GENETICS

Nucleus-independent transgenerational small RNA inheritance in *Caenorhabditis elegans*

Itai Rieger[1]*, Guy Weintraub[1], Itamar Lev[1], Kesem Goldstein[1], Dana Bar-Zvi[1], Sarit Anava[1], Hila Gingold[1], Shai Shaham[2], Oded Rechavi[1]*

In *Caenorhabditis elegans* worms, epigenetic information transmits transgenerationally. Still, it is unknown whether the effects transfer to the next generation inside or outside of the nucleus. Here, we use the tractability of gene-specific double-stranded RNA–induced silencing to demonstrate that RNA interference can be inherited independently of any nuclear factors via mothers that are genetically engineered to transmit only their ooplasm but not the oocytes' nuclei to the next generation. We characterize the mechanisms and, using RNA sequencing, chimeric worms, and sequence polymorphism between different isolates, identify endogenous small RNAs which, similarly to exogenous siRNAs, are inherited in a nucleus-independent manner. From a historical perspective, these results might be regarded as partial vindication of discredited cytoplasmic inheritance theories from the 19th century, such as Darwin's "pangenesis" theory.

INTRODUCTION

In the 19th century, before the chromosomal theory of inheritance, scientists argued over the role that the nucleus plays in the transmission of information to the next generation. Hertwig, Strasburger, von Kolliker, and Weismann hypothesized that the nucleus is the carrier of hereditary properties (*1*, *2*), while other contemporaries, most notably the Swiss botanist Carl Nägeli, believed that the heritable agents ignore cellular and subcellular boundaries (Nägeli claimed that the hereditary substance is somewhere in the "protoplasm") (*3*). Similarly, Darwin believed in the soma-to-germline inheritance of extranuclear information [via "gemmules," reviewed in (*4*–*6*)]. In the second half of the 20th century, the understanding that DNA is the heritable material ended this discussion (although the DNA of the mitochondria and the chloroplast resides of course outside of the nucleus). Most studies on transgenerational epigenetic inheritance focused on the nuclear inheritance of chromatin changes. Nevertheless, it remained unclear whether other types of epigenetic information (non-DNA-encoded), such as small RNA–controlled responses, can be independently inherited in the cytoplasm.

Transgenerational epigenetic inheritance of environmental responses has been described in many organisms [reviewed in (*7*, *8*)] but is especially well-understood in *Caenorhabditis elegans* nematodes. It is relatively straightforward to study transgenerational effects in *C. elegans* as they exhibit gene-specific long-term heritable RNA interference (RNAi) responses. Transgenerational RNAi entails the synthesis, processing, and inheritance of exogenous small interfering RNAs (siRNAs) following administration of exogenous double-stranded RNAs (dsRNAs) (*9*). In addition to being a useful tool for investigating gene functions, exogenous dsRNA-derived siRNA-mediated silencing is physiologically relevant, as it enables antiviral defense and as both animals and plants evolved mechanisms for taking up dsRNA from the environment and even from other organisms (*10*). In addition to exogenous dsRNA-induced siRNAs, worms respond to different environmental challenges by synthesizing endogenous small interfering RNAs (endo-siRNA) that regulate gene expression and affect their physiology across multiple generations (*11*–*14*). In comparison to experiments using gene-specific dsRNA–induced silencing, transgenerational inheritance of endogenous small RNAs in response to stress is more challenging to study, as these complex responses reshape the entire transcriptome and entail many indirect interactions between numerous genes.

The dedicated machinery that enables RNAi inheritance in *C. elegans* has been extensively studied (*15*–*22*), and heritable siRNAs were found to typically be 22 nucleotides (nt) long and to start with the nucleotide guanine (hereafter 22Gs). Inherited 22G small RNAs persist in the progeny despite the dilution of the parental RNAs, as they are synthesized anew in every generation by RNA-dependent RNA polymerases (RdRP) which use the target RNA as a template (*23*). RDE-3, a nucleotidyltransferase, adds untemplated polyUG sequences to the mRNAs of RNAi-targeted genes (*24*), marking them for additional rounds of amplification by RdRPs, and thus leading to the generation of more 22G to induce silencing and perpetuate inheritance (*25*).

Are amplified small RNAs transmitted to the next generation inside or outside of the germline nucleus? Different studies examined the role of different cellular compartments in the transmission of epigenetic effects (*26*–*28*). However, it is still debated whether small RNA–related transgenerational effects transmit via the cytoplasm independently of the nucleus and which heritable agents are involved (see Discussion).

Different RNAi factors, which are key for inheritance, for example, some of the Argonautes that carry amplified small RNAs, reside in the cytoplasm, while others exist in the nucleus. Argonautes can shuttle between the nucleus and the cytoplasm, and both predominantly nuclear Argonautes, such as Heritable RNAi Deficient (HRDE-1) (also known as WAGO-9) and cytoplasmic Argonautes, such as WAGO-4, were shown to be important for RNAi inheritance (*18*, *29*–*32*) [HRDE-1 was recently shown to transfer between the nucleus and cytoplasm, (*33*)]. However, different

[1]Department of Neurobiology, Wise Faculty of Life Sciences and Sagol School of Neuroscience, Tel Aviv University, Tel Aviv, Israel. [2]Laboratory of Developmental Genetics, The Rockefeller University, New York, NY, USA.
*Corresponding author. Email: itairieger@gmail.com (I.R.); odedrechavi@gmail.com (O.R.)

studies showed that neither Argonaute is required for RNAi inheritance (*32*, *34*, *35*).

The capacity of small RNAs to be amplified by RdRPs makes them attractive candidates for the role of agents that can perpetuate and carry the heritable information transgenerationally without diluting it (*11*). However, some chromatin marks can similarly self-template (*36*, *37*) (cytosine methylation can famously self-template but the *C. elegans* genome does not contain this modification). Accordingly, the debate regarding the contribution of chromatin information to RNAi inheritance has yet to be settled. It is challenging to untangle the different mechanisms from one another since, for example, also in worms, heritable small RNAs guide the trimethylation of histone H3 lysine 9 (H3K9me3), H3K27, and H3K23 (*38*–*42*), and in multiple organisms, it has been shown that chromatin modifications feedback to induce additional rounds of small RNA synthesis (*37*). The chromatin on the targeted locus is not required for transmission of RNAi to the next generation and it has been shown that RNAi can be inherited even when the targeted locus is crossed out and reintroduced one generation later (*15*). However, in different organisms, including *C. elegans*, RNAi has been shown to induce heritable chromatin modification footprints that spread many kilobases from the site that was directly targeted by dsRNA (*39*, *40*). It is thus possible that even when the targeted locus is crossed out, some chromatin mark is left in a distant site, and that long-range interactions reinstate the silencing when a naïve locus is reintroduced in the next generation. Moreover, it is possible that other changes to the chromatin landscape persist transgenerationally in the absence of the targeted locus [such as recruitment of heterochromatic areas near the nuclear periphery after RNAi, or other 3D conformation changes (*33*)]. Thus, whether RNAi can be inherited independently of any chromatin contributions remains to be demonstrated.

In this work, we demonstrate that siRNA-mediated silencing and siRNAs can be inherited in the cytoplasm independently of the nucleus and investigate the cytoplasmic inheritance mechanism.

RESULTS

Detection of nucleus-independent RNAi inheritance using mosaic worms

To test whether dsRNA-induced RNAi can be inherited independently of any chromatin changes or the involvement of any other nuclear factor, we used an elegant genetic trick that was engineered by Besseling and Bringmann (*43*). Overexpression of a codon-optimized version of G Protein Regulator (GPR-1), a conserved microtubule force regulator, disrupts the fusion of the maternal and paternal nuclei in the fertilized egg. This leads to uneven segregation of the paternal and maternal nuclei during the first cell division to the blastomeres that will give rise to the germline (P lineage) and the soma (AB lineage). As a result, only one parent contributes its nucleus to the germline of the cross progeny of GPR-1–overexpressing [GPR-1(OE)] hermaphrodites. A fluorescently marked strain that was constructed by Artiles *et al.* (*44*) enables simple and conclusive identification of such mosaic progeny. The entire germline of these chimeric worms derives from a cell with an exclusively paternal or exclusively maternal nucleus and a fused cytoplasm from both origins.

To test for nucleus-independent RNAi inheritance, we exposed GPR-1(OE) mothers to anti–*oma-1* dsRNA, to induce silencing of the redundant germline-expressed gene *oma-1* (*45*). After selfing, the progeny of these hermaphrodites, which were laid on plates without dsRNA-producing bacteria, were crossed to males carrying a temperature-sensitive and dominant lethal *oma-1(zu405)* allele (*45*, *46*). Silencing of this *oma-1* allele is commonly used to test for RNAi inheritance because it rescues from embryonic lethality at 20°C (*16*, *20*, *34*). In other words, only individuals who inherit RNAi survive. In our experiment, we tracked the mosaic progeny that had in their germline only the paternally provided nucleus, and therefore, their germline genome contained only the *oma-1(zu405)* allele. We found that ~85% (85.56 ± 6.65%) of these worms gave rise to viable F3 progeny (Fig. 1), while empty vector (EV) RNAi-treated control worms failed to hatch (3.76 ± 0.18% viable progeny). Since in these experiments, the GPR-1(OE) mothers do not transmit their nuclei to the germline of the progeny, we reasoned that the heritable RNAi response was inherited via the ooplasm. Thus, these results reveal that dsRNA-induced RNAi can be inherited in a nucleus-independent manner.

The nucleus-independent RNAi signal is inherited across generations in the germline and does not result from soma-to-germline transport of dsRNA

In *C. elegans*, RNAi can function noncell autonomously (*47*). Therefore, the silencing response in the paternally derived germline of chimeras could in theory derive from RNA molecules originating in somatic tissues that contain the maternal nuclei. To account for this possibility, we crossed the hermaphrodites to *oma-1(zu405); sid-1(qt9)* mutant males (Fig. 2A). Systemic RNA Interference Defective (SID-1) is a transmembrane dsRNA transporter required for import of RNA and systemic RNAi (*48*). In our experiments, the *sid-1(+)* somatic cells are responsive to RNAi and support noncell autonomous silencing. However, the germline, which contains only the *sid-1(−)* paternal genome, is defective in importing RNAi from cells outside of the germline. We found that SID-1–dependent transport of RNAi from the soma to the germline is not required for nucleus-independent RNAi inheritance (85.44 ± 2.59%), suggesting that the germline already contained sufficient silencing RNA molecules and that the RNAi signal was inherited from the ooplasm (Fig. 2B).

ZNFX-1 functions in nucleus-independent RNAi inheritance

Recent evidence highlights the cytoplasmic germ granule component Zinc finger NFX-type containing homolog (ZNFX-1) as a key player in RNAi inheritance (*21*, *49*). It was suggested that ZNFX-1 participates in a small RNA amplification cycle that might function independently of a parallel small RNA amplification cycle that depends on HRDE-1 (*35*). We tested whether nucleus-independent RNAi inheritance depends on ZNFX-1. Ouyang *et al.* (*35*) found that heritable silencing of *mex-6* and *oma-1* in *znfx-1(gg561)* mutants is weak (partially defective) and therefore concluded that ZNFX-1 and HRDE-1 can compensate for each other (*znfx-1;hrde-1* double mutant were found to be completely defective for RNAi inheritance). Similar redundancy with HRDE-1 was observed for *wago-4* mutants [the Argonaute WAGO-4 colocalizes with ZNFX-1 in the germ granules and the proteins were suggested to work together to promote RNAi inheritance (*21*, *32*)]. We successfully replicated the conclusion of these experiments by showing that *znfx-1(gg561)* mutants are capable of partial (weak) RNAi inheritance (24.49 ± 8.91% viable progeny; Fig. 3B, third bar from the

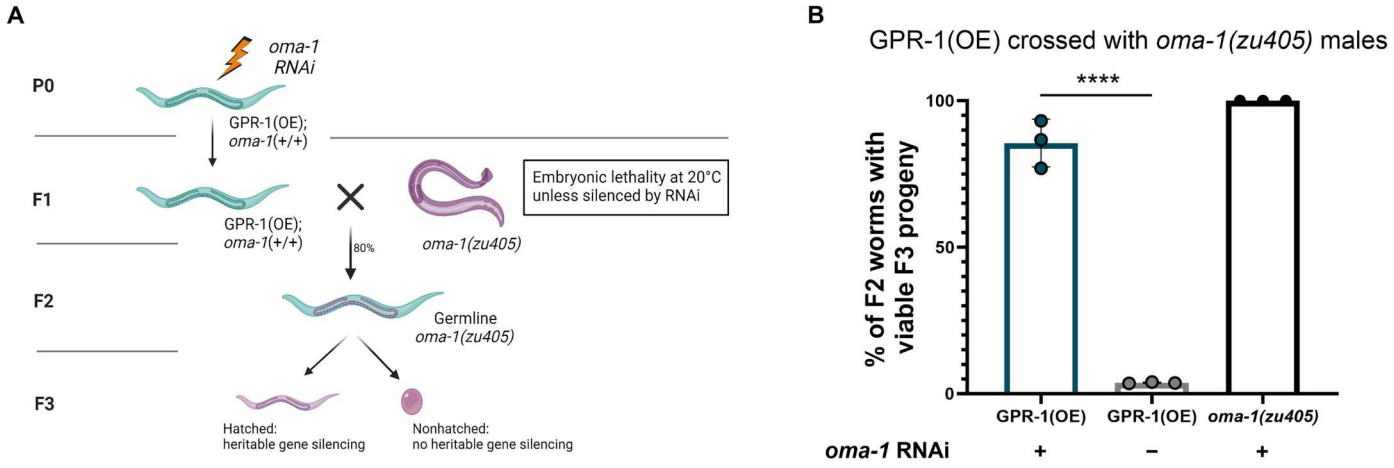

Fig. 1. Detection of nucleus-independent RNAi inheritance using chimeric worms. (**A**) A schematic diagram depicting the experimental procedure. P0 GPR-1(OE) worms were fed bacteria expressing dsRNA complementary to the *oma-1* gene. F1 progeny were then crossed to *oma-1(zu405)* males. Approximately 80% of chimeric progeny carry a paternally derived germline and 2% have a maternally derived germline. We isolated F2 chimeric progeny containing a paternally derived germline and scored for viable F3 progeny. (**B**) Analysis of nucleus-independent RNAi inheritance. Genotypes on the *x* axis refer to the P0, dsRNA-exposed worms. All worms were crossed to *oma-1(zu405)* males at the F1 generation. *oma-1* RNAi (+) worms were exposed to anti–*oma-1* dsRNA or (–) to control empty vector (EV) plasmid. Each dot represents a biological repeat with ~30 individual F2 worms; bars, means ± SD. *oma-1(zu405)* (black dots) are the positive control for the RNAi treatment. Additional controls included *oma-1(zu405)* on EV (none of the F1s hatch), F2s with a maternally derived *oma-1*(+) germline (100% of which had viable progeny). *P* value was determined via Fisher's exact test, ****$P < 0.0001$.

left) by testing a different complementary phenotype (see Materials and Methods). We found that the observed partial weak RNAi inheritance in *znfx-1* mutants is revoked when inheritance is restricted to the cytoplasm [using the GPR-1(OR) system, 6.87 ± 3.54% viable progeny] (Fig. 3). We posited that the residual heritable silencing witnessed in *znfx-1* mutants is mediated by inheritance of epigenetic information in the maternal nucleus and reasoned that ZNFX-1 is involved in nucleus-independent RNAi inheritance.

Germ granule disruption in *pptr-1* mutants potentiates nucleus-independent RNAi inheritance and enables transgenerational silencing even in *znfx-1* mutants

Which cytoplasmic components or organelles are involved in nucleus-independent RNAi inheritance? Since ZNFX-1 localizes to cytoplasmic germ granules, we reasoned that germ granules function in nucleus-independent dsRNA-mediated siRNA inheritance. The germ granules are deposited from the ooplasm to the P lineage

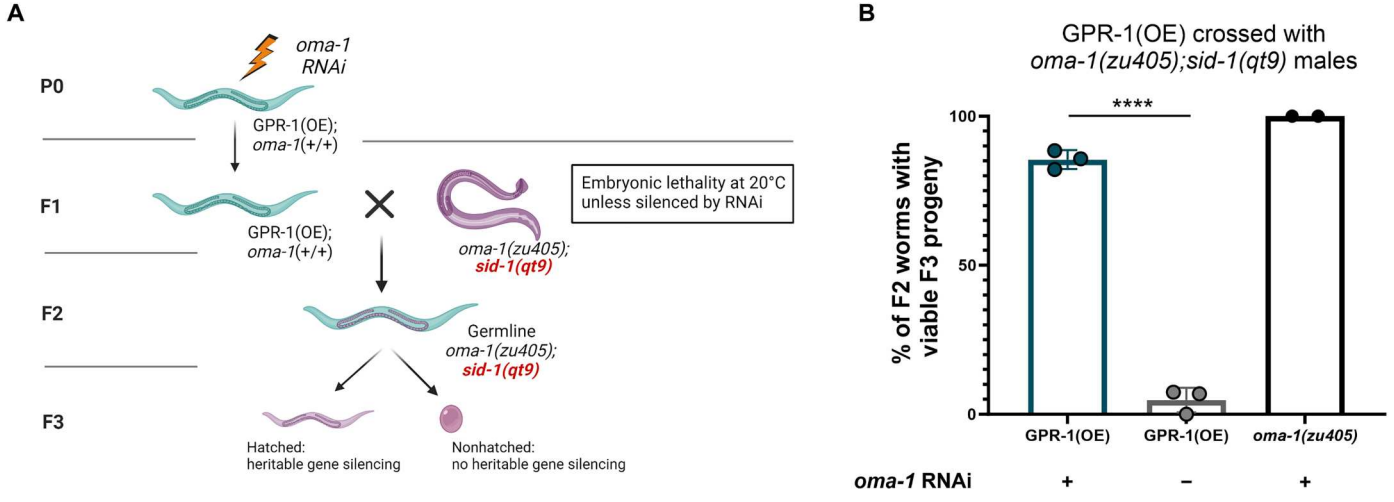

Fig. 2. The nucleus-independent RNAi response is inherited across generations in the germline and does not result from soma-to-germline transport of dsRNA. (**A**) A schematic diagram depicting the experimental procedure. P0 GPR-1(OE) worms were fed bacteria expressing dsRNA complementary to the *oma-1* gene. F1 progeny were then crossed to *oma-1(zu405);sid-1(qt9)* males. F2 chimeric progeny containing paternally derived germline were isolated and scored for viable F3 progeny. (**B**) Analysis of nucleus-independent RNAi inheritance with *sid-1* mutants. Genotypes on the *x* axis refer to the P0, dsRNA-exposed worms. All worms were crossed to *oma-1(zu405);sid-1(qt9)* males at the F1 generation. *oma-1* RNAi (+) worms were exposed to anti–*oma-1* dsRNA or (–) to control EV plasmid. Each dot represents a biological repeat with ~30 individual F2 worms; bars, means ± SD. *oma-1(zu405)* (black dots) are the positive control for the RNAi treatment. Additional controls included *oma-1(zu405)* on EV (none of the F1s hatch), F2s with a maternally derived *oma-1*(+) germline (100% of which had viable progeny). *P* value was determined via Fisher's exact test, ****$P < 0.0001$.

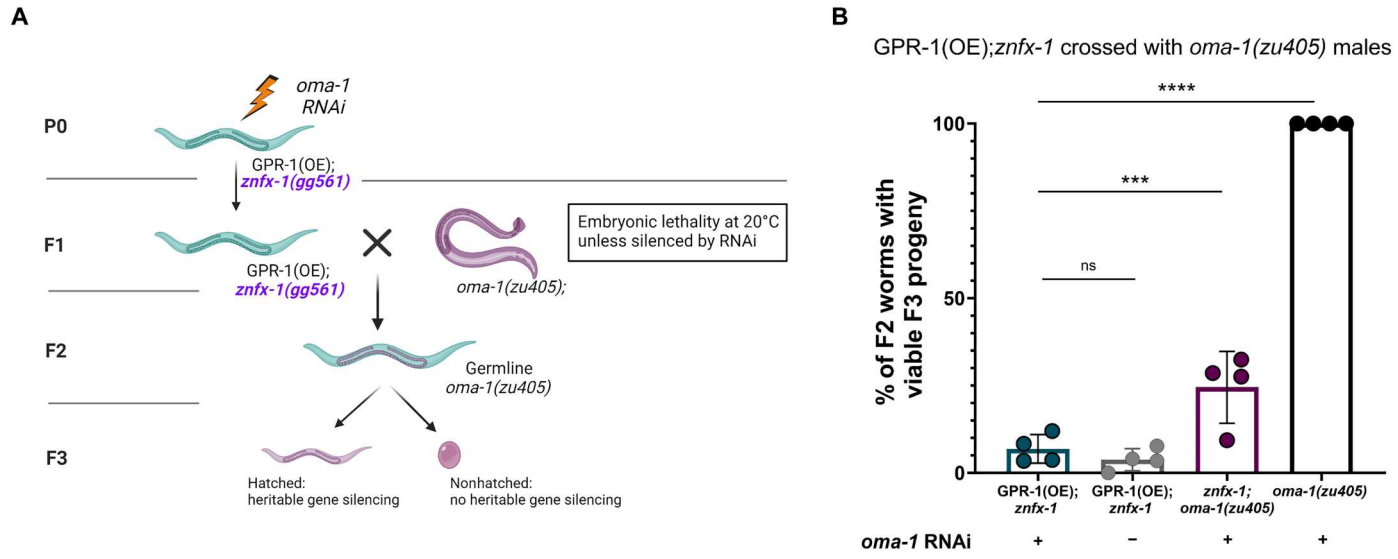

Fig. 3. ZNFX-1 is required for nucleus-independent RNAi inheritance. (**A**) A schematic diagram depicting the experimental procedure. P0 GPR-1(OE);*znfx-1(gg561)* or *oma-1(zu405);znfx-1(gg561)* worms were fed bacteria expressing dsRNA complementary to the *oma-1* gene. F1 progeny were then crossed to *oma-1(zu405)* males. F2 chimeric progeny containing paternally derived germline were isolated and scored for viable F3 progeny. (**B**) Analysis of nucleus-independent RNAi inheritance in *znfx-1* mutants. Genotypes on the *x* axis refer to the P0, dsRNA-exposed worms. All genotypes were crossed with *oma-1(zu405)* males at the F1 generation. *oma-1* RNAi (+) worms were exposed to anti–*oma-1* dsRNA or (–) to control EV plasmid. Each dot represents a biological repeat with ~30 individual F2 worms; bars, means ± SD. *znfx-1; oma-1(zu405)* (maroon dots) show partial RNAi inheritance (potentially via the nuclear, *hrde-1*–dependent pathway), ***$P = 0.0003$. *oma-1(zu405)* (black dots) are the positive control for the RNAi treatment. Additional controls included *oma-1(zu405)* on EV (none of the F1s hatch), F2s with a maternally derived *oma-1*(+) germline (100% of which had viable progeny). *P* values were determined via Fisher's exact test; ****$P < 0.0001$ indicates no inheritance in *znfx-1* mutation background to GPR-1(OE). ns, not significant.

in the early embryo (*50*). While the granules are important for RNAi and for RNAi inheritance (*51*, *52*), it was recently shown that RNAi can nevertheless be inherited even when the deposition of germ granules to the embryo is severely disrupted (*34*, *53*). To examine the role of proper germ-granule segregation in cytoplasmic inheritance, we tested nucleus-independent RNAi inheritance in *pptr-1* mutants [BFF131: GPR-1(OE);*pptr-1(tm3103)*]. Protein Phosphatase Two A (2A) Regulatory subunit (PPTR-1) encodes for a regulatory subunit of the conserved phosphatase PP2A and is necessary for the correct, asymmetrical segregation of P granules to the embryo's P lineage and ultimately to the germline. In *pptr-1(tm3103)* mutants, P granules are incorrectly segregated and disassembled at each division, resulting in germ cells containing P granules that are reduced in both size and number (*54*).

Previously, we found that, unexpectedly, heritable RNAi silencing is much stronger in *pptr-1(tm3103)* mutants compared to wild-type worms (*34*). The effect is marked, as heritable RNAi responses last three to five generations on average in wild-type worms (*16*, *20*), while in *pptr-1* mutants, they last for at least 70 generations (*34*). Here, we show that when *pptr-1* is disabled, nuclear-independent RNAi inheritance is potentiated and lasts longer (Fig. 4, A and B). Furthermore, we found that when nuclear-independent RNAi inheritance is potentiated in *pptr-1* mutants, RNAi is weakly inherited via the cytoplasm even in the absence of ZNFX-1 (19.25 ± 10.94% viable progeny) (Fig. 4C). Together, these results suggest that the fidelity of germ granule segregation determines the strength of cytoplasmic siRNA inheritance in a ZNFX-1–dependent manner; however, ZNFX-1 is not absolutely required for cytoplasmic inheritance.

Endogenous siRNAs are inherited in a nucleus-independent manner

Next, we tested if endogenous small RNAs are also inherited via the cytoplasm, similar to exogenous dsRNA-derived siRNAs. To do so, we crossed different *C. elegans* isolates. We crossed hermaphrodites of the lab strain N2 (isolated in Bristol, UK) which overexpress GPR-1, with MY16 males (isolated in Munster, Germany). These two isolates differ in 111,533 single-nucleotide polymorphisms (SNPs) and 17,410 indels across the genome (*55*) and, moreover, differentially express ~1850 endogenous small RNAs (fig. S1). By combining the non-Mendelian inheritance of GPR-1(OE) mutants and the polymorphism between the two isolates, we were able to search for endogenously derived heritable small RNAs: Overexpression of GPR-1 in N2 resulted in chimeric F1 worms that gave F2 offspring that were genetically identical to either MY16 or N2 (Fig. 5A), which we refer to as F2 MY16* and F2 N2*, respectively. After crossing the worms, we sequenced small RNAs and mRNAs from multiple biological and technical replicates of F2 N2, F2 N2*, F2 MY16, and F2 MY16* worms. Sequencing and analysis of mRNAs confirmed that the nuclear mRNAs from F2 MY16* worms corresponded to the MY16 genome, while the cytoplasmic, mitochondrially encoded mRNAs in MY16* corresponded to the N2 mitochondrial DNA.

We detected cytoplasmic inheritance of endogenous small RNAs that target 129 protein-coding genes (these small RNAs are transmitted via the cytoplasm of P0 N2 to F2 MY16*). We determined that these endogenous small RNAs were inherited via the mother's cytoplasm based on three observations. (i) The small RNA pool of MY16* worms exhibited higher similarity to the small RNA pool of N2 worms than did the small RNA pool of MY16, based on

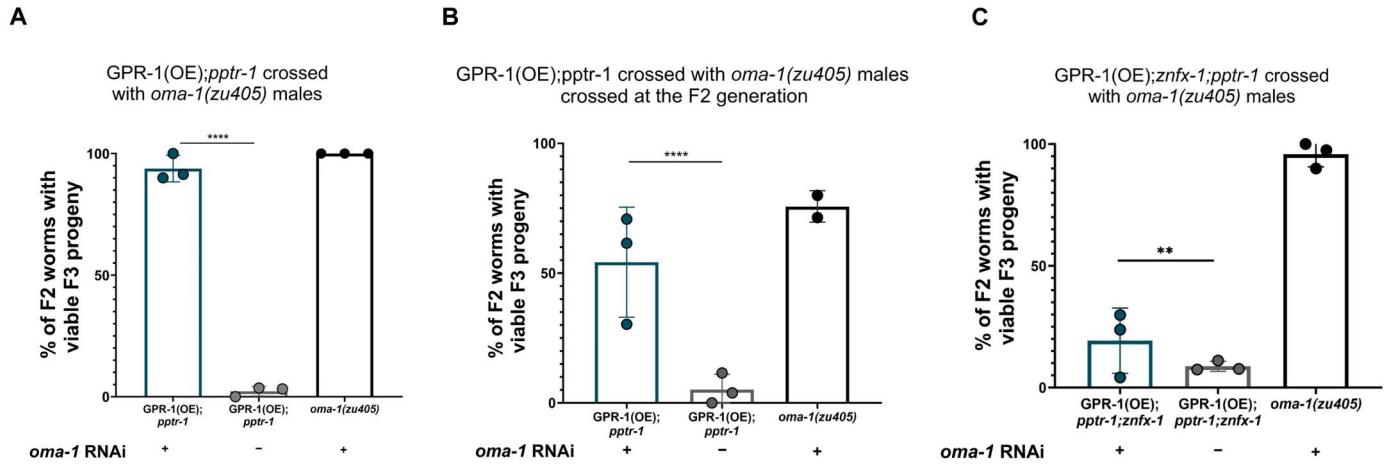

Fig. 4. The involvement of germ granules in nucleus-independent RNAi inheritance. Analysis of nucleus-independent RNAi inheritance in *pptr-1* mutants (**A** and **B**) or *pptr-1;znfx-1* (**C**). Genotypes on the *x* axis refer to the P0, dsRNA-exposed worms. All genotypes were crossed with *oma-1(zu405)* males at the F1 generation (A and C) or F2 generation (B). *oma-1* RNAi (+) worms were exposed to anti–*oma-1* dsRNA or (−) to control EV plasmid. Each dot represents a biological repeat with ~30 individual F2 (A and C) or F3 (B) worms; bars, means ± SD. *oma-1(zu405)* (black dots) are the positive control for the RNAi treatment. Additional controls included *oma-1(zu405)* on EV (none of the F1s hatch), F2s (A and C) or F3s (B) with a maternally derived *oma-1*(+) germline (100% of which had viable progeny). *P* values were determined via Fisher's exact test; ****$P < 0.0001$ indicates inheritance in *pptr-1* mutation background to GPR-1(OE); **$P = 0.0094$ indicates inheritance in *pptr-1;znfx-1* mutation background to GPR-1(OE).

principle components analysis (Fig. 5B). (ii) We expected that accumulation of heritable small RNAs would increase the levels of these RNAs in the worms that inherit them, and the small RNAs that were up-regulated in MY16* compared with MY16 had higher levels in N2 worms than the small RNAs that were down-regulated in MY16* worms compared with MY16 worms (Fig. 5, C and D). (iii) We found that small RNAs that were up-regulated in MY16* were enriched for ZNFX-1 class small RNAs (see Materials and Methods) (fold enrichment of 6.2, $P < 0.0001$) consistent with the results we describe above regarding inheritance of exogenous RNAi responses. Furthermore, we found that the cytoplasmically inherited endo-siRNAs that transmit from the N2 isolate to the MY16 progeny are enriched for siRNAs that target genes that are poorly conserved between *Caenorhabditis* species (fold enrichment of 1.95, $P = 0.0019$).

Which types of RNA molecules are inherited in the germ granules? Unlike 22G siRNAs which can be amplified by RdRPs, mRNAs should get diluted and are therefore not expected to be inherited in large enough quantities to enable information transfer beyond the F1 generation. Still, one might imagine a scenario in which hypothetical "heritable mRNAs" are concentrated in germ granules so that enough parentally transcribed mRNA molecules get deposited in the oocytes and remain functional even after more than one generation (namely, enough remain to serve as templates for RdRP-mediated de novo synthesis of 22G amplified siRNAs that would lead to silencing). To examine this possibility, we analyzed SNPs that differentiate N2 and MY16 animals. Mitochondrial RNA in MY16* contained SNPs that match the N2 genome, as MY16* worms inherited their mitochondria from their N2 grandmothers. In contrast, we found that nuclearly encoded mRNAs in MY16* match the MY16 genome and not the N2 genome. In other words, we could not detect statistically significant levels of N2 nuclear SNPs in RNAs that were inherited by MY16* worms. We reasoned therefore that the majority of the heritable cytoplasmic RNAs are not mRNAs but instead 22G siRNAs which are amplified again and again in every generation based on newly transcribed nuclear mRNA templates (and not based on miniscule amounts of mRNAs that avoid dilution and get inherited via the cytoplasm). This suggests that the heritable agents, the RNA molecules which are inherited in the germline, are siRNAs, which are capable of RdRP-mediated amplification, and not granules-enriched mRNA templates.

DISCUSSION

Perturbing the small RNA pool or the germline chromatin landscape leaves a transgenerational trace in the progeny. Our results suggest that cytoplasmic inheritance of small RNA–mediated silencing can occur independently of any nuclear factors for multiple generations.

Previous studies examined the dependency of different heritable epigenetic effects on the nucleus and reached different conclusions depending on the assay and phenotype tested. For example, starvation-induced effects on gut development were found to be transmitted by nuclear factors, independently of heritable cytoplasmic maternal factors (*27*). Many mutants defective in both cytoplasmic and nuclear factors (both in small RNA and in chromatin genes) display a transgenerational loss of fertility phenotype termed "mortal germline" (Mrt) (*32*, *56*–*63*). Recently, Wahba *et al.* (*28*) suggested that sterility factors that lead to a Mrt phenotype pass exclusively via the cytoplasm. Wahbe *et al.* proposed that these cytoplasmically inherited sterility factors could be small RNAs that regulate ribosomal RNAs. Another study argued that other silencing factors could also have important contributions to the Mrt phenotype (*64*) (it was not directly examined where in the cell these factors localize). A different heritable phenotype, termed mating-induced transgene silencing, was also shown to be nucleus-independent (*26*). Since our results show that siRNAs can transmit

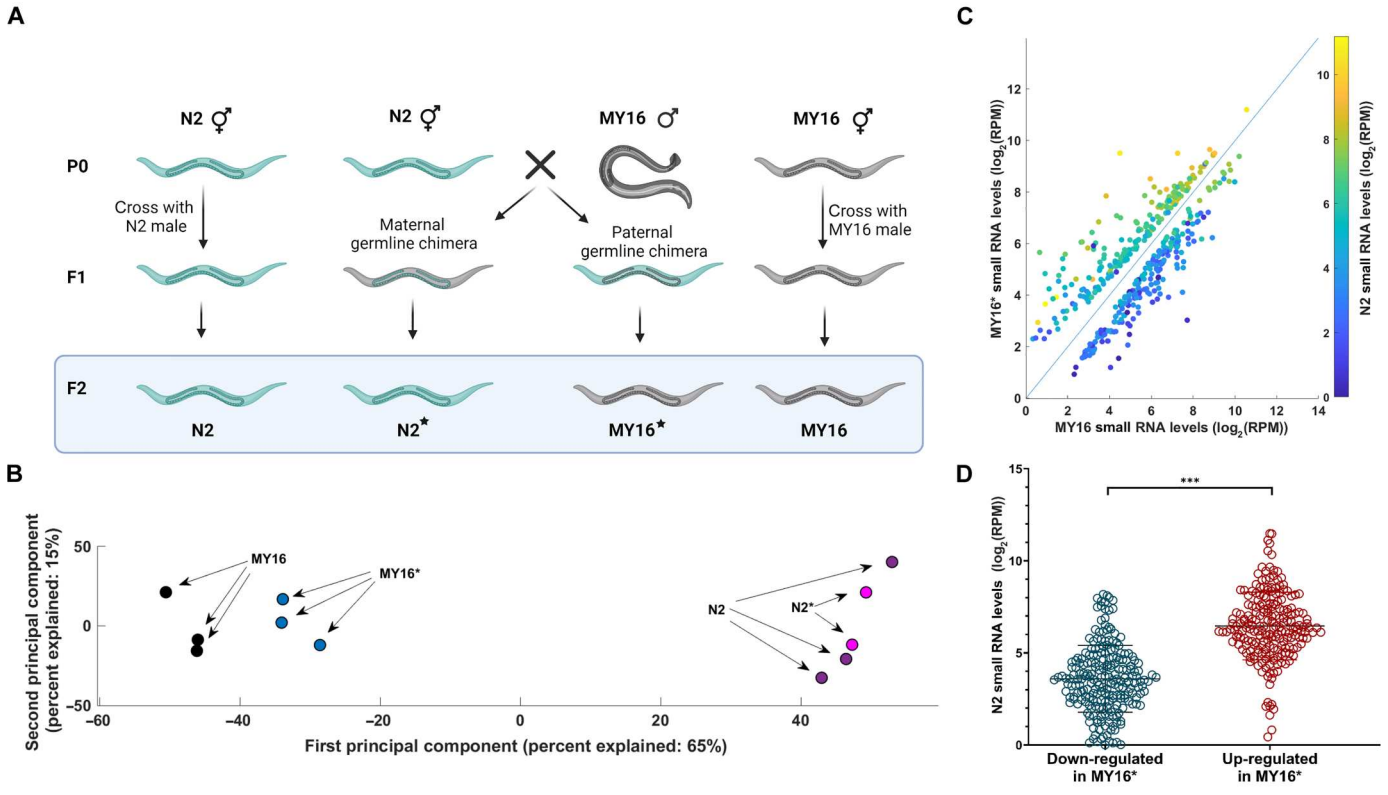

Fig. 5. Endogenous siRNAs are inherited in a nucleus-independent manner. (**A**) A schematic diagram depicting the experimental procedure. P0 N2 worms, overexpressing GPR-1, were crossed to MY16 males (middle) to generate mosaic F1 progeny with either maternal or paternal germlines. F2 progeny coming from F1 with paternal germlines are genotypically MY16 and are named MY16*, while F2 progeny of F1 worms with maternal germlines are genotypically N2 and are named N2*. In addition, P0 N2 worms, overexpressing GPR-1, were crossed to N2 males to ultimately generate F2 N2 (left), and MY16 worms were crossed to MY16 males to generate F2 MY16 worms. Small RNA and mRNA libraries were generated from the F2 generation. (**B**) A principle components analysis (PCA) projection of small RNA targeting protein-coding genes from four conditions. MY16* and MY16 are located distinctly, with MY16* closer to N2 and N2* than MY16 is. The % variance, out of the total original variance in the high-dimensional space, spanned by the first and second PCs is indicated on the *x* and *y* axes, respectively. (**C**) Comparison of small RNA expression levels [$\log_2$ of reads per million (RPM)] of genes that are differentially expressed between MY16 worms (*x* axis) and MY16* worms (*y* axis), with color code indicating the corresponding small RNA levels in N2 worms. (**D**) Comparison of genes that are down-regulated in small RNA reads in MY16* worms relative to MY16 (left) and up-regulated (right). *y* Axis indicates small RNA levels in N2 worms. Genes that are up-regulated in MY16* worms have significantly higher expression of small RNA levels in N2 worms than genes that are down-regulated, suggesting inheritance from P0 N2 to F2 MY16* (Wilcoxon rank sum test, $P = 5.1775 \times 10^{-40}$).

cytoplasmically without the nucleus, it strengthens the possibility that different heritable epigenetic phenotypes, such as Mrt and mating-induced silencing, result from aberrant cytoplasmic inheritance of siRNA molecules.

The germ granules organize the production of heritable small RNAs and are crucial for cytoplasmic RNAi inheritance. Our results show that *znfx-1* mutants are defective in RNAi inheritance via the cytoplasm. However, disrupting proper segregations of the granules in *pptr-1* mutants paradoxically strengthens dsRNA-mediated cytoplasmic RNAi inheritance and enables weak cytoplasmic RNAi inheritance even in *znfx-1* mutants. One possible explanation could be that this potentiation of cytoplasmic inheritance occurs since in *pptr-1* mutants other inherited endogenous small RNAs are not properly synthesized, rendering more small RNA–biogenesis machinery free to produce exogenous siRNAs [as hypothesized in (*34*)]. Competition between different small RNA species has been raised as an explanation for transgenerational effects multiple times in the past (*20*, *22*, *65*, *66*), and an increase in the levels of exogenous small RNAs, coupled with a decrease in the levels of endogenous small RNAs, has been documented in *pptr-1* mutants (*34*).

A recent publication by Schreier *et al.* (*63*) described the epigenetic inheritance of the Mrt phenotype via *C. elegans* males. This inheritance was found to be mediated by the Argonaute protein WAGO-3, which is associated with 22G RNAs and is expressed in sperm cells. In addition, Schreier *et al.* identified and defined the presence of paternal epigenetic inheritance granules, which are specific to sperm cells and get inherited along with WAGO-3. Our sequencing data did not reveal the inheritance of cytoplasmic RNAs from sperm (Fig. 5, A and B, and fig. S2), perhaps because the cytoplasmic content of the sperm is very small relative to the oocytes. It would be interesting in the future to study the pool of paternally inherited endogenous small RNAs.

Small RNA inheritance in *C. elegans* requires amplification of 22G siRNAs using RdRPs. However, small RNAs are not amplified and inherited forever, as it appears that small RNAs cannot themselves serve as templates for amplified small RNA synthesis (*67*). Our analysis of RNA sequencing and SNP data strengthens the hypothesis that heritable small RNAs require de novo synthesis of mRNA templates as neither significant amounts of P0 parental small RNAs nor mRNA templates were found to persist all the

way to the F2 generation. This could be a fundamental limit on the independence of RNA-encoded information from DNA.

"Epigenetic reprogramming" is one of the major theoretical boundaries preventing epigenetic transmission of parental responses across generations (*68*). This process entails the resetting of almost all epigenetic information in the nucleus, including both DNA and histone modifications, presumably so that the next generation can start as a "blank slate." Work in some organisms, notably fish, shows that chromatin reprogramming is not absolutely required (*69*). Studying RNAi-triggered epigenetic inheritance in *C. elegans* is technically easy and thus continues to provide insights into the mechanisms of heredity, including the revelation that gene regulatory responses can transmit via cytoplasmic small RNAs transgenerationally even when the entire nucleus is replaced, regardless of whether the chromatin epigenetic information is modified or "reprogrammed."

MATERIALS AND METHODS

Cultivation of worms

All the experiments were performed at 20°C, except for maintenance of strains containing the *oma-1(zu405)* allele, which was done at 15°C. Worms were cultivated on nematode growth medium (NGM) plates seeded with OP50 bacteria apart from when treated with HT115 bacteria that express dsRNAs for RNAi induction.

These strains were used in this work: PD2218 ccTi1594 [*mex-5p::GFP::gpr-1::smu-1* 3′UTR + *Cbr-unc-119(+)*, III: 680195] III. umnIs7 [*myo-2p::GFP* + NeoR, III:9421936] III, YY996 *znfx-1*(gg561), TX20 *oma-1*(zu405), JH2787 *pptr-1*(tm3103), HC196 *sid-1*(qt9), MY16, N2, and several combinations made by us by crossing strains and validating genotype using polymerase chain reaction and/or Sanger sequencing.

The nematodes were kept well fed and extra care to avoid contamination was taken. Contaminated or starved plates were discarded and not analyzed.

GPR-1(OE) crosses

Hermaphrodites carrying both ccTi1594 and umnIs7 transgenes, containing GPR-1(OE) and a pharyngeal marker, respectively, were crossed to *oma-1(zu405)* or *oma-1(zu405);sid-1(qt9)* males. Non-Mendelian progeny of these crosses were identified using the pharyngeal fluorescent marker to identify F2 chimeric worms containing maternal chromosomes in the AB cell lineage and paternal chromosomes in the P1 cell lineage as described by Artiles *et al.* (*44*). As *C. elegans*′ germline derives entirely from the P1 cell lineage (*70*), the worms′ germline contained the temperature sensitive *oma-1(zu405)* allele. OMA-1 silencing was quantified by scoring the number of worms that lay five or more viable progeny, as previously described (*20*).

RNAi Treatment

HT115 bacteria that transcribe dsRNA targeting *oma-1* or control EV that does not lead to dsRNA transcription and gene silencing were grown in LB with carbenicillin (100 μg/ml). Bacteria were then seeded on NGM plates that contained carbenicillin (25 μg/ml) and isopropyl-β-D-thiogalactopyranoside (1 mM). Worms were put on RNAi plates 24 hours after seeding for two generations. The first generation was put at the L4 stage. Worms were treated on RNAi plates for two consecutive generations. Before transferring worms from RNAi plates to NGM plates, the worms were washed four times in M9 buffer to remove dsRNA-inducing bacteria.

Worm collection and RNA extraction

Hermaphrodites were collected on the first day of adulthood, washed four times in M9 buffer, and collected into an Eppendorf tube before the addition of 4 volumes of TRIzol (Life Technologies) to 1 volume of worm/M9 pellet. To extract RNA, the following protocol was used: three freeze/thaw cycles, −80°C for 30 min followed by 15-min vortex at room temperature. One volume of chloroform was added to 5 volumes of TRIzol/worm/M9 and left in ice for 10 min. The mix was put in a pre-spun 2-ml Heavy Phase Lock Gel tube and centrifuged at 16,000*g* for 5 min at 4°C. The aqueous phase was transferred to a new pre-spun 2-ml Heavy Phase Lock Gel tube, and 1 volume of phenol:chloroform:isoamyl alcohol was added per 1 volume of aqueous phase. The tube was centrifuged at 16,000*g* at room temperature. The aqueous phase was transferred to an Eppendorf tube and precipitated with 1 volume of isopropanol and 1.3 μl of glycogen (20 μg/μl). The tubes were put for 30 min at −20°C before centrifugation at 16,000*g* at 4°C. The pellet was washed with 900 μl of cold 70% ethanol (EtOH) and left at room temperature for 20 min before being left at −20°C overnight. The next morning, the tube was centrifuged at 16,000*g* for 10 min, and the pellet was washed again with cold 70% EtOH. Tubes were centrifuged at 16,000*g* for 10 min, all EtOH was removed and the pellet was resuspended in 12 μl of warm (70°C) ddH_2O. RNA concentration was determined using Qubit and RNA quality was tested using Agilent 2200 TapeStation.

To ensure the capture of small RNA regardless of their 5′ phosphorylation status, 150 to 1000 ng from each sample was treated with RNA 5′ polyphosphatase. Concentrations and quality were assessed using Qubit and Agilent 2200 TapeStation, respectively.

sRNA libraries

The NEBNext Multiplex Small RNA Library Prep Set for Illumina from New England Biolabs was used for small RNA library preparation, following the manufacturer's protocol. The concentration of the samples was determined using Qubit, and their quality was assessed using an Agilent 2200 TapeStation. Subsequently, the samples were pooled and electrophoresed on a 4% agarose E-Gel from Life Technologies. Bands ranging in length from 140 to 160 nt were carefully excised and purified using the MiniElute Gel Extraction Kit (QIAGEN). The purified samples were once again assessed for quality and concentration using the Agilent 2200 TapeStation. Libraries were sequenced on an Illumina NextSeq500 sequencer.

mRNA libraries

The NEBNext Ultra II Directional RNA Library Prep Kit for Illumina from New England Biolabs was used for mRNA library preparation, following the manufacturer's protocol. The concentration of the samples was determined using Qubit, and their quality was assessed using an Agilent 2200 TapeStation. Samples were then pooled together before sequencing on an Illumina NextSeq500 sequencer.

Small RNA seq analysis

The Illumina *.fastq output files were first assessed for quality using FastQC (*71*). The files were then assigned to adapters clipping using Cutadapt (*72*). Next, the clipped reads were aligned against the ce11 version of the *C. elegans* genome using ShortStack (*73*). We counted reads that align in antisense orientation to genes, using the Python-based script HTSeq-count (*74*) and the Ensembl-provided gff file (release-95). We then assigned the summarized counts for differential expression analysis using the R package DESeq2 (*75*) and limited the hits for genes that were shown to have a false discovery rate (FDR) < 0.1.

ZNFX-1-class small RNAs

To generate a list of ZNFX-1–regulated genes, we analyzed available data from Ouyang *et al.* (*35*) as written above. ZNFX-1–regulated genes are genes targeted by small RNAs that were down-regulated in *znfx-1* mutants compared to wild-type N2.

mRNA seq analysis

mRNA libraries were first assessed for quality using the FastQC tool (*71*) and were then aligned to the ce11 version of the genome using HISAT2 (*76*). The aligned reads were then counted using the Python-based script HTSeq-count (*74*) and the Ensembl-provided gff file (release-95). Next, the samples were then compared for differential expression using the R package DESeq2 (*75*). Genes were regarded as differentially expressed if they passed the criterion of FDR < 0.1.

SNP analysis

We first aligned the FASTQ files from small RNA sequencing of N2 and MY16 isolates to the ce11 reference sequence while allowing up to two mismatches. We pooled the aligned reads of each strain and calculated the frequency of each of the four nucleotides in each covered position. We only included reads with a length of >20 nt, which are uniquely aligned to the genome.

For a given genomic position, we defined the occurrence of SNP if (i) it was covered by at least 5 reads per million in each isolate, (ii) it was dominated (frequency, >0.95) by a specific nucleotide in each isolate, and (iii) the identity of the dominant nucleotide differs between N2 and MY16. We further compared the SNPs defined by us to the SNPs reported in CeNDR (Caenorhabditis elegans Natural Diversity Resource) (*55*) and found that more than 80% of the SNPs detected by us also appear in the CeNDR database.

Next, we aligned the FASTQ files from small RNA sequencing of MY16* to the ce11 reference genome under the same conditions and looked for cases in which the identity of the nucleotide in each given position, in at least 5% of the occurrences, deviates from that of the MY16 isolate, but identical to that of the N2 strain. To avoid the possible effect of sequencing error and other biases, we further demand that the representations of the N2-like nucleotide among the nucleotides covering a given position in the MY16* isolate will be higher by at least 1.5-fold from the representations of the N2-like nucleotide observed for the MY16 one. Last, to evaluate the significance of our results, we ran a similar analysis, in which the definition of SNPs was determined using the N2 and MY16* strains while looking for cases in which the identity of the MY16′s nucleotide at a given position follows that of N2 and deviates from the MY16*.

Statistical analyses

Statistical analyses and generation of graphs were performed using GraphPad Prism, R v4.0.0, and RNAlysis (*77*). Statistical details of experiments appear in figure legends. For viable progeny analysis, we used Fisher's exact test, with the two-stage step-up method of Benjamini, Krieger, and Yekutiely correction for multiple comparisons.

Supplementary Materials

This PDF file includes:

Figs. S1 and S2

REFERENCES AND NOTES

1. W. N. Parker, *The Germ-Plasm: A Theory of Heredity, by August Weismann. Translated by W. Newton Parker and Harriet. Rönnfeldt* (Charles Scribner, 1893).
2. F. B. Churchill, From heredity theory to Vererbung: The transmission problem, 1850-1915. *Isis* **78**, 337–364 (1987).
3. K. Rogers, *The Cell* (Britannica Educational Publishing, ed. 2014, 2014).
4. P. J. Bowler, J. Browne, Charles Darwin: The power of place. *Arch. Nat. Hist.* **30**, 362–363 (2003).
5. Y. Liu, A new perspective on Darwin's pangenesis. *Biol. Rev.* **83**, 141–149 (2008).
6. Y. Liu, *In Search of Darwin's Imaginary Gemmules* (Elsevier Ltd, 2018), vol. 101.
7. E. V. A. Jablonka, G. A. L. Raz, Transgenerational epigenetic inheritance: Prevalence, mechanisms, and implications for the study of heredity and evolution. *Q. Rev. Biol.* **84**, 131–176 (2009).
8. E. J. Radford, Exploring the extent and scope of epigenetic inheritance. *Nat. Rev. Endocrinol.* **14**, 345–355 (2018).
9. A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, C. C. Mello, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811 (1998).
10. X. Chen, O. Rechavi, Plant and animal small RNA communications between cells and organisms. *Nat. Rev. Mol. Cell Biol.* **23**, 185–203 (2022).
11. O. Rechavi, G. Minevich, O. Hobert, Transgenerational inheritance of an acquired small RNA-based antiviral response in *C. elegans*. *Cell* **147**, 1248–1256 (2011).
12. O. Rechavi, L. Houri-Ze'evi, S. Anava, W. S. S. Goh, S. Y. Kerk, G. J. Hannon, O. Hobert, Starvation-induced transgenerational inheritance of small RNAs in *C. elegans*. *Cell* **158**, 277–287 (2014).
13. S. Anava, R. Posner, O. Rechavi, The soft genome. *Worm* **3**, e989798 (2014).
14. J. Z. Ni, N. Kalinava, E. Chen, A. Huang, T. Trinh, S. G. Gu, A transgenerational role of the germline nuclear RNAi pathway in repressing heat stress-induced transcriptional activation in *C. elegans*. *Epigenetics Chromatin* **9**, 3 (2016).
15. A. Grishok, H. Tabara, C. C. Mello, Genetic requirements for inheritance of RNAi in *C. elegans*. *Science* **287**, 2494–2497 (2000).
16. R. M. Alcazar, R. Lin, A. Z. Fire, Transmission dynamics of heritable silencing induced by double-stranded RNA in *Caenorhabditis elegans*. *Genetics* **180**, 1275–1288 (2008).
17. W. Gu, M. Shirayama, D. Conte Jr., J. Vasale, P. J. Batista, J. M. Claycomb, J. J. Moresco, E. M. Youngman, J. Keys, M. J. Stoltz, C.-C. G. Chen, D. A. Chaves, S. Duan, K. D. Kasschau, N. Fahlgren, J. R. Yates III, S. Mitani, J. C. Carrington, C. C. Mello, Distinct Argonaute-mediated 22G-RNA pathways direct genome surveillance in the *C. elegans* germline. *Mol. Cell* **36**, 231–244 (2009).
18. B. A. Buckley, K. B. Burkhart, S. G. Gu, G. Spracklin, A. Kershner, H. Fritz, J. Kimble, A. Fire, S. Kennedy, A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. *Nature* **489**, 447–451 (2012).
19. A. Grishok, *Biology and Mechanisms of Short RNAs in Caenorhabditis elegans* (Elsevier Inc., ed. 1, 2013), vol. 83.
20. L. Houri-Ze'evi, Y. Korem, H. Sheftel, L. Faigenbloom, I. A. Toker, Y. Dagan, L. Awad, L. Degani, U. Alon, O. Rechavi, A tunable mechanism determines the duration of the transgenerational small RNA inheritance in *C. elegans*. *Cell* **165**, 88–99 (2016).
21. G. Wan, B. D. Fields, G. Spracklin, A. Shukla, C. M. Phillips, S. Kennedy, Spatiotemporal regulation of liquid-like condensates in epigenetic inheritance. *Nature* **557**, 679–683 (2018).
22. L. Houri-Zeevi, Y. Korem Kohanim, O. Antonova, O. Rechavi, Three rules explain transgenerational small RNA inheritance in C. elegans. *Cell* **182**, 1186–1197.e12 (2020).

23. T. Sijen, J. Fleenor, F. Simmer, K. L. Thijssen, S. Parrish, L. Timmons, R. H. Plastrek, A. Fire, On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* **107**, 465–476 (2001).
24. M. A. Preston, D. F. Porter, F. Chen, N. Buter, C. P. Lapointe, S. Keles, J. Kimble, M. Wickens, Unbiased screen of RNA tailing activities reveals a poly(UG) polymerase. *Nat. Methods* **16**, 437–445 (2019).
25. A. Shukla, J. Yan, D. J. Pagano, A. E. Dodson, Y. Fei, J. Gorham, J. G. Seidman, M. Wickens, S. Kennedy, poly(UG)-tailed RNAs in genome protection and epigenetic inheritance. *Nature* **582**, 283–288 (2020).
26. S. Devanapally, P. Raman, M. Chey, S. Allgood, F. Ettefa, M. Diop, Y. Lin, Y. E. Cho, A. M. Jose, Mating can initiate stable RNA silencing that overcomes epigenetic recovery. *Nat. Commun.* **12**, 4239 (2021).
27. C. K. Ewe, Y. N. Torres Cleuren, S. E. Flowers, G. Alok, R. G. Snell, J. H. Rothman, Natural cryptic variation in epigenetic modulation of an embryonic gene regulatory network. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 13637–13646 (2020).
28. L. Wahba, L. Hansen, A. Z. Fire, An essential role for the piRNA pathway in regulating the ribosomal RNA pool in *C. elegans*. *Dev. Cell* **56**, 2295–2312.e6 (2021).
29. A. Ashe, A. Sapetschnig, E. M. Weick, J. Mitchell, M. P. Bagijn, A. C. Cording, A. L. Doebley, L. D. Goldstein, N. J. Lehrbach, J. Le Pen, G. Pintacuda, A. Sakaguchi, P. Sarkies, S. Ahmed, E. A. Miska, PiRNAs can trigger a multigenerational epigenetic memory in the germline of *C. elegans*. *Cell* **150**, 88–99 (2012).
30. M. Shirayama, M. Seth, H.-C. Lee, W. Gu, T. Ishidate, D. Conte, C. C. Mello, PiRNAs initiate an epigenetic memory of nonself RNA in the *C. elegans* germline. *Cell* **150**, 65–77 (2012).
31. M. J. Luteijn, P. Van Bergeijk, L. J. T. Kaaij, M. V. Almeida, E. F. Roovers, E. Berezikov, R. F. Ketting, Extremely stable Piwi-induced gene silencing in *Caenorhabditis elegans*. *EMBO J.* **31**, 3422–3430 (2012).
32. F. Xu, X. Feng, X. Chen, C. Weng, Q. Yan, T. Xu, M. Hong, S. Guang, A cytoplasmic argonaute protein promotes the inheritance of RNAi. *Cell Rep.* **23**, 2482–2494 (2018).
33. Y.-H. Ding, H. J. Ochoa, T. Ishidate, M. Shirayama, C. C. Mello, The nuclear Argonaute HRDE-1 directs target gene re-localization and shuttles to nuage to promote small RNA-mediated inherited silencing. *Cell Rep.* **42**, 112408 (2023).
34. I. Lev, I. A. Toker, Y. Mor, A. Nitzan, G. Weintraub, O. Antonova, O. Bhonkar, I. Ben Shushan, U. Seroussi, J. M. Claycomb, S. Anava, H. Gingold, R. Zaidel-Bar, O. Rechavi, Germ granules govern small RNA inheritance. *Curr. Biol.* **29**, 2880–2891.e4 (2019).
35. J. P. T. Ouyang, W. L. Zhang, G. Seydoux, The conserved helicase ZNFX-1 memorializes silenced RNAs in perinuclear condensates. *Nat. Cell Biol.* **24**, 1129–1140 (2022).
36. S. E. Castel, R. A. Martienssen, RNA interference in the nucleus: Roles for small RNAs in transcription, epigenetics and beyond. *Nat. Rev. Genet.* **14**, 100–112 (2013).
37. S. I. S. Grewal, The molecular basis of heterochromatin assembly and epigenetic inheritance. *Mol. Cell* **83**, 1767–1785 (2023).
38. N. O. Burton, K. B. Burkhart, S. Kennedy, Nuclear RNAi maintains heritable gene silencing in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 19683–19688 (2011).
39. S. G. Gu, J. Pak, S. Guang, J. M. Maniar, S. Kennedy, A. Fire, Amplification of siRNA in *Caenorhabditis elegans* generates a transgenerational sequence-targeted histone H3 lysine 9 methylation footprint. *Nat. Genet.* **44**, 157–164 (2012).
40. H. Mao, C. Zhu, D. Zong, C. Weng, X. Yang, H. Huang, D. Liu, X. Feng, S. Guang, The Nrde pathway mediates small-RNA-directed histone H3 lysine 27 trimethylation in *Caenorhabditis elegans*. *Curr. Biol.* **25**, 2398–2403 (2015).
41. I. Lev, U. Seroussi, H. Gingold, R. Bril, S. Anava, O. Rechavi, MET-2-dependent H3K9 methylation suppresses transgenerational small RNA inheritance. *Curr. Biol.* **27**, 1138–1147 (2017).
42. I. Lev, H. Gingold, O. Rechavi, H3K9me3 is required for inheritance of small RNAs that target a unique subset of newly evolved genes. *eLife* **8**, e40448 (2019).
43. J. Besseling, H. Bringmann, Engineered non-Mendelian inheritance of entire parental genomes in *C. elegans*. *Nat. Biotechnol.* **34**, 982–986 (2016).
44. K. L. Artiles, A. Z. Fire, C. Frøkjær-Jensen, Assessment and maintenance of unigametic germline inheritance for *C. elegans*. *Dev. Cell* **48**, 827–839 (2019).
45. M. R. Detwiler, M. Reuben, X. Li, E. Rogers, R. Lin, Two zinc finger proteins, OMA-1 and OMA-2, are redundantly required for oocyte maturation in *C. elegans*. *Dev. Cell* **1**, 187–199 (2001).
46. R. Lin, A gain-of-function mutation in oma-1, a *C. elegans* gene required for oocyte maturation, results in delayed degradation of maternal proteins and embryonic lethality. *Dev. Biol.* **258**, 226–239 (2003).
47. J. S. Whangbo, C. P. Hunter, Environmental RNA interference. *Trends Genet.* **24**, 297–305 (2008).
48. W. M. Winston, C. Molodowitch, C. P. Hunter, D. Rna-mediated, Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. 295, 2456–2459 (2002).
49. T. Ishidate, A. R. Ozturk, D. J. Durning, R. Sharma, E. Shen, H. Chen, M. Seth, M. Shirayama, C. C. Mello, ZNFX-1 functions within perinuclear nuage to balance epigenetic signals. *Mol. Cell* **70**, 639–649.e6 (2018).
50. S. Strome, W. B. Wood, Generation of asymmetry and segregation of germ-line granules in early *C. elegans* embryos. *Cell* **35**, 15–25 (1983).
51. I. Lev, O. Rechavi, Germ granules allow transmission of small RNA-based parental responses in the "Germ Plasm". *iScience* **23**, 101831 (2020).
52. A. E. Sundby, R. I. Molnar, J. M. Claycomb, Connecting the dots: Linking *Caenorhabditis elegans* small RNA pathways and germ granules. *Trends Cell Biol.* **31**, 387–401 (2021).
53. A. E. Dodson, S. Kennedy, Germ granules coordinate RNA-based epigenetic inheritance pathways. *Dev. Cell* **50**, 704–715.e4 (2019).
54. C. M. Gallo, J. T. Wang, M. Fumio, G. Seydoux, Cytoplasmic partitioning of p granule components is not required to specify the germline in *C. elegans*. *Science* **330**, 1685–1689 (2010).
55. D. E. Cook, S. Zdraljevic, J. P. Roberts, E. C. Andersen, CeNDR, the *Caenorhabditis elegans* natural diversity resource. *Nucleic Acids Res.* **45**, D650–D657 (2017).
56. S. Ahmed, J. Hodgkin, MRT-2 checkpoint protein is required for germline immortality and telomere replication in *C. elegans*. *Nature* **403**, 159–164 (2000).
57. P. J. Batista, J. G. Ruby, J. M. Claycomb, R. Chiang, N. Fahlgren, K. D. Kasschau, D. A. Chaves, W. Gu, J. J. Vasale, S. Duan, D. Conte, S. Luo, G. P. Schroth, J. C. Carrington, D. P. Bartel, C. C. Mello, PRG-1 and 21U-RNAs interact to form the piRNA complex required for fertility in C. elegans. *Mol. Cell* **31**, 67–78 (2008).
58. D. J. Katz, T. M. Edwards, V. Reinke, W. G. Kelly, A *C. elegans* LSD1 demethylase contributes to germline immortality by reprogramming epigenetic memory. *Cell* **137**, 308–320 (2009).
59. C. Zhang, T. A. Montgomery, H. W. Gabel, S. E. J. Fischer, C. M. Phillips, N. Fahlgren, C. M. Sullivan, J. C. Carrington, G. Ruvkun, mut-16 and other mutator class genes modulate 22G and 26G siRNA pathways in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 1201–1208 (2011).
60. C. C. Conine, J. J. Moresco, W. Gu, M. Shirayama, D. Conte, J. R. Yates, C. C. Mello, Argonautes promote male fertility and provide a paternal memory of germline gene expression in *C. elegans*. *Cell* **155**, 1532–1544 (2013).
61. M. Simon, P. Sarkies, K. Ikegami, A. L. Doebley, L. D. Goldstein, J. Mitchell, A. Sakaguchi, E. A. Miska, S. Ahmed, Reduced insulin/IGF-1 signaling restores germ cell immortality to *Caenorhabditis elegans* piwi mutants. *Cell Rep.* **7**, 762–773 (2014).
62. K. I. Manage, A. K. Rogers, D. C. Wallis, C. J. Uebel, D. C. Anderson, D. A. H. Nguyen, K. Arca, K. C. Brown, R. J. C. Rodrigues, B. F. M. de Albuquerque, R. F. Ketting, T. A. Montgomery, C. M. Phillips, A tudor domain protein, SIMR-1, promotes sirna production at pirna-targeted mrnas in *C. elegans*. *eLife* **9**, 1–33 (2020).
63. J. Schreier, S. Dietz, M. Boermel, V. Oorschot, A. S. Seistrup, A. M. de Jesus Domingues, A. W. Bronkhorst, D. A. H. Nguyen, S. Phillis, E. J. Gleason, S. W. L'Hernault, C. M. Phillips, F. Butter, R. F. Ketting, Membrane-associated cytoplasmic granules carrying the Argonaute protein WAGO-3 enable paternal epigenetic inheritance in *Caenorhabditis elegans*. *Nat. Cell Biol.* **24**, 217–229 (2022).
64. B. E. Montgomery, T. Vijayasarathy, T. N. Marks, C. A. Cialek, K. J. Reed, T. A. Montgomery, Dual roles for piRNAs in promoting and preventing gene silencing in *C. elegans*. *Cell Rep.* **37**, 110101 (2021).
65. J. J. Zhuang, C. P. Hunter, The influence of competition among *C. elegans* small RNA pathways on development. *Genes.* **3**, 671–685 (2012).
66. P. Sarkies, A. Ashe, J. Le Pen, M. A. McKie, E. A. Miska, Competition between virus-derived and endogenous small RNAs regulates gene expression in *Caenorhabditis elegans*. *Genome Res.* **23**, 1258–1270 (2013).
67. J. Pak, J. M. Maniar, C. C. Mello, A. Fire, Protection from feed-forward amplification in an amplified RNAi mechanism. *Cell* **151**, 885–899 (2012).
68. E. Heard, R. A. Martienssen, Transgenerational epigenetic inheritance: Myths and mechannisms. *Change* **29**, 997–1003 (2012).
69. K. Skvortsova, K. Tarbashevich, M. Stehling, R. Lister, M. Irimia, E. Raz, O. Bogdanovic, Retention of paternal DNA methylome in the developing zebrafish germline. *Nat. Commun.* **10**, 3054 (2019).
70. J. E. Sulston, E. Schierenberg, J. G. White, J. N. Thomson, The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100**, 64–119 (1983).
71. S. Andrews, *FastQC: A Quality Control tool for High Throughput Sequence Data* (Babraham Bioinformatics, 2010); www.bioinformatics.babraham.ac.uk/projects/fastqc/.
72. M. Martin, Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. J.* **17**, 10–12 (2011).
73. S. Shahid, M. J. Axtell, Identification and annotation of small RNA genes using ShortStack. *Methods* **67**, 20–27 (2014).
74. S. Anders, P. T. Pyl, W. Huber, HTSeq-A Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169 (2015).
75. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
76. D. Kim, B. Langmead, S. L. Salzberg, HISAT: A fast spliced aligner with low memory requirements. *Nat. Methods* **12**, 357–360 (2015).

77. G. Teichman, D. Cohen, O. Ganon, N. Dunsky, S. Shani, H. Gingold, O. Rechavi, RNAlysis: Analyze your RNA sequencing data without writing a single line of code. *BMC Biol.* **21**, 74 (2023).

Acknowledgments: We thank all the Rechavi and Shaham laboratory members for their helpful comments and fruitful discussions. We thank P. Cherian and O. Wurtzel for assistance. We are grateful to Itai A. Toker for comments and suggestions. Some strains were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). **Funding:** I.R. is supported partly by a fellowship from the Prajs-Drimmer Institute. O.R. is grateful for funding from the Eric and Wendy Schmidt Fund for Strategic Innovation (Polymath Award #0140001000), the Khan Foundation (grant #0604918421), the DFG (grant #0604918111), and the John Templeton Foundation (grant #0604916631). **Author contributions:** I.R. contributed to Figs. 1 to 5 and figs. S1 and S2. G.W. contributed to Fig. 1 and formulated the project. K.G. contributed to Fig. 3. D.B.-Z. contributed to Fig. 5. S.A. contributed to Fig. 5 and figs. S1 and S2. H.G. contributed to Fig. 5 and figs. S1 and S2. O.R., S.S., and I.L. cosupervised the project. I.R., G.W., S.A., H.G., I.L., S.S., and O.R. interpreted results and wrote the paper. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** The raw sequencing files and processed data generated during this study are available under GEO accession number GSE243434. All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Submitted 20 July 2023
Accepted 20 September 2023
Published 25 October 2023
10.1126/sciadv.adj8618

ScienceAdvances

Nucleus-independent transgenerational small RNA inheritance in *Caenorhabditis elegans*

Itai Rieger, Guy Weintraub, Itamar Lev, Kesem Goldstein, Dana Bar-Zvi, Sarit Anava, Hila Gingold, Shai Shaham, and Oded Rechavi

Sci. Adv. **9** (43), eadj8618. DOI: 10.1126/sciadv.adj8618

Science Advances (ISSN 2375-2548) is published by the American Association for the Advancement of Science. 1200 New York Avenue NW, Washington, DC 20005. The title *Science Advances* is a registered trademark of AAAS.