. (2021). High-Temporal-Resolution smFISH
1013 Method for Gene Expression Studies in *Caenorhabditis elegans* Embryos. *Analytical Chemistry*,
1014 93(3), 1369–1376. <https://doi.org/10.1021/acs.analchem.0c02966>

1015 Cook, D. E., Zdraljevic, S., Roberts, J. P., & Andersen, E. C. (2017). CeNDR, the *Caenorhabditis elegans*
1016 natural diversity resource. *Nucleic Acids Research*, 45(D1), D650–D657.
1017 <https://doi.org/10.1093/nar/gkw893>

1018 Crombie, T. A., Zdraljevic, S., Cook, D. E., Tanny, R. E., Brady, S. C., Wang, Y., Evans, K. S., Hahnel,
1019 S., Lee, D., Rodriguez, B. C., Zhang, G., van der Zwagg, J., Kiontke, K., & Andersen, E. C. (2019).
1020 Deep sampling of Hawaiian *Caenorhabditis elegans* reveals high genetic diversity and admixture
1021 with global populations. *ELife*, 8, e50465. <https://doi.org/10.7554/eLife.50465>

1022 Dalzell, J. J., McVeigh, P., Warnock, N. D., Mitreva, M., Bird, D. M., Abad, P., Fleming, C. C., Day, T.
1023 A., Mousley, A., Marks, N. J., & Maule, A. G. (2011). RNAi effector diversity in nematodes. *PLoS*
1024 *Neglected Tropical Diseases*, 5(6), e1176. <https://doi.org/10.1371/journal.pntd.0001176>

1025 De-Souza, E. A., Camara, H., Salgueiro, W. G., Moro, R. P., Knittel, T. L., Tonon, G., Pinto, S., Pinca, A.
1026 P. F., Antebi, A., Pasquinelli, A. E., Massirer, K. B., & Mori, M. A. (2019). RNA interference may
1027 result in unexpected phenotypes in *Caenorhabditis elegans*. *Nucleic Acids Research*, 47(8), 3957–
1028 3969. <https://doi.org/10.1093/nar/gkz154>

1029 Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., &
1030 Gingeras, T. R. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics*, 29(1), 15–21.
1031 <https://doi.org/10.1093/bioinformatics/bts635>

1032 Elvin, M., Snoek, L. B., Frejno, M., Klemstein, U., Kammenga, J. E., & Poulin, G. B. (2011). A fitness
1033 assay for comparing RNAi effects across multiple *C. elegans* genotypes. *BMC Genomics*, 12, 510.
1034 <https://doi.org/10.1186/1471-2164-12-510>

1035 Félix, M.-A. (2008). RNA interference in nematodes and the chance that favored Sydney Brenner.
1036 *Journal of Biology*, 7(9), 34. <https://doi.org/10.1186/jbiol97>

1037 Félix, M.-A., Ashe, A., Piffaretti, J., Wu, G., Nuez, I., Bêlicard, T., Jiang, Y., Zhao, G., Franz, C. J.,
1038 Goldstein, L. D., Sanroman, M., Miska, E. A., & Wang, D. (2011). Natural and experimental
1039 infection of *Caenorhabditis* nematodes by novel viruses related to nodaviruses. *PLoS Biology*, 9(1),
1040 e1000586. <https://doi.org/10.1371/journal.pbio.1000586>

1041 Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and
1042 specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*,
1043 391(6669), 806–811. <https://doi.org/10.1038/35888>

1044 Franz, C. J., Renshaw, H., Frezal, L., Jiang, Y., Félix, M.-A., & Wang, D. (2014). Orsay, Santeuil and Le
1045 Blanc viruses primarily infect intestinal cells in *Caenorhabditis* nematodes. *Virology*, 448, 255–264.
1046 <https://doi.org/10.1016/j.virol.2013.09.024>

1047 Frézal, L., Demoinet, E., Braendle, C., Miska, E., & Félix, M.-A. (2018). Natural genetic variation in a
1048 multigenerational phenotype in *C. elegans*. *Current Biology : CB*, 28(16), 2588-2596.e8.
1049 <https://doi.org/10.1016/j.cub.2018.05.091>

1050 Grishok, A. (2013). Biology and Mechanisms of Short RNAs in *Caenorhabditis elegans*. *Advances in*
1051 *Genetics*, 83, 1–69. <https://doi.org/10.1016/B978-0-12-407675-4.00001-8>

1052 Harris, T. W., Arnaboldi, V., Cain, S., Chan, J., Chen, W. J., Cho, J., Davis, P., Gao, S., Grove, C. A.,
1053 Kishore, R., Lee, R. Y. N., Muller, H.-M., Nakamura, C., Nuin, P., Paulini, M., Raciti, D., Rodgers,
1054 F. H., Russell, M., Schindelman, G., ... Sternberg, P. W. (2020). WormBase: A modern Model
1055 Organism Information Resource. *Nucleic Acids Research*, 48(D1), D762–D767.
1056 <https://doi.org/10.1093/nar/gkz920>

1057 He, F. (2011). Total RNA Extraction from *C. elegans*. *Bio-Protocol*, e47–e47.

1058 Hodgkin, J., & Doniach, T. (1997). Natural variation and copulatory plug formation in *Caenorhabditis*
1059 *elegans*. *Genetics*, 146(1), 149–164.

1060 Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous inference in general parametric models.
1061 *Biometrical Journal. Biometrische Zeitschrift*, 50(3), 346–363.
1062 <https://doi.org/10.1002/bimj.200810425>

1063 Hourri-Zeevi, L., Korem Kohanim, Y., Antonova, O., & Rechavi, O. (2020). Three Rules Explain
1064 Transgenerational Small RNA Inheritance in *C. elegans*. *Cell*, 182(5), 1186-1197.e12.
1065 <https://doi.org/10.1016/j.cell.2020.07.022>

1066 Houri-Zeevi, L., Teichman, G., Gingold, H., & Rechavi, O. (2021). Stress resets ancestral heritable small
1067 RNA responses. *ELife*, 10, e65797. <https://doi.org/10.7554/eLife.65797>

1068 Kamath, R. S., & Ahringer, J. (2003). Genome-wide RNAi screening in *Caenorhabditis elegans*. *Methods*
1069 (*San Diego, Calif.*), 30(4), 313–321. [https://doi.org/10.1016/s1046-2023\(03\)00050-1](https://doi.org/10.1016/s1046-2023(03)00050-1)

1070 Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N.,
1071 Moreno, S., Sohrmann, M., Welchman, D. P., Zipperlen, P., & Ahringer, J. (2003). Systematic
1072 functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature*, 421(6920), 231–
1073 237. <https://doi.org/10.1038/nature01278>

1074 Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G., & Ahringer, J. (2001). Effectiveness
1075 of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis*
1076 *elegans*. *Genome Biology*, 2(1), RESEARCH0002. [https://doi.org/10.1186/gb-2000-2-1-](https://doi.org/10.1186/gb-2000-2-1-research0002)
1077 [research0002](https://doi.org/10.1186/gb-2000-2-1-research0002)

1078 Kamitaki, N., Usher, C. L., & McCarroll, S. A. (2018). Using Droplet Digital PCR to Analyze Allele-
1079 Specific RNA Expression. *Methods in Molecular Biology (Clifton, N.J.)*, 1768, 401–422.
1080 https://doi.org/10.1007/978-1-4939-7778-9_23

1081 Kassambara, A. (2020). *ggpubr: “ggplot2” Based Publication Ready Plots*.
1082 <https://rpkgs.datanovia.com/ggpubr/>

1083 Koren, S., Walenz, B. P., Berlin, K., Miller, J. R., Bergman, N. H., & Phillippy, A. M. (2017). Canu:
1084 Scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation.
1085 *Genome Research*, 27(5), 722–736. <https://doi.org/10.1101/gr.215087.116>

1086 Lee, D., Zdravljjevic, S., Stevens, L., Wang, Y., Tanny, R. E., Crombie, T. A., Cook, D. E., Webster, A. K.,
1087 Chirakar, R., Baugh, L. R., Sterken, M. G., Braendle, C., Félix, M.-A., Rockman, M. V., &
1088 Andersen, E. C. (2021). Balancing selection maintains hyper-divergent haplotypes in
1089 *Caenorhabditis elegans*. *Nature Ecology & Evolution*, 5(6), 794–807.
1090 <https://doi.org/10.1038/s41559-021-01435-x>

1091 Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for
1092 RNA-seq data with DESeq2. *Genome Biology*, 15(12), 550. [https://doi.org/10.1186/s13059-014-](https://doi.org/10.1186/s13059-014-0550-8)
1093 [0550-8](https://doi.org/10.1186/s13059-014-0550-8)

1094 Lüdtke, D. (2018). *sjstats: Statistical Functions for Regression Models*. Zenodo.
1095 <https://doi.org/10.5281/zenodo.1489175>

1096 Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads.
1097 *EMBnet.Journal*, 17(1), 10–12. <https://doi.org/10.14806/ej.17.1.200>

1098 Nuez, I., & Félix, M.-A. (2012). Evolution of susceptibility to ingested double-stranded RNAs in
1099 *Caenorhabditis* nematodes. *PloS One*, 7(1), e29811. <https://doi.org/10.1371/journal.pone.0029811>

1100 Obbard, D. J., Gordon, K. H. J., Buck, A. H., & Jiggins, F. M. (2009). The evolution of RNAi as a
1101 defence against viruses and transposable elements. *Philosophical Transactions of the Royal Society*
1102 *B: Biological Sciences*, 364(1513), 99–115. <https://doi.org/10.1098/rstb.2008.0168>

1103 Obenchain, V., Lawrence, M., Carey, V., Gogarten, S., Shannon, P., & Morgan, M. (2014).
1104 VariantAnnotation: A Bioconductor package for exploration and annotation of genetic variants.
1105 *Bioinformatics (Oxford, England)*, 30(14), 2076–2078.
1106 <https://doi.org/10.1093/bioinformatics/btu168>

- 1107 Paaby, A. B., & Testa, N. D. (2018). Developmental Plasticity and Evolution. In L. Nuno de la Rosa & G.
1108 Müller (Eds.), *Evolutionary Developmental Biology: A Reference Guide* (pp. 1–14). Springer
1109 International Publishing. https://doi.org/10.1007/978-3-319-33038-9_110-1
- 1110 Paaby, A. B., White, A. G., Riccardi, D. D., Gunsalus, K. C., Piano, F., & Rockman, M. V. (2015). Wild
1111 worm embryogenesis harbors ubiquitous polygenic modifier variation. *ELife*, 4.
1112 <https://doi.org/10.7554/eLife.09178>
- 1113 Pagliuso, D. C., Bodas, D. M., & Pasquinelli, A. E. (2021). Recovery from heat shock requires the
1114 microRNA pathway in *Caenorhabditis elegans*. *PLOS Genetics*, 17(8), e1009734.
1115 <https://doi.org/10.1371/journal.pgen.1009734>
- 1116 Paradis, E. (2010). pegas: An R package for population genetics with an integrated-modular approach.
1117 *Bioinformatics (Oxford, England)*, 26(3), 419–420. <https://doi.org/10.1093/bioinformatics/btp696>
- 1118 Pfeifer, B., Wittelsbürger, U., Ramos-Onsins, S. E., & Lercher, M. J. (2014). PopGenome: An efficient
1119 Swiss army knife for population genomic analyses in R. *Molecular Biology and Evolution*, 31(7),
1120 1929–1936. <https://doi.org/10.1093/molbev/msu136>
- 1121 Pollard, D. A., & Rockman, M. V. (2013). Resistance to germline RNA interference in a *Caenorhabditis*
1122 *elegans* wild isolate exhibits complexity and nonadditivity. *G3 (Bethesda, Md.)*, 3(6), 941–947.
1123 <https://doi.org/10.1534/g3.113.005785>
- 1124 R Core Team. (2020). *R: A language and environment for statistical computing*. R Foundation for
1125 Statistical Computing. <https://www.R-project.org/>
- 1126 Raj, A., van den Bogaard, P., Rifkin, S. A., van Oudenaarden, A., & Tyagi, S. (2008). Imaging individual
1127 mRNA molecules using multiple singly labeled probes. *Nature Methods*, 5(10), 877–879.
1128 <https://doi.org/10.1038/nmeth.1253>
- 1129 Richaud, A., Frézal, L., Tahan, S., Jiang, H., Blatter, J. A., Zhao, G., Kaur, T., Wang, D., & Félix, M.-A.
1130 (2019). Vertical transmission in *Caenorhabditis* nematodes of RNA molecules encoding a viral
1131 RNA-dependent RNA polymerase. *Proceedings of the National Academy of Sciences of the United*
1132 *States of America*, 116(49), 24738–24747. <https://doi.org/10.1073/pnas.1903903116>
- 1133 Rual, J.-F., Ceron, J., Koreth, J., Hao, T., Nicot, A.-S., Hirozane-Kishikawa, T., Vandenhaute, J., Orkin,
1134 S. H., Hill, D. E., van den Heuvel, S., & Vidal, M. (2004). Toward improving *Caenorhabditis*
1135 *elegans* phenome mapping with an ORFeome-based RNAi library. *Genome Research*, 14(10B),
1136 2162–2168. <https://doi.org/10.1101/gr.2505604>
- 1137 Sarkies, P., Ashe, A., Le Pen, J., McKie, M. A., & Miska, E. A. (2013). Competition between virus-
1138 derived and endogenous small RNAs regulates gene expression in *Caenorhabditis elegans*. *Genome*
1139 *Research*, 23(8), 1258–1270. <https://doi.org/10.1101/gr.153296.112>
- 1140 Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S.,
1141 Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K.,
1142 Tomancak, P., & Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis.
1143 *Nature Methods*, 9(7), 676–682. <https://doi.org/10.1038/nmeth.2019>
- 1144 Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image
1145 analysis. *Nature Methods*, 9(7), 671–675. <https://doi.org/10.1038/nmeth.2089>
- 1146 Schott, D. H., Cureton, D. K., Whelan, S. P., & Hunter, C. P. (2005). An antiviral role for the RNA
1147 interference machinery in *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences*
1148 *of the United States of America*, 102(51), 18420–18424. <https://doi.org/10.1073/pnas.0507123102>

1149 Seidel, H. S., Ailion, M., Li, J., van Oudenaarden, A., Rockman, M. V., & Kruglyak, L. (2011). A novel
1150 sperm-delivered toxin causes late-stage embryo lethality and transmission ratio distortion in *C.*
1151 *elegans*. *PLoS Biology*, 9(7), e1001115. <https://doi.org/10.1371/journal.pbio.1001115>

1152 Seidel, H. S., Rockman, M. V., & Kruglyak, L. (2008). Widespread genetic incompatibility in *C. elegans*
1153 maintained by balancing selection. *Science (New York, N.Y.)*, 319(5863), 589–594.
1154 <https://doi.org/10.1126/science.1151107>

1155 Shabalina, S. A., & Koonin, E. V. (2008). Origins and evolution of eukaryotic RNA interference. *Trends*
1156 *in Ecology & Evolution*, 23(10), 578–587. <https://doi.org/10.1016/j.tree.2008.06.005>

1157 Shiu, P. K., & Hunter, C. P. (2017). Early Developmental Exposure to dsRNA Is Critical for Initiating
1158 Efficient Nuclear RNAi in *C. elegans*. *Cell Reports*, 18(12), 2969–2978.
1159 <https://doi.org/10.1016/j.celrep.2017.03.002>

1160 Stephens, M. (2017). False discovery rates: A new deal. *Biostatistics (Oxford, England)*, 18(2), 275–294.
1161 <https://doi.org/10.1093/biostatistics/kxw041>

1162 Stiernagle, T. (2006). Maintenance of *C. elegans*. *WormBook: The Online Review of C. Elegans Biology*,
1163 1–11. <https://doi.org/10.1895/wormbook.1.101.1>

1164 Testa, N. D., Kaul, S., Le, K. N., Zhan, M., Lu, H., & Paaby, A. B. (2020). A portable, low-cost device
1165 for precise control of specimen temperature under stereomicroscopes. *PloS One*, 15(3), e0230241.
1166 <https://doi.org/10.1371/journal.pone.0230241>

1167 Tijsterman, M., Okihara, K. L., Thijssen, K., & Plasterk, R. H. A. (2002). PPW-1, a PAZ/PIWI protein
1168 required for efficient germline RNAi, is defective in a natural isolate of *C. elegans*. *Current*
1169 *Biology: CB*, 12(17), 1535–1540. [https://doi.org/10.1016/s0960-9822\(02\)01110-7](https://doi.org/10.1016/s0960-9822(02)01110-7)

1170 Timmons, L., Court, D. L., & Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce
1171 specific and potent genetic interference in *Caenorhabditis elegans*. *Gene*, 263(1–2), 103–112.
1172 [https://doi.org/10.1016/s0378-1119\(00\)00579-5](https://doi.org/10.1016/s0378-1119(00)00579-5)

1173 Timmons, L., & Fire, A. (1998). Specific interference by ingested dsRNA. *Nature*, 395(6705), 854.
1174 <https://doi.org/10.1038/27579>

1175 van de Geijn, B., McVicker, G., Gilad, Y., & Pritchard, J. K. (2015). WASP: Allele-specific software for
1176 robust molecular quantitative trait locus discovery. *Nature Methods*, 12(11), 1061–1063.
1177 <https://doi.org/10.1038/nmeth.3582>

1178 Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag. <http://ggplot2.org>

1179 Wilkins, C., Dishongh, R., Moore, S. C., Whitt, M. A., Chow, M., & Machaca, K. (2005). RNA
1180 interference is an antiviral defence mechanism in *Caenorhabditis elegans*. *Nature*, 436(7053), 1044–
1181 1047. <https://doi.org/10.1038/nature03957>

1182 Wilson, R. C., & Doudna, J. A. (2013). Molecular Mechanisms of RNA Interference. *Annual Review of*
1183 *Biophysics*, 42(1), 217–239. <https://doi.org/10.1146/annurev-biophys-083012-130404>

1184 Winston, W. M., Sutherlin, M., Wright, A. J., Feinberg, E. H., & Hunter, C. P. (2007). *Caenorhabditis*
1185 *elegans* SID-2 is required for environmental RNA interference. *Proceedings of the National*
1186 *Academy of Sciences of the United States of America*, 104(25), 10565–10570.
1187 <https://doi.org/10.1073/pnas.0611282104>

1188 Wu, A. C.-Y., & Rifkin, S. A. (2015). Aro: A machine learning approach to identifying single molecules
1189 and estimating classification error in fluorescence microscopy images. *BMC Bioinformatics*, 16,
1190 102. <https://doi.org/10.1186/s12859-015-0534-z>

1191 Wynant, N., Santos, D., & Vanden Broeck, J. (2017). The evolution of animal Argonautes: Evidence for
1192 the absence of antiviral AGO Argonautes in vertebrates. *Scientific Reports*, 7(1), 9230.
1193 <https://doi.org/10.1038/s41598-017-08043-5>

1194 Yigit, E. (2007). *The Argonaute Family of Genes in Caenorhabditis Elegans: A Dissertation*. University
1195 of Massachusetts Medical School.

1196 Yigit, E., Batista, P. J., Bei, Y., Pang, K. M., Chen, C.-C. G., Tolia, N. H., Joshua-Tor, L., Mitani, S.,
1197 Simard, M. J., & Mello, C. C. (2006). Analysis of the C. elegans Argonaute Family Reveals that
1198 Distinct Argonautes Act Sequentially during RNAi. *Cell*, 127(4), 747–757.
1199 <https://doi.org/10.1016/j.cell.2006.09.033>

1200 Youngman, E. M., & Claycomb, J. M. (2014). From early lessons to new frontiers: The worm as a
1201 treasure trove of small RNA biology. *Frontiers in Genetics*, 5, 416.
1202 <https://doi.org/10.3389/fgene.2014.00416>

1203

SUPPLEMENTAL FIGURES & TABLES

Figure S1. Embryonic lethality following RNAi against *par-1*.

Figure S2. Complementation test for DL238 and the RNAi-sensitive laboratory strain N2, with and without a deletion allele at *ppw-1*. The embryos scored here were laid by pooled hermaphrodites on replicate plates. Significance level (Tukey's contrasts): $p < 0.05$ (*).

Figure S3. RNAi sensitivity for 55 wild isolates, versus genetic distance from the reference strain N2. RNAi data is from (Paaby *et al.*, 2015), which measured embryonic lethality following RNAi by feeding against 29 individual maternal-effect targets. The y-axis plots the coefficients associated with the strain term in the full statistical model, and represents the strain-specific variation in embryonic lethality associated with RNAi responsiveness; increasingly positive values indicate weaker germline RNAi (Paaby *et al.*, 2015). The x-axis gives the proportion of alternate genotype calls, out of the total number of calls, for each strain in the CeNDR database (Cook *et al.*, 2017).

Figure S4. Droplet digital PCR results for *ppw-1* and *sago-2* for all ten tested strains. Colors correspond to strains also evaluated by RNA-seq (Figure 6).

Figure S5. For 62 RNAi genes, mapping coverage (A) and mutational variation (B) in the 403 strain isotypes from CeNDR. Gene names are color coded by classification: AGO Argonaute (dark blue), PIWI Argonaute (blue), WAGO Argonaute (light blue), Argonaute pseudogenes (grey), and other RNAi factor (black or red). Each point corresponds to an individual strain; zeros (no observation of poor coverage or mutation relative to the reference) are not plotted.

Table S1. Statistical results for the ddPCR analysis. The model used for each gene is described in the Methods.

ppw-1

	Df	Deviance	Resid. Df	Resid. Dev
NULL			158	37942
Strain	9	24433.1	149	13509
DevStage	1	910.3	148	12599
Strain:DevStage	9	4271.9	139	8327
Strain:DevStage:BiolRepl	20	2327.6	119	5999

sago-2

	Df	Deviance	Resid. DF	Resid. Dev
NULL			158	4722.0
RunDate	1	29.38	157	4692.6
Strain	9	2932.73	148	1759.9
DevStage	1	5.87	147	1754.0
Strain:DevStage	9	825.20	138	928.8
Strain:DevStage:BiolRepl	20	436.26	118	492.6

Table S2. Candidate genes for weak germline RNAi in the seven strains tested. Only genes with relevant genotypes in at least one strain are displayed, out of 62 RNAi genes queried. “High impact” indicates disruptive mutations like frameshifts or stop-gains. “Poor mapping coverage” indicates a read depth of 3 or less. “Functional diverged allele” indicates at least 1% nucleotide divergence from the reference genome, including at least five moderate mutations, such as amino acid substitutions, and no high impact mutations.

Class	Gene	One or more high impact mutation	Poor mapping coverage at 10% or more of locus	Functional diverged allele
Argonaute (AGO)	<i>alg-4</i>			QX1211
Argonaute (PIWI)	<i>ergo-1</i>	ECA369	ECA369	
Argonaute (WAGO)	<i>hrde-1</i>			QX1211
	<i>nrde-3</i>			QX1211
	<i>ppw-1</i>	CB4856		
	<i>sago-2</i>	DL238	DL238, ECA369, JU1522, KR314, QX1211	
	<i>wago-10</i>			QX1211
Argonaute (pseudogene)	<i>wago-11</i>	CB4852, KR314, QX1211	CB4856	
	<i>wago-2</i>		QX1211	
	<i>ZK218.8</i>		DL238	
Other RNAi factor	<i>dcr-1</i>			QX1211
	<i>drh-1</i>			QX1211
	<i>eri-1</i>			QX1211
	<i>eri-5</i>		JU1522	
	<i>eri-6</i>			QX1211
	<i>eri-7</i>	CB4856, DL238, ECA369	DL238, JU1522, KR314	
	<i>eri-9</i>			QX1211
	<i>lin-15b</i>	QX1211		ECA369
	<i>nyn-2</i>		DL238	QX1211
	<i>rde-10</i>		DL238, ECA369, JU1522, KR314, QX1211	
	<i>rde-12</i>			QX1211
	<i>rsd-2</i>		JU1522, QX1211	CB4856, DL238, ECA369, QX1211
	<i>sid-1</i>		DL238, ECA369, JU1522, QX1211	
	<i>sid-2</i>			QX1211
	<i>sid-3</i>			QX1211
	<i>hsf-1</i>		DL238, ECA369, JU1522, KR314	ECA369, KR314

Table S3. Strains used in this study.

Ref in text	Name	Genotype	Provenance
CB4852	CB4852	wild-type	Gift from Matthew Rockman
CB4856	CB4856	wild-type	Gift from Matthew Rockman
DL238	DL238	wild-type	Gift from Matthew Rockman
ECA369	ECA369	wild-type	Purchased from CeNDR
ECA701	ECA701	wild-type	Purchased from CeNDR
KR314	KR314	wild-type	Gift from Matthew Rockman
EG4348	EG4348	wild-type	Gift from Matthew Rockman
JU561	JU561	wild-type	Purchased from CeNDR
JU1088	JU1088	wild-type	Gift from Matthew Rockman
JU1522	JU1522	wild-type	Gift from Matthew Rockman
N2	N2	wild-type	Gift from Matthew Rockman
N2 ^{peel-1}	PTM377	<i>peel-1(kah126)</i> I	Gift from Patrick McGrath
N2 ^{ppw-1}	QF201	<i>ppw-1(pk1425)</i> I	Derived from backcrossing strain NL3511 5x to N2
N2 ^{ppw-1;peel-1}	QF204	<i>ppw-1(pk1425) peel-1(kah126)</i> I	Derived from PTM377 and QF201
N2 ^{ppw-1 CB4856}	NL2550	<i>ppw-1(pk2505)</i> I	Purchased from CGC
NL3511	NL3511	<i>ppw-1(pk1425)</i> I	Purchased from CGC
QF14	QF14	<i>zuIs178; stIs10024</i> [RW10029>CB4856]	Derived from backcrossing strain RW10029 18x to CB4856
QF15	QF15	<i>zuIs178; stIs10024</i> [RW10029>JU1522]	Derived from backcrossing strain RW10029 16x to JU1522
QF16	QF16	<i>zuIs178; stIs10024</i> [RW10029>ECA369]	Derived from backcrossing strain RW10029 17x to ECA369
QF90	QF90	<i>zuIs178; stIs10024</i> [RW10029>QX1211]	Derived from backcrossing strain RW10029 10x to QX1211
QX1211	QX1211	wild-type	Gift from Matthew Rockman
RW10029	RW10029	<i>zuIs178; stIs10024</i>	Purchased from CGC
XZ1516	XZ1516	wild-type	Purchased from CeNDR