

Defects in Piwi or germ granules trigger a common program of sterility that is independent of transposon activation

Short title: Germ granules may orchestrate Piwi sterility

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Abstract:

Defective Piwi/piRNA genome silencing leads to heterochromatin dysfunction and immediate sterility in many species. Sterile Piwi mutants experience transposon expression and transposon-induced genomic instability, although the cause of Piwi mutant sterility remains uncertain. *C. elegans* germ cells deficient for Piwi pathway genome silencing factors transmit a form of heritable stress that induces sterility after growth for several generations, allowing comparisons of animals that are close to sterility but fertile with sterile siblings. Sterile Piwi pathway mutants displayed inconsistent increases in DNA damage signaling but consistently altered perinuclear liquid droplets termed germ granules. Germ granule dysfunction did not elicit significant levels of transposon expression but was sufficient to induce a range of idiosyncratic phenotypes associated with sterile Piwi pathway genome silencing mutants, including germline atrophy, reproductive arrest and univalents in oocytes. Expression of genes that perturb germ granule structure was not compromised in sterile Piwi pathway mutants, suggesting that a post-transcriptional mechanism regulates the stability of Piwi mutant germ granules. We propose that sterility in response to transgenerational deficiency for Piwi and in response to acute dysfunction of germ granules occur as a consequence of a common reproductive arrest mechanism. The germ granule abnormalities of sterile Piwi pathway mutants therefore suggest germ granule dysfunction as a cause of Piwi mutant sterility.

Keywords: Germ granules, reproductive arrest, small RNAs, heterochromatin, germ cell immortality, Heritable Stress, Piwi

Introduction:

Germ cells give rise to the mortal somatic tissues while maintaining themselves in a pristine condition that allows them to be propagated for an indefinite number of generations. This capacity for self-renewal is termed “germ cell immortality”, and functions to protect the germline from forms of damage that can result in sterility [1]. Defects in *Caenorhabditis elegans* (*C. elegans*) genes that promote germline immortality cause a Mortal Germline (*mrt*) phenotype, where robust fertility occurs for several generations but deteriorates in later generations, finally culminating in complete sterility. The transgenerational sterility of *mrt* mutants implies that their germ cells may age as they proliferate over the generations such that they accumulate a form of damage that ultimately evokes sterility. Identifying the transgenerational damage that accumulates in *mrt* mutants and the cause of sterility are central challenges for understanding the basis of germ cell immortality.

The initial germline immortality pathway defined in *C. elegans* was caused by deficiency for telomerase, a reverse transcriptase that maintains telomere stability by adding telomere repeat sequences to chromosome termini de novo. *C. elegans* mutants that are defective for telomerase-mediated telomere repeat addition display several phenotypes that correlate with telomerase deficiency. The first is progressive telomere erosion as detected by Southern blotting, which represents the transgenerational damage that accumulates in these mutants. The second is that end-to-end chromosome fusions accumulate in these strains as they become sterile [2,3]. The sterility of *C. elegans* telomerase mutants is therefore believed to occur as a result

of genome catastrophe induced by telomere dysfunction, although the precise cause of telomerase mutant sterility remains uncertain [4–7], possibly the creation of circular chromosomes that are toxic to meiosis [4]. Similar to the transgenerational proliferation defects of *C. elegans* telomerase mutants, primary human somatic cells that are cultured in vitro have a limited proliferative capacity that is associated with telomerase dysfunction, progressive telomere erosion and ultimately telomere dysfunction, which triggers entry into an irreversible state of cell cycle arrest termed senescence [5,6]. Confidence that senescence of primary human fibroblasts that proliferate in vitro is caused by telomere dysfunction was obtained by acute knockdown of the telomere capping protein TRF2, which led to immediate telomere deprotection of telomeres whose lengths were normal, accompanied by telomere fusions and senescence [7].

A distinct fertility pathway, P-M Hybrid Dysgenesis, was discovered in the 1970's as a temperature-sensitive *Drosophila* fertility defect that was linked to defects in transposon silencing [8,9]. In these experiments, *Drosophila* M strain females were mated with males of the more recently isolated P strain to yield F1 hybrid progeny with high levels of P element transposition and temperature-sensitive sterility. The reduced fertility of P-M F1 hybrids has been attributed to lack of piRNA-mediated genome silencing for specific transposable elements [10], which are normally repressed by Piwi. Piwi is a conserved Argonaute protein that is enriched in germ cells and interacts with thousands of small RNAs termed piRNAs to repress transposons and foreign nucleic acids that represent parasitic threats to the integrity of the genome [11–13]. Deficiency for Piwi proteins leads to immediate sterility in *Drosophila*, zebrafish and mouse, and this correlates with transposon expression and increased levels of DNA damage in

sterile F2 animals, which implies that transposon-induced genome instability could cause sterility of Piwi mutants [9,13,14]. However, analysis of effects of transposon copy number on P-M hybrid dysgenesis suggests that transposon-induced genome instability may only explain a minor fraction of P-M hybrid sterility [15]. Therefore, the cause of sterility in Piwi mutants is uncertain and remains an unsolved problem in experimental biology.

Deficiency for the *C. elegans* Piwi ortholog *prg-1* compromises germ cell immortality [18], rather than inducing the immediate sterility phenotype that is observed in Piwi mutants in some other species. The reason for the transgenerational delay in the onset of sterility in *prg-1*/Piwi mutants and in related Piwi pathway mutants of *C. elegans* is not clear, but it is possible that genomic silencing marks that are induced by the Piwi/piRNA pathway are more robustly maintained in germ cells of *C. elegans* if the Piwi silencing system is inactivated. The transgenerational damage that accumulates in *C. elegans* Piwi mutants may therefore correspond to progressive deterioration of Piwi-mediated heterochromatin.

Fertility defects that occur in response to heterochromatin dysfunction in *C. elegans* have been associated with increased levels of RNA-DNA hybrids and of transposon-induced DNA damage [16,17]. *C. elegans prg-1* mutants display progressively increased levels of germline apoptosis when grown for successive generations, and apoptosis promotes germ cell atrophy for sterile *rsd-2*, *rsd-6* and *prg-1* mutants [18–20]. However, very low levels of transposition occur in late-generation *prg-1* mutants, and in *C. elegans mutator* mutants, which do not display transgenerational sterility, high levels of transposition do occur suggesting transposition is not sufficient to

induce transgenerational sterility [18]. Furthermore, fertility can be restored to minor fraction of sterile late-generation *prg-1* mutants [18,20]. Together, these results suggest that the sterility of *prg-1*/Piwi mutants is unlikely to be a consequence of debilitating levels of transposon-induced DNA damage. However, it remains possible that significant levels of DNA damage are unleashed when *C. elegans prg-1* mutants become sterile.

The late-generation sterility phenotype of *C. elegans prg-1*/Piwi mutants was recently shown to elicit a striking but pleiotropic germ cell degeneration phenotype at the generation of sterility, as L4 larvae mature into 1 day old adults, such that most animals display ‘atrophied’ germlines that retain only a small population of mitotic germ cells, whereas some germlines are ‘empty’ and lack germ cells altogether, some germlines are ‘short’ such that they have fewer germ cells than normal but possess both mitotic and meiotic germ cells, and some germlines are sterile but ‘normal’ in size such that they are not smaller than germlines of fertile late-generation *prg-1* mutant siblings that are not yet sterile [20]. Consistently, temperature-sensitive Piwi pathway genome silencing mutants *rsd-2* and *rsd-6* give rise to late-generation sterile adults that exhibit germline arms that are normal in size but also give rise to small and atrophied germline arms that are promoted by apoptosis [19]. Moreover, atrophied germ lines of sterile *prg-1* mutants can regrow on day 2 of adulthood, and a small fraction of constitutively sterile 5 day old *prg-1* mutant adults can become fertile again if they are shifted to a distinct food source [20]. The ability of sterile late-generation of *C. elegans prg-1*/Piwi mutant adults to become fertile suggests that Piwi mutant sterility is a reversible form of reproductive arrest termed Adult Reproductive Diapause, which can be induced in

response to environmental stresses such as starvation [22–24]. We therefore proposed that late-generation sterility of *prg-1*/Piwi mutants may occur in response to accumulation of a heritable stress that is transmitted by germ cells [20].

Some Argonaute proteins that regulate epigenomic pathways are located in perinuclear germ granules, which are conserved germline structures akin to liquid droplets that interact with nuclear pore complexes and are necessary for fertility [25]. *C. elegans* germ granules are termed P granules based on antibodies that label the P blastomere that generates the germ cell lineage [26]. Core P granule proteins have been shown to play redundant functions making it difficult to eliminate P granules [27]. Here we study the late-generation sterility phenotype of Piwi pathway genome silencing mutants and find that it is associated with abnormalities of germ granules, and we demonstrate that acute germ granule dysfunction is sufficient to phenocopy the reproductive arrest phenotypes of sterile Piwi mutants.

Results:

Genome instability in Piwi pathway genome silencing mutants

Transgenerational sterility in *C. elegans* telomerase mutants that lack the ability to maintain sequences that cap chromosome ends is caused by high levels of telomere fusions and associated genome damage [2]. Many *mrt* mutants only become sterile when grown at the non-permissive temperature 25°C [28], some of which are deficient for Piwi pathway-mediated genome silencing [1,16,19,21,29–31]. However, the deficiency for the Piwi Argonaute protein PRG-1 or the nuclear RNA interference (RNAi) proteins NRDE-1 or NRDE-4 results in progressive sterility at any temperature

[16,18,30–33]. We studied the sterility of Piwi pathway mutants that were temperature-sensitive for germ cell immortality at 25°C and also non-conditional Piwi pathway mutants at 20°C. We compared fertile late-generation Piwi pathway mutants with late generation siblings that were sterile, in an effort to learn about the cause of their sterility.

Although DNA damage has been suggested not to contribute significantly to the sterility of *prg-1* mutants based on several lines of evidence, the levels of DNA damage signaling in sterile animals have not been previously studied. We therefore examined *prg-1* mutants grown at 20°C across several generations for the presence of the DNA damage response, as detected by an antibody to phosphorylated S/TQ, a protein modification that is created by two sensors of DNA damage, ATM and ATR, which are phosphatadylisoinositol-3-kinase like protein kinases [34,35]. We observed a significant increase in the fraction of germ cells with upregulated DNA damage response for late-generation fertile and sterile *prg-1* mutants (~3- to 6-fold) when compared to either wild-type controls or to early-generation *prg-1* mutants (Fig. 1a,b). However, the fraction of germ cells with an activated DNA damage response was not consistently elevated in sterile *prg-1*/Piwi mutants in comparison to fertile late-generation siblings (Fig. 1b). We also tested temperature-sensitive Piwi pathway mutants and found that the wild-type DNA damage response was upregulated by growth at 25°C but that this level was not further elevated in germlines of sterile late-generation *rsd-6*, *nrde-2* or *hrde-1* mutant adults (Fig. 1b,c).

Piwi pathway genome silencing mutants exhibit P granule defects at sterility

Deficiency for PRG-1 causes delocalization of the P granule protein PGL-1 [36], and combined loss of the epigenomic regulators SPR-5 and LET-418, or loss of H3K9 methylation modifier SET-2 and the nuclear RNAi pathway, results in immediate sterility that is associated with disruption of P granules [37,38]. Whilst characterizing germ line defects of sterile *rsd-6* mutants by immunofluorescence, we found that a P granule antibody exhibited altered staining patterns in sterile but not fertile late-generation *rsd-6* mutant siblings (Fig 2d-d'). We also found that sterile late-generation *hrde-1* and *nrde-2* temperature-sensitive mutant germ lines displayed P granule defects (Fig 2c-c', S1), which were absent or comparatively minor in fertile late-generation mutant adults that were close to the generation of sterility (Fig. 2c-c', S1, Table 1). When compared to wild-type controls, P granule defects in the sterile mutants ranged from a loss of P granules in a subset of cells to varying amounts of P granule disorganization (Fig. 2, S1). Sterile *hrde-1(tm1200)* mutant animals displayed the most severe P granule phenotype, as the majority of sterile *hrde-1* mutant germ cells exhibited little to no staining (Fig. S1). We also examined the temperature sensitive *mrt* mutant *rbr-2(tm1231)*, which encodes a histone 3 lysine 4 demethylase that promotes genomic silencing and likely functions downstream of small RNAs to promote germ cell immortality [39]. Similarly to *hrde-1* and *nrde-2* mutants, late-generation fertile *rbr-2* mutant animals did not exhibit obvious P granule defects, while loss of P granules was observed in many *rbr-2* mutant germ cells at sterility (Fig. 2e-e').

Germ line atrophy occurs in some but not all *prg-1* mutants as L4 larvae develop into sterile adults [20]. Large-scale apoptosis contributes to this germ cell degeneration phenotype [20], and P granules have been shown to vanish in cells that undergo

apoptosis [40,41]. However, we observed widespread P granule disorganization in sterile germlines of *rsd-6* mutants that were wildtype in size and had not undergone significant amounts of apoptosis (Fig. 2d).

nrde-1 and *nrde-4* mutant animals displayed a distinct sterility phenotype, whereby only a subpopulation of sterile germline arms displayed P granule defects (60% and 22% respectively (Fig. S1, Table 1). This suggests that loss of *nrde-1* and *nrde-4*, which act downstream of multiple small RNA genome silencing pathways, may dampen the pronounced P granule defects that are observed in germline arms of most sterile Piwi pathway mutants.

Although most Piwi pathway mutants do not display severe fertility defects until the generation of sterility, late-generation *prg-1*/Piwi mutants display very small brood sizes for many generations prior to sterility [20]. By immunofluorescence, we observed a wide range of P granule defects in both sterile and fertile late-generation *prg-1* mutant germ cells (Fig. 2b, S1), such that many germlines exhibited strong P granule disorganization that either resulted in an irregular distribution of or a total absence of P granules (Fig. 2b-b', S1).

C. elegans P granules are adjacent to Mutator bodies, which house Mutator proteins that recruit RNA-dependent RNA polymerase to create secondary effector siRNA populations in response to primary siRNAs [42]. *mutator* mutants are dysfunctional for secondary siRNA biogenesis and remain fertile indefinitely at low temperatures [18], but display an immediate highly penetrant fertility defect at 25°C that could be caused by dysfunction of Piwi pathway-mediated heterochromatin [43]. We examined germlines of sterile *mut-14* mutant adults at the restrictive temperature of

25°C and observed pronounced P granule defects in 87% of animals (Fig. 2f-f', Table 1).

We conclude that germ granule morphology was consistently altered in most sterile Piwi pathway mutants (Fig. 2, Table 1), even though there was qualitative variability in the nature of the germ granule defects in distinct Piwi pathway mutants. These results contrast with levels of DNA damage signaling that were either unchanged or inconsistently increased in sterile Piwi pathway mutants in comparison to fertile late-generation siblings (Fig. 1).

Germ granule dysfunction is sufficient to elicit phenotypes of sterile Piwi pathway genome silencing mutants

We initially addressed the significance of P granule dysfunction in sterile late-generation small RNA genome silencing mutants using an RNA interference clone that simultaneously targets four P granule subunits *pgl-1*, *pgl-3*, *glh-1*, *glh-4* [27,44]. This resulted in many second generation (G2) sterile adults at 25°C (80%) (Fig. 3a). The germlines of G2 P granule RNAi L4 larvae were normal in size in comparison to control worms. However, a pronounced germ cell atrophy phenotype occurred as many P granule-depleted animals matured into sterile adults, resulting in a significant change in their germline profile (Fig. 3b, $p=3.93E-24$). Furthermore, oocyte univalents were previously observed in sterile but not fertile late-generation *rsd-6* mutants [19], and 45.5% of oocytes of sterile P granule depleted adults contained 7-12 univalents (Fig. 3c, e-h).

Deficiency for *pgl-1* phenocopies of reproductive arrest of sterile *prg-1* mutants

The germline phenotypes observed in P granule-depleted worms mimicked those of sterile *prg-1*/Piwi mutant adults (Fig. 3a-c), which can regrow their atrophied germlines and become fertile, suggesting that germ granule dysfunction might be sufficient to cause a form of reproductive arrest termed Adult Reproductive Diapause [20]. To test this hypothesis, we used a mutant deficient for the P granule component *pgl-1*, which displays a pronounced sterility phenotype if shifted to the restrictive temperature of 25°C [45]. While most first generation (G1) *pgl-1* animals at 25°C displayed normal germlines at the L4 larval stage, there was a significant shift in the germline profiles with 52% of day 1 adults showing germline atrophy (Fig. 3b, $p=1.04E-35$), similar to P granule RNAi knockdown and sterile *prg-1* mutants [20]. Furthermore, when oocytes of sterile *pgl-1* mutant adults were scored, we found that 56.7% of oocytes had univalents, in agreement with the oocyte univalents observed in response to P granule depletion by RNAi (Fig. 3c).

Some G1 progeny of *pgl-1* mutants whose parents were shifted from 20°C to 25°C were fertile and gave rise to G2 25°C animals that were uniformly sterile. 25°C G2 *pgl-1* mutant L4 larvae had germline profiles that displayed more severe germ cell proliferation defects than those of G1 L4 larvae, and day 1 adults showed a striking 54% increase in empty germlines (Fig. 3d, $p=2.44E-05$). Therefore, germlines of G1 progeny of *pgl-1* mutant mothers that were shifted to the restrictive temperature of 25°C for a single generation mimicked the germ cell degeneration phenotypes of sterile Piwi pathway mutants as L4 larvae matured into adults, whereas 25°C G2 generation *pgl-1* mutant larvae displayed more pronounced germ cell proliferation defects.

We asked if the fertility of sterile 25°C G1 *pgl-1* mutants could be restored by shifting them to the permissive temperature of 20°C. Sterile G1 *pgl-1* mutants that lacked embryos were shifted to 20°C on day 1 of adulthood and their germlines were scored after 48 hours, which revealed a significant change in their germline profiles ($p=0.007$), with a shift from atrophied germlines towards large germlines when compared to sterile G1 *pgl-1* sibling controls that remained sterile at 25°C (Fig. 3i). In agreement with the regrowth of some germline arms, we observed that some sterile G1 *pgl-1* mutant adults that were shifted to 20°C for several days had laid oocytes and dead embryos, and that a few even gave rise to progeny (Fig. 3j). In contrast, sterile *pgl-1* mutant control adults that were kept at 25°C never gave rise to oocytes, dead embryos or living progeny (Fig. 3j). The frequency of fertility observed in this circumstance is very similar to that observed for sterile 5 day old *prg-1* mutant adults that exit Adult Reproductive Diapause in response to an alternative food source [20]. Therefore, P granule dysfunction may induce a transient and potentially adaptive form of reproductive arrest termed Adult Reproductive Diapause.

Transcriptional consequences of germ granule dysfunction and small RNA genome silencing mutant sterility

The sterility of Piwi/piRNA mutants correlates with expression of transposons and associated genome instability [9,13,14,46]. In an effort to deduce how germ granule dysfunction might occur in small RNA genome silencing mutants, we sequenced RNA from the nuclear RNAi defective mutants *nrde-1*, *nrde-2* and *nrde-4*. These mutants are all defective in nuclear silencing in response to exogenous dsRNA triggers [30,47,48],

and at 25°C they all have Mortal Germlines [31]. However, at 20°C *nrde-1* and *nrde-4* mutants become progressively sterile but *nrde-2* mutants remain fertile indefinitely [31]. We therefore focused on *nrde-1* and *nrde-4* mutants because they have relatively large brood sizes at sterility at 20°C, such that large cohorts of L4 larvae that are poised to become sterile can be collected. In contrast, *prg-1* mutants develop very low brood sizes in late generations [20], and it is difficult to obtain large numbers of synchronous animals that are poised to become sterile. We therefore prepared RNA from early-generation and sterile-generation *nrde-1* and *nrde-4* mutants at 20°C and compared this with RNA from wildtype and *nrde-2* mutant controls.

We found that sterile generation *nrde-1* and *nrde-4* mutant L4 larvae showed strong upregulation of similar transposon classes in comparison to early generation *nrde-1* or *nrde-4* mutant L4 larvae or to wildtype controls (Fig. 4a). We identified 18 transposon classes that were upregulated at least two-fold in both *nrde-1* and *nrde-4*, 13 of which were CER retrotransposons, which is consistent with previous transgenerational analysis of *hrde-1* mutants [49]. However, we also found that *nrde-2* mutant controls that do not become sterile at 20°C displayed strong upregulation of similar transposon loci, even in early generations. Of the 18 transposons upregulated in both *nrde-1* and *nrde-4*, 17 were also upregulated in *nrde-2* compared with wildtype. As a comparison, we analyzed published RNA-seq data from *prg-1(n4357)* mutant animals [16]. Previous papers have reported that distinct classes of transposons are upregulated in *prg-1* and *nrde-2* mutants [16,18,33,49], and we confirmed this result by showing that MIRAGE1 was the only transposon upregulated in both *nrde-1*, *nrde-4* and *prg-1* mutants (Fig. 4a).

We compared gene expression data from sterile generation *nrde-1* or *nrde-4* mutant L4 larvae with late-generation *prg-1* mutants [16] and compared this with RNA from wildtype or *nrde-2* mutant control L4 larvae in order to identify common genes whose expression is specifically induced in L4 larvae that are poised to become sterile and that might explain the P granule abnormalities at sterility. Late-generation *prg-1* mutant and sterile generation *nrde-1* and *nrde-4* mutant gene expression was very different from wildtype or *nrde-2* mutant controls RNA. Spermatogenic genes were over-represented among genes significantly upregulated in sterile generation *nrde-1* and *nrde-4* mutant larvae ($p = 1e-15$, Chi Square test for both mutants), which is consistent with what has been previously reported for *spr-5* mutants that become progressively sterile [50]. However, there was no general tendency of an up- or down-regulation of any gene category for *prg-1*, *nrde-1* and *nrde-4* mutants. Only 11 genes were significantly upregulated in all three mutants, and there were no commonly downregulated genes (Fig. 4c). We identified a set of 20 genes that were significantly upregulated at least fourfold in *prg-1* and either *nrde-1* or *nrde-4*, which included many *pud* (protein upregulated in dauer) genes that could be an indicator for genes upregulated due to a general stress response of the organism (Table S2). This may be consistent with our recent observation that DAF-16, which promotes dauer formation and stress resistance [51], is responsible for several phenotypes of sterile generation *prg-1* mutant adults, including germ cell atrophy [20].

RNA-seq data sets have been previously obtained for germlines of P granule defective L4 larvae and adults, but gene expression was not significantly different from wildtype for L4 larvae, whereas somatic genes were overexpressed in P granule

defective adults because these animals displayed a germline to soma transformation [44]. We compared sterile generation *nrde-1* and *nrde-4* RNA from L4 larvae with RNA from germlines of P granule defective L4 larvae [44] and found no genes that were up or downregulated in both *nrde-1/nrde-4* and P granule defective larvae. Moreover, only two out of the 18 transposons upregulated in *nrde-1/nrde-4* were also upregulated in P granule defective L4 larvae (Fig. 4a). We conclude that although varying degrees of P granule disorganization occur at the generation of sterility for Piwi pathway genome silencing mutants, and that although acute dysfunction of P granules based on simultaneous knockdown of four P granule components leads to germ line degeneration phenotypes that mimic those of sterile generation Piwi pathway mutants, that acute P granule dysfunction does not cause immediate overt effects on expression of heterochromatic segments of the genome or on genes in a manner that mimics Piwi pathway mutant transcriptomes that are poised to become sterile.

Knockdown of a number of genes has been shown to disrupt *C. elegans* germline P granule structure, in a manner that could be similar to what we observe in sterile Piwi pathway mutant adult germlines [52]. We compared the expression of genes known to perturb P granule structure with genes whose expression is significantly altered in late-generation *prg-1* mutants or in sterile generation *nrde-1* and *nrde-4* mutant L4 larvae. We examined gene expression in late- or sterile-generation *prg-1*, *nrde-1* or *nrde-4* mutants but could find no consistent changes in in genes whose knockdown leads to reduced or eliminated P granule structures in wildtype animals [52].

Our analyses indicate that *prg-1* and *nrde-1/nrde-4* mutants have fundamentally different gene expression profiles at or near sterility. We conclude that although the

sterility phenotype of *prg-1* and *nrde-1/nrde-4* is physiologically similar in terms of germ cell degeneration at the L4 stage of development, it is very different on a molecular level, which could be consistent with our observations that pronounced P granule defects are observed in fertile and sterile late-generation *prg-1* mutant germ lines as opposed to pronounced P granule defects in sterile but not fertile late-generation adults of deficient for other Piwi pathway genes. We also note the relatively mild effects on P granules in sterile *nrde-1* and *nrde-4* mutants, where only a subset of nuclei are affected. Therefore, although deficiency for *prg-1* and *nrde-1/nrde-4* disrupts small RNA-mediated genome silencing and compromises germ cell immortality, these genes have distinct functions with regards to small RNA regulation of the genome such that we observed only a small number of overlapping transcriptional effects.

Discussion:

Previous work in the field of Piwi pathway mutant sterility has demonstrated that the immediate sterility that is observed in several species correlates with transposon expression and genomic instability [9,13,14]. However, analysis of our transgenerational model of Piwi pathway mutant sterility in *C. elegans* indicates that DNA damage, as reflected by the activities of the ATM and ATR DNA damage response kinases, is unlikely to be the central cause of sterility, as levels of DNA damage were not consistently increased in sterile generation animals in comparison to fertile late-generation siblings. These data complement previous observations that suggest that DNA damage may not cause the sterility of *prg-1*/Piwi mutants [18], and provide an important contrast with germ granule morphology of sterile Piwi pathway mutants.

We addressed the mechanism that evokes Piwi sterility by discovering pronounced germ granule defects in most sterile but not fertile late-generation Piwi pathway mutant animals. Disrupting germ granules directly by RNAi-knockdown of four germ granule components or by shifting the *pgl-1* germ granule mutant to restrictive temperature led to germline degeneration phenotypes that phenocopied those of sterile generation Piwi pathway mutants [20]. Furthermore, our data suggest that univalent chromosomes that are specifically observed in oocytes of some sterile Piwi pathway genome silencing mutants are a secondary consequence of P granule dysfunction that indirectly disrupts a meiotic process [53,54]. We also found that some sterile *pgl-1* mutants could recover at permissive temperature, resulting in growth of the germline and fertility (Fig. 3i-j), as previously observed for sterile late-generation *prg-1* mutants whose germlines were in a quiescent state of Adult Reproductive Diapause [20]. This implies that sterility in response to transgenerational dysfunction of the *C. elegans* Piwi pathway and in response to acute dysfunction of germ granules lead to a common reproductive arrest program. Defects in telomerase-mediated telomere repeat addition results in progressive telomere erosion in the context of human somatic cell proliferation, which leads to the accumulation of critically short dysfunctional telomeres and senescence [5,6]. Based on this analogy, and on the ability of acute telomere uncapping to similarly induce senescence [7], we suggest that the common reproductive arrest program caused by acute germ granule dysfunction and by transgenerational deficiency for Piwi suggest that the germ granule morphology defects of sterile Piwi mutants could be responsible for their sterility. However, it is formally possible that the germ granule defects that occur in sterile but not fertile late-generation Piwi pathway

mutants are a consequence, rather than a cause, of the reproductive arrest program that is responsible for Piwi mutant sterility. Even if this were the case, the changes in germ granule morphology of sterile late-generation Piwi pathway mutants are likely to be closely related to the cause of Piwi mutant sterility.

The endogenous functions of cytoplasmic liquid droplet compartments such as germ granules, P bodies and stress granules, which contain common protein components, are not well understood [55]. Although germ granules have been well studied in the context of germ cell biology and fertility, we report the first evidence to our knowledge that connects germ granules to a complex, pleiotropic reproductive arrest mechanism that we recently demonstrated occurs for sterile *C. elegans prg-1*/Piwi mutants [20]. Given that reproductive arrest has been linked to stress responses in biology, for example in response to starvation or seasonal change [23,24], we suggest that it is not coincidental that germ granules share components with other cytoplasmic liquid compartments like P bodies and stress granules, which are well known to respond to stress. We speculate that the release of RNA or protein components from germ granules could promote reproductive arrest in this context.

Lack of commonly upregulated transposons in late-generation Piwi pathway mutants and in germ granule defective animals suggests that the reproductive arrest that they experience is not simply a response to transposon desilencing. Given that *prg-1*/Piwi mutant germ cells may transmit a form of heritable stress that occurs in response to heterochromatin dysfunction [20], and given that we observed germ granule disorganization in Piwi pathway mutants that generally coincided precisely with the generation of sterility, our results suggest two possible models regarding the cause of

Piwi mutant sterility. First, the Piwi/piRNA pathway could play a direct role in the maintenance of germ granules, which are liquid droplet composites of protein and RNA whose biogenesis and stability may depend on protein-RNA interactions [55]. Small RNAs created in response to the Piwi/piRNA pathway could interact with mRNAs in germ granules to facilitate their maintenance in a manner that becomes transgenerationally mis-regulated in Piwi pathway mutants. In this first scenario (Fig. 5b), disruption of germ granules themselves would represent the cause of reproductive arrest, as we found for *pgl-1* mutants (Fig. 3). Some Argonaute proteins that modulate gene expression in response to small RNAs, such as CSR-1, PRG-1 or WAGO-1, are localized to P granules [32,33,56–58]. In addition, previous analysis of sterile *prg-1* mutants, created by crossing *prg-1*; *mutator* double mutants together, resulted in adult germlines with a range of sizes analogous to those observed in sterile late-generation *prg-1* mutants [20,59]. This led to the hypothesis that misrouting of small RNAs associated with pro- and anti-silencing small RNA pathways could be the cause of sterility in Piwi pathway genome silencing mutants [36,59], which may be consistent with our observations of germ granule abnormalities in sterile Piwi pathway mutants. However, the misrouting of small RNAs hypothesis was not previously suggested or shown to be related to changes to germ granule structure or function [36,59].

A second scenario that could explain the sterility of *C. elegans* Piwi pathway mutants is that transgenerational dysfunction of the Piwi/piRNA pathway could lead to heterochromatin defects that result in the misregulation of an endogenous biochemical pathway in late-generation *C. elegans* Piwi pathway mutants, and that this then perturbs germ granule structure or function in a manner that elicits reproductive arrest (Fig. 5b).

When RNA from sterile generation *nrde-1* and *nrde-4* mutant L4 larvae and late-generation *prg-1* mutants was examined, we failed to observe consistent depletion of any gene whose knockdown is known to disrupt P granule formation [52], suggesting a post-transcriptional reproductive arrest mechanism that could be consistent with either scenario depicted in Fig. 5b. Nevertheless, we favor scenario 2 because deficiency for the *C. elegans* H3K4 demethylases *spr-5* or *rbr-2*, which function within nuclei downstream of small RNAs to promote genomic silencing, elicit a transgenerational sterility phenotype that is similar to that of Piwi pathway silencing mutants [39]. However, we also note that heterochromatin itself is transcribed in a manner that facilitates the maintenance of small RNA populations [60,61].

Possibly consistent with our observations, sterile mouse mutants with defects in piRNA biogenesis have been reported to display germ granule defects [62,63]. We suggest that germ granule dysfunction could explain the sterility observed in P-M hybrid dysgenesis and the immediate sterility phenotype of Piwi mutants in several species [9,13,14]. Environmental stresses such as starvation can also evoke reproductive arrest [22,23], and our data suggest that germ granule dysfunction could represent a general mechanism for orchestrating stress-induced reproductive arrest. We note that there has been a rise in human infertility in recent decades that has been attributed to factors such as psychological stress, caffeine consumption and pollutants [64,65].

The sterility of *prg-1*/Piwi mutants is a pleiotropic, non-uniform phenotype that involves dramatic levels of germ cell atrophy and can be followed by germ line regrowth and reproduction for a small fraction of sterile animals [20]. We suggest that this phenotype, while non-uniform and inefficient in terms of its reversibility, is an effective

form of reproductive arrest that promotes survival of wild nematode populations that are composed of numerous individuals. Therefore, even if the reproductive arrest mechanism of sterile Piwi mutants is inefficient, it is likely to be under significant evolutionary pressure.

Our results establish a novel framework for considering inheritance in the context of epigenetic stress and how this can evoke transgenerational sterility. We suggest that the sterility we describe in response to epigenome dysfunction represents a developmental arrest mechanism to protect the genome or epigenome in times of stress. We note that transposons and viruses are likely to be in a continuous arms race between parasite and host, where components of germ granules that promote Piwi pathway genome silencing and protect the host are likely to be targeted [52]. It may therefore be reasonable to hypothesize the evolution of a reproductive arrest mechanism in the context of an attack on the Piwi silencing system that is predicated on disrupting the integrity of germ granules themselves.

Materials and Methods

Strains

All strains were cultured at 20°C or 25°C on Nematode Growth Medium (NGM) plates seeded with *E. coli* OP50. Strains used include Bristol N2 wild type, *hrde-1(tm1200) III*, *rsd-6(yp11) I*, *nrde-1(yp4) III*, *nrde-2(gg95) II*, *nrde-4 (gg131) IV*, *prg-1 (n4357) I*, *prg-1 (tm872) I*, *mut-14 (pk730) V*, and *rbr-2(tm1231) IV*.

RNAi Assay

Feeding RNAi plates harboring host bacteria HT115(DE3) engineered to express “quad” dsRNA (targeting P granules) were obtained from Susan Strome [27,44]. L1 larvae were placed onto freshly prepared feeding RNAi plates with dsRNA induced by 1 mM IPTG (isopropyl- β -D(-)-thiogalactopyranoside) and were transferred after 1 generation at 25°C and collected at G2 adults as described in Knutson et al 2017 [44] for DAPI staining, oocyte and germline analysis.

DAPI staining and Scoring

DAPI staining was performed as previously described [28]. Briefly, 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 48 hours after collection. Univalents were scored by counting DAPI bodies in the -1 to -4 oocytes. Germline profiles were scored using the method outlined in Heestand et al 2018 [20].

Statistical Analysis

Statistical analysis was performed as previously described [20]. Briefly, statistical analysis was performed using the R statistical environment [66]. For germline phenotypes, contingency tables were constructed and pairwise Chi Square tests with Bonferroni correction was used to determine significant differences in in germline phenotype distributions. The significance of the DNA damage response analysis was determined by a Kruskal-Wallis test, followed by a Mann-Whitney test between individual samples. P-values were adjusted with Bonferroni correction when multiple comparisons were performed.

RNA extraction and sequencing

Animals were grown at 20°C on 60 mm NGM plates seeded with OP50 bacteria. RNA was extracted using Trizol (Ambion) followed by isopropanol precipitation. Library preparation and sequencing was performed at the UNC School of Medicine High-Throughput Sequencing Facility (HTSF). Libraries were prepared from ribosome-depleted RNA and sequenced on an Illumina Hiseq 2500.

RNA-seq Analysis

The following publicly available RNA-seq datasets were download from the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>): GSE92690 (P granule RNAi experiment) and GSE87524 (*prg-1* experiment). Adapter trimming was performed as required using the bbdut.sh script from the BBmap suite [67] and custom scripts. Reads were then mapped to the *C. elegans* genome (WS251) using hisat2 [68] with default settings and read counts were assigned to protein-coding genes using the featureCounts utility from the Subread package [69]. For multimapping reads, each mapping locus was assigned a count of 1/n where n=number of hits. Differentially expressed genes were identified using DESeq2, and were defined as changing at least 2-fold with FDR-corrected p-value < 0.01. For analysis of transposon RNAs, reads were mapped to the *C. elegans* transposon consensus sequences downloaded from Repbase (<http://www.girinst.org/repbase/>) with bowtie [70] using the options -M 1 -v 2. Transposons with fewer than 10 counts in each sample were excluded from further analysis. Counts were normalized to the total number of mapped reads for each library

for the prg-1 dataset, or to the total number of non-ribosomal mapped reads for all other datasets. A pseudocount of 1 was added to each value to avoid division by zero errors. Analysis of sequencing data and plot creation was performed using the R statistical computing environment [66].

Accession numbers: RNA-seq data reported in this study have been submitted to the GEO database and will be available at the time of publication.

Immunofluorescence

Adult hermaphrodites raised at 20°C or 25°C were dissected in M9 buffer and flash frozen on dry ice before fixation for 1 min in methanol at -20°C. After washing in PBS supplemented with 0.1% Tween-20 (PBST), primary antibody diluted in in PBST was used to immunostain overnight at 4 °C in a humid chamber. Primaries used were 1:50 OIC1D4 (Developmental Studies Hybridoma Bank). Secondary antibody staining was performed by using a Cy3 donkey anti-mouse or Cy-5 donkey anti-rabbit overnight at 4°C. All images were obtained using a LSM 710 laser scanning confocal and were taken using same settings as control samples. Images processed using ImageJ.

DNA Damage Assay

Worms that were close to sterility were isolated and defined as sterile if they did not have any offspring as day 3 adults at 20°C or day 2 adults at 25°C. Fertile siblings of sterile worms were defined as ‘close to sterility’. The presence of the DNA damage response was determined by using a phospho-specific antibody targeting the

phosphorylated consensus target site of ATM and ATR kinases (pS/TQ) (Cell Signaling Technology). This antibody has only been shown to stain a DNA damage response in the germline and was used as previously described [35].

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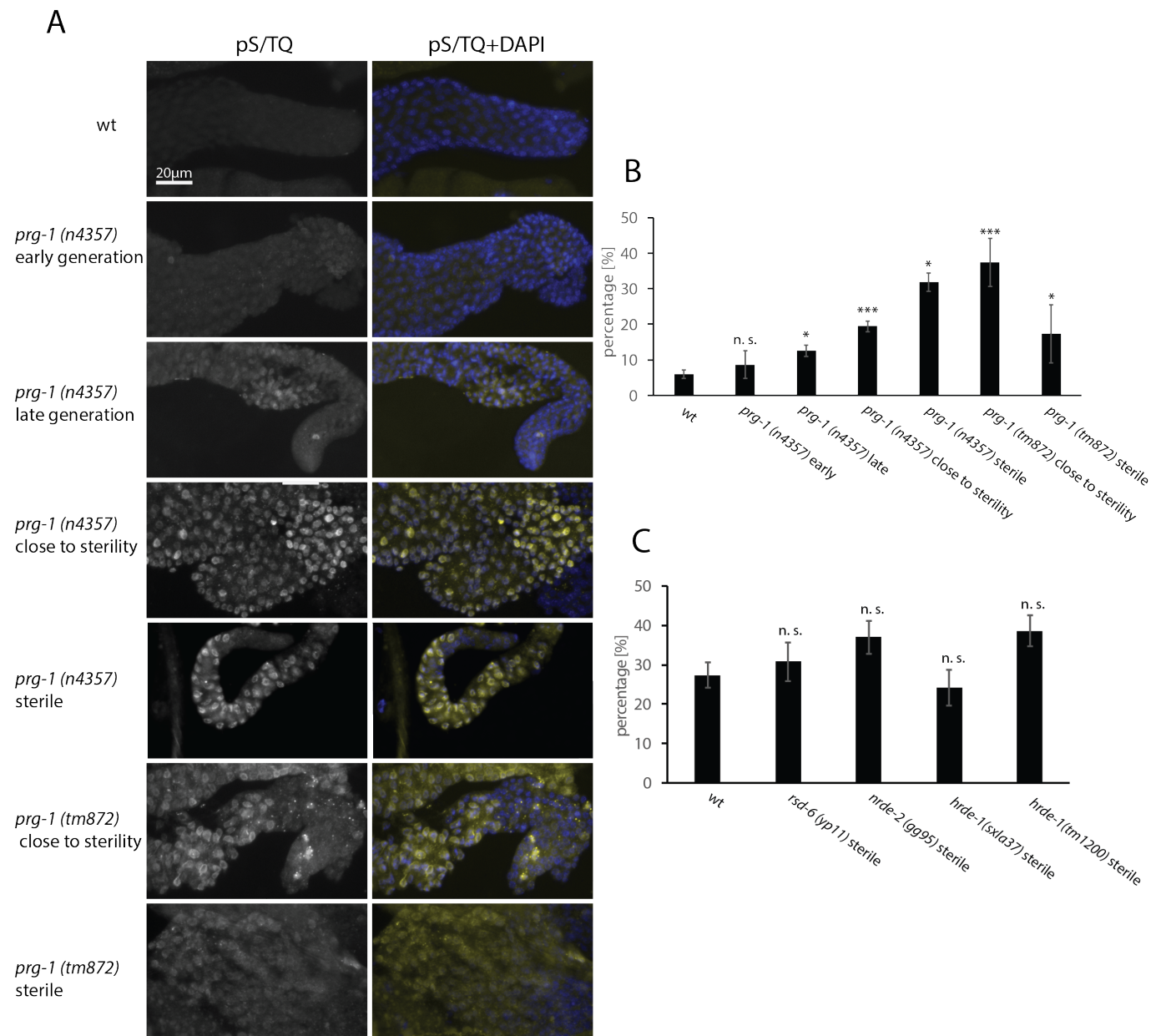


Figure 1: DNA Damage Response is variable in Piwi pathway genome silencing

mutants prior to and at sterility. (A) Staining of pS/TQ in wildtype, *prg-1(n4357)* and *prg-1(tm876)* mutants. pS/TQ(grey and yellow) and DAPI(blue) **(B)** Quantification of pS/TQ staining in 20°C wildtype and *prg-1* mutants at different levels of fertility. **(C)** Quantification of pS/TQ staining in 25°C wildtype and small RNA genomic silencing

782 mutants at sterility. P-values were obtained by comparing sample groups in a Mann-
 783 Whitney U test (p-value: $P > 0.05 = \text{n. s.}$, $P < 0.05 = *$, $P = < 0.01 = **$, $P = < 0.001 = ***$
 784 compared to wildtype)

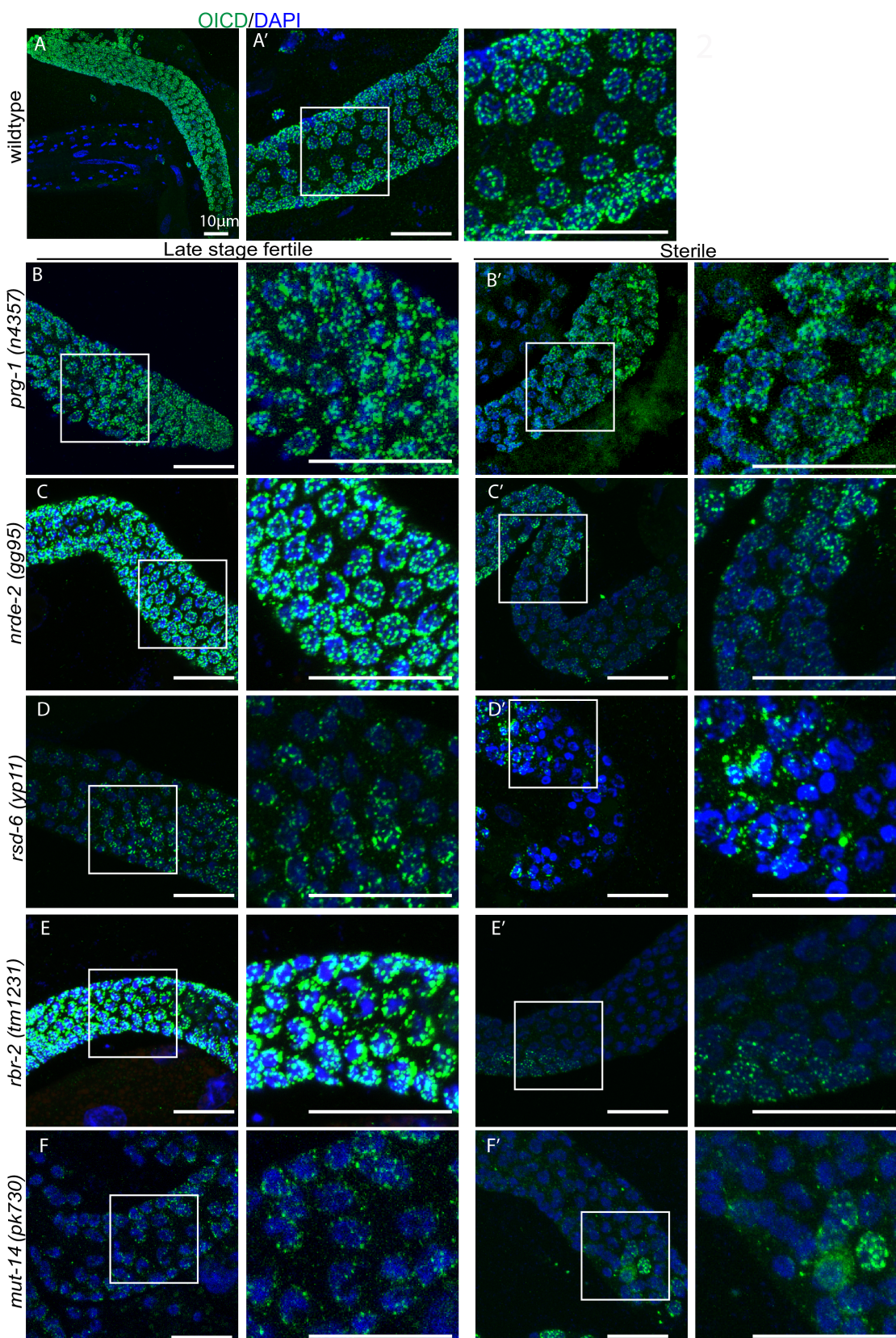


Figure 2: At sterility, partial loss of P granule staining occurred in *prg-1* and temperature-sensitive mutants. Germlines of sterile Day 2-3 adult animals were stained using the OIC1D4 antibody against P granules (green) and DAPI (blue). **(A-A')** Control animals contain uniform puncta of P granule staining surrounding each nuclei. **(B-F)** Late generation fertile *prg-1*, *rsd-6*, *nrde-2*, *rbr-2*, and *mut-14* animals displayed relatively normal P granule staining in the germlines. **(B'-F')** *prg-1*, *nrde-2*, *rsd-6*, *rbr-2*, and *mut-14* sterile animals all exhibited some degree of germ cell P granule loss.

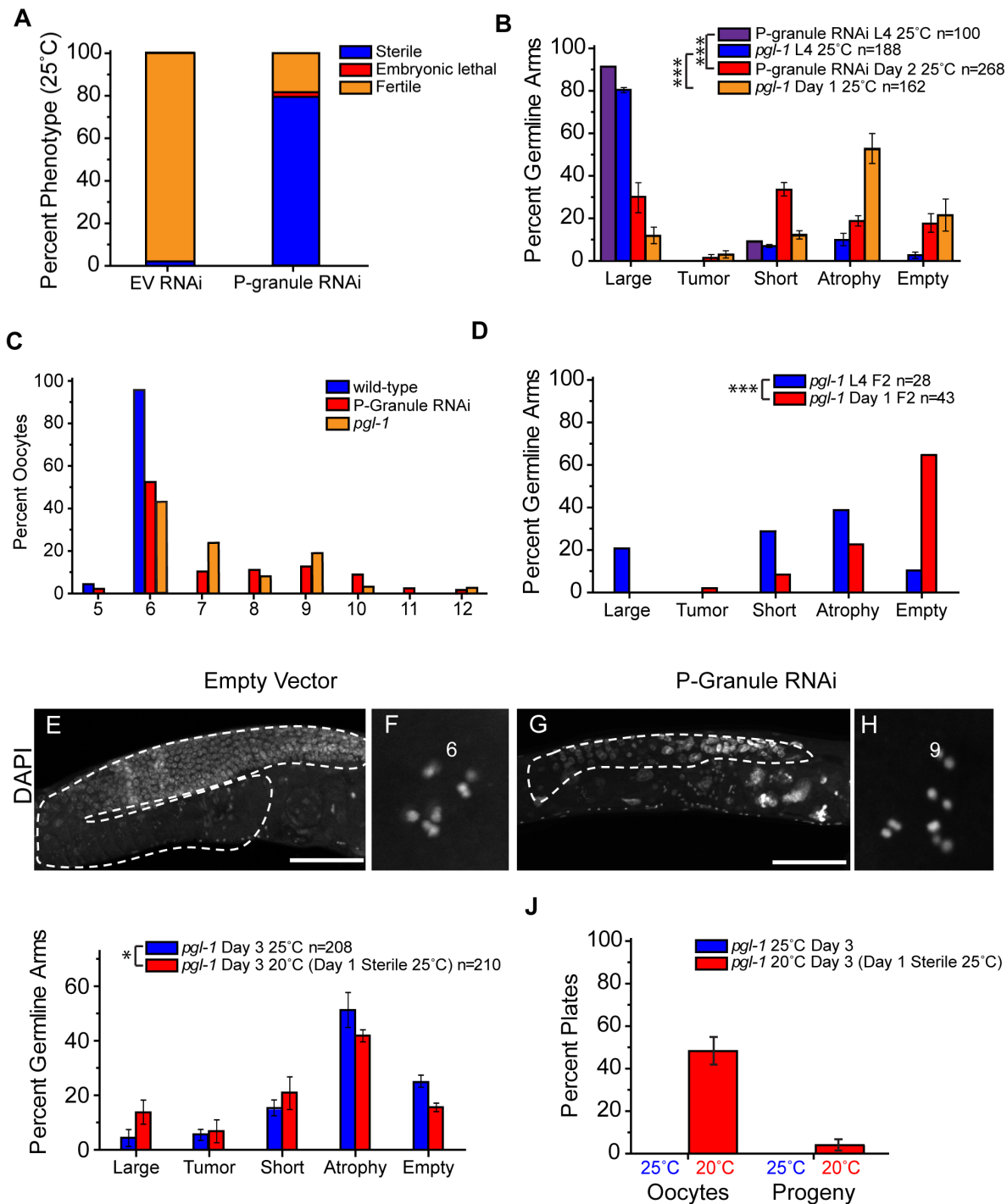
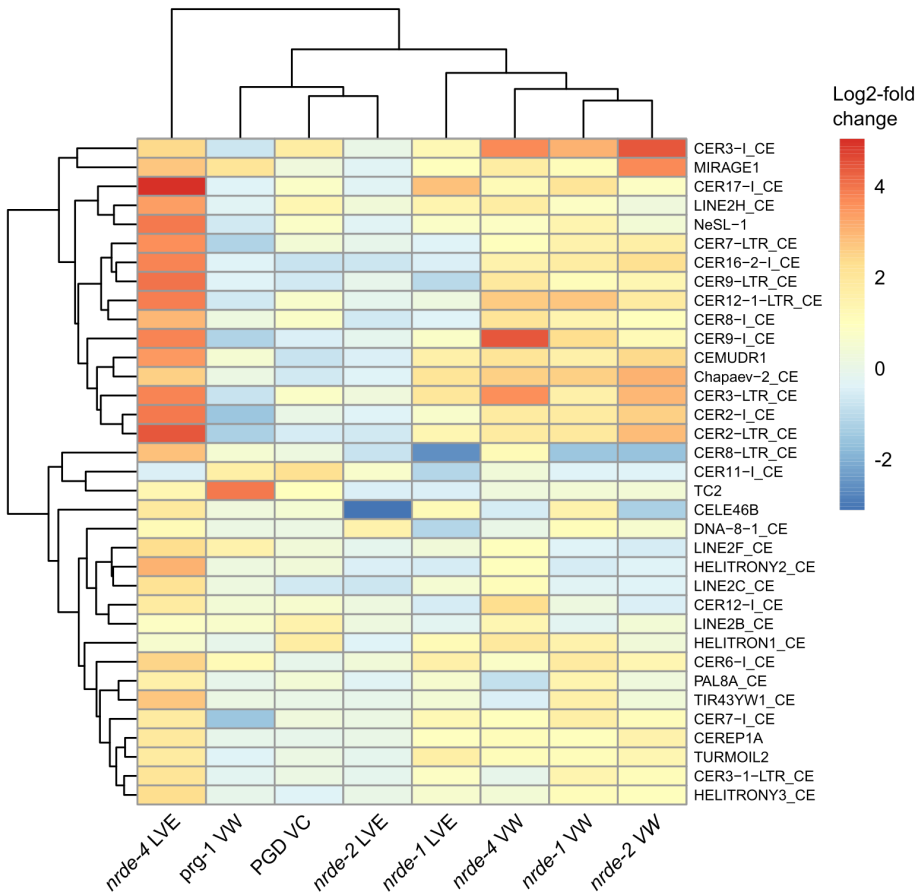


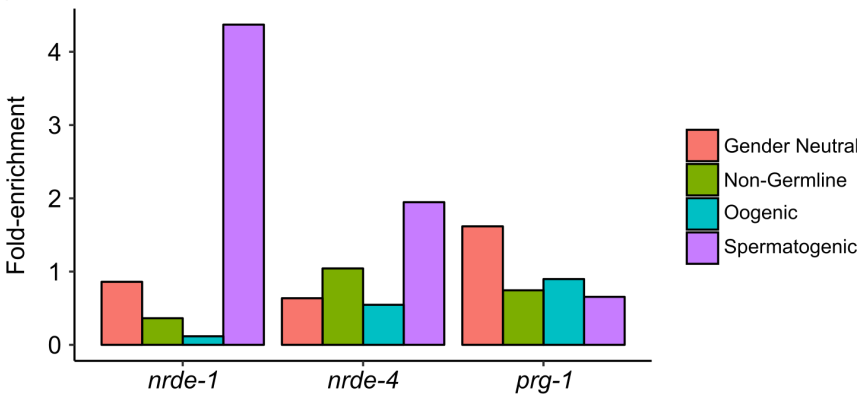
Figure 3: Disruption of P granules by RNAi or loss of *pgl-1* resulted in similar phenotypes. (A) G2 wild-type (N2) worms on Empty Vector (EV) or P granule RNAi at 25°C were scored for sterility, embryonic lethality, or fertility. n=16-30 worms per

condition, 2 independent experiments. **(B)** G1 L4 *pgl-1* mutants and G2 L4 P granule RNAi treated animals stained by DAPI contained mostly large germlines while adults exhibited a range of germline phenotypes from empty to large. **(C)** G1 *pgl-1* mutants and G2 P granule RNAi treated animals contained oocytes with increased numbers of univalents at sterility as measured by DAPI bodies n=151 wild-type, 81 P granule RNAi, 37 *pgl-1*. **(D)** Stained DAPI germlines of sterile G2 L4 and Day 1 *pgl-1* mutants. **(E-H)** Representative DAPI images of germline and oocyte defects in empty vector treated animals **(E,F)** and P granule RNAi treated animals **(G,H)** scale bar=10µm. **(I)** *pgl-1* G1 worms at 25°C were either DAPI stained as Day 3 adults (blue) or shifted to 20°C at Day 1 and DAPI stained at Day 3 (red). **(I)** No sterile day 1 *pgl-1* G1 at 25°C laid oocytes or progeny (n=35 plates, 350 worms). Sterile day 1 *pgl-1* shifted to 20°C (n=50 plates, 500 worms) gave rise to plates with oocytes and rarely progeny. Plates with oocytes contained both oocytes and dead embryos. Plates with progeny contained embryos that hatched to L1. For B,D,I *** p<0.0001, * p=0.007 Fisher's Chi Square test with Bonferroni correction, n=total germlines scored, error bars are S.E.M.

A



B



C

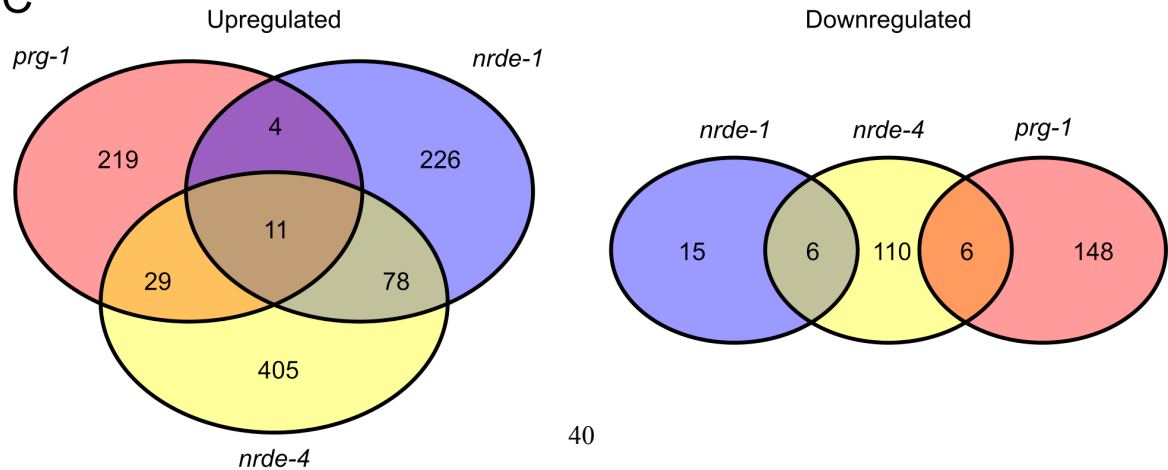


Figure 4: Transcriptome profiling of germ granule deficient larvae of Piwi pathway genome silencing mutants *nrde-1* and *nrde-4*. **(A)** Log2-fold changes in transposon transcript abundance in mutant vs wild-type (VW), late vs early generation (LVE) or P granule RNAi-treated vs control (PGD VC). Transposons upregulated at least two-fold in *nrde-1*, *nrde-4* or *prg-1(n4357)* are shown. **(B)** Enrichment of germline-expressed genes in genes upregulated in *nrde-1* and *nrde-4* mutants. **(C)** Number of genes up and downregulated in *nrde-1* and *nrde-4* mutants.

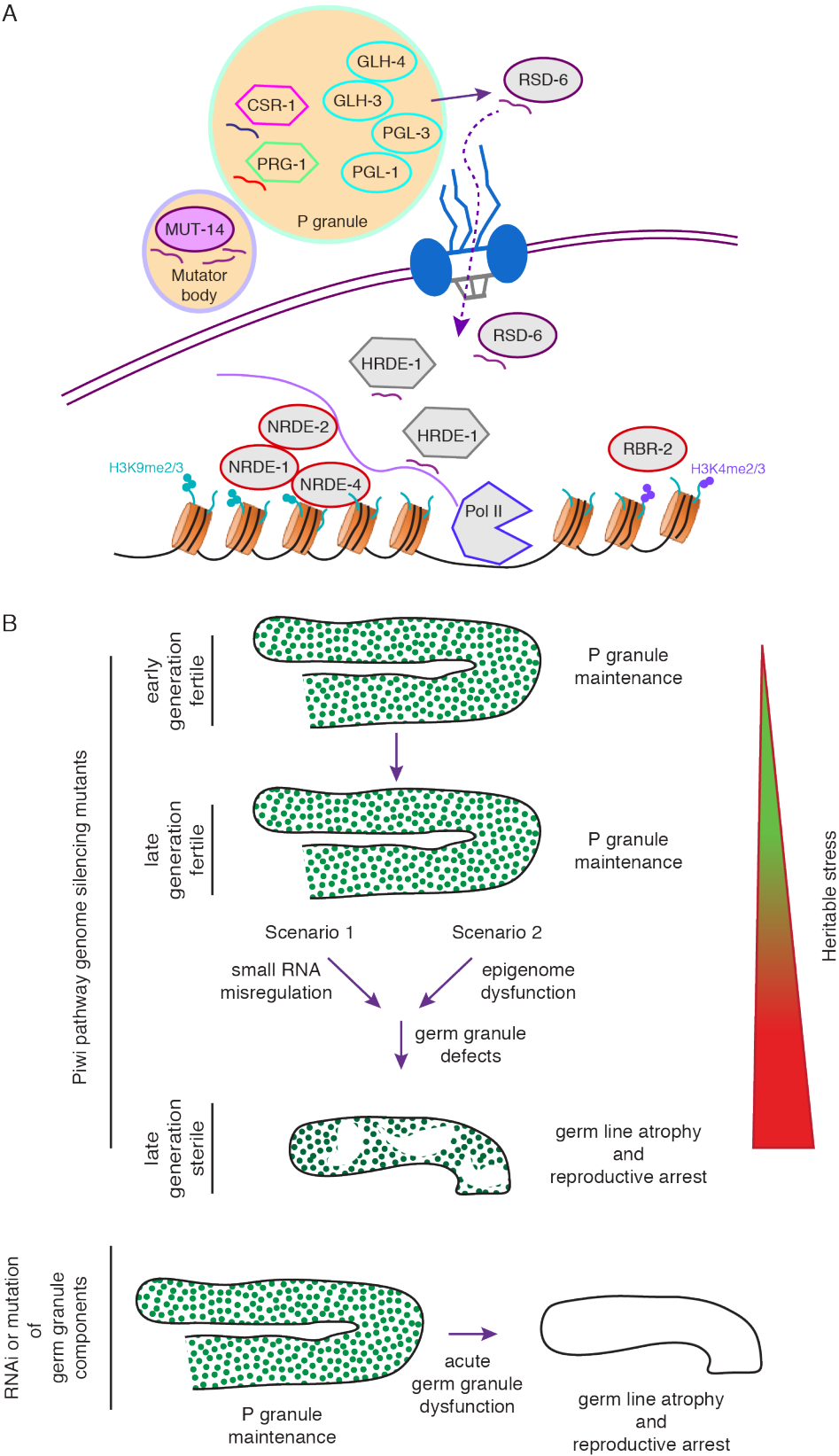


Figure 5: Model for P granule dysfunction in Piwi pathway genome silencing

mutants. (A) Model of P granules and associated small RNA-mediated genome silencing proteins. Green circles contain P granule components. Mutants in this paper that are temperature-sensitive for germ cell immortality have blue outlines. Non-conditional mutants have red outlines. **(B)** Model for transgenerational sterility in response to accumulation of Heritable Stress in Piwi pathway genome silencing mutants, which ultimately compromises P granule integrity (top), and model for induction of an analogous form of reproductive arrest by acute dysfunction of P granule components (bottom).

Table 1

Genotype	<20% loss	>20%loss	p-value
wt	32	0	
<i>prg-1 (tm872)</i>	1	7	<.0001
<i>prg-1 (n4357)</i>	3	1	=.11
<i>hrde-1 (tm1200)</i>	0	31	<.0001
<i>rsd-6 (yp11)</i>	0	32	<.0001
<i>nrde-2(gg95)</i>	0	25	<.0001
<i>rbr-2 (tm1231)</i>	0	32	<.0001
<i>nrde-4 (gg131)</i>	17	5	=.008
<i>mut-14 (pk730)</i>	1	6	<.0001
<i>nrde-1 (yp4)</i>	4	6	<.0001

Table 1: Quantification of P granule defects in the germline.

Sterile Piwi pathway genome silencing mutants were stained with P granule antibody. Germlines arms were semi-qualitatively scored for P granules. If at least 20% of the arm was missing P granules it was scored as loss and if 80% or more of the arm contained P granules on DAPI-stained nuclei it was considered normal. Statistics were performed using Fishers exact test.

Supplemental Information

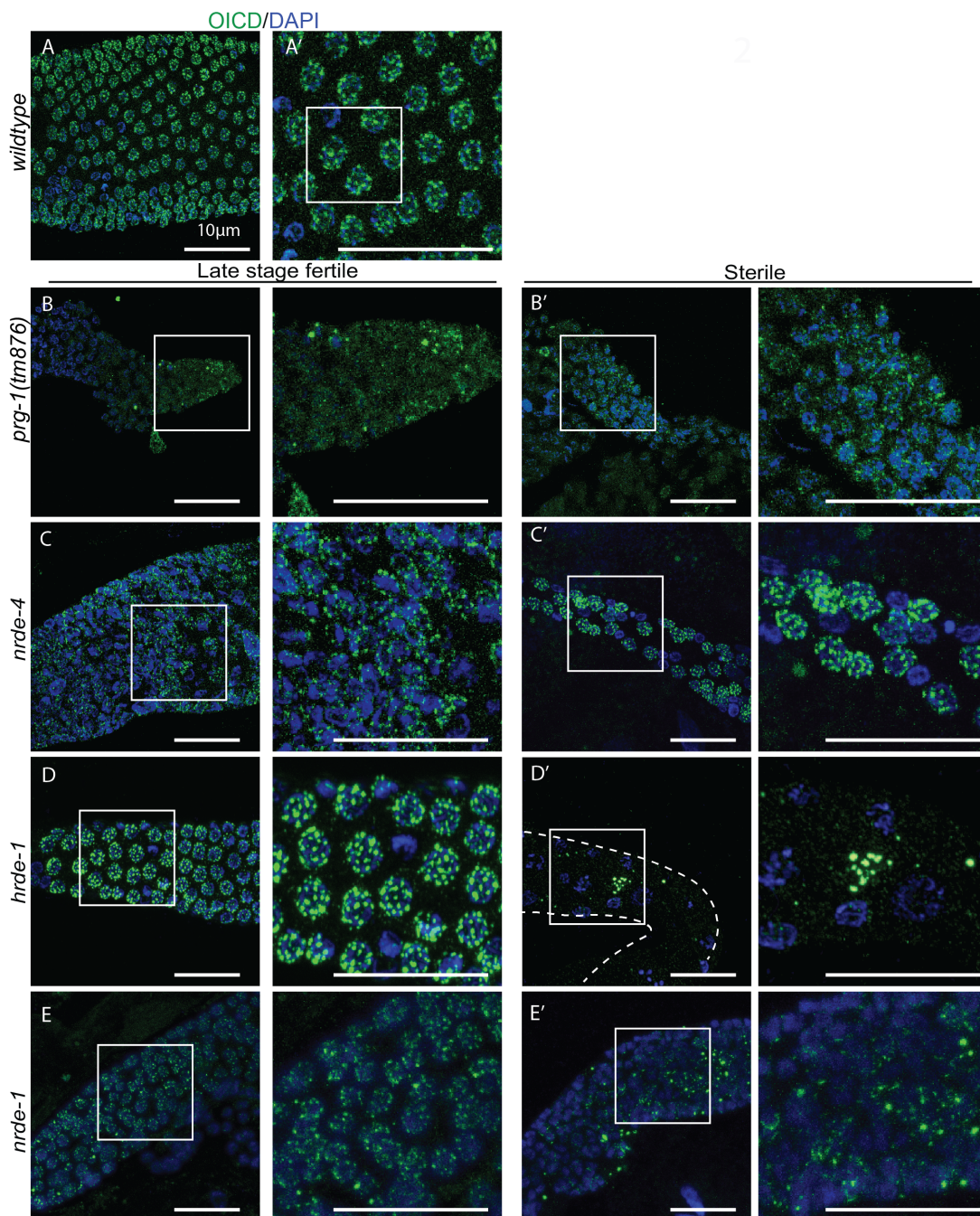


Fig. S1. Related to Figure 2. P granule defects in *prg-1*, *nrde-4*, *hrde-1* and *nrde-1*.
Germlines of sterile Day 2-3 adult animals were stained using the OIC1D4 antibody against P

granules (green) and DAPI (blue). (A-A') Control animals contain uniform puncta of P granule staining surrounding each nuclei. (B-E) Late generation fertile *prg-1*, *nrde-4*, *hrde-1*, and *nrde-1* animals displayed relatively normal P granule staining in the germlines. (B'-E') sterile *prg-1*, *nrde-4*, *hrde-1*, and *nrde-1* germ cell P granules.

	Total Worms Scored	% Fertile	% Sterile
<i>pgl-1</i> G1 at 25°C	91	22%	78%
<i>pgl-1</i> G2 at 25°C	71	0%	100%

Table S1: Related to Figure 3. Fertility of *pgl-1* mutants at 25°C. 20°C P0 *pgl-1* mutants were shifted to 25°C and fertility was measured for the G1 generation. Fertile G1 worms were cloned and the fertility of the G2 generation was measured.

gene	<i>prg-1</i>	<i>nrde-1</i>	<i>nrde-4</i>
<i>C06B3.7</i>	4.331128036	1.732445354	2.669978253
<i>dhs-26</i>	3.88587979	3.752818032	1.793937273
<i>bath-45</i>	3.604851731	5.5002102	2.319444032
<i>fbxb-97</i>	3.555301139	3.002992933	3.278539846
<i>irg-2</i>	3.519633623	2.605415087	2.189754742
<i>pud-4</i>	3.226292885	2.956152303	4.407697185
<i>pud-3</i>	3.097392415	3.143142026	4.328678969
<i>R09E10.2</i>	2.745900789	5.100997358	4.251177196
<i>Y53F4B.5</i>	2.636725232	5.821227047	4.162462556
<i>K08D12.6</i>	2.631068263	2.955437046	0.277803871
<i>R03H10.6</i>	2.469258519	4.82098578	3.295830487
<i>Y58A7A.5</i>	2.397260966	1.795895	2.143861408
<i>pud-1.1</i>	2.299685196	2.556376909	3.17781014
<i>T22B7.7</i>	2.276777478	3.01143186	2.207919501
<i>fbxa-224</i>	2.248998131	4.226992478	1.979961678
<i>swt-6</i>	2.144598564	3.153313277	0.277029378
<i>pud-2.1</i>	2.119890425	2.781615959	2.880747229
<i>pud-2.2</i>	2.094123631	2.779337879	2.796566876
<i>pud-1.2</i>	2.080250623	2.559790088	3.000944771
<i>catp-3</i>	2.014970724	2.436698711	2.674464594

Table S2: Related to Figure 4. Genes upregulated in late-generation *prg-1* mutants and in sterile *nrde-1* and *nrde-4* mutant L4 larvae.