LETTER

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A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality

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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[3–8]. 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 an Argonaute protein that associates with small interfering RNAs in the germ cells of progeny of animals exposed to double-stranded RNA. In the nuclei of these germ cells, HRDE-1 engages the nuclear RNAi defective pathway to direct the trimethylation of histone H3 at Lys 9 (H3K9me3) at RNAi-targeted genomic loci and promote RNAi inheritance. Under normal growth conditions, HRDE-1 associates with endogenously expressed short interfering RNAs, which direct nuclear gene silencing in germ cells. In *hrde-1*- or nuclear RNAi-deficient animals, germline silencing is lost over generational time. Concurrently, these animals exhibit steadily worsening defects in gamete formation and function that ultimately lead to sterility. These results establish that the Argonaute protein HRDE-1 directs gene-silencing events in germ-cell nuclei that drive multigenerational RNAi inheritance and promote immortality of the germ-cell lineage. We propose that *C. elegans* use the RNAi inheritance machinery to transmit epigenetic information, accrued by past generations, into future generations to regulate important biological processes.

We conducted a genetic screen to identify factors required for multigenerational RNAi inheritance. We mutagenized animals carrying a germline green fluorescent protein (*gfp*) reporter gene, and screened for mutant animals that retained the ability to silence *gfp* when exposed directly to *gfp* double-stranded RNA (dsRNA), but failed to silence *gfp* in subsequent generations. Among fourteen mutant alleles fulfilling these criteria, four alleles defined a gene we term here *heritable RNAi defective 1* (*hrde-1*) (Supplementary Fig. 2). *hrde-1* mutant animals silenced GFP expression when exposed to *gfp* dsRNA, but failed to transmit this silencing to subsequent generations (Fig. 1a and Supplementary Fig. 3). Similarly, *hrde-1* mutants silenced the germline-expressed *oma-1* gene when treated directly with *oma-1* dsRNA, but were defective for silencing inheritance (Supplementary Fig. 4). We conclude that *hrde-1* promotes multigenerational RNAi silencing in the germ line.

We mapped *hrde-1* to a genomic region containing the *c16c10.3* (also known as *wago-9*) gene, which is predicted to encode an Argonaute (AGO) protein not known to contribute to gene silencing[9,10] (see note added in proof). *c16c10.3* encodes a predicted bipartite nuclear localization signal (NLS) and PAZ and PIWI domains (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[9]. 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 nucleus.

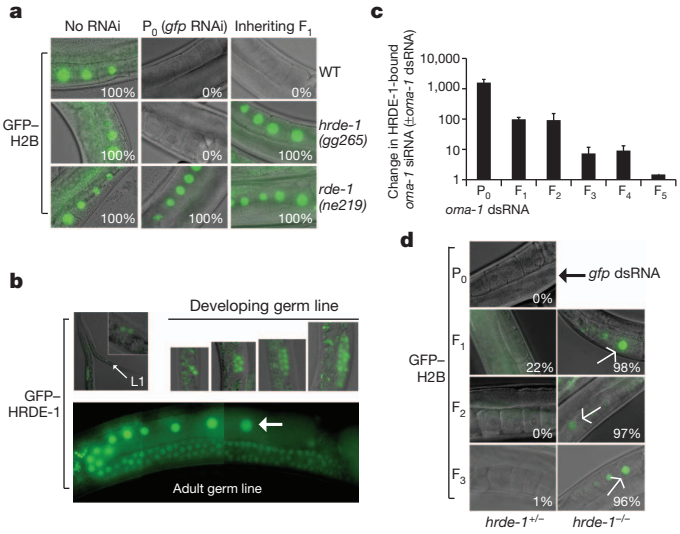

Figure 1 | *hrde-1* encodes a nuclear AGO protein that acts in inheriting generations to promote multigenerational germline RNAi inheritance. **a**, *pie-1*::*gfp*::*h2b*-expressing animals were exposed to *gfp* dsRNA. F_1 progeny were grown in the absence of dsRNA, and GFP expression in oocytes was 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 fluorescent microscopy. Small arrow, L1 animal; inset, magnifications showing GFP–HRDE-1 in primordial germ cells. Large arrow, oocyte nucleus. **c**, 3×Flag–HRDE-1 was immunoprecipitated with an anti-Flag antibody and co-precipitating *hrde-1* RNA was isolated, and *oma-1* siRNAs were quantified with an *oma-1* TaqMan probe set. Data are expressed as fold change (± *oma-1* RNAi), '1' 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.

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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 (RdRPs) (see Supplementary Discussion). The following genetic analyses confirmed that HRDE-1 acts in inheriting generations. Animals that were *hrde-1*$^{+/-}$ in the parental (P_0) RNAi generation, but were *hrde-1*$^{-/-}$ in the F_1 inheriting generation, failed to inherit RNAi silencing (Fig. 1d and Supplementary Table 1). Similarly, HRDE-1 activity was required in the F_2 generation for F_1-to-F_2 RNAi inheritance, and in the F_3 generation for F_2-to-F_3 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 RNAs[11–13]. 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 elongation[11–13]. The NRDE factors contribute to heritable gene silencing events that are manifest in somatic cells[7]. 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. 9). 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-1/2/4*$^{-/-}$ 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 *nrde-2/3/4*$^{-/-}$ animals, conducted H3K9me3 chromatin immunoprecipitation (ChIP), and subjected H3K9me3 co-precipitating nucleosome core DNA to high-throughput sequencing[8]. We identified 320 predicted genes that were depleted for H3K9me3 more than twofold in both *nrde-2*$^{-/-}$ and *nrde-4*$^{-/-}$

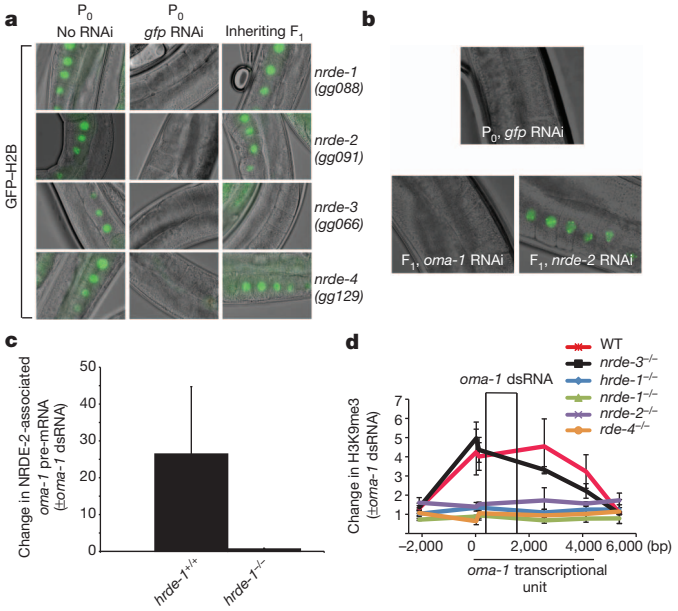

Figure 2 | HRDE-1 engages the NRDE nuclear RNAi pathway to direct multigenerational RNAi inheritance. **a**, *pie-1::gfp::h2b* fluorescence is shown. More than 98% of animals of each genotype exhibited phenotypes similar to that of the image shown. **b**, P_0 animals expressing *pie-1::gfp::h2b* were exposed to *gfp* dsRNA. F_1 progeny were exposed to *oma-1* or *nrde-2* dsRNA ($n = 3$). **c**, Flag–NRDE-2 was precipitated with an anti-Flag antibody and NRDE-2 co-precipitating *oma-1* pre-mRNA was quantified by rtPCR using exon/intron primer sets. Data are expressed as a ratio ± *oma-1* RNAi, and the mean and s.e.m. are shown ($n = 3$). **d**, The F_1 progeny of *oma-1* dsRNA-treated animals were subjected to H3K9me3 chromatin immunoprecipitation. Co-precipitating *oma-1* DNA was quantified by rtPCR. Data were normalized to co-precipitating *eft-3* DNA and expressed as a ratio ± *oma-1* RNAi (1 = no change). On the *x*-axis, 0 denotes the predicted start codon of *oma-1*. *rde-4* is required for RNAi[18]. Data are mean ± s.e.m. ($n = 3$–4). bp, base pair. Original magnification, ×630.

animals relative to wild type (Fig. 3a and Supplementary Table 2). H3K9me3 ChIP, followed by directed quantitative real-time PCR (rtPCR) analysis, confirmed the *nrde-2/4* dependence of H3K9me3 at four out of four of these loci (data not shown). NRDE-dependent H3K9me3 was present in germ cells; in temperature-sensitive *glp-4*(*ts*) mutants[14], which lack most germ cells, H3K9me3 was significantly reduced at most *nrde-2/4*-dependent sites (Fig. 3a, $P = 2 \times 10^{-13}$). Together, these data show that *nrde-2* and *-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 templates[10,15], 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 NRDE 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-2/4*$^{-/-}$ animals ($P = 2 \times 10^{-16}$) (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

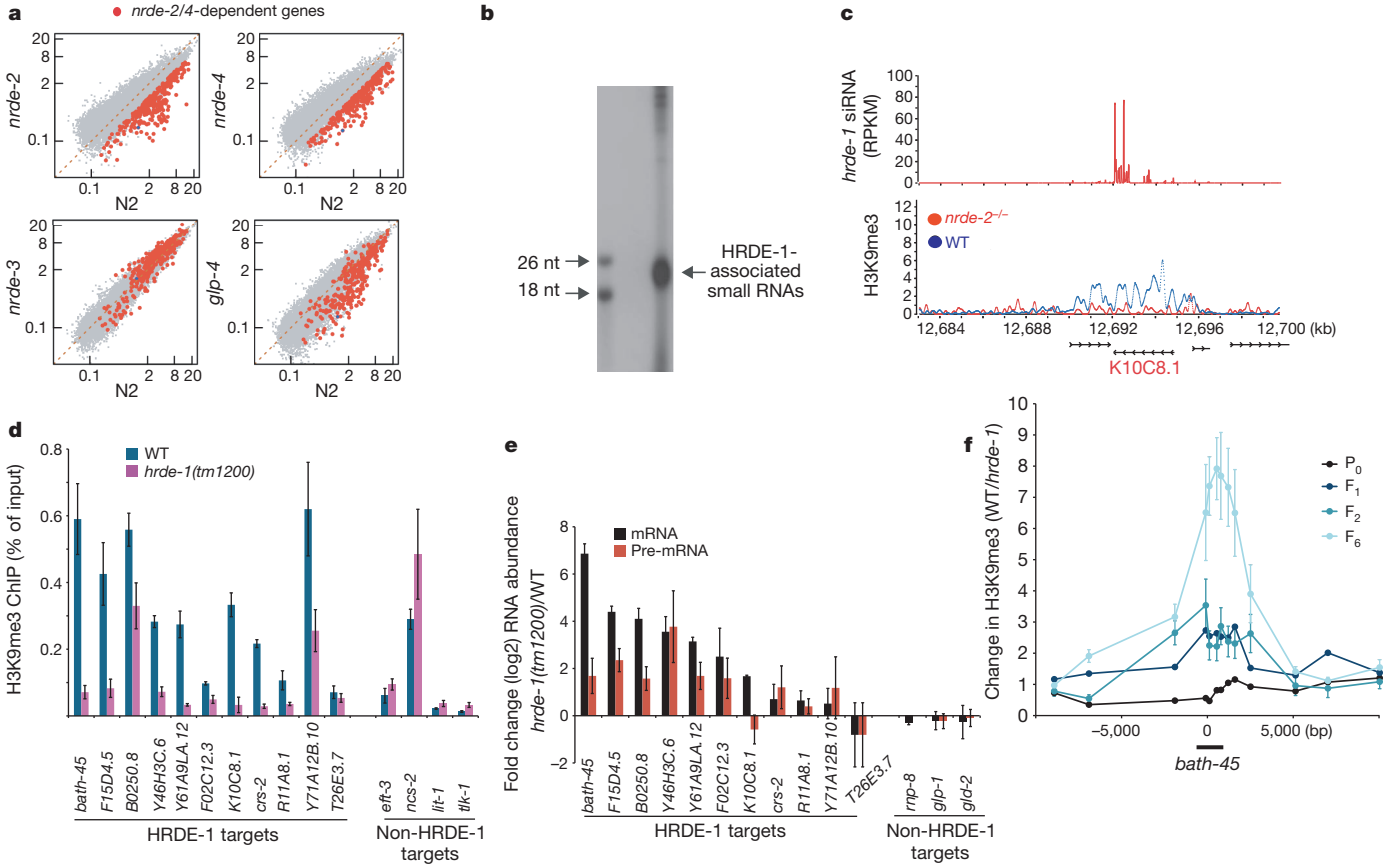

Figure 3 | The RNAi inheritance machinery transmits endogenous epigenetic information across generations. **a**, H3K9me3 in mutant (*y*-axis) versus wild type (N2, *x*-axis). Levels of H3K9me3 at *C. elegans* genes are the ratio of immunoprecipitated nucleosomes to input nucleosomes. *smg-1* (positive 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 ^{32}P-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 (wild-type or *hrde-1*$^{-/-}$ animals grown at 25 °C) at 11 genes targeted by *hrde-1* siRNAs (HRDE-1 targets). Four genes, which are not targeted by *hrde-1* siRNAs and exhibit NRDE-independent H3K9me3 in the germ line (non-HRDE-1 targets), do not exhibit H3K9me3 loss, showing that 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 out-crossed five times, and dumpy (Dpy) adult animals (P_0) and adult progeny (F_1, F_2, F_6) 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 P_0 and F_1, and $n = 3$ for F_2 and F_6). Note, in this panel increased H3K9me3 signal means loss of H3K9me3 in *hrde-1*$^{-/-}$ animals.

pre-mRNA expression from many germline target genes in *hrde-1*$^{-/-}$ 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 RNAi in germ cells.

We asked whether HRDE-1-mediated nuclear RNAi at germline target genes was heritable. We out-crossed *hrde-1*$^{-/-}$ animals with wild-type animals, isolated *hrde-1*$^{-/-}$ progeny, and conducted H3K9me3 ChIP on these *hrde-1*$^{-/-}$ animals and their progeny. H3K9me3 at germline target genes was progressively lost over generations in *hrde-1*$^{-/-}$ animals (Fig. 3f and Supplementary Fig. 14). Similar results were seen with *nrde-1*$^{-/-}$ animals (Supplementary Fig. 14). Coincident with a loss of H3K9me3, germline target gene overexpression became more pronounced in late generation *hrde-1*$^{-/-}$ animals (Supplementary Fig. 15). These data show that the RNAi inheritance machinery transmits endogenous gene regulatory information across generational boundaries.

Why an organism might transmit gene regulatory information across generations is an intriguing question. During the course of our studies, we noticed that our RNAi inheritance-defective strains would periodically become sterile; stock plates would contain hundreds of adults, but no progeny. We proposed that the RNAi inheritance machinery might be required to maintain the integrity of the germ-cell lineage. To test this idea, we out-crossed two independently isolated alleles each of *hrde-1*$^{-/-}$ and *nrde-1/2/4*$^{-/-}$ to wild-type and then monitored fertility across generations. After out-crossing, *hrde-1*$^{-/-}$ and *nrde-1/2/4*$^{-/-}$ animals exhibited near wild-type fertility (early generations), but became sterile in subsequent generations (late generations) (Fig. 4a and Supplementary Fig. 16). These data show that RNAi inheritance-defective animals exhibit a mortal germline (Mrt) phenotype[16]. Animals lacking the somatic AGO NRDE-3 were not Mrt (Supplementary Fig. 17). The Mrt phenotype of *hrde-1*$^{-/-}$ animals was temperature sensitive: *hrde-1*$^{-/-}$ animals were Mrt at 25 °C, but not at 20 °C, indicating that growth at higher temperatures is required to reveal defects associated with HRDE-1 loss (Supplementary Fig. 18). Most late-generation *hrde-1*$^{-/-}$ mutants (grown at 25 °C) failed to produce mature oocytes or sperm, showing that one reason *hrde-1*$^{-/-}$ animals do not produce progeny is owing to defects in gametogenesis (Fig. 4b and Supplementary Fig. 19). Twenty-six per cent of late generation *hrde-1*$^{-/-}$ animals were able to produce sperm

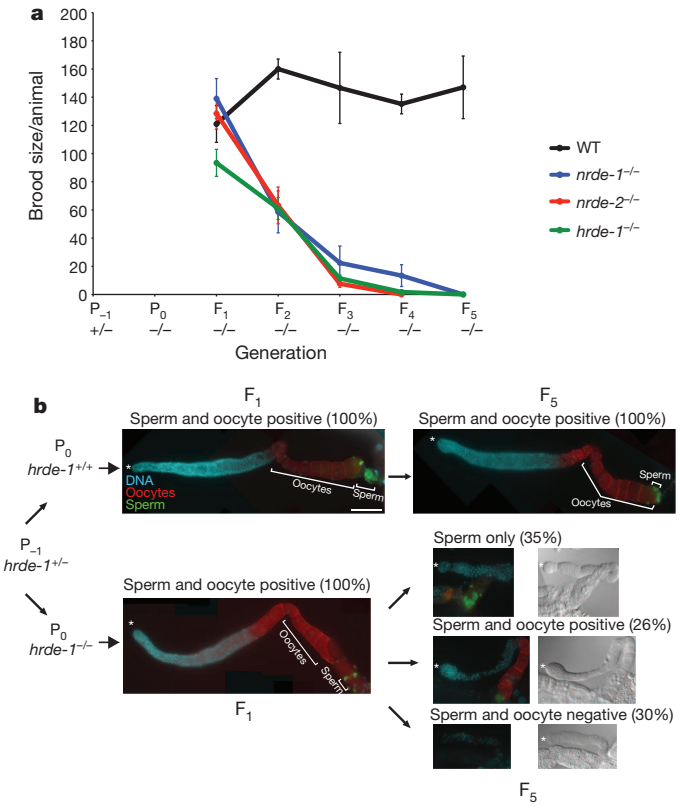

Figure 4 | The RNAi inheritance machinery promotes germline immortality. a, Animals of indicated genotypes were out-crossed to wild-type two to four times, and brood sizes scored across generations at 25 °C. Data are mean ± s.e.m. ($n = 5$). Note, for unknown reasons, *nrde-1* and *nrde-4* mutants are Mrt at both 20 °C and 25 °C, but *hrde-1* and *nrde-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; DNA), and immunofluorescence was used to detect sperm (green) and oocytes (red) (see Methods) in F_1 and F_5 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[7], 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). We note, however, that both processes depend on the same complement of factors (*hrde-1* and *nrde-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[11]. The *oma-1* and *pos-1* constructs were taken from the Ahringer library.

RNA immunoprecipitation. RNA immunoprecipitations (RIPs) were performed as described previously[11], 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[12], except that gravid adult animals were used. Worms were frozen before cross-linking and were dounced 10 times before sonicating. The H3K9me3 antibody was from Upstate (07-523).

***oma-1* siRNA TaqMan assay.** The TaqMan assay was performed as described previously[7]. 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*), *nrde-1*(*gg088*), *nrde-2*(*gg091*), *nrde-3*(*gg066*) and *nrde-4*(*gg129*).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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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).