GERM CELL IMMORTALITY IN C. ELEGANS: REDUCED INSULIN SIGNALING RESTORES GERMLINE IMMORTALITY IN PIWI ARGONAUTE PRG-1 MUTANTS

Matthew Alan Simon

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Approved by:

Shawn Ahmed

Jeff Sekelsky

Kerry Bloom

Bob Goldstein

Dave Reiner
ABSTRACT

Matthew Alan Simon: GERM CELL IMMORTALITY IN C. ELEGANS: REDUCED INSULIN SIGNALING RESTORES GERMLINE IMMORTALITY IN PIWI ARGONAUTE PRG-1 MUTANTS
(Under the direction of Shawn Ahmed)

A key distinction between germ and somatic cells is the capacity for indefinite self-renewal. Somatic cells typically exist for a single generation, whereas germ cells are effectively immortal as they proliferate from one generation to the next. Thus, germ cells must overcome damage that can contribute to aging of the soma, such as telomere erosion, DNA damage or proteotoxicity. It is unknown how many pathways contribute to germ cell immortality and what connections there might be between germline and somatic aging. Understanding the role of genes that maintain germ cell immortality is of interest to the fields of aging and cancer, as it may provide insight into the aging of disposable somatic tissues and understanding into how normally mortal cells might enter into the immortal state, as in the case of various cancers.

To gain insight into germ cell immortality, I report here the role of the C. elegans PIWI Argonaute paralog PRG-1 in maintaining the immortal state. In the Chapters 1-3, I demonstrate that dysfunction of prg-1 results in a mortal germline phenotype. We provide evidence that increased misexpression of repetitive elements results in toxic stress that causes sterility. Additionally, I show that decreased signaling in the DAF-2/IGF-1 insulin signaling somatic longevity pathway restores germline immortality to prg-1 mutant animals and decreases repetitive element misexpression.

Microscopy revealed that sterile prg-1 animals display atrophied germlines reminiscent of a
starvation-induced phenotype known as adult reproductive diapause (ARD). Through genetic characterization, I demonstrate the prg-1 atrophy phenotype is, indeed, the ARD respond. Furthermore, we describe a new role for the FOXO transcription factor, DAF-16, in prg-1-induced ARD. Continued exploration of the daf-16-mediated rescued identified a critical role for the dsRNA uptake channel, SID-1, in both the rescue of prg-1’s mortal germline (mrt) phenotype and in normal prg-1-induced ARD. I go on to show that components of the rrf-3 endogenous RNAi pathway are required for daf-16-mediated rescue of the mrt phenotype in prg-1. I conclude that prg-1 functions to protect the integrity of the germline and that the insulin signaling somatic longevity pathway can also function in this capacity. I conclude that daf-16-mediated rescue of prg-1 progressive sterility and its role in prg-1-induced ARD requires a previously unidentified endogenous RNAi pathway.

The fourth section covers a collaborative project in which I played a significant role, focusing on the role of the RNAi spreading genes, rsd-2 and rsd-6 in germline immortality. We found that these two genes were responsible for maintaining germline immortality at the stressful temperature of 25°C through a small RNA-mediated transcriptional silencing pathway. Similar to prg-1, stressed rsd mutants displayed desilencing of repetitive loci, as well as misregulation of spermatogenesis genes and chromosome missegregation.

In the fifth and final section, I summarize the major findings of my work. I cover the advances my research has contributed to the field of germline immortality, aging, and small RNA biology and their implications on future work. I also describe ongoing work and proposed future experiments for answering some of the exciting questions still to be answered regarding my work.
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<tr>
<td>ARD</td>
<td>Adult Reproductive Diapause</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>mrt</td>
<td>mortal germline</td>
</tr>
<tr>
<td>IIR</td>
<td>Insulin/IGF Receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>piRNA</td>
<td>Piwi-interacting RNA</td>
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<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
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<tr>
<td>CGH</td>
<td>Comparative Genome Hybridization</td>
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<tr>
<td>NGM</td>
<td>Nematode Growth Medium</td>
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CHAPTER I: REDUCED INSULIN/IGF-1 SIGNALING RESTORES GERM CELL IMMORTALITY TO CAENORHABDITIS ELEGANS PIWI MUTANTS

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 for *prg-1* became sterile after growth for many generations. This phenotype did not occur for RNA interference 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 up-regulating 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 the activities of RNA interference 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.

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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). Although deficiency for telomerase in humans is likely to contribute to proliferative aging of somatic cells (Armanios and Blackburn, 2012), other pathways that promote germ cell immortality could be specific to the germ cells, or could reveal new connections between the germline and somatic aging.

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 in 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 was then maintained by a number of factors, including small interfering RNA proteins that are responsible for silencing many active transposons in C. elegans.

Here we report that outcrossed prg-1 mutants display a previously undescribed 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/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 RNA interference 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 1-1A), and a minority of *prg-1* mutants displayed >80% embryonic lethality at 25°C (Figures 1-1A and 1-S1A). We saw robust levels of fertility during propagation of *prg-1* and *prg-2* for 8 generations at either 20°C or 25°C based on Mortal Germline (Mrt) assays, where 6 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* strains resulted in drops in fertility and ultimately complete sterility (Figures 1-1B and 1-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 1-1C). 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* mutations were the cause of progressive sterility in the above experiments by rescuing *prg-1* mutation with a transgene that expresses wild-type PRG-1 (Figure 1-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 1-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) .

Figure 1-1. Progressive sterility of prg-1 mutants. (A) Levels of fertility for different prg-1 mutants at 20°C and 25°C (mean±s.d., n=10 lines per strain). Individuals from independently derived lines were singed and their progeny counted. (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.

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+/-.3 generations to sterility) than at 20°C (24.9+/-.2 generations to sterility; P=3.38e-5 Mantel-Cox log-rank test; n=40 strains per genotype) (Figure 1-1B). 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 or 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 1-1D). 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 1-1D), 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 1-2A and 1-52A).
*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 1-2B). Transgenerational lifespan of *prg-1* was reduced by ~30% for *prg-1 daf-16* or *prg-1 daf-16; daf-2* strains (P=5.05E-03 and 1.56E-03, respectively, Mantel-Cox log-rank test) (Figure 1-2B). 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 lifespan (Figure 1-2B). 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 RNA interference 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 1-2B).

Taken together these data show that a specific response downstream of inactivation of the *daf-2* pathway 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* mutant males and selected 16 *prg-1 -/-; daf-2 +/-* lines descended from *prg-1 -/-; daf-2 +/-* F1 animals (Figure 1-52E). These lines maintained fertility for 10 generations and then became progressively sterile in a manner similar to *prg-1* single mutants (Figure 1-2C), suggesting that *daf-2* deficiency directly prevents damage accumulation in *prg-1* mutants.
Figure 1-2. 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 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 m41, e1368 and e1370 (n=10 strains scored per triple mutant 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 triple mutant genotype). Data for all independent alleles was 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-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 10 generations while RNAi is maintained.

**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), and the temperature-sensitive hybrid dysgenesis phenotype of *Drosophila* is caused by deficiency for a set of piRNAs and 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 (CGH) 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 1-S2B and 1-S2D), which was not observed for genes and simple repetitive regions (Figure 1-S2C). However, several lines of evidence suggest that this is unlikely to explain the defects in prg-1 fertility. 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 1-2D and E, Table 1-S1). Second, RNA interference 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 1-2D, 1-2E and Table 1-S1). Third, we did not observe any evidence of chromosomal instability as measured by DAPI staining in late-generation prg-1 animals compared to either early-generation prg-1 or wild-type animals (Figure 1-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, which also negatively regulates daf-16, and found that either RNAi treatment allowed some infertile adults to produce viable offspring again (Figure 1-2F). Further, fertility could be maintained for at least 10 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.

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 as well as by the Mutator-class secondary 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 (Figure 1-S2F-K). Since outcrossed mut-2, mut-7 and rde-2 mutants are not Mrt (Figure 1-5F), this implies 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 that was placed in a prg-1 mutant background (Ashe et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012) and remained silent in sterile late-generation adults (Figure 1-S2N-O).
Disrupted silencing of repetitive loci in prg-1 mutants

PRG-1 interacts with ~16,000 21 nucleotide (nt) RNAs possessing 5’ uracil that represent the C. elegans piRNA 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 1-3A). 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 1-3B). No such progressive reduction was seen in microRNA levels. We tested the hypothesis that expression of 22G-RNA target genes might be deregulated 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 1-3C), an intersection significantly greater than that predicted by 1000 simulations of random overlap (Z=35) and all but 3 of these genes changed in the same direction in all alleles, more than 10-fold greater than expected by chance (P<2e-16, χ² 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 Log₂ units) in late-generation prg-1; daf-2 mutants (P<0.05, Student’s T-test, two-sided, log scale) and only 3 were piRNA targets (Figures 1-3D and 1-S3, Table 1-S3), implying that daf-2 deficiency does not suppress the fertility defects of prg-1 mutants by restoring piRNA-dependent gene expression changes.
Figure 1-3. piRNA loss affects expression of few genes targeted by piRNAs. (A) Boxplots 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, crosses indicate outliers. (B) Boxplots 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.

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 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 1-4A and 1-4B). The most up-regulated were the Mariner class transposons, which include the previously characterized piRNA target Tc3 (Figure 1-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 was also significantly larger than for genes (Z=214 for simple repeats, Z=35 for genes) (Figures 1-3C and 1-4C). This was not accompanied by increased DNA copy number from these regions (Figure 1-52B-D). 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 1-4D-I and 1-54D-E) (P<2e-16, two-sided paired T-test), including most of the 101 longest tandem repeat tracts found in the *C. elegans* genome (Figure 1-4I). 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 1-54F). 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.
Figure 1-4. 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. (B) Density plot of the transposon expression changes shown in Figure 4A 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 that 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. (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.

We asked whether progressive loss of 22G-RNAs might account for loss of silencing of transposons and tandem repeats. 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, 1 sample T-test, two-sided) (Figures 1-S4A and 1-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 wildtype (Figure 1-S4A). Transposons with increased 22G-RNAs in late-generation prg-1 animals were enriched for the Tc4 family of transposons whilst transposons with reduced 22G-RNAs were enriched for the Mariner family (*P* <0.05, Fisher’s Exact Test) (Figures 1-S4B and 1-S4C). Transposons showing increased 22G-RNAs in late-generation prg-1 animals were accompanied by increased 22G-RNA to 22A-RNA ratios (Figure 1-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 1-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/RNA interference proteins that target transposons for silencing; thus the increased small RNAs against transposons in late-generation prg-1 is dependent on mut-7 and rde-2/mut-8 (Figure 1-S5B) (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 whilst 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 mutant were much more similar to the mut-7 single mutant than to the prg-1 single mutant (Figure 1–S5A). Importantly out of all the transposon consensus sequences, there were no transposons with more than 5 antisense 22G-RNA reads per million in mut-7 that had fewer than 5 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 1-S5 and 1-S6). We therefore examined 22G-RNAs against simple repeats in prg-1 mutants. The number of 22G-RNAs against simple repeat regions was smaller in prg-1 mutants than in wild-type animals (P=0.002, 1 sample T-test, two-sided), and overall 22G-RNA levels decreased further in late-generation prg-1 animals (P=0.00026, 1 sample T-test, two-side) (Figure 1-5C). 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=6e-6 to wild-type and P= 8e-5 to early-generation prg-1, Kolmogorov-Smirnov test for different distributions) (Figures 1-5C and 1-S5B). Again, increased 22G-RNAs were dependent on the Mutator pathway (Figure 1-SSC). 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 1-SSD). 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 1-S5E) and transposons (Figure 1-S5F) that were upregulated in late-generation prg-1 mutants, performing RNAi from early generations onwards, but none repressed the sterility phenotype of prg-1 mutants (P>0.191) (Figures 1-S5E and 1-S5F). We created repetitive extrachromosomal arrays containing histone loci, a tandem repeat CeRep59 or the Helitron transposon by microinjection of prg-1 mutants. Strikingly, an array overexpressing CeRep59 (ypEx3) shortened transgenerational lifespan (P=2.06E-05), whereas Helitron transposon or histone locus arrays had no effect (P>0.29) (Figure 1-SD, 1-SG and 1-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 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 (Figure 1-S6C-R). We also found that low levels of repetitive RNA were expressed in wild-type embryos (Figure 1-S6A-B), suggesting that repetitive loci are normally transiently expressed during development. Taken together, our results imply that expression of repetitive loci contributes to the transgenerational fertility defects of prg-1 mutants.
**Figure 1-5. 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. (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 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 repression 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 \textit{daf}-2(e1370) and \textit{prg}-1(n4357) \textit{spr}-5 was combined with \textit{daf}-2(e1368) (n=10 strains per genotype).

**A small RNA pathway restores germ cell immortality \textit{prg}-1 mutants**

Having established that progressive loss of 22G-RNAs against tandem repeats and transposons occurred in \textit{prg}-1, we tested whether \textit{daf}-2 might suppress increased repeat expression via siRNAs. We examined small RNA libraries from \textit{prg}-1; \textit{daf}-2 mutants at early- and late-generations. As for \textit{prg}-1 single mutants, piRNAs were lost from both early- and late-generation \textit{prg}-1; \textit{daf}-2. We therefore examined protein-coding genes, transposons and simple repeats showing decreased small RNAs in late-generation \textit{prg}-1 relative to early-generation for suppression of transgenerational decrease by \textit{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 1-1 and 1-S6). 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 \textit{rde}-2, \textit{prg}-1; \textit{daf}-2 triple mutants compared to early-generation \textit{prg}-1 mutants (Table 1-1). In addition, sequencing of small RNAs from progeny of sterile \textit{prg}-1 mutants that were restored by \textit{daf}-2 RNAi revealed that 22G-RNAs targeting repetitive sequences were partially restored in comparison to late-generation \textit{prg}-1 controls, though not to early-generation levels (P<1e-16 to late generation, two-tailed paired T-test) (Figure 1-SSH). We therefore hypothesized that \textit{daf}-2 may suppress activation of repetitive loci by upregulating an alternative \textit{prg}-1-independent silencing pathway.

We therefore tested whether the suppression of \textit{prg}-1 fertility defects by \textit{daf}-2 might be dependent on small RNAs. Deficiency for \textit{rde}-2 abolished the ability of \textit{daf}-2 mutations to ameliorate the germ cell immortality defects of \textit{prg}-1 mutants (Figure 1-5F), and late-generation \textit{rde}-2 \textit{prg}-1; \textit{daf}-2 strains expressed high levels of repetitive RNA (Figures 1-5G, 1-SSJ and 1-SSK). Consistently, another Mutator class gene \textit{mut}-7 was vital for suppression of \textit{prg}-1 by \textit{daf}-2, even though fertility was ameliorated for \textit{prg}-1; \textit{mut}-7 double mutants (Figure 1-5F). We also found that an Argonaute protein
required for efficient germline RNAi, PPW-1 (Tijsterman et al., 2002), is required for suppression of prg-1 by daf-2 (Figure 1-5F). 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 1-6).

Small RNA pathways can promote gene silencing by degrading RNA in the cytoplasm or by silencing loci within the nucleus. We tested the hypothesis that 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, the rbr-2 locus is regulated by reduced daf-2 signaling (Lee et al., 2003). We found that rbr-2 is required for suppression of prg-1 by daf-2 mutation (Figure 1-5H). Late-generation prg-1; daf-2; rbr-2 strains expressed high levels of RNA from repetitive loci (Figures 1-5L-N), 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 1-5H).

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 1-6).
Figure 1-6. 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.

Table 1-1. Effect of daf-2 on transgenerational alterations in 22G-RNA levels in prg-1.

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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 several generations before becoming progressively sterile. Second, we show that the sterility of *prg-1* animals is not due to increased transposition. Instead, our data supports a transgenerational epigenetic cause of sterility in *prg-1* mutants.

A role for PRG-1 in transgenerational epigenetic maintenance of fertility in *C. elegans* connects 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 *(Figure 1-6A-B)* (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) and maintain silencing of repetitive loci that become de-repressed in late-generation *prg-1* mutants *(Figures 1-5F, 1-S5I)*, despite being essential for maintenance of transgene silencing *(Figures 1-52F-K, Table 1-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 1-S2N). We suggest that at least two classes of ‘non-self’ 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 1-4 and 1-S4D-E), an alternative PRG-1-independent silencing pathway can be activated by reduced insulin signaling (Figure 1-2). 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 1-5A-C, 1-S4G-I and 1-S5B-D). This led us to define components of an endogenous small RNA silencing pathway that are required for daf-2 to suppress prg-1 (Figures 1-5F and 1-6C, Table 1-S2). This small RNA pathway may act upstream of a chromatin-based silencing pathway involving rbr-2- and spr-5-mediated demethylation of histone H3 K4 (Figure 1-5H). 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 anti-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 1-6C).
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 1-2B*). 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 wildtype 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 1-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, and if these 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 also in the aging-related disorder cancer (Ting et al., 2011; Zhu et al., 2011). Further, 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.
MATERIALS AND METHODS

Germline Mortality assays

Worms were assessed for the Mortal Germline phenotype using the assay previously described (Ahmed and Hodgkin, 2000). Once per week, 6 L1 or L2 animals would be placed on fresh NGM 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.

Small RNA Sequence Analysis

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/](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.

**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 1000 repeat sequences were 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 1000 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 between gene expression and 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 1000 times and a mean and standard deviation of random overlap computed. The size of the observed overlap could thus be compared to simulated random overlap using the Z statistic.

**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.

**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 (chrI:4281435, chrIII:7405366) and a 174-mer simple repeat (chrIV:6682640) were used. An additional experimental array consisting of a Helitron transposon sequence (chrIV:16880484). A control array was created using primers flanking a stretch of genomic sequence containing the H2A, H2B, H3 and H4 histone locus *his-13, his-14, his-15*, and *his-16*.

**RNA Fluorescence In Situ Hybridization**

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 4281435-4294595 nt). The *CeRep59* probe was tttctgaaggcagtaattct.
RNA FISH was performed with mixed stage animals from non-starved plates. Animals were washed off plates 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 RNAs-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 counterstained 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.
Figure 1-S1. 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 Germline 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 Slicer-dead gfp::prg-1 transgene (SD). (D) DAPI image of sterile prg-1 oocytes. Arrows indicate 6 chromosomes in oocytes.
Figure S2. 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 compared to randomly selected control sequences with the same length distribution (C). (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 (+/+)* lines. (F) The silenced GFP piRNA sensor transgene in wildtype. (G) DIC image for F. (H) Desilenced GFP piRNA sensor in mutant *mut-2* background. (I) DIC for H. (J) Disilenced GFP piRNA sensor in mutant *rde-2* background. (K) DIC image for J. (L) Image of GFP piRNA sensor; *nrde-*
1 strain reveals transgene expression in a \textit{nrde-1} nuclear RNAi-deficient background. (M) DIC image for L. (N) The silenced GFP piRNA sensor transgene remains silent in for fertile \textit{prg-1} mutant adults in early and late generations as well as for sterile late generation \textit{prg-1} adults (shown in M). (O) DIC image for N. The GFP piRNA sensor transgene shown in panels L and N was initially created in a \textit{prg-1} mutant background, then silenced by crossing away into a wildtype \textit{prg-1}(+) background, and then crossed back into either \textit{nrde-1} or \textit{prg-1} mutant backgrounds. Dotted lines outline the germline. Signal outside the germlines is due to autofluorescence from the intestine.

**Figure 1-S3. Altered gene expression can reflect piRNA targeting and can be affected by \textit{daf-2}.** For genes that changed more than 2-fold in late-generation \textit{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 nucleosome 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 \textit{daf-2} in \textit{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:000786, GO:0007186, GO:0042302, GO:0007606 enriched in gene expression changes for \textit{prg-1} mutants.
Figure 1-S4. 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 1-S5. 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 wildtype 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 ypEx3 extrachromosomal
array (+) or sibling control lines lacking the array (-) reveals increased expression of repetitive sequences on ypEx3 using primers targeting CeRep59 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 daf-2 RNAi. (I) mut-2, mut-7 and rde-2 display no increasing 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; daf-2 mutants that are deficient for either rde-2 or rbr-2.
Figure 1-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 (H, arrows), due to the incorporation of a Pmyo-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 plane. 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.

Tables 1-S1 through 1-S8 can be viewed at: http://www.cell.com/cell-reports/abstract/S2211-1247%2814%2900253-8
CHAPTER II: ADULT REPRODUCTIVE DIAPAUSE IN RESPONSE TO A HERITABLE EPIGENETIC STRESS

SUMMARY

Environmental cues such as starvation or seasonal change can transiently suspend reproduction in response to stress. Deficiency for the *C. elegans* Argonaute Piwi *prg-1* results in strains that can reproduce for many generations but then become sterile in response to a heritable epigenetic defect. Late-generation *prg-1* mutants matured into sterile adults that displayed germ cell atrophy. This developmental program was reversible and depended on the apoptosis protein CED-3 and the nuclear hormone receptor NHR-49, which remodel the germline in response to starvation. Germ cell atrophy also required CEP-1, whose mammalian homolog p53 regulates senescence in the context of proliferative aging, and the DAF-16/Foxo stress response transcription factor. We propose that *prg-1* mutants transmit a heritable, epigenetic stress that induces Adult Reproductive Diapause, a developmental program where germ cells enter a reversible state of suspended animation that could reflect an ancestral interface between aging and senescence.

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2 The following chapter contains work done in collaboration with Jacinth Mitchell, Dr. Peter Sarkies, Dr. Eric A. Miska and Dr. Shawn Ahmed. I contributed significantly to all aspects of the work.
INTRODUCTION

Diapause is a state of developmental arrest that promotes survival in response to harsh environmental conditions such as seasonal change or limited nutrient availability and has been documented for a variety of invertebrates (Schiesari and O’Connor, 2013; Saunders et al., 2002). Vertebrates, possibly including humans, can also enter a state of suspended animation in response to anoxia (Blackstone and Roth, 2005). One form of diapause in insects is Adult Reproductive Diapause (ARD), where larvae mature into sterile adults that can become fertile when environmental conditions improve. While environmental cues can modulate ARD, it is unclear if this developmental program can be triggered in response to an endogenous stress.

PRG-1 is the C. elegans orthologue of Piwi, a conserved Argonaute protein that interacts with small piRNAs in germ cells to promote silencing of transposons and some genes (Batista et al., 2008; Das et al., 2008). Although mutation of Piwi in many organisms results in immediate sterility (Klattenhoff et al., 2008; Lin et al., 2007), C. elegans prg-1 mutants are defective for germ cell immortality and become completely sterile after growth for many generations as a consequence of a heritable epigenetic defect (See Chapter I).
RESULTS

Adult Reproductive Diapause in sterile prg-1 mutants

Late-generation prg-1 mutant populations were identified by scoring for populations where all young adults present were sterile and younger sibling animals, likely to become sterile, were available for analysis. In both early generation and sterile generation animals, germline development was comparable to wildtype for L4 larvae, the stage immediately preceding adulthood (Figure 2-1A, B, Figure 2-2A, B, and Figure 2-S1A). Although a fraction of sterile prg-1 adults contained germlines of normal sizes (Figure 2-S1F), germline remodeling events occurred in most sterile prg-1 worms during the L4 to adult transition, resulting in severe germ cell atrophy or lack of germ cells altogether (Figures 2-1D, E). Additionally, a small fraction of the population displayed a shortened germline, considerably more flush and populated than the atrophy phenotype, but not as robust as those displaying a “normal” phenotype (Figure 2-1F). Extensive scoring revealed that the majority of sterile young adults displayed germline atrophy, where small populations of germ cells remained in distal arms of the germline and mature oocytes were absent (Figure 2-2B). While germ cells of early-generation prg-1 mutant adults displayed normal progression from mitosis to meiosis, germ cell atrophy in sterile prg-1 adults resulted in germ cell nuclei with condensed chromosomes, suggesting mitotic arrest, and lacked a transition zone where chromosomes pair during initiation of meiosis or more differentiated meiotic pachytene cells undergoing homologous recombination (Figure 2-1G-I, and Figure 2-S1D) (Dernburg et al., 1998).
Figure 2-1. Germline atrophy in sterile prg-1 animals. DAPI-stained photos of (A) an N2 wildtype L4, (B) a late generation prg-1 L4 likely to become sterile, (C) an N2 wildtype adult, (D) a late generation prg-1 mutant sterile adult with germline atrophy, (E) a late generation prg-1 mutant sterile adult with two empty germline arms, (F) a late generation prg-1 sterile adult with shortened germline arms, (G) the mitotic region of an early generation prg-1 adult, (H) the meiotic region of an early generation prg-1 adult, (I) mitotic cells in a late generation prg-1 worm with germline atrophy, (J) meiotic cells in a sterile prg-1, daf-16 adult.

Based on the analysis above, most sterile prg-1 mutants displayed stereotypical pattern of germline development that was grossly normal until the L4 larval stage but then followed by germ cell...
rarrangement during the transition into adulthood. This suggests a programmed developmental response to the heritable epigenetic defect that causes sterility in prg-1 mutants. Additionally, the prevalent phenotype of atrophied germlines in sterile animals resembles a germline remodeling event that occurs when C. elegans L4 larvae undergo starvation and enter a state of adult reproductive diapause, termed ARD (Angelo et al., 2009). Starvation-induced ARD requires the apoptosis gene ced-3 and the nuclear hormone receptor nhr-49 (Angelo et al., 2009). A role for apoptotic cell death in the dramatic reduction in germ cell numbers that occurs as most sterile prg-1 mutants was assessed using ced-3 and ced-4 mutations that eliminate apoptosis (Metzstein et al., 1998). Deficiency for ced-3 or ced-4 failed to rescue the progressive sterility phenotype of prg-1 (Figure 2-2C). However, sterile prg-1; ced-3 and prg-1; ced-4 double mutants displayed reduced levels of germline atrophy in comparison to prg-1 single mutant controls (P=1.0E-04 for ced-3, P=2.0E-08 for ced-4) (Figure 2-2D). Thus, apoptotic cell death contributes to germline atrophy in sterile prg-1 animals.

nhr-39 encodes a nuclear hormone receptor responsible for fat metabolism and lifespan by activating transcription of target genes in conjunction with MDT-15 (Van Gilst et al., 2005). Additionally, nhr-49 has been found to function in the starvation-induced ARD pathway (Angelo et al., 2009). Similar to the ced-3 and ced-4, we found that nhr-49 failed to rescue the progressive sterility of prg-1 (Figure 2-2C). prg-1, nhr-49 sterile animals displayed altered germline phenotypes, with a significant increase in animals with short germlines (p<1.0E-06) (Figure 2-2D). Taken together with the ced-3 and ced-4 data, the heritable epigenetic defect of prg-1 mutants triggers a developmental response that resembles starvation-induced ARD.
Figure 2-2. Cell death potentiates germline remodeling of sterile prg-1 animals. (A) Phenotypes of fertile, early generation (F4) germlines in L4 larva and young adults. (B) Phenotypes of sterile, late-generation germlines in L4 larva and young adults. Sterile animals were isolated as L4s from sterile sister plates for 24 hours to confirm sterility, DAPI stained, and then scored. (C) prg-1; ced-3, prg-1; ced-4, and dpy-5, prg-1, nhr-49 double mutants become progressively sterile. prg-1; ced-3 and prg-1; ced-4 double mutants were made for three prg-1 alleles, pk2298, n4357 and tm872 (n=10 lines were propagated per genotype). Additionally, prg-1; cep-1 and dpy-5, prg-1, nhr-49 double mutants were made for two prg-1 alleles, n4357 and tm872 (n=10 lines were propagated per genotype). Generational lifespan for combined prg-1 alleles is shown in comparison to prg-1 single mutant controls. (D) Effects of starvation-induced ARD genes and stress response transcription factors on sterile germline phenotypes of prg-1.

Stress response transcription factors promote germ cell atrophy

We hypothesized that the epigenetic defect that triggers sterility in late-generation prg-1 mutants could represent a form of heritable stress. To test this hypothesis, we examined transcription factors with conserved roles in responding to stress for roles in the germ cell atrophy phenotype of sterile prg-1 mutants. Previously, we have shown that suppression of the DAF-2/DAF-16 insulin signaling pathway can rescue the progressive sterility in prg-1 mutants (See Chapter I). The C. elegans DAF-16/Foxo transcription factor can promote resistance to diverse stresses such as heat shock,
proteotoxicity, oxygen radicals, pathogenic bacteria and high metal concentrations (Zhou et al., 2011). Based on these results, we hypothesized that DAF-16 activity likely plays a role in prg-1-induced ARD. A role for DAF-16 in germline atrophy of prg-1 mutants was directly evaluated by examining sterile late-generation prg-1, daf-16 double mutant adults, which displayed strongly reduced frequencies of germline atrophy (Figure 2-S2A). Although germline arms of sterile prg-1, daf-16 double mutants were primarily underproliferated (short) in comparison to those of wildtype, most contained many more cells than atrophied germlines of prg-1 single mutants (Figure 2-S1G). Short prg-1, daf-16 germline arms also displayed hallmarks normally observed during meiosis such as a transition zone (chromosome pairing) and synapsed homologous chromosomes (Dernburg et al., 1998), as well as mature oocytes at the proximal end of the germline, despite a lack of fertilized embryos (Figure 2-1J and Figure 2-S1G). These phenotypes were recapitulated in prg-1, daf-16; daf-2 triple mutants (Figure 2-S2A). Thus, DAF-16 promotes proper germline remodeling in sterile late-generation prg-1 animals via the daf-2/daft-16 insulin signaling pathway.

DAF-16 can function downstream of C. elegans PTEN tumor suppressor homologue DAF-18 to promote longevity in response to reduced DAF-2 signaling but can also respond to distinct signaling from other pathways (Ogg et al., 1998; Vowels et al., 1992). In order to evaluate if whether DAF-16 was function in ARD through the DAF-2/DAF-16 insulin signaling pathway, prg-1; daf-18 double mutants were created. The double mutants became progressively sterile, with late-generation sterile adults displaying short germlines with differentiated germ cells, at levels similar to the prg-1, daf-16 double mutants (Figure 2-S2A). As with daf-16 triple mutants with daf-2, prg-1; daf-2; daf-18 triple mutants displayed phenotypes similar to the prg-1; daf-18 mutants (Figure 2-S2A). Thus, DAF-16/FOXO functions downstream of DAF-18/PTEN to promote germline remodeling in late-generation prg-1 L4 larvae that are poised to become sterile, indicating that DAF-16 responds to reduced daf-2 insulin/IGF1-signaling cues in this context.
Having established a role for daf-16 in prg-1-induced ARD, we set out to further elucidate the role of DAF-16 in the germline remodeling program of ARD. DAF-16 can translocate from the cytoplasm into the nucleus in response to various stresses as well as reduced daf-2 signaling (Friedman, 1998; Kenyon, 1993). We crossed independent DAF-16::GFP transgenes into prg-1 mutant backgrounds, and found that DAF-16::GFP was primarily cytoplasmic for early generation and fertile late-generation adults (Figure 2-3A-C, G-I), indicative of low DAF-16 activity. In sterile late-generation adults, DAF-16::GFP translocated into nuclei of intestinal cells (Figure 2-3D-F, J-L), which supports the observation that reduced daf-2 signaling promotes germ cell remodeling in sterile prg-1 adults.

The nuclear localization of daf-16 in the intestines of sterile generation prg-1 animals suggests that daf-16’s role in ARD may be non-cell autonomous and facilitated by daf-16 activity in somatic tissue. kri-1 encodes a FERM domain protein that is required for daf-16 localization to the nucleus in intestine cells (Berman and Kenyon, 2006). prg-1, kri-1 mutants were constructed and found to display germline phenotype distributions unlike prg-1, daf-16 sterile animals, but also unlike prg-1 single mutants (Figure 2-S2A). These results indicated that daf-16 signaling from the intestines does play a role in proper ARD formation in prg-1 sterile animals, but is not the sole source of daf-16 signaling involved in this process.
DAF-16 is known to have numerous downstream targets that are regulated by the nuclear, active form of the transcription factor (McElwee, et al. 2003). Many of these downstream target genes are susceptible to regulation by other transcription factors in addition to DAF-16. One such factor is HSF-1, a heat shock factor known to induce stress-response gene expression (Hsu et al. 2003). Due to its roles in response to both external stress (heat) and internal stress (proteotoxicity), we asked if hsf-1 activity influenced ARD in prg-1 sterile animals. Sterile prg-1, hsf-1 animals displayed an altered germline phenotype distribution, primarily favoring the small germline phenotype. Thus, hsf-1 activity promotes...
germline rearrangement in sterile prg-1 animals, suggesting a large role for stress response pathways in ARD.

The involvement of ced-3 and ced-4 in germline rearrangement in sterile prg-1 animals suggests a strong role for the apoptotic cell death pathway. Mammalian p53 are well as the C. elegans homolog of p53, CEP-1, can promote cell death or cell cycle arrest in response to a variety of stresses, including DNA damage, heat shock, oxidative, anoxia, osmotic stresses (Gartner et al. 2008). CEP-1 acts upstream of CED-3 and CED-4 to promote germ cell apoptosis in response to genotoxic stress as well as other stresses (Gartner et al., 2000). Based on this information, we hypothesized that cep-1 may play a large role in facilitation germline rearrangement, perhaps more so than either ced-3 or ced-4 by themselves.

cep-1 failed to rescue progressive sterility of prg-1 and significantly enhanced the onset of sterility (p = 6.35E-05) (Figure 2-2C). Additionally, prg-1, cep-1 sterile animals displayed a dramatically altered ARD response, with a substantial increase in germlines of normal sizes, though these animals lacked embryos (Figure 2-2D). This robust shift favoring larger germlines was a significantly larger percent of the population of sterile animals than found in either ced-3 or ced-4 double mutants with prg-1 (p <1.0E-06 vs. prg-1; ced-3, p<4.32E-04 vs. prg-1; ced-4). Thus, cep-1 strongly promotes germline rearrangement during ARD.

The role of several transcription factors and stress-response genes in prg-1-induced ARD suggests several pathways might be involved in facilitation germline rearrangement in sterile prg-1 animals. In order to investigate the relationship between these pathways, we generated triple mutants and examined the germline phenotypes of the sterile generation animals. prg-1, daf-16; ced-4 mutants displayed germlines that favored the small phenotype, similar to prg-1, daf-16 single mutants and suggesting that daf-16 functions upstream of ced-4 in the germline rearrangement pathway(Figure 2-S2B). Additionally, we found that prg-1, cep-1; daf-18 mutants displayed predominantly normal germline arms, similar to prg-1 cep-1 animals (Figure 2-2SB). Along with significantly fewer atrophied
germlines, this data indicates that cep-1 functions upstream of daf-18 activity and suggests that it may be epistatic to the input from the insulin signaling pathway. Finally, we looked at the germlines of prg-1, nhr-49; ced-4 animals and found that the distribution did not favor either of the double mutants (Figure 2-S2B). While not as informative as a clear epistatic relationship, this result (in conjunction the discrepancies in the other phenotypes observed for the other triple mutants) suggests that the interaction between these genes and their corresponding pathways is complex and may involve several genes interactions that affect each pathway at several intersections.

**Germ cell atrophy represents a form of reversible sterility**

Germline remodeling of sterile prg-1 L4 larvae that mature into adults resembles starvation-induced ARD. For starvation-induced ARD, addition of food can restore fertility to animals by inducing the germline to exit ARD, demonstrating that ARD is a facultative stress response that suspends reproduction in response to starvation until favorable nutritional cues are encountered (Angelo et al., 2009). We previously showed that strong reductions in daf-2 signaling can abolish the progressive sterility phenotype of prg-1 mutants, and that RNA interference (RNAi)-mediated knockdown of daf-2 or the downstream PI3 kinase subunit age-1 can restore fertility to sterile prg-1 adults (See Chapter I). To determine if daf-2 RNAi can restore fertility to animals possessing the germ cell atrophy phenotype, sterile prg-1 adults were sorted into two pools: those with germlines of normal size and those with atrophied. A fraction of animals from each pool became fertile when treated with daf-2 RNAi (Figure 2-4A). Thus, germline atrophy represents a state of reversible sterility akin to ARD, in which fertility could be restored in sterile animals containing either normal or atrophied germlines.

For starvation-induced ARD, sterility can be reversed for old adults that are fed many days after starvation (Angelo et al., 2009). Normal worms can reproduce until day 8 of adulthood. We found that if sterile prg-1 adults with atrophied germlines were aged 10 days, a small but significant frequency
became fertile upon treatment with \textit{daf-2} RNAi (\textbf{Figure 2-4A}). These results are consistent with restoration of fertility to a small fraction of aged adults subjected to starvation-induced ARD.

Genetic or physical ablation of the germline extends adult lifespan by 60\% (Hsin and Kenyon, 1999), and starvation also extends longevity (Lakowski et al. 1998). We therefore asked if the sterility or accompanying germ cell atrophy phenotypes of late-generation \textit{prg-1} adults affects longevity. We found that early-generation \textit{prg-1} mutants had lifespans similar to wildtype, whereas fertile late-generation and sterile adults displayed a 30.9\% and 69.3\% increase in lifespan, respectively (P=3.6E-06 and P<1.0E-06, respectively) (\textbf{Figure 2-4B}). The dramatic increase in somatic lifespan in sterile generation animals may be due to signaling from the germline and not the endogenous stress, directly. However, the increase seen in the late-generation fertile animals indicates that somatic longevity is being increased in response to an endogenous signal. Thus, the endogenous stress present in \textit{prg-1} mutants is capable of inducing somatic stress response pathways, ultimately triggering an ARD response in sterile generation animals.
Figure 2-4. Reduced daf-2 signaling restores fertility to sterile prg-1 adults. (A) Reduced daf-2 signaling rescues sterile prg-1 animals in ARD. (B) Lifespan extension in late generation and sterile prg-1 mutants. N2=89, glp-1 n=98, Early n=94, Late n=102, Sterile n=111. (C) Model of PRG-1 suppressing a heritable form of stress that triggers adult germ cell remodeling. Stress response pathways, especially DAF-16 and components of the cell death pathway, orchestrate germ cell atrophy. Dysfunction in no single pathway eliminates germ cell atrophy. High levels of DAF-16 activity allow for exit from Adult Reproductive Diapause.
DISCUSSION

Harsh environmental conditions are known to cause a reversible form of sterility termed ARD (Angelo et al., 2009; Ragland et al., 2010). The timing of the germline remodeling events of prg-1 mutants at the L4/adult transition, the genetic requirements of ced-3 and nhr-49, and restoration of fertility to old sterile prg-1 adults, match previously established criteria for starvation-induced ARD (Angelo et al., 2009). We conclude that the heritable epigenetic defect of prg-1 mutants triggers a developmental response that also occurs in response to severe exogenous stresses. We found that fertility could be restored for sterile prg-1 mutants that displayed germlines of normal size or dramatic germ cell atrophy. Thus, germline atrophy is not necessary for animals to enter ARD and instead represents one form of reversible reproductive diapause.

DAF-16/Foxo activity has previously been shown to promote a 60% increase in somatic longevity of adults in response to germline ablation (Berman et al., 2006; Hsin, 1999), and our data demonstrate that DAF-16 can reprogram development of the adult germline (Figure 2-S2A). We found that nuclear DAF-16 was observed in intestinal cells of sterile prg-1 adults which accompanied strong increases in adult lifespan in comparison to fertile late-generation siblings (Figure 2-4B). We also found that three major stress response transcription factors, CEP-1/p53, HSF-1 and DAF-16/Foxo promote germ cell atrophy in sterile prg-1 adults. Together, these results suggest that prg-1 mutants transmit a form of ‘heritable stress’ that accumulates transgenerationally to cause sterility and that triggers somatic longevity extension when it becomes severe.

Restoration of fertility to sterile prg-1 mutants with germline atrophy at day 10 of adulthood indicates that this developmental program is capable of transiently suspending reproduction for long periods of time. We previously showed that prg-1 mutants display desilencing of repetitive segments of the genome in late generations and that repeat desilencing is repressed by daf-2 which restores fertility to prg-1 mutants via a small RNA silencing pathway (See Chapter I), implying that repeat expression may
cause sterility in prg-1 mutants. Repetitive elements play major roles in genome evolution. For example, rapid evolution of tandem repeats that specify vertebrate centromeres promotes genomic conflict and speciation (Malik, 2002). We propose that ARD could occur in the context of genomic conflict, or when transposons or retroviruses that enter the germline in horizontal transfer events and create bursts of genomic stress. Thus, ARD may integrate development with genome evolution, possibly allowing time for the genome to adapt to or shape genomic incidents (Figure 2-4C). Consistently, we show that DAF-16/Foxo is stimulated to promote entry into ARD, and that a further increase in DAF-16 activity via daf-2 RNAi restores fertility to prg-1 mutants. Thus, the activity of at least one pathway that promotes ARD is capable of resolving the stress that causes ARD.

We propose that ARD represents a reversible state of germ cell arrest or quiescence that can occur in response to heritable stress. Our results imply that prg-1 mutants become ‘stressed to sterility’ as their germ cells proliferate over the generations. Consistently, increased lifespans were observed in post-mitotic sterile adults, likely due to the presence of DAF-16 in intestinal nuclei. Thus, the heritable stress transmitted by prg-1 mutants can affect post-mitotic aging as well as aging as cells proliferate, suggesting that it could be relevant for understanding how gametes influence aging in mammals (De Cecco et al. 2013). ARD can be triggered by exogenous stresses (Angelo et al., 2009; Ragland et al., 2010), whereas Lamarckian inheritance can also occur in response to environmental cues. These observations suggest a possible interface between stress-induced decisions that affect transgenerational inheritance and ARD, which could represent a cornerstone of the evolutionary biology of aging in metazoans.
MATERIALS AND METHODS

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, *dpy-5(e61) I, unc-13(e450) I, prg-1(n4357) I, prg-1(tm876), prg-1(pk2298), unc-55(e402) I, unc-29(e193) I, daf-16(mg50) I, daf-16(mu86) I, hsf-1 (sy441) I, kri-1 (ok1251) I, nhr-49 (nr2041) I, nhr-49 (ok2165) I, daf-2 (e1368) III, daf-2(e1370) III, daf-2(m41) III, ced-4(n1162) III, dpy-17(e164) III, daf-18(e1375), daf-18(ok480) IV, dpy-9(e12) IV, unc-24(e120) IV, ced-3(n717) IV.

*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(e61) unc-13(e450)* 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-17* for *ced-4*, or *dpy-9* for *daf-18*, or *unc-24* for *ced-3*, 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*.

The TJ356 strain expresses from a *daf-16* promoter and contains a cDNA derived *daf-16* sequence containing both *a* and *b* isoforms, whereas CF1407 only expresses a sequence derived a isoform with the *b* knocked out.
**Lifespan analysis**

Animals were singled from non-starved plates and transferred every two days until the end of their reproductive cycles. Animals were kept in pools of 5 and moved to new plates once per week. All animals were maintained at 20°C.

**Germline Mortality Assay**

*(See Chapter I)*

**DAPI Staining**

DAPI staining was performed as previously described (Ahmed et al., 2000). L4 larvae were selected from sibling plates and sterile adults were singled as late L4s, observed 24 hours later for confirmed sterility, and then stained.

**RNAi rescue**

Sterile prg-1 worms (F0) were selected and transferred to fresh NGM plates with OP50 bacteria for a period no less than 24 hours and no greater than 48 hours to confirm that they were sterile. Worms were then singled to NGM plates with 50 mg/ml Ampicillin, 12.5 mg/ml Tetracycline and 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and seeded with daf-2 RNAi clone *E. coli* (Kamath et al., 2001). The worms were then allowed to grow for 7 days at 20°C and then scored for live progeny (F2 and beyond).
Figure 2-S1. Germline atrophy in sterile *prg-1* animals. DAPI-stained photos of (A) a *prg-1* early generation L4, (B) *prg-1* early generation fertile adult, (C) an N2 adult, (D) a *prg-1* early generation adult with mitotic-miotic transition and normal oocyte production, (E) mitotic cells in a likely sterile *prg-1* L4, (F) a rare sterile *prg-1* animal with intact germline lacking embryo production, (G) a *prg-1, daf-16* sterile adult with oocytes.
Figure 2-S2. The daf-2/daf-16 insulin signaling pathway affects ARD and interacts with other ARD genetic factors. (A) Triple mutants containing mutant alleles of daf-2 produce sterile animals with phenotypic ARD distributions similar to the corresponding double mutants combining prg-1 with either daf-16 or daf-18. (B) Interplay between various stress response pathways in prg-1 mutants.
CHAPTER III: DAF-2/DAF-16 INSULIN SIGNALING FUNCTIONS THROUGH A SMALL RNA PATHWAY TO DIRECT GERMLINE HEALTH IN PRG-1 MUTANTS

SUMMARY

Many organisms can alter gene expression in response to both internal and external stimuli via RNAi silencing. In *C. elegans*, several pathways exist that regulate this response depending on the origin of the signaling stimulus. Deficiency for the Piwi Argonaute *prg-1* results in progressive sterility, during which the germline of the animals is drastically restructured as the animals enter a form of Adult Reproductive Diapause (ARD), partially regulated by Foxo transcription factor, DAF-16. Nuclear localization of DAF-16 to intestinal nuclei is the primary source for the signaling that directs germline rearrangement and facilitates DAF-16’s role in progressive sterility of *prg-1* mutants. The dsRNA channel protein, SID-1, is required for *daf-2* knockdown rescue and normal ARD response in *prg-1* mutants. Additional RNAi genes, including *mut-7, rde-2, rde-4*, and *rrf-3* are also required for *daf-2* knockdown rescue. We propose the existence of a previously undescribed pathway through which DAF-16 signaling in the soma functions through a dsRNA in response to an endogenous stress in *prg-1* deficient animals.

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3The following chapter contains work on a partially completed manuscript. Many of the experiments that follow up the results described in this chapter can be found in the Future Directions section. Current follow-up work expanding on the results described here is being performed in collaboration with Dr. Peter Sarkies and Dr. Eric Miska. I performed all work currently described in this section.
INTRODUCTION

The ability for tissues to communicate to each other within an organism is essential for metazoan homeostasis. These pathways that allow molecular signals to transmit from one tissue to another allow for coordinated responses to both external and internal stimuli, improving the organism's survivability to a variety of phenomena. RNA interference (RNAi) encompasses a number of such pathways, capable of regulating post-transcriptional gene activity within a cell and with the ability to propagate signals to other tissues (Fire et al., 1998; Gent et al., 2010). In C. elegans, the endogenous RNAi pathway responsible for self-gene regulation involves the production of primary siRNAs that require the RdRP RRF-3 and associate with the ERGO-1 Argonaute (Gent et al., 2009; Lee et al., 2006).

In C. elegans, the role of the daf-2/daf-16 insulin signaling pathways in somatic aging has been shown to rely on the nuclear localization of DAF-16 FOXO transcription factor (Murphy et al., 2003). DAF-16 can act both cell-autonomously and non-autonomously to regulate lifespan and gene expression (Libina et al., 2003, Murphy et al., 2007, and Zhang et al., 2012). The ability for daf-16 signals to extend beyond the effected cells serves an evolutionary mechanism of coordinated communication between tissues, allowing the organism to direct metabolic and developmental changes efficiently. Previously, it has been shown that elevated DAF-16 activity in the reduction of insulin/IGF receptor (IIR) activity inhibits germline proliferation cell-nonautonomously (Qi et al., 2012). However, a positive role for DAF-16 activity on germline health has been demonstrated (See Chapter I). These data suggest that different tissues contribute to the global affect of DAF-16 activity.

Here, we show that cell-nonautonomous DAF-16 functions to facilitate both the execution of the adult reproductive diapause (ARD) program in sterile prg-1 mutants and the rescue of progressive sterility induced by prg-1 loss. Additionally, we show that the role of a cell autonomous dsRNA channel,
*sid-1*, is necessary for both ARD and reduced IIR-mediated *daf-16* rescue of germline mortality. We go on to show that rescue of the progressive sterility requires components of the endogenous *rrf-3* RNAi pathway and contribution of DAF-16 signaling from intestinal tissue. In the context of prg-1 deficiency, a genetic background that has been previously shown to suffer from the accumulation of a toxic stress transgenerationally (*See Chapter II*), the data presented here indicates that the contribution of a cell-nonautonomous small RNA signal from somatic tissues is required for *daf-16*'s role in germline protection.
RESULTS

*sid-1 facilitates daf-16 rescue of prg-1 mutants*

The progressive sterility of *prg-1* mutants can be rescued by elevated *daf-2/daf-16* signaling ([See Chapter I](#)). Further characterization of this relationship revealed that DAF-16 localized to the nucleus of intestinal cells in sterile animals, indicating that the endogenous stress accumulating in *prg-1* mutants could trigger a stress response in a somatic tissue ([See Chapter II](#)). Given *prg-1*’s role in germline RNAi, we hypothesized that a small RNA signal produced by DAF-16 activity was responsible for the rescue of *prg-1* by *daf-2* knockdown. *sid-1* encodes a dsRNA channel that functions cell-autonomously in systemic RNAi ([Winston, et al. 2002](#)). We found that *prg-1; sid-1* double mutants did not show a reduced transgenerational lifespan, indicating that normal *daf-16* activity was not affected by the inability of a dsRNA signal to spread cell non-autonomously ([Figure 3-1A](#)). However, we found that *prg-1; daf-2; sid-1* triple mutants possessed a progressive sterility phenotype, indicating a failure of *daf-2* knockdown to rescue ([Figure 3-1A](#)). We conclude that the rescue of *prg-1* progressive sterility by increased *daf-16* signaling occurs cell non-autonomously and requires the dsRNA uptake channel, *sid-1*.

Having observed a role for systemic RNAi spreading on the rescue of *prg-1* by *daf-2* knockdown, we then asked if a small RNA signal was also responsible for *prg-1* sterile animals to enter ARD. We observed a 41.5% decrease in the number of animals with atrophied germlines in the *prg-1; sid-1* double mutants ([Figure 3-1B](#)). Additionally, we found slight increases in the other phenotypes associated with *prg-1* sterility. Curiously, we did not see a significant shift favoring any of the other germline phenotypes. This result might suggest that a small RNA signal from more than one pathway involved with ARD might rely on *sid-1* activity. Thus, *sid-1* is required for proper execution of ARD in *prg-1* mutant animals. Together with the role in progressive sterility, this data suggests that a RNAi signal is facilitated by *daf-16* activity and plays a role in both ARD and germline immortality.
**Figure 3-1: sid-1 is required for daf-2 rescue and ARD in prg-1 mutants.** (A) Deficiency in sid-1 activity disrupts rescue of prg-1 sterility by daf-2 knockdown. (B) Absence of sid-1 significantly alters sterile germline phenotype ratios compared to prg-1 single mutants. Animals with typical atrophy phenotype were drastically reduced in prg-1; sid-1 double mutants, while other phenotypes were slightly elevated.

**daf-16 rescue functions through an RNAi pathway**

In addition to the involvement of the germline Argonaute ppw-1 required for RNAi signal spreading (See Chapter I), the implication of sid-1 in the relationship between daf-16 activity and prg-1 deficiency suggests that an endogenous RNAi pathway plays a role in prg-1 progressive sterility and the ARD response in sterile animals.

Endogenous somatic RNAi is believed to involve two distinct phases of activity, of which the primary requires several proteins including the RNA dependent RNA polymerase, rrf-3, as well as the ERGO-1 Argonaute (Gent et al., 2010). Other genes, including rde-4 and eri-1 also play a role in this pathway. We hypothesized that the daf-16 signal affecting prg-1 transgenerational lifespan might use this endogenous RNAi pathway to effect germline health. We created triple mutants with prg-1; daf-2 to explore the requirements of prg-1 progressive sterility rescue in this vain (Figure 3-2A). We found that daf-16 mediated rescue of prg-1 progressive sterility required rde-4 and rrf-3, indicating that dsRNA production is necessary. Curiously, neither ergo-1 nor eri-1 was required to mediate rescue. This would suggest that the RNAi signal relies on another Argonaute to elicit rescue.
Figure 3-2: An endogenous RNAi pathway functions to enable somatic tissue-derived daf-16 rescue of prg-1 progressive sterility. (A) Components of the rrf-3 endogenous RNAi pathway play a role in daf-2 mutant rescue. All strains were grown at 20°C with n>20 for each genotype. (B) Tissue-specific daf-16 transgenes indicate roles of specific somatic tissues in daf-16 mediated rescue of prg-1 progressive sterility. muEx211 contains a GFP-tagged, cDNA-derived daf-16 transcript driven by the gut-specific ges-1 promoter, whereas muEx212 is driven by the muscle specific myo-3 promoter.

Multiple somatic tissues contribute to daf-16 signal input

Previously, we’ve shown that daf-16 mediated rescue requires the germline Argonaute, ppw-1, and the systemic RNAi spreading dsRNA channel, sid-1 (Figure 3-1A and Chapter I). Together, these data indicate that rescue occurs via a non-cell autonomous, somatic signal. Many tissues, including intestines and neurons, contribute to daf-16-mediated somatic longevity (Libina, et al. 2003). We generated prg-1,
*daf-16* mutants that contained *daf-16* transgenes driven by tissue specific promoters. We found that the triple mutant expressing the intestine-specific *daf-16* transgene (*muEx211*) had a transgenerational lifespan comparable to *prg-1* mutants, while the mutant expressing a muscle-specific transgene (*muEx212*) had a significantly shortened transgenerational lifespan (p=3.4E-03 vs. *prg-1*, *daf-16* double mutants. Additionally, we found that *muEx212* was also short lives when *daf-2* was mutant, while *muEx211* containing lines demonstrated a slightly extended transgenerational lifespan. Thus, intestinal expression of *daf-16* is sufficient to rescue *prg-1, daf-16* shortened transgenerational lifespan. Additionally, the inability of intestinal *daf-16* expression to restore *daf-2* mutant rescue indicates that *daf-16* activity in other tissues may be necessary for complete rescue.

*Figure 3-3: DAF-16 functions through a cell non-autonomous RNAi pathway to rescue prg-1 dysfunction.* DAF-16 activity in the soma can stimulate the rescue of prg-1 via the RNAi signal reliant on the the RdRP RRF-3 and the dsRNA binding protein RDE-4. The production of this rescuing signal occurs cell non-autonomously and spreads via the dsRNA uptake channel, SID-1.
DISCUSSION

With these experiments we demonstrate that somatically expressed \textit{daf-16} mediates \textit{prg-1} transgenerational lifespan and \textit{prg-1}-mediated ARD. The requirement of \textit{sid-1} for both the mutant \textit{daf-2} rescue of progressive sterility and normal germline ARD phenotypes shows that neither can occur without the spread of a somatic RNAi signal. Additionally, we show that the rescue of progressive sterility by increased \textit{daf-16} activity requires components of an endogenous RNAi pathway and \textit{daf-16} activity in somatic tissue, specifically the intestines.

In \textit{C. elegans}, animals can alter their germlines and suspend reproduction by entering ARD in response to starvation, demonstrating that an exogenous stress sensed by somatic tissue can trigger alterations to germline structure and activity (Angelo et al., 2009). Here, our results suggest that the accumulating endogenous stress in \textit{prg-1} deficient animals is capable of inducing a classically somatic stress response pathway outside of the germline (Figure 3-3). This pathway is then capable of triggering an ARD response in the germline and, if the signaling is sufficiently strong, can ameliorate the effects of the stress all together via an RNAi pathway.

We found that the RdRP \textit{rrf-3} and dsRNA channel \textit{sid-1} were necessary for this elevated \textit{daf-16} signaling to rescue \textit{prg-1} progressive sterility, which suggests that a dsRNA signal is the likely source of the initial response. The requirement of \textit{rde-4}, but not \textit{rde-1}, suggest that production of one or several dsRNA siRNA signals is necessary for rescue (Parrish et al., 2001). The expendability of \textit{rde-1} and \textit{ergo-1}, suggests that a currently unidentified Argonaute and a possible second protein are needed to transport the siRNA and remove the passenger strand. There is also the possibility that the dsRNA signal diffuses systemically to the germline, precluding an additional Argonaute.

\textit{daf-16} activity in the intestines was found to have a significant effect in \textit{prg-1} mutants, both in terms of the ARD response to stress and the transgenerational lifespan of the animals. The signal for intestine tissue was sufficient to rescue the transgenerational lifespan of \textit{prg-1}, \textit{daf-16} mutants and was
found to contribute to the formation of atrophied germlines in sterile animals. Currently, we have only
tested these two tissues daf-16 activity contributions and the non-complete rescue of the ARD
phenotype suggests that daf-16 activity in other tissues may play a role. In the daf-2/daf-16 somatic
aging pathway, activity from both intestines and neurons were found to affect lifespan (Libina, et al.
2003). The numerous parallels between the daf-16 somatic aging and its roles in the prg-1
transgenerational aging pathway make this an attractive hypothesis to test.

The link between a somatic longevity pathway and germline immortality demonstrated that
germline health and somatic health can be addressed without sacrificing one for the other. Our data
takes this link further by identifying a previously undescribed RNAi pathway through which DAF-16 can
function in the soma to affect germline health and development (Figure 3-3). This raises exciting
possibilities for the fields of aging and reproductive health, as both pathways are highly conserved in
humans. Additionally, the identification of a RNAi pathway and the implications that a siRNA or small
group of siRNAs suggests that both somatic aging and germline integrity could be regulated by a group
of downstream genes, effectively identifying new targets for intervention therapies.
MATERIALS AND METHODS

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, *dpy-5(e61) I, unc-13(e450) I, prg-1(n4357) I, prg-1(tm876) I, unc-55(e402) I, unc-29(e193) I, daf-16(mg50) I, daf-16(mu86) I, rrf-3(pk1326) II, dpy-10(e128) II, unc-4(e120), rde-4(ne301) III, daf-2(e1368) III, daf-2(e1370) III, daf-2(m41) III, dpy-17(e164) III, dpy-9(e12) IV, unc-24(e120) IV, eri-1(mg366), sid-1(pk3321) V, ergo-1(gg98) V, ergo-1(tm1860) V, rde-1(ne219) V, unc-60(e677) V, CF1514, CF1515

Germline Mortality Assay

(See Materials and Methods, Chapter I)

DAPI Staining

(See Materials and Methods, Chapter II)
CHAPTER IV: C. ELEGANS RSD-2 AND RSD-6 PROMOTE GERM CELL IMMORTALITY BY MAINTAINING SMALL INTERFERING RNA POPULATIONS

SUMMARY

Germ cells are maintained in a pristine non-aging state as they proliferate over generations. Here we show that a novel function of the C. elegans RNA interference proteins RSD-2 and RSD-6 is to promote germ cell immortality at high temperature. rsd mutants cultured at high temperatures became progressively sterile and displayed loss of small interfering RNAs (siRNAs) that target spermatogenesis genes, simple repeats and transposons. Desilencing of spermatogenesis genes occurred in late-generation rsd mutants, though defective spermatogenesis failed to explain the majority of sterility. Increased expression of repetitive loci occurred in both germ and somatic cells of late-generation rsd mutant adults, suggesting that desilencing of many heterochromatic segments of the genome contributes to sterility. NRDE-2 promotes nuclear silencing in response to exogenous double-stranded RNA, and our data imply that RSD-2, RSD-6 and NRDE-2 function in a common transgenerational nuclear silencing pathway that responds to endogenous siRNAs. We propose that RSD-2 and RSD-6 promote germ cell immortality at stressful temperatures by maintaining transgenerational epigenetic inheritance of endogenous siRNA populations that promote genome silencing.

The following chapter contains work on a submitted manuscript. The following chapter contains work done in collaboration with Dr. Aisa Sakaguchi, Dr. Peter Sarkies, Anna-Lisa Doebley, Dr. Leonard D. Goldstein, Ashley Hedges, Dr. Kohta Ikegami, Dr. Stacey M. Alvares, Lewei Yang, Dr. Jeannine R. LaRocque, Dr. Julie Hall, Dr. Eric A. Miska and Dr. Shawn Ahmed. I share the first authorship with Dr. Peter Sarkies, who performed much of the small RNA analysis and tiling array analysis, and Dr. Aisa Sakaguchi, who was the principle researcher for the majority of the project and did much of the writing. I contributed to the tiling array experiments, FISH experiments and analysis, karyotype and germline morphology experiments, and manuscript preparation.
INTRODUCTION

Cellular lifespan is regulated by developmental fate. Somatic cells typically have a limited lifespan of a single generation. In vertebrates, proliferation of somatic cells is governed by an irreversible state of cell cycle arrest that can occur in response to cellular stresses, termed senescence. Senescence is a powerful tumor suppressor mechanism, but it may also contribute to aging. Endogenous stresses have been clearly shown to accumulate with age to cause p53-dependent senescence include telomere attrition as well as irreparable telomere-associated DNA damage (Fumagalli et al., 2012 and Sharpless et al., 2007). Although distinct sources of endogenous stress accumulate as somatic cells proliferate and induce p16-mediated senescence, natural triggers of this pathway remain unclear (Sharpless et al., 2007).

One approach to address forms of stress that could contribute to proliferative aging of somatic cells is to study germ cell immortality. Germ cells have an effectively unlimited proliferative capacity as they are transmitted through the generations (Weismann, 1882). Germ cell immortality can be studied using C. elegans mortal germline (mrt) mutants that initially possess normal levels of fertility but become progressively sterile. Consistent with telomere attrition as a cause of proliferative aging in humans (Shay & Wright, 2005), mrt mutants with highly penetrant progressive sterility phenotypes can suffer from progressive telomere shortening as a consequence of defects in telomerase-mediated telomere replication (Ahmed and Hodgkin, 2000 and Meier et al., 2006), and these mutants typically become sterile at any temperature that they are propagated (Hofmann et al., 2002).

We report that the RNAi spreading proteins RSD-2 and RSD-6 (Tijsterman et al., 2004) are required for germ cell immortality at elevated temperatures. RSD-6 is a Tudor domain protein that is homologous to TDRD5 of mammals, which plays a role in spermatogenesis and suppression of
transposons (Yabuta et al., 2011), whereas RSD-2 does not have any clear mammalian homologs (Tijsterman et al., 2004). We found that the transgenerational fertility defects of rsd-2 and rsd-6 mutants are not restricted to spermatogenesis and are accompanied by desilencing of transposons as well as other repetitive loci, though transposition does not appear to be the trigger of sterility.
RESULTS

RSD-2 and RSD-6 promote germ cell immortality at stressful temperatures

Many *C. elegans* mrt mutants are temperature-sensitive and remain fertile indefinitely at low temperatures but become sterile after growth for multiple generations at the restrictive temperature of 25°C (Ahmed & Hodgkin, 2000). Of sixteen mrt mutants that were previously identified (Ahmed & Hodgkin, 2000), we found that yp10 and yp11 were defective in responding to RNA interference, as assessed by feeding *E. coli* expressing double-stranded RNA triggers (Figure 4-S1A). yp10 and yp11 only became sterile at 25°C and can be propagated indefinitely at lower temperatures. Outcrosses of yp10 and yp11 mutations revealed tight linkage of the exogenous RNAi and Mrt defects. Genetic mapping (Figure 4-S1B), non-complementation tests (Figure 4-1B) and DNA sequence analysis reveal that yp10 is an allele of rsd-2 whereas yp11 is an allele of rsd-6, two genes with roles in RNAi (Figure 4-1A) (Tijsterman et al., 2004). An independent allele of rsd-6, pk3300, was previously reported to become sterile immediately at 25°C (Han et al., 2008), but we found that this phenotype was due to a linked mutation (Figure 4-S1B). Instead, outcrossed rsd-6(pk3300) lines became progressively sterile at 25°C, similar to rsd-6(yp11) and rsd-2(pk3307) (Figure 4-1B). Thus, mutations in rsd-2 and rsd-6 elicit a highly penetrant Mrt phenotype at 25°C, in which strains display healthy levels of fertility for a number of generations prior to becoming completely sterile. In contrast, these strains remain completely fertile at the less stressful temperature of 20°C.

RSD-2 and RSD-6 may function in germ cells to promote germ cell immortality

RSD-6 and RSD-2 form a complex that was previously suggested to promote RNAi spreading from somatic to germ cells (Tijsterman et al., 2004). However, rsd-6 and rsd-2 mutants fail to respond to some somatic RNAi triggers (Han et al., 2008) and may simply be deficient for responding to low doses of exogenous dsRNA (Tijsterman et al., 2004 and Zhang et al., 2012). To test whether rsd-2 might function in the germline (Figure 4-1E), we performed immunofluorescence using a specific anti-RSD-2
antibody. RSD-2 was found at high levels in the cytoplasm of wild-type embryos (Figure 4-1F and 4-S1C-E), where it adopts a perinuclear position (Figure 4-1G). RSD-2 also localized to nuclei of most wild-type adult germ cells (Figure 4-1H, I). These results are consistent with previous observations of cytoplasmic and nuclear GFP-tagged RSD-2 in embryos (Han et al., 2008), though the specific localization to adult germ cell nuclei observed with our anti-RSD-2 antibody may more precisely reflect endogenous RSD-2. Thus RSD-2 could function in the germline and/or in early embryos to promote germ cell immortality.

To test this further, we generated rsd-6 strains carrying single copy rsd-6 transgenes driven by the germline-specific pgl-3 or by the ubiquitous dpy-30 promoter (Ashe et al., 2012 and Frokjaer-Jensen et al., 2008). Ppgl-3::rsd-6 and Pdpy-30::rsd-6 transgenes rescued the Mortal Germline defects of rsd-6(pk3300), indicating that rsd-6 functions within germ cells to promote germ cell immortality (Figure 4-1C). We confirmed this result by creating repetitive extrachromosomal arrays in wild-type animals, which results in ‘cosuppression’ or germline-specific silencing of the transgene and the corresponding endogenous gene (Dernburg et al., 2000 and Ketting & Plasterk 2000). Cosuppression of rsd-6 caused a temperature-sensitive Mrt phenotype (Figure 4-S1F, G).
Figure 4-1. rsd-2 and rsd-6 promote germ cell immortality. A-B, yp10 and yp11 mutations in rsd-2 and rsd-6 (A) fail to complement known rsd-2(pk3307) or rsd-6(pk3300) mutations for progressive sterility at 25°C (n=9 for WT and rsd-6 mutants, n=8 for rsd-2 mutants and for yp11/pk3300 and yp10/pk3307 trans-heterozygotes) (B). The RSD-2.a isoform is shown in panel a, but yp10 and pk3307 also affect RSD-2.b and .d isoforms (http://www.wormbase.org/). (C), A germline-specific pgl-3 promoter-driven rsd-6 genomic transgene Is[Ppgl-3::rsd-6] as well as a ubiquitous dpy-30 promoter-driven rsd-6 transgene Is[Pdpy-30::rsd-6] rescued progressive sterility of rsd-6(pk3300) at 25°C (n=4 for WT and pk3300, n=10 for rescued lines). (D), Lack of an additive fertility defect at 25°C in rsd-6; nrde-2 double mutants indicates that these genes act in the same pathway to promote germ cell immortality. Deficiency for rde-4 did not result in progressive sterility at 25°C, suggesting 26G-RNAs are dispensable for germ cell
immortality. (E), Model of the *C. elegans* germline. TZ; transition zone. (F-I), Anti-RSD-2 antibody immunofluorescent images assessed using widefield (F, H, I) and confocal (G) microscopy. RSD-2 localizes to cytoplasm of wild-type embryos (F), where it adopts a perinuclear position (G, arrows) and adult germ cell nuclei in wild type (H) but not in *rsd-2(pk3307)* (I). The germline is outlined with white dashes lines for panels G and I, which are images of the distal germline that reflect staining throughout the germline.

**Massive germline apoptosis and chromosome missegregation occur in sterile *rsd* mutants**

Cohorts of completely sterile late-generation *rsd-2* and *rsd-6* adults were stained with 4',6-diamidino-2-phenylindole (DAPI) to reveal that many contained germlines that were either reduced in size or absent (Figure 4-2A, B). Additionally, unlike wild type, a fraction of late-generation *rsd* mutant germlines were tumorous and showed excessive germ cell proliferation (Figure 4-2A, B). Mutations in the core cell death genes *ced-3* and *ced-4* (Metzstein et al., 2008) were used to test if apoptosis might affect germline development in sterile *rsd-2* or *rsd-6* animals. Atrophy and absence of germline phenotypes were significantly ameliorated for late-generation *rsd-6(yp11); ced-3* and *rsd-6(yp11); ced-4* double mutants, although the tumorous phenotype persisted (Figure 4-2B). In contrast, early generation animals maintained at both 20°C and 25°C displayed germline comparable to wildtype (Figure S2A). Thus, germ cell remodeling events promoted by apoptosis occurred in sterile *rsd* hermaphrodites, whereas tumorous germ cell overproliferation occurred independently of apoptosis.

Analysis of DAPI-stained oocytes in both early and late-generation sterile *rsd-2* or *rsd-6* adults revealed 6 spots, indicative of a normal karyotype in most of oocytes (Figure 4-2C, D and Figure S2B). However, some oocytes displayed an increase or decrease in the number of DAPI-positive spots, whereas N2 wild-type siblings propagated for the same number of generations did not (Figure 4-2C, D). Thus, the strong chromosome segregation defects of *rsd-2* and *rsd-6* mutations at sterility are consistent with exacerbation of a previously reported mild chromosome non-disjunction phenotype at high temperature (Han et al., 2008). Chromosome missegregation has been noted for other small RNA pathway mutants in *C. elegans* such as CSR-1, an Argonaute protein with roles in promoting chromosome segregation, histone mRNA maturation, and germline gene expression (Avgousti et al.,
In addition, RNAi defects lead to desilencing of repetitive DNA in pericentric heterochromatin in S. pombe, resulting in centromere cohesion defects and chromosome instability (Volpe et al. 2002).

In contrast to late-generation rsd mutants (Figure 4-2C, D), late-generation mrt mutants with telomerase defects display consistent decreases in the number of DAPI-positive spots (Ahmed & Hodgkin 2000 and Meier et al., 2006). Furthermore, most telomerase mutants are not temperature-sensitive and become sterile at all temperatures (Ahmed & Hodgkin 2000 and Meier et al., 2006). Thus, defects in telomerase-mediated telomere repeat addition are unlikely to explain the fertility defects of rsd mutants.

Figure 4-2. Germ cell proliferation defects and non-disjunction in late-generation rsd mutants. (A), DAPI images of wild type reveals normal germ cell proliferation (arrowheads), whereas sterile late-generation rsd-6 mutants can display germ cell atrophy (asterisk) or overproliferation (arrow). (B), Frequency of germline phenotypes in size in sterile late-generation rsd mutants as well as fertile wild-type controls grown at 25°C for the same number of generations (wild type n=40, yp11 n=70, pk3300 n=30, yp10 n=60, yp11; ced-3 n=30, yp11; ced-4 n=46, ced-3 n=30). (C, D), Images (C) and quantification (D) of DAPI-positive chromosome spots in oocytes of sterile late-generation rsd-6 or rsd-2 adults at 25°C, as well as matched fertile wild-type controls (arrows correspond to 6 chromosomes in diakinesis, dotted
circle surrounds a single oocyte nucleus) (wild type n=165, yp11 n=31, pk3300 n=9, yp10 n=45). Bars; 100 μm(A), 10 μm(C).

**Small RNAs targeting spermatogenesis genes are perturbed in rsd mutants**

We hypothesized that RSD-2 and RSD-6 might protect germ cell integrity based on their roles in endogenous RNA silencing pathways. To verify this hypothesis, we fed dsRNA against GFP to strains that carried a germline-expressed Ppie-1-gfp transgene and examined small RNA populations against the gfp transgene by RNA sequencing. We found that rsd-2 mutants are defective for secondary 22G-RNA production or maintenance in the germline, rather than for primary siRNA biogenesis (Figure 4-S3A-F), consistent with a previous report that RSD-2 and RSD-6 promote secondary siRNA amplification (Zhang et al., 2012).

We next examined small RNA populations from mixed developmental stages of early- and late-generation rsd-2 and rsd-6 mutant strains grown in parallel at 20°C and 25°C. RNA was prepared from late-generation populations that were close to sterility but had very few sterile adults, such that comparable amounts of germline tissue would be present in early- and late-generation RNA samples. Deep sequencing of single rsd-2 or rsd-6 samples was performed to define consistent molecular changes that occurred at 25°C (two independent alleles tested per genotype), but did not occur in wild-type controls at 25°C. We found that 21U-RNAs corresponding to known piRNA genes (Batista et al., 2008 and Ruby et al., 2006) were still present and even increased in abundance in late-generation rsd-2 and rsd-6 animals (Figure 4-S3G), as were 26G-RNAs which are also involved in endogenous silencing pathways (Figure 4-S3H) (Han et al., 2009). By contrast, 22G-RNAs antisense to protein-coding genes were reduced at a subset of genes after repeated passages of rsd-2 animals at 25°C (Figure 4-S3I), and the number of genes showing this reduction was significantly larger in rsd-2 animals than that in wild-type animals, as revealed by a Q-Q plot where all points would follow the X=Y axis if there were no difference between wild type and rsd-2 (Figure 4-3A). We identified 481 protein-coding genes that were >16-fold depleted for 22G-RNA reads in late-generation rsd-2 relative to early-generation rsd-2 grown at 25°C.
22G-RNAs mapping to these genes were also reduced in *rsd-6* late- versus early-generation (*P*<2e-16, Wilcoxon Signed Rank Test) (Figure 4-S3J). Genes targeted by these depleted 22G-RNAs overlapped strongly with genes downstream of ALG-3/-4-dependent 26G-RNAs (*P*=1e-16, Fisher’s Exact Test) (Figure 4-3B), which have been shown to target spermatogenesis genes (Conine et al., 2010) (see below). Consistently, the set of RSD-2-dependent genes also overlapped strongly with spermatogenesis genes (Reinke et al., 2004) (*P*<2e-16, Fisher’s Exact Test) (Figure 4-3B). We compared these genes to a set of 248 genes that show reduced 22G-RNAs in *rsd-2* mutants grown at 20°C relative to WT (Zhang et al., 2012). There was a statistically significant overlap between the two sets of genes (*P*<1e-3, Fisher’s Exact Test); however, none of the spermatogenesis genes affected in *rsd-2* mutants at 25°C were also affected at 20°C (Figure 4-S3K), thus the progressive reduction in small RNAs mapping to spermatogenesis genes in *rsd* mutants only occurs at higher temperatures.

The reduction in small RNAs mapping to spermatogenesis genes in late-generation *rsd* mutants was intriguing because increased expression of spermatogenesis genes has been implicated in the transgenerational sterility phenotype of SPR-5 histone demethylase mutants (Katz et al., 2009). At the same time, a recent report suggests that transgenerational depletion of small RNAs dependent on the Argonautes ALG-3/-4 and CSR-1 leads to sterility accompanied by reduced spermatogenesis gene expression (Conine et al., 2013). We therefore tested whether the expression of spermatogenesis genes in *rsd-2/-6* was altered in late-generation animals. Systematic examination of the expression of 760 spermatogenesis genes (Reinke et al., 2004) using tiling microarrays across the *C. elegans* genome demonstrated strong upregulation in late-generation versus early-generation 25°C *rsd-2* and *rsd-6* animals (for both *rsd-2* and *rsd-6* *P*<2e-16, Mann Whitney Unpaired Test) (Figure 4-3C, Dataset 4-S2). This upregulation was not seen at 20°C (*rsd-2* *P*<2e-16; *rsd-6* *P*<2e-16, Wilcoxon Signed Rank Test) (Figure 3C), and downregulation of spermatogenesis genes occurred infrequently. Thus, in contrast to the effect of ALG-3/-4 and CSR-1, loss of *rsd-2/-6* results in increased expression of spermatogenesis
genes. However, the spermatogenesis genes targeted by *rsd*-2 are not exactly the same as those targeted by SPR-5 (Dataset 4-S2); moreover, there was no upregulation of non-spermatogenesis genes targeted by *spr*-5 (Katz et al., 2009) in late-generation *rsd*-2 mutants (Figure 4-3D). This implies that although RSD-2, RSD-6 and SPR-5 repress spermatogenesis gene expression, SPR-5 regulates a distinct repertoire of targets.

We tested if the sterile phenotype of late-generation *rsd*-6 hermaphrodites was due to disrupted spermatogenesis by mating wild-type males with sterile *rsd*-6 hermaphrodites, but found that fertility was rarely restored (n=0/98, 2/132, 2/36, 1/48 and 1/40 sterile *rsd*-6 hermaphrodites mated with wild-type males gave rise to progeny in 5 independent trials). Thus, the transgenerational sterility of *rsd* mutants primarily arises from more widespread germline dysfunction than defects in spermatogenesis alone.
Figure 4-3. Gene expression changes in rsd mutants associated with loss of small RNAs. (A), Q-Q plot to identify differences from normal distribution shows overrepresentation of genes with reduced antisense small RNAs at 25°C in late versus early-generation rsd-2 mutants compared to late- versus early-generation wild type. Arrow indicates deflection from the expected y=x line distribution. (B), Overlap of 22G-RNA-depleted genes in late-generation rsd-2 strains at 25°C with spermatogenesis and ALG-3/-4 regulated genes (Conine et al., 2010). The total number of genes in each set including subsets is shown inside the relevant section. (C), Tiling array analysis shows upregulation of spermatogenesis genes in late- versus early-generation rsd strains at 25°C but not at 20°C. 0 indicates no change in expression. Shift to the right indicates increased expression. Density reflects the number of genes at a specific expression level relative to other segments. (D), Tiling array analysis indicates spermatogenesis genes
that are upregulated by spr-5 show higher expression in late- versus early-generation rsd-2 mutants at 25°C but non-spermatogenesis genes that are upregulated by spr-5 does not. (E-F), RNA sequencing analysis shows wider spread of 22G-RNA reads, normalized to total library size, mapping with up to two mismatches to transposons (E) or tandem repeats (F) in late-generation rsd-2 strains at 25°C. (G), Expression of tandem repeats increases in late-generation rsd mutants, compared to control sequences of the same length drawn randomly from non-repetitive regions of the genome at 25°C. (H), Expression of the 19 longest tandem repeat tracts in the C. elegans genome in late- versus early-generation rsd mutants analyzed by tiling arrays. (I), Tiling array data from UCSC genome browser reveals upregulation of a 172-mer repeat tract in late- versus early-generation rsd strains grown at 25°C but not at 20°C, and also in late-generation rsd-6 versus late-generation wild-type RNA at 25°C. (J), RT-PCR analysis confirms results shown in panel I. E, early generation. L, late generation. M, size marker. act-1 PCR is shown as loading control.

Desilencing of repetitive loci in stressed late-generation rsd mutants

In addition to targeting 22G-RNAs, vertebrate and Drosophila Tudor domain proteins are known to target repetitive regions of the genome such as transposons (Yabuta et al., 2011; Patil & Kai 2010; Liu et al., 2011; Bagijn et al., 2012; Huang et al., 2011; Shoji et al., 2009). We therefore mapped 22G-RNAs in rsd mutants against these repetitive loci. In late-generation rsd-2 animals grown at 25°C, we found significantly wider distribution of differences for 22G-RNAs mapping against transposons (Figure 4-3E) and tandem repeats (Figure 4-3F) such that some showed increased 22G-RNA reads and some showed strongly reduced 22G-RNA reads (both P<2e-16, Kolmogorov-Smirnov test). We also found that rsd-2 and rsd-6 showed highly comparable changes at individual transposon loci (Figure 4-S4). Moreover, similarly to rsd-2, the wider distribution of differences was seen for 22G-RNA reads against tandem repeats and transposons in 25°C rsd-6 late-generation animals (tandem repeats P<0.05, transposons P<2e-16, Kolmogorov-Smirnov test) (Figure 4-S3L, M). We also mapped 22G-RNAs from previously published sequencing data of rsd-2 mutants grown at 20°C (Zhang et al., 2012) to transposons and tandem repeats. Whilst 22G-RNA reads against transposons showed little difference from wild type (Figure 4-S3N) and whilst a small subset of repeats showed reduced reads (Figure 4-S3O) at 20°C, there was no evidence of the widespread dysfunction that was seen at 25°C. Thus, RSD-2 and RSD-6 proteins promote accumulation of 22G-RNAs targeting repetitive regions of the genome at the stressful temperature of 25°C, an activity that is consistent with a silencing defect that promotes sterility in rsd
mutants, as discussed below. Consistently, genome-wide, we saw a dramatic increase in the expression of repetitive repeat regions compared to control regions of the genome in both rsd-2 and rsd-6 late-generation animals at 25°C, but not for wild type (Figure 4-3G, H), but not at 20°C (Figure 4-3H, Figure 4-S5A) (P<2e-16, Wilcoxon signed rank test). The upregulated loci included transposons (Figure 4-S5E) and short direct tandem repeat tract loci, composed of 25- to 180-mer repeat tract and total lengths up to 50 kb (Figure 4-3I, Figure 4-S5B, C), as confirmed by RT-PCR analysis of rsd samples (Figure 4-3J, and Figure 4-S5D). These data show clearly that up-regulation of repeats is much greater relative to random selections of regions in the genome and is systematically seen across the genome.

As the microarray analysis was performed using RNA from mixed stages of development, we examined the cellular localization of repetitive RNA expression in adults and early embryos using RNA Fluorescence In Situ Hybridization (FISH). We detected RNA from independent tandem repeat sequences in embryos for all strains examined at 25°C (Figure 4-4A-C, G-I, M-R, and 4-Figure S6P-R) and also at 20°C for wild type, including both antisense and sense probes of CeRep59 (Figure 4-S6S-U). We did not detect tandem repeat RNA in the adult germline or somatic cells of wild-type animals (Figure 4-4G-I and P-R, and Figure 4-S6P-U) or in control mid-generation rsd-6 mutants at 25°C (Figure 4-4M-O), suggesting that tandem repeats are silenced there. However, we saw a strong signal for tandem repeat probes in both the germline and somatic cells of late-generation rsd-2 and rsd-6 mutants grown at 25°C (Figure 4-4A-F and J-I, and Figure 4-S6A-C and J-O). This signals was considerably elevated in sterile animals compared to fertile, early generation controls (Figure S8). Control RNA FISH probes for repetitive loci that express 26S ribosomal RNA were detected at high levels in the germline of all strains examined, and this did not change in late-generation rsd-6 animals (Figure 4-S6D-I). Thus RNA from repetitive regions accumulates in both somatic and germ cells of rsd mutants concomitantly with the onset of sterility.
Expression of RNA from both strands of repetitive loci during embryogenesis could promote production of siRNAs that silence repeats in the germline and soma of wild-type adults (Figure 4-4G-I and P-R, and Figure 4-S6P-U). Although it was previously shown that the 26G-RNA class of primary siRNAs that target endogenous loci for silencing are generated during embryogenesis by RDE-4 (Vasale et al., 2010), we found that deficiency for rde-4 did not result in progressive sterility at 25°C (Figure 4-1D). Thus, 26G-RNAs are dispensable for germ cell immortality.

As repetitive elements including transposon loci were desilenced in rsd-2 and rsd-6 animals, increased levels of transposition might account for the progressive sterility phenotype. Transposition of a few DNA transposons can be scored based on established excision assays (Vasale et al., 2010), but these tests represent a limited analysis of the diversity of RNA and DNA transposons present in the C. elegans genome. We therefore employed unbiased tests that would detect increased activity for any transposon and obtained three independent lines of evidence that increased levels of transposition are unlikely to occur in late-generation rsd mutants (Figure 4-S5F-J).
Figure 4-4. Germline and somatic expression of tandem repeats in late-generation rsd mutants. (A-R), Confocal images of Cy5-labeled RNA FISH probes (A, D, G, J, M, P), DAPI-stained nuclei of the corresponding images (B, E, H, K, N, Q), and merged images containing Cy5 and DAPI signals (C, F, I, L, O, R) for animals derived from strains grown at 25°C. RNA FISH probes reveal expression of CeRep59 antisense tandem repeat (A-I) or CeRep59 sense tandem repeat (J-R) in late-generation rsd-2 (A-C), rsd-6 (D-F, J-L) or control mid-generation rsd-6 (M-O), control wild-type (G-I, P-R) two-day old adults. All images were taken under the same condition. The germline is outlined with white dashes line and embryo is outlined with white solid line for merge panels. (S, T), Models of RSD-2/-6 and NRDE-2 function in transgenerational silencing. RSD-2/-6 and NRDE-2 function via independent RNA silencing pathways with immediate effects in response to exogenous dsRNA (S, top). However, they function in a common transgenerational nuclear silencing pathway that responds to endogenous siRNAs and is revealed only at high temperature (S, bottom). Expression of forward (purple) and reverse (red) strands of RNA from repetitive loci occurs in early embryos (T, top cell), which may lead to siRNA production in the cytoplasm. In adult germ cells, RSD proteins translocate to the nucleus (T, bottom cell), where they may act with NRDE-2 to facilitate a temperature-sensitive process that promotes transgenerational siRNA-mediated silencing of repetitive loci.

RSD-6 acts via small RNA-mediated transcriptional silencing

Since RSD-2 localized to nuclei of adult germ cells (Figure 4-1H) where tandem repeats were ectopically expressed in late-generation rsd-2 and rsd-6 mutants grown at 25°C (Figure 4-4 and Figure 4-S6), we hypothesize that RSD-2 and RSD-6 promote germ cell immortality in nuclei of adult germ cells. It
was recently reported that three genes that promote RNAi-mediated silencing at genomic loci within nuclei are required for germ cell immortality (Buckley et al., 2012). Mutation of one of these nuclear RNAi genes, nrde-2, results in sterility if propagated at 25°C, similar to rsd-2 and rsd-6 (Figure 4-1D), whereas deficiency for nrde-1 and nrde-4 compromises germ cell immortality at all temperatures (Buckley et al., 2012). To determine how nrde-2 and rsd mutations interact in the context of germ cell immortality, we constructed rsd-6; nrde-2 double mutants, which displayed a transgenerational lifespan (6.66±0.18 generations) that was slightly longer than that of nrde-2 mutants (5.72±0.14 generations) (Figure 4-1D and Table 4-S1). Lack of an additive fertility defect in rsd-6; nrde-2 double mutants, as compared to single mutant controls, indicates that these genes act in the same pathway to promote germ cell immortality. Consistently, late-generation nrde-2 mutant adults expressed high levels of repeat RNA in both germ and somatic cells, comparable with rsd-6 mutants (Figure 4-S7).
DISCUSSION

We demonstrate that *rsd-2* and *rsd-6* are required for germ cell immortality at 25°C, where strains display robust fertility for many generations and then yield a highly penetrant sterile phenotype. This Mortal Germline phenotype implies gradual accumulation of a heritable defect. Our data argue that perturbed transgenerational maintenance of 22G secondary siRNA populations occurs as *rsd* mutants are propagated at 25°C (*Figure 4-3E-F, Figure 4-S3L-M*), resulting in a genome-wide failure of epigenetic silencing and infertility.

*C. elegans* harbors both pro- and anti-silencing secondary siRNA systems, where pro-silencing 22G-RNAs associate with WAGO-class Argonaute proteins and anti-silencing 22G-RNAs associate with ALG-3/-4 and CSR-1 Argonaute proteins. Both these pathways have been implicated in transgenerational sterility phenotypes (Conine et al., 2013 and Simon et al., 2014). Conine *et al.* reported that heterozygosity for *alg-3/-4* or *csr-1* mutations in males can elicit a temperature-sensitive Mortal Germline phenotype, apparent only at 25°C (Conine et al., 2013). This was accompanied by transgenerational depletion of anti-silencing 22G-RNA populations associated with CSR-1, and by reduced expression of spermatogenesis genes. Further, late-generation sterility of *alg-3/-4* or *csr-1* heterozygotes was caused by a highly penetrant spermatogenesis defect that was frequently and strongly rescued by wild-type sperm (Conine et al., 2013). Our finding that *rsd* mutants lose 22G-RNAs against spermatogenesis genes is reminiscent of this effect; however, *rsd* mutants show transgenerational increase in expression of spermatogenesis genes rather than the reduced expression presumed to underlie the phenotype of *csr-1* heterozygosity. Moreover, the fertility of *rsd* mutants cannot be rescued by wild-type sperm. Our data therefore suggest that RSD-2 and RSD-6 do not act in the same pathway as CSR-1/ALG-3/-4 to maintain spermatogenesis gene expression.

Our study instead suggests that a key function of RSD-2, RSD-6 and NRDE-2 is to promote germ cell immortality via a common transgenerational endogenous small RNA-mediated nuclear silencing
process (Figure 4-1D and 4-S5). Since rsd-2/-6 and nrde-2 mutants only became sterile at 25°C, RSD-6 and RSD-2 may function in conjunction with NRDE-2 at the crux of an intrinsically temperature-sensitive process that is essential for germ cell immortality, possibly involving maintenance of pro-silencing endogenous 22G-RNAs populations that promote maintenance of heterochromatin (Figure 4-4T). Such a model is reminiscent of small RNA-mediated heterochromatin silencing in yeast, Drosophila and plants (Slotkin & Martienssen 2007).

Despite the involvement of the nuclear RNAi pathway in silencing repetitive sequences, we detected both forward and reverse strands of tandem repeat sequences in the cytoplasm in wild-type embryos (Figure 4-4 and Figure 4-S6). Thus, bursts of transcription from repetitive loci during embryogenesis may generate both primary and secondary siRNAs against these sequences. These data suggest a developmentally regulated mechanism of siRNA production that promotes transgenerational maintenance of pro-silencing siRNA populations associated with heterochromatin. Interestingly, RSD-2 was cytoplasmic in embryos, whilst in adults we observed RSD-2 in the nuclei of germ cells. This suggests that RSD-2 could couple cytoplasmic siRNA production with maintenance of NRDE-2-dependent nuclear silencing in the adult germline and soma (Figure 4-1F-H, 4, Figure 4-S6 and Figure 4-S7), thus acting to link cytoplasmic and nuclear RNAi pathways. Alternatively, expression of repetitive loci during embryogenesis may represent a developmentally conserved stage of reprogramming of silent chromatin that is separable from the function of RSD-2/RSD-6 in siRNA maintenance at repetitive loci, which could be restricted to nuclei of adult germ cells (bottom cell of Figure 4-4T), consistent with RSD-2 localization (Figure 4-1H).

Our data imply that RSD-2 and RSD-6 promote transgenerational maintenance of siRNA populations that repress expression of repetitive loci such as transposons and tandem repeats (Figure 4-5S, T). Despite increased expression of transposons, we found no evidence for transposition in late-generation rsd mutants (Figure 4-5SF-J). This may be because there are several layers of defense against
transposons, so increased expression of transposon RNA could occur without significant increases in transposition. However, even without increased transposition, increased expression of repetitive regions of the genome including transposons or tandem repeats or associated effects on chromosome architecture may contribute to the transgenerational fertility defect of rsd mutants.

One goal of studying germ cell immortality in C. elegans is to create models for forms of stress that may be relevant to aging of mammalian somatic cells as they proliferate (50 to 100 cell divisions). Though the over- and under-proliferation phenotypes of sterile rsd adult germ cells may appear to be effects of an opposite nature (Figure 4-2), proliferative aging of mammalian somatic cells has potentially analogous consequences, where age-related stress can trigger senescence or apoptosis but can also lead to inappropriate overproliferation in the context of tumor development. Our data suggest that a malleable siRNA-based repeat silencing system in germ cells, which may be subject to stochastic, genetic or epigenetic effects in human populations, could explain some of the transgenerational variation in the penetrance of aging-related disorders.
MATERIALS AND METHODS

Strains

Unless noted otherwise, all strains were cultured at 20°C on Nematode Growth Medium (NGM) plates seeded with E. coli OP50 (Brenner, 1974). Strains used include Bristol N2 wild type, dpy-5(e61) I, mut-2(r459) I, unc-13(e450) I, unc-13(e51) I, rsd-6(yp11) I, rsd-6(pk3300) I, daf-8(m85ts) I, unc-29(e193) I, EG4322 ttTi5605 II (Mos1); unc-119(ed3) III, nrde-2(gg95) II, rde-4(ne301) III, sma-2(e502) III, unc-32(e189) III, ced-4(n1162) III, unc-30(e191) IV, ced-3(n717) IV, unc-26(e205) IV, rsd-2(yp10) IV, rsd-2 (pk3307) IV, dpy-4(e1166) IV, dpy-11(e224) V, unc-58(e665) X.

RNAi mutants mut-2 and rsd-6 were outcrossed using unc-13 as a marker. RNAi mutant rde-4 was outcrossed using unc-32 as a marker. rsd-2 was outcrossed using unc-30 and dpy-11 as markers, respectively.

Mutator assays were performed at 25°C with late-generation unc-58 single mutant and rsd-6; unc-58 or rsd-2; unc-58 or mut-2; unc-58 double mutants as previously described (Harris et al., 2006).

Germ Line Mortality Assay

(See Chapter I Materials and Methods)

Feeding-RNAi Assay

Feeding RNAi plates harboring host bacteria HT115(DE3) engineered to express pop-1, par-6 or pos-1 dsRNA were prepared from the Ahringer RNAi library (Kamath et al., 2003). L4 larvae were placed onto freshly prepared feeding RNAi plates with dsRNA induced by 1 mM IPTG (Isopropyl-β-D(-)-thiogalactopyranoside), and were transferred animals every 24 hours (Kamath et al., 2001). Assays were performed at 20°C.

Gene silencing via cosuppression

A fragment from 2 kb upstream to 1 kb downstream of rsd-6 gene including 1st and 2nd exons was amplified from N2 wild-type genomic DNA for the co-suppression experiment. The amplified
fragment was injected into N2 wild-type animals with pCes1943[rol-6(su1006)] marker, and animals with transgenes were distinguished by the Roller phenotype of su1006.

**DAPI Staining**

DAPI staining was performed as previously described (Ahmed & Hodgkin 2000). 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. The stained animals were observed by Nikon Eclipse E800 microscope.

**RSD-2 immunofluorescence**

Adult hermaphrodites raised at 20°C were dissected in M9 buffer and flash frozen on dry ice before fixation for 20 minutes in Methanol at -20°C. After washing in Phosphate Buffered Saline (PBS) supplemented with 0.1% Tween-20 (PBST), a Rabbit polyclonal antibody against amino acids 581-730 of RSD-2 (F52G2.2) (SDIX, Windham, ME) at a 1/3000 dilution in PBST was used to immunostain overnight at 4°C in a humid chamber. Secondary antibody staining was performed using an Alexa-Fluor 488-coupled mouse anti-rabbit, for one hour at 37°C. Slides were visualized using a Zeiss (Figure 4-1F, H, I, Figure 4-S3A-C) or Olympus Upright Confocal microscope (Figure 4-1G). Staining was also carried out in parallel on rsd-2(pk3307) animals and showed no reactivity of the antibody with embryos, germline or oocytes.

**RNA Preparation and cDNA Synthesis**

RNA for tiling arrays and RT-PCR was prepared at each generation. Mixed staged animals were collected with M9 buffer and total RNA was purified by a guanidinium thiocyanate-phenol-chloroform (TRIzol) extraction method (Chomczynski & Sacchi, 1987). First-strand cDNA was synthesized using Super Script II or III Reverse Transcriptase (Invitrogen). Second-strand cDNA for tiling array was synthesized using Second Strand Buffer (Invitrogen), *E. coli* DNA ligase (NEB), *E. coli* DNA polymerase I (Promega) and T4 DNA polymerase (NEB). Synthesized cDNA were fragmented by sonication for tiling arrays.
**Tiling arrays**

Tiling microarrays were performed as previously described (Ikegami et al., 2010). The detection platform was Roche NimbleGen Custom 2.1M Tiling arrays, with 50-mer probes, tiled every 50 bp for the WS170 (ce4) genome build, providing even and almost gap-free coverage across the whole *C. elegans* genome. Samples were amplified using Ligation-Mediated PCR (LM-PCR) as previously described (Selzer et al., 2005). Late-generation mutant samples were labeled with Cy5 and their input reference late-generation wild type or early-generation mutant with Cy3 following the methods described (Selzer et al., 2005). Single microarrays were performed for each early- versus late- or late- versus late-generation experiment, using RNA samples from independent alleles of *rsd-2* and *rsd-6*, which play very similar roles in RNA interference and germ cell immortality, and whose gene products have previously been shown to physically interact (Tijsterman et al., 2004).

**Microarray data analysis**

Data normalized for gene bodies was analyzed using custom scripts in R to identify spermatogenesis genes as previously annotated (Reinke et al., 2004). Statistical testing for spermatogenesis gene expression changes was performed by using the entire set of spermatogenesis genes as a sample so that a paired test could be carried out between changes at 20°C and at 25°C, and an unpaired test between changes in spermatogenesis genes and all genes. Repeat expression was analyzed by mapping probes to either repetitive regions downloaded from the UCSC genome browser website (http://genome.ucsc.edu/), or to control regions randomly selected from each chromosome to have the same distribution of lengths as the repeats on the same chromosome. Statistical testing for repeat upregulation was performed as for gene expression changes. Non-parametric methods were used in both cases to avoid assumptions of normality.

**Analysis of copy number changes**
Z-scores comparing Comparative Genome Hybridization intensity from early- and late-generation *rsd-6* animals grown at 25°C were interrogated for regions annotated as transposons or tandem repeats using the ce6 genome (WS190) annotation. Each set was compared to control regions randomly selected with the same length distribution as the test set as described for microarray data analysis. Statistical testing for increased copy number analysis was performed using non-parametric methods. This method was successful in identifying increased copy number of transposons in late-generation mutants lacking *prg-1* (Simon et al., 2014).

**Small RNA sequencing analysis**

5′ independent small RNA sequencing was performed as described previously (Ashe et al., 2012), using one repeat for each time-point of N2 WT, *rsd-2* and *rsd-6* at 25°C. Custom Perl scripts were used to select different small RNA species from the library. To map small RNA sequences to genes, reads were aligned to the *C. elegans* ce6 genome using Bowtie, Version 0.12.7, requiring perfect matches. Data was normalized to the total number of aligned reads and 1 was added to the number of reads mapping to each gene to avoid division by zero errors. To map 22G sequences to transposons and tandem repeats, direct alignment to the transposon consensus sequences, downloaded from Repbase (Ver 17.05) or repeats obtained from the ce6 genome (WS190) annotations downloaded from UCSC as above, was performed using Bowtie allowing up to two mismatches and reporting only the best match. Uncollapsed fasta files were used for these alignments to compensate for the problem of multiple identical matches. Data was normalized to the total library size and 1 was added to the number of reads mapping to each feature to avoid division by zero errors. In order to analyze data from *rsd-2* mutants grown at 20°C (Zhang et al., 2012), Fasta files were downloaded from the Gene Expression Omnibus and uncollapsed using a custom Perl script before aligning to transposons or tandem repeats as above. Analysis of data was carried out using the R statistical package.

**RT-PCR Analysis**
Total RNA was isolated as described above. RT-PCR was accomplished using the Anchor T primer (ATACCCGCTAATTTTTTTTTTTT) with Superscript® III First Strand Synthesis System (Invitrogen). The resulting cDNA was then normalized using serial cDNA dilutions with act-1 specific primers with Ex Taq polymerase (TaKaRa), a 64°C annealing temperature and 30 PCR cycles. cDNA concentrations were normalized based on ethidium bromide fluorescence of samples separated on 1% agarose gels. Tandem repeats were then analyzed using PCR amplification with a 65°C annealing temperature and 28 PCR cycles. PCR primers used were:

*act-1* Fwd: GATATGGAGAAGATCTGGCATCA
*act-1* Rev: GGGCAAGAGCGGTGATT
TanRepI Fwd: CGATGCTCTTTGTAGACAAATCA
TanRepI Rev: GCACCCAATATTTAGAGACAGAG
TanRepII Fwd: CATAGGGCATCGAAAGCAG
TanRepII Rev: GAAAATCATCAATTTCTGGAGGC
TanRepIV Fwd: GAACCTTTGAACATGCTCCAAAC
TanRepIV Rev: GCCATGCCTTTGTACATATC

**Genetic mapping and complementation tests of *sma*-3 lines**

Each of the five *sma* mutations were outcrossed away from the original *rsd* mutations in order to analyze what might have caused the Small or Dumpy phenotype. In order to determine whether the Small/Dumpy phenotype occurred in the same gene for all five mutations, *sma; unc-3* double mutants were created and crosses with F1 *sma -/+* males that were generated from crosses of *sma -/-* hermaphrodites with wild-type males. Sma animals were observed in non-Unc cross progeny for all combinations of the 5 independent *sma* alleles, indicated that they failed to complement one another and are likely to be alleles of the same gene.
We used a SNP mapping approach (Davis et al., 2005) to genetically map the *sma* mutations and found that they were located on Chromosome III, between -7 and +1.55 map units. Mutations in three genes within this genetic interval, *sma*-2, *sma*-3, and *daf*-4, are known to cause small body size (Patterson & Padgett 2000). Heterozygous *sma*-2(e502)/+ or *sma*-3(e491)/+ males were crossed with each *sma*; unc-3 double mutant lines and progeny were scored for the Small phenotype. All F1 cross progeny sired by *sma*-2(e502)/+ males were wild type while a proportion of F1 cross progeny sired by *sma*-3(e491)/+ males exhibited a Small phenotype, indicating failure to complement *sma*-3. Primers were generated to amplify the entire genomic region of *sma*-3, and PCR products created with these primers were sequenced in both directions for each allele and for wild type to clearly show that each *sma* mutation occurred as a consequence of the same single nucleotide insertion. Primers used to amplify the *sma*-3 coding region were: AATTCAGGTGGTGCGAGAAG and TTTCCGCTCAGTTTACCCAC. Sequencing primers were: AATTCAGGTGGTGCGAGAAG, ACTTTGACCAGTGGCATGTTC, CGTTGAGCTTCCACTAGACTGC, AACTTCATAAGCGCGTCGAG, TTGAGCCTCCTCCTCCTTAGTC, TTTCCGCTCAGTTTACCCAC and AAGCTTCAATCAGCTATTCT. 

**RNA Fluorescence In Situ Hybridization**

DNA oligonucleotide probes coupled with a 5' Cy5 fluorophore were used to detect repetitive element expression. The four probes used in this study were:

- tttctgaagcccagaatct - CeRep59 on Chromosome I (located at 4281435-4294595 nt)
- agaattctgcttcagaaa - antisense CeRep59 on Chromosome I
- caactgaatcctcctca - Chromosome V tandem repeat (located at 8699155-8702766 nt)
- gcctaagttcagcggttaat - 26S rRNA

Probes were diluted in RNase free TE buffer immediately before use to produce a working 25 mM stock. The strains used for RNA FISH experiments were *rds-6*(*yp11*), *rds-2*(pk3307), and N2 Bristol wild type. Mixed stage animals were washed off from non-starved plates into microcentrifuge tubes, then washed
once in M9 buffer and three times in 1mL of 1x DEPC-treated PBS. Fixation was performed by suspending worms in 1 mL of fixation buffer (3.7% formaldehyde in 1x DEPC-treated PBS) for 45 minutes rotating at room temperature. After fixation, worms were washed twice in 1mL 1x DEPC-treated PBS. Fixed animals were permeabilized overnight at 4°C in 70% Ethanol in DEPC-treated H₂O. Following permeabilization, worms were washed once in 1mL wash buffer (10% formamide in 2x RNase-free SSC) then hybridized in 100 μL hybridization buffer (0.2 g dextran sulfate, 200 mL 20x RNase-free SSC, 200 mL deionized formamide, 1.5mL DEPC-H₂O) with 1.25 μM probe. Worms were incubated at 30°C overnight. After these animals were washed in 1 mL wash buffer for 30 min at room temperature and washed again in 1 mL wash buffer with 25 ng/mL DAPI counterstain for 30 min at room temperature. Animals were mounted for imaging on glass slides using VECTASHIELD mounting media (Vector Laboratories, Inc) prior to epifluorescence or confocal microscopy.
Figure 4-S1. RSD-2 and RSD-6 function in germ cells to promote germ cell immortality. (A), Defect of RNA interference in rsd-2 and rsd-6 mutants grown at 20°C. yp10 and yp11 were defective in responding to RNA interference, as assessed by feeding E. coli expressing double-stranded RNA triggers that target pop-1 or par-6, which are essential for embryonic development. (B), Mapping of yp10 and yp11. yp10 was initially mapped to Chromosome IV based on difficulty constructing unc-22; yp10 double mutants. yp10 was positioned right of unc-30 in three-factor crosses of + + yp10 / dpy-20 unc-30 + heterozygotes. yp10 was then shown to lie between unc-22 and dpy-4 based on Dpy-non-Unc recombinants from three-factor crosses using + yp10 + / unc-22 + dpy-4 heterozygotes. yp11 was initially mapped to Chromosome I, and then three-factor crosses of yp11 with dpy-5 unc-13 or dpy-5 unc-29 were used to define the map position of +2.6 on Chromosome I. Two- and three-factor mapping crosses revealed that yp11 had a genetic map position of +2.6 on Chromosome I, whereas yp10 was located at +9.2 on Chromosome IV. Mutations in two genes with roles in RNAi, rsd-6 and rsd-2, failed to complement yp11 and yp10,
respectively, for RNAi deficiency. Sequencing revealed that yp10 contained a G to A transition mutation in rsd-2 that is predicted to result in a G570E substitution in a conserved amino acid, whereas yp11 contained a G to A mutation in rsd-6 that is predicted to result in a premature stop codon at amino acid 71. It was previously reported that rsd-6(pk3300) becomes sterile immediately at 25°C(3). We confirmed this observation, which is inconsistent with sterility of rsd-6(yp11) after growth for 6 to 10 generations at 25°C. The rapid temperature-sensitive Sterile phenotype of rsd-6(pk3300) was due to a linked mutation, which was removed by isolating unc-13 rsd-6(pk3300) recombinants from unc-13 daf-8 / rsd-6(pk3300) heterozygotes. rsd-6(pk3300) was then re-isolated from unc-13 rsd-6(pk3300), and this outcrossed version of pk3300 became sterile at 25°C after growth for 6 to 12 generations. An independent allele of rsd-6, pk3300 displayed progressive sterility at 25°C, and dpy-5 + rsd-6(yp11) unc-29 / + unc-13 rsd-6(pk3300) + as well as unc-30 + rsd-2(yp10) dpy-4 / + unc-26 rsd-2(pk3307) + transheterozygotes became progressively sterile at 25°C (Figure 4-1B). (C-E), Anti-RSD-2 antibody immunofluorescent images assessed using widefield microscopy. RSD-2 localizes to cytoplasm of embryos, where it adopts a perinuclear position (arrows) in wild type (C,D) but not in rsd-2(pk3307) (E). (F, G), Cell autonomy test for rsd-6 by creating repetitive extrachromosomal arrays, which can result in silencing of the transgene and the corresponding endogenous gene in germ cells but not in somatic cells(4, 5). Repetitive Extrachromosomal extrachromosomal arrays consisting of 2.0 kb upstream and 1.0 kb downstream region from the start codon of the rsd-6 gene were introduced into wild-type worms, and cosuppression of rsd-6 (indicated as rsd-6sup which was injected with rol-6d Roller marker) resulted in progressive sterility at 25°C in comparison to control extrachromosomal arrays (indicated as rol-6d) (n=9 for N2 WT and rsd-6 mutants, n=8 for cosuppression lines) (F). Thus, the Tudor domain protein RSD-6 functions in the germline to promote germ cell immortality. However, cosuppression of rsd-6 did not affect embryonic lethality of pop-1, par-6, pos-1 RNAi at 20°C (G), suggesting that the response to exogenous RNAi and Mrt functions of rsd-6 may be separable. In agreement, rsd-2 and rsd-6 mutants are strongly defective for RNAi triggers that target germline genes at both 20°C and 25°C, whereas germ cell immortality is only compromised at 25°C.
Supplemental Figure 4-2: Germline phenotypes and chromosome counts in early generation animals. (A), Animals grown at both the normal temperature of 20°C and at the stressful temperature of 25°C displayed normal germline morphologies. At 25°C, all lines, including wildtype N2, had a small population (<2%) of animals displaying short germline arms. Early generation rsd mutants as well as N2 controls were DAPI stained at generation F4 after being maintained at either 20°C or 25°C for 2 generations (At 20°C, n=218 WT, 108 yp11, 72 pk3300, 170 yp10). (At 25°C, n= 226 WT, 261 yp11, 213 pk3300, 268 yp10). (B), Early generation rsd mutants display similar chromosome counts in oocytes at both 20°C and 25°C. Early generation rsd mutants as well as N2 controls were DAPI stained at generation F4 after being maintained at either 20°C or 25°C for 2 generations (At 20°C, n=71 WT, 66 yp11, 65 pk3300, 69 yp10). (At 25°C, n= 56 WT, 68 yp11, 58 pk3300, 54 yp10).
Figure 4-S3. Additional small RNA analysis. (A-F), RSD-2 promotes production or maintenance of secondary 22G-RNAs. RNA was isolated from strains that carried a germline expressed Ppie-1-GFP transgene and fed dsRNA against GFP. 5’ linker ligation-dependent small RNA library assesses levels of primary siRNAs which are 5’ monophosphorylated Dicer-dependent. 5’ linker ligation-independent small RNA library assesses levels of total siRNAs including highly abundant secondary siRNAs which are 5’ triphosphorylated. A, B, 5’ linker ligation-dependent siRNA reads (5’D) antisense to GFP for rsd-2 mutant (A) and wild type (B). C, D, 5’ linker ligation-independent siRNA reads (5’I) antisense to GFP for rsd-2 mutant (C) and wild type (D). E, F, Distribution of siRNAs possessing a 5’ G (E) or 5’ A, C or T (F) nucleotides. In C. elegans, RNA-dependent RNA polymerases can generate secondary siRNAs (22G-RNAs) in response to primary exogenous or endogenous siRNA triggers(6). The 22G-RNAs against the gfp transgene are reduced in rsd-2 mutant samples C-F, whilst the primary siRNAs against the transgene showed a similar distribution of length and the first nucleotide for rsd-2 and wild-type samples A and B. (G), Mapping of 21U nucleotide sequences annotated as piRNAs to the genome in early- and late-generation rsd-2 animals grown at 25°C shows no reduction in piRNA levels in later generations (F10). (H), 26G nucleotide sequences with more than 25 reads in N2 wild-type animals show no consistent reduction in late-generation (F8) rsd-2 or rsd-6 animals grown at 25°C. (I), Density plot showing an increased number of genes with reduced small RNA reads in late-generation rsd-2 animals compared to wild-type animals grown at the same temperature (alternative plot for Fig. 3a). (J), 22G-RNAs mapping to the genes that were reduced in rsd-2 late- versus early-generation (indicated as ‘rsd-2 dependent’) were also reduced in rsd-6 late- versus early-generation compared to 22G-RNAs mapping to all genes (P<2e-16, Wilcoxon Signed Rank Test). (K), Venn diagram comparing genes with reduced 22G-RNA reads in late-generation rsd-2 compared to early-generation rsd-2 at 25°C to genes identified to have reduced siRNAs in rsd-2 animals at 20°C(7). (L-M), Global analysis of 22G nucleotide sequences from 25°C-grown early- (F0) and late-generation (F8) rsd-6 mapping to tandem repeats and transposons, respectively, demonstrating comparable patterns to those shown by rsd-2. (N-O), Distribution of changes in 22G-RNAs extracted from sequencing data, deposited at the Gene Expression Omnibus (GEO) for rsd-2 animals raised at 20°C(7), for transposons and tandem repeats, respectively.
Figure 4-S4. 22G-RNA reads mapping to individual transposon loci for late-generation rsd-2 and rsd-6 animals. (A), Heatmap showing highly comparable changes in 22G-RNA reads. Vertical clusters indicate similarity between different mutants. Horizontal clusters indicate sets of transposons that behave similarly. Importantly, late-generation rsd-6 and rsd-2 mutants (rsd-6 F8 and rsd-2 F10, respectively), show similar behavior for the majority of transposons and cluster together more closely than either clusters to the early-generation samples. (B, C), Bar charts showing enrichment of types of transposons with either decreased (B) or increased (C) 22G-RNA reads. Transposons with decreased 22G-RNAs in late-generation rsd-2 mutants were enriched for LINE and LONGPAL, whilst depleted for TC1/MARINER elements (B), consistent with a primary role for the piRNA pathway in controlling TC1(8). Transposons with increased 22G-RNAs were not enriched for LONGPAL or LINE elements but showed a similar depletion for TC1/MARINER elements as for transposons with decreased 22G-RNAs (C).
Expression of tandem repeat tracts and transposition assays in *rsd-2/-6* strains. (A) Tiling array analysis shows expression of tandem repeats (indicated as all repeats) does not increase in late-generation *rsd-2* mutants grown at 20°C, compared to control sequences (indicated as control). 0 of the X axis indicates no change in read. Density of the Y axis reflects the number of segments. (B, C) Tiling array data from UCSC genome browser reveals upregulation of a 29-mer tandem repeat tract adjacent to the telomere of Chromosome IV (B), 94-mer repeat tracts on Chromosome I (C) in late- versus early-generation *rsd-2* and *rsd-6* strains grown at 25°C but not at 20°C, and also in late-generation *rsd-6(yp11)* versus late-generation wild-type RNA at 25°C. (D) RT-PCR analysis confirms results shown in panel C, showing high levels of expression of a large tandem repeat tract in late-generation (L) but not early-generation (E) *rsd-2/-6* strains at 25°C but not at 20°C, and little expression in late-generation wild-type strains at either temperature. (E) Tiling array analysis indicates a non-autonomous Helitron transposon is upregulated at 25°C in late-generation *rsd-2/-6* strains. Although non-autonomous Helitron transposons form short tandem arrays whose genomic structure appears similar to non-coding tandem repeats such as that shown in B, coding segments of full length Helitron transposons are similarly desilenced in *rsd* mutants (for example, see ChrlII:1,953,000-1,993,000). (F, G) CGH Copy number analysis for *rsd-6*. Shown is Z scores for comparative genome hybridization for transposons (F) and tandem repeats (G) compared to randomly generated control sequences with the same length distribution as the test set. Neither transposons nor tandem repeats show significant enrichment for increased copy number compared to the control sequences (P>0.1 for each, Wilcoxon unpaired test). (H, I) Five independent mutations in the *sma-3* gene isolated from late-generation *rsd-2* and *rsd-6* lines. To detect increased activity for any transposon, we examined spontaneous mutation directly. *C. elegans* strains with DNA damage response defects mutate at a 5- to 10-fold higher frequency than wild type, and these strains give rise to mutations in many genes that can cause visible phenotypes that can be readily identified in large populations of nematodes (9, 10). We examined many late-generation 25°C *rsd-2* and *rsd-6* mutant lines in an effort to identify lesions in genes that cause visible mutant phenotypes but very few mutations were found, suggesting a very low frequency of spontaneous mutation. In these observation, five independent mutations that caused a Small phenotype were identified from late-generation 25°C *rsd* mutant lines (four from *rsd-6* and one from *rsd-2*). Genetic mapping and complementation tests indicated that these were all mutations in the *sma-3* gene. Thus, mutations in a variety of genes were not recovered from stressed late-generation *rsd* mutants, as might be expected if transposon activity were high. However, sequencing analysis revealed that all five mutations occurred as a consequence of a single nucleotide addition in a small polypurine tract within the seventh exon of *sma-3*. Thus, the *sma-3* lesions that were recovered from *rsd* mutants did not correspond to those expected from transposon activity, such as small deletions that result from hit-and-run events or large transposon insertions (11). (H) All 5 mutations occurred as a consequence of addition of a thymine nucleotide found in a small polypurine tract of *sma-3* exon 7 (capital T). Sequence shown is the 32 nucleotides that include the polypurine tract and neighboring sequence for both wild-type (+) and *rsd* mutant strains. Red star indicates where the 5 mutations were found. Even though the *sma-3* gene contains a non-autonomous DNA transposon in the last intron of the *sma-3* gene (grey line), this segment of *sma-3* was wild-type in all 5 mutations isolated from *rsd* mutant backgrounds. Similar polypurine tracts are found in exons of many *C. elegans* genes, including those that cause strong visible phenotypes when mutated, but only *sma-3* mutations were recovered from *rsd* mutant backgrounds. (I) Analysis for SMA-3 reveals that the additional thymine causes a frameshift at amino acid 176 and is predicted to cause a premature stop codon in exon 8 (denoted by *), likely resulting in deletion of most of the conserved SMAD domain of SMA-3. (J) Normal frequency of spontaneous mutation in late-generation *rsd-2* and *rsd-6* lines. Transposon activity test was performed by measuring the frequency of forward mutation of several independent loci using an *unc-58* mutant, which can be suppressed by mutations in two loci (10).
Unc-58 reversion assays reveal lack of an increased frequency in spontaneous mutation for rsd mutants grown at 25°C, whereas mut-2(r459) mutator mutant displays an elevated frequency.

**Figure 4-S6.** Additional RNA FISH images. Confocal images of Cy5-labeled RNA FISH probes (A, D, G, J, M, P), DAPI-stained nuclei of the corresponding images (B, E, H, K, N, Q), and merged images containing Cy5 and DAPI signals (C, F, I, L, O, R) for animals derived from strains grown at 25°C. RNA FISH probes reveal expression of CeRep59 antisense tandem repeat (a-c) or 26S ribosomal RNA (D-I).
Chromosome V tandem repeat probe (J-R) in late-generation rsd-2 (A-C, J-L), rsd-6 (D-F, M-O) or control wild-type (G-I, P-R) two-day old adults. Widefield epifluorescence images of a 20°C wild-type adult reveals CeRep59 expression in embryos (S-U). Dashed lines outline germline, dotted lines outline embryos.
Figure 4-S7. FISH staining in sterile *rsd-6* and *nrde-2* animals reveals broad expression of repetitive elements. Sterile animals isolated from plates grown at 25°C were stained with Cy5-labeled probes targeting the *CeRep59* tandem repeat. Both *rsd-6* and *nrde-2* animals displayed widespread expression of the element throughout both germline and somatic tissue. Both sense and antisense sequences were detected in both genotypes. Data representative of *n*>80 animals from 4 sister lines per genotype and probe. Bars, 100μm.
Supplemental Figure 4-S8: Sterile nrde-2 and rsd-6 animals display elevated levels of CeRep59 RNA. Sterile nrde-2 and rsd-6 animals from lines maintained at 25° displayed increased FISH staining for CeRep59 repetitive RNA. Sister lines maintained for an identical number of generations at 20° remained fertile and displayed lower CeRep59 levels. Each line was isolated and stained within the same day (n=20+ for all strains). Images were captured using identical exposure times and settings in NIS Elements.
CHAPTER V: SUMMARY AND FUTURE DIRECTIONS

SECTION ONE

Listed below are the general conclusions from section one:

-prg-1 dysfunction results in mortal germline (mrt) phenotype under all conditions

-prg-1 can be rescued by reducing daf-2 IIR signaling and upregulating the DAF-16/Foxo transcription factor

-Misregulation of repetitive elements, including satellite repeats and transposons, increases transgenerationally in prg-1 mutants, but is rescued by reduced daf-2 signaling

-Taken together, these results indicate prg-1 functions to regulate selfish and repetitive elements in the germline to maintain germ cell immortality, and that daf-16 can aid in this function

The ability for germ cells to pass information along from one generation to the next without succumbing to mutations and replicative death demonstrates immortality within a non-cancerous tissue. In the study of this phenomenon, dozens of genes have been identified as necessary to maintain this immortal state. Here, we identify one of those genes as prg-1, the Piwi Argonaute ortholog in C. elegans. prg-1 mutants express a mortal germline phenotype at both the stressful temperature of 25°C
and at the normal growth temperature of 20°C (Figure 1D). We found that growth at high
temperature significantly extended the transgenerational lifespan of prg-1 mutants, due to accelerated
food depletion resulting in brief periods of starvation. These data suggested an ability for a starvation
response pathway to interface with germline health in these animals.

Pursing the results of the effects of starvation on prg-1 transgenerational lifespan led us to test
the daf-2/daf-16 insulin signaling pathway, a major pathway in starvation response and somatic
longevity, in the context of prg-1. We found that mutation in daf-2 could rescue prg-1 progressive
sterility via increased DAF-16 activity (Figure 1-2A and 1-2B). Further testing revealed that the function
of daf-2 and daf-16 in the rescue was facilitated by DAF-16 activity within the insulin signaling pathway
(Figure 1-2B and 1-2F). This interface between a typically somatic longevity pathway and a germline
immortality pathway reveals an intricate survival program, benefiting not only the organism itself, but
the genetic integrity of the offspring as well.

Microarray analysis of prg-1 animals revealed that transposons and other repetitive elements,
such as satellite repeats, were strongly misregulated in late generation animals. We found that
mutagenesis rates were not increased in prg-1 late generation animals and that sterile animals could be
rescued by RNAi knockdown of daf-2 and age-1, indicating that transposition-mediated mutation was
not the cause of progressive sterility (Figure 1-2D-F). However, we found that increased expression of
other repetitive elements could accelerate sterility, suggesting that misregulation of such elements
constitutes an aging stress that accumulates with successive generations (Figure 1-4E-I and Figure 1-5F).

Finally, we provide evidence that daf-16-mediated rescue requires components of an RNAi
pathway as well as the chromatin remodeling genes, spr-5 and rbr-2 (Figure 1-5H). Taken together, this
data demonstrates that daf-16 can affect chromatin remodeling via an RNAi signal intermediate. To
date, the ability of daf-16 to initiate or interface with an RNAi regulatory mechanism has not been
described, leaving us with the exciting implications this revelation has for the fields of both replicative and post-mitotic aging.

**Ongoing and future work:**

**Molecular identity of trigger(s) of prg-1 progressive sterility**

In Chapter I, we demonstrated the role for prg-1 in germline immortality and go to great lengths to characterize the genetic relationships and molecular changes involved in the transition from fertile animals to sterile animals. While we were able to recover a great deal of evidence implicating the misexpression of repetitive elements as the cause, the identification of a single element or small group of elements directly responsible has yet to be achieved. There are currently hundreds of such elements we identified in our microarrays that show misregulation in the absence of prg-1 and there exists the possibility that only some or perhaps one of them is the actual toxic element. We also have yet to determine whether it is the RNA or an aberrant protein transcribed from the RNA that functions as the toxic element.

In order to address these lingering questions, we have begun working on the following experimental set ups:

- **Aggregate protein staining:** We intend to employ the use of protein aggregate stains, such as the amyloid-binding dye Thioflavin, on early and late generation prg-1 animals to determine whether or not repetitive elements lead to excessive, non-function protein accumulation within cells. Positive results from this experiment would likely be followed up by treatments of late generation animals with aggregate-disrupting pharmacological agents in an attempt to rescue sterility or prolong transgenerational lifespan

- **RNA aggregates:** FISH staining has indicated that misexpression of some repetitive elements increases in late generation prg-1 animals (Figure 1-S6). FISH staining did not reveal any specific foci or aggregation of the RNA targets used. However, this does not preclude the possibility that
other RNAs do not form structures that may indicate stress, such as RNA stress granules. We propose to use immunostaining to probe for such structures in late generation animals.

-Small RNA analysis: In collaboration with Dr. Peter Sarkies and Dr. Eric Miska, we have begun small RNA analysis of several strains to narrow down the pool of misexpressed elements that trigger germline mortality. In addition to analysis of several new allelic combinations of prg-1 and prg-1; daf-2, we have begun exploring the siRNA and 22G RNA populations in both prg-1; daf-2; rde-2 and prg-1; daf-2, mut-7. As the latter two genotypes contain mutations in small RNA machinery necessary for rescue and accumulation of sub-populations of secondary siRNAs, we hope to gain insight into the nature of the elements still present in these ablated rescue lines.

-Co-immunoprecipitation of ribosomes: Due to the misexpression of a considerable number of repetitive elements in late generation prg-1 animals, it stands to reason that RNA-processing machinery may be stressed due to increased load. Even if only a fraction of these repetitive RNAs make their way to translation, it would represent competition with RNAs needed for homeostasis at ribosomes. To explore this possibility, fixation and immunoprecipitation of ribosomes in early and late generation prg-1 animals could provide insight into the state of RNA translation in these animals. Once precipitated, the RNA transcripts could be isolated and sequenced, revealing if there are any repeat RNAs making it to translation and the relative percent change in this translation between populations. This experiment is attractive in that it would answer the question if repeat RNAs are making it to translation, which repeat sequences they are, and if increased expression results in increased competition at ribosomes.
SECTION TWO

Listed below are the general conclusions from section two:

- Sterile prg-1 animals undergo germline rearrangement between L4 and adult animals, with most adults displaying a form adult productive diapause (ARD)

-prg-1-induced ARD relies on the same genetic components required for starvation-induced ARD

-daf-16 influences prg-1-induced ARD

-Germ cell atrophy is influenced by stress-response factors and represents a reversible arrested state

Organisms have evolved a plethora of genetic programs to respond to adverse conditions in their environment. This extends to both the external environment in which the organism lives and the internal environment of its tissues and cells. In C. elegans, the Piwi Argonaute PRG-1 protects the germline from an endogenous stress that ultimately triggers sterility. We found that the germlines of prg-1 deficient animals respond to this stress by initiating a massive germline rearrangement. This germline restructuring occurred in sterile animals during the transition between the L4 larval state and adulthood and was characterized by an atrophied phenotype, wherein the germline arms retract and contain a small cluster of germ cells in the distal tip regions (Figure 2-2B).

The restructuring of germlines in sterile animals was reminiscent of a stress response program typically induced by starvation, termed adult reproductive diapause (ARD). We found that the same genetic components required for starvation-induced ARD, specifically the cell death pathway component ced-3 and the nuclear hormone receptor nhr-49, were required for proper execution of
germline atrophy in prg-1 sterile animals (Figure 2-2C and 2-2D). Another hallmark of ARD is the ability to reverse the diapause state, in which the animals regain normal germlines and continue their reproductive lifestyle. We found that fertility could be restored to prg-1 sterile animals by RNAi knockdown of daf-2. With the meeting of these criteria, we established that prg-1 sterile animals were undergoing an ARD response triggered by an endogenous stress. These results demonstrate that ARD is a broad-response program induced by both internal and external sources of stress.

The presence of endogenous stress coupled with a massive tissue rearrangement led us to explore the function of the daf-2/daf-16 insulin signaling pathway on ARD. Based on work described in Chapter I and ARD’s described activation in response to starvation, we examined the role of daf-16 and daf-18 in germline rearrangement. We found that daf-16 signaling contributed to the normal ARD response in prg-1 animals (Figure 2-S2A). Further investigation found that daf-16 localized to the nuclei of intestinal cells in sterile animals, a hallmark of daf-16 activation. These data indicate daf-16 activation in response to an endogenous stress, drawing another parallel with its role regarding exogenous stressors (i.e. starvation). Furthermore, it implies that daf-16 activation by the toxic stress contributes to the ARD response addressing the stress.

The role of ARD as a response to both endogenous and exogenous sources of stress suggested that other stress response pathways may influence germline rearrangement. We found that other stress response factors, including the heat shock factor hsf-1 and the p53 homolog cep-1, were required for ARD (Figure 2-2D). With at least three separate stress response pathways implicated in ARD execution, we attempted to tease out the relationships between these genes. Triple mutants were made, combining prg-1 with members of the cell death pathway, the insulin signaling pathway, and the canonical starvation-induced ARD pathway (Figure 2-S2B). Our initial results suggest that cep-1 is epistatic to the insulin signaling pathway in the execution of ARD. However, other results did not
indicate a clear distinction in the genetic hierarchy of the ARD response, suggesting that there exists a
great deal of interconnection between the genes and pathways described thus far.

Ultimately, our results identify ARD as the programmed response to critical levels of an
endogenous stress present in \( prg-1 \) animals. This is the first instance of ARD responding to an
endogenous stress and establishment of a heritable toxic stress present in \( prg-1 \) mutants. We further
implicate an intimate role for the insulin signaling pathway and \( daf-16 \) in \( prg-1 \) biology, as well as
expand upon the complex genetic interplay involved in the execution of ARD. We propose that ARD is an
evolutionary response to environmental and endogenous stress factors designed to protect the
germline until such time in which it is favorable to resume reproduction.

**Ongoing and future work:**

**Establishment of the ARD genetic pathway**

In Chapter II, I demonstrate that \( prg-1 \) sterile animals enter a state of ARD due to the
accumulation of a heritable endogenous stress. This stress response is reliant on several stress response
genes, including \( daf-16, nhr-49, ced-3, ced-4, cep-1 \) and \( hsf-1 \). However, the exact nature of the
relationship between these genes and their corresponding pathways remains unclear. Our preliminary
results from exploring the genetic interactions revealed that, while there appeared to be some instances
of epistasis, the interaction between these genes does not suggest a clear, linear genetic pathway. In
fact, the data suggests that there exist parallel levels of interaction from genes at different points in
their respective pathways.

Currently, I am performing genetic crosses to generate additional triple mutants. These include
the following genotypes:

\[
\begin{align*}
prg-1, hsf-1; ced-3 & \quad prg-1, hsf-1; ced-4 & \quad prg-1, hsf-1; daf-18 \\
prg-1, cep-1; ced-3 & \quad prg-1, cep-1; ced-4 & \quad prg-1, cep-1; daf-18 \\
prg-1, daf-16; ced-3 & \quad prg-1, nhr-49; ced-3
\end{align*}
\]
With the data gained from these lines, I intend to develop a more complete genetic map of the interactions that regulate the ARD response.
SECTION THREE

Listed below are the general conclusions from section three:

-daf-16 mediated rescue of prg-1 progressive sterility and prg-1 ARD requires the dsRNA channel, sid-1

-daf-16 functions through a endogenous RNAi pathways partially dependent on components of the rrf-3 endogenous RNAi pathway

-Contributions of daf-16 signaling from somatic tissues influence prg-1 progressive sterility and facilitate rescue by reduced daf-2 insulin/IGF receptor (IIR) signaling

Many multicellular organisms, including C. elegans, contain the mechanisms by which cells and tissues can communicate with each other in order to execute system-wide programs in response to stimuli. Such abilities allow such forms of life adaptability to a chaotic universe, increasing their likelihood of survival. One such mechanism is RNA interference (RNAi), which allows for post-transcriptional gene regulation. Small RNA signals can pass from cell to cell and tissue to tissue to execute gene regulation programs. We found that the reduced daf-2/IIR rescue of prg-1 deficient animals and the effect of daf-16 on prg-1-induced ARD function through a small RNA pathway.

Previously, it was shown that active daf-16 translocated to the nuclei of intestinal cells in sterile and late generation prg-1 mutants (See Chapter II). Additionally, the role of a germline Argonaute, PPW-1, and components of the second phase RNAi pathway in the rescue of prg-1 progressive sterility suggested a small RNA signal was responsible for executing rescue. We found that the dsRNA channel, sid-1, was also required for this rescue (Figure 3-1A). The role of sid-1 in the passive uptake of dsRNA was also found necessary for the ARD response in sterile prg-1 animals. Collectively, these results point
to a dsRNA signal that functions cell-nonautonomously to facilitate ARD and daf-16-mediated rescue as the source of daf-16’s activity in rescue.

The origin of the daf-16 RNAi signal in prg-1; daf-2 rescue suggests the role of an endogenous RNAi pathway. We explored this possibility by testing the roles of genes associated with the rrf-3 endogenous RNAi pathway. A role was identified for the RdRP rrf-3 and rde-4 in daf-16-mediated rescue, further indicating a small RNA signal produced by DAF-16 activity (Figure 3-2A). However, the lack of a requirement for the Argonaute, ergo-1, as well as several other components of the rrf-3 pathway suggests that there yet remains a few pieces missing from the pathway through which daf-16 functions to restore fertility and influence ARD. This, along with the other data discussed here indicates a currently unidentified RNAi pathway responsible for daf-16 cell-nonautonomous signaling.

Multiple tissues express DAF-16, but only a few are required for the somatic longevity associated with increased DAF-16 activity, specifically intestines and neurons. Having identified the daf-16 signal to be cell-nonautonomous, we sought to identify the tissue(s) responsibly for signaling the rescue of the mortal germline. We found that contribution from intestinally expressed daf-16, but not muscle-derived daf-16, was sufficient to rescue prg-1, daf-16 shortened transgenerational lifespan (Figure 3-2B). This data suggests that the intestines play a vital role in prg-1; daf-2 rescue and draws an additional parallel between daf-16 signaling in somatic longevity and germline health.

Ongoing and future work:
Identification of missing components of daf-16 RNA signaling

In Chapter III, we demonstrate that daf-16 functions cell-nonautonomously through a small RNA intermediate to influence germline integrity and ARD in prg-1 mutants. The identification of the sid-1 dsRNA channel, as well as the rrf-3 RdRP, indicate an endogenous RNAi pathway functions to execute daf-16 signaling in the germline. However, the lack of requirement of ergo-1, eri-1 and rde-1 raise questions about the pathway? How does the signal move from cell to cell? Is the signal truly a dsRNA or
a similar structure? In order to answer these questions we have begun work on the following experiments:

**-RNA sequencing and analysis of small RNA species:** As *sid-1* has been shown to be vital to *prg-1; daf-2* rescue of progressive sterility, we hope to examine the differences in RNA populations between these strains and *prg-1* lines in order to identify those displaying significant changes. In collaboration with Dr. Peter Sarkies and Dr. Eric Miska, we have begun analysis of the small RNA populations of *prg-1; sid-1* and *prg-1; daf-2; sid-1* strains. This analysis includes RNA sequencing and analysis of 22G RNA, in addition to pRNA analysis. Identification of a sub-type of siRNA would allow us to narrow down the possible Argonaute candidates.

**-Isolation and sequencing of dsRNA species:** Another line of experimentation we propose is the isolation and sequencing of dsRNA species. As RDE-4 was found to be required for rescue and has a defined role in processing dsRNA into primary siRNAs, we intend to examine the dsRNA populations from *prg-1* single mutants, *prg-1; daf-2* double mutants and *prg-1; daf-2; rde-4* triple mutants. We will sequence large amounts of total RNA from these strains and purify them over RNeasy spin columns, removing all small RNAs with lengths less than 200 base. We will then remove single stranded RNA species with single strand-specific RNA endonucleases. Finally, we will use RNase III to digest the remaining dsRNA transcripts into discrete 21nt siRNAs and then sequence. Sequencing of these transcripts would reveal the differences in the precursor sequences used to generate primary siRNAs. By comparing these results to the known targets of DAF-16 transcription initiation, we could determine the genes and precursor sequences targeted for regulation by the *daf-16*-signaling pathway responsible for *prg-1* rescue and ARD execution.
SECTION FOUR

Listed below are the general conclusions from section FOUR:

- RSD-2 and RSD-6 function to protect germline immortality at stressful temperatures via small RNA-mediated transcriptional silencing

- Stressed rsd mutants display disrupted small RNAs targeting spermatogenesis genes and desilencing of repetitive loci

- Sterile rsd mutants display germline apoptosis and chromosome missegregation

The adverse effects of stressful environments on homeostasis precipitate an evolutionary pressure to develop means to deal with such conditions. A key aspect to such defenses is the ability to protect the integrity of germ cells in order to ensure survival of the genome to the next generations. We found that RSD-2 and RSD-6 function to protect the germline under stressful temperatures, demonstrating one such mechanism by which C. elegans adapts to environmental changes.

From a previous screen, we mapped and cloned two mrt mutations that manifest only at the stressful temperature of 25°C. These genes were identified as the RNAi spreading genes, rsd-2 and rsd-6, which function in the germline (Figure 4-1A and 4-1C). Separate mutations in rsd-2 and rsd-6 recapitulated these findings, demonstrating a clear role for these two genes in promoting germline immortality. These results suggest an intriguing new role for RNAi in maintaining a tissue-wide signal required for responding to stress that threatens germline or germ cell health and integrity.

With the advent of sterility, rsd mutants were found to undergo a profound alteration of their germlines, signifying a potential role for apoptosis in this severe reduction of germ cells (Figure 4-2A, B). Curiously, a contrasting phenotype of tumorous germlines was observed in a small fraction of animals.
Follow up experiments revealed a role for the cell death genes ced-3 and ced-4, as they were found to ameliorate this germline rearrangement, although the tumorous phenotype persisted. Additionally, analysis of the karyotype of these late generation and sterile animals revealed chromosome missegregation, indicating chromosome instability (Figure 4-2D).

The role of the rsd genes in endogenous RNAi suggests that their role in germline immortality may lie within this function. We examined both early and late generation animals and found that a subset of secondary 22G siRNAs were perturbed, gradually diminishing over the generations (Figure 4-3). Further, many of these siRNAs were found to target spermatogenesis genes, which have been shown to affect fertility when misregulated in other genetic backgrounds, including csr-1 and spr-5 (Figure 4-3D). Defects in spermatogenesis appeared an attractive culprit for the observed mrt phenotype in these mutants. However, sterile animals were rarely rescuable by outcrossing to WT males and the trends in spermatogenesis gene misregulation were not congruent with other infertile and mrt strains with spermatogenesis defects, suggesting another source for the transgenerational loss of fertility.

In our analysis of the small RNA populations, we observed that rsd mutants displayed a progressive increase in the expression of repetitive elements and transposons, similar to what was observed in prg-1 mutants (See Chapter I). 22G RNA reads revealed a wider distribution in late generation rsd mutants, with higher reads against some transposons and repeat sites and lower reads against others (Figure 4-3E, F). As the RNA for these experiments came from whole animals at mixed developmental stages, we examined the expression of some of these repeats via RNA FISH in order to determine if specific tissues were contributing to these observations. Curiously, we found that only the embryos of wildtype and early generation rsd mutant animals express these repeats, but stressed rsd mutants had elevated systemic expression throughout all tissues (Figure 4-4). While these results indicate systemic misexpression of repetitive elements may contribute to rsd progressive sterility, the expression of these elements in embryos of wildtype animals raises intriguing questions. In Arabidopsis,
repetitive and retroviral elements are expressed transiently in early embryos, allowing the silencing machinery to identify and silence the corresponding parts of the genome in the next generation. A possible explanation for this observation may be that C. elegans permits relaxation of silencing mechanisms for these elements in early development to reestablish strong silencing markers, thus preventing a loss of memory of non-self elements over generations.

With the observation of rsd-2 localizing to the nuclei and the ectopic expression of repeat elements in late generation mutant animals, we suspected a role for RSD-2 and RSD-6 in nuclear transcriptional silencing (Figure 4-1H and Figure 4-4). Another mrt gene, nrde-2, was shown to have a similar phenotype at 25°C to the rsd mutants. NRDE-2 functions in a nuclear RNAi pathway and we found that no additive effect was observed on the transgenerational lifespan of the double mutants compared to the single mutant controls (Figure 4-1D). Follow up analysis found that late generation nrde-2 mutants display increased levels of repeat expression in all tissues, comparable to the rsd mutants. These results indicate that the rsd genes and nrde-2 function in the same RNAi pathway to facilitate germline immortality at stressful temperatures.
REFERENCES


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