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 Horvitz, 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.
at 25°C per genotype). The germ cell immortality function of previously unrecognized Piwi phenotype: transgenerational replication.

progressive sterility of germ cells. The germ cell immortality function of tm872

prg-1

singled and their progeny counted. Error bars represent SD. n = 10 lines per strain). Individuals from independently derived lines were

(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).

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), the type of sterility varied across strains. Consistent with previous observations, loss of daf-2 activity suppressed progressive sterility caused by prg-1 (Figure 1C, 1D). These results suggest that daf-2 acts within these mutants to rescue progressive sterility in response to the stresses of transgenerational genome silencing by PRG-1.
25°C to 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...
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 d41, e1388, and e1370 (n = 10 strains scored per genotype) and examined for progressive sterility. Four prg-1; daf-2(e1388); daf-18 lines were constructed from the four allelic combinations of prg-1; daf-18 and examined for progressive sterility (Figure 5F). 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 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 life span 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 strains per genotype). Four prg-1; daf-18 double-mutant strains were 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 d41, e1388, and e1370 (n = 10 strains scored per genotype) and examined for progressive sterility. Four prg-1; daf-2(e1388); 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 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.
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 \times 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.

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**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.
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* alleles e1370 or e1368 (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* alleles e1370 (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 *n4357* (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 *ppw-1* *prg-1* double-mutant strains were studied where *prg-1* alleles tm872 and *n4357* were combined with *ppw-1* alleles e1370 or *tm1231* (n = 10 strains per genotype). Eight *prg-1*; *rbr-2* double-mutant strains 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* alleles e1347 (n = 10 strains per genotype). Three *prg-1*; *rbr-2* double-mutant strains were studied where each *prg-1*; *rbr-2* mutant was combined with *daf-2* alleles e1370 and *prg-1* alleles *n4357*; *spr-5* was combined with *daf-2* alleles 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...
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 mutant 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 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 MRT-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^{-6} 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^{-3}), whereas Helitron transposon or histone locus arrays had no effect (p > 0.29) (Figures S5D, S5E, 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.

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

<table>
<thead>
<tr>
<th>2-Fold Down</th>
<th>Late prg-1 versus Early prg-1</th>
<th>Suppressed</th>
<th>Significance</th>
<th>Suppressed</th>
<th>Dependent</th>
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<tbody>
<tr>
<td>Transposons</td>
<td>54</td>
<td>4</td>
<td>1.00</td>
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<td>50</td>
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<tr>
<td>Genes</td>
<td>356</td>
<td>71</td>
<td>5.80 × 10^{-29}</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Simple repeats</td>
<td>497</td>
<td>92</td>
<td>1.66 × 10^{-12}</td>
<td>56</td>
<td></td>
</tr>
</tbody>
</table>

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 promotor pathway, as the majority of suppressed genes, transposons, and repeat sequences 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
late-generation *prg-1* controls, though not to early-generation levels (*p* < 1 × 10⁻⁶ 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, SSJ, and SSK). 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, 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 instead 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...
continuous 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*/pRNAs 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 aging 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 functions 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, 2003). 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**

*Caenorhabditis 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 C. elegans PRG-1 loci (chr IV: 4281435; chr I: 7405368) and a 174-mer simple repeat (chrII: 6682640) were used. An additional experimental array consisted of a Helitron transposon sequence (chrII: 16880484). A control array was created using primers flanking a stretch of genomic sequence containing the H2A, H2B, H3, and H4 histone loci (chr III: 6682640; chr IV: 4281435–4294,595 nt). The C. elegans probe was tttctgaaggcagtaattct.

**RNA FISH**

Freshly outcrossed alleles of prg-1, n4357, and tm872 and prg-1 lines containing the extrachromosomal array probe 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 ttctgagagcctgatcctt.

**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**


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**REFERENCES**


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

EXPERIMENTAL PROCEDURES

Strains

Unless noted otherwise, all strains were cultured at 20°C on Nematode Growth Medium (NGM) plates seeded with *E. coli* OP50. Strains used include Bristol N2 wild type, *ppw-1 (pk1425) I, dpy-5(e61) I, rde-2(ne221) I, unc-13(e450) I, prg-1(n4357) I, prg-1(tm876) I, prg-1(pk2298) I, unc-55(e402) I, unc-29(e193) I, daf-16(mg50) I, daf-16(mu86) I, spr-5(by134) I, unc-54(r293) I, daf-2(e1368) III, daf-2(e1370) III, daf-2(m41) III, dpy-17(e164) III, daf-18(e1375) IV, daf-18(ok480) IV, dpy-9(e12) IV, prg-2(n4358) IV, prg-2(tm1094) IV, prg-2(ok1328) IV, prg-2(nDf57) IV, unc-24(e120) IV, rbr-2(tm1231) IV, rbr-2(ok2544) IV, unc-58(e665) X.*

*prg-1* mutations were outcrossed versus an outcrossed stock of *dpy-5(e61), unc-13(e450),* and freshly isolated homozygous F2 lines were established for analysis. *dpy-5, unc-55; daf-2* triple mutant were crossed with *prg-1 / dpy-5, unc-13* males which were then selected based on Dauer phenotype at 25°C and loss of *dpy-5, unc-55* to create *prg-1; daf-2*. Analogous crosses using marker mutations *dpy-9* for *daf-18* or *unc-24* for *prg-2* as balancers to create double mutant strains. To create the linked *prg-1, daf-16* double mutant, *prg-1, dpy-24* and *unc-13, daf-16* double mutants were first created, then progeny of *prg-1, dpy-24 / unc-13, daf-16* heterozygotes that lost *unc-13* were identified, and the resulting putative *prg-1, daf-16* recombinant chromosomes were made homozygous and PCR genotyped to verify the presence of *prg-1* or *daf-16* deletions. *prg-1, daf-16* doubles were crossed with *unc-13, dpy-24; daf-2 / +, +; +
heterozygous males and then selected for Daf and against Dpy Unc phenotypes to create prg-1, daf-16; daf-2 triples.

For Germline Mortality assays, although daf-2 mutants grown at high temperature give rise to dauer larvae, a highly stress resistant state of larval arrest, dauers were not transferred during transgenerational fertility assays. Thus, constitutively reduced DAF-2 signaling immortalizes prg-1 mutant strains independent of dauer formation.

For Mutator assays, Unc-non-Dpy recombinants from dpy-5, unc-54 / prg-1 heterozygotes were identified, made homozygous, or dpy-5, unc-55; unc-58 triples were crossed with prg-1 and unc-58 / + F2 that failed to segregate Dpy Unc F3 were identified to create prg-1; unc-58 doubles.

To create ppw-1, prg-1 lines, ppw-1, unc-13 and dpy-5, prg-1 lines were produced and then crossed to make dpy-5, prg-1/ppw-1, unc-13 heterzygotes. Non-Dpy, non-Unc F2 animals with Dpy and Unc siblings were isolated and ppw-1, prg-1 recombinants were isolated from lines with only one marker displayed in F3 progeny by selecting against the marker.

For rde-2, prg-1 animals, dpy-5, prg-1, unc-29 triple mutants were constructed and genotyped to confirm the presence of prg-1 between the flanking markers. The triple mutants were crossed into rde-2 and F2 recombinants lacking the Dpy phenotype were isolated. pop-1 RNAi plates were used to test for the presence of rde-2, which is RNAi-resistant. Resistant lines were then genotyped for prg-1. Further, dpy-5, unc-13 was crossed to the rde-2, prg-1, unc-29 and F2 recombinants lacking unc-29 were isolated, homozygosed, and re-tested for RNAi resistance and prg-1 presence. rde-2,
prg-1; daf-2 lines were constructed using dpy-5, unc-55; daf-2, which were then selected based on Dauer phenotype at 25°C and loss of dpy-5, unc-55 to create rde-2, prg-1; daf-2.

To create prg-1, spr-5 lines, prg-1, unc-29 and unc-13, spr-5 double mutants were produced and then crossed to make prg-1, unc-29/unc-13, spr-5 heterozygotes. Non-Unc F2 animals with unc-13 and unc-29 siblings were isolated and prg-1, spr-5 recombinants were isolated from lines with only one marker displayed by F3 progeny by selecting against the marker. Final lines were genotyped for both prg-1 and spr-5. Additionally, prg-1, spr-5; daf-2 was created by crossing dpy-5, unc-55; daf-2 into prg-1, spr-5, followed by selection based on Dauer phenotype at 25°C and loss of dpy-5, unc-55. Genotyping for prg-1 and spr-5 was performed to ensure no loss during the cross.

Creation of the prg-1; rbr-2 strains was accomplished by first generating unc-13; rbr-2 and prg-1; dpy-20. Crosses were made to produce unc-13; rbr-2/ prg-1; dpy-20 heterzygotes. Non-Dpy, non-Unc F2 animals with Dpy and Unc siblings were isolated and prg-1; rbr-2 recombinants were isolated from lines with only one marker displayed in F3 progeny by selecting against the marker. Triple mutants of prg-1; daf-2; rbr-2 were created by crossing unc-13; daf-2; dpy-20 into prg-1; rbr-2, producing heterozygous unc-13; daf-2; dpy-20 /prg-1; rbr-2 F1 animals. Selection based on Dauer phenotype at 25°C and loss of unc-13 and dpy-20 was performed to isolate prg-1; daf-2; rbr-2 triple mutants.

For the silent transgene experiment shown in Figure S6, prg-1; piRNA sensor strains were propagated for many generations until significant numbers of sterile animals were observed. L4 larvae were then singled and allowed to develop into adults.
Adults were then scored for complete sterility for 1 day and imaged using a Nikon E800 epifluorescent microscope. Control nrde-1; piRNA sensor animals were propagated under the same conditions and fertile mid-generation adults were imaged to show expression of the piRNA sensor transgene, whose silencing depends on nrde-1 (Ashe et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012).

**Small RNA Sequence Analysis**

Fastq sequence data were processed using custom Perl scripts. Sequence reads with missing bases or barcodes not matching any of the expected nucleotide sequences were excluded. Reads were trimmed by removing 3’ A nucleotides (As). Inserts in the expected size range (18-30 nucleotides) were collapsed to unique sequences, retaining the number of reads for each sequence. Sequences were aligned to the *C. elegans* genome (ce6/WS190) downloaded from the UCSC genome browser website (Karolchik et al., 2008; Kent et al., 2002). Perfect genomic matches were obtained using Bowtie allowing for an arbitrary number of multiple matches for each sequence (Langmead et al., 2009). Read counts for individual piRNAs were obtained by matching sequences against 16,003 piRNAs annotated previously (after trimming 3’ As) (Bagijn et al., 2012). Read frequencies were defined as read counts divided by the total number of aligned reads from the same library.

High-throughput sequencing of whole animal small RNAs was performed after generating 5’ independent 18-30 nucleotide small RNA libraries as described previously (Das et al., 2008). Alignment to the genome and matching to known piRNA loci was performed as described previously (Bagijn et al., 2012, see Extended Experimental
Procedures. For analysis of 22G-RNAs mapping to transposons or simple repeats, 22G-RNAs were first selected from the libraries using a custom Perl script. Transposon consensus sequences were downloaded from RepBase (version 17.05) and repeat sequences were extracted from the ce6 genomic sequence using the genomic coordinates of simple repeats, downloaded from the UCSC genome browser website (simpleRepeats.txt). Mapping was carried out using Bowtie reporting the best single match with up to two mismatches, to avoid the problem of multiple matches potentially exaggerating differences between samples. With these parameters, if there are multiple “best” matches, as is likely to be the case for repeats, Bowtie will report one match randomly, therefore mapping was carried out using un-collapsed fasta files so that the effect of this would be minimized. Repeats with fewer than 5 reads and transposons with fewer than 20 reads mapping to them in N2 wild-type were discarded, and mapped reads were normalized to the levels of the abundant somatic microRNA miR-52. Data analysis was carried out using the R statistical package (Gentelman, 2005). In the analysis of 22G-RNAs mapping antisense to genes, 22G-RNAs mapping uniquely to the ce6 genome with no mismatches were selected from the libraries using custom Perl scripts, and those mapping antisense to genes were identified by comparing coordinates with the coordinates of genes (sangerGene.txt) downloaded from the UCSC genome browser website. For comparison, sequences perfectly matching C. elegans mature microRNA sequences downloaded from miRbase (http://www.mirbase.org/) (Kozomara and Griffiths-Jones, 2011) were selected using a custom Perl script.

**Microarray analysis of RNA expression and CGH**
Total RNA was harvested from whole plates of worms that had been allowed to deplete their food supply, but not enter starvation. Standard phenol/chloroform RNA extraction was used to recover whole animal RNA. DNA for CGH was harvested from animals grown on NGM agar plates using Qiagen DNeasy Blood and Tissue Kit. cDNA and genomic DNA was processed and used for tiling microarray analysis following the Nimblegen protocols for HX1 microarrays (Ikegami et al., 2010). Scanning of arrays was performed using calibrations for repeat elements.

**CGH analysis**

Comparison between early- and late-generation *prg-1* alleles *n4357* and *tm872* for copy number across the genome was used to specifically interrogate regions mapping to genes, transposons and tandem repeats downloaded from Wormbase (ce6 assembly; WS190). Tandem repeat copy number changes were compared to randomly selected genomic regions generated as for expression analysis above.

**DAPI Staining**

DAPI staining was performed as previously described (Meier et al., 2006). L4 larvae were selected from sibling plates and sterile adults were single as late L4s, observed 24 hours later for confirmed sterility, and then stained. Animals were washed with M9 buffer to remove excess bacteria. Excess M9 was drained and the animals were treated with 200 ng/ul DAPI in a 75% ethanol solution for 30 minutes, followed by destaining in M9 buffer for an additional 30 minutes. Animals were then picked to slides containing NPG glycerol and imaged for DAPI fluorescence.
RNA Fluorescence In Situ Hybridization protocol

RNA FISH was performed with mixed stage animals from non-starved plates. Animals were washed off plates with M9 buffer into microcentrifuge tubes then washed once in 1mL M9 buffer followed by three washes in 1mL of 1x DEPC-treated PBS. Animals were then fixed for 45 minutes at room temperature in 1 mL of fixation buffer (3.7% formaldehyde in 1x DEPC-treated PBS). Following fixation, animals were washed twice in 1mL 1x DEPC-treated PBS and permeabilized overnight at 4 degrees in 1mL of 70% ethanol in DEPC-treated H2O.

The following day, hybridization buffer was prepared (0.2 g dextran sulfate, 200 mL 20x RNAse-free SSC, 200 mL deionized formamide, 1.5mL DEPC-treated H2O). Dry probes were diluted in RNAse-free TE buffer to a concentration of 25 mM. Probes were then further diluted by mixing into hybridization buffer for a final concentration of 1.25 µM. Permeabilized animals were washed for 5 minutes at room temperature in 1mL wash buffer (10% formamide in 2x RNAse-free SSC). Wash buffer was removed and 100 mL of probe in hybridization buffer was added to each sample. Then the samples were incubated overnight at 30 degrees. The next day, samples were washed once in 1mL wash buffer for 30 minutes at room temperature then a second time in 1mL wash buffer with 25 ng/mL DAPI counterstain for 30 minutes at room temperature. Animals were mounted on glass slides using VECTASHIELD mounting media (Vector Laboratories, Inc.) and imaged by epifluorescence microscopy using the same exposure times relative to imaging filter and DIC images were acquired in the same plane of focus.
Mutator Assay

For *unc*-58 and *unc*-54 assays, experimental lines were established using genetic crosses. Six L1 animals were placed on plates that were fully seeded with OP50 E. coli bacteria and allowed to propagate on the same plate until it was completely starved. Half of a chunked plate was placed on a fresh NGM agar plate with a single straight streak of bacteria opposite of the placed chunk. The plates were then scored for the presence of animals presenting restored mobility (Harris et al., 2006).

RNA isolation and RT-PCR

Total RNA (prepared as above) was reverse transcribed using the Anchor T primer and Superscript® III First Strand Synthesis System (Invitrogen), using identical quantities of RNA per sample. cDNA was then treated with RNase A to remove excess RNA and column purified. Serial dilutions of each sample were then normalized using *act-1* specific primers (Ex Taq, 64°C Annealing, 90 sec extension, 30 cycles) such that the PCR products were within the linear range of amplification and could be assessed for ~0.25- to 0.5-fold changes in quantity, based on ethidium fluorescence of bands separated on 1% agarose gels. PCR analysis of tandem repeats were carried out at identical PCR settings for all samples using Ex Taq polymerase (Takara), 65°C annealing and 28 PCR cycles. Primers used in these experiments were:

Anchor T : 5'-ATACCCGCTTAATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
TanRepI Fwd: CGATGCTCTTTGTAGACAAATCA
TanRepI Rev: GCACCCAATATTTAGAGAACAAG
TanRepIII Fwd: CATAGGGCATCGAAAAGC
TanRepIII Rev: GAAAATCATCAATTTCTGGAGGC
TanRepIV Fwd: GAACCTTTGAAAACATGTCCCAAC
TanRepIV Rev: GCCATGCGTTTGACATATCATAC

act-1 was used as a normalization control due to its stability and lack of significant change in expression based on microarray analysis.

**Extrachromosomal arrays**

Extrachromosomal arrays were created by microinjection of a wildtype background. Subsequently, unc-13; array strains were created, which were then crossed to prg-1 to yield F1 that contained the array, from which prg-1 -/- F2 progeny that either contained (ypEx) or lacked (ypEx control) the array were used to establish experimental and control lineages for analysis of effects on transgenerational lifespan. Pmyo-2::mCherry was used as a transgenic marker.

For the experimental array containing the CeRep59 loci and 174 mer tandem repeat, and injection mix containing equal amounts of the sequences and 10 ng/ul of the pmyo-2::mcherry reporter (pCFJ90) were injected into N2 animals. For CeRep59, a range of concentrations of the repetitive sequences were used, including 90, 120, 150, and 240 ng/ul. While all concentrations yielded animals possessing the array in the F1 generation, only 150 ng/ul concentrations produced stable array transmission (n= 60, 40, 75 and 70 F1 animals containing the array tested, respectively). F2 animals
transmitting the CeRep59 loci and 174 mer extrachromosomal array (ypEx3) were crossed with an unc-13 to generate unc-13 F2 animals containing the array, which were subsequently crossed with prg-1/unc-13 males. F2 animals were singled to generate an experimental series of prg-1 mutant homozygotes transmitting the ypEx3 array and as well as sibling controls that did not. Transmission was scored based on mCherry fluorescence and the animals were subjected to the Mrt assay.

For the Helitron experimental array (ypEx4), a concentration of 40 ng/ul was used in conjunction with 10 ng/ul of pCFJ90 was injected into N2 animals to produce stable transmission of the array. Generation of prg-1 lines containing the ypEx4 array was performed as described above.

An array consisting of the his-13, his-14, his-15, and his-16 histone locus (ypEx5) was generated by injection of a mix containing 40 ng/ul of PCR product corresponding to the histone locus and 10 ng/ul pCFJ90 (of n=60 F1 containing the histone locus array, 3 transmitted arrays containing the histone locus to the F2, one of which was chosen for experimental crosses, ypEx5). For the histone arrays, higher concentrations of 110 ng/ul were attempted but failed to produce F2 progeny containing the array (n=80 F1 containing the array examined). Construction of prg-1 lines containing the ypEx5 array was performed as described above.

Primers used to create PCR products of genomic loci incorporated into extrachromosomal arrays:

TanRepl Fwd (CeRep59 ChrI): CGATGCTCTTTGTAGACAAATCA
TanRepl Rev (CeRep59 ChrI): GCACCCAATATTTAGACACAGAAG
TanReplIII Fwd (CeRep59 ChrIII): CATAGGGCATCGAAAAGCAC
TanRepIII Rev (CeRep59 ChrIII): GAAAATCATCAATTCTGGAGGC

TanRepIV Fwd (174 mer ChrIV): GAACCTTTGAAACATGTCCCAAC

TanRepIV Rev (174 mer ChrIV): GCCATGCGTTTGTACATATCATAC

Helitron Y1A Fwd: CCTCAAATTTGACTAGTTGGAAA

Helitron Y1A Rev: GAATGTTCTGATTTTTCTACACTCAA

Histone locus Fwd: TTATTCCTTATCTCCACCGGTCTTC

Histone locus Rev: GTTCGGTTAGATTTCAGATCAAGCTG

REFERENCES


Figure S1, Related to Figure 1. Analysis of fertility for prg-1 and prg-2 mutants. (A) Individual embryonic lethality counts for prg-1 at 20°C and 25°C. High temperature increases average embryonic lethality to high levels in some individuals. (B) prg-2 mutants do not display a Mortal Germine phenotype. Four independent mutant alleles of prg-2 were propagated for 60 generations to determine if deficiencies progressive sterility similar to that of prg-1 mutants occurred (n=10 lines per allele). (C) prg-1 progressive sterility is rescued by introduction of a gfp:prg-1 transgene and a Silence-dead gfp:prg-1 transgene (SD). (D) DAPI image of sterile prg-1 oocytes. Arrows indicate 6 chromosomes in oocytes.
Figure S2. Related to Figure 2. Analysis of transgenerational fertility and copy number changes in prg-1 mutants.
(A) Deficiency for daf-2 restores germ cell immortality for an independent prg-1 mutation tm872. Red lines indicate strains that became sterile, blue lines indicate strains that remain fertile. (B,C) Array CGH of late-generation prg-1 compared to early-generation prg-1 for genes compared to transposons (B) and tandem repeats (C) compared to randomly selected control sequences with the same length distribution. (D) Enrichment for families of transposons showing increased copy number in late-generation prg-1, with statistical significance of enrichment indicated by the color of the bar. (E) Outcrossing scheme for generating prg-1(-); daf-2(+). (F) The silenced GFP plrRNA sensor transgene in wildtype. (G) DIC image for F. (H) Desilenced GFP plrRNA sensor in mutant mut-2 background. (I) DIC for H. (J) Desilenced GFP plrRNA sensor for mutant rde-2 background. (K) DIC image for J. (L) Image of GFP plrRNA sensor; rde-1 strain reveals transgene expression in a rde-1 nuclear RNAI-deficient background. (M) DIC image for L. (N) The silenced GFP plrRNA sensor transgene remains silent in for fertile prg-1 mutant adults in early and late generations as well as for sterile late generation prg-1 adults (shown in M). (O) DIC image for N. The GFP plrRNA sensor transgene shown in panels L and N was initially created in a prg-1 mutant background, then silenced by crossbreeding into a wildtype prg-1(+/-) background, and then crossed back into either rde-1 or prg-1 mutant backgrounds. Dotted lines outline the germline. Signal outside the germlines is due to autofluorescence from the intestine.
**Figure S3.** Related to Figure 3. Altered gene expression can reflect piRNA targeting and can be affected by *daf-2*. For genes that changed more than 2-fold in late-generation *prg-1* strains, these were not significantly enriched for germline-specific genes (*P*>0.1, Fisher’s Exact Test). Gene Ontology analysis showed the set to be highly enriched (*P*<0.005, Fisher’s exact test after Benjamani and Hochberg multiple test correction) for nucleosomal components (mostly histone genes; expression generally increased), cuticle formation (expression generally decreased) and sensory perception pathways (expression generally decreased). The genes that were “cured” by *daf-2* in *prg-1; daf-2* mutants were not highly represented for cuticle formation or nucleosome-associated genes relative to the original set of 205 genes. Gene Ontology categories GO:0000786, GO:0007186, GO:0042302, GO:0007606 enriched in gene expression changes for *prg-1* mutants.
Figure S4, Related to Figure 4. Supplementary analysis of transposon families, tandem repeat tracts and 22G RNAs. (A) Transposon categories enriched or depleted for increased expression in late-generation prg-1 relative to early-generation. (B) Transposon categories enriched or depleted for increased 22G reads in late-generation prg-1 relative to early-generation. (C) Transposon categories enriched or depleted for decreased 22G reads in late-generation prg-1 relative to early-generation. In B and C, the Y axis is truncated at -5 for ease of visualization. (D) A complex tandem repeat tract on Chromosome III, where some repeats are homologous to the tract shown in Figure 4E. Upregulated expression of a tandem repeat tract on Chromosome III in prg-1 mutants does not occur in prg-1; daf-2 strains. (E) Independent primers were used to confirm the microarray results shown in panel D by RT-PCR. (F) Silencing to tandem repeat loci restored when late-generation sterile prg-1 adults are rescued by RNAi of daf-2 in comparison to late-generation strains fed on OP50 bacteria. (G) Progressively reduced median expression of transposons in late generation prg-1 mutants. (H) Transposons showing higher 22G reads in late-generation prg-1 animals do not have reduced 22G/22A ratio compared to either transposons for which reads do not change. (I) A smaller increase in expression for transposons targeted by 22G RNAs that are increased in late-generation prg-1 mutants.
Figure S5. Related to Figure 5. Analysis of 22G siRNAs mapping to repetitive regions and manipulation of loci in prg-1 mutants.

(A) 22G RNA reads from prg-1 and mut-7 single mutants in comparison to prg-1; mut-7 double mutants. (B) Progressively increased spread of normalized 22G reads in late-generation prg-1 animals compared to wild type. (C) Increased 22G reads in late-generation prg-1 strains mapping to simple repeats are reduced in prg-1 mutants deficient for the Mutator pathway genes rde-2 or mut-7. (D) Repeats showing both increased and decreased reads relative to wild-type show increased expression in late-generation prg-1 animals relative to all repeats. (E and F) Knockdown of genes and repetitive loci by RNAi feeding reveals that little effect on transgenerational lifespan of prg-1 mutants. dod = cocktail of dod-16-20. (G) RT-PCR for cDNA prepared from prg-1 lines containing the ppEx3 extrachromosomal array (x) or sibling control lines lacking the array (†) reveals increased expression of repetitive sequences on ppEx3 using primers targeting CepRep59 or 174 mer repeats in early or late generations. (H) 22G-RNAs are partially restored when sterile prg-1 adults where fertility is rescued by das-2 RNAi. (I) mut-2, mut-7 and rde-2 display no increased expression of tandem repeats. RT-PCR of Tandem Repeat III (J), Tandem Repeat IV (K), Tandem Repeat I (L), Tandem Repeat III (M), Tandem Repeat IV (N) for cDNA created from late-generation strains reveals increased expression for independent tandem repeats for prg-1, das-2 mutants that are deficient for either rde-2 or rbr-2.
Figure S6. Increased FISH staining CeRep59 transgenic animals carrying the ypEx3 array. Antisense Cy5 probe against the CeRep59 repeat was used to evaluate the expression levels and pattern of the sequence encoded by the array injected into prg-1 mutants. (A,B) Low expression observed in N2 wild-type animals observed in the embryos. (C-F) Probe staining in prg-1(n4357) shows expression in embryos and low, diffuse staining throughout the animals. (G-J) Increased CeRep59 expression in all tissues and embryos of prg-1(n4357) animals containing the ypEx3 array encoding copies of the CeRep59 repetitive sequence. Animals also displayed strong staining in the pharynx (image H, arrows), due to the incorporation of a Pyro-2:mCherry plasmid to track the injected array, where the myo-2 promoter drives expression in the pharynx. (K-N) Probe staining for prg-1(tm872). (O-R) Increased staining in prg-1(tm872) lines containing ypEx3 array. All paired images were taken in the same focal plan. DAPI images and Cy5 images all taken with equal exposure. When compared to wildtype, prg-1 mutant lines displayed a 1.23-fold and 1.02-fold increase in staining in the embryos and body, respectively. prg-1 lines containing ypEx3 displayed a 5.05-fold increase in embryo staining over prg-1 controls and a 5.35-fold increase for general body staining.
Table S1. Raw data and statistical analysis of unc-54 and unc-58 reversion assays.

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