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1 # Natural cryptic epigenetic variation in an embryonic gene
2 regulatory network

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13

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19 **Abstract**

20 Gene regulatory networks (GRNs) that direct animal embryogenesis must respond to varying
21 environmental and physiological conditions to ensure robust construction of organ systems. While GRNs
22 are evolutionarily modified by natural genomic variation, the roles of epigenetic processes in shaping
23 plasticity of GRN architecture are not well-understood. The endoderm GRN in *C. elegans* is initiated by the
24 maternally supplied SKN-1/Nrf2 bZIP transcription factor; however, the requirement for SKN-1 in endoderm
25 specification varies widely among distinct *C. elegans* wild isolates owing to rapid developmental system
26 drift driven by accumulation of cryptic genetic variants. We report here that heritable epigenetic factors that
27 are stimulated by transient developmental diapause also underlie cryptic variation in the requirement for
28 SKN-1 in endoderm development. This epigenetic memory is inherited from the maternal germline,
29 apparently through a nuclear, rather than cytoplasmic, signal, resulting in a parent-of-origin effect (POE),
30 in which the phenotype of the progeny resembles that of the maternal founder. The occurrence and
31 persistence of POE varies between different parental pairs, perduring for at least ten generations in one
32 pair. This long-perduring POE requires piwi-piRNA function and the germline nuclear RNAi pathway, as
33 well as MET-2 and SET-32, which direct histone H3K9 trimethylation and drive heritable epigenetic
34 modification. Such non-genetic cryptic variation between wild isolates may provide a resource of additional
35 phenotypic diversity through which adaptation may facilitate evolutionary change and shape developmental
36 regulatory systems.

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37 **Introduction**

38 The "Modern Synthesis" of the early 20th Century articulated how biological traits shaped by
39 Darwinian forces result from random mutations following the rules of Mendelian inheritance (1). Since that
40 formulation, it has become clear that non-genetic heritable mechanisms can underlie substantial differences
41 in traits between individuals (reviewed in ref. 2). Extensive epigenetic reprogramming occurs in germline
42 and in gamete pronuclei after fertilization to maintain the totipotent state of the zygote. In mammals,
43 disruption of this process often leads to lethal consequences (reviewed in refs. 3, 4). In *C. elegans*, aberrant
44 reprogramming of epigenetic memory can result in transgenerational accumulation of inappropriate
45 epigenetic marks and a progressive sterile mortal germline (Mrt) phenotype (5, 6). In many cases, the Mrt
46 phenotype is exacerbated by heat stress, demonstrating that environmental factors may influence
47 epigenetic reprogramming in the germline, and that these epigenetic modifications may be passed to
48 subsequent generations (7, 8). Interestingly, *C. elegans* wild isolates, each carrying a unique haplotype,
49 exhibit variation in the temperature-induced Mrt phenotype, suggesting differential stress response and
50 distinct epigenetic landscapes in natural populations of the species (9).

51 Many of the documented instances of epigenetic inheritance in mammals are parental or
52 *intergenerational* effects (less than three generations for female transmission and two generations for male
53 transmission), which can be attributed to direct exposure of the developing embryos *in utero* to the triggers
54 that alter epigenetic states (2). Parental traumatic experience can trigger heritable behavioral changes and
55 nutritional status of the parents can cause metabolic remodeling in the offspring, which often lasts for one
56 or two generations (10–13). Epidemiological analyses on different human cohorts demonstrate that paternal
57 grandfather's food access during pre-puberty period affects the mortality of the grandsons (14–16),
58 revealing the potential for long-term *transgenerational* epigenetic inheritance (TEI) that is induced by
59 environmental conditions.

60 Studies over the past decade on *C. elegans* have provided convincing evidence for TEI that persists
61 for at least three generations. Small RNAs are prime candidates for mediators of epigenetic memory, as
62 their expression undergoes only minimal reprogramming in the germline and embryos (17, 18). Primary
63 siRNAs, processed from exogenous dsRNAs or endogenous small RNAs by DICER, are loaded onto the

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64 RNA-induced silencing complex (RISC) and mediate degradation of mRNA targets, thereby silencing gene
65 expression. In addition, primary siRNAs, including PIWI-interacting RNA (piRNA – 21U) in the germline,
66 can guide RNA-dependent RNA polymerases (RdRPs) to particular target mRNAs and then amplify
67 silencing signals by producing an abundance of secondary 22G siRNAs. In the germline, HRDE-1 binds to
68 these secondary siRNAs and localizes to the nucleus, where it recruits NRDE-1/2/4 to the nascent
69 transcripts and genomic sites targeted by the small RNAs. This complex then inhibits RNA polymerase II
70 elongation and directs deposition of repressive H3K9me3 marks on the corresponding genomic loci,
71 mediated by histone methyltransferases MET-2 (H3K9me1/2), SET-23 (H3K9me1/2/3) and SET-32
72 (H3K9me3). Amplification of secondary siRNAs by RdRPs prevents loss of epigenetic memory over multiple
73 generations and, therefore, may permit long-term heritable epigenetic responses (reviewed in refs. 19, 20).
74 We have uncovered natural epigenetic variation in the gene regulatory network (GRN) that directs
75 development of the embryonic endoderm in *C. elegans*. The maternal SKN-1/Nrf2 transcription factor
76 activates the mesendoderm GRN in the EMS blastomere at the four-cell stage. EMS subsequently divides
77 to produce the E founder cell, which gives rise exclusively to the intestine, and its anterior sister, the MS
78 founder cell, which produces much of the mesoderm. A triply redundant (Wnt, MAP kinase, and Src)
79 extracellular signal sent from the neighboring P_2 blastomere is received by EMS and acts in parallel with
80 SKN-1 to activate endoderm development in the E lineage. In the laboratory N2 strain, elimination of
81 maternal SKN-1 function causes fully penetrant embryonic lethality and a partially penetrant loss of gut:
82 while the majority of the E cells in embryos lacking SKN-1 adopt the mesoectodermal fate of the normal C
83 founder cell, ~30% undergo normal gut differentiation as a result of this parallel signaling input (SI Appendix,
84 Fig. S1) (reviewed in refs. 21–23).
85 We recently found that the requirement for SKN-1 shows widespread natural variation across
86 genetically distinct *C. elegans* wild isolates. While removal of SKN-1 in some of the isotypes causes loss
87 of intestine in virtually 100% of embryos, in other isotypes a majority of the embryos differentiate endoderm.

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88 Thus, the architecture of the early stages in the endoderm GRN appears to have undergone rapid change
89 during *C. elegans* evolution (24).
90 We report here that, although much of the variation in SKN-1 requirement results from genetic
91 differences between the wild isolates (24), it is also determined in part by cryptic, stably heritable epigenetic
92 variation. This effect is uncovered from reciprocal crosses between wild isotypes with quantitatively different
93 phenotypes. This parent-of-origin effect (POE) is transmitted exclusively through the maternal germline.
94 When mothers experience dauer diapause, an alternative developmental stage in *C. elegans* that confers
95 resistance to environmental insults and longevity, the POE appears to be transmitted through the maternal
96 nucleus, rather than cytoplasmic factors, and can persist through many generations. We further show that
97 this stress-induced POE requires factors that direct H3K9 methylation and the nuclear RNAi machinery.
98 These findings reveal that heritable epigenetic states underlie differences between natural wild isolates and
99 can influence developmental plasticity in early embryos. Such cryptic epigenetic variation provides a
100 potential resource upon which natural selection might act, thus contributing to evolution of GRN architecture
101 (25).
102

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103 **Results**

104 **Transgenerational parent-of-origin effect alters the SKN-1 dependence of endoderm formation**

105 The requirement for SKN-1 in endoderm specification varies dramatically across *C. elegans*
106 isotypes (24). Depending on the isotype tested, between 0.9% and 60% of arrested *skn-1(RNAi)* embryos
107 undergo gut differentiation when maternal SKN-1 function is eliminated. The behavior of each isotype is
108 quantitatively highly reproducible, showing low variability through many generations, when analyzed by
109 different laboratories and researchers, and from independent lines established from different founder
110 animals (24).

111 While performing crosses between isotypes with quantitatively different SKN-1 requirements, we
112 found that the outcomes differed depending on the sex of the parent in reciprocal crosses (SI Appendix,
113 Fig. S2). We initially tested two isotypes in which we observed dramatically different *skn-1(RNAi)*
114 phenotypes: the laboratory N2 strain (29 ±0.4% sd with gut; n = 1320) and the wild isolate JU1491 (1.2 ±
115 0.4% sd; n = 1228) (SI Appendix, Fig. S3A, $p < 0.001$). These results agree well with our previous findings
116 (24). Consistent with variation at the level of maternal components, we found that in reciprocal crosses (i.e.,
117 male N2 x JU1491 hermaphrodite, and *vice-versa*), the quantitative requirement for SKN-1 reliably followed
118 that of the maternal line (Fig. 1A). Unexpectedly, however, we found that this non-reciprocality persisted in
119 subsequent generations: the average phenotype of F2 and F3 embryos continued to follow more closely
120 the behavior of their grandmothers and great-grandmothers than their paternal ancestors (Fig. 1B), despite
121 the fact that, with the exception of the mitochondrial genome, the F1 progeny genotypes should be identical
122 regardless of the sex of the founder P0. Thus, these two strains showed a strong parent-of-origin effect
123 (POE) that persists through multiple generations.

124

125 **Dauer diapause stimulates long-term transgenerational POE through the maternal line**

126 As epigenetic inheritance can be environmentally triggered, it was conceivable that the POE we
127 observed might be influenced by the experience of the parents. Indeed, we found that POE was seen only
128 when the P0 parents had been starved and experienced an extended period (~2 weeks) of dauer diapause
129 with the N2 x JU1491 crosses. In contrast, the progeny of P0's that were continuously well fed showed an

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130 intermediate average phenotype that was not significantly different between descendants of reciprocal
131 crosses (Fig. 1B), consistent with the known multigenic characteristic of the phenotype (24).
132 We sought to determine whether this environmentally triggered POE extends to other wild isolates.
133 Isolates MY16 (in which only 2.2 ±1% sd (n = 1169) of *skn-1(-)* embryos make endoderm) and JU1172 (40
134 ± 3% sd; n = 1491) (SI Appendix, Fig. S3B, $p < 0.001$) show widely different quantitative phenotypes (24).
135 Consistent with our previous findings, in reciprocal crosses of MY16 and JU1172, we observed a strong
136 maternal effect in the requirement for SKN-1: F1 embryos from mated *skn-1(RNAi)* mothers followed the
137 maternal phenotype (Fig. 1C). In control experiments with well-fed founder P0 worms, this maternal effect
138 quickly dissipated and was not detectable in F2 embryos (Fig. 1C and D). However, when the parental
139 worms experienced dauer diapause, the average *skn-1(RNAi)* phenotype of their descendants reliably
140 followed that characteristic of the maternal line through at least ten generations (Fig. 1C and D), a strongly
141 perduring effect.
142 While dauer development enhances POE, we found that it is not an absolute requirement in all
143 cases. Specifically crosses of JU1491 and JU1172 revealed weak POE even without the diapause trigger,
144 although the effect was stimulated by dauer development (Fig. 2C). This observation suggests that cryptic
145 epigenetic differences between some natural isolates may exist even in the absence of an environmental
146 or physiological trigger. Finally, we found that this effect does not appear to be general to all isotype pairs
147 that show very different phenotypes: for example, diapause-induced POE was not detectable with N2 and
148 MY16 (SI Appendix, Fig. S4).
149 As expected for successful crosses, in all cases ~50% of the F1 offspring were males (SI Appendix,
150 Fig. S5A and D; one-sample t-test, $p > 0.05$). Further, cultures established from at least eight randomly
151 selected F1s of successful crosses (with ~50% F1 males) all showed POE (SI Appendix, Fig. S5C), thus
152 ruling out the possibility that the maternal-line bias of the phenotype might result from frequent selfing.
153 To distinguish between the paternal and maternal contributions to the POE, we starved either the
154 P0 male or hermaphrodite and traced the POE for five generations following reciprocal crosses. These
155 experiments demonstrated that the diapause-induced POE is inherited exclusively through the maternal
156 germline (Fig. 1E; SI Appendix, Fig. S5B). This stable non-reciprocality cannot be explained by long-

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157 perduring maternal factors in the cytoplasm: each animal produces ~250 progeny and after five
158 generations, this would result in a dilution factor of ~10^{11}.
159

160 **Heritability of POE is associated with the maternal nucleus, not heritable mitochondrial or**
161 **cytoplasmic factors**

162 Dauer larvae and post-dauer adults exhibit a metabolic shift which may reflect changes in
163 mitochondrial function (26–29). Further, starvation has been shown to impact mitochondrial structure and
164 function (30). Thus, the observed maternally directed POE results might arise from differences between the
165 mitochondrial genome sequences in the two strains (31) or might be driven by other cytoplasmically
166 inherited factors. Indeed, wild isolates MY16 and JU1172 contain 13 single nucleotide polymorphisms
167 (SNPs) in mitochondrial protein coding genes (SI Appendix, Table S1), which could alter energy metabolism
168 and stress responses (32, 33). To test whether the POE we observed is attributable to maternal inheritance
169 of mitochondria with particular genomic characteristics, we performed reciprocal crosses in which progeny
170 were repeatedly backcrossed to the paternal strain to obtain lines with primarily the MY16 nuclear genome
171 and mitochondria from the JU1172 line and *vice-versa* (Fig. 2A). While a strong POE was initially observed
172 in F2 *skn-1(RNAi)* embryos, this effect was rapidly eliminated as more paternal nuclear DNA was
173 introduced. By the F5 generation, the phenotype was indistinguishable from that of the respective paternal
174 strain (Fig. 2B), suggesting that POE is attributable to the nuclear, rather than mitochondrial, genome.
175 Moreover, the transgenerational POE observed with JU1491 and JU1172 (Fig. 2C) cannot result from
176 variation in mitochondrial DNA, as these two strains carry identical mitotypes. Collectively, our results
177 suggest that the POE we observe is not likely to be caused by mitochondrial inheritance.

178 To further assess whether nuclear or cytoplasmic/mitochondrial factors underlie the observed POE,
179 we took advantage of a genetic system that generates germlines containing a nucleus derived fully from
180 the paternal line and maternally derived cytoplasm (including mitochondria). In zygotes overexpressing
181 GPR-1 ($N2^{GPR\text{-}1\ OE}$) (34, 35), which is required to modulate microtubule-based pulling forces (36), excessive
182 pulling forces cause the maternal and paternal pronuclei to be drawn to opposite poles before nuclear
183 envelope breakdown. This effect generates mosaic embryos in which the anterior daughter (AB) inherits

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184 only the maternal chromosomes, while the posterior (P_1) receives only the paternal chromosomes.These
185 non-Mendelian events can be scored with the appropriate fluorescent markers (Fig. 2D; SI Appendix, Fig.
186 S6) (35). We found that 72% (n = 230) of the viable F1 progeny from crosses of N2$^{GPR\text{-}1\ OE}$ hermaphrodites
187 with JU1491 males contained an exclusively paternally derived P_1 lineage. If cytoplasmic maternal factors
188 were responsible for the observed diapause-induced POE, the effect would be expected to follow the
189 cytoplasm of the founder P0 worms in the F1 hybrids. In contrast, however, we found that the *skn-1(RNAi)*
190 phenotypes of F2 and F3 descendants of F1 mosaic animals (those with an N2-derived AB and JU1491-
191 derived P_1; SI Appendix, Fig. S6; see Materials and Methods) were indistinguishable from that of the
192 JU1491 strain, regardless of the feeding status of the parents (Fig. 2D'). This finding suggests that the
193 diapause-induced POE is associated with heritable changes in the nucleus, not heritable cytoplasmic
194 maternal factors, including the mitochondrial genome.
195
196 **POE is not the result of competition in fitness or maternal incompatibility**
197 Parental age has been shown to affect progeny phenotypes in *C. elegans* and other organisms
198 (37–40). To test the possibility that the POE is influenced by differences in maternal age, we synchronized
199 day-one adults (Fig. 1C) and day-two adults (Fig. 1B, D and E; Fig. 2C). We detected a strong POE in all
200 cases. Moreover, despite large variation in the *skn-1(RNAi)* phenotype that arises from genetic variation,
201 we observed POE in F5 cultures that were established from very late broods (the last few progeny)
202 produced by senescent F1 animals (Fig. 3A). These findings indicate that parental age does not contribute
203 substantially to the POE observed.
204 The differences in SKN-1 requirement seen as the result of the POE might reflect maternal
205 incompatibility, which favors particular genetic regions as a result of lethality or slow growth (41, 42). If such
206 regions included those known to influence the requirement for SKN-1 in the endoderm GRN (24), there
207 could be selection for the trait following recombination of the two parental genomes. We note that such a
208 possibility would also require that any such selection is triggered only after starvation and dauer
209 development for the cases in which we observed such an essential requirement. Further, we observed
210 strong diapause-induced POE in embryos from *skn-1* RNAi-treated F1 heterozygous mothers, whose

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211 genotypes would be identical in the two reciprocal crosses. Thus, the effect at this stage is not attributable
212 to maternal incompatibility resulting in selection against particular allelic combinations that arise by
213 recombination (Fig. 1B-D).
214 To further investigate whether POE might be driven by genetic incompatibility that is
215 environmentally triggered by starvation/dauer development, we also characterized lethality and fecundity
216 of F2 progeny from the reciprocal crosses. Two mechanisms involving selfish genetic elements that result
217 in maternal incompatibilities were previously described in *C. elegans*: the *peel-1/zeel-1* (41) and *sup-*
218 *35/pha-1* (42) toxin/antidote systems. The wild isolate JU1172 does not carry the paternal selfish *peel-*
219 *1/zeel-1* element (41). When mated, MY16 sperm deliver PEEL-1 toxin, causing F2 embryos that are
220 homozygous for the JU1172 *zeel-1* haplotype (~25%) to arrest. We found that, indeed, crosses between
221 JU1172 and MY16 are associated with embryonic lethality. However, although this lethality was slightly
222 lower (~13-16%) when JU1172 was the paternal strain compared to the reciprocal crosses (20-26%; Fig.
223 3B), the difference is insufficient to explain the strong POE we have observed. Furthermore, this effect does
224 not change appreciably regardless of the experience of the P0 (fed or starved/dauer), which is not
225 consistent with selection induced by this experience. As both MY16 and JU1172 harbor the active *sup-*
226 *35/pha-1* maternal toxin/antidote element (42), the lethality may reflect an unidentified maternal-effect toxin
227 in the MY16 strain. In addition, the progeny of crosses between MY16 and JU1172 in either direction both
228 showed somewhat reduced fecundity/viability, presumably owing to genomic incompatibility between the
229 two strains (Fig. 3C). However, the parental origin of the P0s did not influence the degree of larval lethality
230 or sterility in the F2 animals (Fig. 3C, Fisher-exact test $p > 0.05$). Together these results indicate that genetic
231 incompatibility alone cannot account for the strong POE we observed. Rather, POE appears to result from
232 perduring epigenetic inheritance reflecting the experience of the original founding parents of the cross.
233

234 **Maintenance of POE involves the nuclear RNAi pathway and histone H3K9 trimethylation**

235 The findings noted above suggest that POE is mediated through nuclear signals. The nuclear RNAi
236 pathway has been implicated in a number of examples of TEI (7). To assess whether this pathway might
237 be involved in transmitting the POE we have observed, we analyzed the F4 progeny of reciprocal crosses

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238 of post-dauer P0's in which *nrde-4* was knocked down by RNAi (strategy shown in Fig. 4A). While a strong
239 POE was observed in the control animals containing functional NRDE-4, *nrde-4(RNAi)* abrogated the POE
240 in the F5 embryos (Fig. 4B): i.e., the requirement for SKN-1 was not significantly different in the descendants
241 of reciprocal crosses. It was conceivable that this effect might simply reflect a direct role for NRDE-4 in the
242 requirement for SKN-1 *per se*. However, we found that the *skn-1(RNAi)* phenotypes of the MY16 and
243 JU1172 isolates treated with *nrde-4* RNAi were indistinguishable from those treated with control RNAi
244 (Fisher-Exact test $p > 0.05$; Fig. 4C). Thus, these findings implicate NRDE-4, and hence the nuclear RNAi
245 pathway, in the POE process.

246 Gene silencing though the nuclear RNAi pathway that results in TEI is mediated through the Piwi-
247 encoding homologue, *prg-1*, and piRNAs that trigger biosynthesis of secondary 22G RNAs; (a second
248 homologue, *prg-2*, is likely to be a pseudogene) (43–46). We knocked down PRG-1/2 in F4 animals from
249 MY16 and JU1172 reciprocal crosses and found that, in contrast to their siblings treated with control RNAi,
250 POE was abrogated in the F5 embryos (Fig. 4B). Control experiments demonstrated that *prg-1/2* RNAi
251 does not affect *skn-1* RNAi efficacy in either parent line ($p > 0.05$; Fig. 4C). Loss of nuclear RNAi factors
252 lowers the efficacy of RNAi targeting of nuclear-localized RNAs (47–49); however, maternal *skn-1* mRNA
253 in the early embryos is localized in the cytoplasm, and the silencing effect of *skn-1* RNAi would be expected
254 to depend primarily on RISC in the cytoplasm (50).

255 NRDE-4 is required for the recruitment of NRDE-1 to the targeted loci and subsequent deposition
256 of the repressive H3K9me3 mark, which results in gene silencing (49). Furthermore, H3K9me3 has been
257 implicated in transgenerational silencing of transgenes or endogenous loci mediated by exogenous RNAi
258 (43, 51–53). These observations, and our findings that knockdown of *nrde-4* abolishes POE, led us to
259 hypothesize that H3K9 methylation might function as a mediator of POE. Indeed, we found that treating F4
260 animals that showed POE with RNAi against *met-2* or *set-3*2, in contrast to their control siblings, eliminated
261 POE in the F5 embryos (Fig. 4B). Although loss of MET-2 has been shown to enhance RNAi sensitivity
262 (52), we found that neither *met-2* RNAi nor *set-32* RNAi significantly modifies the *skn-1(RNAi)* phenotypes
263 of MY16 and JU1172 wild isolates ($p > 0.05$; Fig. 4C). Thus, the loss of POE in the F5 generation with *met-*
264 *2* or *set-32* RNAi is not attributable to modified RNAi response. Collectively, these results suggest that, in

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265 response to dauer diapause, piRNAs in the germline direct histone methylation through the nuclear RNAi
266 pathway, thereby maintaining POE across generations (Fig. 4D).

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267 **Discussion**

268 While massive epigenetic reprogramming ensures totipotency of the germline during animal
269 development, some epigenetic marks escape erasure, leading to stable epigenetic inheritance that can
270 persist through many generations. Such long-term epigenetic inheritance has the potential to provide a
271 source of cryptic variation upon which evolutionary processes might act; however, little is known about
272 natural epigenetic variation within a species, how it is influenced by environmental conditions, and the
273 degree to which it influences GRN plasticity. In this study, we report four major findings that reveal cryptic
274 natural epigenetic variation and it mechanistic action in a core embryonic GRN in *C. elegans*: 1) dauer
275 diapause can trigger POE that alters the output of the endoderm GRN. 2) This effect is transmitted through
276 the maternal germline across multiple generations apparently through nuclear signals. 3) Different
277 combinations of wild isolates exhibit variation in their capacity for establishing and maintaining these
278 transgenerational epigenetic states. 4) This POE requires components of the piRNA-nuclear RNAi pathway
279 and H3K9 trimethylation. These findings indicate that maintenance of an acquired epigenetic state in
280 response to environmental stimulus can confer substantial plasticity to a core developmental program and
281 may provide additional natural variation that may be subject to evolutionary selection.

282

283 **Dauer diapause induces persistent epigenetic inheritance**

284 The perduring epigenetic effect that we have observed is triggered in parents that have experienced
285 dauer diapause. Dauer entry and formation require extensive epigenetic remodeling and some of these
286 changes are retained throughout the remainder of development: post-dauer adults contain distinct
287 chromatin architecture and particular pools of small RNA that differ from animals that have not experienced
288 dauer diapause (29, 54). In addition, the progeny of starved animals show increased starvation resistance
289 and lifespan (55–57). Consistent with the model that the effect of ancestral developmental history is carried
290 across generations, we found that dauer diapause leads to TEI that modifies the quantitative SKN-1 input
291 in endoderm development.

292 The TEI we have observed varies in its long-term perdurance, depending on the wild isolates
293 involved. In crosses between the laboratory strain N2 and wild isolate JU1491, dauer diapause-induced

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294 POE lasted for three generations, but was subsequently lost, similar to the transmission dynamics of the
295 silencing effect induced by exogenous RNAi (58, 59). This progressive transgenerational loss in the effect
296 may result from passive dilution of regulatory small RNAs and active restoration of an epigenetic "ground
297 state" over generations, although the detailed mechanisms for such a process are not well understood (59).
298 In contrast, in crosses between the MY16 and JU1172 wild isolates, we observed stable TEI that lasted for
299 at least 10 generations and which conceivably persists longer. Consistent with a recent study that identified
300 genetic determinants of efficient germline maintenance and epigenetic reprogramming among *C. elegans*
301 wild isolates (9), our results showed that the generational duration of epigenetic inheritance may also be
302 influenced by genetic background, suggesting an interplay between genetics and epigenetics.

303 The transmission of the silencing effects of exogenous RNAi in *C. elegans* has provided an
304 excellent paradigm for revealing mechanisms of epigenetic inheritance (19, 20). While inheritance of
305 exogenous RNAi and physiological responses triggered by changing environment share overlapping
306 machinery, our results reveal two key differences between the two processes: 1) we demonstrated that
307 epigenetic memory triggered by dauer diapause is transmitted exclusively through the maternal germline.
308 This contrasts with the inheritance of exogenous RNAi (59) and transgenes (60), which show paternal bias.
309 2) We found that PRG-1 is required for the *maintenance* of POE. In contrast, the piwi-piRNA pathway had
310 previously been shown to be required for the *initiation*, but not *maintenance* of transgene silencing in the
311 germline. Once established, this silencing state depends on the nuclear RNAi pathway, which promotes
312 deposition of H3K9me3 marks on the transgene (43, 44). Supporting our findings, however, Simon *et al.*
313 demonstrated that PRG-1 is important for maintaining germline mortality through a mechanism that is
314 independent from its action in transgene silencing. Animals that lack PRG-1 exhibit dysregulation of gene
315 expression and reactivation of transposons and tandem repeats, showing that piRNAs are required to
316 maintain silencing of at least some endogenous loci (61, 62).

317

318 **Relationship of POE to genomic imprinting in *C. elegans***

319 Genomic imprinting is perhaps the best-studied example of epigenetic inheritance. Differential DNA
320 methylation or histone modifications on the two parental chromosomes, established during gametogenesis

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321 or post-fertilization, escape epigenetic reprogramming, causing genes to be expressed in a parent-of-origin
322 manner (63, 64). However, in the case of *C. elegans*, animals that inherit the entire paternal genome are
323 fertile and viable, as we and others have shown (34, 35). This observation reveals that genomic imprinting
324 is not essential for normal development or survival in *C. elegans*, consistent with an early study in which
325 animals containing individual chromosomes from only one parent were analyzed (65). Nevertheless, the X
326 chromosome of sperm, unlike that of oocytes, is devoid of H3K4me2 activation marks, a pattern that persists
327 through several rounds of cell division cycles during early embryogenesis (66). In addition, the expression
328 of sperm-derived autosomal transgenes is greater than that in oocytes, which may result from differential
329 epigenetic remodeling in sperm and oocyte chromatin upon fertilization (60). While these findings
330 demonstrate the imprinting capacity of *C. elegans*, endogenous imprinted genes have not yet been
331 reported.

332 We propose that passage through dauer diapause may induce paternal-specific silencing through
333 deposition of repressive H3K9me3 on paternal loci that affect the endoderm GRN and the SKN-1
334 requirement (24), leading to the POE we observed. Although imprinting is not essential for viability in *C.*
335 *elegans*, its effects may become significant in response to environmental stimuli. For example, maternal
336 dietary restriction elevates vitellogenin oocyte provisioning (67). Both yolk-associated fatty acids and small
337 RNAs, which have been proposed to be associated with yolk particles, promote epigenetic changes in the
338 nucleus, and might thereby direct establishment of parent-of-origin epigenetic marks (68–71). Dauer-
339 favoring conditions also reduce insulin/insulin-like growth factor signaling and enhance starvation stress
340 resistance in the progeny (67). With recent advances in techniques for examining transcriptional regulatory
341 landscapes (64, 72), it will be of interest to identify loci that are responsive to environmental stimuli and that
342 may be differentially imprinted across generations.

343

344 **The potential role of cryptic epigenetic variation in accelerating evolutionary change**

345 It is clear that in *C. elegans*, stress responses can be transmitted transgenerationally and influence
346 physiology adaptively in the offspring (45, 55–57). In *Arabidopsis*, it has been shown that experimentally
347 induced, or naturally occurring epigenetic variations, once stabilized, can be subjected to artificial selection

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348 (73, 74), highlighting the potential capacity of TEI to facilitate adaptation and evolution. While TEI is
349 prominent in worms and plants with a short life cycle, and hence environmental conditions may be relatively
350 constant through multiple generations (58, 75), there is evidence that TEI may also occur in mammals with
351 much longer reproductive cycles (76). Epigenetic inheritance may be especially important in organisms with
352 low genetic diversity, such as those, including *C. elegans* and *Arabidopsis*, that propagate by self-
353 fertilization. Many *C. elegans* strains isolated from neighboring locations are near-identical and
354 polymorphism rates are low even among genetically distinct isotypes (77). In such homozygous, genetically
355 non-diverse populations, epigenetic variations may provide a particularly rich resource upon which natural
356 selection may act.

357 Environmental factors can induce plastic phenotypic changes that are subjected to Darwinian
358 selection. Over time, the phenotypic variants may become genetically fixed, a process known as "genetic
359 assimilation" (25). As the rate of genetic mutations is low in *C. elegans* (2.1×10^{-8} per nucleotide site per
360 generation)(78), heritable epigenetic variants may act as a buffer to cope with rapid environmental change
361 before adaptive mutations arise. Alterations in epigenetic states can also affect mutation rates and trait
362 evolution (79) and TEI might therefore accelerate the rate of evolution by facilitating genetic assimilation.
363 We propose that epigenetic inheritance affecting SKN-1 dependence may contribute towards the rapid
364 change in the endoderm gene regulatory network architecture that we previously observed among *C.*
365 *elegans* wild isolates (24). SKN-1 acts in pleiotropic functions, including mesendoderm specification,
366 oxidative stress and unfolded protein responses, promoting longevity, and modulating metabolism during
367 starvation (reviewed in ref. 80). It is conceivable that SKN-1 is particularly susceptible to plastic changes in
368 its regulatory outputs as a means of adapting to frequently varying environmental conditions. In the wild, *C.*
369 *elegans* experience a boom-and-bust cycle and most worms isolated in the wild are present in the dauer
370 stage (81). As we have shown, dauer diapause is associated with strong heritable epigenetic responses
371 (55) that may, therefore, influence developmental plasticity and adaptive evolution in response to the local
372 environment. We believe that our findings may provide among the first example of environmentally-induced
373 heritable epigenetic changes that modulate developmental inputs into an embryonic GRN.

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374 **Materials and Methods**

375 *C. elegans* strains and maintenance

376 N2 (Bristol, UK), MY16 (Mecklenbeck, Germany), JU1491 (Le Blanc, France), and JU1172 (Concepcion,
377 Chile). JR3336*, (elt-2::GFP) X; (ifb-2::GFP) IV*. PD2227 (35), *oxIs322 II; ccTi1594 III. oxIs322 contains*
378 *[myo-2p::mCherry::H2B + myo-3p::mCherry::H2B + Cbr-unc-119(+)] II. ccTi1594* contains *[mex-*
379 *5p::GFP::gpr-1::smu-1 3'UTR + Cbr-unc-119(+), III: 680195] III.*

380 Worm strains were maintained as described (82) and all experiments were performed at 20°C
381 unless noted otherwise. To ensure no carry-over of parental stress response, fresh worm stock was
382 obtained from -80°C and maintained in 150mm NGM plate seeded with *E. coli* OP50 for at least five
383 generations prior to beginning experiments. To avoid genetic drift and lab domestication, a fresh worm
384 stock was obtained every ~30 generations.

385 To obtain males for crosses, 20–30 L4 hermaphrodites were picked into 7% ethanol solution in
386 microcentrifuge tubes and rotated for an hour (83). Worms were pelleted by centrifugation at 2000 rpm for
387 30 seconds. They were then transferred to NGM plates seeded with *E. coli* OP50. F1 male progeny were
388 mated with sibling hermaphrodites to establish male stocks.

389

390 Dauer induction and POE assays

391 The animals were maintained on NGM plates seeded with OP50. Once the cultures became crowded and
392 exhaust their food supply, they were incubated for an extra two weeks at 20°C. The worms were then
393 washed with M9 buffer and incubated in 1% sodium dodecyl sulfate (SDS) for 30-60 minutes with gentle
394 agitation to select for dauer larvae (84). Isolated dauer larvae were then washed with M9 to remove all SDS
395 and allowed to recover overnight on 60 mm NGM plates seeded with OP50.

396 Reciprocal crosses were set up using L4s and the animals were allowed to mate overnight. Control
397 experiments using well-fed animals were performed in parallel. Mated hermaphrodites, as indicated by the
398 presence of copulatory plugs (except for crosses involved N2 males which do not deposit plugs), were
399 transferred to a fresh NGM plate to lay eggs for ~5-7 hours and early brood was discarded to avoid
400 contamination of self-progeny. The hermaphrodites were then transferred to a fresh seeded NGM plate to

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401 lay eggs overnight. The hermaphrodite (P0) was then removed, leaving the F1s alone. Once the F1 worms
402 reached early or mid-adulthood, they were treated with 15% alkaline hypochlorite solution to obtain F2
403 embryos which were allowed to hatch on food. This procedure was repeated until the F4 generation (F10
404 for the experiment shown in Fig. 1B) was obtained. At each generation, L4 worms were used to determine
405 *skn-1* RNAi phenotype (SI Appendix, Fig. S2).

406 For crosses between PD2227 hermaphrodites and JU1491 males, the POE assay was performed
407 as described above. F1 L4s were immobilized on 5% agar pad with 5 mM levamisole diluted in M9 and
408 observed using Nikon Eclipse Ti-E inverted microscope. Mosaic worms that expressed *myo-2*::mCherry,
409 but not *myo-3*::mCherry and *mex-5*::GFP (i.e., PD2227-derived AB and JU1491-derived P1), were
410 recovered on seeded NGM plate in the presence of M9. 20 F2 animals were then randomly selected and
411 observed to ensure no worms expressed fluorescent markers, i.e. JU1491 nuclear genotype (Fig. 2D, SI
412 Appendix, Fig. S6).
413

414 Viability and embryonic lethality scoring

415 To score viability, young hermaphrodites (F1 progeny of the reciprocal crosses) were allowed to lay eggs
416 on an NGM plate seeded with OP50. The next day, newly hatched L1s (F2) were transferred to individual
417 seeded plates. “Larval lethal” was defined as the percentage of worms that arrested as L1s. Worms that
418 reached adulthood but failed to reproduce in five days were scored as sterile. To score embryonic lethality,
419 individual young hermaphrodites (F1) were allowed to lay eggs on an NGM plate seeded with a small drop
420 of OP50 for ~4-8 hours. The hermaphrodites were then removed, leaving the embryos. The fraction of
421 unhatched embryos were counted and scored ~24 hours later. At least two independent broods were
422 scored.
423

424 RNAi

425 Feeding-based RNAi experiments were performed as described (24). RNAi clones were obtained from
426 either the Vidal (85) or Ahringer (86) libraries. RNAi bacterial strains were grown at 37°C in LB containing
427 50 µg/ml ampicillin. The overnight culture was then diluted 1:10. After four hours of incubation at 37°C, 1

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428 mM IPTG was added and 60-100 µl was seeded onto 35 mm agar plates containing 1 mM IPTG. Seeded
429 plates were allowed to dry and used within five days when kept at room temperature. For *skn-1* RNAi, five
430 to 10 L4 animals were placed on RNAi plate. 24 hours later, they were transferred to another RNAi plate to
431 lay eggs for 12 hours. The adults are then removed, leaving the embryos to develop for an extra 5-7 hours.
432 Embryos expressing birefringent gut granules are quantified and imaged on an agar pad using a Nikon Ti-
433 E inverted microscope under dark field with polarized light (SI Appendix, Fig. S1B).

434 For *met-2*, *set-32*, *nrde-4* and *prg-1/2* RNAi, 15-30 F3 L4 animals showing POE were placed on
435 plates of *E. coli* containing an empty control vector (L4440) or expressing double stranded RNA. 24 hours
436 later, they were transferred to another RNAi plate to lay eggs for about seven hours. The adults were then
437 removed and the F4 animals were allowed to develop on RNAi bacteria. F4 L4 larvae were used for *skn-1*
438 RNAi assay for POE as described above (Fig. 4A).

439

440 <u>Statistics and figure preparation</u>

441 Statistics were performed using R software v3.4.1 (https://www.r-project.org/). Two-sample two-tailed t-
442 tests were used to compare *skn-1* RNAi phenotype between two groups, unless stated otherwise. Welch's
443 t-tests were performed if the variances of the two groups being compared are not equal. Plots were
444 generated using R package ggplot2.

445

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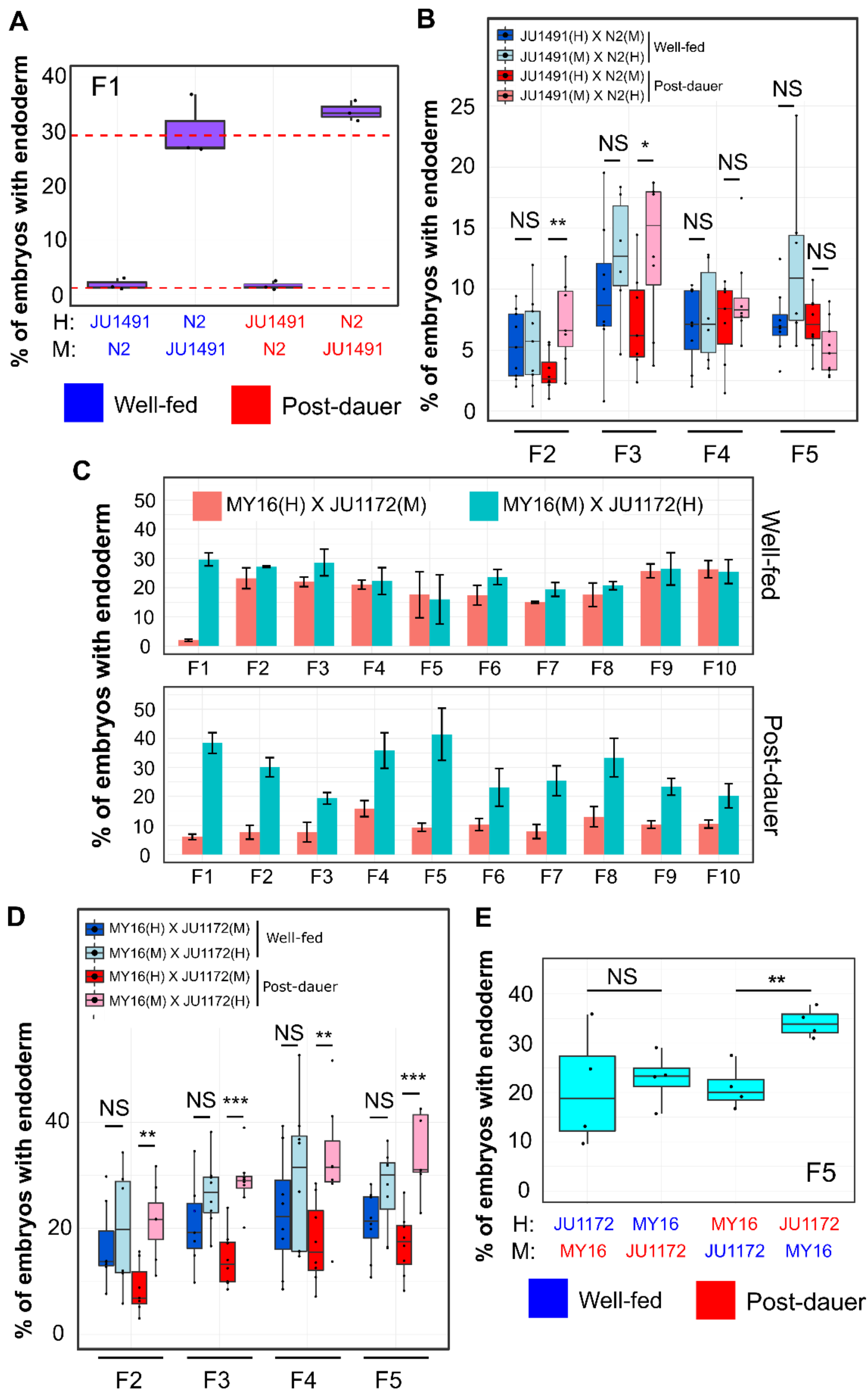
628 **Figures and Tables**



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630 **Fig. 1: Dauer diapause stimulates POE.**

631 A) Strong maternal effect in F1 embryos from mated *skn-1(RNAi)* mothers in N2 x JU1491 crosses. At least
632 three independent crosses were performed. Red dashed lines indicate phenotypes of N2 (29.2%) and
633 JU1491 (1.2%). B) POE in *skn-1(RNAi)* embryos from four generations: F2, F3, F4 and F5 derived from
634 reciprocal N2 x JU1491 crosses. At least five independent crosses were performed for each treatment. C)
635 Dauer diapause-induced POE persists for at least 10 generations in JU1172 x MY16 progeny. Three
636 independent crosses were performed. Error bars represent +/- standard error of the mean. D) Robust POE
637 in *skn-1(RNAi)* embryos from four generations: F2, F3, F4 and F5 derived from reciprocal JU1172 x MY16
638 crosses. At least five independent crosses were performed for each treatment. E) POE shown by *skn-*
639 *1(RNAi)* F5 embryos from reciprocal crosses between MY16 and JU1172. Data points represent
640 independent crosses. For panels A and E, blue text represents experiments performed using well-fed
641 animals while red text represents experiments performed using post-dauer animals. Two sample t-test (NS
642 $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Boxplot represents median with range bars showing upper
643 and lower quartiles. H: hermaphrodite, M: male.

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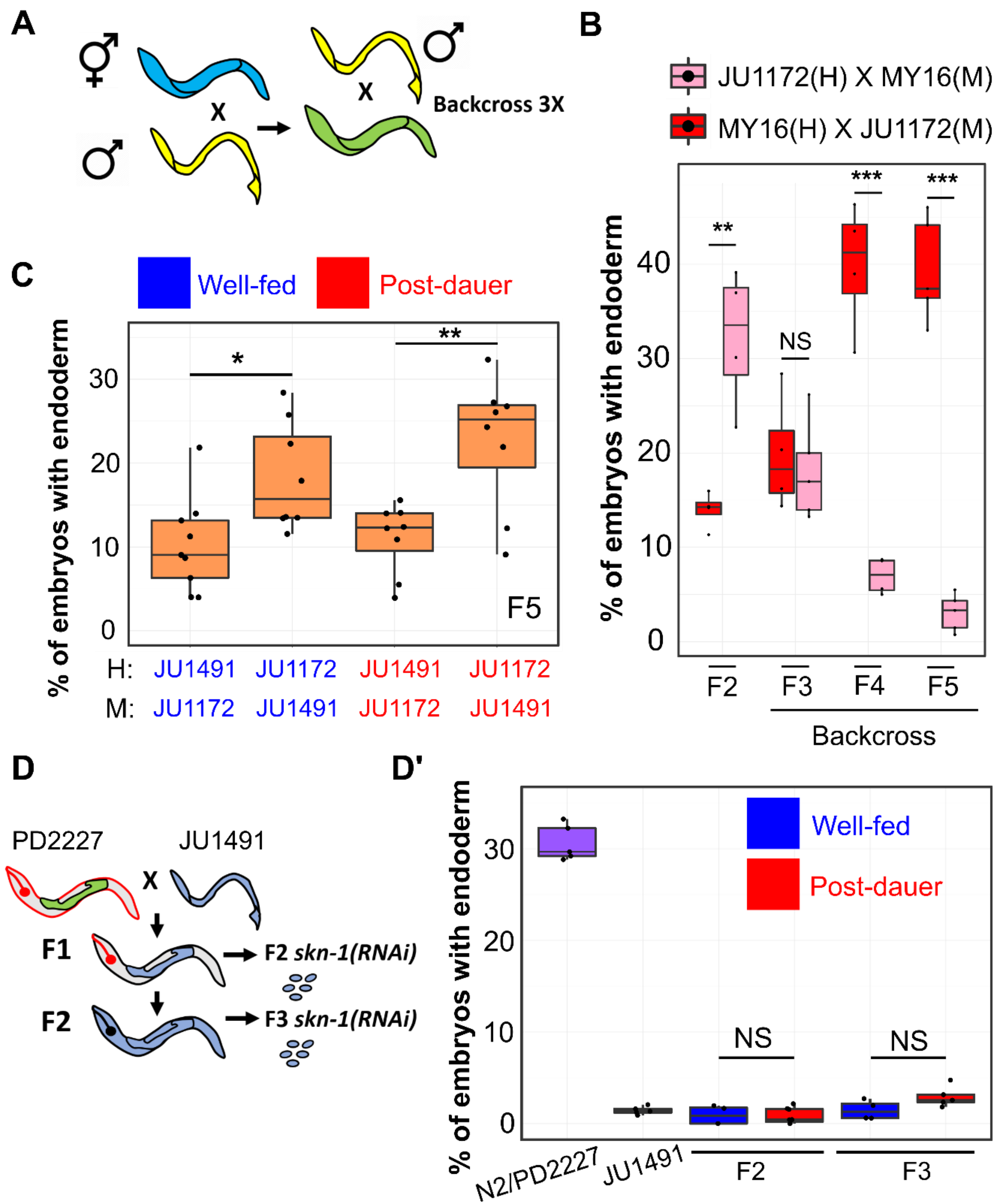

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645 **Fig. 2: POE is not attributable to cytoplasmic inheritance.**

646 A) Schematic of mitochondria transfer experiment. Blue and yellow represent different wild isolates. Five to
647 ten hermaphrodites were used for the backcrossing at every generation for three generations. B) POE in
648 *skn-1(RNAi)* embryos. Data points represent replicates from a single reciprocal cross using post-dauer
649 animals. C) POE shown by *skn-1(RNAi)* F5 embryos of reciprocal crosses performed between JU1491 and
650 JU1172. Data points represent independent crosses. D) When PD2227 ($N2^{GPR\text{-}1\ OE}$) hermaphrodites are
651 mated to JU1491, non-mendelian segregation of maternal and paternal chromosomes result in some F1
652 mosaic animals that express the maternally-derived pharyngeal marker (*myo-2*::mCherry) but lose the body
653 wall muscle (*myo-3*::mCherry) and germline (*mex-5*::GFP) markers. F2 self-progeny of the F1 mosaic
654 animals contain only the JU1491 nuclear genome (see SI Appendix, Fig. S6). D') *skn-1(RNAi)* phenotype
655 of N2/PD2227, JU1491, and F2 and F3 embryos from the mosaic animals. For panels C and D', Blue
656 represents experiments performed using well-fed animals while red represents experiments performed
657 using post-dauer animals. Two sample t-test (NS $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Boxplot
658 represents median with range bars showing upper and lower quartiles. For panel B and C, H:
659 hermaphrodite; M: male.

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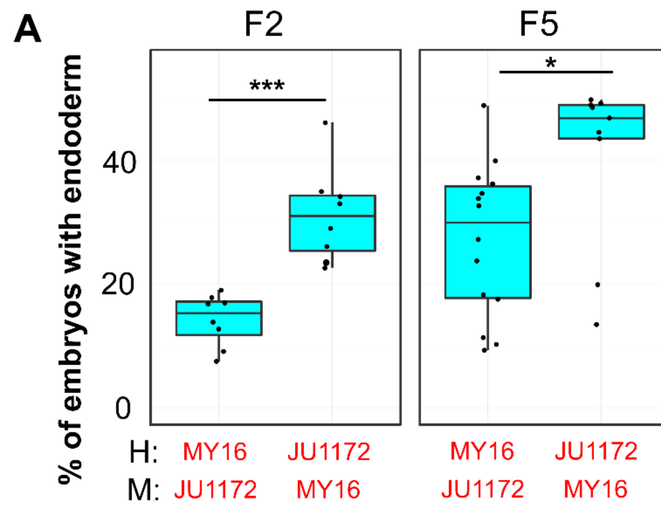
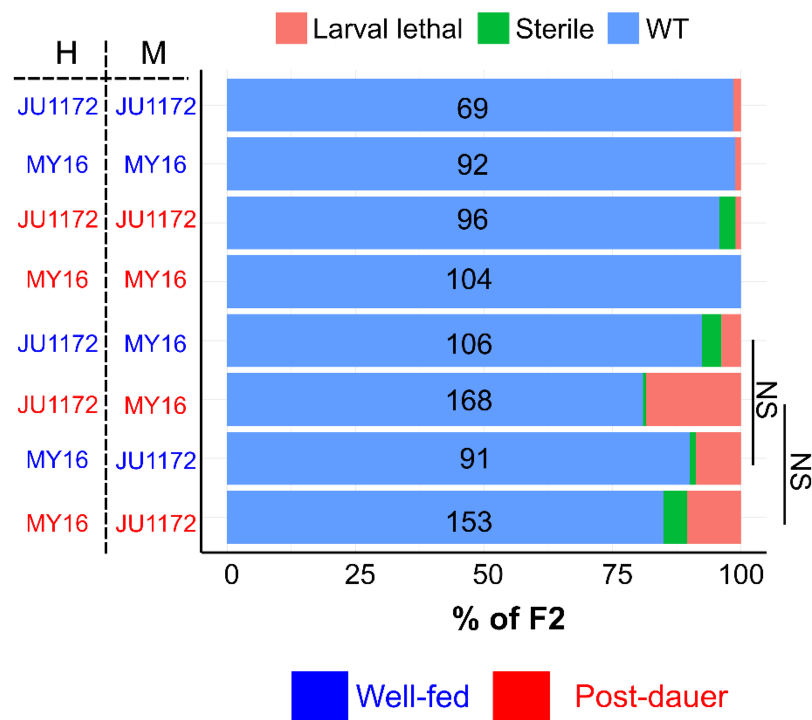


B

⚥	MY16	JU1172	MY16	JU1172
♂	MY16	JU1172	JU1172	MY16
Well-fed	0.0% (102)	0.0% (77)	15.6% (84)	25.7% (171)
Post-dauer	0.0% (45)	0.0% (48)	13.0% (115)	20.1% (144)



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664 **Fig. 3: POE is not due to competitive fitness or maternal incompatibility.**

665 A) Left: POE shown by *skn-1(RNAi)* F2 embryos of reciprocal crosses performed between MY16 and
666 JU1172. Right: Individual data points represent lines derived from late F2s from senescent F1 animals. Two
667 independent crosses were performed for each direction. Two sample t-test (NS $p > 0.05$, * $p \leq 0.05$, ** $p \leq$
668 0.01, *** $p \leq 0.001$). Boxplot represents median with range bars showing upper and lower quartiles. H:
669 hermaphrodite; M: male. B) Embryonic lethality in F2s. Two independent broods were examined. Total
670 number of embryos scored is shown in brackets. C) Sterility and larval lethality of F2 progeny. Identity and
671 the sex (H: hermaphrodite; M: male) of the parents are indicated on the left. Total number of animals scored
672 is indicated. Fisher-exact test (NS $p > 0.05$). For panels A and C, blue represents experiments performed
673 using well-fed animal while red represents experiments performed using post-dauer animals.

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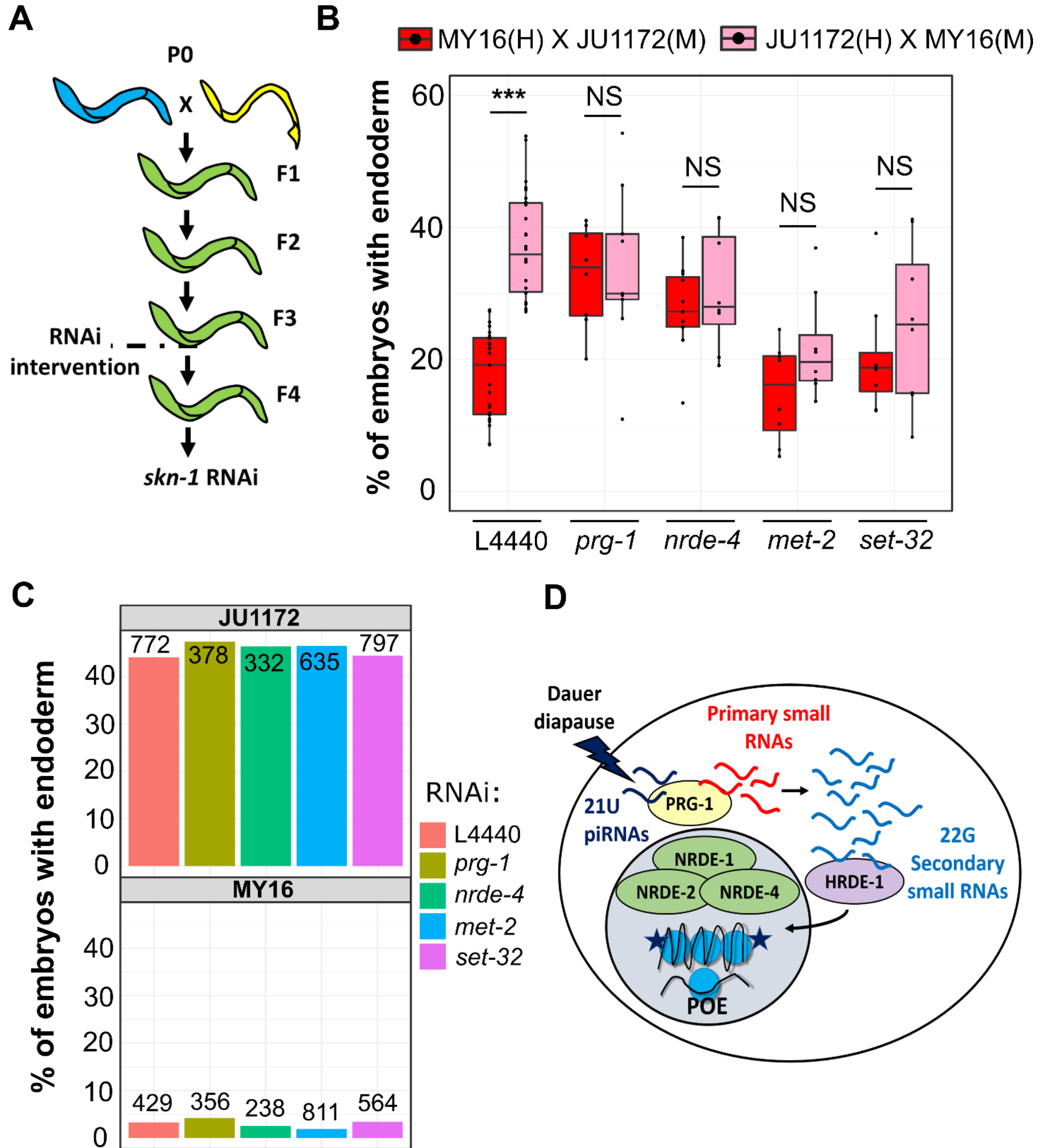

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676 **Fig. 4: POE requires both the piRNA/nuclear RNAi pathway and factors that make H3K9me3**
677 **chromatin marks.**
678 A) Schematic of RNAi experiments examining epigenetic regulation of POE. F3 L4 animals were treated
679 with the indicated RNAis and F4 L4s were used for the *skn-1* RNAi assay. Animals in the control and
680 treatment groups were siblings (see Materials and Methods). B) Data points are replicates from at least two
681 independent crosses. Boxplot represents median with range bars showing upper and lower quartiles. H:
682 hermaphrodite; M: male. Two sample t-test (NS $p > 0.05$, *** $p \le 0.001$). C) The effect of RNAi treatments
683 on *skn-1(RNAi)* phenotype. MY16 and JU1172 L4s were exposed to L4440 (control), *prg-1*, *nrde-4*, *met-2*
684 or *set-32* feeding RNAi and the *skn-1(RNAi)* phenotype of the F1 were quantified. No difference between
685 different RNAi treatments was detected (Fisher-exact test, $p > 0.05$). Total number of embryos scored is
686 indicated. D) Model for POE (see text).