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

45

46 **Introduction:**

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

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

68 of genome catastrophe induced by telomere dysfunction, although the precise cause of
69 telomerase mutant sterility remains uncertain [4–7], possibly the creation of circular
70 chromosomes that are toxic to meiosis [4]. Similar to the transgenerational proliferation
71 defects of *C. elegans* telomerase mutants, primary human somatic cells that are
72 cultured in vitro have a limited proliferative capacity that is associated with telomerase
73 dysfunction, progressive telomere erosion and ultimately telomere dysfunction, which
74 triggers entry into an irreversible state of cell cycle arrest termed senescence [5,6].
75 Confidence that senescence of primary human fibroblasts that proliferate in vitro is
76 caused by telomere dysfunction was obtained by acute knockdown of the telomere
77 capping protein TRF2, which led to immediate telomere deprotection of telomeres
78 whose lengths were normal, accompanied by telomere fusions and senescence [7].
79 A distinct fertility pathway, P-M Hybrid Dysgenesis, was discovered in the 1970's
80 as a temperature-sensitive *Drosophila* fertility defect that was linked to defects in
81 transposon silencing [8,9]. In these experiments, Drosophila M strain females were
82 mated with males of the more recently isolated P strain to yield F1 hybrid progeny with
83 high levels of P element transposition and temperature-sensitive sterility. The reduced
84 fertility of P-M F1 hybrids has been attributed to lack of piRNA-mediated genome
85 silencing for specific transposable elements [10], which are normally repressed by Piwi.
86 Piwi is a conserved Argonaute protein that is enriched in germ cells and interacts with
87 thousands of small RNAs termed piRNAs to repress transposons and foreign nucleic
88 acids that represent parasitic threats to the integrity of the genome [11–13]. Deficiency
89 for Piwi proteins leads to immediate sterility in *Drosophila*, zebrafish and mouse, and
90 this correlates with transposon expression and increased levels of DNA damage in

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

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

106 Fertility defects that occur in response to heterochromatin dysfunction in *C.*
107 *elegans* have been associated with increased levels of RNA-DNA hybrids and of
108 transposon-induced DNA damage [16,17]. *C. elegans prg-1* mutants display
109 progressively increased levels of germline apoptosis when grown for successive
110 generations, and apoptosis promotes germ cell atrophy for sterile *rsd-2*, *rsd-6* and *prg-1*
111 mutants [18–20]. However, very low levels of transposition occur in late-generation *prg-*
112 *1* mutants, and in *C. elegans mutator* mutants, which do not display transgenerational
113 sterility, high levels of transposition do occur suggesting transposition is not sufficient to

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

120 The late-generation sterility phenotype of *C. elegans prg-1*/Piwi mutants was
121 recently shown to elicit a striking but pleiotropic germ cell degeneration phenotype at
122 the generation of sterility, as L4 larvae mature into 1 day old adults, such that most
123 animals display 'atrophied' germlines that retain only a small population of mitotic germ
124 cells, whereas some germlines are 'empty' and lack germ cells altogether, some
125 germlines are 'short' such that they have fewer germ cells than normal but possess both
126 mitotic and meiotic germ cells, and some germlines are sterile but 'normal' in size such
127 that they are not smaller than germlines of fertile late-generation *prg-1* mutant siblings
128 that are not yet sterile [20]. Consistently, temperature-sensitive Piwi pathway genome
129 silencing mutants *rsd-2* and *rsd-6* give rise to late-generation sterile adults that exhibit
130 germline arms that are normal in size but also give rise to small and atrophied germline
131 arms that are promoted by apoptosis [19]. Moreover, atrophied germ lines of sterile *prg-*
132 *1* mutants can regrow on day 2 of adulthood, and a small fraction of constitutively sterile
133 5 day old *prg-1* mutant adults can become fertile again if they are shifted to a distinct
134 food source [20]. The ability of sterile late-generation of *C. elegans prg-1*/Piwi mutant
135 adults to become fertile suggests that Piwi mutant sterility is a reversible form of
136 reproductive arrest termed Adult Reproductive Diapause, which can be induced in

137 response to environmental stresses such as starvation [22–24]. We therefore proposed
138 that late-generation sterility of *prg-1*/Piwi mutants may occur in response to
139 accumulation of a heritable stress that is transmitted by germ cells [20].
140 Some Argonaute proteins that regulate epigenomic pathways are located in
141 perinuclear germ granules, which are conserved germline structures akin to liquid
142 droplets that interact with nuclear pore complexes and are necessary for fertility [25]. *C.*
143 *elegans* germ granules are termed P granules based on antibodies that label the P
144 blastomere that generates the germ cell lineage [26]. Core P granule proteins have
145 been shown to play redundant functions making it difficult to eliminate P granules [27].
146 Here we study the late-generation sterility phenotype of Piwi pathway genome silencing
147 mutants and find that it is associated with abnormalities of germ granules, and we
148 demonstrate that acute germ granule dysfunction is sufficient to phenocopy the
149 reproductive arrest phenotypes of sterile Piwi mutants.
150

151 **Results:**

152 **Genome instability in Piwi pathway genome silencing mutants**

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

160 [16,18,30–33]. We studied the sterility of Piwi pathway mutants that were temperature-
161 sensitive for germ cell immortality at 25°C and also non-conditional Piwi pathway
162 mutants at 20°C. We compared fertile late-generation Piwi pathway mutants with late
163 generation siblings that were sterile, in an effort to learn about the cause of their sterility.
164 Although DNA damage has been suggested not to contribute significantly to the
165 sterility of *prg-1* mutants based on several lines of evidence, the levels of DNA damage
166 signaling in sterile animals have not been previously studied. We therefore examined
167 *prg-1* mutants grown at 20°C across several generations for the presence of the DNA
168 damage response, as detected by an antibody to phosphorylated S/TQ, a protein
169 modification that is created by two sensors of DNA damage, ATM and ATR, which are
170 phosphatadylisonisol-3-kinase like protein kinases [34,35]. We observed a significant
171 increase in the fraction of germ cells with upregulated DNA damage response for late-
172 generation fertile and sterile *prg-1* mutants (~3- to 6-fold) when compared to either wild-
173 type controls or to early-generation *prg-1* mutants (Fig. 1a,b). However, the fraction of
174 germ cells with an activated DNA damage response was not consistently elevated in
175 sterile *prg-1*/Piwi mutants in comparison to fertile late-generation siblings (Fig. 1b). We
176 also tested temperature-sensitive Piwi pathway mutants and found that the wild-type
177 DNA damage response was upregulated by growth at 25°C but that this level was not
178 further elevated in germlines of sterile late-generation *rsd-6, nrde-2* or *hrde-1* mutant
179 adults (Fig. 1b,c).
180
181 **Piwi pathway genome silencing mutants exhibit P granule defects at sterility**

182 Deficiency for PRG-1 causes delocalization of the P granule protein PGL-1 [36], and
183 combined loss of the epigenomic regulators SPR-5 and LET-418, or loss of H3K9
184 methylation modifier SET-2 and the nuclear RNAi pathway, results in immediate sterility
185 that is associated with disruption of P granules [37,38]. Whilst characterizing germ line
186 defects of sterile *rsd-6* mutants by immunofluorescence, we found that a P granule
187 antibody exhibited altered staining patterns in sterile but not fertile late-generation *rsd-6*
188 mutant siblings (Fig 2d-d'). We also found that sterile late-generation *hrde-1* and *nrde-2*
189 temperature-sensitive mutant germ lines displayed P granule defects (Fig 2c-c', S1),
190 which were absent or comparatively minor in fertile late-generation mutant adults that
191 were close to the generation of sterility (Fig. 2c-c', S1, Table 1). When compared to
192 wild-type controls, P granule defects in the sterile mutants ranged from a loss of P
193 granules in a subset of cells to varying amounts of P granule disorganization (Fig. 2,
194 S1). Sterile *hrde-1(tm1200)* mutant animals displayed the most severe P granule
195 phenotype, as the majority of sterile *hrde-1* mutant germ cells exhibited little to no
196 staining (Fig. S1). We also examined the temperature sensitive *mrt* mutant *rbr-*
197 *2(tm1231)*, which encodes a histone 3 lysine 4 demethylase that promotes genomic
198 silencing and likely functions downstream of small RNAs to promote germ cell
199 immortality [39]. Similarly to *hrde-1* and *nrde-2* mutants, late-generation fertile *rbr-2*
200 mutant animals did not exhibit obvious P granule defects, while loss of P granules was
201 observed in many *rbr-2* mutant germ cells at sterility (Fig. 2e-e').
202 Germ line atrophy occurs in some but not all *prg-1* mutants as L4 larvae develop
203 into sterile adults [20]. Large-scale apoptosis contributes to this germ cell degeneration
204 phenotype [20], and P granules have been shown to vanish in cells that undergo

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

208 *nrde-1* and *nrde-4* mutant animals displayed a distinct sterility phenotype,
209 whereby only a subpopulation of sterile germline arms displayed P granule defects
210 (60% and 22% respectively (Fig. S1, Table 1). This suggests that loss of *nrde-1* and
211 *nrde-4*, which act downstream of multiple small RNA genome silencing pathways, may
212 dampen the pronounced P granule defects that are observed in germline arms of most
213 sterile Piwi pathway mutants.

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

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

228 25°C and observed pronounced P granule defects in 87% of animals (Fig. 2f-f', Table
229 1).
230 We conclude that germ granule morphology was consistently altered in most
231 sterile Piwi pathway mutants (Fig. 2, Table 1), even though there was qualitative
232 variability in the nature of the germ granule defects in distinct Piwi pathway mutants.
233 These results contrast with levels of DNA damage signaling that were either unchanged
234 or inconsistently increased in sterile Piwi pathway mutants in comparison to fertile late-
235 generation siblings (Fig. 1).
236

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

239 We initially addressed the significance of P granule dysfunction in sterile late-generation
240 small RNA genome silencing mutants using an RNA interference clone that
241 simultaneously targets four P granule subunits *pgl-1*, *pgl-3*, *glh-1*, *glh-4* [27,44]. This
242 resulted in many second generation (G2) sterile adults at 25˚C (80%) (Fig. 3a). The
243 germlines of G2 P granule RNAi L4 larvae were normal in size in comparison to control
244 worms. However, a pronounced germ cell atrophy phenotype occurred as many P
245 granule-depleted animals matured into sterile adults, resulting in a significant change in
246 their germline profile (Fig. 3b, p=3.93E-24). Furthermore, oocyte univalents were
247 previously observed in sterile but not fertile late-generation *rsd-6* mutants [19], and
248 45.5% of oocytes of sterile P granule depleted adults contained 7-12 univalents (Fig. 3c,
249 e-h).
250

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

252 The germline phenotypes observed in P granule-depleted worms mimicked those of
253 sterile *prg-1*/Piwi mutant adults (Fig. 3a-c), which can regrow their atrophied germlines
254 and become fertile, suggesting that germ granule dysfunction might be sufficient to
255 cause a form of reproductive arrest termed Adult Reproductive Diapause [20]. To test
256 this hypothesis, we used a mutant deficient for the P granule component *pgl-1*, which
257 displays a pronounced sterility phenotype if shifted to the restrictive temperature of 25°C
258 [45]. While most first generation (G1) *pgl-1* animals at 25°C displayed normal germlines
259 at the L4 larval stage, there was a significant shift in the germline profiles with 52% of
260 day 1 adults showing germline atrophy (Fig. 3b, p=1.04E-35), similar to P granule RNAi
261 knockdown and sterile *prg-1* mutants [20]. Furthermore, when oocytes of sterile *pgl-1*
262 mutant adults were scored, we found that 56.7% of oocytes had univalents, in
263 agreement with the oocyte univalents observed in response to P granule depletion by
264 RNAi (Fig. 3c).

265 Some G1 progeny of *pgl-1* mutants whose parents were shifted from 20°C to
266 25°C were fertile and gave rise to G2 25°C animals that were uniformly sterile. 25°C G2
267 *pgl-1* mutant L4 larvae had germline profiles that displayed more severe germ cell
268 proliferation defects than those of G1 L4 larvae, and day 1 adults showed a striking 54%
269 increase in empty germlines (Fig. 3d, p=2.44E-05). Therefore, germlines of G1 progeny
270 of *pgl-1* mutant mothers that were shifted to the restrictive temperature of 25°C for a
271 single generation mimicked the germ cell degeneration phenotypes of sterile Piwi
272 pathway mutants as L4 larvae matured into adults, whereas 25°C G2 generation *pgl-1*
273 mutant larvae displayed more pronounced germ cell proliferation defects.

274 We asked if the fertility of sterile 25°C G1 *pgl-1* mutants could be restored by
275 shifting them to the permissive temperature of 20°C. Sterile G1 *pgl-1* mutants that
276 lacked embryos were shifted to 20°C on day 1 of adulthood and their germlines were
277 scored after 48 hours, which revealed a significant change in their germline profiles
278 (p=0.007), with a shift from atrophied germlines towards large germlines when
279 compared to sterile G1 *pgl-1* sibling controls that remained sterile at 25°C (Fig. 3i). In
280 agreement with the regrowth of some germline arms, we observed that some sterile G1
281 *pgl-1* mutant adults that were shifted to 20°C for several days had laid oocytes and
282 dead embryos, and that a few even gave rise to progeny (Fig. 3j). In contrast, sterile
283 *pgl-1* mutant control adults that were kept at 25°C never gave rise to oocytes, dead
284 embryos or living progeny (Fig. 3j). The frequency of fertility observed in this
285 circumstance is very similar to that observed for sterile 5 day old *prg-1* mutant adults
286 that exit Adult Reproductive Diapause in response to an alternative food source [20].
287 Therefore, P granule dysfunction may induce a transient and potentially adaptive form
288 of reproductive arrest termed Adult Reproductive Diapause.
289

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

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

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

306 We found that sterile generation *nrde-1* and *nrde-4* mutant L4 larvae showed
307 strong upregulation of similar transposon classes in comparison to early generation
308 *nrde-1* or *nrde-4* mutant L4 larvae or to wildtype controls (Fig. 4a). We identified 18
309 transposon classes that were upregulated at least two-fold in both *nrde-1* and *nrde-4*,
310 13 of which were CER retrotransposons, which is consistent with previous
311 transgenerational analysis of *hrde-1* mutants [49]. However, we also found that *nrde-2*
312 mutant controls that do not become sterile at 20°C displayed strong upregulation of
313 similar transposon loci, even in early generations. Of the 18 transposons upregulated in
314 both *nrde-1* and *nrde-4*, 17 were also upregulated in *nrde-2* compared with wildtype. As
315 a comparison, we analyzed published RNA-seq data from *prg-1(n4357)* mutant animals
316 [16]. Previous papers have reported that distinct classes of transposons are upregulated
317 in *prg-1* and *nrde-2* mutants [16,18,33,49], and we confirmed this result by showing that
318 MIRAGE1 was the only transposon upregulated in both *nrde-1*, *nrde-4* and *prg-1*
319 mutants (Fig. 4a).

320 We compared gene expression data from sterile generation *nrde-1* or *nrde-4*
321 mutant L4 larvae with late-generation *prg-1* mutants [16] and compared this with RNA
322 from wildtype or *nrde-2* mutant control L4 larvae in order to identify common genes
323 whose expression is specifically induced in L4 larvae that are poised to become sterile
324 and that might explain the P granule abnormalities at sterility. Late-generation *prg-1*
325 mutant and sterile generation *nrde-1* and *nrde-4* mutant gene expression was very
326 different from wildtype or *nrde-2* mutant controls RNA. Spermatogenic genes were over-
327 represented among genes significantly upregulated in sterile generation *nrde-1* and
328 *nrde-4* mutant larvae (p = 1e-15, Chi Square test for both mutants), which is consistent
329 with what has been previously reported for *spr-5* mutants that become progressively
330 sterile [50]. However, there was no general tendency of an up- or down-regulation of
331 any gene category for *prg-1*, *nrde-1* and *nrde-4* mutants. Only 11 genes were
332 significantly upregulated in all three mutants, and there were no commonly
333 downregulated genes (Fig. 4c). We identified a set of 20 genes that were significantly
334 upregulated at least fourfold in *prg-1* and either *nrde-1* or *nrde-4*. which included many
335 *pud* (protein upregulated in dauer) genes that could be an indicator for genes
336 upregulated due to a general stress response of the organism (Table S2). This may be
337 consistent with our recent observation that DAF-16, which promotes dauer formation
338 and stress resistance [51], is responsible for several phenotypes of sterile generation
339 *prg-1* mutant adults, including germ cell atrophy [20].
340 RNA-seq data sets have been previously obtained for germlines of P granule
341 defective L4 larvae and adults, but gene expression was not significantly different from
342 wildtype for L4 larvae, whereas somatic genes were overexpressed in P granule

343 defective adults because these animals displayed a germline to soma transformation
344 [44]. We compared sterile generation *nrde-1* and *nrde-4* RNA from L4 larvae with RNA
345 from germlines of P granule defective L4 larvae [44] and found no genes that were up or
346 downregulated in both *nrde-1/nrde-4* and P granule defective larvae. Moreover, only two
347 out of the 18 transposons upregulated in *nrde-1/nrde-4* were also upregulated in P
348 granule defective L4 larvae (Fig. 4a). We conclude that although varying degrees of P
349 granule disorganization occur at the generation of sterility for Piwi pathway genome
350 silencing mutants, and that although acute dysfunction of P granules based on
351 simultaneous knockdown of four P granule components leads to germ line degeneration
352 phenotypes that mimic those of sterile generation Piwi pathway mutants, that acute P
353 granule dysfunction does not cause immediate overt effects on expression of
354 heterochromatic segments of the genome or on genes in a manner that mimics Piwi
355 pathway mutant transcriptomes that are poised to become sterile.
356 Knockdown of a number of genes has been shown to disrupt *C. elegans*
357 germline P granule structure, in a manner that could be similar to what we observe in
358 sterile Piwi pathway mutant adult germlines [52]. We compared the expression of genes
359 known to perturb P granule structure with genes whose expression is significantly
360 altered in late-generation *prg-1* mutants or in sterile generation *nrde-1* and *nrde-4*
361 mutant L4 larvae. We examined gene expression in late- or sterile-generation *prg-1*,
362 *nrde-1* or *nrde-4* mutants but could find no consistent changes in in genes whose
363 knockdown leads to reduced or eliminated P granule structures in wildtype animals [52].
364 Our analyses indicate that *prg-1* and *nrde-1/nrde-4* mutants have fundamentally
365 different gene expression profiles at or near sterility. We conclude that although the

366 sterility phenotype of *prg-1* and *nrde-1/nrde-4* is physiologically similar in terms of germ
367 cell degeneration at the L4 stage of development, it is very different on a molecular
368 level, which could be consistent with our observations that pronounced P granule
369 defects are observed in fertile and sterile late-generation *prg-1* mutant germ lines as
370 opposed to pronounced P granule defects in sterile but not fertile late-generation adults
371 of deficient for other Piwi pathway genes. We also note the relatively mild effects on P
372 granules in sterile *nrde-1* and *nrde-4* mutants, where only a subset of nuclei are
373 affected. Therefore, although deficiency for *prg-1* and *nrde-1/nrde-4* disrupts small
374 RNA-mediated genome silencing and compromises germ cell immortality, these genes
375 have distinct functions with regards to small RNA regulation of the genome such that we
376 observed only a small number of overlapping transcriptional effects.
377

378 **Discussion:**

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

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

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

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

428 Lack of commonly upregulated transposons in late-generation Piwi pathway
429 mutants and in germ granule defective animals suggests that the reproductive arrest
430 that they experience is not simply a response to transposon desilencing. Given that *prg-*
431 *1/*Piwi mutant germ cells may transmit a form of heritable stress that occurs in response
432 to heterochromatin dysfunction [20], and given that we observed germ granule
433 disorganization in Piwi pathway mutants that generally coincided precisely with the
434 generation of sterility, our results suggest two possible models regarding the cause of

435 Piwi mutant sterility. First, the Piwi/piRNA pathway could play a direct role in the
436 maintenance of germ granules, which are liquid droplet composites of protein and RNA
437 whose biogenesis and stability may depend on protein-RNA interactions [55]. Small
438 RNAs created in response to the Piwi/piRNA pathway could interact with mRNAs in
439 germ granules to facilitate their maintenance in a manner that becomes
440 transgenerationally mis-regulated in Piwi pathway mutants. In this first scenario (Fig.
441 5b), disruption of germ granules themselves would represent the cause of reproductive
442 arrest, as we found for *pgl-1* mutants (Fig. 3). Some Argonaute proteins that modulate
443 gene expression in response to small RNAs, such as CSR-1, PRG-1 or WAGO-1, are
444 localized to P granules [32,33,56–58]. In addition, previous analysis of sterile *prg-1*
445 mutants, created by crossing *prg-1; mutator* double mutants together, resulted in adult
446 germlines with a range of sizes analogous to those observed in sterile late-generation
447 *prg-1* mutants [20,59]. This led to the hypothesis that misrouting of small RNAs
448 associated with pro- and anti-silencing small RNA pathways could be the cause of
449 sterility in Piwi pathway genome silencing mutants [36,59], which may be consistent
450 with our observations of germ granule abnormalities in sterile Piwi pathway mutants.
451 However, the misrouting of small RNAs hypothesis was not previously suggested or
452 shown to be related to changes to germ granule structure or function [36,59].
453 A second scenario that could explain the sterility of *C. elegans* Piwi pathway
454 mutants is that transgenerational dysfunction of the Piwi/piRNA pathway could lead to
455 heterochromatin defects that result in the misregulation of an endogenous biochemical
456 pathway in late-generation *C. elegans* Piwi pathway mutants, and that this then perturbs
457 germ granule structure or function in a manner that elicits reproductive arrest (Fig. 5b).

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

468 Possibly consistent with our observations, sterile mouse mutants with defects in
469 piRNA biogenesis have been reported to display germ granule defects [62,63]. We
470 suggest that germ granule dysfunction could explain the sterility observed in P-M hybrid
471 dysgenesis and the immediate sterility phenotype of Piwi mutants in several species
472 [9,13,14]. Environmental stresses such as starvation can also evoke reproductive arrest
473 [22,23], and our data suggest that germ granule dysfunction could represent a general
474 mechanism for orchestrating stress-induced reproductive arrest. We note that there has
475 been a rise in human infertility in recent decades that has been attributed to factors
476 such as psychological stress, caffeine consumption and pollutants [64,65].

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

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

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

495

496 **Materials and Methods**

497

498 Strains

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

503

504 RNAi Assay

505 Feeding RNAi plates harboring host bacteria HT115(DE3) engineered to express "quad"
506 dsRNA (targeting P granules) were obtained from Susan Strome [27,44]. L1 larvae were
507 placed onto freshly prepared feeding RNAi plates with dsRNA induced by 1 mM IPTG
508 (isopropyl-β-D(-)-thiogalactopyranoside) and were transferred after 1 generation at 25°C
509 and collected at G2 adults as described in Knutson et al 2017 [44] for DAPI staining,
510 oocyte and germline analysis.
511

512 DAPI staining and Scoring

513 DAPI staining was performed as previously described [28]. Briefly, L4 larvae were
514 selected from sibling plates and sterile adults were singled as late L4s, observed 24
515 hours later for confirmed sterility, and then stained 48 hours after collection. Univalents
516 were scored by counting DAPI bodies in the -1 to -4 oocytes. Germline profiles were
517 scored using the method outlined in Heestand et al 2018 [20].
518

519 Statistical Analysis

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

528

529 RNA extraction and sequencing

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

535

536 RNA-seq Analysis

537 The following publicly available RNA-seq datasets were download from the Gene
538 Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/): GSE92690 (P granule RNAi
539 experiment) and GSE87524 (*prg-1* experiment). Adapter trimming was performed as
540 required using the bbduk.sh script from the BBmap suite [67] and custom scripts. Reads
541 were then mapped to the *C. elegans* genome (WS251) using hisat2 [68] with default
542 settings and read counts were assigned to protein-coding genes using the
543 featureCounts utility from the Subread package [69] . For multimapping reads, each
544 mapping locus was assigned a count of 1/n where n=number of hits. Differentially
545 expressed genes were identified using DESeq2, and were defined as changing at least
546 2-fold with FDR-corrected p-value < 0.01. For analysis of transposon RNAs, reads were
547 mapped to the *C. elegans* transposon consensus sequences downloaded from
548 Repbase (http://www.girinst.org/repbase/) with bowtie [70] using the options -M 1 -v 2.
549 Transposons with fewer than 10 counts in each sample were excluded from further
550 analysis. Counts were normalized to the total number of mapped reads for each library

551 for the prg-1 dataset, or to the total number of non-ribosomal mapped reads for all other
552 datasets. A pseudocount of 1 was added to each value to avoid division by zero errors.
553 Analysis of sequencing data and plot creation was performed using the R statistical
554 computing environment [66].
555
556 Accession numbers: RNA-seq data reported in this study have been submitted to the
557 GEO database and will be available at the time of publication.
558
559 Immunofluorescence
560 Adult hermaphrodites raised at 20°C or 25°C were dissected in M9 buffer and flash
561 frozen on dry ice before fixation for 1 min in methanol at -20°C. After washing in PBS
562 supplemented with 0.1% Tween-20 (PBST), primary antibody diluted in in PBST was
563 used to immunostain overnight at 4 °C in a humid chamber. Primaries used were 1:50
564 OIC1D4 (Developmental Studies Hybridoma Bank). Secondary antibody staining was
565 performed by using a Cy3 donkey anti-mouse or Cy-5 donkey anti-rabbit overnight at
566 4°C. All images were obtained using a LSM 710 laser scanning confocal and were
567 taken using same settings as control samples. Images processed using ImageJ.
568
569 DNA Damage Assay
570 Worms that were close to sterility were isolated and defined as sterile if they did not
571 have any offspring as day 3 adults at 20˚C or day 2 adults at 25˚C. Fertile siblings of
572 sterile worms were defined as ‘close to sterility’. The presence of the DNA damage
573 response was determined by using a phospho-specific antibody targeting the

574 phosphorylated consensus target site of ATM and ATR kinases (pS/TQ) (Cell Signaling
575 Technology). This antibody has only been shown to stain a DNA damage response in
576 the germline and was used as previously described [35].
577
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584
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586
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589
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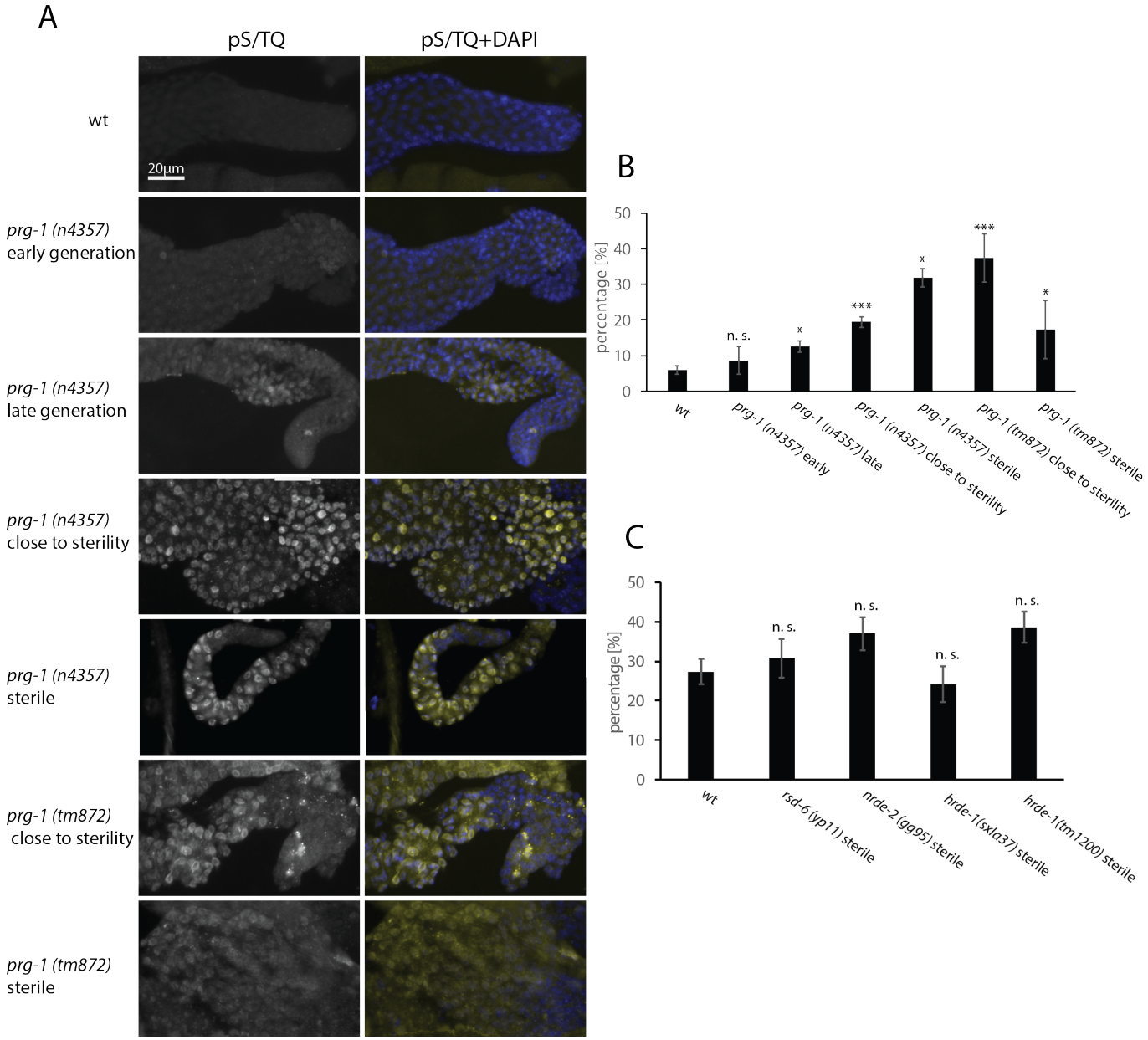
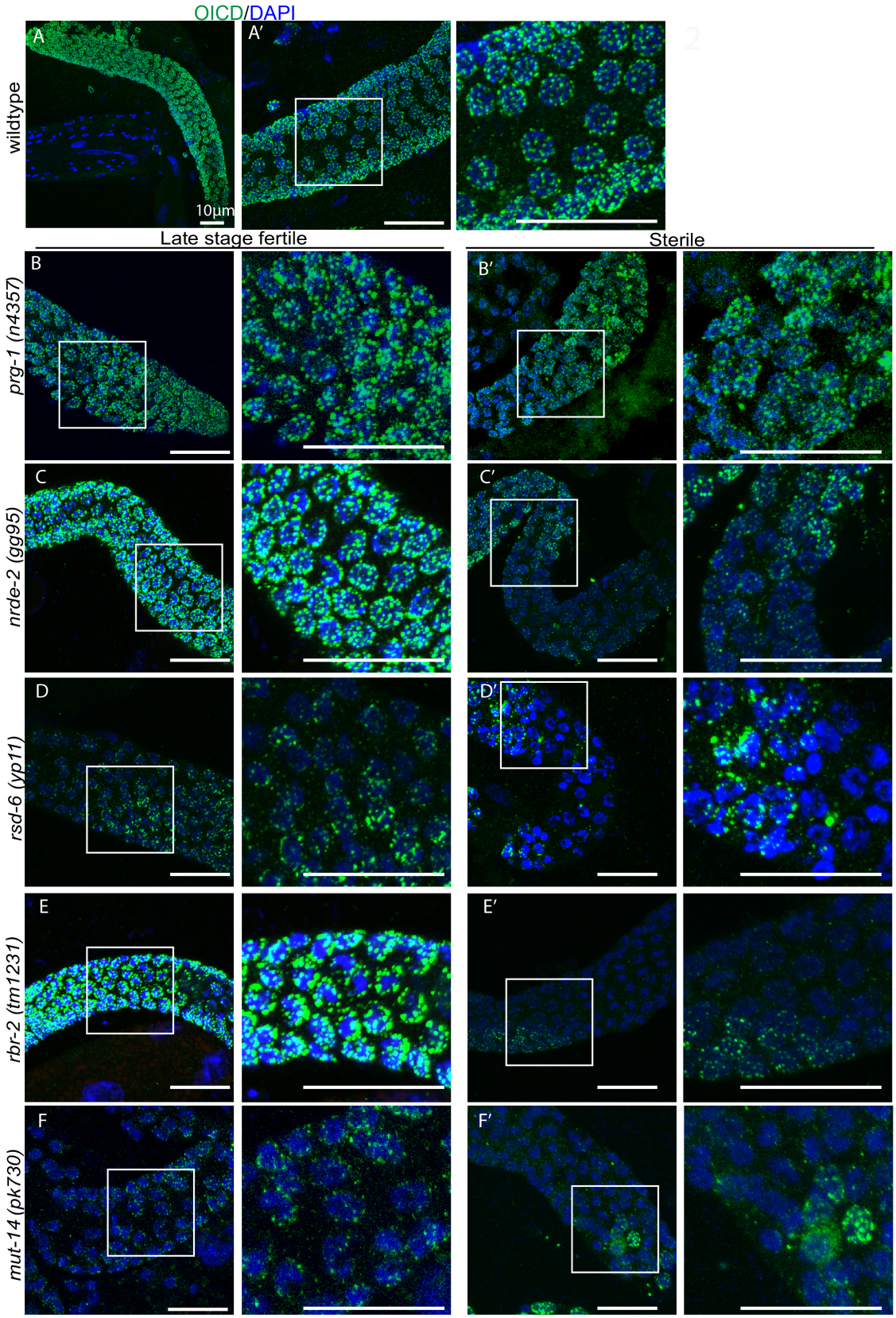


Figure 1: DNA Damage Response is variable in Piwi pathway genome silencing mutants prior to and at sterility. (A) Staining of pS/TQ in wildtype, *prg-1(n4357) and prg-1(tm876)* mutants. pS/TQ(grey and yellow) and DAPI(blue) **(B)** Quantification of pS/TQ staining in 20°C wildtype and *prg-1* mutants 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 = n. s., P < 0.05 = *, P = <0.01 = **, P =< 0.001 = ***

784 compared to wildtype)


OICD/DAPI
A
A′
wildtype
10µm
Late stage fertile
Sterile
B
B′
prg-1 (n4357)
C
C′
nrde-2 (gg95)
D
D′
rsd-6 (yp11)
E
E′
rbr-2 (tm1231)
F
F′
mut-14 (pk730)

785

786 **Figure 2: At sterility, partial loss of P granule staining occurred in *prg-1* and**
787 **temperature-sensitive mutants.** Germlines of sterile Day 2-3 adult animals were
788 stained using the OIC1D4 antibody against P granules (green) and DAPI (blue). **(A-A')**
789 Control animals contain uniform puncta of P granule staining surrounding each nuclei.
790 **(B-F)** Late generation fertile *prg-1, rsd-6*, *nrde-2*, *rbr-2*, and *mut-14* animals displayed
791 relatively normal P granule staining in the germlines. **(B'-F')** *prg-1*, *nrde-2*, *rsd-6, rbr-2,*
792 and *mut-14* sterile animals all exhibited some degree of germ cell P granule loss.
793

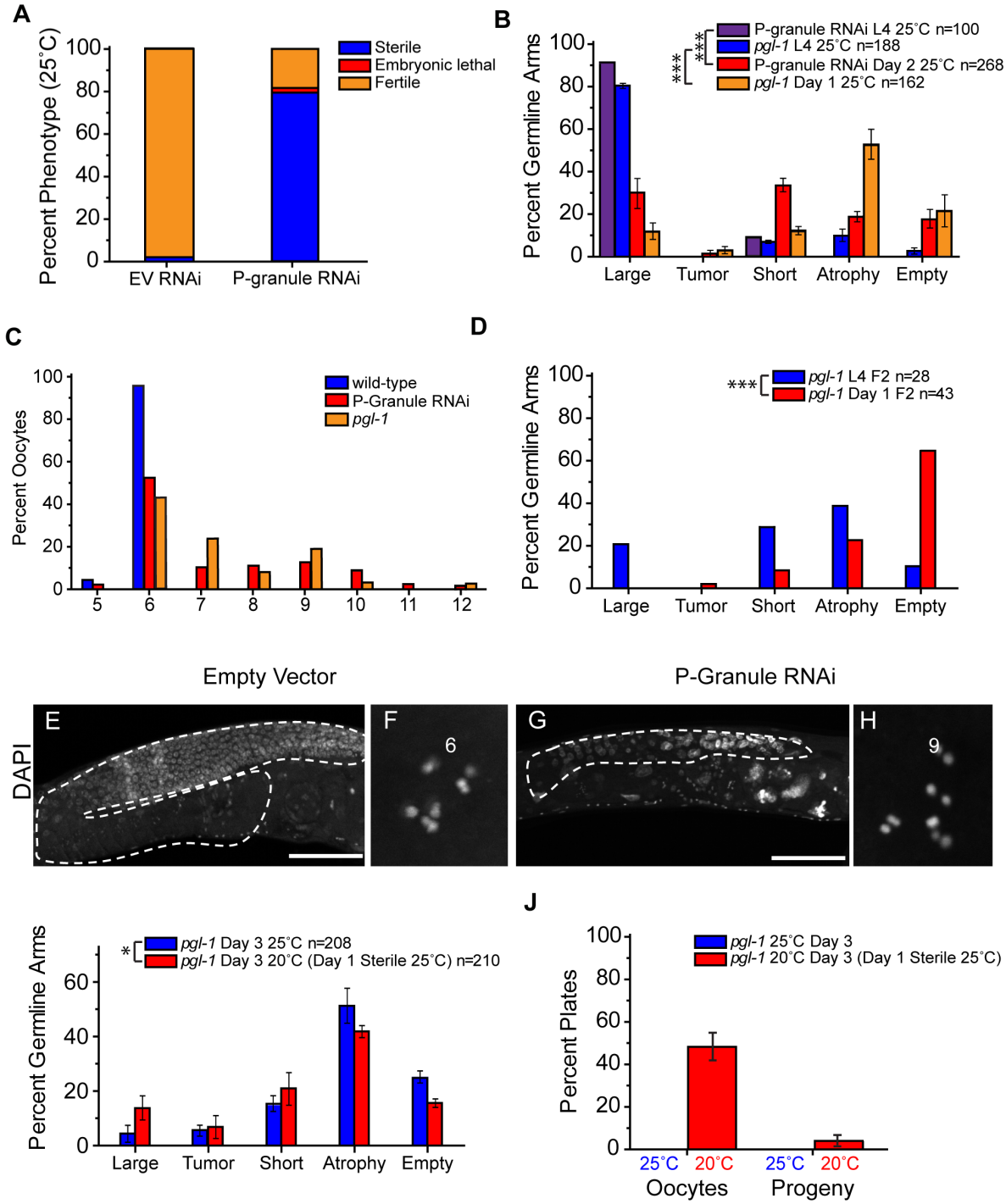
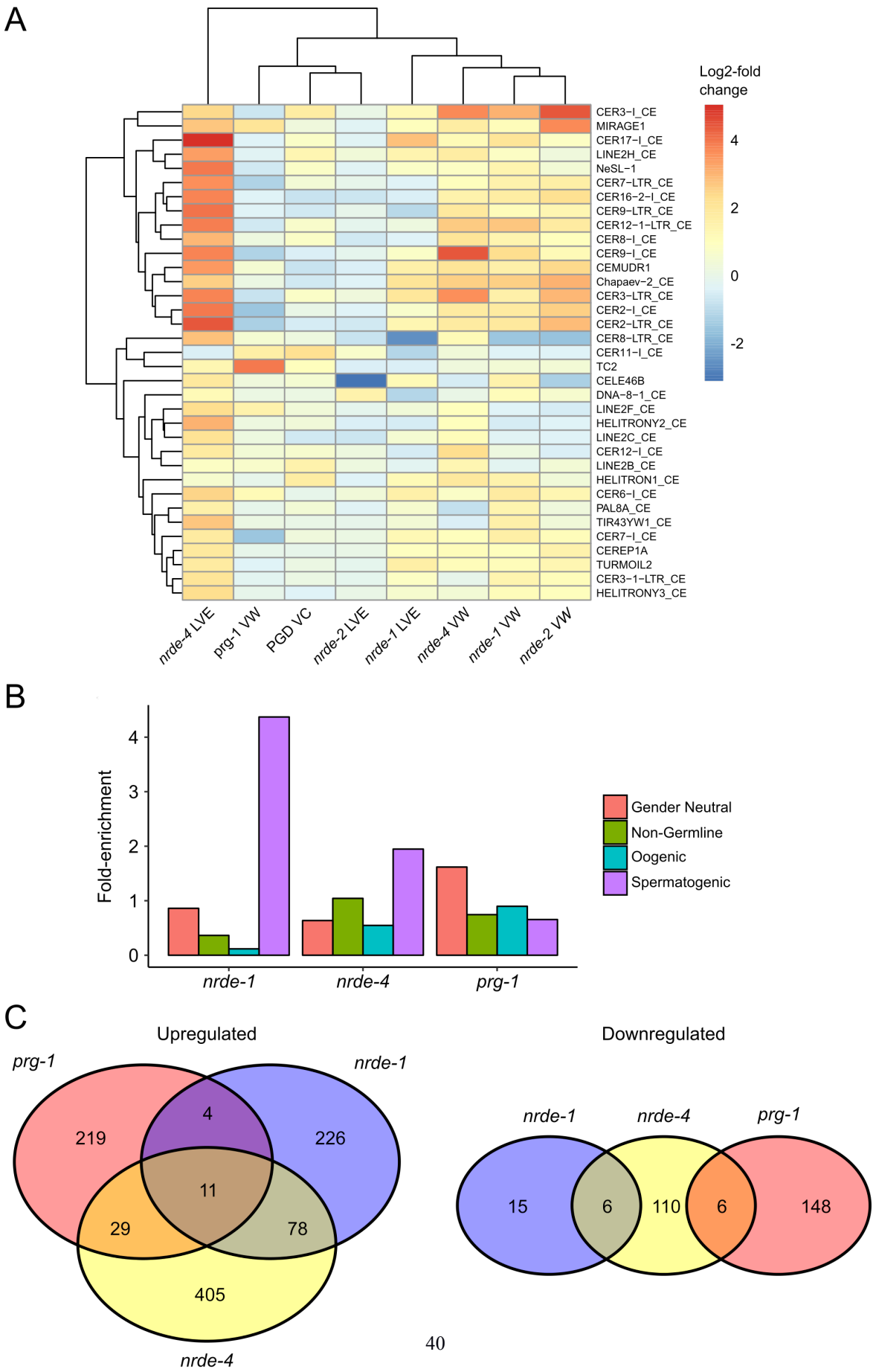
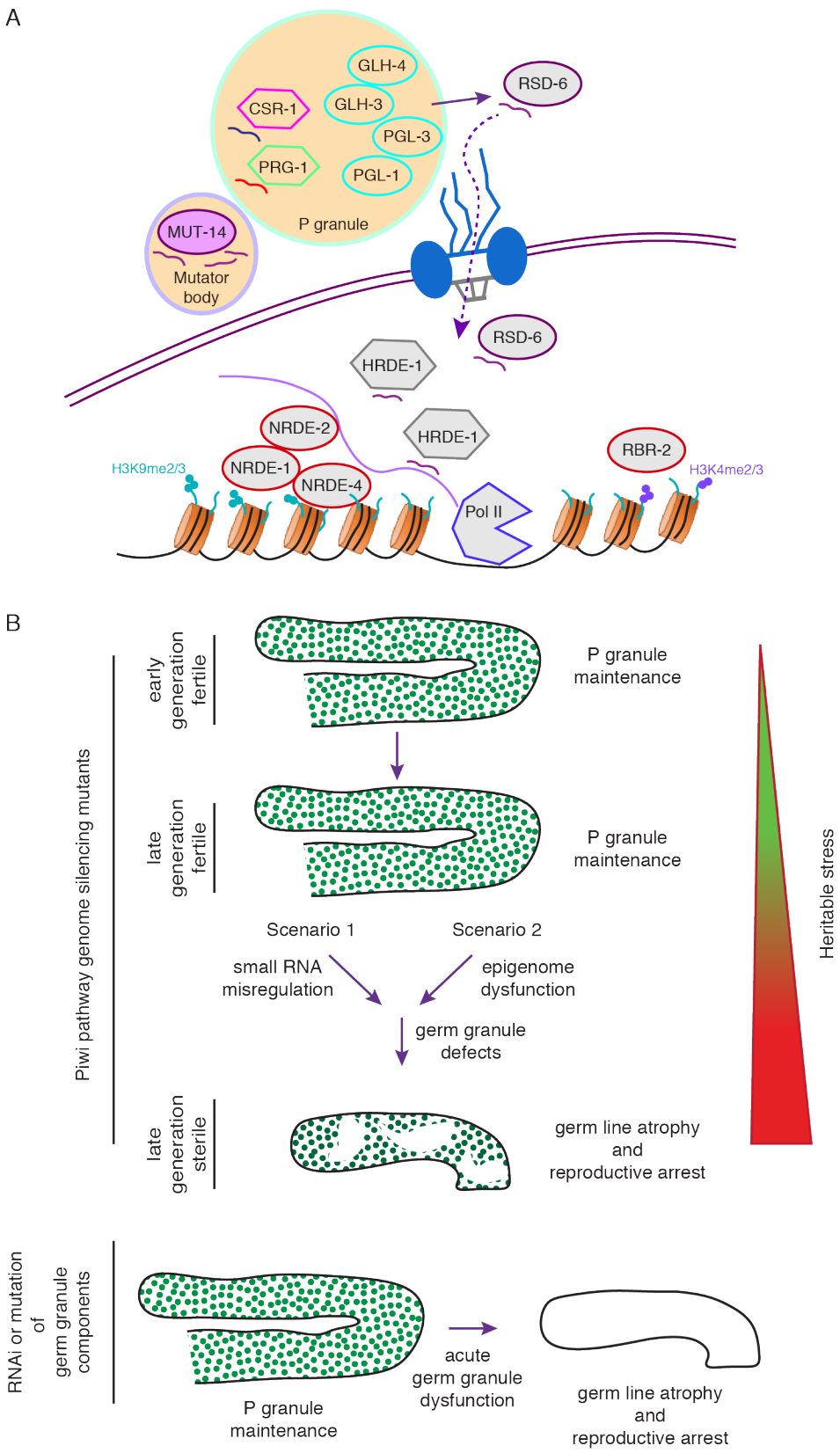


Figure 3: Disruption of P granules by RNAi or loss of *pgl-1* resulted in similar phenotypes. (A) G2 wild-type (N2) worms on Empty Vector (EV) or P granule RNAi at 25°C were scored for sterility, embryonic lethality, or fertility. n=16-30 worms per

798 condition, 2 independent experiments. **(B)** G1 L4 *pgl-1* mutants and G2 L4 P granule
799 RNAi treated animals stained by DAPI contained mostly large germlines while adults
800 exhibited a range of germline phenotypes from empty to large. **(C)** G1 *pgl-1* mutants
801 and G2 P granule RNAi treated animals contained oocytes with increased numbers of
802 univalents at sterility as measured by DAPI bodies n=151 wild-type, 81 P granule RNAi,
803 37 *pgl-1*. **(D)** Stained DAPI germlines of sterile G2 L4 and Day 1 *pgl-1* mutants. **(E-H)**
804 Representative DAPI images of germline and oocyte defects in empty vector treated
805 animals **(E,F)** and P granule RNAi treated animals **(G,H)** scale bar=10μm. **(I)** *pgl-1* G1
806 worms at 25°C were either DAPI stained as Day 3 adults (blue) or shifted to 20°C at
807 Day 1 and DAPI stained at Day 3 (red). **(I)** No sterile day 1 *pgl-1* G1 at 25°C laid
808 oocytes or progeny (n=35 plates, 350 worms). Sterile day 1 *pgl-1* shifted to 20°C (n=50
809 plates, 500 worms) gave rise to plates with oocytes and rarely progeny. Plates with
810 oocytes contained both oocytes and dead embryos. Plates with progeny contained
811 embryos that hatched to L1. For B,D,I *** $p<0.0001$, * $p=0.007$ Fisher's Chi Square test
812 with Bonferroni correction, n=total germlines scored, error bars are S.E.M.
813


A
Log2-fold change
4
2
0
-2
CER3−I_CE
MIRAGE1
CER17−I_CE
LINE2H_CE
NeSL−1
CER7−LTR_CE
CER16−2−I_CE
CER9−LTR_CE
CER12−1−LTR_CE
CER8−I_CE
CER9−I_CE
CEMUDR1
Chapaev−2_CE
CER3−LTR_CE
CER2−I_CE
CER2−LTR_CE
CER8−LTR_CE
CER11−I_CE
TC2
CELE46B
DNA−8−1_CE
LINE2F_CE
HELITRONY2_CE
LINE2C_CE
CER12−I_CE
LINE2B_CE
HELITRON1_CE
CER6−I_CE
PAL8A_CE
TIR43YW1_CE
CER7−I_CE
CEREP1A
TURMOIL2
CER3−1−LTR_CE
HELITRONY3_CE
nrde-4 LVE
prg-1 VW
PGD VC
nrde-2 LVE
nrde-1 LVE
nrde-4 VW
nrde-1 VW
nrde-2 VW
B
Fold-enrichment
0
1
2
3
4
nrde-1
nrde-4
prg-1
Gender Neutral
Non-Germline
Oogenic
Spermatogenic
C
Upregulated
prg-1
nrde-1
nrde-4
219
4
226
11
29
78
405
Downregulated
nrde-1
nrde-4
prg-1
15
6
110
6
148

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


A
GLH-4
CSR-1
GLH-3
PGL-3
PRG-1
PGL-1
P granule
RSD-6
MUT-14
Mutator body
RSD-6
HRDE-1
HRDE-1
NRDE-2
NRDE-1
NRDE-4
H3K9me2/3
RBR-2
H3K4me2/3
Pol II
B
Piwi pathway genome silencing mutants
early generation fertile
P granule maintenance
late generation fertile
P granule maintenance
Scenario 1
Scenario 2
small RNA misregulation
epigenome dysfunction
germ granule defects
late generation sterile
germ line atrophy and reproductive arrest
Heritable stress
RNAi or mutation of germ granule components
P granule maintenance
acute germ granule dysfunction
germ line atrophy and reproductive arrest

822

823 **Figure 5: Model for P granule dysfunction in Piwi pathway genome silencing**
824 **mutants.** (**A**) Model of P granules and associated small RNA-mediated genome
825 silencing proteins. Green circles contain P granule components. Mutants in this paper
826 that are temperature-sensitive for germ cell immortality have blue outlines. Non-
827 conditional mutants have red outlines. **(B)** Model for transgenerational sterility in
828 response to accumulation of Heritable Stress in Piwi pathway genome silencing
829 mutants, which ultimately compromises P granule integrity (top), and model for
830 induction of an analogous form of reproductive arrest by acute dysfunction of P granule
831 components (bottom).

832

833

834 **Table 1**

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

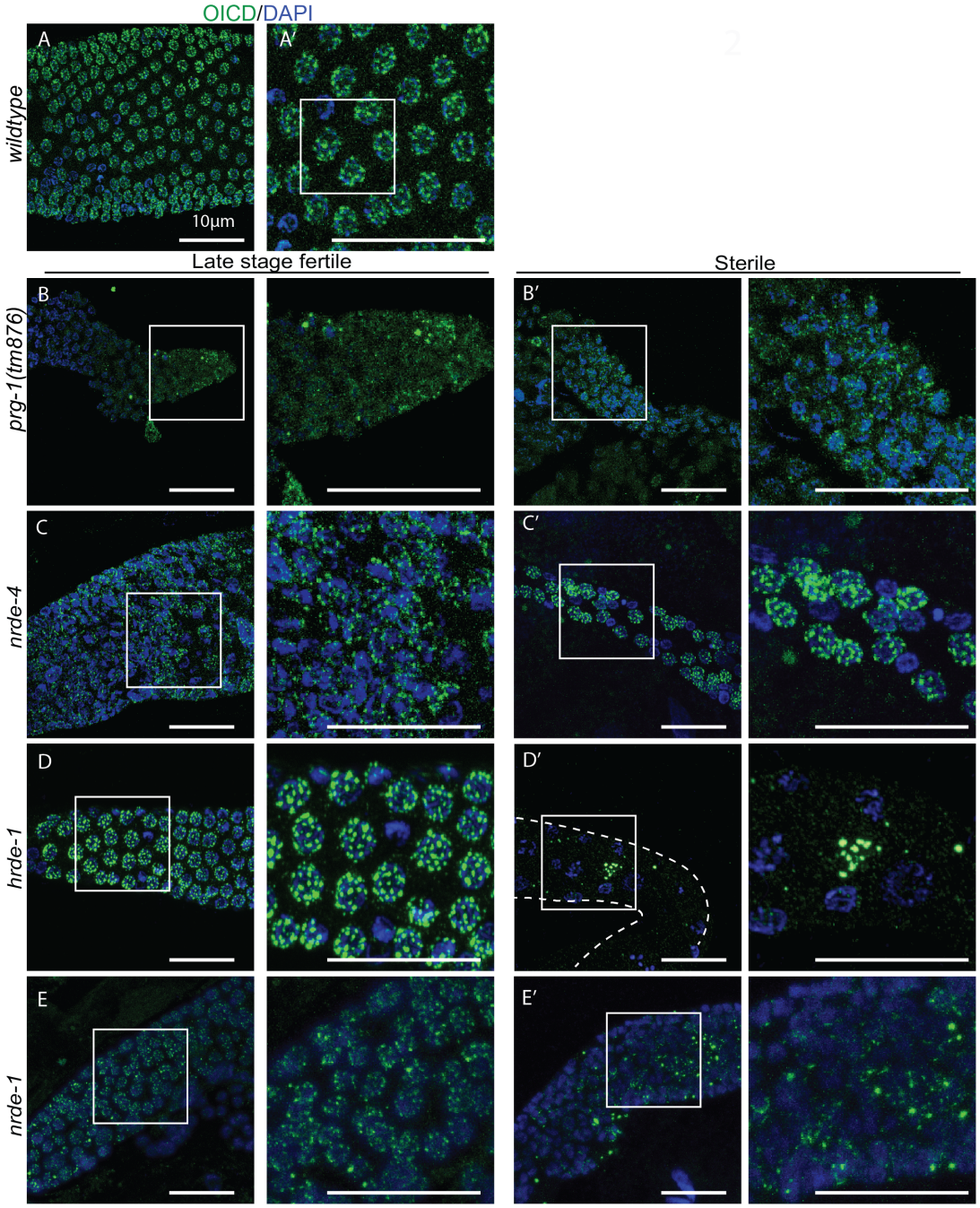
835

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

837 Sterile Piwi pathway genome silencing mutants were stained with P granule antibody.
838 Germlines arms were semi-qualitatively scored for P granules. If at least 20% of the arm
839 was missing P granules if was scored as loss and if 80% or more of the arm contained
840 P granules on DAPI-stained nuclei it was considered normal. Statistics were performed
841 using Fishers exact test.
842

843
844

845 ## Supplemental Information



846

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

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

853

854

855

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

856

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

860

861

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

862

863 **Table S2: Related to Figure 4. Genes upregulated in late-generation *prg-1* mutants**
864 **and in sterile *nrde-1* and *nrde-4* mutant L4 larvae.**
865