piRNAs Can Trigger a Multigenerational Epigenetic Memory in the Germline of *C. elegans*

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http://dx.doi.org/10.1016/j.cell.2012.06.018

SUMMARY

Transgenerational effects have wide-ranging implications for human health, biological adaptation, and evolution; however, their mechanisms and biology remain poorly understood. Here, we demonstrate that a germline nuclear small RNA/chromatin pathway can maintain stable inheritance for many generations when triggered by a piRNA-dependent foreign RNA response in *C. elegans*. Using forward genetic screens and candidate approaches, we find that a core set of nuclear RNAi and chromatin factors is required for multigenerational inheritance of environmental RNAi and piRNA silencing. These include a germline-specific nuclear Argonaute HRDE1/WAGO-9, a HP1 ortholog HPL-2, and two putative histone methyltransferases, SET-25 and SET-32. piRNAs can trigger highly stable long-term silencing lasting at least 20 generations. Once established, this long-term memory becomes independent of the piRNA trigger but remains dependent on the nuclear RNAi/chromatin pathway. Our data present a multigenerational epigenetic inheritance mechanism induced by piRNAs.

INTRODUCTION

Since August Weismann (1834–1914) formulated the distinction between innate and acquired characteristics at the end of the 19th century, the debate relating to the inheritance of acquired traits has raised many controversies in the scientific community (Weismann, 1891; Bateson, 1919; Haig, 2006). August Weismann himself theoretically rejected this type of hereditability, arguing that, even though environmental stimuli could provoke adaptive responses in the somatic lineage, no evidence suggested that these changes could be communicated to the germline (Weismann, 1891). However, a number of epigenetic phenomena involving RNA, histone modification, or DNA methylation in many organisms have renewed interest in this area (Varmuza, 2003; Haig, 2006; Daxinger and Whitelaw, 2012). Paramutation is a prime example. In this phenomenon, a silenced allele can act in trans on a homologous sequence to cause stable and heritable silencing. This newly silenced allele can now itself act in a paramutagenic fashion to silence other alleles. Paramutation has been described in multiple species, and it seems likely that small RNAs play a key role in the process, although the full mechanisms involved still remain unclear (Stam and Mittelsten Scheid, 2005; Chandler, 2010; Suter and Martin, 2010). *C. elegans* has emerged as a key model for the analysis of several related pathways that regulate genes via small RNAs. *C. elegans* is well suited to the analysis of multigenerational effects, due to its short generation time (~3 days) and the ease with which they can be maintained under tightly controlled experimental conditions. In eukaryotes, 20–30 nucleotide (nt) RNAs bound to Argonaute (AGO) protein cofactors are the effectors of a number of gene regulation pathways (Carmell et al., 2002). The discovery of the process of RNA interference (RNAi) has been a major milestone (Fire et al., 1998). While 21–22 nt small interfering RNAs (siRNAs) are the small RNA effectors of RNAi, RNAi can be induced by injection of long double-stranded RNA (dsRNA) or by providing dsRNA environmentally in the food of *C. elegans* (Timmons et al., 2001). In both instances, dsRNA is processed by the RNase Dicer to give rise to primary siRNAs. RNAi effects are generally systemic (soma and germline) and...
are observed in the F1 generation, but the latter requires the generation of secondary siRNAs (Grishok et al., 2000; Pak and Fire, 2007; Sijen et al., 2007; Gu et al., 2009). Secondary siRNAs represent the most abundant class of endogenous small RNAs in C. elegans, are RNA-dependent RNA polymerase products, have a 5’ phosphate, and are predominantly 22 nt in length with a 5’ guanosine (22G-RNAs). Secondary siRNA pathways and RNA-dependent RNA polymerases (RdRPs) have not been found in vertebrates or Drosophila, but have been found in many other organisms, including nematodes, plants, fungi, and viruses. Secondary siRNA pathways in C. elegans are complex, can involve many different AGO proteins, and are only partly understood (Yigit et al., 2006).

Several studies have reported inheritance of environmental RNAi beyond the F1 generation (Fire et al., 1998; Grishok et al., 2000; Vastenhouw et al., 2006; Alcazar et al., 2008; Gu et al., 2012). In one transgenerational paradigm, small RNA inheritance and histone H3K9Me3 marks were observed for at least two generations (Gu et al., 2012). In addition, transgenerational inheritance of viral immunity (Rechavi et al., 2011) and longevity (Greer et al., 2011) were recently reported for C. elegans. These data suggest that the biological roles of transgenerational inheritance could be diverse but remain largely speculative. In addition, whether this transmission involves transgenerationally transmitted RNAs or modifications of chromatin is still controversial.

Piwi-interacting RNAs (piRNAs) are distinct from siRNAs and have an evolutionarily conserved role in transposon silencing in the germline in many animals, including nematodes (Malone and Hannon, 2009; Bagijn et al., 2012). C. elegans encodes two Piwi clade, AGO superfamily proteins, PRG-1 and PRG-2, although PRG-2 has likely little or no function (Bhatia et al., 2008; Das et al., 2008; Bagijn et al., 2012). C. elegans piRNAs are absent in prg-1 mutant animals, which exhibit fertility defects. PRG-1 and piRNA expression is restricted to the male and female germline (Bhatia et al., 2008; Das et al., 2008; Bagijn et al., 2012). The piRNAs of C. elegans are 21 nucleotides in length with a 5’ uracil (21U-RNAs) (Ruby et al., 2006; Batista et al., 2008; Das et al., 2008; Wang and Reinke, 2008). C. elegans piRNAs derive from two broad clusters on chromosome IV (Ruby et al., 2006) and act in trans to regulate endogenous targets in the germline (Bagijn et al., 2012).

Here, we report how transgenerational inheritance of environmental RNAi and the piRNA pathway converge on the same germline nuclear RNAi/chromatin pathway. Both nuclear RNAi factors and chromatin regulators are essential for silencing. This pathway can elicit a long-term epigenetic memory for more than 24 generations. Once established, the initial silencing trigger is no longer required.

RESULTS

A Reporter-Based System to Investigate Transgenerational Gene Silencing in C. elegans

To genetically dissect multigenerational gene silencing in C. elegans, we developed a heritable environmental RNAi (Hrde) sensor. The Hrde receptor consists of a reporter transgene expressing a histone-GFP fusion protein in the germline of C. elegans (Figure 1A and Figure S1 available online). The use of such a defined artificial locus combines the ability to map small RNA populations, which was previously not possible using multicolor transgenes (Vastenhouw et al., 2006), with a high-throughput, quantifiable approach that was not possible using an endogenous locus (Alcazar et al., 2008). Eliciting environmental RNAi by feeding transgenic animals with bacteria expressing dsRNA corresponding to the GFP mRNA results in gene-specific silencing of this GFP transgene (P0, Figures 1A and 1B), as expected (Timmons et al., 2001). Transfer of these animals to a neutral environment results in a high level of silenced animals in the F1 generation, again as expected (Figure 1B) (Fire et al., 1998; Grishok et al., 2000). Furthermore, silencing of the transgene is maintained for at least four additional generations in a subpopulation of animals. We quantified this phenomenon in thousands of animals for each generation using flow cytometry and found that inheritance of transgene silencing was maintained in more than 60% of animals for at least four generations (Figures 1C and S2). We conclude that we have established a reporter-based paradigm for the investigation of transgenerational inheritance, the “heritable RNAi defective,” or “Hrde,” sensor.

Multigenerational Gene Silencing Is Associated with Continued Small RNA Expression

As the mechanisms of transgenerational inheritance are currently not understood, we first asked whether the Hrde sensor silencing that we observed is due to posttranscriptional regulation of mRNA or (co-)transcriptional gene regulation. Using quantitative RT-PCR, we tested whether Hrde sensor silencing in the F2 generation affected either. We were able to robustly detect both primary transcript (pre-mRNA) and Hrde transgene mRNA. However, mRNA levels were significantly repressed (p < 0.05) in silenced animals as compared to nonsilenced animals (Figure 1D). pre-mRNA levels showed a similar trend. These data suggest that posttranscriptional mechanisms of silencing are required in the Hrde paradigm. We postulated that Hrde sensor transcript availability might result in continued small RNA pathway activity in silenced animals. Therefore, we profiled small RNAs using high-throughput sequencing from animals undergoing environmental RNAi (P0), control RNAi (P0), or at the F4 generation after RNAi. Small RNA libraries were prepared using protocols that did not necessitate the presence of a 5’ monophosphate to capture primary and secondary siRNAs. We detected abundant sense and antisense small RNAs during environmental RNAi (P0 generation) (Figure 1E). These small RNAs had a peak length of 21–22 nt and little bias for the 5’-most nucleotide and likely represent Dicer cleavage products (primary siRNAs) (Figure 1F). In contrast, four generations later, only antisense small RNAs remain with the characteristic signature of secondary siRNAs (22 nucleotide length with a 5’ guanosine bias, 22G RNAs). Given that each generation represents at least a hundred-fold dilution in volume (with more than 200 offspring generated by each hermaphrodite), these secondary siRNAs must be generated de novo in each generation. Animals undergoing control RNAi displayed a peak of small RNAs homologous to cloning sequences flanking the GFP minigene. These
are homologous to cloning sequences that are present in the RNAi vectors and have no apparent effect on Hrde sensor activity (Figure 1C).

Multigenerational Gene Silencing and piRNA Silencing Depend on Common Nuclear Factors

We recently reported that piRNA-mediated silencing in the C. elegans germline results in secondary siRNA-dependent silencing of a “piRNA sensor” and endogenous piRNA targets (Bagijn et al., 2012). Thus, piRNA-mediated silencing might converge on a common downstream multigenerational gene silencing pathway. To this end, we carried out forward genetic screens to identify genes required for either phenomenon using the Hrde and piRNA sensors. Using these two distinct sensors (Figure S1), one silenced by a single endogenous piRNA (piRNA sensor) and the other silenced by heritable environmental RNAi (Hrde sensor), we identified, mapped, and cloned new alleles of three known genes in small RNA pathways: nrde-2, nrde-4,
and hrde-1/wago-9 (Table 1). Next, combining forward genetic screens with a candidate gene approach, we were surprised to identify a total of eight small RNA or chromatin pathway genes to be required (Table 1). For example, the Hrde sensor was disabled in \textit{nrde-2}, \textit{hrde-1/wago-9}, and \textit{set-25} mutants (Figure 2A and Table 1). The products of all of these genes are either known to be, or are predicted to be, nuclear. NRDE-2 is a conserved protein involved in nuclear RNAi that is expressed in the nucleus (Guang et al., 2010); SET-25 is a putative histone H3 lysine-9 methyltransferase with a C-terminal SET domain. To our knowledge, this is the first time that a histone-modifying enzyme has been identified as required for multigenerational inheritance. HRDE-1/WAGO-9 is an Argonaute protein. Using immunostaining, we show that it is expressed in the germline (Figure 2B), where it localizes to the nucleus (Figures 2 C and 2D). NRDE-2 was recently shown to be important in a similar inheritance paradigm (Gu et al., 2012). However, some genes that were previously reported to be involved in transgenerational effects appeared not to be required for our transgenerational inheritance paradigm, including \textit{hda-4}, \textit{mrg-1} (Vastenhouw et al., 2006) or \textit{spr-5}, \textit{lsd-1}, and \textit{amx-1} (Katz et al., 2009) (Table 1). For the piRNA sensor, aside from proteins that were defined in Hrde

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### Table 1. Multigenerational Environmental RNAi and piRNA Silencing Require a Common Nuclear Pathway

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Gene Product(s)</th>
<th>Reference Allele Tested</th>
<th>Alleles Described in This Study</th>
<th>Heritable Environmental RNAi Defective</th>
<th>piRNA Silencing Defective</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>\textit{nrde-2}</td>
<td>novel</td>
<td>gg91, gg95</td>
<td>\textit{mj168 (Q135Stp)}</td>
<td>+</td>
<td>+(^a)</td>
</tr>
<tr>
<td>\textit{nrde-1}</td>
<td>novel</td>
<td>gg88, yp4, yp5</td>
<td>NA</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>\textit{nrde-4}</td>
<td>novel</td>
<td>mj249 (Q707Stp) mj259 (Q683Stp)</td>
<td>NA</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>\textit{hrde-1/wago-9}</td>
<td>nuclear Argonaute</td>
<td>tm1200</td>
<td>mj278 (P720L)</td>
<td>+</td>
<td>+(^a)</td>
</tr>
<tr>
<td>\textit{nrde-3}</td>
<td>nuclear Argonaute</td>
<td>tm1116</td>
<td>NA</td>
<td>ND</td>
<td>–(^b)</td>
</tr>
<tr>
<td>\textit{sago-1}</td>
<td>Argonaute</td>
<td>tm1195</td>
<td>NA</td>
<td>–(^b)</td>
<td></td>
</tr>
<tr>
<td>\textit{sago-2}</td>
<td>Argonaute</td>
<td>tm894</td>
<td>NA</td>
<td>–(^b)</td>
<td></td>
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<tr>
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<td>chromo domain</td>
<td>tm1489</td>
<td>NA</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>\textit{hpl-1}</td>
<td>chromo domain</td>
<td>tm1624</td>
<td>NA</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>\textit{hpl-1; hpl-2}</td>
<td>chromo domains</td>
<td>tm1489; tm1624</td>
<td>NA</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>\textit{set-25}</td>
<td>SET domain</td>
<td>n5021</td>
<td>NA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>\textit{set-32}</td>
<td>SET domain</td>
<td>ok1457</td>
<td>NA</td>
<td>+</td>
<td></td>
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<tr>
<td>\textit{met-2}</td>
<td>SET domain</td>
<td>n4256</td>
<td>NA</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>\textit{met-1}</td>
<td>SET domain</td>
<td>n4337</td>
<td>NA</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>\textit{lin-59}</td>
<td>SET domain</td>
<td>n3192</td>
<td>NA</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>\textit{set-2}</td>
<td>SET domain</td>
<td>n4589</td>
<td>NA</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>\textit{set-6}</td>
<td>SET domain</td>
<td>ok2195</td>
<td>NA</td>
<td>–</td>
<td></td>
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<tr>
<td>\textit{set-9}</td>
<td>SET domain</td>
<td>n4949</td>
<td>NA</td>
<td>–</td>
<td></td>
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<tr>
<td>\textit{set-11}</td>
<td>SET domain</td>
<td>n4488</td>
<td>NA</td>
<td>–(^a)</td>
<td></td>
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<tr>
<td>\textit{set-12}</td>
<td>SET domain</td>
<td>n4442</td>
<td>NA</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>\textit{hda-4}</td>
<td>histone deacetylase</td>
<td>ok518</td>
<td>–</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>\textit{mrg-1}</td>
<td>chromo domain</td>
<td>qa6200</td>
<td>–</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>\textit{spr-5}</td>
<td>histone demethylase</td>
<td>by134</td>
<td>–</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>\textit{lsd-1}</td>
<td>histone demethylase</td>
<td>vr12</td>
<td>–</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>\textit{amx-1}</td>
<td>amine oxidase</td>
<td>ok659</td>
<td>–</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>\textit{prg-1; prg-2}</td>
<td>Piwi</td>
<td>n4357, n4358</td>
<td>–</td>
<td>+(^b)</td>
<td></td>
</tr>
<tr>
<td>\textit{prg-1}</td>
<td>Piwi</td>
<td>n4357</td>
<td>ND</td>
<td>+(^b,d)</td>
<td></td>
</tr>
<tr>
<td>\textit{rsd-2}</td>
<td>novel</td>
<td>pk3307</td>
<td>ND</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>\textit{rsd-6}</td>
<td>Tudor domain</td>
<td>pk3300</td>
<td>ND</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>\textit{mut-7}</td>
<td>RNase D</td>
<td>pk204</td>
<td>+(^c)</td>
<td>+(^b)</td>
<td></td>
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</tbody>
</table>

For heritable environmental RNAi and piRNA silencing assays, intrachromosomal single-copy transgenes were used as reporters. For heritable environmental RNAi, animals were scored at the F2 generation (see Figure 1A). For piRNA silencing, a sensor for the endogenous piRNA 21UR-1, the “piRNA sensor,” on chromosome II was used (Bagijn et al., 2012), ND, not done.

\(^a\)Tested using the piRNA cherry sensor on chromosome I, as described in Bagijn et al., 2012.

\(^b\)Previously reported in Bagijn et al., 2012.

\(^c\)These mutants were already defective in silencing in the F1 generation.

\(^d\)Results are dependent on multigenerational ancestry of the animals (this study).
screens such as NRDE-2, HRDE-1/WAGO-9, and SET-25, we also identified additional nuclear small RNA components and chromatin factors, including NRDE-1, NRDE-4, SET-32, and one of the C. elegans heterochromatin protein 1 (HP1) orthologs, HPL-2 (Table 1 and Figure S3). We conclude that there exists a common and specific nuclear RNA/chromatin pathway in the germline that is required for environmentally induced heritable RNAi- and piRNA-induced silencing.

The Nuclear RNAi/Chromatin Silencing Pathway Acts Downstream of Small RNA Expression in Gene Silencing
To establish a hierarchy in the silencing pathways described here, we asked whether nuclear RNA/chromatin components are upstream or downstream of secondary siRNA expression and/or stability. First, we analyzed small RNA expression in the Hrde sensor paradigm in wild-type and a nde-2 mutant background. In both cases, we find abundant 22G secondary siRNAs that map to the Hrde sensor (Figure 3A). Thus, NRDE-2 is not required for secondary siRNA generation. Next, we tested a chromatin factor using the piRNA sensor. The HP-1 ortholog HPL-2, but not HPL-1, is required for silencing of the piRNA sensor (Table 1 and Figure 3B). Therefore, we asked whether secondary siRNAs are expressed and stable in hpl-2 mutant animals. Using northern blotting, we show that the piRNA 21UR-1 and a piRNA-sensor-specific 22G RNA (siR22G-1) are dependent on the Piwi protein PRG-1 (Figure 3C). However, both RNAs are present in hpl-2 and hpl-2; hpl-1 mutant backgrounds, although possibly at reduced levels for siR22G-1.

These observations are in agreement with similar observations made for siRNAs in S. pombe lacking Swi6/HP1 (Bühler et al., 2006). In addition, we analyzed endogenous targets of the piRNA pathway that we recently identified (Bagijn et al., 2012). We generated small RNA libraries from wild-type and prg-1 or hpl-2 mutant animals. 22G secondary siRNAs at endogenous piRNA targets bath-45 and zfp-1 are dependent on PRG-1, but not HPL-2 (Figures 3D and 3E). Again, we observed some reduction in RNA levels, consistent with a positive interaction between nuclear RNAi and chromatin regulation. We conclude that the nuclear RNAi/chromatin pathway described here is not essential for secondary siRNA expression or stability.

Multigenerational Gene Silencing and piRNA Silencing Does Not Spread into the Soma
As the nuclear RNAi/chromatin pathway that we describe here utilizes small RNAs, it might act in trans on transcripts that share significant sequence similarity. Indeed, using the piRNA sensor, we were able to test this directly. The piRNA sensor is under the transcriptional control of a germline-specific promoter (mex-5). Silencing of the piRNA sensor is established through an endogenous piRNA 21-UR-1 with perfect complementarity to a corresponding sequence in the piRNA sensor (Bagijn et al., 2012). A cross of the piRNA sensor strain to a different transgenic strain with a ubiquitously expressed GFP transgene that is not regulated by piRNAs (Figure 4A) results in dominant silencing of both transgenes in the germline of heterozygous F1 animals (Figure 4B), likely via a process termed transitive RNAi (Alder

Figure 2. Transgenerational Inheritance Requires NRDE-2 and the Germline-Specific Nuclear Argonaute HRDE-1/ WAGO-9
(A) Biosorter analysis of WT, nde-2, and hrde-1/ wago-9 animals showing the failure of heritable silencing in these mutant strains. GFP fluorescence of the transgene and the percentage of silenced animals per plate were determined using a large particle biosorter and FlowJo. Ten silenced worms were selected where possible from each plate to produce the next generation. At least 500 worms were analyzed per plate with the following number of replicates (empty vector, GFP RNAi, F1, F2, respectively): WT, n = 7, 6, 4, 4; nde-2, n = 3, 3, 3; hrde-1/wago-9, n = 12, 6, 6, 6. Error bars represent the SEM.
(B) HRDE-/WAGO-9 is expressed in the germline. Wild-type dissected germlines (adults) were stained with anti-HRDE-1/WAGO-9 (green) and a P-granule-specific antibody (OIC1D4, red). DNA was stained with DAPI (blue). Images on the right are merged from all three channels. (C and D) HRDE-/WAGO-9 is a nuclear protein. Immunostainings were performed on dissected gonads from adult wild-type (N2) or hrde-1/wago-9 (tm1200) animals using anti-HRDE-1/WAGO-9 (green) and anti-a-tubulin antibodies (red). DNA was stained with DAPI (blue). Images on the right are merged from all three channels. Images shown are germ cells in the transition zone/ pachytene region (C) and oocytes (D).

Cell 150, 1–12, July 6, 2012 ©2012 Elsevier Inc. 5
Thus, the nuclear RNAi/chromatin pathway can silence in trans. We postulate that this effect is mediated via secondary siRNAs. As exogenous and endogenous RNAi are systemic in C. elegans (Fire et al., 1998; Winston et al., 2002), we therefore wondered whether the germline nuclear RNAi/chromatin silencing pathway that we describe here could transcend the germline/soma boundary. We do not find this to be the case, as GFP expression in the trans-heterozygous animals (dpy-30::his-58::gfp::tbb-2/piRNA sensor) described above remains unaffected in the soma (Figure 4B). We made the same observation using the Hrde-1 sensor and another somatic transgene in an analogous experiment (data not shown). We conclude that, though the nuclear RNAi/chromatin pathway that we describe here can be vertically transmitted, it does not trigger systemic RNAi. This is consistent with recent work demonstrating that secondary siRNAs are not systemically transmitted in the soma of C. elegans (Jose et al., 2011). We note that results based on multicopy transgenes that possibly involve dsRNA intermediates could be different from those reported here (Jose et al., 2011).
piRNAs Can Trigger Long-Term Multigenerational Gene Silencing

Our data demonstrate that environmentally induced multigenerational gene silencing and piRNA silencing converge on a common germline silencing pathway. Can a piRNA therefore trigger multigenerational gene silencing? To address this question, we carried out genetic crosses in which we removed PRG-1 and thereby piRNA function from the piRNA sensor strain (Das et al., 2008; Bagijn et al., 2012). In these circumstances, the piRNA trigger is removed but silencing might be maintained. In a cross of animals homozygous for the piRNA sensor (GFP silenced) with an animal homozygous for the piRNA sensor but in a prg-1 mutant background (GFP expressed), we generated F1 animals homozygous for the piRNA sensor but heterozygous for the recessive mutation in prg-1 (Figure S4). Such animals are GFP silenced for several generations, as expected. These heterozygous animals segregate progeny that are homozygous, heterozygous, or wild-type with respect to prg-1. We observed piRNA sensor reactivation in prg-1 homozygous mutants or their immediate offspring. Because all piRNAs are eliminated in prg-1 mutants (Batista et al., 2008; Das et al., 2008), these data suggested that a piRNA trigger may be required to maintain multigenerational silencing memory.

Next, we recreated a piRNA sensor strain that was mutant for prg-1 by outcrossing the piRNA sensor and then performing several crosses using mutations that cause visible phenotypes to mark the positions of prg-1 or the piRNA sensor transgene (see Experimental Procedures). Unexpectedly, 11 prg-1; piRNA sensor strains failed to reactiviate the piRNA sensor (n = 8 or 3 independent strains created per trial for 2 trials) (Figures 5A and 5B). GFP expression of these prg-1; piRNA sensor strains failed to materialize even though many successive generations were scored, which were last analyzed at F16, F17 (three strains), F20, and F24 (six strains) generations. We also observed that silencing can become PRG-1 independent using a second piRNA sensor construct integrated on a different chromosome (the piRNA mCherry sensor; Figure S5). We conclude that germline silencing can persist for many generations even in the absence of a piRNA trigger. It is of interest to note that all crosses that led to trigger-independent maintenance of silencing involved the piRNA sensor transgene being heterozygous for 3–5 generations due to outcrossing.

In contrast to multigenerational silencing of piRNA sensor transgenes in the absence of prg-1, mutation of nrde-1 (yp4 or yp5) or mutation of nrde-2 (gg95) triggered reactivation of outcrossed piRNA sensors (n = 3, 2, and 3, respectively, independently isolated F3 or F4 strains scored) (Figure 5C). All nrde-2 mutant lines expressed bright GFP from F3 onward. Of five piRNA sensor; nrde-1 lines, three lines expressed weak GFP signal in all germ cells in the F3 generation, whereas germ cells of all animals scored in piRNA sensor; nrde-1 lines were uniformly positive for a weak GFP signal by the F4 generation. We conclude that nuclear small RNA factors are required to maintain the silenced state over many generations, whereas the piRNA trigger that initiates silencing becomes dispensable if the silent locus is outcrossed multiple times.

A Tudor domain protein RSD-6 and a novel protein RSD-2 have previously been shown to be required for RNAi responses to environmental dsRNA triggers that target genes expressed in the germline and are proficient for RNAi to some somatic targets, possibly due to dose-dependent RNAi defects (Tijsterman et al., 2004; Merritt et al., 2008; Zhang et al., 2012). The strong germline RNAi defects of rsd-6 or rsd-2 suggested that they could function to promote systemic spreading of RNAi from soma to germline (Tijsterman et al., 2004). To determine where rsd-6 functions to promote germline RNAi, single-copy

Figure 4. The Germline Nuclear RNAi/Chromatin Pathway Acts in trans but Cannot Exit the Germline

(A and B) trans-heterozygous animals were generated by crossing SX1866 hermaphrodites with piRNA sensor males. Strain SX1866 expressing H2B-GFP under control of the ubiquitous dpy-30 promoter was generated by MosSCI into tt5660 on chromosome II (np31[dpy-30::his-58::gfp::tbb-2]; DIC and fluorescence microscopy of animals from the parental line (A) or of trans-heterozygous animals from the cross (B). Note that the parental line expresses H2B-GFP from two copies in the genome and is therefore brighter. Yellow arrowheads indicate germ cell nuclei; red arrowheads indicate somatic (intestinal) cell nuclei.
rsd-6 transgenes driven by the germline-specific pgl-3 promoter or by the ubiquitous promoter dpy-30 were created (Frokjaer-Jensen et al., 2008; Han et al., 2008). Both transgenes rescued an rsd-6 mutant for the response to dsRNAs targeting the germline-expressed genes pop-1 or par-6 (Figure S6), indicating that RSD-6 functions in a cell-autonomous manner within the germline. We created rsd-6; piRNA sensor and piRNA sensor; rsd-2 strains using outcrossed sensor transgenes and observed that these strains were GFP negative when initially created and for many generations thereafter (Figure 5C). These results suggest that piRNA sensor silencing may not depend on systemic RNAi effects (possibly mediated by expression of dsRNA in somatic cells). They also suggest that the response to dsRNA generated in germ cells is unlikely to promote sensor silencing (Tabara et al., 1999; 2002).

**DISCUSSION**

Here, we show that piRNA and environmental RNAi pathways converge on a common germline nuclear RNAi/chromatin pathway. This pathway can induce stable, multigenerational inheritance. Previous work found evidence for inheritance of small RNAs, chromatin, or both in related transgenerational inheritance paradigms (Burton et al., 2011; Rechavi et al., 2011; Gu et al., 2012). However, here we demonstrate that both small RNA and chromatin factors are essential for multigenerational inheritance and do not act redundantly (Table 1 and Figures 2, 3, S3). We also show that small RNA biogenesis occurs upstream of nuclear RNAi and chromatin factors (Figures 3). Recent work has proposed that somatic nuclear RNAi acts at the level of transcriptional elongation (Tabara et al., 1999; 2002).
required (Table 1 and Figures 3 and S3). SET-25/32 are putative chromatin factors, such as HPL-2 and SET-25/32, are without being functional in silencing. However, our data show that chromatin changes observed in transgenerational inheritance paradigms (Gu et al., 2012) might simply be correlative without being functional in silencing. These observations opened the possibility that the original RNAi model of transgene silencing (N.J.L., A.S., and E.A.M., unpublished data) (Frøkjaer-Jensen et al., 2008). Indeed, multigenerational silencing of transgenes that remain silenced (Shirayama et al., 2012) is also required for the establishment of nuclear RNAi/chromatin pathway. Maintenance of silencing requires nuclear RNAi factors, including the germline-specific nuclear Argonaute HRDE-1/WAGO-9 and chromatin proteins such as the HP1 ortholog HPL-2 and the putative histone methyltransferases SET-25 and SET-32. Silencing can be maintained into the F1 for multiple generations (F1–F5) or can become epiallelic with multigenerational, nonstochastic inheritance. Silencing might be suppressed by a germline licensing pathway that recognizes bona fide germline transcripts (CSR-1 22G-RNA pathway) or might be enforced through the recognition of unpaired DNA during meiosis.

These observations suggest that the chromatin states involved in multigenerational silencing might be complex and could include a hierarchy, which merits further investigation. We have summarized a model of our current understanding of this pathway in Figure 6.

**Multicopy versus Single-Copy Transgenes**

Multicopy transgenes, intra- or extrachromosomal, are generally efficiently silenced in the germline of C. elegans (Kelly et al., 1997). This has been interpreted as an example of the RNAi machinery distinguishing self from nonself (Vastenhouw and Plasterk, 2004). In this model, repetitive DNA such as endogenous transposable elements or multicopy transgenes would give rise to dsRNA that is processed by Dicer to generate siRNA triggers to induce silencing. As the pathways silencing multicopy transgenes and transposable elements share common factors, this phenomenon is of biological interest. However, it has also been a major technical roadblock for researchers studying germ cell biology who rely on reproducible transgene expression in the germline. The advent of MosSCI technology to produce single-copy transgenes has the promise to overcome this problem (Frøkjaer-Jensen et al., 2008, 2012). Interestingly, we and others observed that, in some cases, individual transgenes remain silenced even when present as single, intrachromosomal entities (N.J.L. and E.A.M., unpublished data) (Frøkjaer-Jensen et al., 2008, 2012). In this model, repetitive DNA is processed by Dicer to generate siRNA triggers to induce silencing. As the pathways silencing multicopy transgenes and transposable elements share common factors, this phenomenon is of biological interest. However, it has also been a major technical roadblock for researchers studying germ cell biology who rely on reproducible transgene expression in the germline. The advent of MosSCI technology to produce single-copy transgenes has the promise to overcome this problem (Frøkjaer-Jensen et al., 2008, 2012). Interestingly, we and others observed that, in some cases, individual transgenes remain silenced even when present as single, intrachromosomal entities (N.J.L. and E.A.M., unpublished data) (Frøkjaer-Jensen et al., 2008). Indeed, an accompanying paper reports a collection of MosSCI transgenes that remain silenced (Shirayama et al., 2012). Generating MosSCI transgenes in animals in which germline nuclear RNAi pathways are impaired, such as *mut-7*, or “curing” silenced transgenes by outcrossing first to germline RNAi mutants strains and then back to wild-type often results in loss of transgene silencing (N.J.L., A.S., and E.A.M., unpublished data). These results suggest that the original RNAi model of
multicopy transgene silencing needs to be revised. Indeed, there appears to be no requirement for dsRNA intermediates in the silencing phenomena reported here either, as factors required for dsRNA-induced RNAi in the germline such as RSD-2 and RSD-6 (Figure 5 and Table 1) or RDE-1 and RDE-4 (Shirayama et al., 2012) are dispensable for single-copy transgene silencing.

Self versus Nonself
How does C. elegans detect single-copy transgenes and target them for silencing in the germline, or how does the animal distinguish self from nonself? The answer might lie in a combination of three factors: scanning germline gene expression by the piRNA pathway (nonself RNA recognition), licensing of germline transcripts (self RNA recognition), and unpaired genomic DNA in meiosis. Based on this and related work, we propose that the piRNA pathway can detect transgenes as sources of foreign transcripts (self RNA recognition), and unpaired genomic DNA in meiosis. The piRNA sensor to be silenced or active depending on its multigenerational ancestry. In our crosses, the piRNA sensor can trigger dispensable (Figure 6). Unpaired DNA silencing responses have been observed in C. elegans in the case of the unpaired X chromosome (Kelly et al., 2002; Bean et al., 2004) and for a mutant fem-1 locus (Johnson and Spence, 2011) and has also been found in other organisms (Hynes and Todd, 2003; Lee, 2005; Matzke and Birchler, 2005). Establishment of heritable silent chromosome domains that can be robustly maintained in the absence of the original piRNA trigger could be relevant to populations in which sources of piRNAs are polymorphic and may evolve rapidly in response to novel transposons or retroviruses.

Related Phenomena in Other Phyla
The core molecular pathway described here is reminiscent of related (co-) transcriptional pathways both in yeasts and plants (Moazed, 2009). Many yeasts and all plants and nematodes share key factors, such as the RNA-dependent RNA polymerases involved in secondary siRNA generation. Though transgenerational phenomena have been reported in many animals, including humans (Hitchins et al., 2007), this class of polymerases and secondary siRNAs appears to be absent in Drosophila and vertebrates. However, it is interesting to note that Drosophila and vertebrates have a more complex piRNA system that includes an amplification loop termed “ping-pong,” which could function in a manner analogous to secondary siRNA pathways (Brennecke et al., 2007; Gunawardane et al., 2007). Despite differences in details of piRNA and secondary siRNA systems, common downstream silencing mechanisms may exist.

One, Few, or Many Generations?
Transgenerational phenomena have been observed over one or multiple generations (Grishok et al., 2000; Vastenhouw et al., 2006; Alcazar et al., 2008; Burton et al., 2011; Rechavi et al., 2011; Gu et al., 2012). In some cases, inheritance is stochastic; in others, Mendelian. Here, we report that piRNAs can trigger silencing that lasts for more than 20 generations (Figures 5A, 5B, and S5). Although maintenance of this memory is observed in 100% of offspring, establishment of strong piRNA-independent memory is not obligatory (Figure S5) and only occurs if a silent locus is heterozygous for several generations. This is reminiscent of ubiquitous yet stochastic inactivation of repetitive germline transgenes in many organisms, including C. elegans. Our study of transgenes targeted by an endogenous piRNA may recapitulate the fate of transposons that are transmitted in rare horizontal transfer events, in which a single transposon insertion could be subjected to dual layers of silencing, as the locus would likely remain heterozygous for a number of generations before potentially becoming fixed. It will be of great interest to identify the factor(s) that determines these distinct states of silencing.

Experimental Procedures

Genetics
C. elegans were grown under standard conditions at 20°C unless otherwise indicated. The food source used was E. coli strain HB101 (Caenorhabditis Genetics Center, University of Minnesota, Twin Cities, MN, USA). Bleaching followed by starvation-induced L1 arrest was used to generate synchronized cultures. The wild-type strain was var. Bristol N2 (Brenner, 1974). All strains used are listed in Table S1. For details about genetic crosses, see Supplemental Information.
Transgenics
To generate transgenic animals, germline transformation was performed as described (Mello and Fire, 1995). Injection mixes contained 2–20 ng/μl of MosSCI plasmid and 5–10 ng/μl of marker plasmid DNA (see Supplemental Information for details). Single-copy transgenes were generated by transposase-mediated integration (MosSCI), as described (Frøkjaer-Jensen et al., 2008, 2012).

COPAS Biosort Analysis
A COPAS Biosort instrument (Union Biometrica, Holliston, MA, USA) was used to simultaneously measure length (time of flight), absorbance (extinction), and fluorescence. Data handling and analysis were performed using FlowJo (Tree Star, Inc.) and R.

ACCESSION NUMBERS
The sequence data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, http://www.ncbi.nlm.nih.gov/geo, with accession numbers GSE37433 and GSE38813.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.06.018.

ACKNOWLEDGMENTS
We thank Sylviane Moss for excellent laboratory management and help with genetic crosses and high-throughput sequencing. We thank Craig Mello and Scott Kennedy for sharing unpublished information. We thank Andrew Bannister and Anne Ferguson-Smith for helpful comments on the manuscript. This work was supported by Cancer Research UK, the Wellcome Trust, and an ERC Starting Grant to E.A.M. and by NIH grant GM083048 to S.A.

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