RESEARCH ARTICLE

# Tissue- and sex-specific small RNAomes reveal sex differences in response to the environment

Alexandra Bezler[1☯¤a], Fabian Braukmann[2,3,4☯], Sean M. West[5¤b], Arthur Duplan[1], Raffaella Conconi[1], Frédéric Schütz[6], Pierre Gönczy[7], Fabio Piano[5,8], Kristin Gunsalus[5,8], Eric A. Miska[2,3,4]*, Laurent Keller[1]*
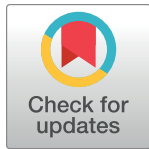
1 Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland, 2 Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Cambridge, United Kingdom, 3 Department of Genetics, University of Cambridge, Cambridge, United Kingdom, 4 Wellcome Sanger Institute, Wellcome Trust Genome Campus, Cambridge, United Kingdom, 5 Center for Genomics & Systems Biology, New York University, New York, New York, United States of America, 6 Bioinformatics Core Facility; SIB Swiss Institute of Bioinformatics and Centre for Integrative Genomics, University of Lausanne, Lausanne, Switzerland, 7 Swiss Institute for Experimental Cancer Research (ISREC), Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland, 8 Center for Genomics & Systems Biology, New York University Abu Dhabi, Abu Dhabi, United Arab Emirates

☯ These authors contributed equally to this work.
¤a Current address: Swiss Institute for Experimental Cancer Research (ISREC), Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland
¤b Current address: Biologics Discovery California, Biologics Discovery Center, Redwood City, United States of America
* eam29@cam.ac.uk (EAM); laurent.keller@unil.ch (LK)

## Abstract

RNA interference (RNAi) related pathways are essential for germline development and fertility in metazoa and can contribute to inter- and trans-generational inheritance. In the nematode *Caenorhabditis elegans*, environmental double-stranded RNA provided by feeding can lead to heritable changes in phenotype and gene expression. Notably, transmission efficiency differs between the male and female germline, yet the underlying mechanisms remain elusive. Here we use high-throughput sequencing of dissected gonads to quantify sex-specific endogenous piRNAs, miRNAs and siRNAs in the *C. elegans* germline and the somatic gonad. We identify genes with exceptionally high levels of secondary 22G RNAs that are associated with low mRNA expression, a signature compatible with silencing. We further demonstrate that contrary to the hermaphrodite germline, the male germline, but not male soma, is resistant to environmental RNAi triggers provided by feeding, in line with previous work. This sex-difference in silencing efficacy is associated with lower levels of gonadal RNAi amplification products. Moreover, this tissue- and sex-specific RNAi resistance is regulated by the germline, since mutant males with a feminized germline are RNAi sensitive. This study provides important sex- and tissue-specific expression data of miRNA, piRNA and siRNA as well as mechanistic insights into sex-differences of gene regulation in response to environmental cues.

## Author summary

Environmental factors such as heat or pathogens influence living organisms, and in some
cases these experiences can be passed on to the next generation. One such environmental
factor is double stranded RNA that can be taken up by some animals and induce changes
in gene expression via small RNA mediated silencing. Here, we use the roundworm *Cae-
norhabditis elegans* to show that the female and male germline react differently to the
presence of such an environmental factor that may affect transmission to following gener-
ations. Sequencing of gonadal small RNA suggests that the underlying sex-differences
affect downstream processing but not uptake. Furthermore, we describe the naturally
occurring small RNA populations of female and male gonads and use genetic mutants to
assign small RNAs to the two tissues of the gonad, namely the somatic gonad or the germ-
line. These sex-and tissue-specific small RNA expression data provide an important
resource for future research. In addition, this work reveals mechanistic insights into sex-
differences in response to environmental cues.

## Introduction

The environment can induce changes in phenotype and gene expression that persist across
multiple generations [reviewed in 1]. Such intra- and trans-generational inheritance can pass
both through the male and female germlines. Several studies have revealed sex-differences in
transmission efficiency of heritable phenotypic changes, yet the underlying molecular mecha-
nisms remain unknown [2–6].

In the nematode *Caenorhabditis elegans*, environmental cues such as starvation, viral RNA
or environmental RNA can trigger heritable phenotypic changes that are transmitted via small
RNA [7–9]. The mechanisms of trans-generational inheritance are best understood from stud-
ies of environmental RNA [5–7,10–12]. In *C. elegans* phenotypic changes induced by environ-
mental RNAi require a double stranded RNA (dsRNA) entering the animal via a dedicated
dsRNA transporter [13,14]. Thereafter, exogenous dsRNAs are processed by the conserved
nuclease Dicer into ~22 nucleotide (nt) primary small interfering RNAs (siRNA) and incorpo-
rated into Argonaute proteins to form the RNA-induced silencing complex (RISC) [15,16].
This protein-RNA complex binds complementary mRNA sequences and initiates the produc-
tion of secondary siRNA by RNA-dependent RNA polymerases (RdRP) (Fig 1A) [17–19].
Such secondary siRNAs are 22 nt with a 5' guanine, thus named 22G RNAs [20]. Depending
on the Argonaute, primary and secondary siRNA may trigger systemic gene silencing of com-
plementary sequences through destabilization of mRNA or translational repression (Fig 1A)
[21–23]. Once established, phenotypic changes induced by exogenous dsRNA can be transmit-
ted over multiple generations [10,12,23,24].

On top of their role in trans-generational inheritance, some endogenous small RNA types–
such as miRNA, piRNA and siRNA–are essential for development and fertility in both sexes of
*C. elegans*. Albeit distinguishable, those small RNA types share some biogenesis and silencing
mechanisms (Fig 1A). One major class of small RNA, miRNAs, regulates translation of mRNA
targets via partially complementary base-pairing. Many miRNAs are expressed in a tissue- and
sex-specific specific manner reflecting functional sex-differences [25–28]. For instance, miR-
35 family activity in the female germline is important for progeny viability and fecundity [29–
31]. Furthermore, distinct Argonaute proteins associate with additional types of endogenous
siRNAs in the male and female germline, suggesting functional sex-differences [20,32–36].
Importantly, germline-expressed PIWI-bound small RNA (piRNA) populations target mRNA
and thus maintain genome stability in hermaphrodites and males [37–40]. Such piRNAs come
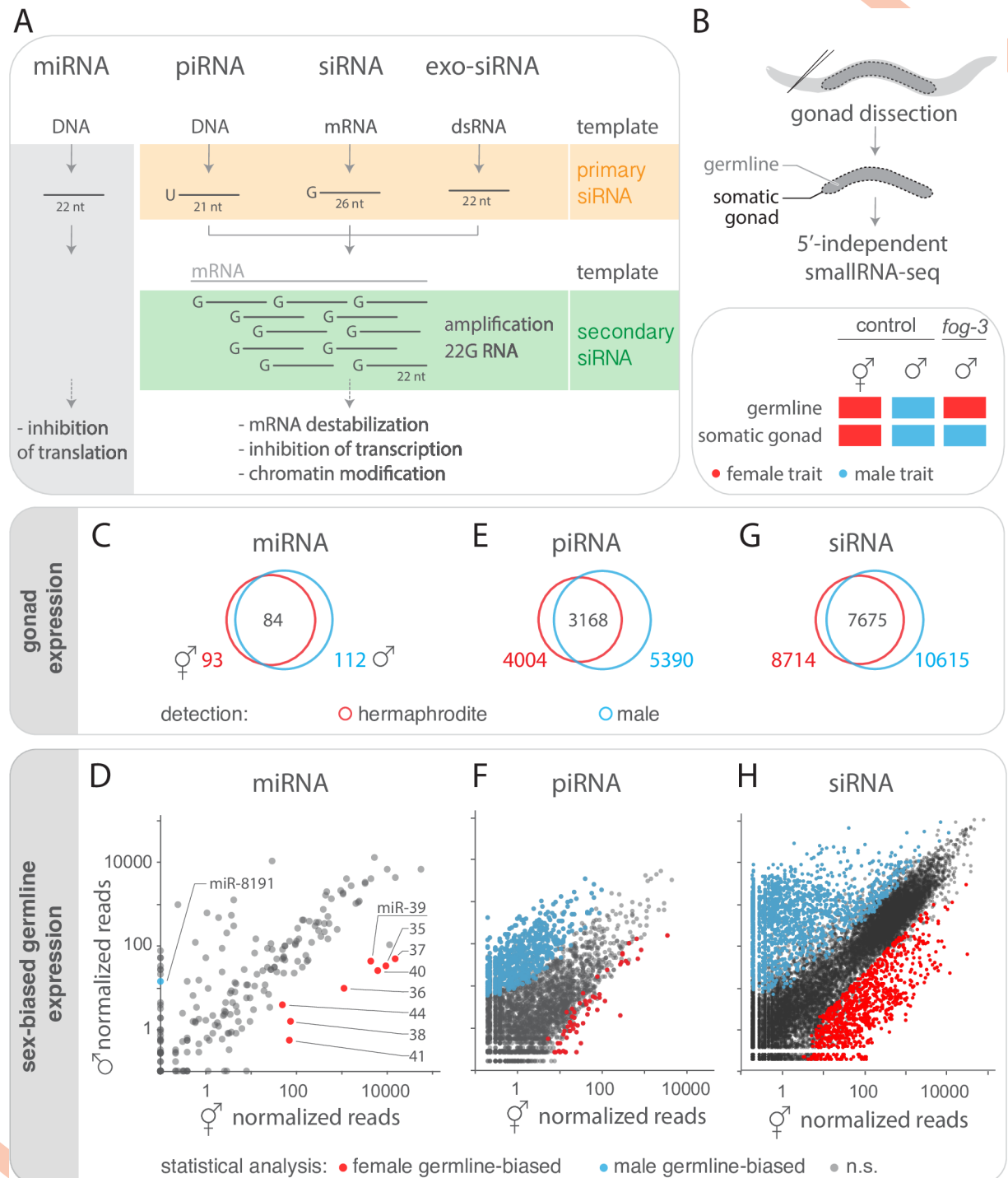
**Fig 1. Tissue- and sex-specific small RNA profiling.** A) Small RNA types in *C. elegans* categorized by template (DNA or RNA), length and first nucleotide (nt). Distinct primary small RNA types (orange) induce the amplification of secondary 22G RNAs (green) by RNA-dependent RNA polymerase (RdRP) using mRNA as a template. B) Small RNAs were sequenced from dissected adult *C. elegans* gonads containing germline and somatic gonad tissue. Control hermaphrodites and males have sex-specific germline and somatic gonad tissue (red: female traits; blue: male traits). Mutant *fog-3* males have a 'female' germline (red) and a male somatic gonad (blue). C, E, G) Gonad expression of miRNA (C), piRNA (E) and endogenous siRNA (G, >5 mean reads) in control hermaphrodites (red circle, n = 6 replicates) and males (blue circle n = 3). Total number of gonadal small RNAs in each sex is shown as well as the overlap. D, F, H) Sex-differences in expression of mean normalized miRNA (D), piRNA (F) and endogenous siRNA (H) reads in hermaphrodite (n = 6 replicates) and male gonads (n = 3). (D, H) miRNA and siRNA expression-differences with four-fold difference in abundance that were statistically different (Wilcoxon rank sum test with continuity correction; p adjusted<0.01) between hermaphrodites and males as well as between control males and *fog-3* males (n = 2) are

highlighted in red (female germline-biased) and blue (male germline-biased). (F) Since piRNA are germline-specific, expression-differences between hermaphrodites and males (n = 2) are highlighted in red (female germline-biased) and blue (male germline-biased) without comparison to *fog-3*. See S1 Fig for sex-differences in somatic gonad expression.

in two flavours: type I piRNA are expressed from two genomic loci and have a conserved upstream motif, whereas type II piRNAs lack an apparent upstream motif and are dispersed throughout the genome [41]. In *C. elegans*, another group of endogenous small RNAs are expressed in the gonads of both sexes. Such siRNAs can be distinguished by length and are either (i) primary products of the RNAse III enzyme Dicer/DCR-1 (e.g., 26G RNA) or (ii) secondary products of RNA-dependent RNA polymerases (e.g., 22G RNA) (Fig 1A). Both types of endogenous siRNAs modulate gene expression and are essential for fertility, but very little is known about potential sex-differences in their gonadal expression [32,34,35].

The reproductive tissue of *C. elegans* consists of the germline surrounded by the somatic gonad, and development of both tissues is coordinated by multiple mechanisms [42]. Notably, miRNA activity in the somatic gonad is essential for gonad development and germline proliferation, and thus fertility [43,44]. However, a comprehensive tissue- and sex-specific expression study of gonadal siRNA is lacking.

Males and females also present germline sex-specific differences in response to environmental cues. First, RNAi in hermaphrodite gonads induces very strong knockdown phenotypes [2,45–47], in contrast to anecdotal evidence for mostly no detectable RNAi phenotypes in *C. elegans* sperm [6,45,48,49]. Second, siRNA induced trans-generational silencing is often more efficient through the female than the male germline [2–6]. The underlying mechanisms causing these sex-differences are not well understood.

Here we provide a comprehensive study of both the sex-determined small RNAome and environmentally-induced siRNA silencing. First, we quantify sex-biased expression of miRNA, piRNA and siRNA in isolated male and female gonads. We further ascribe small RNA sex-differences to the germline or somatic gonad by comparing gonads of each sex to mutant male gonads (*fog-3*) with a female germline. Quantitative analysis of gonadal expression of mRNA and 22G RNA identified genes with low mRNA expression and high 22G RNA levels, a signature compatible with silencing. Moreover, there was lower mRNA expression of WAGO than non-WAGO targets, further supporting the notion of endogenous silencing. Second, using environmental RNAi targeting a GFP-sensor we show that germline RNAi silencing efficacy is determined by germline sex, in line with previous work [6,45,48,49]. This tissue-specific sex-difference in silencing efficacy was associated with lower levels of RNAi amplification products in male than female gonads. These data provide a mechanistic basis for sex-differences in germline RNAi efficacy with implications for trans-generational inheritance.

## Results

### The small RNAome of male and hermaphrodite gonadal tissues

To identify small RNAs expressed in hermaphrodite and male gonads, we quantified small RNA populations (Fig 1A) from dissected *C. elegans* gonads by high-throughput sequencing (Fig 1B). This approach allowed us to simultaneously analyse and compare tissue-specific expression of miRNA, piRNA and siRNAs of the *C. elegans* gonads.

### miRNAs with sex-biased germline or somatic gonad expression

Of the 257 annotated miRNAs in the *C. elegans* genome (assembly WS235, miRBase release 21) we detected 93 in hermaphrodite gonads and 112 miRNAs in male gonads (Fig 1C;

minimum 5 mean sense reads across replicates, S1 Table). To uncover quantitative differences in miRNA expression between sexes, we compared normalized read counts between hermaphrodite and male gonads. By using a cut-off of four-fold difference in abundance and a false-discovery rate of 1%, we identified 37 miRNAs with sex-biased gonad expression (Fig 1C).

Since the gonads consist of two tissue types, the germline and the somatic gonad, the observed difference between hermaphrodite and male may stem from expression differences in either tissue. To determine the contribution of the germline, we made use of mutant males that have a feminized germline and a male somatic gonad (i.e., the loss of function allele *fog-3(q849 [E126K])* called *fog-3* or 'feminized male' for clarity) [50,51]. The comparison of the control male and feminized male gonads allows one to uncover differences that stem only from the germline, since both types of individuals have identical male somatic gonads (Fig 1B). Of the 37 miRNAs with sex-biased gonad expression, nine were differentially expressed between the male and feminized male gonads (Fig 1D). Eight of them (including all from the miR-35-41 cluster and miR-44) were expressed more highly in the feminized germline (*fog-3* gonads) whereas only one (miR-8191) was more highly expressed in the male germline (male gonads) (Fig 1D). This result shows for the first time sex-specific expression of miRNAs in the *C. elegans* germline.

The comparison of hermaphrodite and feminized males allows one also to identify the contribution of the somatic gonad to differences in sex expression between hermaphrodite and male gonads, since germlines are identical (both female). This comparison revealed that 16 of the 37 miRNAs with sex-biased gonad expression were differentially expressed between the hermaphrodite and feminized gonads. One miRNA was more highly expressed in hermaphrodites, and 15 miRNAs were more highly expressed in feminized males, indicating sex-differences that stem from the somatic gonad (see S1 Fig, S1 Table).

## piRNAs with sex-biased expression

Next, we compared hermaphrodite and male gonads to identify type I and II piRNAs with sex-biased expression. Our cloning technique allowed us to capture (5'-monophosphate-) piRNAs, yet at lower frequency than other types of (5'-triphosphate-) siRNA. We detected sense reads of 4004 annotated piRNAs [38,52] in hermaphrodite gonads and 5390 in male gonads, including 3168 piRNAs present in both sexes (Fig 1E, S1 Table). To uncover putative quantitative differences in piRNA expression between sexes, we compared normalized read counts between hermaphrodite and male gonads. Using a cutoff of four-fold difference in abundance and a false-discovery rate of 1%, 66 piRNAs with hermaphrodite-biased expression and 919 piRNAs with male-biased expression were revealed (Fig 1F). piRNAs are germline specific, and previous studies have identified male- and hermaphrodite-specific piRNAs by sequencing of whole animals or purified gametes [38,39,53]. We identified 93% of the previously identified male- and 32% of the female-biased piRNAs in another study [53] despite differences between the two (i.e., cell type, genotype, cloning technique and statistics). Thus, our method captures differences in piRNA expression with more sex-specific piRNAs in males than in hermaphrodites, further suggesting sex-specific regulatory mechanisms.

## Endogenous siRNAs with sex-biased germline expression

As a third group of endogenous small RNA, we focused on siRNAs expressed in gonads of both sexes. We analysed all siRNAs that map to protein coding genes with a minimum of five mean antisense reads, thus excluding mRNA degradation products with sense orientation. This revealed 8714 genes with siRNAs in hermaphrodite gonads and 10615 genes in male gonads (Fig 1G). 7675 of these siRNA targeted genes were found in both sexes (Fig 1G; S1 Table). Additional expression data of small RNA (antisense and sense) mapping to other

annotated features in the genome such as pseudogenes, transposons and different types of small RNA for gonads of both sexes is available as S1 Table.

To detect potential quantitative expression differences between sexes, we compared normalized antisense read counts per gene between hermaphrodite and male gonads, selecting only genes with at least a four-fold difference in siRNA abundance and a false-discovery rate of 1%. 4508 genes exhibited a sex-biased expression of siRNAs: 1138 were higher expressed in hermaphrodites and 3370 were higher expressed in males. siRNAs of 68 genes were detected exclusively in hermaphrodite and 748 only in male gonads.

To determine whether these expression differences stem from the germline or the somatic gonad, we again made use of the *fog-3* mutants. Of the 4508 genes with sex-biased siRNAs expression in the gonad, 3173 were also differentially expressed between the gonads of control males and feminized males. siRNAs mapping to 2245 genes were more highly expressed in the male gonads, and 928 were more highly expressed in the feminized male gonads (Fig 1H, S1 Table). Thus, most of the sex-differences between hermaphrodite and male gonads stem from expression differences in the germline proper.

To relate sex-differences in germline siRNA expression to biological processes, we carried out gene set enrichment analysis using WormExp v1.0 [54]. This tool queries *C. elegans* mRNA and siRNA expression data in functional groups called 'gene sets' based on individual experiments for statistically significant overlap with a given gene list. We predicted that genes with sex-biased germline siRNA expression (i.e., 928 hermaphrodite- and 2245 male-biased genes) may overlap with gene sets corresponding to germline mRNA or siRNA expression. For statistical analysis, we compared enrichment to all 18106 genes with siRNAs detected (S2 Table). From the selected 2293 gene sets queried (WormExp categories: mutants, tissue, other), there was significant overlap in both sexes with multiple gene sets related to germline mRNA expression (such as down-regulation in germline-less *glp-1* mutant; here and in the following comparisons FDR<0.001) and siRNA regulation (such as alteration in *csr-1*, *rde-1* and *rrf-3* mutants). In addition, this analysis revealed sex-specific overlap with several prior siRNA experiments probing siRNA pathways. Genes with hermaphrodite-biased germline siRNA expression overlapped with siRNA targets detected in whole animals, for example those regulated by *rde-8*, *ergo-1*, *eri-6/-7*, *mut-16*. Likewise, genes with male-biased germline siRNA expression overlapped with spermatogenesis specific *alg-3/-4* targets. Thus, as expected, gonadal siRNAs with tissue-and sex-specific expression are involved in germline and siRNA functions, confirming the specificity of our approach.

## High 22G RNA levels are associated with low mRNA expression, compatible with silencing

Since 22G RNAs of several biogenesis pathways induce gene silencing, we determined the relative abundance of 22G RNAs in the gonads of both hermaphrodites and males. 22G RNAs represented 27.4% of reads in the hermaphrodite gonads and 27.9% of reads in the male gonads (S2A Fig). The 22G RNA levels per gene were not significantly different between the male (mean reads 234±1433) and hermaphrodite gonads (173±858; two-sided Wilcoxon rank sum test p<0.19, S2B Fig). Comparison of our expression data for 22G RNA and the level of gonadal mRNA measured in a previous study [55] revealed that the vast majority of mRNAs were targeted by 22G RNAs (83% in male gonads and 81% in hermaphrodite gonads; minimum 10 reads of each RNA type). Moreover, 22G RNA levels were not consistently different between hermaphrodites and males when comparing genes with similar mRNA levels (15 of the 17 bins were not significant, and the two that were significant (both p<0.041) were among the three with the lowest mRNA levels, S2C Fig).

To gain insights into 22G RNA-mediated mRNA silencing, we tested if there was an association between the levels of 22G RNAs that we measured in the gonads of hermaphrodites and males and the corresponding mRNA template expression measured in a previous study [55]. We hypothesized that there should be a positive correlation between the levels of mRNA and 22G RNA if mRNA induces 22G RNA synthesis. Alternatively, if 22G RNA expression causes mRNA silencing, a negative correlation between the levels of mRNA and 22G RNA is expected. We first grouped genes in 20 bins according to their 22G RNA expression level. Across bins, there was a positive association between the levels of 22G RNA and gonadal mRNA for groups of genes with low and medium levels of 22G RNA (Fig 2A, S3 Table). In contrast, in both hermaphrodites and males, the level of mRNA decreased with extremely high 22G RNA levels (Fig 2A), a pattern suggestive of silencing. For statistical analyses we next grouped genes in 5% intervals with equal number of genes again according to 22G RNA expression (S3A Fig). The most significant negative association between the levels of 22G RNA and mRNA was found only for the 5% of the genes with highest level of 22G RNA expression (hermaphrodites $p<1\times10^{-12}$, males $p<1\times10^{-16}$; S3B Fig). These data indicate that mRNA expression is globally decreased for genes with very high levels of 22G RNAs, a signature compatible with siRNA mediated gene silencing. Because 22G RNA and mRNA engage in a feedback loop with unknown dynamics, other interpretations remain possible.

We next asked whether 22G RNA of distinct small RNA pathways may have distinct effects on target gene regulation. To investigate the role of individual small RNA pathways, we used three datasets: published germline small RNA targets (WAGO/WAGO-1 [20], CSR-1 [35,56], ALG-3/-4 [34]), gonadal 22G RNA expression from hermaphrodites and males (this study), and gonadal mRNA expression from hermaphrodites and males [55] (see S3 Table). Using those three independent datasets, we grouped genes in 20 bins based on their 22G RNA expression, and next compared within bins the mRNA expression of Argonaute-target and non-target categories (some bins had only genes of a single category and were therefore not included in the analyses). We first analysed the WAGO/WAGO-1 dependent 22G RNAs that are involved in mRNA silencing [20]. mRNA levels were significantly lower for WAGO/WAGO-1 target genes than non-target genes in 11 of the 19 bins in hermaphrodites and 14 of the 20 bins in males (Fig 2B; S3C Fig), in line with the known function of WAGO/WAGO-1 in silencing. In addition, this analysis revealed that a wide range of 22G RNA levels mediate endogenous gene silencing in gonadal tissues (Fig 2B). Thus, genes with intermediate 22G RNA levels are also subject to silencing.

To investigate how other germline small RNA pathways may impact gonadal mRNA expression, we next analysed CSR-1 dependent 22G RNAs, which license expression of germline transcripts [36]. We found that, for both sexes, the level of mRNA expression was higher for CSR-1 targets [35,56] than for non-CSR-1 targets (significant difference in 16 bins (of 17) in hermaphrodite and 16 bins (of 16) in males, Fig 2C), a signature consistent with licensing, as shown previously [36]. Likewise, mRNA expression in males was higher for spermatogenesis specific ALG-3/-4 target genes [34] than non-target genes (14 of 14 bins; S3D Fig). In conclusion, germline-specific Argonautes CSR-1 and ALG-3/-4 do not appear to be involved in global mRNA silencing. Furthermore, these results highlights that small RNA sorting into distinct Argonaute complexes affects mRNA expression beyond 22G RNA levels.

## Germline sex regulates environmental siRNA accumulation

Since endogenous siRNA pathways share secondary siRNAs owing to similar biogenesis routes [17,19,32], we cannot experimentally determine the fraction of 22G RNAs induced by a specific primary siRNA type. To compare expression levels of both primary and secondary siRNA
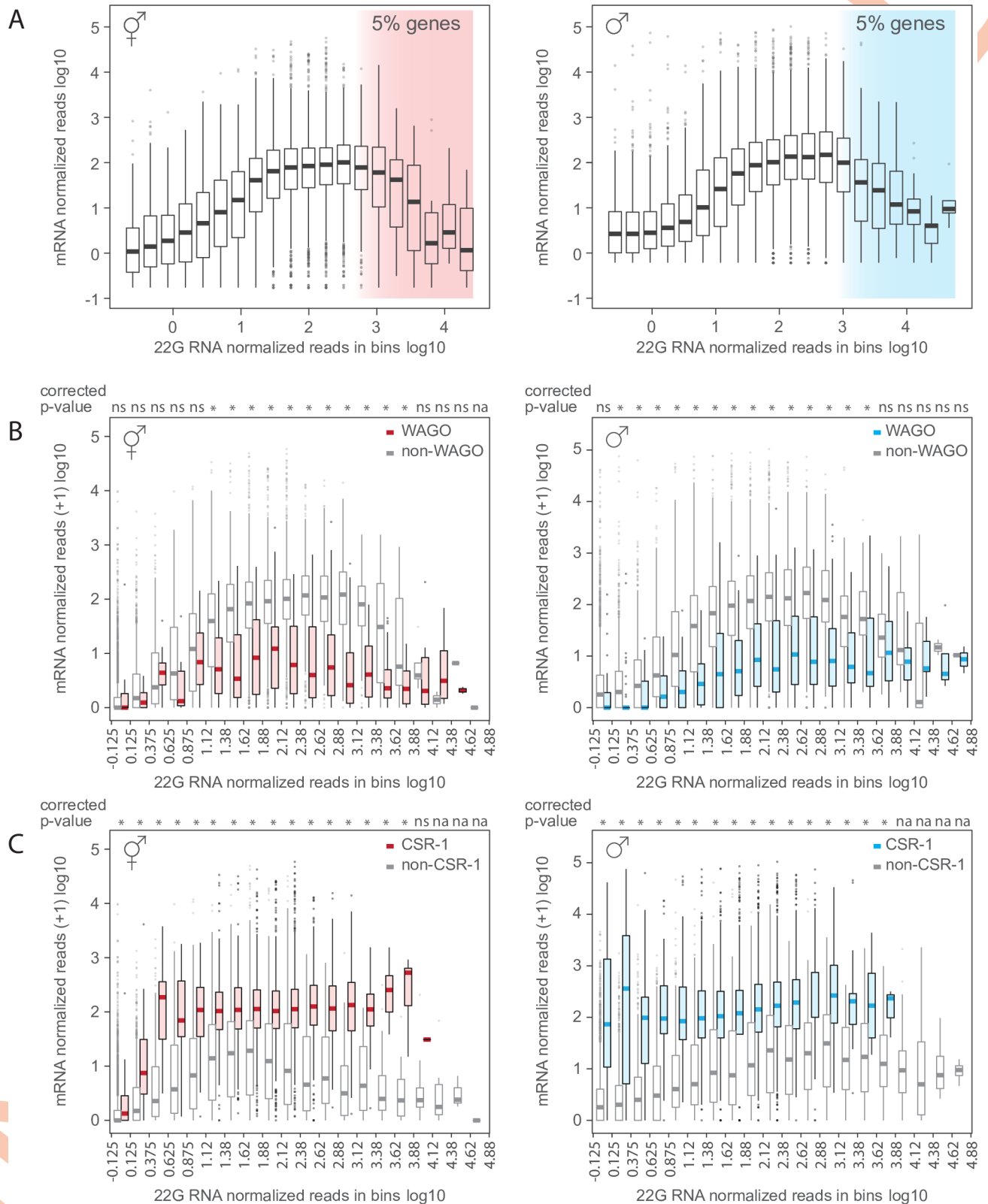
**Fig 2. High 22G RNA levels are associated with low mRNA expression, compatible with silencing.** A) Mean normalized mRNA [55] and 22G RNA reads detected in hermaphrodite and male gonads (data in 20 bins according to 22G RNA level; number of genes per bin: 4–1101 in hermaphrodites, 5–1343 in males; mean: solid line, outliers: open circles). mRNA expression is globally decreased for the top 5% of genes (shaded area) with high levels of

22G RNA. See S3A Fig and S3B Fig for statistical analysis. B) mRNA expression for WAGO-target genes and non-target genes (Bonferroni corrected p-values of the Wilcoxon rank sum test q<0.01 indicated by stars, ns = not significant, n/a = not applicable). Data are the same as for Fig 2A but plotted as WAGO targets [20] and non-WAGO targets in hermaphrodites and males. WAGO targets were available only for hermaphrodites and interpolated to male samples. C) mRNA expression for CSR-1-target genes and non-target genes (Bonferroni corrected p-values of the Wilcoxon rank sum test q<0.01 indicated by stars, ns = not significant, n/a = not applicable). Data are the same as for Fig 2A but plotted as CSR-1 targets and non-CSR-1 targets in hermaphrodite and male [35,56]. CSR-1 targets were available for each sex and analyzed separately.

and their impact on protein expression, we developed a sensor-based assay. To this end, we used a germline expressed GFP-sensor [57] targeted by *gfp(RNAi)*. Environmental *gfp* dsRNA provided by feeding allows one to manipulate primary siRNA levels and thus directly measure the impact of altered levels of primary siRNA on secondary siRNA levels. To distinguish exogenous primary siRNAs from worm-generated secondary siRNAs, we generated *gfp* dsRNA with single-nucleotide-polymorphisms (SNPs) every 21 nucleotides relative to the GFP-sensor transgene. Thus, primary siRNAs can be discriminated from secondary siRNAs (Fig 3A). To investigate potential sex-specific regulation of siRNA levels during exogenous RNAi, we quantified primary and secondary siRNAs by sequencing siRNAs from gonads of males and hermaphrodites expressing the GFP-sensor. Uptake and primary siRNA processing were active in both male (9 siRNA/ $10^6$ reads) and hermaphrodite gonads (6 siRNA/ $10^6$ reads). Moreover, primary siRNA levels were not statistically different between the sexes (t-test p<0.08, Fig 3B). By contrast, the secondary siRNA level was significantly lower in the male (82 siRNA/$10^6$ reads) than hermaphrodite gonads (280 siRNA/$10^6$ reads, t-test p<4.3x$10^{-3}$, Fig 3B). Accordingly, the ratio of secondary siRNA/ primary siRNA was significantly lower in the male gonads compared to the hermaphrodite gonads (respectively, 9 and 56 secondary siRNA/primary
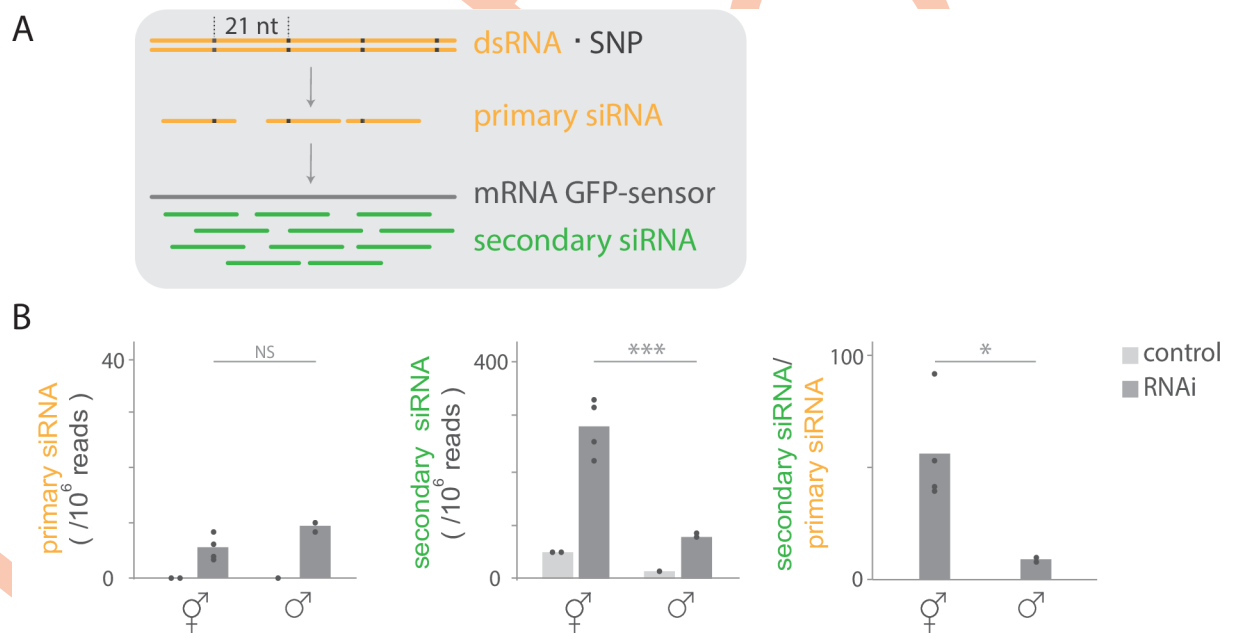


**Fig 3. Sex-differences in gonadal siRNA levels during germline RNAi silencing.** A) Assay to quantify primary and secondary siRNA levels (from dissected gonads from hermaphrodites and males; see Fig 1B). Animals expressing the GFP-sensor in the germline were raised on *gfp (RNAi)* containing SNPs (black square) each 21 nucleotides. Primary siRNAs (orange) are identified by the presence of the SNPs, while secondary siRNAs (green) lack SNPs. B) Mean primary and secondary siRNA reads normalized to total reads in gonads from hermaphrodites (circles indicate replicates, n = 4) and males (n = 2) and mean secondary siRNA/ primary siRNA ratio (only shown for *gfp(RNAi)* treated animals). Animals were raised on *gfp(RNAi)* or control empty vector (hermaphrodite n = 2, male n = 1). Means were compared with t-tests; * = p<0.05, *** = p<0.001. Low levels of secondary siRNA are generated from the transgene in animals raised on control RNAi.

siRNA, t-test p<0.03, Fig 3B). Taken together, we conclude that environmental dsRNA triggers primary and secondary siRNA production in the germline, with notably higher levels of secondary siRNA products in hermaphrodites than in males.

Our data do not allow one to formally ascribe primary siRNA production to either somatic gonad or germline tissue. However, several indirect lines of evidence support the presence of primary siRNAs specifically in the male germline. The GFP-sensor is expressed specifically in the germline and dsRNA feeding increases the level of 22G RNAs targeting the GFP-sensor (Fig 3B). Secondary siRNAs are not mobile [58] and therefore must be generated in the germline. Since the generation of secondary siRNAs requires primary siRNAs in the tissue of secondary siRNA production [59], we conclude that primary siRNAs are present in the male germline. Thus, uptake and transport of silencing RNA (dsRNA and/or primary siRNA) from the environment across male somatic tissues, notably the somatic gonad, is functional.

## RNAi efficacy is germline sex dependent

Anecdotal evidence suggests that germline RNAi silencing may differ between C. elegans hermaphrodites and males [6,45,48,49]. RNAi in hermaphrodite germlines is extremely potent as depletion phenotypes often appear within 24 hours of dsRNA exposure [47], even at reduced dsRNA dose [46]. In contrast, similar experiments generally cause suspiciously few phenotypic changes in the male germline, likely because of inefficient RNAi depletion [45,48,49]. Presumably stronger RNAi depletion occurs in male germlines directly injected with high doses of dsRNA [60], or by exposing the parental generation [61–64]. However, direct comparison of silencing efficacy of endogenous genes solely based on phenotypes in germlines of hermaphrodites and males is insufficient, notably due to sex differences in physiology and gene function. The GFP-sensor targeted by RNAi feeding allows one to measure both siRNA levels and the resulting silencing in hermaphrodites and males raised in the same environment. The difference in siRNA amplification products observed above (see Fig 3B) prompted us to ask whether germline sex affects RNAi silencing. To assess RNAi silencing in both sexes, we monitored the presence or absence of GFP-sensor fluorescence upon *gfp(RNAi)* (Fig 4A). These analyses revealed that germline silencing by dsRNA is sex specific. Of the 307 hermaphrodites analysed, 305 (99.4%) silenced the germline GFP-sensor in the entire germline (Fig 4B), in contrast to only 3 (3.7%) of the 81 males analysed (chi-square p<0.001; Fig 4B). This difference pertained to the entire germline of males since GFP-sensor expression was visible in proliferating germ cells and differentiated spermatocytes (Fig 4A). Contrary to the germline, the soma of males was RNAi sensitive, as evidenced by the complete silencing of a ubiquitous GFP-sensor [65] in all non-neuronal somatic cells (Fig 4C; 60/60 males). This is in line with successful RNAi-based screens targeting somatic tissues in male, notably the somatic gonad [66,67] and shows that somatic RNAi efficacy is sex independent. Thus, isogenic worms raised in the same environment show phenotypic differences in response to environmental cues. Since RNAi silencing is dependent on the dsRNA levels and because mutants defective in siRNA amplification are partly RNAi resistant [17,68–70], the observed sex-difference in secondary siRNA abundance in gonads (Fig 3B) may explain the difference between sexes in germline RNAi efficacy (Fig 4A and 4B).

To determine whether the observed differences in germline RNAi silencing between male and hermaphrodites was induced by differences in the germline or the soma, we again made use of the feminized males. All other traits of the feminized males such as soma, karyotype and feeding-behaviour are undistinguishable from wild-type males (Fig 4B). Upon feeding *gfp (RNAi)*, all 106 feminized males silenced the GFP-sensor (Fig 4A and 4B), indicating that germline-intrinsic factors regulate RNAi silencing, rather than other male traits such as the soma, karyotype or behaviour.

To test whether differences in germline RNAi silencing between control and feminized mutant male gonads could stem from altered siRNA levels or altered downstream silencing activities, for instance target slicing by Argonautes, we compared siRNA levels in gonads. The gonads of feminized males contained higher levels of primary *gfp* siRNAs ($74/10^6$ reads, t-test $p<9.5\times10^{-4}$) and secondary *gfp* siRNAs ($798/10^6$ reads, t-test $p<0.04$) than control male gonads (S4 Fig). Since RNAi silencing is dose-dependent, the higher siRNA levels in feminized mutant to male (or hermaphrodite) gonads are a plausible explanation for the sex-difference in germline RNAi silencing and may indicate increased uptake/ processing or stability. Exogenous siRNA levels may also depend on turnover of germline tissue: while control animals regularly release gametes containing siRNA, feminized males do not ovulate and thus may accumulate higher siRNA levels. Intriguingly, the ratio of secondary siRNA/ primary siRNA was not significantly different between control and feminized male gonads, suggesting
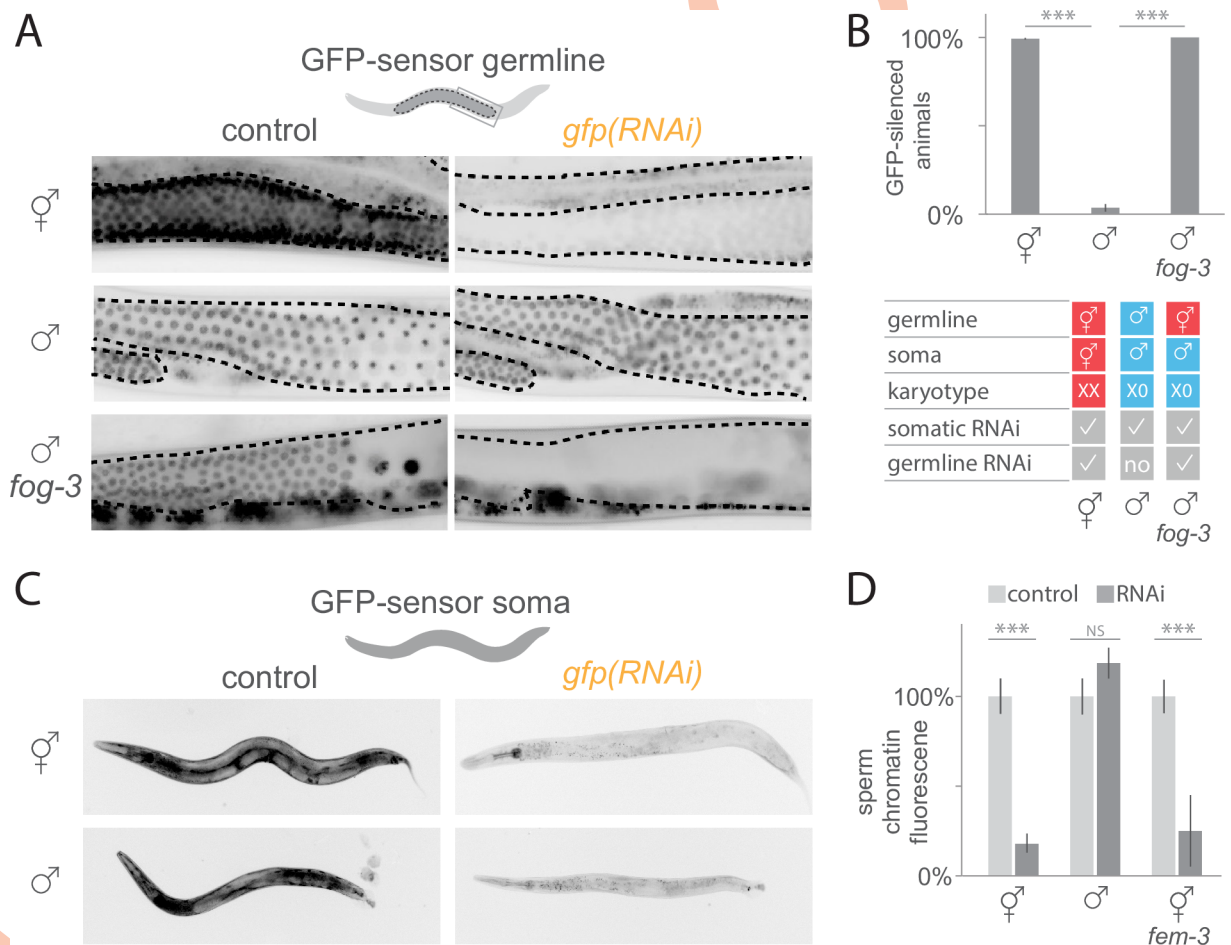


**Fig 4. Sex-differences in germline RNAi efficacy.** A) Fluorescence microscopy images of germline (dotted line) GFP-sensor expression in hermaphrodites, males and *fog-3* males (left). Silencing of the GFP-sensor upon *gfp(RNAi)* in hermaphrodite and *fog-3* male germlines, while control male germlines are RNAi resistant (right). Gut autofluorescence is visible outside the germline. B) Percentage of worms (±SEM) silencing the GFP-sensor in the germline upon *gfp(RNAi)* (n = 307 hermaphrodites, 81 males, 106 *fog-3* males). Chi-square test was applied; *** = p<0.001. Table with hermaphrodite/ female (red) and male (blue) traits in hermaphrodites, males and *fog-3* males. C) Fluorescence microscopy images of ubiquitous GFP-sensor expression in the soma of hermaphrodites and males (left, for both n = 54). *gfp(RNAi)* silencing of somatic GFP in non-neuronal cells was observed in all hermaphrodites and males (right, respectively n = 57 and n = 60). D) Background subtracted sperm GFP-sensor chromatin signal intensity (mean ±SE) upon *gfp(RNAi)* in hermaphrodites (n = 10), males (n = 9) and *fem-3* hermaphrodites producing only sperm (n = 15). Signal intensity was normalized to animals not exposed to dsRNA (n = 10 hermaphrodites, 11 males, 12 *fem-3* hermaphrodites). Note that silencing efficiency may be underestimated because quantification was based on residual chromatin fluorescence. T-test was applied for significance test; *** = p<0.001.

that the soma, which is male in both types of gonads, regulates accumulation of siRNA amplification products.

Male germlines are constantly undergoing spermatogenesis, therefore we asked if sperm producing tissue is generally RNAi resistant. We tested RNAi silencing efficiency in hermaphrodite germlines in the L4 larval stage prior to and during spermatogenesis. The GFP-sensor expression was variable in animals of the same developmental stage raised in the same environment. We observed 22% of animals without GFP-sensor expression, 48% with intermediate and variable expression (stronger fluorescence intensity in proximal nuclei compared to distal nuclei), 15% with intermediate and uniform expression and 15% with high GFP expression (S5A Fig). This suggests that developing male germ cells are partially susceptible to RNAi silencing.

To further examine whether the male germline RNAi resistance was inherent to sperm or influenced by the surrounding germline environment, we quantified RNAi silencing between the sperm of males and hermaphrodites. If the germline environment regulates RNAi, silencing should differ between the sperm of males and hermaphrodites. By contrast, if the sperm physiology confers RNAi resistance, both types of sperm should be RNAi resistant. We measured fluorescence intensities of a ubiquitous GFP-sensor [71] on chromatin using both types of sperm imaged in whole worms fed with *gfp(RNAi)* (S5B Fig). This experiment revealed RNAi silencing in hermaphrodite sperm, since *gfp(RNAi)* significantly decreased sperm GFP signal intensity by a mean of 82%±5% compared to animals not exposed to dsRNA (Fig 4D; t-test $p < 1.8x10^{-6}$). By contrast, the sperm of males was completely RNAi resistant, with the GFP-sensor signal intensity being not significantly different between males fed with *gfp(RNAi)* and males not being exposed to dsRNA (Fig 4D; t-test $p < 0.2$). The finding that, in contrast to sperm in males, sperm from hermaphrodites is RNAi sensitive, indicates that the surrounding germline environment plays a crucial role in RNAi silencing of sperm. An alternative interpretation is that intrinsic differences between the sperm produced by males and hermaphrodites may directly affect RNAi silencing [72].

Because germ cell precursors for sperm and oocytes grow in the same syncytium in hermaphrodites, silencing agents such as siRNAs or RNAi proteins produced in oocytes may diffuse in the germline and thus influence silencing in sperm. To test this hypothesis, we investigated whether mutant hermaphrodites producing only sperm initiate RNAi independently of oogenic germ cells using the gain-of-function allele *fem-3(q96)* [73]. We found that such sperm were RNAi sensitive, since fluorescence GFP-sensor [71] signal intensity was on average 75%±20% lower in *fem-3* worms fed *gfp(RNAi)* compared to animals not exposed to RNAi (Fig 4D; t-test $p < 3.0x10^{-7}$, S5B Fig). Thus, the sperm of hermaphrodites is RNAi sensitive even in the complete absence of oogenic germ cells in the syncytium.

## Sex-biased gonadal mRNA expression of RNAi genes

In an attempt to identify candidate genes that may regulate sex-differences in germline RNAi efficacy, we analysed mRNA expression of 178 RNAi genes in hermaphrodite and male gonads [55]. While several essential RNAi genes (*rde-1*, *rde-2*, *rde-4*) were not differentially expressed between sexes, 60 genes had sex-biased gonad expression using a cut-off of four-fold difference in abundance and a false-discovery rate of 1%. Forty-two RNAi genes were expressed more highly in the hermaphrodite gonads and 18 RNAi genes were expressed more highly in the male gonads (Fig 5). This candidate gene list serves as a starting point for future functional investigations of sex-differences in germline RNAi efficacy.

## Discussion

Here we provide the first comprehensive small RNA profile of isolated *C. elegans* male and hermaphrodite gonads and show qualitative as well as quantitative sex-differences in siRNA
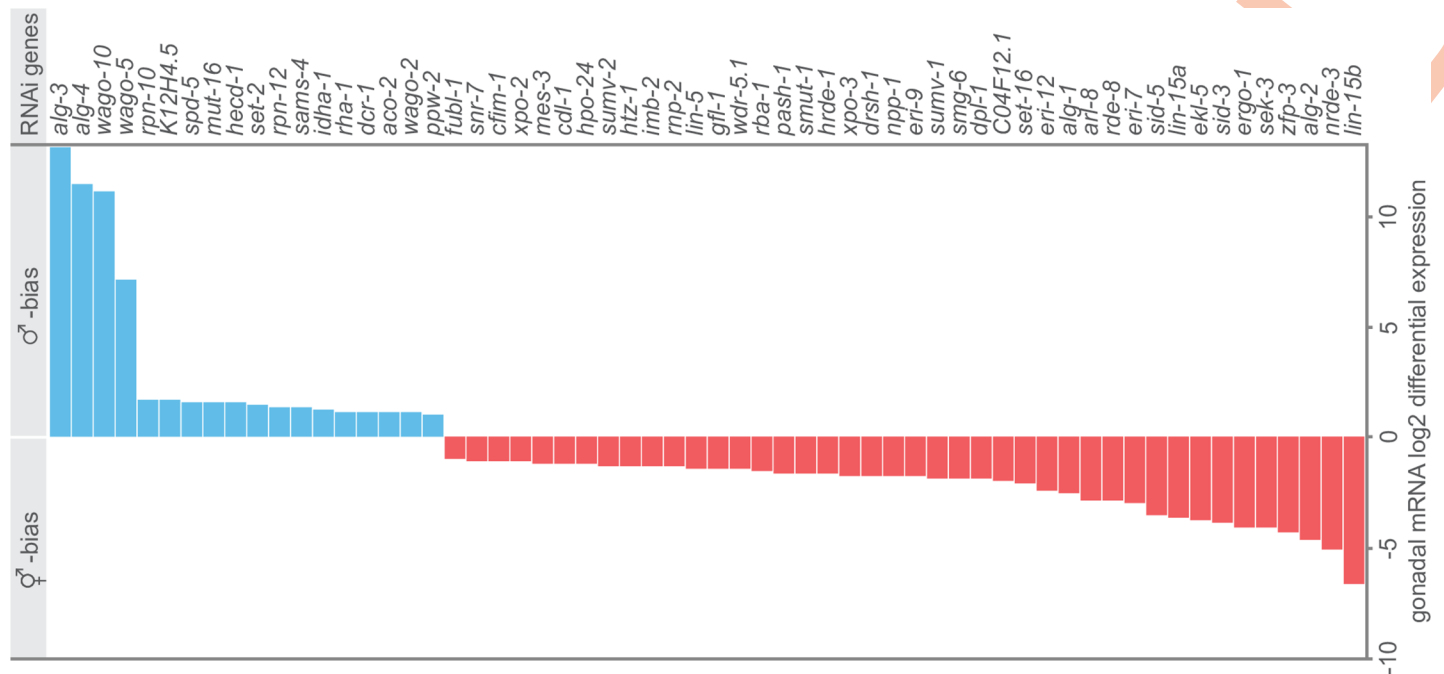
**Fig 5. RNAi genes with sex-biased gonadal mRNA expression.** Gonadal mRNA expression data from [55]. Blue: male-biased genes, red: hermaphrodite-biased genes. We selected 178 candidate RNAi genes from WormMine based on their phenotype (classification contains "RNAi") as well as all RNAi related genes from wormbase (such as argonautes, *sid*, *rde*, *rsd* and *eri*, see S4 Table).

expression for both somatic gonad and germline tissue. We demonstrate tissue-specific sex-differences in response to environmental RNAi triggers: in contrast to the hermaphrodite germline, the male germline is resistant to silencing and accumulates lower levels of RNAi amplification products. Taken together, these results provide a mechanistic explanation for sex-differences in RNAi efficacy in response to the environment with implications for trans-generational inheritance.

## Sex-differences in gonadal miRNA, piRNA and siRNA expression

miRNA regulation, notably via the conserved miR-35 family is crucial for embryonic viability, proliferation of the germline and is implicated in sex-determination [29,30,43,74]. In addition, miRNAs expressed in the *C. elegans* somatic gonad maintain germline proliferation and differentiation [43]. We identified female germline-biased expression of the miR35-41 cluster in isolated gonads, which is in line with studies conducted on whole animals [29,31,75].

The transgene reporter for miR-246 expression, which typically escapes germline expression, was previously observed in hermaphrodite gonadal sheath cells [25]. Here we establish sex-biased expression of endogenous miR-246 specifically in the hermaphrodite somatic gonad as opposed to the germline or the male gonad. Since miR-246 expression positively correlates with lifespan [76,77], it will be interesting to identify miR-246 targets in the somatic gonad or germline. Thus, small RNA sequencing of isolated gonads and comparison of expression differences between wild-type and sex-transformed mutant gonads captures known sex-differences in expression and in addition provides more detailed, tissue-specific information.

Our data provide the first tissue-specific analysis of miRNA expression in males. Of the 46 male-biased miRNAs with searchable names in miRBase detected in whole animals [26], 13 miRNAs showed male-biased gonad expression, underlining the importance of a tissue-specific approach. Thus, sex-biased miRNA expression occurs in both soma and gonad. Some

miRNAs with male-biased gonad expression were previously not detected as sex-biased [26] and could have been masked by somatic expression in both sexes.

In contrast to miRNAs, the piRNA pathway acts exclusively in the germline. Since the germline makes up about half of the adult *C. elegans* cells [78], whole worm sequencing is a good proxy for germline-restricted piRNA expression. Our analysis of sex-biased piRNA expression thus complements previous studies on whole animals that reported distinct piRNA expression in hermaphrodites and males [26,39]. While the cloning method applied here causes relatively low (5'-monophasphate) piRNA detection level compared to a previous study [39], it also causes preferential cloning of 5'-triphosphate RNA species and thus high coverage of endogenous siRNA. The tissue-specific analysis of endogenous 22G RNA and mRNA identified transcripts that are potentially regulated by RNAi. Importantly, hermaphrodites and males share common as well as distinct siRNA targeted gene sets in gonadal tissue. Most gonadal sex-differences in siRNA expression stem from the germline, which represents the vast majority of the gonadal tissue. Further comparison of gonadal siRNA and corresponding mRNA uncovers uncoupling of siRNA and mRNA levels, indicative of RNAi silencing. It will be interesting to investigate this regulation in the future.

## RNAi efficacy is regulated by germline sex

Our study provides novel insights into how the sex of an animal influences RNA-induced environmental changes in the phenotype. In particular, using environmental dsRNA triggers, we demonstrated that RNAi resistance affects exclusively the male germline. Sequencing primary and secondary siRNA with unique SNPs in isolated gonads from males and hermaphrodites allowed us to quantitatively compare steady-state levels. We detected similar levels of primary siRNAs in gonads of both sexes, suggesting functional transport of RNAi trigger from the environment to the soma and processing of primary siRNA in the male germline. In contrast, we discovered low levels of secondary siRNA amplification products in males compared to hermaphrodites. Thus, RNAi resistance in the male germline affects downstream processes that likely impact sex-differences in secondary siRNA processing or siRNA stability. It follows that RNAi is generally not suitable for functional studies of the *C. elegans* male germline if provided during one generation only. Since RNAi silencing is efficient in sperm of hermaphrodites, such animals are more suitable for reverse genetics targeting the germline.

What determines the sex-differences of gene regulation in response to environmental cues? It is conceivable that sex-differences in gonadal mRNA expression of RNAi regulators impact RNAi efficiency. In hermaphrodite gonads, RNAi genes having diverse functions–such as transport, processing or negative regulators–were expressed more highly than in male gonads. Notably, the endoribonuclease RDE-8, which is essential for amplification of siRNA products and silencing, was expressed predominantly in hermaphrodite gonads [70]. Thus, RNAi resistance in male germlines may possibly be linked to reduced RDE-8 activity.

Alternatively, competition between endogenous and exogenous RNAi pathways may cause male-specific germline RNAi resistance. Intriguingly, male-specific gonad expression affected most prominently four Argonautes. In addition to ALG-3/-4 that are implicated in spermatogenesis [34], we detected male-specific expression of WAGO-10 and WAGO-5. Although the molecular functions of WAGO-10 and WAGO-5 remain to be determined, their sex-specific expression suggests a role in male small RNA regulation.

Also, sex-differences in localization may affect RNAi efficacy. Notably CID-1, a poly(U)-polymerase modulating germline RNAi efficacy shows distinct subcellular localization in male and female germlines–perinuclear and chromosomal, respectively—that may affect function [79]. Intriguingly, in addition, multiple sex-differences are well described for the Argonaute

CSR-1 that targets endogenous germline transcripts via secondary 22G RNAs [20,36]. First, males and hermaphrodites express distinct CSR-1 isoforms [36,78]. Second, *csr-1* mutant hermaphrodites are mostly sterile, while *csr-1* mutant males show only modestly reduced fertility over multiple generations [35,56]. Thus, sex-differences in CSR-1 function may contribute to the observed sex-difference in RNAi sensitivity, in line with CSR-1 function in germline RNAi [80]. Overall, multiple mechanisms may contribute to sex-specific gene regulation and untangling the individual contributions will provide exiting answers on male and female biology.

In conclusion, this study provides the tissue-specific small RNAome of *C. elegans* hermaphrodite and male gonads and identifies quantitative sex-differences in miRNA, piRNA and siRNA expression. Furthermore, we demonstrate that the male germline is resistant to RNAi triggers taken up from the environment and accumulates lower levels of RNAi amplification products. We thus provide mechanistic insights into sex-differences of gene regulation in response to environmental cues that may play a role in transgenerational inheritance.

## Materials and methods

### Nematode strains

*C. elegans* strains of the following genotype were cultured according to standard procedures [81]: *him-8(e1489)* IV [81], *fog-3(q849[E126K])* I/ hT2*[qIs48]* (I;III); (kind gift from Scott Aoki and Judith Kimble) and *fem-3(q96)* IV [73]. The following GFP-sensors were used: single copy *mjIs145* [mex-5p::GFP::his-58::21UR-1sense::tbb-2 3'UTR] II ([57]; Figs 1–3, 4A and 4B, S4 Fig and S5A Fig), multicopy *adIs2122* [lgg-1p::GFP::lgg-1 *3'UTR* + rol-6(su1006)] [65]; Fig 4C) and multicopy *zuIs178* [his-72p::his-72::GFP:his-72 3'UTR] ([71]; Fig 4D and S5B Fig). All strains were wild-type for *mut-16(mg461)* by PCR [82]. *him-8(e1489)* was used to generate otherwise wild-type males and hermaphrodites, for simplicity we refer to such animals as 'control males' and 'control hermaphrodites'.

### RNA interference

RNAi feeding plates where prepared from freshly streaked HT115 bacteria containing L4440 vector according to [83]. Those were used to feed synchronized L1 worms 40h at 20˚C until L4 stage (S5A Fig) or minimum 48h at 20˚C for adults unless otherwise stated. Initial *gfp(RNAi)* experiments were carried out with a construct targeting the full *gfp* coding sequence including three introns. The recoded *gfp(RNAi)* with SNP every 21 nucleotides targets only exons and was synthesized by Integrated DNA Technologies and cloned into the L4440 plasmid. Silencing efficiency of recoded *gfp(RNAi)* is slightly reduced compared to full length *gfp(RNAi)*, which may be caused by SNPs or lacking introns.

### Microscopy

RNAi silencing was scored in live adults mounted on slides with reaction wells (Paul Marienfeld) using 20x or 40x objective on a Zeiss Axio Scope.A1 microscope. Worms scored as RNAi sensitive on a binary scale if GFP-sensor was absent or greatly diminished as compared to animals treated with empty vector RNAi. Differential interference contrast (DIC) and fluorescence images were acquired on a Axiocam 506 mono CCD camera and processed with Fiji software [84]. Outlines of the germlines where drawn on the DIC image in Adobe Illustrator.

### Gonad isolation and replicates

Bleach-synchronized L1 worms were grown on RNAi bacteria 48h at 20˚C until L4/ young adult, then washed 5 times in 15mL M9 to remove bacteria and grown overnight at 20˚C on

OP50 bacteria. Prior dissection, adults were picked on empty plates and transferred in groups of 3–4 in a drop of 0.01% levamisole + sperm buffer [85] to wash and paralyze animals. Animals were cut with a 21G needle behind the pharynx to liberate the gonad in a new drop of buffer on a depression slide (cavity 15-18mm, depth 0.6–0.8 mm; Marienfeld). Non-gonadal somatic tissue was removed, including the intestine; the spermatheca was removed from hermaphrodite gonads. Using a mouth pipette with glass capillary gonads where transferred to a tube with RNAlater (50–100μl) and after TRIzol (Thermo Fisher) addition frozen at -80˚C.

Gonads from hermphrodites (100), males or *fog-3* males (each 200) were processed as independent replicates for sequencing as follows (# replicates; including # recoded *gfp(RNAi)* samples): hermaphrodites (6;4), males (3;2) and *fog-3* males (2;2). Since endogenous siRNAs levels were not different from germlines treated with or without *gfp(RNAi)*, both were used for sex-biased expression analysis.

## RNA extraction

RNA was extracted with 1 ml of TRIzol according to manufactor's protocol. Briefly, RNA was precipitated adding 1 volume of isopropanol and 20 μg glycogen (Roche). Samples forming a white precipitate at this point were cleared by addition of 500 μL isopropanol: water 50% (v/v). Samples were frozen at -80˚C, thawed on ice and RNA was pelleted by centrifugation for 30 min at +4˚C, 16000 g. The pellet was washed in ice cold 70% (v/v) ethanol and recovered by centrifugation for 20 min at +4˚C, 16000 g and finally resuspended in 10 μL water. RNA concentration was determined by Qubit RNA BR Assay Kit (Thermo Fisher).

## 5' independent library preparation and sequencing

For RNA dephosphorylation 400 ng of RNA were treated with 20 Units of 5' polyphosphatase (Epicenter) in 20 μL reaction volume, purified using acid-phenol-chloroform, pH 4.5 (Thermo Fisher) and isopropanol precipitated using 20 μg of glycogen. Subsequently, RNA was suspended in 6 μL water and directly used for TruSeq Small RNA library kit (Illumina) following the manufacturer's instructions with exception that 15 cycles of PCR amplification were used. The cDNA libraries were separated on 6% TBE PAGE gels (Life Technologies) and bands with 147–157 nucleotides were cut from the gel. The gel matrix was broken by centrifugation through gel breaker tubes (IST Engineering Inc.) and size-selected cDNA eluted with 400 μl of 0.3M Na-Chloride. cDNA was purified by centrifugation through Spin-X 0.22μm cellulose acetate filter columns (Costar) followed by isopropanol precipitation. Libraries were sequenced on a HiSeq 1500 Sequencer (Illumina).

## Computational analysis of small RNA high-throughput sequencing data

Small RNA sequencing results were obtained from https://basespace.illumina.com/ as fastq files after demultiplexing. Sequencing data is available in the European Nucleotide Archive under accession number PRJEB12010 (S5 Table). Firstly, adapter sequences, reads shorten than 21 nt and reads longer than 34 nt were removed using cutadapt v1.9.1. Secondly, the remaining reads were aligned using bowtie 1.1.2 with at most 2 mismatches to *Escherichia coli* str. K-12 MG1655. Thirdly, the remaining reads were aligned with at most 2 mismatches to *C. elegans* WS235 tRNAs and rRNAs. Fourthly, the remaining reads were aligned with no mismatches to the worm *gfp* sequence. Fifthly, the remaining reads were aligned with no mismatches to the recoded *gfp* sequence. Sixthly, the remaining reads were aligned with no mismatches to *C. elegans* miRNAshairpins (miR-base release 21). Seventhly, the remaining reads were aligned with no mismatches to *C. elegans* genome cel235. Overall, we obtained ~2.3–12.8 million trimmed reads mapping to the genome (WS235) for each sex. endo-siRNAs

was quantified per gene, antisense reads mapping to coding exons, which account for more than 96% of reads in this class.

## Silencing of mRNA targeted by very high levels of 22G RNA

Fastq-mcf was used to trim adapters from single-end reads [86]. Bowtie 1.1.2 (parameters "—best—strata—tryhard -m 1") was used to map single-ended reads to *C. elegans* WS250 genome [87]. Reads were classified as 22G/26G based on length and starting base. HTSeq 0.6.0 was used to generate counts for reads that map to WS250 features in the sense and antisense directions [88]. Reads mapping against transposons were retrieved using c_elegans.PRJNA13758. WS250.annotations.exons.genes.with_transposons.gff. Features counts from separate samples were normalized and differences in expression were determined using DESeq2 1.13.8 [89]. Because alternative biological processes generate sense and antisense mapping reads, reads mapping in the sense direction from each library were normalized together, likewise reads mapping in the antisense direction were separately normalized. The cutoff for sex-biased expression was >4 fold difference in abundance and adjusted p-value <0.01. For correlations with gonad transcriptome and differential expression of RNAi genes, we used data from [55] (Sequence Read Archive, accession number SRP096640). For miRNAs both star and non-star sequences from the same gene were summed.

Assay and quantification of primary and secondary *gfp* siRNA levels: Reads were trimmed with cutadapt v1.9.1 [90]. Bacteria, tRNA and rRNA reads contaminations were removed using bowtie 1.1.2 alignment to the *Escherichia coli* str. K-12 MG1655 and to *C. elegans* WS235 tRNAs and rRNAs [87]. Remaining reads were aligned with no mismatches to the worm *gfp* sequence. Then the remaining reads were aligned with no mismatches to the recoded *gfp* sequence.

## Supporting information

**S1 Fig. Somatic gonad sex-differences in expression of miRNA and endogenous siRNA.** A) Mean normalized miRNA reads (sense) in hermaphrodite (n = 6 replicates) and male gonads (n = 3). miRNA expression-differences with four-fold difference in abundance that were statistically different (Wilcoxon rank sum test with continuity correction; p adjusted<0.01) between gonads of hermaphrodites and males as well as between hermaphrodites and *fog-3* males (n = 2) are highlighted in red (female somatic gonad-biased) and blue (male somatic gonad-biased). Related to Fig 1D. B) Mean normalized endogenous siRNA reads (antisense) in hermaphrodite and male gonads; same analyses and representation used as in S1A Fig. Related to Fig 1H.
(TIF)

**S2 Fig. Hermaphrodite and male gonadal 26G RNA and 22G RNA.** A) Percentage of antisense reads in hermaphrodite and male gonads classified as primary 26G RNA, secondary 22G RNA or other siRNA types. B) Normalized mean 22G RNA reads in hermaphrodite and male gonads per gene, median dotted line, mean full line. C) Mean 22G RNA reads targeting mRNAs of similar abundance in hermaphrodite (red) and male (blue) gonads. Boxes represent first and second quartile, 1.5 interquartile range: vertical line, mean: horizontal line, outliers: circles. Bonferroni corrected p-value of Wilcoxon rank sum test give the probability that samples from a continuous distribution have equal medians.
(TIF)

**S3 Fig. Statistical analysis of gonadal mRNA and siRNA expression levels.** A) Mean normalized mRNA reads [55] and siRNAs in percentiles in hermaphrodite and male gonads. For

20 bins (each representing 5% of data i.e. 427 genes per bin in hermaphrodites and 504 in males) a linear model was fitted (black line). Related to Fig 2A. B) p-values indicate whether the linear model deviates from zero. The most significant negative correlation between the levels of 22G RNA and mRNA was found for the 5% of the genes with highest level of 22G RNA expression (shaded). C) mRNA expression for WAGO-1 target genes and non-target genes (Bonferroni corrected p-values of the Wilcoxon rank sum test q<0.01 indicated by stars, ns = not significant, n/a = not applicable). Data are the same as for Fig 2A but plotted as WAGO-1 targets [20] and non-WAGO-1 targets in hermaphrodites and males. Data were only available for hermaphrodites and interpolated to male samples. D) mRNA expression for ALG-3/-4 target genes and non-target genes (Bonferroni corrected p-values of the Wilcoxon rank sum test q<0.01 indicated by stars, ns = not significant, n/a = not applicable). Data are the same as for Fig 2A but plotted as ALG-3/-4 targets [34] and non-ALG-3/-4 targets in males.
(TIF)

**S4 Fig. Sex-differences in RNAi efficacy in female germlines.** Mean primary and secondary siRNA reads normalized to total reads in gonads from *fog-3* males (n = 2), control hermaphrodites (n = 4) and control males (n = 2) raised on *gfp(RNAi)*. Data for control hermaphrodites and control males are taken from Fig 3B. The right panel shows the ratio of secondary siRNA/primary siRNA. Means were compared with t-tests w* = p<0.05, *** = p<0.001.
(TIF)

**S5 Fig. RNAi silencing susceptibility of sperm producing tissue and sperm.** A) Fluorescence microscopy images of GFP-sensor expression of L4 hermaphrodite germlines (dotted line, distal end indicated by star); animals fed *(gfp)*RNAi or control RNAi. Percentage of animals with germline expression classified as: high, intermediate uniform, intermediate variable and no GFP-sensor expression (from bottom to top) for *(gfp)*RNAi (n = 27). 100% of animals raised on control RNAi showed high GFP-sensor expression (n = 24). B) Fluorescence microscopy images of sperm chromatin marked with a ubiquitous GFP-sensor in adult hermaphrodites, males and *fem-3* hermaphrodites producing only sperm (top). Partial silencing of the GFP-sensor upon *gfp(RNAi)* at 24°C in control and *fem-3* hermaphrodites, while control male germlines are RNAi resistant (bottom). Images were acquired with the same exposure time in control and RNAi condition, but are different between sexes due to different expression levels and physiology. For illustration purposes images are background subtracted, as explained in Fig 4D.
(TIF)

**S1 Table. Summary gonadal small RNA expression.** Analyses of sex-biased miRNA, piRNA, germline siRNA, sense and antisense reads.
(XLSX)

**S2 Table. Overlap with gene sets.** WormExp input and output.
(XLSX)

**S3 Table. Summary 22G RNA, mRNA and siRNA pathways.** Expression levels of 22G RNA and mRNA and classification as WAGO, WAGO-1, CSR-1, ALG-3/-4.
(XLSX)

**S4 Table. Gonadal mRNA expression of RNAi regulators.**
(XLSX)

**S5 Table. Sequencing details.** Accession and genotype of each replicate.
(XLSX)

## Acknowledgments

## Author Contributions

**Conceptualization:** Alexandra Bezler, Fabian Braukmann, Pierre Gönczy, Fabio Piano, Kristin Gunsalus, Eric A. Miska, Laurent Keller.

**Data curation:** Fabian Braukmann, Sean M. West.

**Formal analysis:** Alexandra Bezler, Fabian Braukmann, Sean M. West, Frédéric Schütz.

**Funding acquisition:** Sean M. West, Fabio Piano, Kristin Gunsalus, Eric A. Miska, Laurent Keller.

**Investigation:** Alexandra Bezler, Fabian Braukmann, Arthur Duplan, Raffaella Conconi.

**Methodology:** Alexandra Bezler, Fabian Braukmann, Eric A. Miska.

**Resources:** Arthur Duplan, Raffaella Conconi.

**Software:** Fabian Braukmann, Sean M. West, Frédéric Schütz.

**Supervision:** Pierre Gönczy, Fabio Piano, Kristin Gunsalus, Eric A. Miska, Laurent Keller.

**Validation:** Alexandra Bezler, Fabian Braukmann, Sean M. West.

**Visualization:** Alexandra Bezler, Fabian Braukmann, Sean M. West, Frédéric Schütz.

**Writing – original draft:** Alexandra Bezler, Fabian Braukmann, Eric A. Miska, Laurent Keller.

**Writing – review & editing:** Alexandra Bezler, Fabian Braukmann, Sean M. West, Pierre Gönczy, Fabio Piano, Kristin Gunsalus, Eric A. Miska, Laurent Keller.

## References

1. Miska EA, Ferguson-Smith AC. Transgenerational inheritance: Models and mechanisms of non-DNA sequence-based inheritance. Science. 2016. pp. 59–63. https://doi.org/10.1126/science.aaf4945 PMID: 27846492

2. Grishok A, Tabara H, Mello CC. Genetic requirements for inheritance of RNAi in *C. elegans*. Science. 2000; 287: 2494–2497. https://doi.org/10.1126/science.287.5462.2494 PMID: 10741970

3. Luteijn MJ, van Bergeijk P, Kaaij LJT, Almeida MV, Roovers EF, Berezikov E, et al. Extremely stable Piwi-induced gene silencing in *Caenorhabditis elegans*. EMBO J. 2012; 31: 3422–3430. https://doi.org/10.1038/emboj.2012.213 PMID: 22850670

4. Shirayama M, Seth M, Lee HC, Gu W, Ishidate T, Conte D, et al. PiRNAs initiate an epigenetic memory of nonself RNA in the *C. elegans* germline. Cell. 2012; 150: 65–77. https://doi.org/10.1016/j.cell.2012.06.015 PMID: 22738726

5. Schott D, Yanai I, Hunter CP. Natural RNA interference directs a heritable response to the environment. Sci Rep. 2014; 4: 7387. https://doi.org/10.1038/srep07387 PMID: 25552271

6. Marré J, Traver EC, Jose AM. Extracellular RNA is transported from one generation to the next in *Caenorhabditis elegans*. Proc Natl Acad Sci. 2016; 113: 12496–12501. https://doi.org/10.1073/pnas.1608959113 PMID: 27791108

7.  Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 1998; 391: 806–811. https://doi.org/10.1038/35888 PMID: 9486653

8.  Rechavi O, Minevich G, Hobert O. Transgenerational Inheritance of an Acquired Small RNA-Based Antiviral Response in *C. elegans*. Cell. 2011; 147: 1248–1256. https://doi.org/10.1016/j.cell.2011.10.042 PMID: 22119442

9.  Jobson MA, Jordan JM, Sandrof MA, Hibshman JD, Lennox AL, Baugh LR. Transgenerational Effects of Early Life Starvation on Growth, Reproduction, and Stress Resistance in *Caenorhabditis elegans*. Genetics. 2015; 201: 201–12. https://doi.org/10.1534/genetics.115.178699 PMID: 26187123

10. Vastenhouw NL, Brunschwig K, Okihara KL, Muller F, Tijsterman M, Plasterk RHA. Gene expression: long-term gene silencing by RNAi. Nature. 2006; 442: 882. https://doi.org/10.1038/442882a PMID: 16929289

11. Alcazar RM, Lin R, Fire AZ. Transmission dynamics of heritable silencing induced by double-stranded RNA in *Caenorhabditis elegans*. Genetics. 2008; 180: 1275–1288. https://doi.org/10.1534/genetics.108.089433 PMID: 18757930

12. Lev I, Seroussi U, Gingold H, Bril R, Anava S, Rechavi O. MET-2-Dependent H3K9 Methylation Suppresses Transgenerational Small RNA Inheritance. Curr Biol. 2017; 27: 1138–1147. https://doi.org/10.1016/j.cub.2017.03.008 PMID: 28343968

13. Winston WM, Molodowitch C, Hunter CP. Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. Science. 2002; 295: 2456–9. https://doi.org/10.1126/science.1068836 PMID: 11834782

14. Winston WM, Sutherlin M, Wright AJ, Feinberg EH, Hunter CP. *Caenorhabditis elegans* SID-2 is required for environmental RNA interference. Proc Natl Acad Sci. 2007; 104: 10565–10570. https://doi.org/10.1073/pnas.0611282104 PMID: 17563372

15. Tabara H, Yigit E, Siomi H, Mello CC. The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in *C. elegans*. Cell. 2002; 109: 861–71. PMID: 12110183

16. Parker GS, Eckert DM, Bass BL. RDE-4 preferentially binds long dsRNA and its dimerization is necessary for cleavage of dsRNA to siRNA. RNA. 2006; 12: 807–18. https://doi.org/10.1261/rna.2338706 PMID: 16603715

17. Sijen T, Fleenor J, Simmer F, Thijssen KL, Parrish S, Timmons L, et al. On the Role of RNA Amplification in dsRNA-Triggered Gene Silencing. Cell. 2001; 107: 465–476. https://doi.org/10.1016/S0092-8674(01)00576-1 PMID: 11719187

18. Sijen T, Steiner FA, Thijssen KL, Plasterk RHA. Secondary siRNAs Result from Unprimed RNA Synthesis and Form a Distinct Class. Science. 2007; 315: 244–247. https://doi.org/10.1126/science.1136699 PMID: 17158288

19. Pak J, Fire A. Distinct Populations of Primary and Secondary Effectors During RNAi in *C. elegans*. Science. 2007; 315: 241–244. https://doi.org/10.1126/science.1132839 PMID: 17124291

20. Gu W, Shirayama M, Conte D, Vasale J, Batista PJ, Claycomb JM, et al. Distinct Argonaute-Mediated 22G-RNA Pathways Direct Genome Surveillance in the *C. elegans* Germline. Mol Cell. 2009; 36: 231–244. https://doi.org/10.1016/j.molcel.2009.09.020 PMID: 19800275

21. Grishok A, Sinskey JL, Sharp PA. Transcriptional silencing of a transgene by RNAi in the soma of *C. elegans*. Genes Dev. 2005; 19: 683–696. https://doi.org/10.1101/gad.1247705 PMID: 15741313

22. Guang S, Bochner AF, Burkhart KB, Burton N, Pavelec DM, Kennedy S. Small regulatory RNAs inhibit RNA polymerase II during the elongation phase of transcription. Nature. 2010; 465: 1097–101. https://doi.org/10.1038/nature09095 PMID: 20543824

23. Buckley BA, Burkhart KB, Gu SG, Spracklin G, Kershner A, Fritz H, et al. A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. Nature. 2012; 489: 447–51. https://doi.org/10.1038/nature11352 PMID: 22810588

24. Devanapally S, Ravikumar S, Jose AM. Double-stranded RNA made in *C. elegans* neurons can enter the germline and cause transgenerational gene silencing. Proc Natl Acad Sci U S A. 2015; 112: 2133–8. https://doi.org/10.1073/pnas.1423333112 PMID: 25646479

25. Martinez NJ, Ow MC, Reece-Hoyes JS, Barrasa MI, Ambros VR, Walhout AJM. Genome-scale spatiotemporal analysis of *Caenorhabditis elegans* microRNA promoter activity. Genome Res. 2008; 18: 2005–15. https://doi.org/10.1101/gr.083055.108 PMID: 18981266

26. Kato M, de Lencastre A, Pincus Z, Slack FJ. Dynamic expression of small non-coding RNAs, including novel microRNAs and piRNAs/21U-RNAs, during *Caenorhabditis elegans* development. Genome Biol. 2009; 10: R54. https://doi.org/10.1186/gb-2009-10-5-r54 PMID: 19460142

27. Alberti C, Manzenreither RA, Sowemimo I, Burkard TR, Wang J, Mahofsky K, et al. Cell-Type specific sequencing of microRNAs from complex animal tissues. Nat Methods. 2018; 15: 283–289. https://doi.org/10.1038/nmeth.4610 PMID: 29481550

28. Diag A, Schilling M, Klironomos F, Ayoub S, Rajewsky N. Regulation of spatial and temporal gene expression in an animal germline. bioRxiv. Cold Spring Harbor Laboratory; 2018; 348425. https://doi.org/10.1101/348425

29. Alvarez-Saavedra E, Horvitz HR. Many Families of C. elegans MicroRNAs Are Not Essential for Development or Viability. Curr Biol. 2010; 20: 367–373. https://doi.org/10.1016/j.cub.2009.12.051 PMID: 20096582

30. McJunkin K, Ambros V. The embryonic mir-35 family of microRNAs promotes multiple aspects of fecundity in Caenorhabditis elegans. G3. 2014; 4: 1747–54. https://doi.org/10.1534/g3.114.011973 PMID: 25053708

31. McEwen TJ, Yao Q, Yun S, Lee C-Y, Bennett KL. Small RNA in situ hybridization in Caenorhabditis elegans, combined with RNA-seq, identifies germline-enriched microRNAs. Dev Biol. 2016; 418: 248–257. https://doi.org/10.1016/j.ydbio.2016.08.003 PMID: 27521456

32. Han T, Manoharan AP, Harkins TT, Bouffard P, Fitzpatrick C, Chu DS, et al. 26G endo-siRNAs regulate spermatogenic and zygotic gene expression in Caenorhabditis elegans. Proc Natl Acad Sci U S A. 2009; 106: 18674–9. https://doi.org/10.1073/pnas.0906378106 PMID: 19846761

33. Vasale JJ, Gu W, Thivierge C, Batista PJ, Claycomb JM, Youngman EM, et al. Sequential rounds of RNA-dependent RNA transcription drive endogenous small-RNA biogenesis in the ERGO-1/Argonaute pathway. Proc Natl Acad Sci U S A. 2010; 107: 3582–7. https://doi.org/10.1073/pnas.0911908107 PMID: 20133583

34. Conine CC, Batista PJ, Gu W, Claycomb JM, Chaves DA, Shirayama M, et al. Argonautes ALG-3 and ALG-4 are required for spermatogenesis-specific 26G-RNAs and thermotolerant sperm in Caenorhabditis elegans. Proc Natl Acad Sci U S A. 2010; 107: 3588–93. https://doi.org/10.1073/pnas.0911685107 PMID: 20133686

35. Conine CC, Moresco JJ, Gu W, Shirayama M, Conte D, Yates JR, et al. Argonautes Promote Male Fertility and Provide a Paternal Memory of Germline Gene Expression in C. elegans. Cell. 2013; 155: 1532–1544. https://doi.org/10.1016/j.cell.2013.11.032 PMID: 24360276

36. Gerson-Gurwitz A, Wang S, Sathe S, Green R, Yeo GW, Oegema K, et al. A Small RNA-Catalytic Argonaute Pathway Tunes Germline Transcript Levels to Ensure Embryonic Divisions. Cell. 2016; 165: 396–409. https://doi.org/10.1016/j.cell.2016.02.040 PMID: 27020753

37. Wang G, Reinke V. A C. elegans Piwi, PRG-1, Regulates 21U-RNAs during Spermatogenesis. Curr Biol. 2008; 18: 861–867. https://doi.org/10.1016/j.cub.2008.05.009 PMID: 18501605

38. Batista PJ, Ruby JG, Claycomb JM, Chiang R, Fahlgren N, Kasschau KD, et al. PRG-1 and 21U-RNAs Interact to Form the piRNA Complex Required for Fertility in C. elegans. Mol Cell. 2008; 31: 67–78. https://doi.org/10.1016/j.molcel.2008.06.002 PMID: 18571452

39. Shi Z, Montgomery TA, Qi Y, Ruvkun G. High-throughput sequencing reveals extraordinary fluidity of miRNA, piRNA, and siRNA pathways in nematodes. Genome Res. 2013; 23: 497–508. https://doi.org/10.1101/gr.149112.112 PMID: 23363624

40. Das PP, Bagijn MP, Goldstein LD, Woolford JR, Lehrbach NJ, Sapetschnig A, et al. Piwi and piRNAs Act Upstream of an Endogenous siRNA Pathway to Suppress Tc3 Transposon Mobility in the Caenorhabditis elegans Germline. Mol Cell. 2008; 31: 79–90. https://doi.org/10.1016/j.molcel.2008.06.003 PMID: 18571451

41. Gu W, Lee HC, Chaves D, Youngman EM, Pazour GJ, Conte D, et al. CapSeq and CIP-TAP identify pol ii start sites and reveal capped small RNAs as C. elegans piRNA precursors. Cell. 2012; 151: 1488–1500. https://doi.org/10.1016/j.cell.2012.11.023 PMID: 23260138

42. Killian DJ, Hubbard EJA. Caenorhabditis elegans germline patterning requires coordinated development of the somatic gonadal sheath and the germ line. Dev Biol. 2005; 279: 322–335. https://doi.org/10.1016/j.ydbio.2004.12.021 PMID: 15733661

43. Bukhari SIA, Vasquez-Rifo A, Gagné D, Paquet ER, Zetka M, Robert C, et al. The microRNA pathway controls germ cell proliferation and differentiation in C. elegans. Cell Res. 2012; 22: 1034–1045. https://doi.org/10.1038/cr.2012.31 PMID: 22370633

44. Rios C, Warren D, Olson B, Abbott AL. Functional analysis of microRNA pathway genes in the somatic gonad and germ cells during ovulation in C. elegans. Dev Biol. 2017; 426: 115–125. https://doi.org/10.1016/j.ydbio.2017.04.007 PMID: 28461238

45. Fraser AG, Kamath RS, Zipperlen P, Martinez-Campos M, Sohrmann M, Ahringer J. Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. Nature. 2000; 408: 325–330. https://doi.org/10.1038/35042517 PMID: 11099033

46.  Gönczy P, Echeverri C, Oegema K, Coulson A, Jones SJ, Copley RR, et al. Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. Nature. 2000; 408: 331–6. https://doi.org/10.1038/35042526 PMID: 11099034

47.  Green RA, Kao H-L, Audhya A, Arur S, Mayers JR, Fridolfsson HN, et al. A High-Resolution *C. elegans* Essential Gene Network Based on Phenotypic Profiling of a Complex Tissue. Cell. 2011; 145: 470–482. https://doi.org/10.1016/j.cell.2011.03.037 PMID: 21529718

48.  Reinke V, Gil IS, Ward S, Kazmer K. Genome-wide germline-enriched and sex-biased expression profiles in *Caenorhabditis elegans*. Development. 2003; 131: 311–323. https://doi.org/10.1242/dev.00914 PMID: 14668411

49.  Ma X, Zhu Y, Li C, Xue P, Zhao Y, Chen S, et al. Characterisation of *Caenorhabditis elegans* sperm transcriptome and proteome. BMC Genomics. 2014; 15: 168. https://doi.org/10.1186/1471-2164-15-168 PMID: 24581041

50.  Ellis RE, Kimble J. The fog-3 gene and Regulation of Cell Fate in the Germ line of *Caenorhabditis elegans*. Genetics. 1995; 139: 561–577. PMID: 7713418

51.  Noble DC, Aoki ST, Ortiz MA, Kim KW, Verheyden JM, Kimble J. Genomic Analyses of Sperm Fate Regulator Targets Reveal a Common Set of Oogenic mRNAs in *Caenorhabditis elegans*. Genetics. 2016; 202: 221–34. https://doi.org/10.1534/genetics.115.182592 PMID: 26564160

52.  Ruby JG, Jan C, Player C, Axtell MJ, Lee W, Nusbaum C, et al. Large-Scale Sequencing Reveals 21U-RNAs and Additional MicroRNAs and Endogenous siRNAs in *C. elegans*. Cell. 2006; 127: 1193–1207. https://doi.org/10.1016/j.cell.2006.10.040 PMID: 17174894

53.  Billi AC, Freeberg MA, Day AM, Chun SY, Khivansara V, Kim JK. A conserved upstream motif orchestrates autonomous, germline-enriched expression of *Caenorhabditis elegans* piRNAs. Copenhaver GP, editor. PLoS Genet. 2013; 9: e1003392. https://doi.org/10.1371/journal.pgen.1003392 PMID: 23516384

54.  Yang W, Dierking K, Schulenburg H. WormExp: a web-based application for a *Caenorhabditis elegans*-specific gene expression enrichment analysis. Bioinformatics. 2016; 32: 943–945. https://doi.org/10.1093/bioinformatics/btv667 PMID: 26559506

55.  West SM, Mecenas D, Gutwein M, Aristizábal-Corrales D, Piano F, Gunsalus KC. Developmental dynamics of gene expression and alternative polyadenylation in the *Caenorhabditis elegans* germline. Genome Biol. 2017; 19: 8. https://doi.org/10.1186/s13059-017-1369-x PMID: 29368663

56.  Claycomb JM, Batista PJ, Pang KM, Gu W, Vasale JJ, van Wolfswinkel JC, et al. The Argonaute CSR-1 and Its 22G-RNA Cofactors Are Required for Holocentric Chromosome Segregation. Cell. 2009; 139: 123–134. https://doi.org/10.1016/j.cell.2009.09.014 PMID: 19804758

57.  Bagijn MP, Goldstein LD, Sapetschnig A, Weick E-M, Bouasker S, Lehrbach NJ, et al. Function, targets, and evolution of *Caenorhabditis elegans* piRNAs. Science. 2012; 337: 574–578. https://doi.org/10.1126/science.1220952 PMID: 22700655

58.  Jose AM, Garcia GA, Hunter CP. Two classes of silencing RNAs move between *Caenorhabditis elegans* tissues. Nat Struct Mol Biol. 2011; 18: 1184–1188. https://doi.org/10.1038/nsmb.2134 PMID: 21984186

59.  Raman P, Zaghab SM, Traver EC, Jose AM. The double-stranded RNA binding protein RDE-4 can act cell autonomously during feeding RNAi in *C. elegans*. Nucleic Acids Res. 2017; 45: 8463–8473. https://doi.org/10.1093/nar/gkx484 PMID: 28541563

60.  Chen P-J, Singal A, Kimble J, Ellis RE. A Novel Member of the Tob Family of Proteins Controls Sexual Fate in *Caenorhabditis elegans* Germ Cells. Dev Biol. Academic Press; 2000; 217: 77–90. https://doi.org/10.1006/dbio.1999.9521 PMID: 10625537

61.  Luitjens C, Gallegos M, Kraemer B, Kimble J, Wickens M. CPEB proteins control two key steps in spermatogenesis in *C. elegans*. Genes Dev. 2000; 14: 2596–609. https://doi.org/10.1101/GAD.831700 PMID: 11040214

62.  Sumiyoshi E, Sugimoto A, Yamamoto M. Protein phosphatase 4 is required for centrosome maturation in mitosis and sperm meiosis in *C. elegans*. J Cell Sci. 2002; 115: 1403–10. PMID: 11896188

63.  Zhu G, Salazar G, Zlatic SA, Fiza B, Doucette MM, Heilman CJ, et al. SPE-39 family proteins interact with the HOPS complex and function in lysosomal delivery. Mol Biol Cell. 2009; 20: 1223–40. https://doi.org/10.1091/mbc.E08-07-0728 PMID: 19109425

64.  von Tobel L, Mikeladze-Dvali T, Delattre M, Balestra FR, Blanchoud S, Finger S, et al. SAS-1 Is a C2 Domain Protein Critical for Centriole Integrity in *C. elegans*. Dutcher SK, editor. PLoS Genet. 2014; 10: e1004777. https://doi.org/10.1371/journal.pgen.1004777 PMID: 25412110

65.  Kang C, You Y -j., Avery L. Dual roles of autophagy in the survival of *Caenorhabditis elegans* during starvation. Genes Dev. 2007; 21: 2161–2171. https://doi.org/10.1101/gad.1573107 PMID: 17785524

66. Kalis AK, Kroetz MB, Larson KM, Zarkower D. Functional genomic identification of genes required for male gonadal differentiation in *Caenorhabditis elegans*. Genetics. 2010; 185: 523–535. https://doi.org/10.1534/genetics.110.116038 PMID: 20308279

67. Nelson MD, Zhou E, Kiontke K, Fradin H, Maldonado G, Martin D, et al. A bow-tie genetic architecture for morphogenesis suggested by a genome-wide RNAi screen in *Caenorhabditis elegans*. Chisholm AD, editor. PLoS Genet. 2011; 7: e1002010. https://doi.org/10.1371/journal.pgen.1002010 PMID: 21408209

68. Pak J, Maniar JM, Mello CC, Fire A. Protection from Feed-Forward Amplification in an Amplified RNAi Mechanism. Cell. 2012; 151: 885–899. https://doi.org/10.1016/j.cell.2012.10.022 PMID: 23141544

69. Zhang C, Montgomery TA, Fischer SEJ, Garcia SMDA, Riedel CG, Fahlgren N, et al. The *Caenorhabditis elegans* RDE-10/RDE-11 Complex Regulates RNAi by Promoting Secondary siRNA Amplification. Curr Biol. 2012; 22: 881–890. https://doi.org/10.1016/j.cub.2012.04.011 PMID: 22542102

70. Tsai H-Y, Chen C-CG, Conte D, Moresco JJ, Chaves DA, Mitani S, et al. A Ribonuclease Coordinates siRNA Amplification and mRNA Cleavage during RNAi. Cell. 2015; 160: 407–419. https://doi.org/10.1016/j.cell.2015.01.010 PMID: 25635455

71. Ooi SL, Priess JR, Henikoff S. Histone H3.3 Variant Dynamics in the Germline of *Caenorhabditis elegans*. PLoS Genet. 2006; 2: e97. https://doi.org/10.1371/journal.pgen.0020097 PMID: 16846252

72. LaMunyon CW, Ward S. Larger sperm outcompete smaller sperm in the nematode *Caenorhabditis elegans*. Proc R Soc B Biol Sci. 1998; 265: 1997–2002. https://doi.org/10.1098/rspb.1998.0531 PMID: 9821364

73. Barton MK, Schedl TB, Kimble J. Gain-of-Function Mutations of fem-3, a Sex-Determination Gene in *Caenorhabditis elegans*. Genetics. 1987;115.

74. McJunkin K, Ambros V. A microRNA family exerts maternal control on sex determination in *C. elegans*. Genes Dev. 2017; 31: 422–437. https://doi.org/10.1101/gad.290155.116 PMID: 28279983

75. Lim LP, Lau NC, Weinstein EG, Abdelhakim A, Yekta S, Rhoades MW, et al. The microRNAs of *Caenorhabditis elegans*. Genes Dev. 2003; 17: 991–1008. https://doi.org/10.1101/gad.1074403 PMID: 12672692

76. de Lencastre A, Pincus Z, Zhou K, Kato M, Lee SS, Slack FJ. MicroRNAs Both Promote and Antagonize Longevity in *C. elegans*. Curr Biol. 2010; 20: 2159–2168. https://doi.org/10.1016/j.cub.2010.11.015 PMID: 21129974

77. Pincus Z, Smith-Vikos T, Slack FJ. MicroRNA Predictors of Longevity in *Caenorhabditis elegans*. Kim SK, editor. PLoS Genet. 2011; 7: e1002306. https://doi.org/10.1371/journal.pgen.1002306 PMID: 21980307

78. Ortiz MA, Noble D, Sorokin EP, Kimble J. A new dataset of spermatogenic vs. oogenic transcriptomes in the nematode *Caenorhabditis elegans*. G3. 2014; 4: 1765–72. https://doi.org/10.1534/g3.114.012351 PMID: 25060624

79. van Wolfswinkel JC, Claycomb JM, Batista PJ, Mello CC, Berezikov E, Ketting RF. CDE-1 Affects Chromosome Segregation through Uridylation of CSR-1-Bound siRNAs. Cell. 2009; 139: 135–148. https://doi.org/10.1016/j.cell.2009.09.012 PMID: 19804759

80. Yigit E, Batista PJ, Bei Y, Pang KM, Chen C-CG, Tolia NH, et al. Analysis of the *C. elegans* Argonaute Family Reveals that Distinct Argonautes Act Sequentially during RNAi. Cell. 2006; 127: 747–757. https://doi.org/10.1016/j.cell.2006.09.033 PMID: 17110334

81. Hodgkin J, Horvitz HR, Brenner S. Nondisjunction Mutants of the Nematode *Caenorhabditis elegans*. Genetics. 1979; 91: 67–94. PMID: 17248881

82. Zhang C, Montgomery TA, Gabel HW, Fischer SEJ, Phillips CM, Fahlgren N, et al. mut-16 and other mutator class genes modulate 22G and 26G siRNA pathways in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A. 2011; 108: 1201–8. https://doi.org/10.1073/pnas.1018695108 PMID: 21245313

83. Kamath RS, Martinez-Campos M, Zipperlen P, Fraser AG, Ahringer J. Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. Genome Biol. 2001; 2: RESEARCH0002. https://doi.org/10.1186/gb-2000-2-1-research0002 PMID: 11178279

84. O'Connell J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012; 9: 676–682. https://doi.org/10.1038/nmeth.2019 PMID: 22743772

85. Peters N, Perez DE, Song MH, Liu Y, Muller-Reichert T, Caron C, et al. Control of mitotic and meiotic centriole duplication by the Plk4-related kinase ZYG-1. J Cell Sci. 2010; 123: 795–805. https://doi.org/10.1242/jcs.050682 PMID: 20144993

86. Aronesty E. Comparison of Sequencing Utility Programs. Open Bioinforma J. 2013; 7: 1–8. https://doi.org/10.2174/1875036201307010001

87. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009; 10: R25. https://doi.org/10.1186/gb-2009-10-3-r25 PMID: 19261174

88. Anders S, Pyl PT, Huber W. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015; 31: 166–169. https://doi.org/10.1093/bioinformatics/btu638 PMID: 25260700

89. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014; 15: 550. https://doi.org/10.1186/s13059-014-0550-8 PMID: 25516281

90. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal. 2011; 17: 10. https://doi.org/10.14806/ej.17.1.200