Reduced Insulin/IGF-1 Signaling Restores Germ Cell Immortality to *Caenorhabditis elegans* Piwi Mutants

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SUMMARY

Defects in the Piwi/piRNA pathway lead to transposon desilencing and immediate sterility in many organisms. We found that the *C. elegans* Piwi mutant *prg-1* became sterile after growth for many generations. This phenotype did not occur for RNAi mutants with strong transposon-silencing defects and was separable from the role of PRG-1 in transgene silencing. Brief periods of starvation extended the transgenerational lifespan of *prg-1* mutants by stimulating the DAF-16/FOXO longevity transcription factor. Constitutive activation of DAF-16 via reduced *daf-2* insulin/IGF-1 signaling immortalized *prg-1* strains via RNAi proteins and histone H3 lysine 4 demethylases. In late-generation *prg-1* mutants, desilencing of repetitive segments of the genome occurred, and silencing of repetitive loci was restored in *prg-1*; *daf-2* mutants. This study reveals an unexpected interface between aging and transgenerational maintenance of germ cells, where somatic longevity is coupled to a genome-silencing pathway that promotes germ cell immortality in parallel to the Piwi/piRNA system.

INTRODUCTION

Somatic cells accumulate stress that limits proliferation within a single generation, whereas germ cells are effectively immortal as they proliferate from one generation to the next. Genetic studies of *C. elegans* have revealed that telomerase-mediated telomere maintenance is essential for germ cell immortality (Meier et al., 2006) and that several histone modification enzymes contribute to germline maintenance over generations (Andersen and Hor-

vitz, 2007; Buckley et al., 2012; Katz et al., 2009; Xiao et al., 2011). Deficiency for telomerase in humans is likely to contribute to proliferative aging of somatic cells (Armanios and Blackburn, 2012), and other pathways that promote germ cell immortality could reveal new connections between the germline and somatic aging, or could be specific to germ cells.

Piwi is an Argonaute protein that associates with a diverse class of small RNAs that are abundant in germ cells termed Piwi-interacting RNAs (piRNAs) (Juliano et al., 2011). Conserved functions for Piwi include suppression of transposons and self-renewal of germline or meristematic stem cells. Deficiency for Piwi and Piwi-like genes in *Drosophila*, *Arabidopsis*, and vertebrate males results in immediate sterility (Juliano et al., 2011). Further, mating of *Drosophila* females that lack piRNAs targeting a transposon class with males that possess the transposon yields F1 progeny with a temperature-sensitive embryonic lethal phenotype termed hybrid dysgenesis, accompanied by transposon-induced genome instability (Juliano et al., 2011; Kidwell et al., 1977). Hybrid dysgenesis may be related to the strong immediate sterility phenotype that is accompanied by large-scale desilencing of transposons in Piwi mutants.

C. elegans has two closely related Piwi homologs, PRG-1 and PRG-2, but only deficiency for PRG-1 has phenotypic consequences (Bagijn et al., 2012; Batista et al., 2008; Das et al., 2008). PRG-1 is expressed in germ cells, and *prg-1* mutants were previously reported to display temperature-sensitive sterility accompanied by transposition of the Tc3 transposon, but not other DNA transposons (Bagijn et al., 2012; Batista et al., 2008; Das et al., 2008). These phenotypes could conceivably be related to hybrid dysgenesis in *Drosophila* (Juliano et al., 2011; Kidwell et al., 1977). In addition, PRG-1 was recently shown to initiate silencing of foreign transgenes (Ashe et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012), though silencing is then maintained by a number of factors, including small interfering RNA proteins that are responsible for silencing active transposons in *C. elegans*.
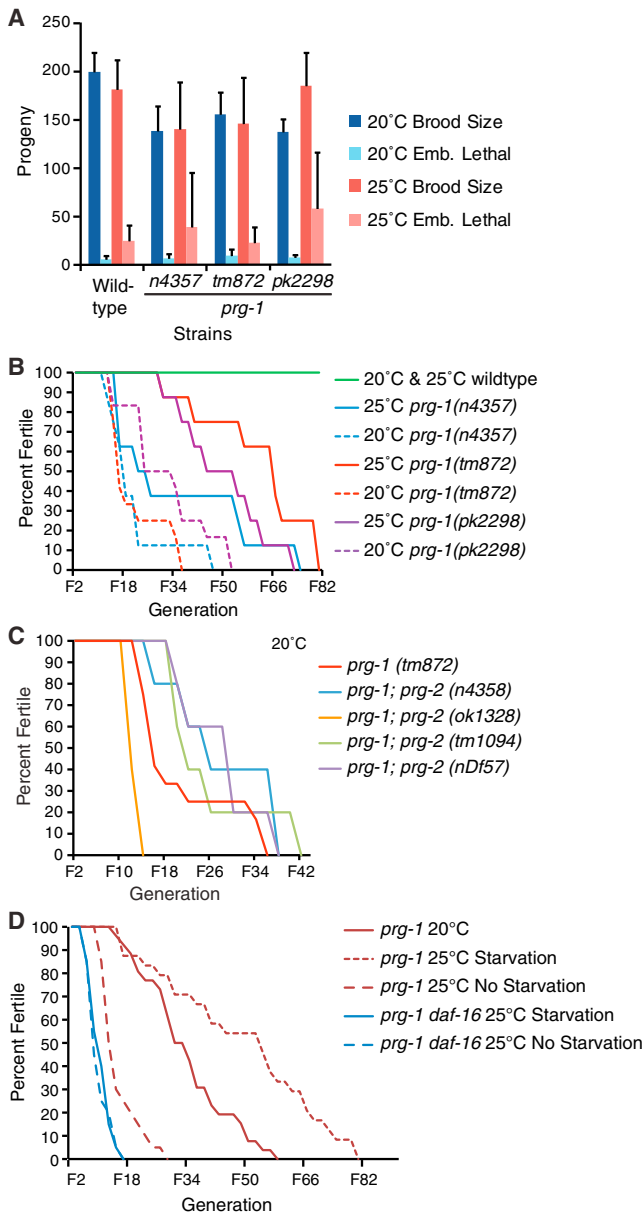
Figure 1. Progressive Sterility of *prg-1* Mutants

(A) Levels of fertility for different *prg-1* mutants at 20°C and 25°C (mean ± SD; n = 10 lines per strain). Individuals from independently derived lines were singled and their progeny counted. Error bars represent SD.

(B) *prg-1* exhibits progressive sterility at both 20°C and 25°C. Animals at 25°C appear more robust than siblings at 20°C (Mantel-Cox log rank test; p = 0.001 for *tm872*; p = 0.030 for *pk2298*; p = 0.144 for *n4357*; n = 12).

(C) Progressive sterility of *prg-1(tm872)*; *prg-2* double mutants (n = 5 strains per genotype).

(D) Starvation extends transgenerational lifespan of *prg-1* strains propagated at 25°C. Extension by starvation is dependent on *daf-16*.

Here, we report that outcrossed *prg-1* mutants display a previously unrecognized Piwi phenotype: transgenerational replicative aging of germ cells. The germ cell immortality function of Piwi occurs at multiple temperatures, is separable from its role in transgene silencing, and is not observed for strains that display high levels of transposition. Reduced *daf-2*/insulin/insulin growth factor 1 (IGF-1) signaling, which extends somatic lifespan in a variety of species (Kenyon, 2010), restores germ cell immortality to *prg-1* mutants by activating an endogenous RNAi pathway that silences repetitive loci. Together, our results place the stem cell self-renewal function of Piwi in the context of transgenerational replicative lifespan of germ cells, suggesting a heritable epigenetic factor that could regulate the rate of aging in stem cells.

RESULTS

Deficiency for *prg-1* Results in Progressive Sterility

To study the effects of Piwi on fertility in *C. elegans*, we backcrossed three alleles of *prg-1* and four alleles of *prg-2* (Batista et al., 2008; Wang and Reinke, 2008), thereby removing unlinked mutations and/or epigenetic effects of the parental backgrounds. In contrast to previous findings (Bagijn et al., 2012; Batista et al., 2008; Das et al., 2008), we did not generally observe strong defects in fertility at high temperature for outcrossed *prg-1* mutants. Instead, slightly reduced brood sizes occurred for maternally depleted F3 *prg-1* homozygotes at both 20°C and 25°C in comparison to N2 wild-type controls (Figure 1A), and a minority of *prg-1* mutants displayed >80% embryonic lethality at 25°C (Figures 1A and S1A). We saw robust levels of fertility during propagation of *prg-1* and *prg-2* for eight generations at either 20°C or 25°C based on mortal germline (Mrt) assays, where six L1 larvae were transferred to freshly seeded plates once per week (Ahmed and Hodgkin, 2000; Meier et al., 2006). No strain displayed even a moderate reduction in fertility during this period, based on complete consumption of the *E. coli* lawn (Ahmed and Hodgkin, 2000; Meier et al., 2006). Continued propagation of *prg-1*, but not *prg-2*, mutants resulted in drops in fertility and ultimately complete sterility (Figures 1B and S1B). This drop in fertility occurred both at 20°C and 25°C. Deficiency for three of four *prg-2* alleles failed to exacerbate the fertility defects of *prg-1* (Figure 1C). Although accelerated sterility was observed for *prg-1*; *prg-2(ok1328)* double mutants, this likely reflects an effect of a background mutation linked to *ok1328*. Thus, loss of function of *prg-1* leads to progressive loss of fertility over several generations, independent of *prg-2*.

We confirmed that *prg-1* deficiency was the cause of progressive sterility in the above experiments with a rescuing transgene that expresses wild-type PRG-1 (Figure S1C) (Bagijn et al., 2012; Batista et al., 2008; Das et al., 2008). Some Argonaute proteins, including PRG-1, contain "slicer" domains that possess nuclease activity. We therefore tested a transgene that expresses slicer-dead PRG-1 (Bagijn et al., 2012; Batista et al., 2008; Das et al., 2008), which also rescued the progressive sterility phenotype caused by *prg-1* deficiency (Figure S1C). Consistently, slicer nuclease activity is not required for piRNA-dependent genome silencing by PRG-1 (Bagijn et al., 2012; Batista et al., 2008; Das et al., 2008).

Deficiency for *daf-2* Suppresses Progressive Sterility of *prg-1* Mutants

Although *prg-1* mutants have been previously reported to display sterility at 25°C (Bagijn et al., 2012; Batista et al.,

2008; Das et al., 2008), we found that outcrossed *prg-1* mutants became sterile more slowly at 25°C (48.4 ± 4.3 generations to sterility) than at 20°C (24.9 ± 2 generations to sterility; p = 3.38 × 10^{-5}; Mantel-Cox log rank test; n = 40 strains per genotype) (Figure 1B). Thus, growth at high temperature doubled the transgenerational lifespan of *prg-1* mutants (the mean number of generations that *prg-1* mutant strains reproduce prior to becoming sterile). The term "transgenerational lifespan" reflects the proliferative capacity of germ cells across generations and was inspired by studies of "replicative lifespan" that assess cellular aging in yeast and mammalian cells (Polymenis and Kennedy, 2012; Smelick and Ahmed, 2005). In contrast, "adult lifespan" concerns aging that occurs in a single generation (Kenyon, 2010).

While studying the transgenerational lifespan of *prg-1* mutants, we noticed that propagation at 25°C led to frequent starvation of the plates prior to transfer, which was rare at 20°C. We therefore repeated the assay ensuring that *prg-1* strains did not starve at 25°C and found that this eliminated the extension of transgenerational lifespan that was observed for *prg-1* mutants that starve transiently (Figure 1D). Typically, *C. elegans* stocks are subjected to long periods of starvation and are infrequently outcrossed. We hypothesize that these conditions suppress the Mrt phenotype of *prg-1* mutants and instead cause a distinct and possibly related epigenetic defect that is manifest as temperature-sensitive sterility (Batista et al., 2008; Wang and Reinke, 2008).

Many effects of starvation in *C. elegans* are triggered by activation of the DAF-16/FOXO transcription factor that promotes stress resistance and longevity (Kenyon, 2010; Lin et al., 1997; Ogg et al., 1997). We therefore constructed *prg-1 daf-16* double mutants and found that their transgenerational lifespan was not extended by starvation (Figure 1D), implying that transient activation of DAF-16 in response to weekly bouts of starvation extends the transgenerational lifespan of *prg-1* mutants. We next constitutively activated DAF-16 using three independent alleles of *daf-2*, which encodes the sole *C. elegans* homolog of mammalian insulin or IGF-1 receptors and negatively regulates DAF-16 (Kimura et al., 1997). We found that *daf-2* mutations strongly suppressed the progressive sterility phenotype of *prg-1*. Remarkably, almost all *prg-1; daf-2* double mutant strains could be propagated indefinitely (n = 53/54 total) (Figures 2A and S2A).

daf-2 mutations promote stress resistance and dauer formation through DAF-16/FOXO (Kenyon, 2010). *prg-1 daf-16; daf-2* triple mutants became progressively sterile, indicating that *daf-2* deficiency suppresses the fertility defects of *prg-1* by activating DAF-16 (Figure 2B). Transgenerational lifespan of *prg-1* was reduced by ∼30% for *prg-1 daf-16* or *prg-1 daf-16; daf-2* strains (p = 5.05 × 10^{-3} and 1.56 × 10^{-3}, respectively; Mantel-Cox log rank test; Figure 2B). We confirmed these observations using independent mutations in *daf-18*, which functions upstream of DAF-16 to promote longevity in response to reduced DAF-2 signaling (Ogg and Ruvkun, 1998), and found that *prg-1; daf-18* double mutants also displayed shortened transgenerational lifespans (Figure 2B). Neither *daf-16* nor *daf-18* single mutants become sterile in Mortal Germline assays (Ahmed, 2006).

Reduced *daf-2* activity is associated with an enhanced response to exogenous RNAi and a soma-to-germline transformation (Curran et al., 2009; Wang and Ruvkun, 2004). However, these phenotypes also occur when *lin-15B* is deficient (Wang et al., 2005), and *lin-15B* did not suppress progressive sterility of *prg-1* (Figure 2B).

Together, our data reveal a specific response downstream of inactivation of *daf-2* that allows animals to remain fertile in the absence of *prg-1*. As *prg-1; daf-2* lines can be propagated indefinitely, we wondered whether these animals accumulate defects that cause them to become sterile immediately when *daf-2* activity is restored—in other words, whether *daf-2* protects against accumulation of damage or simply allows animals to tolerate high levels of damage. To test this, we crossed late-generation *prg-1; daf-2* double mutants with early-generation *prg-1* single-mutant males and selected 16 *prg-1* −/−; *daf-2* +/+ lines descended from *prg-1* −/−; *daf-2* +/− F1 animals (Figure S2E). These lines did not become sterile immediately, but instead became progressively sterile in a manner similar to *prg-1* single mutants (Figure 2C). Thus, *daf-2* deficiency directly prevents damage accumulation in *prg-1* mutants.

Progressive Sterility of *prg-1* Does Not Result from Transposition

Having established that *daf-2* represses the source of progressive sterility of *prg-1* directly, we next wished to consider what the initial source of the damage might be. A conserved function of the Piwi/piRNA pathway is suppression of transposons (Juliano et al., 2011). The temperature-sensitive hybrid dysgenesis phenotype of *Drosophila* is caused by deficiency for a set of piRNAs, which elicits a form of sterility accompanied by high levels of transposon expression and activity (Kidwell et al., 1977). Thus, *prg-1* mutants could become sterile as a consequence of transposition (Batista et al., 2008; Das et al., 2008). We carried out comparative genomic hybridization arrays to assay for increased transposon copy number in late-generation *prg-1* mutants and found that indeed there was overall slightly increased transposon DNA in late-generation *prg-1* compared to early generation (Figures S2B and S2D), which was not observed for genes and simple repetitive regions (Figure S2C). However, several lines of evidence suggest that this is unlikely to explain the *prg-1* fertility defects. First, inspection of many independent late-generation *prg-1* strains failed to reveal frequent de novo mutations that cause visible phenotypes, which are readily observed for *C. elegans* strains with weak (10-fold) increases in the frequency of spontaneous mutation (Harris et al., 2006). To confirm this, forward mutation assays were conducted using *unc-54(r293)*, which can be suppressed by mutation of any of seven *smg* genes, and using *unc-58(e665)*, which can be suppressed by mutations in two loci (Harris et al., 2006). The frequency of forward mutation for *prg-1 unc-54(r293)* or *prg-1; unc-58(e665)* in either early-generation (F4) or late-generation strains very close to sterility (F24) was comparable to *unc-54(r293)* and *unc-58(e665)* single-mutant controls (Figures 2D and 2E; Table S1). This rules out an increased spontaneous mutation frequency in *prg-1* mutants. Second, RNAi mutants *rde-2* or *mut-2*, which are known to confer elevated levels of transposon mobility (Ketting and Plasterk, 2000; Tabara
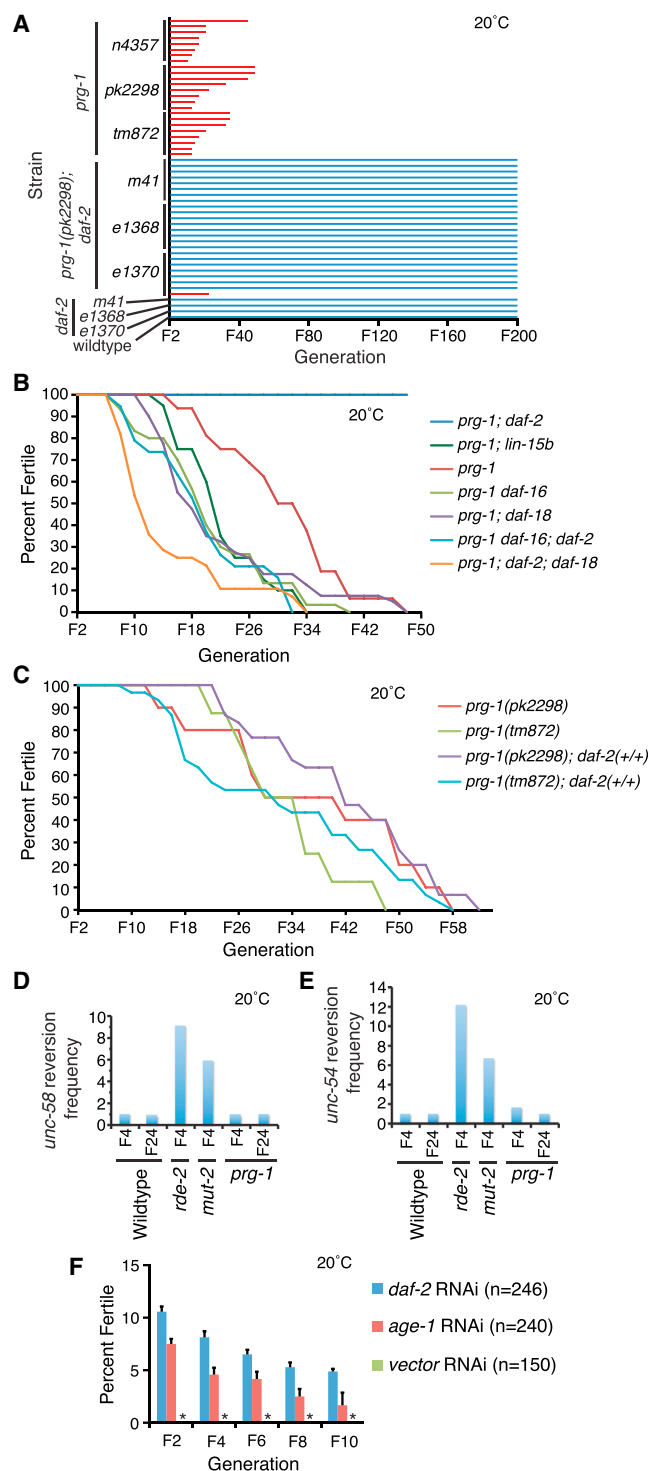
Figure 2. *daf-2* Signaling Can Suppress Fertility Defects of *prg-1* and Modulates Germline Remodeling at Sterility

(A) Reduced *daf-2* signaling suppresses *prg-1*-mediated progressive sterility.

(B) *lin-15B* does not suppress transgenerational lifespan of *prg-1* (n = 30 strains per genotype). *daf-16* and *daf-18* are required for suppression of *prg-1* by *daf-2*. Four *prg-1 daf-16* double-mutant strains were studied where *prg-1* alleles *tm872* and *n4357* were combined with *daf-16* alleles *mgDf50* or *mu86* (n = 10 per genotype). Four *prg-1; daf-18* double-mutant strains were

et al., 1999), did not become sterile when propagated at 20°C, despite causing ∼6- to 12-fold increases in the frequency of spontaneous mutation (Figures 2D and 2E; Table S1). Third, we did not observe any evidence of chromosomal instability based on oocyte chromosome counts in late-generation *prg-1* animals compared to either early-generation *prg-1* or wild-type animals (Figure S1D) (Ahmed and Hodgkin, 2000).

Finally, if the *prg-1* defects were caused by increased transposition or associated genomic instability, we would not expect them to be reversible. We therefore treated late-generation sterile *prg-1* adults with RNAi against either *daf-2* or *age-1*, both of which negatively regulate *daf-16*, and found that RNAi of either gene allowed some infertile adults to produce viable offspring (Figure 2F). Further, fertility could be maintained for at least ten additional generations by maintaining lines with RNAi treatment. The reversibility of the sterility phenotype makes it highly unlikely that sterility is caused by accumulation of deleterious transposon insertions. Our results instead suggest that the sterility phenotype of *prg-1* mutants is likely due to accumulated epigenetic changes that are reversible.

Transgene Silencing Is Separable from Germ Cell Immortality

PRG-1 can initiate silencing of foreign transgenes in *C. elegans*, which is then maintained by nuclear silencing proteins and by the mutator-class secondary small interfering RNA (siRNA) biogenesis protein MUT-7 (Ashe et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). We confirmed that mutator-class genes *rde-2* and *mut-2* were required for transgene silencing (Figures S2F–S2K). Outcrossed *mut-2*, *mut-7*, and *rde-2* mutants are not Mrt (Figure 5F), implying that silencing of foreign transgenes may not be linked to germ cell mortality of *prg-1* mutants. Direct evidence for a distinction between transgene silencing and the Mrt phenotype of *prg-1* was obtained using a silent transgene placed in a *prg-1* mutant background (Ashe et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012), which remained silent in sterile late-generation adults (Figures S2N and S2O).

Disrupted Silencing of Repetitive Loci in *prg-1* Mutants

PRG-1 interacts with roughly sixteen thousand 21-nucleotide (nt) RNAs possessing 5′ uracil that represent the *C. elegans* piRNA

studied where *prg-1* alleles *tm872* and *n4357* were combined with *daf-18* alleles *e1375* and *ok480* (n = 10 strains per genotype). For *prg-1 daf-16; daf-2* triple mutants, *prg-1*(*tm872*) *daf-16*(*mgDf50*) and *prg-1*(*n4357*) *daf-16*(*mu86*) were each combined with three *daf-2* alleles *m41*, *e1368*, and *e1370* (n = 10 strains scored per genotype) and examined for progressive sterility. Four *prg-1; daf-2*(*e1368*); *daf-18* lines were constructed from the four allelic combinations of *prg-1; daf-18* and examined for progressive sterility (n = 10 strains scored per genotype). Data for all independent alleles were combined to show transgenerational lifespan for strains of the same genotype.

(C) Lack of immediate sterility upon removal of *daf-2* suggests suppression of the heritable epigenetic defect that causes sterility in *prg-1* mutants. *prg-1; daf-2*(+/+) strains represent cross-progeny of late-generation *prg-1; daf-2* double mutants where the *daf-2* mutation has been removed.

(D and E) Levels of spontaneous mutation assessed by reversion frequencies of *unc-58* and *unc-54*.

(F) Treatment of sterile late-generation *prg-1* adults with *daf-2* or *age-1* RNAi restores fertility, and fertility can be maintained on these RNAi strains for at least ten generations while RNAi is maintained. Error bars represent SD.
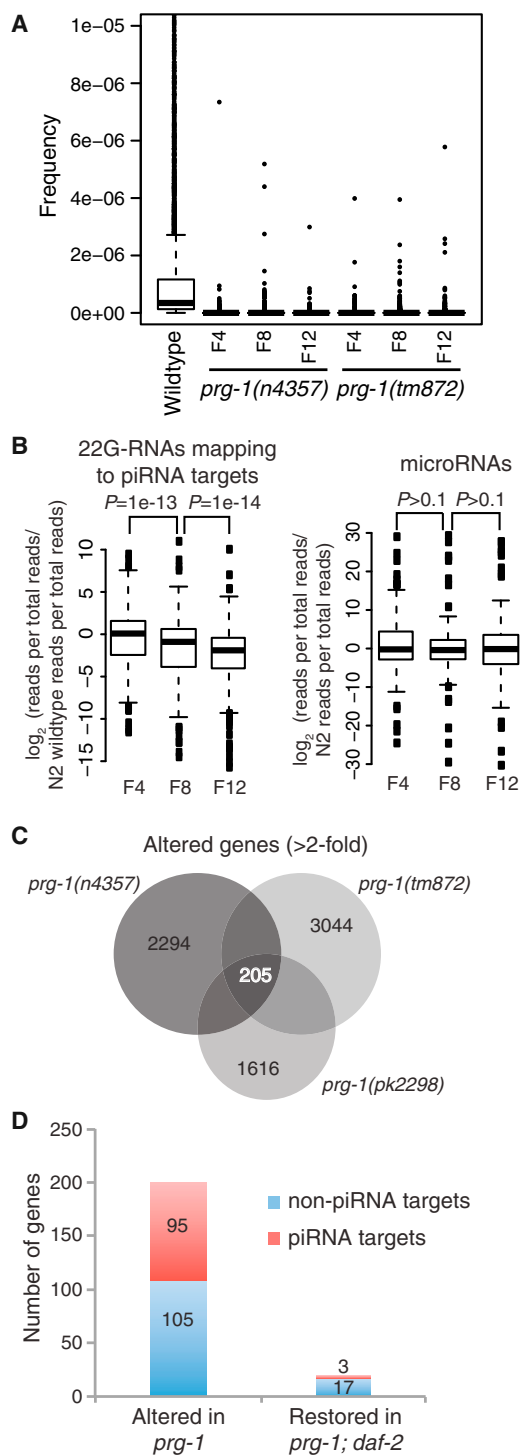
Figure 3. piRNA Loss Affects Expression of Few Genes Targeted by piRNAs

(A) Box plots are based on read frequencies for 4,839 piRNAs sequenced in one or more libraries. Boxes indicate interquartile ranges, horizontal bars medians, whiskers extend to the most extreme data points with distance from the box no more than 1.5 times the interquartile range, and crosses indicate outliers.

(B) Box plots showing reduced median levels of 22G-RNAs for piRNA targets in later generation *prg-1* animals. Levels of microRNAs do not

repertoire and silence many segments of the genome (Bagijn et al., 2012; Batista et al., 2008; Das et al., 2008). PRG-1 has been implicated previously in small-RNA-mediated silencing pathways (Batista et al., 2008; Das et al., 2008) and indeed can trigger transgenerational silencing that can persist in the absence of *prg-1* (Ashe et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). Therefore, to search for possible determinants of the epigenetic defects responsible for sterility, we examined the functional consequences of *prg-1* deficiency on small-RNA-mediated silencing pathways. We searched for changes in small RNAs or their targets that would become more severe over continued propagation and could be suppressed by deficiency in *daf-2*.

In *Drosophila*, piRNAs can be inherited through the female germline (Brennecke et al., 2008). We therefore asked first whether a progressive loss of piRNAs occurred during propagation of *prg-1* mutants. We prepared small RNA libraries from wild-type animals and *prg-1* mutant animals at different generations. We found that piRNAs are essentially absent from *prg-1* strains at generations 4 and 8, as well as generation 12, which was close to sterility for both alleles examined (Figure 3A). As piRNA annotation is based on a genomic motif likely associated with piRNA biogenesis (Ruby et al., 2006), the few remaining small RNAs that fit our piRNA criteria are likely misannotated rather than persistent piRNA species. Progressive loss of piRNAs is therefore unlikely to explain the delay in loss of fertility in *prg-1* animals.

Another possible cause of transgenerational sterility might be progressive changes in gene expression due to loss of piRNA-mediated silencing. In *C. elegans*, 21 nt piRNAs silence their targets by engaging an endogenous secondary siRNA-silencing pathway that utilizes so called 22G-RNAs that are 22 nt in length and possess a 5′ guanine nucleotide (Bagijn et al., 2012). For genes targeted by piRNAs, we observed a progressive reduction in the number of 22G-RNA reads, normalized to library size, mapping to these genes in later generation *prg-1* animals (Figure 3B). No such progressive reduction was seen in microRNA levels. We tested the hypothesis that expression of 22G-RNA target genes might be improperly regulated by preparing RNA from early- and late-generation *prg-1* strains and using cDNA created from this RNA to perform tiling microarrays that interrogate the entire *C. elegans* genome. Analysis of microarrays for three independent alleles of *prg-1* showed little consistent change in the expression of genes targeted by 22G-RNAs (data not shown). Instead, we identified a set of 205 genes whose expression changed by more than 2-fold in late-generation *prg-1* strains (Figure 3C), an intersection significantly greater than that predicted by 1,000 simulations of random overlap (Z = 35), and all but three of these genes changed in the same direction in all alleles, more than 10-fold greater than expected by chance (p < 2 × 10^{-16}; χ^2 test; two-tailed). However, the expression changes of only 20 of the 205 genes were significantly reduced by more than 1.4-fold (0.5 log$_2$ units) in late-generation *prg-1*;

show the same progressive reduction. p values are for Wilcoxon signed rank tests.

(C) Analysis of genes whose expression changed more than 2-fold in late- versus early-generation *prg-1* strains reveals few common genes are altered for *prg-1* alleles.

(D) Few altered genes that are cured by *daf-2* are predicted piRNA targets.
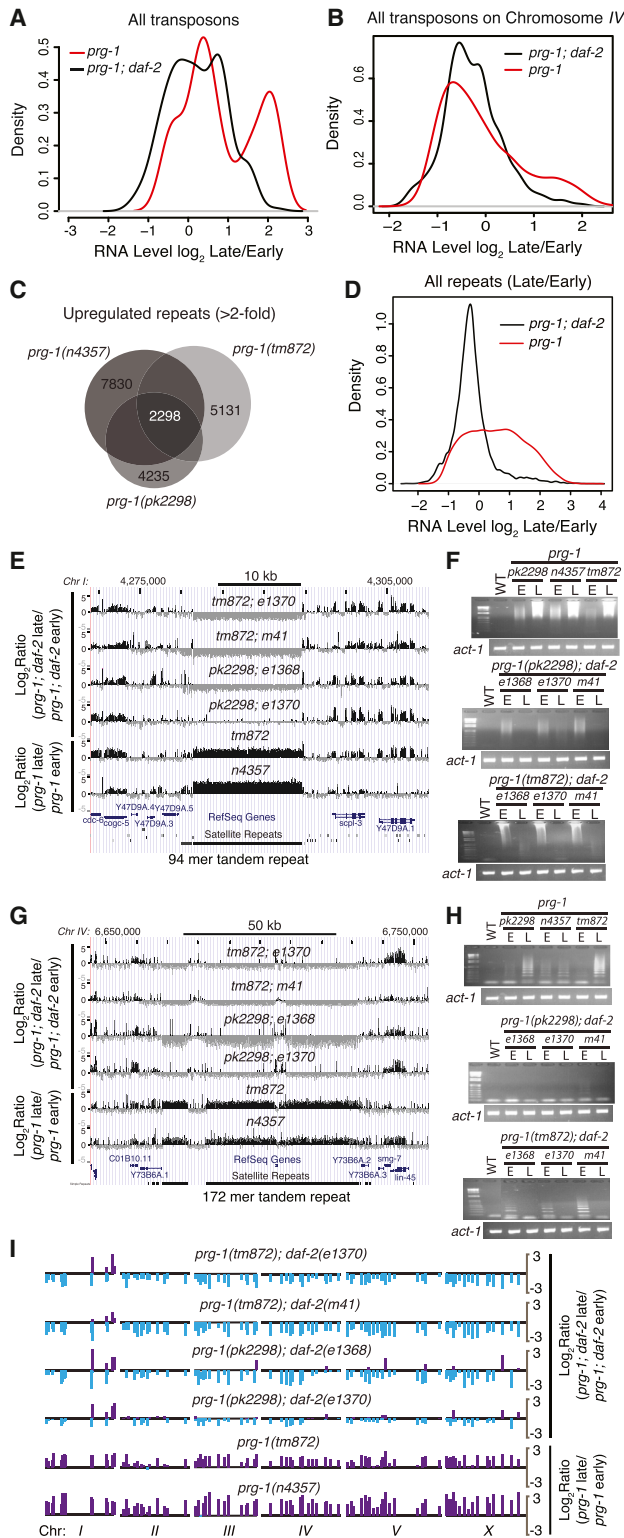
Figure 4. Derepression of Repetitive Elements in *prg-1* Mutants Is Cured by *daf-2*

(A) Late-generation *prg-1* mutants show a mean increase in transposon expression, compared to the expression of transposons in *prg-1; daf-2* double mutants.

daf-2 mutants (p < 0.05; Student's t test; two sided; log scale), and only three were piRNA targets (Figures 3D and S3; Table S3), implying that *daf-2* deficiency does not suppress the fertility defects of *prg-1* mutants by restoring piRNA-dependent gene-expression changes.

As mentioned above, piRNAs in a number of organisms, including *C. elegans*, have been shown to target transposons for silencing (Bagijn et al., 2012; Batista et al., 2008; Das et al., 2008; Juliano et al., 2011). Thus, the epigenetic defect of *prg-1* mutant germ cells could be due to derepression of repetitive genomic loci that are targeted by PRG-1 piRNAs. Using genome-wide tiling arrays, we observed an increase in the expression of a subset of transposons in late-generation *prg-1* mutants (Figures 4A and 4B). The most upregulated were the mariner-class transposons, including the previously characterized piRNA target Tc3 (Figure S4A; Bagijn et al., 2012; Batista et al., 2008; Das et al., 2008). Intriguingly, we also observed increased expression of both simple repeat regions and tandem repeat tracts (hereafter referred to as simple repeats) across the genome in late generations for three different alleles of *prg-1*, with the overlap between upregulated repeat tracts highly statistically significant relative to random simulation. The statistical significance of this overlap in simple repeats was also significantly larger than for genes (Z = 214 for simple repeats; Z = 35 for genes) (Figures 3C and 4C). This was not accompanied by increased DNA copy number from these regions (Figures S2B–S2D). In contrast to the general lack of suppression of gene-expression changes by *daf-2* mutation, changes in expression of repetitive regions, including transposons and simple repeats, were all robustly suppressed for four different allelic combinations of *prg-1; daf-2* (Figures 4D–4I, S4D, and S4E) (p < 2 × 10^{-16}; two-sided paired t test), including most of the 101 longest tandem repeat tracts found in the *C. elegans* genome (Figure 4I). Further, reduced levels of tandem repeat RNA were observed for independent repetitive loci for lines derived from sterile *prg-1* mutant adults whose fertility was restored by *daf-2* RNAi (Figure S4F). Thus, the expression of repetitive elements rather than the increased expression of genes might be a crucial factor in the acquisition of the sterility phenotype.

We asked whether progressive loss of 22G-RNAs might account for loss of silencing of transposons and tandem repeats.

(B) Density plot of the transposon expression changes shown in (A) reveal increased expression of a subset of transposon sequences in late-generation *prg-1* animals that is not seen in *prg-1; daf-2* double mutants.

(C) Simple repeats are upregulated in late-generation *prg-1* mutants.

(D) Simple repeats upregulated in *prg-1* mutants are repressed in late-generation *prg-1; daf-2* mutants.

(E–H) cDNA prepared from RNA was hybridized to microarrays and revealed upregulation of tandem repeat tracts expression in late-generation *prg-1* mutants, but not in late-generation *prg-1; daf-2* mutants (E and G), as confirmed by RT-PCR analysis for late-generation wild-type as well as early (E)- and late (L)-generation mutant strains (F and H). (F) and (H) show expression of tandem repeats corresponding to (E) and (G), respectively. WT, wild-type.

(I) Genome-wide plots of 101 longest tandem repeat tracts defined by visually scanning the *C. elegans* genome in 70 kb sliding windows. Typically, tandem repeats display increased expression in late-generation *prg-1(tm872)* and *prg-1(n4357)* single mutants but silencing in *prg-1* double mutants with *daf-2* alleles *e1368*, *e1370*, or *m41*.
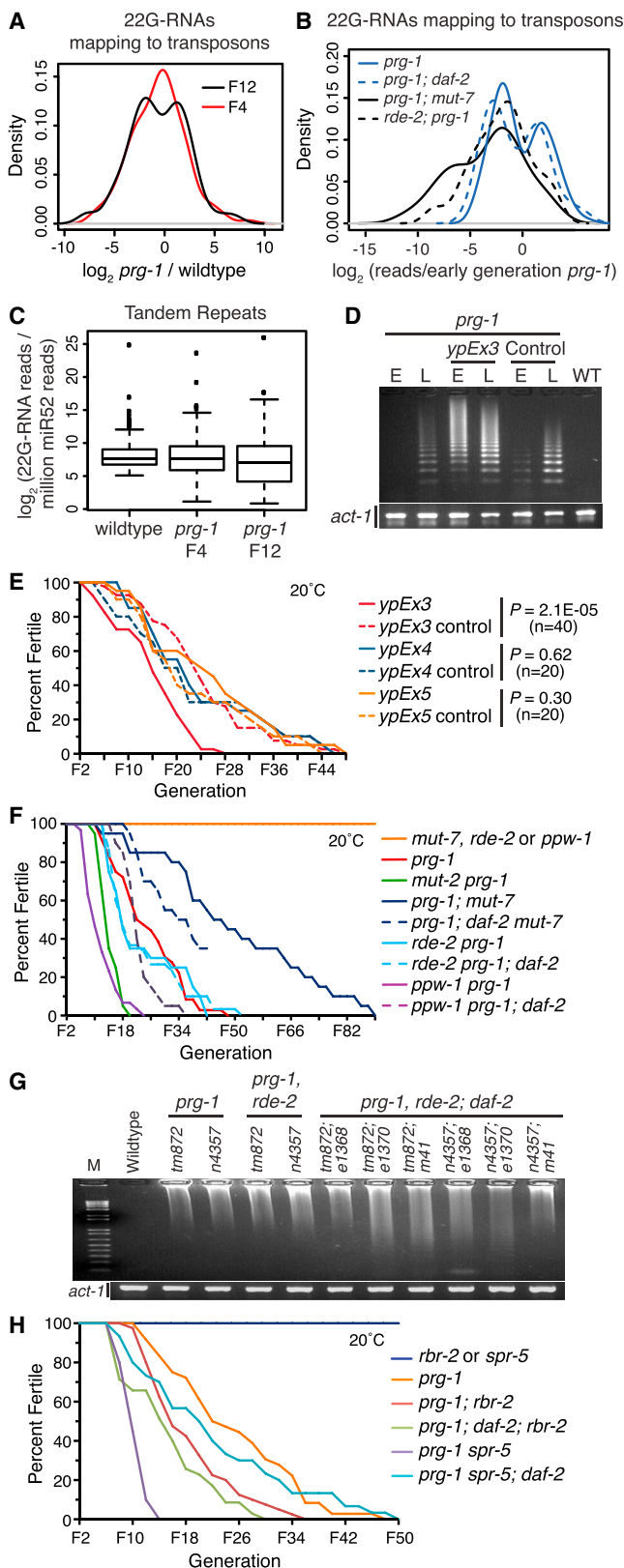
Figure 5. Altered 22G-RNA Frequencies and Gene-Expression Changes in *prg-1* Mutants

(A) 22G-RNAs targeting transposons in *prg-1* versus wild-type strains.

(B) Increased 22G reads in late-generation *prg-1* strains mapping to transposons are reduced in *prg-1* mutants deficient for the mutator pathway genes *rde-2* or *mut-7*.

(C) 22G-RNAs targeting tandem repeats in *prg-1* versus wild-type strains. Note that it is easier to clearly identify transposon 22G-RNAs, because many permutations of each tandem repeat are found, so tandem repeat 22G-RNA data are almost certainly underestimated. Boxes indicate interquartile ranges, horizontal bars medians, whiskers extend to the most extreme data points with distance from the box no more than 1.5 times the interquartile range, and crosses indicate outliers.

(D) RT-PCR reveals increased expression of 174-mer tandem repeat for strains carrying the *ypEx3* extrachromosomal array in comparison to sibling control strains lacking this array or *prg-1* single-mutant controls.

(E) Repetitive extrachromosomal arrays containing *CeRep59* and a 174-mer tandem repeat (*ypEx3* array) or a histone locus cluster containing *his-13*, *his-14*, *his-15*, and *his-16* genes (*ypEx4* array) or a *Helitron* transposon (*ypEx5* array) reveal that *ypEx3* array accelerates the progressive sterility phenotype of *prg-1*.

(F) *rde-2* and *ppw-1* are required for suppression of *prg-1* by *daf-2*. Two *ppw-1 prg-1* double-mutant strains were studied where *prg-1* alleles *tm872* and *n4357* were combined with *ppw-1(pk1425)* (n = 10 strains per genotype). Four *ppw-1 prg-1*; *daf-2* triple mutants were studied where both *ppw-1 prg-1* mutants were combined with *daf-2* alleles *e1370* or *e1368* (n = 5 strains per genotype). Two *rde-2 prg-1* double-mutant strains were studied where *prg-1* alleles *tm872* and *n4357* were combined with *rde-2(ne221)* (n = 10 strains per genotype). Six *rde-2 prg-1*; *daf-2* triple mutants were studied where both *rde-2 prg-1* mutants were combined with *daf-2* alleles *e1370*, *e1368*, and *m41* (n = 5 strains per genotype).

(G) *rde-2* is required for restoration of tandem silencing in *prg-1* mutants by *daf-2*.

(H) *rbr-2* and *spr-5* are required for suppression of *prg-1* by *daf-2*. Four *prg-1*; *rbr-2* double-mutant strains were studied where *prg-1* alleles *tm872* and *n4357* were combined with *rbr-2* alleles *ok2544* or *tm1231* (n = 10 strains per genotype). Eight *prg-1*; *daf-2*; *rbr-2* triple mutants were studied where each *prg-1*; *rbr-2* mutant was combined with *daf-2* alleles *e1370* or *e1368* (n = 5 strains per genotype). Two *prg-1 spr-5* double-mutant strains were studied where *prg-1* alleles *tm872* and *n4357* were combined with *spr-5(by134)* (n = 10 strains per genotype). Three *prg-1*; *daf-2*; *rbr-2* triple mutants were studied where each *prg-1*; *rbr-2* mutant was combined with *daf-2(e1370)* and *prg-1(n4357) spr-5* was combined with *daf-2(e1368)* (n = 10 strains per genotype).

Overall levels of secondary 22G-RNAs mapping with up to two mismatches to transposon consensus sequences, normalized to the levels of a somatic microRNA, were reduced in early-generation *prg-1* strains (p = 0.0008; one sample t test; two sided) (Figures 5A and S4G). In later generations, 22G-RNAs targeting some transposons were further reduced, whereas 22G-RNAs for other transposon classes were restored to high levels, reflected by an apparently bimodal distribution of read differences relative to N2 wild-type (Figure 5A). Transposons with increased 22G-RNAs in late-generation *prg-1* animals were enriched for the Tc4 family of transposons, whereas transposons with reduced 22G-RNAs were enriched for the mariner family (p < 0.05; Fisher's exact test; Figures S4B and S4C). Transposons showing increased 22G-RNAs in late-generation *prg-1* animals were accompanied by increased 22G-RNA to 22A-RNA ratios (Figure S4H), suggesting that these increases are not due to degraded transposon RNA. Moreover, RNA levels of transposons showing increased 22G-RNAs tended to show smaller increases in late-generation *prg-1* than those with reduced

Table 1. Effect of *daf-2* on Transgenerational Alterations in 22G-RNA Levels in *prg-1*

	2-Fold Down Late *prg-1* versus Early *prg-1*	Suppressed *daf-2*	Significance (Fisher's Exact Test)	Suppressed Dependent on *rde-2*
Transposons	54	4	1.00	4
Genes	356	71	5.80×10^{-29}	50
Simple repeats	497	92	1.66×10^{-12}	56

22G-RNAs (p < 0.05; two-tailed unpaired t test; Figure S4I), suggesting that the increased 22G-RNAs contributed to repression of their targets. The bimodal distribution was suppressed in double mutants lacking both *prg-1* and either *mut-7* or *rde-2/mut-8*, which encode mutator/RNAi proteins that target transposons for silencing; thus, the increased small RNAs against transposons in late-generation *prg-1* are dependent on *mut-7* and *rde-2/mut-8* (Figure 5B) (Ketting et al., 1999; Tabara et al., 1999). This suggests that, in the absence of *prg-1*, an alternative silencing pathway dependent on the mutator proteins is induced, which silences the Tc4 family of transposons in particular while leaving mariner transposons with reduced levels of secondary siRNAs.

Outcrossed *mut-7* and *rde-2* mutator mutants are not Mrt (Figure 5F), despite being required for general amplification of 22G-RNAs in response to exogenous and endogenous primary siRNAs (Zhang et al., 2011). By comparing the abundance of 22G-RNAs from the *prg-1* and *mut-7* single mutants with *prg-1*; *mut-7* double mutants, the *prg-1*; *mut-7* double mutants were much more similar to the *mut-7* single mutant than to the *prg-1* single mutant (Figure S5A). Importantly, out of all the transposon consensus sequences, there were no transposons with more than five antisense 22G-RNA reads per million in *mut-7* that had fewer than five reads per million in the *prg-1* single mutant, showing that *prg-1* is unlikely to operate in parallel to *mut-7* at transposons. There were some isolated cases where there were fewer reads in *prg-1*; *mut-7* than in *mut-7*; however, because these had a larger number of reads in *prg-1*, it is not straightforward to argue that loss of these small RNAs is related to the Mrt phenotype. Overall, the vast majority of PRG-1-dependent 22G-RNAs are also dependent on MUT-7 and are dispensable for germ cell immortality.

Several upregulated repeat tracts are also predicted targets of at least one piRNA (Tables S4 and S5). We therefore examined 22G-RNAs against simple repeats in *prg-1* mutants. The number of 22G-RNAs against simple repeat regions was less in *prg-1* mutants than in wild-type animals (p = 0.002; one sample t test; two sided), and overall 22G-RNA levels decreased further in late-generation *prg-1* animals (p = 0.00026; one sample t test; two sided) (Figure 5C). Furthermore, similarly to transposons, normalized 22G-RNA levels mapping to simple repeats were more widely distributed in late-generation *prg-1* animals than in either N2 wild-type or early-generation *prg-1* animals (p = 6 × 10^{-6} to wild-type and p = 8 × 10^{-5} to early-generation *prg-1*; Kolmogorov-Smirnov test for different distributions) (Figures 5C and S5B). Again, increased 22G-RNAs were dependent

on the mutator pathway (Figure S5C). Simple repeats with increased or decreased 22G-RNA levels in late-generation *prg-1* animals were highly upregulated compared to simple repeats with weak or no change in 22G-RNA levels (Figure S5D). Taken together with the analysis of transposons, these data imply that increased expression of repetitive regions of the genome in late-generation *prg-1* mutants is accompanied by progressive dysfunction of 22G-RNAs targeting these regions, reflecting both loss of 22G-RNAs downstream of piRNAs and, potentially, upregulation of a *prg-1*-independent 22G-RNA pathway that can silence a subset of transposons and simple repeats.

We used RNAi to knock down a number of protein-coding genes (Figure S5E) and transposons (Figure S5F) that were upregulated in late-generation *prg-1* mutants, performing RNAi from early generations onward, but none repressed the sterility phenotype of *prg-1* mutants (p > 0.191) (Figures S5E and S5F). We created three repetitive extrachromosomal arrays containing either histone loci, a tandem repeat *CeRep59*, or the Helitron transposon by microinjection of *prg-1* mutants. Strikingly, an array that overexpressed *CeRep59* (*ypEx3*) shortened transgenerational lifespan (p = 2.06 × 10^{-5}), whereas Helitron transposon or histone locus arrays had no effect (p > 0.29) (Figures 5D, 5E, and S5G). Although extrachromosomal arrays can be silenced in the *C. elegans* germline by cosuppression (Dernburg et al., 2000; Ketting and Plasterk, 2000), RNA fluorescence in situ hybridization (FISH) revealed that *prg-1* strains containing *CeRep59* arrays expressed *CeRep59* RNA at ~5-fold higher levels than *prg-1* single-mutant controls in both early embryos and throughout the animals, including in germ cells (Figures S6C–S6R). We also found low levels of repetitive RNA expressed in wild-type embryos (Figures S6A and S6B), suggesting that repetitive loci are normally transiently expressed during development. These results imply that expression of repetitive loci contributes to the transgenerational sterility of *prg-1* mutants.

A Small RNA Pathway Restores Germ Cell Immortality *prg-1* Mutants

Having established that progressive loss of 22G-RNAs against tandem repeats and transposons occurred in *prg-1*, we tested whether *daf-2* might suppress increased repeat expression via siRNAs. We examined small RNA libraries from *prg-1*; *daf-2* mutants at early and late generations. As for *prg-1* single mutants, piRNAs were lost from both early- and late-generation *prg-1*; *daf-2*. We therefore examined protein-coding genes, transposons, and simple repeats showing decreased small RNAs in late-generation *prg-1* relative to early generation for suppression of transgenerational decrease by *daf-2*. The number of suppressed genes was statistically significant for 22G-RNAs mapping to genes and simple repeats, but not transposons, although individual examples of transposons in this category were found (Tables 1, S6, S7, and S8). In all cases, this suppression involved the mutator pathway, as the majority of suppressed genes, transposons, and repeats showed reduced 22G-RNA reads mapping to them in *rde-2 prg-1*; *daf-2* triple mutants compared to early-generation *prg-1* mutants (Table 1). In addition, sequencing of small RNAs from progeny of sterile *prg-1* mutants treated with *daf-2* RNAi revealed that 22G-RNAs targeting repetitive sequences were partially restored in comparison to
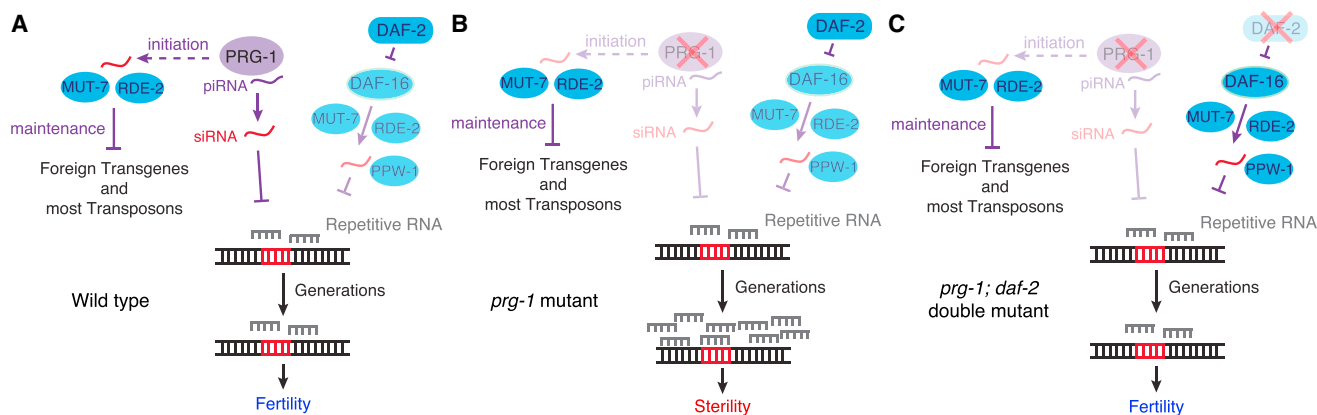
Figure 6. Model of Parallel Small-RNA-Silencing Pathways that Can Repress Transgenerational Fertility Defects
(A) Wild-type PRG-1 maintains transgenerational fertility by silencing repetitive RNA expression and functions separately to initiate silencing of foreign transgenes and most transposons.
(B) Deficiency for *prg-1* results in transgenerational desilencing of repetitive loci and sterility. Transposon and transgene silencing is maintained by mutator proteins.
(C) Increased DAF-16 signaling via *daf-2* mutation suppresses progressive sterility and desilencing of repetitive loci when *prg-1* is mutant. Mutations are indicated by a red X. Light color tints indicate pathway dysfunction for *prg-1* mutants or low levels of DAF-16 activity in response to wild-type DAF-2 signaling.

late-generation *prg-1* controls, though not to early-generation levels (p < 1 × 10^{-16} to late generation; two-tailed paired t test) (Figure S5H). We therefore hypothesized that *daf-2* may suppress activation of repetitive loci by upregulating an alternative *prg-1*-independent silencing pathway.

We therefore tested whether the suppression of *prg-1* fertility defects by *daf-2* might be dependent on small RNAs. Deficiency for the *rde-2* mutator-class gene abolished the ability of *daf-2* mutations to ameliorate the germ cell immortality defects of *prg-1* mutants (Figure 5F), and late-generation *rde-2 prg-1*; *daf-2* strains expressed high levels of repetitive RNA (Figures 5G, S5J, and S5K). Consistently, another mutator-class gene *mut-7* was vital for suppression of *prg-1* by *daf-2*, even though fertility was ameliorated for *prg-1*; *mut-7* double mutants (Figure 5F). We also found that an Argonaute protein required for efficient germline RNAi, PPW-1 (Tijsterman et al., 2002), is necessary for suppression of *prg-1* by *daf-2* (Figure 5F). We conclude that an endogenous RNA interference pathway that requires RDE-2, MUT-7, and PPW-1 can restore germ cell immortality to *prg-1* mutants (Figure 6).

Small RNA pathways can promote gene silencing by degrading RNA in the cytoplasm or silencing loci within the nucleus. The histone H3 lysine 4 (H3K4) demethylase RBR-2, which removes trimethyl H3K4 marks and promotes transcriptional silencing (Christensen et al., 2007), mediates the effects of transgenerational epigenetic marks that regulate somatic longevity in *C. elegans* (Greer et al., 2010). Further, reduced *daf-2* signaling regulates *rbr-2* expression (Lee et al., 2003). We found that *rbr-2* is required for suppression of *prg-1* by *daf-2* mutation (Figure 5H). Late-generation *prg-1*; *daf-2*; *rbr-2* strains expressed high levels of RNA from repetitive loci (Figures S5L–S5N), implying that RBR-2 demethylase suppresses the transgenerational fertility defects of *prg-1* mutants by silencing these loci in response to reduced *daf-2* signaling. We then tested a second demethylase, SPR-5, which removes dimethyl H3K4 marks.

Although one allele of *spr-5* has been reported to be Mrt at 20°C (Katz et al., 2009), we tested another null allele of *spr-5*, *by134*, which does not display fertility defects at 20°C. We then used *spr-5* to confirm that H3K4 demethylation is required for suppression of *prg-1* by *daf-2* (Figure 5H).

Taken together, therefore, these data suggest that the transgenerational silencing defects of *prg-1* can be suppressed by a small-RNA-mediated genome silencing that is activated by reduced insulin/IGF-1 signaling (Figure 6).

DISCUSSION

Here, we demonstrate that *C. elegans prg-1* is required for germ cell immortality. This phenotype shows two clear distinctions from the role of Piwi proteins in promoting fertility in other organisms. First, *prg-1* mutant animals do not become sterile immediately and indeed maintain wild-type levels of fertility for a number of generations before becoming progressively sterile. Second, we show that the sterility of *prg-1* animals is not due to increased transposition. Instead, our data support a transgenerational epigenetic cause of sterility in *prg-1* mutants.

A role for PRG-1 in transgenerational epigenetic maintenance of fertility in *C. elegans* could be relevant to recent data showing that *prg-1* acts upstream of epigenetic silencing of foreign transgenes in *C. elegans* germ cells (Ashe et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). In the case of transgenes however, the silent state is maintained independently of PRG-1 activity by proteins that mediate downstream 22G-RNA production, such as RDE-2, by nuclear RNAi factors, and by chromatin-silencing proteins (Figures 6A and 6B) (Ashe et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). Several nuclear RNAi factors were recently reported to promote germ cell immortality (Buckley et al., 2012), and PRG-1 and associated piRNAs could function upstream of these proteins to direct silencing of endogenous nuclear loci. However, we show that PRG-1 is also required

continuously for germ cell immortality, suggesting that ultimately *prg-1* is indispensable for silencing of some endogenous loci. A second contrast with transgene silencing is that mutator mutants such as *rde-2* and *mut-7*, which display a strongly reduced secondary siRNA response, are wild-type for germ cell immortality at low temperatures (20°C). These mutants also maintain silencing of repetitive loci that become derepressed in late-generation *prg-1* mutants (Figures 5F and S5I), despite being essential for maintenance of transgene silencing (Figures S2F–S2K; Table S2) (Ashe et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). Thus, continuous initiation of silencing by PRG-1/ piRNAs may be sufficient to promote germ cell immortality in the absence of an RDE-2-mediated secondary siRNA response. We present direct evidence for a distinction between transgene silencing and the Mrt phenotype of *prg-1* by showing that transgene silencing is not disrupted in sterile late-generation *prg-1* mutant adults (Figure S2N). We suggest that at least two classes of "nonself" DNA exist: recently introduced foreign transgenes, whose permanent silencing can rapidly become independent of PRG-1 (Ashe et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012), and a distinct class of loci, possibly rapidly evolving tandem repeats or a recently introduced transposable element, whose long-term silencing requires the activity of PRG-1.

Despite the continuous requirement for PRG-1 in a wild-type background for maintenance of silencing of repetitive loci (Figures 4, S4D, and S4E), an alternative PRG-1-independent silencing pathway can be activated by reduced insulin signaling (Figure 2). Analysis of small RNA populations in a variety of *prg-1* mutant backgrounds suggested that a small-RNA-silencing pathway could be the mechanism by which reduced insulin signaling suppresses the transgenerational fertility defects of *prg-1* mutants (Figures 5A–5C, S4G–S4I, and S5B–S5D). This led us to define components of an endogenous small-RNA-silencing pathway that are required for *daf-2* to suppress *prg-1* (Figures 5F and 6C; Table S2). This small RNA pathway may act upstream of a chromatin-based silencing pathway involving *rbr-2*- and *spr-5*-mediated demethylation of histone H3K4 (Figure 5H). Thus, our results indicate that activation of a small RNA genome-silencing pathway that protects germ cell immortality, in addition to its known role in initiating antiaging gene expression (Balch et al., 2008; Kenyon, 2010), is a significant consequence of *daf-2* deficiency. Piwi proteins promote silencing of transposable elements, transcriptional activation, imprinting, heterochromatin formation, and modulation of protein function via heat shock proteins (Juliano et al., 2011; Rangan et al., 2011; Watanabe et al., 2011), and our results suggest that a genome-silencing function of Piwi that is independent of suppression of many transposons and independent of most 22G-effector-silencing RNAs promotes transgenerational germ cell maintenance (Figure 6E).

We found that *daf-16* and *daf-18* mutations shortened the transgenerational lifespan of *prg-1* mutants and *prg-1*; *daf-2* double mutants (Figure 2B). Thus, basal levels of DAF-16 activity contribute to the transgenerational lifespan of *prg-1* mutants, revealing an intriguing parallel with the established role for low levels of DAF-16 activity in promoting the adult lifespan of wild-type animals (Kenyon et al., 1993; Larsen et al., 1995). Although many mutations in the small-RNA-silencing pathway that func-

tions downstream of DAF-16 to suppress deficiency for *prg-1* also resulted in reduced transgenerational lifespan when combined with *prg-1*, this effect did not occur for all such mutations (Table S2). The reason for the shortened transgenerational lifespan of *prg-1 daf-16* double mutants therefore remains uncertain.

Our observations defy a prediction of the antagonistic pleiotropy theory of aging, which suggests that prolonged lifespan might result in compromised fertility (Williams, 1957). Instead, some interventions that repress aging in somatic cells may be beneficial to germ cells. Whether the heritable epigenetic defects that result from *prg-1* deficiency impact somatic lifespan, if these defects are related to the germline function of RBR-2 that can extend adult lifespan (Greer et al., 2011), and how DAF-16 regulates the small RNA pathway that suppresses deficiency for *prg-1* are intriguing questions raised by this study.

Progressive sterility implies transgenerational accumulation of defects that could be relevant to proliferative aging of somatic cells. Our data suggest that epigenetic desilencing of transposons and tandem repeats could contribute to loss of germ cell immortality in *prg-1* mutants. Drawing a parallel to human genetic diseases such as Huntington's chorea, this implies that *prg-1* is subject to "epigenetic anticipation," as each generation will inherit increased levels of repetitive RNA expression, which, combined with inefficient silencing, eventually causes failure of normal germ cell function. A fascinating prospect therefore is whether epigenetic anticipation might occur in human cells. It has recently been shown that repetitive segments of the genome become desilenced when mammalian cells undergo senescence (De Cecco et al., 2013) and in the aging-related disorder cancer (Ting et al., 2011; Zhu et al., 2011). Furthermore, increased expression of Alu retrotransposons may contribute to adult-onset macular degeneration as well as proliferative aging of human stem cells grown in vitro (Kaneko et al., 2011; Wang et al., 2011). We speculate that Piwi-dependent regulation of repetitive rapidly evolving segments of genomes, such as transposons and tandem repeats, creates an epigenetic landscape in germ cells that is transmitted by human gametes, that could affect proliferative aging of somatic cells and that could be modulated in future generations by repression of insulin/ IGF-1-like signaling.

EXPERIMENTAL PROCEDURES

Germline Mortality Assays

Worms were assessed for the mortal germline phenotype using the assay previously described (Ahmed and Hodgkin, 2000). Once per week, six L1 or L2 animals would be placed on fresh nematode growth medium plates seeded with OP50 *E. coli* bacteria. Each passage would be recorded, and plates that yielded no additional L1 animals were marked as sterile. Mantel-Cox log rank analysis was used to determine differences of transgenerational lifespan between strains.

Microarray Data Analysis

C. elegans tiling arrays normalized to gene models were used to compare gene-expression changes in *prg-1* and *prg-1*; *daf-2*. Repeat expression was analyzed by mapping the probes to repeat positions downloaded from the UCSC Genome Browser as described above. Control regions for each chromosome were generated using a custom script in R. First, a set of 1,000 repeat sequences was sampled from the total complement of repeats on each

chromosome (with replacement) and the length of these sequences stored. A random number generator was used to provide 1,000 starting positions across the chromosome, and these starting positions were paired with the lengths at random to calculate the end positions. Expression differences for these control regions were then calculated as for the repeats. Analysis of the significance of overlap in gene expression or repeat expression between alleles was also carried out using a custom script in R. A random sample of either genes or repeats was generated for each allele by sampling n members of the total set of either genes or repeats, where n is the number of altered genes or repeats for the allele in question. The overlap for the three random samples was then calculated and stored. The entire process was repeated 1,000 times, and a mean and SD of random overlap computed. The size of the observed overlap could thus be compared to simulated random overlap using the Z statistic.

Extrachromosomal Arrays

Primers were designed to amplify repetitive sequences directly from the genome. For an experimental array consisting of direct repeats, sequences from two *CeRep59* loci (chr*I*: 4281435; chr*III*: 7405366) and a 174-mer simple repeat (chr*IV*: 6682640) were used. An additional experimental array consisted of a *Helitron* transposon sequence (chr*IV*: 16880484). A control array was created using primers flanking a stretch of genomic sequence containing the H2A, H2B, H3, and H4 histone loci *his-13*, *his-14*, *his-15*, and *his-16*.

RNA FISH

Freshly outcrossed alleles of *prg-1*, *n4357*, and *tm872* and *prg-1* lines containing the extrachromosomal array *ypEx3* were created, and RNA FISH was performed on F4 animals to visualize repetitive RNA expression. A DNA oligonucleotide probe coupled with a 5′ Cy5 fluorophore was designed to detect RNA transcripts of *CeRep59* on chromosome *I* (located at 4,281,435–4,294,595 nt). The *CeRep59* probe was tttctgaaggcagtaattct.

ACCESSION NUMBERS

Small-RNA-sequencing and microarray data sets have been deposited at the Gene Expression Omnibus under accession numbers GSE40569, for expression analysis of *C. elegans* Bristol N2 *prg-1* and Bristol N2 *prg-1; daf-2* double mutant, and GSE40572, for transgenerational changes in small RNA profiles of *C. elegans* mutants lacking PRG-1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.056.

AUTHOR CONTRIBUTIONS

M.S., P.S., A.-L.D., J.M., A.S., and S.A. designed and performed experiments. P.S. and L.D.G. designed and performed small-RNA-sequencing experiments. M.S., P.S., K.I., A.-L.D., L.D.G., A.S., E.A.M., and S.A. analyzed data and wrote the paper. E.A.M. and S.A. supervised the research.

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