Epigenetic information is frequently erased near the start of each new generation\(^1\). In some cases, however, epigenetic information can be transmitted from parent to progeny (multigenerational epigenetic inheritance)\(^2\). A particularly notable example of this type of epigenetic inheritance is double-stranded RNA-mediated gene silencing in *Caenorhabditis elegans*. This RNA-mediated interference (RNAi) can be inherited for more than five generations\(^3\). To understand this process, here we conduct a genetic screen for nematodes defective in transmitting RNAi silencing signals to future generations. This screen identified the *heritable RNAi defective 1* (*hrde-1*) gene. *hrde-1* encodes a gene when treated directly with *gfp* double-stranded RNA, but were defective for silencing inheritance (Supplementary Fig. 5). We found that *hrde-1* is *c16c10.3* (Supplementary Fig. 5), and a member of the worm-specific clade of AGO (WAGO) family\(^6\). HRDE-1 seemed to be relatively unique among the WAGO proteins in its contribution to germline RNAi inheritance (Supplementary Fig. 6). We constructed a fusion gene between *gfp* and a full-length genomic copy of *hrde-1* (*gfp::hrde-1*). *gfp::hrde-1* rescued RNAi inheritance in *hrde-1*\(^{-/-}\) animals, indicating that GFP–HRDE-1 is functional (Supplementary Fig. 5). GFP–HRDE-1 was expressed in the nuclei of male and female germ cells (Fig. 1b and Supplementary Fig. 7). These data indicate that *hrde-1* encodes a germline AGO that localizes to the nuclei.

**Figure 1** *hrde-1* encodes a nuclear AGO protein that acts in inheriting generations to promote multigenerational germline RNAi inheritance. a, *pie-1::gfp::h2b* expressing animals were exposed to *gfp* dsRNA. *F*\(_1\) progeny were grown in the absence of dsRNA, and GFP expression in oocytes was visualized by fluorescence microscopy. The percentage of fluorescent animals is indicated. *rde-1* is required for RNAi\(^17\). *P*\(_0\), parental generation; WT, wild-type (*n > 100*). b, GFP–HRDE-1 was visualized by fluorescence microscopy. Small arrow, *L*\(_1\) animal; inset, magnifications showing GFP–HRDE-1 in primordial germ cells. c, 3×Flag–HRDE-1 was immunoprecipitated with an anti-Flag antibody and co-precipitating siRNAs were quantified with an *oma-1* TaqMan probe set. Data are expressed as fold change (±: *oma-1* RNAi). \(1^\prime\) denotes no change, and the mean and s.d. are shown (*n = 3*). d, *pie-1::gfp::h2b* fluorescence was scored in *hrde-1*\(^{-/-}\) and *hrde-1*\(^{+/-}\) (scored as one group) or in *hrde-1*\(^{-/-}\) inheriting progeny. The *hrde-1*\(^{-/-}\) chromosome was marked with *unc-93* (*unc-93* is ~1.3 cm from *hrde-1*), and *hrde-1* genotypes were inferred by Unc phenotypes. The percentage of fluorescent animals is indicated. Original magnification for all images, ×630.

**LETTER**

*A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality*

Bethany A. Buckley\(^1\), Kirk B. Burkhart\(^1\), Sam Guoping Gu\(^2\), George Spracklin\(^1\), Aaron Kershen\(^1\), Heidi Fritz\(^1\), Judith Kimble\(^1,3\), Andrew Fire\(^5\) & Scott Kennedy\(^3\)

Epigenetic information is frequently erased near the start of each new generation\(^1\). In some cases, however, epigenetic information can be transmitted from parent to progeny (multigenerational epigenetic inheritance)\(^2\). A particularly notable example of this type of epigenetic inheritance is double-stranded RNA-mediated gene silencing in *Caenorhabditis elegans*. This RNA-mediated interference (RNAi) can be inherited for more than five generations\(^3\). To understand this process, here we conduct a genetic screen for nematodes defective in transmitting RNAi silencing signals to future generations. This screen identified the *heritable RNAi defective 1* (*hrde-1*) gene. *hrde-1* encodes a gene when treated directly with *gfp* double-stranded RNA, but were defective for silencing inheritance (Supplementary Fig. 5). We found that *hrde-1* is *c16c10.3* (Supplementary Fig. 5), and a member of the worm-specific clade of AGO (WAGO) family\(^6\). HRDE-1 seemed to be relatively unique among the WAGO proteins in its contribution to germline RNAi inheritance (Supplementary Fig. 6). We constructed a fusion gene between *gfp* and a full-length genomic copy of *hrde-1* (*gfp::hrde-1*). *gfp::hrde-1* rescued RNAi inheritance in *hrde-1*\(^{-/-}\) animals, indicating that GFP–HRDE-1 is functional (Supplementary Fig. 5). GFP–HRDE-1 was expressed in the nuclei of male and female germ cells (Fig. 1b and Supplementary Fig. 7). These data indicate that *hrde-1* encodes a germline AGO that localizes to the nuclei.

**Figure 1** *hrde-1* encodes a nuclear AGO protein that acts in inheriting generations to promote multigenerational germline RNAi inheritance. a, *pie-1::gfp::h2b* expressing animals were exposed to *gfp* dsRNA. *F*\(_1\) progeny were grown in the absence of dsRNA, and GFP expression in oocytes was visualized by fluorescence microscopy. The percentage of fluorescent animals is indicated. *rde-1* is required for RNAi\(^17\). *P*\(_0\), parental generation; WT, wild-type (*n > 100*). b, GFP–HRDE-1 was visualized by fluorescence microscopy. Small arrow, *L*\(_1\) animal; inset, magnifications showing GFP–HRDE-1 in primordial germ cells. c, 3×Flag–HRDE-1 was immunoprecipitated with an anti-Flag antibody and co-precipitating siRNAs were quantified with an *oma-1* TaqMan probe set. Data are expressed as fold change (±: *oma-1* RNAi). \(1^\prime\) denotes no change, and the mean and s.d. are shown (*n = 3*). d, *pie-1::gfp::h2b* fluorescence was scored in *hrde-1*\(^{-/-}\) and *hrde-1*\(^{+/-}\) (scored as one group) or in *hrde-1*\(^{-/-}\) inheriting progeny. The *hrde-1*\(^{-/-}\) chromosome was marked with *unc-93* (*unc-93* is ~1.3 cm from *hrde-1*), and *hrde-1* genotypes were inferred by Unc phenotypes. The percentage of fluorescent animals is indicated. Original magnification for all images, ×630.

1Laboratory of Genetics, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA. 2Departments of Pathology and Genetics, Stanford University, Stanford, California 94305, USA. 3Howard Hughes Medical Institute, and Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA. *These authors contributed equally to this work.*
HRDE-1 could conceivably promote multigenerational RNAi inheritance by acting in animals directly exposed to dsRNA (the RNAi generation) or in the progeny of these animals (the inheriting generation). In *C. elegans*, dsRNA exposure induces the expression of short interfering RNAs (siRNAs) in inheriting generations (refs 7, 8 and Supplementary Fig. 8). HRDE-1 co-precipitated with siRNAs for several generations after exposure to RNAi, consistent with the idea that HRDE-1 acts in inheriting generations to promote RNAi inheritance (Fig. 1c). Note, the maintenance of HRDE-1-associating siRNA populations over generations is probably mediated by RNA-dependent RNA polymerases (RdRs) (see Supplementary Discussion).

The following genetic analyses confirmed that HRDE-1 acts in inheriting generations. Animals that were *hrde-1*+/− in the parental (P0) RNAi generation, but were *hrde-1*−/− in the F1 inheriting generation, failed to inherit RNAi silencing (Fig. 1d and Supplementary Table 1). Similarly, HRDE-1 activity was required in the F2 generation for F1-to-F2 RNAi inheritance, and in the F3 generation for F2-to-F3 RNAI inheritance (Fig. 1d). Conversely, animals that lacked HRDE-1 in the RNAi generation, but expressed HRDE-1 in the inheriting generation, were able to inherit RNAi silencing (Supplementary Table 1). Thus, HRDE-1 acts in inheriting progeny to facilitate the memory of RNAi silencing events that occurred in previous generations. Altogether, these data establish that *C. elegans* possess machinery dedicated to propagating epigenetic information across generational boundaries.

The nuclear RNAi defective factors 1–4 (NRDE-1–4) comprise a sub-branch of the *C. elegans* RNAi silencing machinery that is required for dsRNA-based silencing of nuclear-localized RNAs11–13. According to our current model, siRNAs bound to the somatically expressed AGO NRDE-3 recognize and bind nascent RNA transcripts and recruit NRDE-1, -2 and -4 (termed downstream NRDE factors) to genomic sites of RNAi in somatic cells. Together, the NRDE factors direct nuclear gene-silencing events, which include the deposition of the repressive chromatin mark H3K9me3, and the inhibition of RNA polymerase II elongation11–13. The NRDE factors contribute to heritable gene silencing events that are manifest in somatic cells12. Five lines of evidence indicate that HRDE-1 engages the downstream NRDE factors to direct nuclear RNAi, and, consequently, RNAi inheritance in germ cells. First, the downstream NRDE factors were required for *gfp* and *pos-1* germline RNAi inheritance (Fig. 2a and Supplementary Fig. 6). Second, like HRDE-1, the downstream NRDE factor NRDE-2 acted in inheriting generations to promote the memory of RNAi in germ cells (Fig. 2b). Third, HRDE-1 was required for RNAi-mediated recruitment of NRDE-2 to a germline pre-messenger RNA, indicating that HRDE-1 acts as a specificity factor in germ cells to recruit a downstream NRDE factor to genomic sites of RNAi (Fig. 2c). Fourth, the ability of dsRNA to induce H3K9me3 was lost in mutant strains that eliminate *hrde-1* or the downstream NRDE factors (Fig. 2d, Supplementary Fig. 10 and Supplementary Discussion). Fifth, consistent with the idea that *hrde-1* and the downstream NRDE factors act together in the germ line, *hrde-1*−/− animals share a germline mortality phenotype with *nrde-12/4−/−* animals (see later). These data indicate that NRDE-1, -2 and -4 are required for multigenerational RNAi inheritance, and support a model in which HRDE-1 and NRDE-1, -2 and -4 comprise a germline RNAi pathway that drives RNAi inheritance by inducing gene silencing in the nuclei of inheriting progeny. Henceforth, we refer to HRDE-1 and NRDE-1, -2 and -4 as the germline RNAi inheritance machinery.

We asked whether, under normal reproductive conditions, the germline RNAi inheritance machinery transmits endogenous RNAi silencing signals across generations. To test this idea, we first used H3K9me3 as a read-out for endogenous nuclear RNAi in germ cells. We isolated wild-type or *hrde-1*−/− animals, conducted H3K9me3 chromatin immunoprecipitation (ChIP), and subjected H3K9me3 co-precipitating nucleosome core DNA to high-throughput sequencing. We identified 320 predicted genes that were depleted for H3K9me3 in the F1 inheriting generation, but were H3K9me3-dependent sites (Fig. 3a, Supplementary Table 2). These data indicate that NRDE-1, -2 and -4 are required for RNAi inheritance in germ cells. First, the downstream NRDE factors were recruited NRDE-1, -2 and -4 (termed downstream NRDE factors) to genomic sites depleted for H3K9me3 in inheriting generations (refs 7, 8 and Supplementary Fig. 13). Third, we observed increased animals relative to wild type (Fig. 3a and Supplementary Table 2). H3K9me3 ChIP, followed by directed quantitative real-time PCR (qRT-PCR) analysis, confirmed the dependence of H3K9me3 on the nuclear RNAi Machinery (Fig. 3d and Supplementary Fig. 13). Our results demonstrate that HRDE-1 engagement of the NRDE nuclear RNAi pathway to direct multigenerational RNAi inheritance is lost in germ cells that lack most germ cells, which lack most germ cells, H3K9me3 was significantly depleted at four out of four of these loci (data not shown). NRDE-dependent H3K9me3 was present in germ cells; in temperature-sensitive *gfp* mutants14, which lack most germ cells, H3K9me3 was significantly reduced at most *nrde-12/4−/−* dependent sites (Fig. 3a, Supplementary Table 2). Together, these data show that *nrde-2* and *nrde-4* contribute to H3K9me3 at several loci in germ cells. Henceforth, we refer to these loci as the endogenous NRDE germline target genes.

HRDE-1 co-precipitated with endogenous small RNAs (Fig. 3b). We sequenced these small RNAs and found that HRDE-1 bound endogenous 22G-siRNAs (22 nucleotides in length with a 5′ guanosine residue), which were expressed in germ cells, and were antisense to ~1,500 predicted coding genes (Supplementary Table 2 and Supplementary Fig. 11). HRDE-1 22G-siRNAs also targeted pseudogenes and cryptic loci (Supplementary Table 2). 22G-siRNAs are synthesized by RdRPs acting on cellular RNA templates10,13, suggesting that the HRDE-1 22G-siRNAs are synthesized by RdRP activity in germ cells (see Supplementary Discussion). Three lines of evidence link HRDE-1 to the regulation of gene expression at the RNAi germline target genes. First, we observed a correlation between genomic sites homologous to the most abundant (top 200) HRDE-1-bound siRNAs and genomic sites depleted for H3K9me3 in *nrde-12/4−/−* animals (*P* = 2 × 10−16) (Fig. 3c, Supplementary Fig. 12 and Supplementary Table 2). Second, we conducted H3K9me3 ChIP on HRDE-1−/− animals and quantified H3K9me3 expression at fourteen NRDE germline target genes. At thirteen of these loci, H3K9me3 was depleted in HRDE-1−/− animals (Fig. 3d and Supplementary Fig. 13). Third, we observed increased
Figure 3 | The RNAi inheritance machinery transmits endogenous epigenetic information across generations. a, H3K9me3 in mutant (y-axis) versus wild type (N2, x-axis). Levels of H3K9me3 at C. elegans genes are the ratio of immunoprecipitated nucleosomes to input nucleosomes. smg-1 (positive control)8 (blue dot) and nrde-2/4-dependent genes (red) are highlighted. N2 and nrde-2 ChIP-seq data were published previously (Gene Expression Omnibus (GEO) accession number GSE32631)8. b, Flag–HRDE-1 co-precipitating RNA was 3P-radiolabelled and analysed by polyacrylamide gel electrophoresis (PAGE). nt, nucleotide. c, Example of an HRDE-1 target gene. RPKM, reads per kilobase (kb) per million mapped reads. d, rtPCR quantification of H3K9me3 ChIP (% of input) of late generation animals, indicating that the RNAi inheritance machinery silences germline target genes co-transcriptionally during the normal course of reproduction (Fig. 3e). These data indicate that HRDE-1 contributes to H3K9me3 in the germ line and support a model in which HRDE-1 uses endogenous 22G-siRNAs as specificity factors to direct nuclear epigenetic information across generations. a, H3K9me3 loss in hrde-1 mutants is not simply due to loss of germ cells. Data are expressed as the percentage of input DNA recovered by ChIP; the mean ± s.d. are shown (n = 3). e, Total RNA was isolated from wild-type or hrde-1/−/− animals (25 °C) and rtPCR was used to quantify mRNA or pre-mRNA levels from 11 HRDE-1-targeted genes. Three genes not targeted by HRDE-1 siRNAs, but expressed in germ cells, are also shown. Data are normalized to nos-3 mRNA (germ line only) and expressed as a ratio (hrde-1/−/−/wild type); the mean ± s.d. are shown (n = 3). f, dpy-17/−/− or hrde-1/−/−/dpy-17/−/− animals were analyzed five times, and dumpy (Dpy) adult animals (P0) and adult progeny (F1, F2, F3) were isolated (20 °C) and H3K9me3 was quantified by rtPCR. Data are expressed as a ratio (wild-type/hrde-1/−/−), and mean ± s.e.m. are shown (n = 1 for P0 and F1, and n = 3 for F2 and F3). Note, in this panel increased H3K9me3 signal means loss of H3K9me3 in hrde-1/−/− animals.
and promote RNAi inheritance in germ cells. Nuclear RNAi also occurs in the germline at a rate that is two to four times, and brood sizes scored across generations at 25°C. Data are mean ± s.e.m. (n = 5). Note, for unknown reasons, hrde-1 and ndre-4 mutants are Mrt at both 20°C and 25°C, but hrde-1 and ndre-2 mutants are Mrt only at ~25°C (n = 4). hrde-1/- animals were out-crossed three times, and hrde-1/+/+ or hrde-1/−/− siblings were isolated. Gonads were isolated and stained with 4′,6-diamidino-2-phenylindole (DAPI), and immunofluorescence was used to detect sperm (green) and oocytes (red) (see Methods) in F1 and F5 generations. Scale bar denotes 100 μm, and this is applicable to all images in b.

We note, however, that both processes depend on the same complement of factors (hrde-1 and ndre-1/2/4), and in animals lacking these factors, defects in epigenome maintenance and defects in germ-cell viability are coincident over generational time. Therefore, we propose that one biological function of the RNAi inheritance machinery is to transmit ‘germline immortality’ small RNAs, selected in previous generations for their ability to promote fertility, across generational boundaries to promote fertility in future generations.

Note added in proof: HRDE-1 was recently shown to act downstream of piRNAs to direct a multigenerational epigenetic memory of piRNA silencing in the germ line.19–21

METHODS SUMMARY

RNAi. RNAi experiments were conducted as described previously.22 The oma-1 and pos-1 constructs were taken from the Ahhringer library.

RNA immunoprecipitation. RNA immunoprecipitations (RIPs) were performed as described previously, with the exception that adult animals were used for all RIPs. Adult animals were frozen and dounced 10 times before RIP. Flag-NRDE-2 protein was immunoprecipitated with an anti-Flag M2 antibody (Sigma, A2220). ChIP. ChIP experiments were performed as described previously, except that gravid adult animals were used. Worms were frozen before cross-linking and were dounced 10 times before sonication. The H3K9me3 antibody was from Upstate.

oma-1 sIRNA TaqMan assay. The TaqMan assay was performed as described previously.23 TaqMan probe set 1 was used in Fig. 2a (see Supplementary Methods). Unless indicated otherwise, the following mutant alleles were used in this study: hrde-1(tm1200), ndre-1(gg088), ndre-2(gg091), ndre-3(gg066) and ndre-4(gg129).

Figure 4 | The RNAi inheritance machinery promotes germline immortality. a. Animals of indicated genotypes were out-crossed to wild-type two to four times, and brood sizes scored across generations at 25°C. Data are mean ± s.e.m. (n = 5). Note, for unknown reasons, hrde-1 and ndre-4 mutants are Mrt at both 20°C and 25°C, but hrde-1 and ndre-2 mutants are Mrt only at ~25°C (n = 4). b, hrde-1/- animals were out-crossed three times, and hrde-1/+/+ or hrde-1/−/− siblings were isolated. Gonads were isolated and stained with 4′,6-diamidino-2-phenylindole (DAPI), and immunofluorescence was used to detect sperm (green) and oocytes (red) (see Methods) in F1 and F5 generations. Scale bar denotes 100 μm, and this is applicable to all images in b.

and oocytes (Fig. 4b). Most of these gametes, however, are unlikely to be functional as the fecundity of hrde-1/- animals in this late generation was only 1% of that of wild-type animals (Fig. 4a). Finally, late generation hrde-1/- animals exhibited a high incidence of male (Him) phenotype, suggesting that loss of RNAi inheritance may cause defects in chromosome pairing and/or segregation (Supplementary Fig. 19).

We conclude that the RNAi inheritance machinery is required to maintain the immortality of the germ line and that, over generations, disabling the RNAi inheritance machinery causes progressive and diverse defects in germ-cell formation and function.

Here we show that C. elegans possess dedicated regulatory machinery that promotes an epigenetic memory of RNAi-silencing events that occurred in distant ancestors (Supplementary Fig. 1). The AGO HRDE-1 is at the heart of this process, binding heritably expressed specificity determinants (siRNAs) to direct nuclear RNAi and promote RNAi inheritance in germ cells. Nuclear RNAi also promotes RNAi inheritance in somatic tissues, indicating that nuclear gene silencing events are key determinants of RNAi memory in diverse cell types. Finally, we show that the germline RNAi inheritance machinery transmits endogenous epigenetic information across generational boundaries while promoting germline immortality (Supplementary Fig. 1). Our data suggest a model in which endogenous heritable RNAs that engage HRDE-1 act as specificity factors to direct epigenomic maintenance and immortality of the germ-cell lineage. Further work is needed to determine how defects in epigenomic maintenance relate to germline mortality (see Supplementary Discussion).
Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank P. Anderson, H. Opalicious and D. Wassarman for discussions. We thank S. Ahmed and members of the Ahmed laboratory for sharing unpublished data concerning the role of nrde-1 in germline immortality. This work was supported by grants from the Pew and Shaw scholar’s programs, and the National Institutes of Health, GM08289 (S.K.), GM37706 (A.F.) and GM069454 (J.K).

Author Contributions B.B. contributed to Figs 1a–c, 2b–d and Supplementary Figs 3, 4, 5b, 6, 8, 10 and 13. K.B. contributed to Figs 2c, 3d–f, 4a, b and Supplementary Figs 13–15, 16a–c, 17, 18 and 19c. S.G.G. and A.F. contributed to Fig. 3a–c, Supplementary Table 2 and Supplementary Figs 11 and 12. G.S. contributed to Supplementary Figs 2, 5a and 16d. A.K. and J.K contributed to Fig. 4b and Supplementary Figs 7 and 19a. H.F. contributed to Fig. 4a and Supplementary Figs 16a–c, 17, 18 and 19c. S.K. contributed to Figs 1a–d, 2a, 3b, Supplementary Table 1 and Supplementary Figs 2, 6b, 9, 10b and 19b. S.K., B.B. and K.B. wrote the manuscript.

Author Information ChIP-seq and hrde-1 siRNA data have been submitted to the Gene Expression Omnibus (GEO) under accession number GSE38041. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to S.K. (sgkennedy@wisc.edu).