

1 **The chromatin modifiers SET-25 and SET-32 are required for initiation but not long-term**  
2 **maintenance of transgenerational epigenetic inheritance**

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13 **Summary**

14 Some epigenetic modifications are inherited from one generation to the next, providing a potential  
15 mechanism for the inheritance of environmentally acquired traits. Transgenerational inheritance of  
16 RNA interference phenotypes in *Caenorhabditis elegans* provides an excellent model to study this  
17 phenomenon, and whilst studies have implicated both chromatin modifications and small RNA  
18 pathways in heritable silencing their relative contributions remain unclear. Here we demonstrate  
19 that the histone methyltransferases SET-25 and SET-32 are required for the establishment of a  
20 transgenerational silencing signal but not for long-term maintenance of this signal between  
21 subsequent generations, suggesting that transgenerational epigenetic inheritance is a multi-step  
22 process with distinct genetic requirements for establishment and maintenance of heritable silencing.  
23 Furthermore, small RNA sequencing reveals that the abundance of secondary siRNAs (thought to be  
24 the effector molecules of heritable silencing) does not correlate with silencing phenotypes.  
25 Together, our results suggest that the current mechanistic models of epigenetic inheritance are  
26 incomplete.

27 **Introduction**

28 Despite the wide-held belief for the past hundred years that only information encoded in the  
29 genome of an organism can be inherited, it has recently become clear that epigenetic signals can  
30 sometimes be passed between generations (reviewed in Miska and Ferguson-Smith, 2016). Due to

31 its short generation time and easily manipulated germline, *Caenorhabditis elegans* has emerged as  
32 one of the leading organisms with which to study this phenomenon, with a plethora of studies being  
33 reported over the last few years (Devanapally et al., 2015; Greer et al., 2011; Klosin et al., 2017;  
34 Shirayama et al., 2012).

35 One important tool that has been used to study transgenerational inheritance is RNAi silencing. RNAi  
36 was discovered in *C. elegans* (Fire et al., 1998) and from some of the first reports it was observed  
37 that occasionally the RNAi phenotype was detected in unexposed offspring for one or two  
38 generations (Fire et al., 1998; Grishok et al., 2000). Since then, several studies have shown  
39 inheritance of RNAi phenotypes for multiple generations (Alcazar et al., 2008; Ashe et al., 2012;  
40 Buckley et al., 2012; Gu et al., 2012; Houry-Ze'evi et al., 2016; Vastenhouw et al., 2006). In *C. elegans*  
41 RNAi is usually induced by feeding animals double stranded RNA (dsRNA) targeting the gene of  
42 interest. The double stranded RNA is processed by Dicer and accessory proteins to give primary  
43 small interfering RNAs (siRNAs) (Bernstein et al., 2001). 1° siRNAs have a 5' monophosphate, are 21-  
44 23nt long, and are both sense and antisense to the target gene. The 1° siRNAs are bound by the  
45 argonaute protein RDE-1 (Tabara et al., 1999; Yigit et al., 2006) and are used to guide the production  
46 of secondary siRNAs. 2° siRNAs are almost exclusively 22 nucleotides long, predominantly have a  
47 5'G, and a terminal triphosphate group (Pak and Fire, 2007; Sijen et al., 2001, 2007). 2° siRNAs  
48 (called 22Gs), which also exist in complex with an argonaute protein, are responsible for the  
49 degradation of the target mRNA in the cytoplasm (Aoki et al., 2007) or transcriptional gene silencing  
50 in the nucleus (Guang et al., 2010).

51 Genetic screens to find the components of the RNAi-initiated transgenerational epigenetic  
52 inheritance (TEI) pathway have implicated small RNA pathway components, especially the germline  
53 nuclear RNAi machinery, as well as histone modifying enzymes, implying that there may be some  
54 interplay between small RNAs and chromatin. During nuclear RNAi, the germline-specific nuclear  
55 Argonaute heritable RNAi defective 1 (HRDE-1) binds cytoplasmic 2° siRNAs and translocates to the  
56 nucleus (Ashe et al., 2012; Buckley et al., 2012; Shirayama et al., 2012). Upon interaction with  
57 complementary nascent mRNA transcripts, the nuclear RNAi defective (NRDE) factors NRDE-1, -2 and  
58 -4 are recruited (Ashe et al., 2012; Burton et al., 2011). The NRDE machinery then mediates gene  
59 silencing by inhibiting RNA polymerase II during transcriptional elongation (Guang et al., 2010), and  
60 by promoting chromatin modifications that are associated with gene silencing (Burkhart et al., 2011;  
61 Burton et al., 2011; Gu et al., 2012; Guang et al., 2010; Mao et al., 2015). HRDE-1 and NRDE-1, -2 and  
62 -4 are all required for TEI (Ashe et al., 2012; Buckley et al., 2012).

63 Repressive chromatin modifications such as H3K27me3 and H3K9me3 have been implicated in TEI in  
64 *C. elegans*. RNAi induces robust accumulation of H3K9me3, the hallmark of constitutive  
65 heterochromatin in most eukaryotes, at endogenous genes lasting for two generations (Gu et al.,  
66 2012; Kalinava et al., 2017). SET-25, an H3K9 methyltransferase (Snyder et al., 2016; Towbin et al.,  
67 2012), and SET-32, a putative H3K9 methyltransferase (Kalinava et al., 2017; Snyder et al., 2016;  
68 Spracklin et al., 2017), have both been implicated in TEI. Ashe et al. showed SET-25 is required for  
69 RNAi-initiated heritable silencing of a GFP transgene, and showed SET-32 is required in an analogous  
70 system triggered by PIWI-interacting small RNAs (piRNAs) (Ashe et al., 2012). Spracklin et al. have  
71 since shown that SET-32 is also required for RNAi-initiated heritable transgene silencing (Spracklin et  
72 al., 2017). SET-25, SET-32 and another H3K9 methyltransferase MET-2 are required for heritable  
73 accumulation of H3K9me3 in response to RNAi, but in contrast to TEI studies utilising transgene  
74 silencing, loss of SET-25 and SET-32 does not result in loss of heritable gene silencing of the  
75 endogenous *oma-1* gene (Kalinava et al., 2017), or promoter-mediated heritable germline silencing  
76 of the endogenous *sid-1* gene (Minkina and Hunter, 2017). Thus, there is still considerable debate as  
77 to the requirement of chromatin modifiers in TEI.

78 This study aims to determine the requirements for SET-25 and SET-32 in TEI. We use the same  
79 system as that used by Ashe and colleagues (Ashe et al., 2012), involving RNAi silencing of a  
80 germline-expressed GFP transgene. The visible nature of this phenotype provides an exquisitely  
81 sensitive system, whereby we can separate individual animals according to their silencing status and  
82 measure effects in these distinct groups. This approach has enabled us to probe the genetic  
83 requirements of TEI in each generation, and here we show that SET-25 and SET-32 are required for  
84 the establishment of a long-term silencing signal but not for its maintenance over subsequent  
85 generations, suggesting that TEI is a multi-step process. We also show that small RNAs are not as  
86 closely correlated with the presence of TEI as expected, and further characterise the phenotypes  
87 associated with mutations in *set-32* and *set-25*.

## 88 **Results**

### 89 *set-25* and *set-32* are required for transgenerational epigenetic inheritance to the F1 generation only

90 The requirement for *set-25* and *set-32* in multigenerational silencing in some studies led us to test  
91 whether they are required for transgenerational silencing of the germline-expressed *pie-1::gfp::h2b*  
92 transgene. We fed animals containing this transgene ('sensor') bacteria expressing anti-*gfp* dsRNA,  
93 triggering 100% silencing of the GFP transgene (P0 generation). In all strains, 0% of P0 control  
94 animals fed on empty vector bacteria were GFP-silenced (data not shown). Subsequent generations

95 were produced by isolating silenced individuals, and were fed on regular OP50 bacteria. Selection of  
96 silenced individuals distinguishes this study from others that use RNAi transgene silencing models  
97 (Buckley et al., 2012; Burton et al., 2011; Houriz-Ze'evi et al., 2016; Lev et al., 2017; Spracklin et al.,  
98 2017), and allows us to differentiate between effects in individual animals which inherit silencing  
99 and those which do not, and interrogate the requirements of genes at each generation. The  
100 percentage of GFP-silenced animals was measured at each generation. In wild-type sensor animals  
101 exposed to anti-*gfp* dsRNA, GFP silencing persisted for at least three generations after the dsRNA  
102 trigger was removed, as previously described (Ashe et al. 2012) (Figure 1A). Strikingly, *set-25(n5021)*  
103 and *set-32(ok1457)* F1 animals displayed a highly significant reduction in silencing proportions  
104 (Figure 1A). Surprisingly, the F2 and F3 offspring of animals that successfully inherited the silencing  
105 signal then displayed silencing proportions comparable to the sensor strain (Figure 1A). A  
106 CRISPR/Cas-9 generated mutant, *set-32(smb11)* (Figure S1), displayed the same inheritance pattern  
107 (Figure 1A). These data suggest that both *set-25* and *set-32* are required for transgenerational  
108 silencing in the F1 offspring, but are not required from the F2 generation onwards. The *set-*  
109 *32(ok1457); set-25(n5021)* double mutant shows the same inheritance pattern as *set-25(n5012)*  
110 alone, suggesting that *set-32* and *set-25* act in the same pathway (Figure 1A).

111 Failure of silencing of the GFP transgene in the F1 offspring could be due to a requirement for *set-25*  
112 or *set-32* in either the P0 generation (*i.e.* helping to establish a heritable silencing signal) or the F1  
113 generation (*i.e.* receiving or propagating a silencing signal). We sought to distinguish between these  
114 two possibilities by performing the silencing assay on *set-32* or *set-25* heterozygous individuals (P0)  
115 and assaying the inheritance of silencing in the F1 generation (Figure 1B). If *set-32* or *set-25* are  
116 required in the F1 generation we would expect the homozygous F1 mutants to display a failure of  
117 silencing (*i.e.* more homozygous mutants would express GFP than heterozygous or wild-type).  
118 Alternatively, if the proteins are required in the P0 generation only, the absence of functional  
119 protein in the F1 generation should not matter and we would expect to see wild-type levels of  
120 silencing amongst all offspring genotypes. Strikingly, we did not see an increased proportion of GFP-  
121 positive animals amongst the F1 homozygous mutants for either *set-25* or *set-32*; F1 offspring of *set-*  
122 *25* and *set-32* heterozygous parents displayed silencing proportions comparable to wild-type (Figure  
123 1C), and homozygous mutants were not over-represented amongst GFP positive F1s (Figure 1D). This  
124 indicates that neither *set-25* nor *set-32* is required in the F1 generation, and therefore their role in  
125 epigenetic inheritance must be in the P0 generation. These results lead us to propose a three-step  
126 model of TEI consisting of initiation, establishment and maintenance phases, each requiring distinct  
127 factors (see Discussion) (Figure 1E).

128 *22Gs do not correlate with heritable silencing*

129 Since small RNA molecules, in particular 22Gs, have been implicated in heritable RNAi-induced  
130 silencing, we sequenced small RNAs in P0 animals and F1 animals in *set-25*, *set-32*, *hrde-1* and *nrde-2*  
131 mutant strains. P0 animals exhibited 100% silencing (Figure 1A) and so were collected in one pool,  
132 whilst F1 animals were separated into GFP-silenced (GFP-off) and GFP-expressing (GFP-on) pools,  
133 allowing us to investigate differences in small RNAs between these populations. Our hypothesis was  
134 that since all strains display GFP silencing in the P0 generation, they would have equal amounts of  
135 small RNAs mapping to the GFP transgene. Furthermore, we expected that F1 GFP-off worms would  
136 have anti-*gfp* small RNAs and that GFP-on worms would not. We first focussed our analysis on the  
137 wild-type animals. As expected, 1° siRNAs were present in the P0 generation but were essentially  
138 absent in the F1 generation (Figure 2Ai). 22Gs were present in the F1 GFP-off animals but were  
139 dramatically less abundant than in the P0 generation (Figure 2Aii,B), as expected (Ashe et al., 2012;  
140 Gu et al., 2012; Houri-Ze'evi et al., 2016). Surprisingly, 22Gs were also present in the GFP-on animals  
141 and the numbers of 22Gs did not differ significantly between GFP-off and GFP-on animals in wild-  
142 type (fold change off/on = 1.77, ns paired t-test) (Figure 2C,D). This is surprising because 22Gs are  
143 the effector molecules of silencing, and current models suggest that they are the molecule carried  
144 between generations by HRDE-1 (Minkina and Hunter, 2017); thus one would expect a substantial  
145 difference between GFP-off and GFP-on animals.

146 We next focussed on the various mutant strains. *set-25* animals have previously been shown to be  
147 defective in heritable siRNAs at the F3 generation (Lev et al., 2017). In our hands, we could detect  
148 GFP-targeting siRNAs in both the GFP-off and GFP-on F1 animals, at similar levels to those detected  
149 in wild-type (Figure 2A-D). Again, the difference in small RNAs between GFP-off and GFP-on animals  
150 was small. *set-32* and *hrde-1* animals showed slightly more anti-GFP 22Gs in the GFP-off animals  
151 compared to GFP-on animals (Figure 2C,D). Strikingly, the most abundant 22Gs were found in GFP-  
152 off *nrde-2* animals (Figure 2B-D), despite the defect in heritable silencing that these animals display.  
153 Why is there such an abundance of 22Gs in GFP-off animals in the *nrde-2* strain, which is defective in  
154 heritable silencing, when there is essentially no difference in 22G abundance between GFP-off and  
155 GFP-on animals in wild-type? It is tempting to speculate that 22Gs may not be the main heritable  
156 agent (see Discussion).

157 *Endo siRNAs are perturbed in TEI mutants*

158 It has previously been shown that RNAi can alter levels of endogenous siRNAs both in the exposed  
159 P0 generation and in the F1 generation in wild-type animals (Houri-Ze'evi et al., 2016; Lev et al.,

160 2017). We first wanted to verify this finding. We took the small RNAs from control, P0 and F1 wild-  
161 type animals and divided them into seven classes of small RNAs based on the genomic annotation to  
162 which they map; transposable elements, protein coding, pseudogenic transcripts, repetitive regions,  
163 piRNAs, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). Contrary to previous reports  
164 (Houriz-Ze'evi et al., 2016; Lev et al., 2017), we did not observe any differences in siRNA families  
165 between control and GFP RNAi exposed animals in any subset of small RNAs in wild-type animals  
166 (Figure S2A). We also did not observe any differences between F1 GFP-off and GFP-on animals. We  
167 performed the same analysis in the *set-25*, *set-32*, *nrde-2*, and *hrde-1* mutant strains and also saw no  
168 difference between control or RNAi-treated animals in the P0 generation, or between GFP-off and  
169 GFP-on animals in the F1 generation.

170 We did, however, observe some significant differences between the P0 and F1 generations. miRNA-  
171 mapping siRNAs were decreased in the F1 generation compared to the P0 generation (Figure 2E), in  
172 direct contrast to previous reports (Houriz-Ze'evi et al., 2016) where an increase in miRNAs was  
173 observed in the F1 generation. lnc-RNA-mapping siRNAs also displayed a decrease in F1 (Figure 2F).  
174 These patterns were observed in wild-type and most mutant strains (Figure 2E,F), suggesting that  
175 there may be a heritable response elicited by RNAi exposure in wild-type animals that decreases the  
176 levels of these classes of endo siRNAs in the F1 generation, perhaps due to competition for  
177 components of the RNAi machinery. *hrde-1* animals had no difference between P0 and F1  
178 generations, suggesting that in this strain the heritable endo siRNAs pathways are perturbed. A  
179 lower amount of endo siRNAs in F1 compared to P0 was detected over all strains for piRNAs (further  
180 enhanced in *set-32* mutants) (Figure 2G), transposable elements and pseudogenic-mapping siRNAs  
181 (Figure S2). *nrde-2* animals had globally higher levels of pseudogenic-mapping endo siRNAs in both  
182 generations compared to wild-type.

183 Repeat-mapping siRNAs increased greatly in the F1 generation compared to the P0 generation in  
184 wild-type animals (Figure 2H). This increase was lost in all mutant strains except *set-32(smb11)*,  
185 again suggesting that a heritable response to RNAi exposure is perturbed in these mutant strains,  
186 consistent with our previous results. A role for *set-25* and *nrde-2* in the silencing of repetitive  
187 elements has recently been reported (McMurphy et al., 2017). It is interesting that we do not detect  
188 an overall decrease in siRNA mapping in these strains, and only see the effect in the F1 generation.  
189 Perhaps a subtle effect in the mutant strains is exacerbated by competition with the heritable RNA  
190 response.

191 Finally, we looked at siRNAs that map to protein coding genes. We saw a slight increase in these  
192 endo siRNAs in *nrde-2* compared to wild-type but otherwise found no obvious differences (Figure

193 S2). However when we took a subset of genes – those that are involved in epigenetic processes  
194 (Hourri-Ze’evi et al., 2016) – we saw that siRNAs mapping to those genes were upregulated in the F1  
195 generation in wild-type, but that this effect was lost in all strains except *hrde-1* (Figure 2I).

196 Overall, we saw that endo siRNAs alter following RNAi exposure in the F1 generation in wild-type  
197 animals, and that this heritable endo RNAi response was changed in all of the TEI mutant strains that  
198 we analysed. Our data suggest that heritable siRNA pathways are disturbed in these strains globally  
199 and not only at the GFP locus that we targeted in the RNAi.

#### 200 *SET-25 and SET-32 have distinct germline expression patterns*

201 In order to further characterise SET-32 and SET-25 we used CRISPR/Cas9 to insert *mCherry*  
202 immediately downstream of the start codon of *set-25* and immediately upstream of the stop codon  
203 of *set-32* to generate N- and C-terminal tagged proteins respectively. These strains displayed wild-  
204 type heritable silencing in the RNAi inheritance assay, indicating that the tagged proteins are  
205 functional (data not shown). For mCherry::SET-25 we saw expression in the nuclei of the mitotic  
206 zone of the germline. This expression was detected from larval stage 2 (L2) onwards (Figure 3A,B and  
207 data not shown). Faint expression was also detected in all nuclei of embryos from about the 8-16 cell  
208 stage. Although expression was weak, mCherry::SET-25 was seen to be associated with the  
209 chromatin throughout mitotic cell divisions in the embryos (Figure 3C).

210 Very weak SET-32::mCherry expression was also observed in the germline, but was not localised  
211 exclusively to nuclei, nor to the mitotic zone. Instead, expression was detected throughout the  
212 germline from L1/2 onwards, with maximum expression detected at L4 (Figure 3D and Figure S3A).  
213 No expression could be detected in embryos. As the expression of SET-32::mCherry was so weak, we  
214 imaged SET-32::mCherry and wild-type animals under identical conditions and quantified the  
215 amount of fluorescence in whole adult germlines using ImageJ. There was significantly more  
216 fluorescent signal detected in SET-32::mCherry animals compared to wild-type (Figure 3E).

217 Cytoplasmic expression of a histone methyltransferase is not without precedence, as a previous  
218 study showed the H3K9 methyltransferase MET-2 to be enriched in the cytoplasm (Towbin et al.,  
219 2012). It is worth noting that SET-32::mCherry does not appear to be excluded from the nucleus,  
220 implying that it may have both nuclear and cytoplasmic roles. mCherry::SET-32 was not detectable  
221 by Western blot (data not shown), thus we cannot rule out the possibility that the mCherry tag is  
222 cleaved from the SET-32 protein. Despite this caveat, our data show that *set-32* is expressed  
223 exclusively in the germline.

#### 224 *set-25 and set-32 mutants have perturbed germline H3K9me3*

225 Since SET-25 and SET-32 are both expressed in the germline and have been implicated in H3K9  
226 methylation (Kalinava et al., 2017; Snyder et al., 2016; Spracklin et al., 2017; Towbin et al., 2012), we  
227 performed immunofluorescence against H3K9me3 and H3K9me2 in dissected gonads of mutant  
228 hermaphrodite adults. We observed a significant reduction in H3K9me3 staining in *set-32(ok1457)*;  
229 *set-25(n5021)* mutants throughout the germline compared with wild-type (Figure 3F,G) and a  
230 corresponding increase in H3K9me2 staining (Figure S3B,C). No other strains displayed a difference  
231 in H3K9me2 staining (Figure S3B,C).

232 Surprisingly, no difference was observed in H3K9me3 intensity in the mitotic or pachytene zones of  
233 *set-25(n5021)* or *set-32(smb11)* germlines compared with wild-type (Figure 3F,G). However, in *set-*  
234 *25(n5021)* mutant germlines we observed a significant decrease in intensity in the mitotic region  
235 relative to the pachytene region compared with wild-type (Figure 3H), consistent with a role for SET-  
236 25 function in the mitotic region. Both of these alleles are putative null mutants with predicted loss  
237 of the SET domain responsible for addition of methyl groups to histones; *set-25(n5-21)* carries a  
238 1979 base pair deletion which removes over half of the SET domain coding sequence, and *set-*  
239 *32(smb-11)* carries a 32 base pair frameshift mutation in exon 2 resulting in a predicted premature  
240 stop codon before the SET domain (Figure S1). Thus, the lack of large differences in H3K9me3 in  
241 these putative null mutant strains implies a level of redundancy of H3K9 trimethyltransferases in the  
242 germline (in contrast to the role of SET-25 previously shown in embryos (Towbin et al., 2012)),  
243 and/or that the role of SET-25 and SET-32 in H3K9me3 deposition is at specific loci or developmental  
244 stages, or in response to particular stimuli and therefore not detected in this experiment. The latter  
245 model is consistent with the results of Spracklin et al. who recently showed that loss of SET-32  
246 results in decreased H3K9me3 specifically at genes targeted by HRDE-1 and not at non-HRDE-1  
247 target genes (Spracklin et al., 2017).

248 Interestingly the *set-32(ok1457)* allele behaved differently to the *set-32(smb11)* allele; H3K9me3  
249 intensity in *set-32(ok1457)* mutants was significantly higher than wild-type (Figure 3F,G) and highly  
250 variable between nuclei (Figure 3F, Figure S3D). Variation in H3K9me3 has also been observed  
251 between *set-32(ok1457)* intestinal nuclei (Snyder et al., 2016) (although this is inconsistent with our  
252 observation of SET-32 expression exclusively in the germline). This variation could be explained by  
253 the *set-32(ok1457)* allele; it carries a 514 base pair in frame deletion which removes most of exons 2  
254 and 3 but potentially leaves the SET domain intact and functional (Figure S1). The deletion may  
255 remove important sequences required for correct targeting of the protein, resulting in misregulation  
256 and hence aberrant methyltransferase activity, manifesting as variable germline H3K9me3. It is  
257 interesting that we do not observe this aberrant H3K9me3 in the double mutant strain, which



258 contains the *set-32(ok1457)* allele but lacks SET-25. This suggests that *set-32(ok1457)* requires the  
259 presence of a functional SET-25 protein to exert its aberrant effect.

260 Strikingly, all mutant strains including the double mutant displayed some level of H3K9me3 in the  
261 germline (Figure 3F), suggesting that there is at least one other H3K9 trimethyltransferase acting in  
262 the germline or that the antibodies bind non-specifically at low levels. One possible H3K9  
263 trimethyltransferase candidate is SET-26, which has previously been implicated in H3K9me3  
264 deposition by *in vitro* methyltransferase assay (Greer et al., 2014).

265 *SET-32 does not affect H3K9 methylation in whole worms*

266 Some reports have linked SET-32 to H3K9me3 accumulation, but each showed a correlation between  
267 a loss of SET-32 and decrease in H3K9me3 (Kalinava et al., 2017; Snyder et al., 2016; Spracklin et al.,  
268 2017). Since we observed no decrease in H3K9me3 in *set-32* mutant germlines by  
269 immunofluorescence, we performed bottom-up proteomics on histones extracted from wild-type  
270 and *set-32* mutant strains as an unbiased approach to identifying the modification(s) for which SET-  
271 32 is responsible.

272 While we could see clear evidence for the role of SET-25 in trimethylation of H3K9 (as previously  
273 shown by Towbin and colleagues (Towbin et al., 2012)), we could see no evidence for SET-32 playing  
274 a similar role (Figure 3I). Our analysis was performed on whole animals from mixed-stage  
275 populations. Given the germline localisation of SET-32 determined by our mCherry expression strain  
276 and low expression levels, it is possible that a role of SET-32 in H3K9 methylation at specific loci  
277 and/or developmental times is obscured.

278 We detected a trend towards a decrease in H3K23 mono-methylation in *set-32(smb11)* mutants and  
279 a corresponding increase in H3K23 acetylation (Figure 3I). These changes were not detected in the  
280 *set-32(ok1457)* allele. We also detected an increase in H3K27 tri-methylation in *set-32(smb11)*  
281 (Figure 3I). It is unlikely that a putative methyltransferase mutant would directly cause an increase in  
282 methylation, so this is most likely an indirect effect indicating a general disruption of histone  
283 modifications in *set-32* mutants. A similar trend was observed in the *set-32(ok1457)* strain. We  
284 detected a decrease in H3K79 di-methylation in the *set-32(ok1457); set-25(n5021)* strain, with a  
285 similar trend evident in both single mutant strains (Figure S3E). It has been suggested that H3K79  
286 methylation occurs in a nucleosomal context and is regulated by cross-talk between histone  
287 modifications (Farooq et al., 2016), so this additive effect may be an indirect result of changes to the  
288 overall histone modification pattern in the double mutant.

289 *set-25 mutants do not display a mortal germline phenotype*

290 A recent report (Spracklin et al., 2017) showed that *set-32* mutants display a mortal germline (Mrt)  
291 phenotype – that is, after just two generations at 25°C the brood size dropped by 75%, and by 3-5  
292 generations the animals became sterile. We were interested in testing if this was also the case for  
293 *set-25* mutants. We shifted *set-32(ok1457)*, *set-32(smb11)* and *set-25(n5021)* mutants to 25°C and  
294 serially passaged individual animals for 12 generations, counting brood size at each generation. We  
295 observed a Mrt phenotype in *set-32* mutants, although it was milder than previously reported  
296 (Spracklin et al., 2017); even after twelve generations at 25°C, *set-32(ok1457)* and *set-32(smb11)*  
297 were not fully sterile (Figure 4A). Surprisingly however, we did not observe a Mrt phenotype in *set-*  
298 *25* mutants (Figure 4A). We continued following the brood size of the wild-type and *set-25* mutant  
299 lines and found no difference between them at the 20<sup>th</sup> and 25<sup>th</sup> generation (data not shown). We  
300 also counted the number of sterile individuals which arose during maintenance at 25°C for 12  
301 generations, and again observed no difference between *set-25* mutants and wild-type (Figure S4A).

#### 302 *set-32(ok1457)* animals have defective sperm

303 As SET-32 mutants have not been widely studied, we were interested in further characterising our  
304 mutant strains. We noticed that *set-32(ok1457)* animals did not starve plates as quickly as wild-type  
305 animals grown under the same conditions and investigated this further by performing brood size  
306 assays, counting the numbers of live progeny and unfertilised eggs. *set-32(ok1457)* animals displayed  
307 a dramatically reduced brood size compared with wild-type, producing 59 and 287 live self-progeny  
308 on average respectively (Figure 4B). *set-25(n5021)* animals displayed a small but statistically  
309 significant reduction in brood size compared with wild-type (mean of 287 vs 261 progeny). *set-*  
310 *32(ok1457); set-25(n5021)* double mutants (mean of 62 progeny) displayed the same phenotype as  
311 *set-32(ok1457)* alone. Interestingly, the putative null mutant *set-32(smb11)* displayed no brood size  
312 defects, implying that the defects exhibited by *set-32(ok1457)* are due to the nature of the allele. As  
313 proposed in the immunofluorescence results, protein expressed from the *set-32(ok1457)* allele may  
314 be misregulated. This deleterious gain of function could be responsible for the observed brood size  
315 defects.

316 Production of live progeny from wild-type and *set-25(n5021)* animals peaked at day 2 of adulthood,  
317 and animals continued to lay live progeny until day 4 (Figure S4B). In contrast, *set-32(ok1457)* and  
318 *set-32(ok1457); set-25(n5021)* live progeny production peaked at day 1 of adulthood, with animals  
319 laying very few live progeny from day 2 onwards (Figure S4B). In both these strains animals did not  
320 cease to lay eggs at day 1, but instead continued laying a large number of unfertilised oocytes  
321 (Figure S4C). In total, *set-32(ok1457); set-25(n5021)* animals produced significantly more unfertilised  
322 oocytes than wild-type (mean of 143 vs 68) (Figure 4C). *set-32(ok1457)* animals also displayed a

323 strong trend of increased production of unfertilised oocytes (mean of 112), that was not significantly  
324 different from the number of *set-32(ok1457); set-25(n5021)* unfertilised oocytes (Figure 4C).  
325 Together, these data strongly suggest that *set-32(ok1457)* animals lay more unfertilised oocytes than  
326 wild-type, and that this is not greatly enhanced in the double mutant.

327 The large numbers of unfertilised eggs suggested a sperm defect. In order to test this hypothesis, we  
328 crossed wild-type male animals with *set-32(ok1457)* hermaphrodites. We observed that the brood  
329 size from *set-32(ok1457)* females mated with wild-type males was indistinguishable from wild-type  
330 mated brood size (Figure 4D, Figure S4D), suggesting that *set-32(ok1457)* hermaphrodites have male  
331 germline defects only.

332 In order to further characterise the male germline defects, we performed DAPI staining on wild-type  
333 and *set-32(ok1457)* hermaphrodites 12 and 24 hours after the L4 stage, and counted the number of  
334 sperm nuclei. At 12 hours post L4, there was no difference in the number of sperm per spermatheca  
335 between the two strains (wild-type = 133 sperm, *set-32(ok1457)* = 135 sperm) (Figure 4E,F).  
336 However, at 24 hours post L4 there was a striking, highly significant reduction in the number of  
337 sperm in the *set-32(ok1457)* spermathecas (wild-type = 135 sperm, *set-32(ok1457)* = 17 sperm)  
338 (Figure 4E,F). Furthermore, at 24 hours post L4 we detected large numbers of sperm throughout the  
339 uterus of *set-32(ok1457)* animals which were not present in wild-type animals (Figure 4G). In wild-  
340 type animals some sperm are pushed from the spermatheca into the uterus by the passage of  
341 fertilised oocytes but quickly migrate back, resuming positions in the spermatheca before the  
342 passage of the next oocyte (Ward and Carrel, 1979). Thus, the presence of large numbers of sperm in  
343 the uterus suggests that *set-32(ok1457)* self-sperm are motility-defective.

344 We wanted to test whether *set-32(ok1457)* male sperm are also defective, so we crossed *set-*  
345 *32(ok1457); him-8(e1489)* males or control *him-8(e1489)* males, both carrying a neuronal GFP  
346 transgene, to wild-type hermaphrodites. The *him-8* mutation produces high incidence of males and  
347 was used to aid in obtaining large quantities of males. Cross-progeny express the GFP transgene  
348 whilst self-progeny do not, allowing us to distinguish between the two. Male sperm typically  
349 outcompete hermaphrodite self-sperm (Ward and Carrel, 1979), and we observed that wild-type  
350 mated controls produced predominantly cross-progeny as expected (Figure S4E). In contrast, wild-  
351 type hermaphrodites mated to *set-32(ok1457)* males produced significantly less cross-progeny  
352 (Figure S4E), indicating a male sperm defect. Cross- and self-progeny were produced in parallel  
353 throughout the first few days of adulthood (data not shown) suggesting that mutant male sperm fail  
354 to outcompete wild-type hermaphrodite self-sperm, possibly due to a motility defect as observed in  
355 mutant hermaphrodite self-sperm.

356 *Loss of SET-32 extends lifespan*

357 We were also interested to see whether loss of SET-25 or SET-32 altered lifespan. We performed  
358 lifespan assays in the absence of FUDR on wild-type, *set-25(n5021)*, *set-32(ok1457)* and *set-*  
359 *32(smb11)* mutants. In data representative of two independent assays, *set-25(n5021)* lifespan was  
360 not significantly different to wild-type (median lifespan of 22 vs 21 days) (Figure 4H). However, both  
361 *set-32(ok1457)* and *set-32(smb11)* animals displayed significant lifespan extension compared with  
362 wild-type (median lifespan 24 vs 21 days, 14% increase) (Figure 4H, Figure S4F,J). There is a well-  
363 known inverse relationship between fertility and lifespan (Partridge et al., 2005). The fact that we  
364 see a lifespan extension in both *set-32* mutant strains and reduction of fertility in only one argues  
365 that reduced fertility cannot be the cause of the lifespan extension observed here. Nonetheless, in  
366 order to rule out this possibility we also performed the lifespan assays in the presence of FUDR  
367 which induces sterility. Again, we saw an extension of lifespan in *set-32(ok1457)* animals compared  
368 to wild-type (median lifespan of 28 vs 24, 17% increase) (Figure S4G-J), indicating lifespan extension  
369 is independent of reduced brood size.

370 **Discussion**

371 *SET-25 and SET-32 establish a heritable silencing signal*

372 Here we have shown that the histone methyltransferases SET-25 and SET-32 are required only in the  
373 generation exposed to the initial silencing trigger to establish silencing in the next generation, and  
374 that when established, transgenerational silencing can be efficiently propagated in all subsequent  
375 generations in the absence of SET-32 and SET-25. In stark contrast, HRDE-1 and NRDE -2 are required  
376 to maintain heritable silencing in subsequent generations (Ashe et al., 2012; Buckley et al., 2012;  
377 Minkina and Hunter, 2017). Even when silenced animals are selected to create the next generations,  
378 *hrde-1* and *nrde-2* mutants display heritable silencing failure in increasing proportions in successive  
379 generations (Ashe et al., 2012). None of these genes are required to silence the generation initially  
380 exposed to the RNAi trigger (Ashe et al., 2012; Buckley et al., 2012; Spracklin et al., 2017, this study).  
381 Thus, we propose a three-step model of TEI consisting of initiation of silencing by canonical RNAi  
382 pathway genes, establishment of heritable silencing by *set-25* and *set-32*, and ongoing maintenance  
383 of heritable silencing requiring small RNA-associated genes such as *hrde-1* and *nrde-2* (Figure 1E).  
384 Further work will need to be performed to place other recently identified TEI genes correctly into  
385 this model (Akay et al., 2017; Hourri-Ze'evi et al., 2016; Spracklin et al., 2017; Weiser et al., 2017).  
386 This model is highly complementary to the results of Kalinava et al. ("The establishment of nuclear

387 RNAi is a transgenerational process and is promoted by a putative H3K9 methyltransferase SET-32 in  
388 *Caenorhabditis elegans*”, co-submitted).

389 How might this three-step model work? As SET-25 and SET-32 are histone methyltransferases, it  
390 follows that the establishment of a heritable silencing signal involves the deposition of H3K9me3 at  
391 the targeted locus. Indeed, SET-32 is required for accumulation of H3K9me3 at loci targeted by both  
392 exogenous and endogenous siRNAs (Kalinava et al., 2017; Spracklin et al., 2017). However, previous  
393 studies have demonstrated that silencing can be inherited across generations in the absence of the  
394 targeted DNA locus (Grishok et al., 2000; Houry-Ze’evi et al., 2016; Minkina and Hunter, 2017;  
395 Rechavi et al., 2011; Sapetschnig et al., 2015), suggesting that H3K9me3 cannot be the signal  
396 inherited between generations. In each of these experiments at least one copy of the target locus is  
397 present in animals exposed to the silencing trigger. We propose that the heritable silencing signal  
398 established by SET-25 and SET-32 is established in the mitotic zone of the germline of exposed  
399 animals when at least one copy of the targeted locus is present in every cell, triggering a locus-  
400 independent mechanism that then maintains heritable silencing throughout the subsequent zones of  
401 the germline and into the inheriting generations. This is consistent with the expression of SET-25 in  
402 the mitotic zone of the germline.

403 What could this locus-independent mechanism be? In its role as a H3K9 methyltransferase, SET-25 is  
404 required for complete anchoring of heterochromatic arrays to the nuclear periphery (Towbin et al.,  
405 2012). Furthermore, SET-25 co-localises with its own methylation product in perinuclear foci,  
406 suggesting that following anchoring it mediates propagation of heterochromatin to neighbouring loci  
407 (Towbin et al., 2012), consistent with observations of the spread of H3K9me3 to loci neighbouring  
408 RNAi targeted genes (Burton et al., 2011; Gu et al., 2012). Potentially then, the locus-independent  
409 silencing mechanism could involve localisation of the silenced region to the nuclear periphery, a  
410 region associated with gene silencing (Meister and Taddei, 2013). In this model, SET-25 and SET-32  
411 could deposit H3K9me3 at the target locus, resulting in the anchoring of this locus to the nuclear  
412 periphery. Subsequently, SET-25 and SET-32 could mediate the propagation of heterochromatin to  
413 neighbouring loci, including to homologous regions adjacent to an absent locus on the other  
414 chromosome, tethering the local region to the nuclear periphery and establishing a silencing signal  
415 which is independent of the specific locus. SET-32 may act to maintain the position of these loci at  
416 the nuclear periphery during meiosis, since SET-32 expression was observed throughout the  
417 germline.

418 Silencing in subsequent generations is maintained by HRDE-1 and NRDE-2, and 22Gs. However, our  
419 results suggest that siRNAs cannot be the main heritable agent, because abundance of 2<sup>o</sup> siRNAs did

420 not correlate with heritable silencing in wild-type or mutant strains. Potentially, 2° siRNAs mediate  
421 heritable silencing in conjunction with another mechanism, which may involve maintenance of the  
422 silenced locus at the nuclear periphery, or a different type of RNA molecule. Future research should  
423 investigate the nuclear localisation of the targeted locus during heritable RNAi to determine whether  
424 it is anchored to the nuclear periphery, and investigate how nuclear localisation might interact with  
425 RNAs and associated factors to maintain heritable silencing across generations.

#### 426 *Chromatin modifications mediated by SET-32*

427 Several reports have linked SET-32 to H3K9me3 accumulation (Kalinava et al., 2017; Snyder et al.,  
428 2016; Spracklin et al., 2017). We have investigated the chromatin modifications for which SET-32 is  
429 responsible using two approaches. Using bottom up proteomics we have shown that *set-32* mutants  
430 display no global decrease in H3K9me3 compared with wild-type in whole worms. We did, however,  
431 see evidence for a role for SET-32 in directly or indirectly mediating several other modifications,  
432 including H3K23me, H3K23ac, H3K27me3, and H3K79me2. Using immunofluorescence we have  
433 shown that *set-25* and *set-32* putative null mutants exhibit germline H3K9me3 comparable to wild-  
434 type, although a significant decrease was observed in a double mutant. Furthermore, the potentially  
435 misregulated *set-32(ok1457)* mutant showed increased germline H3K9me3. These latter results  
436 further support the hypothesis that SET-32 mediates H3K9me3 accumulation. However, it is clear  
437 that its role is complex; the lack of H3K9me3 loss at the whole germline and whole worm level in *set-*  
438 *32* mutants suggests that SET-32 may be functioning at least partially redundantly with SET-25 in  
439 H3K9me3 accumulation in the germline, but the presence of severe defects in heritable silencing,  
440 germline maintenance, and lifespan regulation in the single mutant implies that it must have non-  
441 redundant function in these processes. Future work should address which modification(s) SET-32 is  
442 directly modifying and which are indirectly affected, and whether SET-32 operates in conjunction  
443 with other methyltransferases to carry out its methyltransferase activity.

#### 444 *The role of the RNAi inheritance machinery in maintaining germline immortality and implications for* 445 *lifespan*

446 In *C. elegans* several genes found to be involved in TEI have also been reported to have Mrt  
447 phenotypes, leading to the hypothesis that germline immortality maintenance is a general role of  
448 the RNAi inheritance machinery (Buckley et al., 2012; Spracklin et al., 2017). In *set-25* we show the  
449 first example, to our knowledge, of a gene required in TEI which does not exhibit a Mrt phenotype;  
450 after 25 generations at 25°C, *set-25* mutants displayed comparable fertility to wild-type animals. Our  
451 results suggest two potential explanations; *set-25* mutants exhibit a mild Mrt phenotype which may

452 take many generations to appear and hence was not detected in our assay, or SET-25 is not required  
453 for maintaining germline immortality. It is interesting that TEI mutants display Mrt phenotypes of  
454 very different severity, from complete sterility in just a few generations (Spracklin et al., 2017), to  
455 little or no Mrt phenotype (this study). Additionally, *nrde-1* and *nrde-4* mutants become  
456 progressively sterile when maintained at 20°C whilst the phenotype in *nrde-2* and *hrde-1* mutants  
457 only becomes apparent at 25°C (Buckley et al., 2012). These variations do not appear to correlate  
458 with the severity of the TEI defect. Further research is required to explain these differences and  
459 determine the precise roles for the TEI genes in germline maintenance.

460 It has been suggested that a decreased cost of germline maintenance may cause lifespan extension  
461 through the reallocation of resources from germline maintenance to somatic maintenance  
462 (Maklakov and Immler, 2016). Our results are consistent with this model; we have shown that *set-32*  
463 mutants exhibit a Mrt phenotype implying decreased germline maintenance activity, and extended  
464 lifespan. Additionally, *set-25* mutants have apparently normal germline maintenance and normal  
465 lifespan. It would be interesting to investigate the lifespan of other TEI mutants displaying Mrt  
466 phenotypes to determine whether they also show lifespan extension, and hence whether the  
467 germline maintenance activity of the TEI genes has a general link to lifespan.

#### 468 *Conclusion*

469 We have shown that the chromatin modifiers SET-25 and SET-32 are required only to establish a  
470 heritable silencing signal and are dispensable for the maintenance of silencing across subsequent  
471 generations, implying a chromatin-independent mechanism maintains heritable silencing. Whilst 2°  
472 siRNA-associated factors including HRDE-1 and NRDE-2 are required for this maintenance, we have  
473 shown that the abundance of 2° siRNAs does not correlate with heritable silencing potential. This  
474 opens the field to the search for additional mechanisms responsible for the maintenance of  
475 transgenerational epigenetic inheritance.

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484 **Author Contributions**

485 R.W. and A.A. conceived and designed the study and wrote the manuscript. R.W., G.B., M.H., D.H.,  
486 M.L., P.R., and A.A. performed the experiments. R.W., M.L., and A.A. analysed the data. M.L., P.R.,  
487 and A.A. provided expertise and feedback.

488 **Declaration of Interests**

489 The authors declare no competing interests.

490 **References**

491 Akay, A., Di Domenico, T., Suen, K.M., Nabih, A., Parada, G.E., Larance, M., Medhi, R., Berkuyrek,  
492 A.C., Zhang, X., Wedeles, C.J., et al. (2017). The Helicase Aquarius/EMB-4 Is Required to Overcome  
493 Intronic Barriers to Allow Nuclear RNAi Pathways to Heritably Silence Transcription. *Dev. Cell* **42**,  
494 241–255.e6.

495 Alcazar, R.M., Lin, R., and Fire, A.Z. (2008). Transmission dynamics of heritable silencing induced by  
496 double-stranded RNA in *Caenorhabditis elegans*. *Genetics* **180**, 1275–1288.

497 Aoki, K., Moriguchi, H., Yoshioka, T., Okawa, K., and Tabara, H. (2007). In vitro analyses of the  
498 production and activity of secondary small interfering RNAs in *C. elegans*. *EMBO J.* **26**, 5007–5019.

499 Ashe, A., Sapetschnig, A., Weick, E.M., Mitchell, J., Bagijn, M.P., Cording, A.C., Doebley, A.L.,  
500 Goldstein, L.D., Lehrbach, N.J., Le Pen, J., et al. (2012). PiRNAs can trigger a multigenerational  
501 epigenetic memory in the germline of *C. elegans*. *Cell* **150**, 88–99.

502 Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate  
503 ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366.

504 Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71–94.

505 Buckley, B. a., Burkhart, K.B., Gu, S.G., Spracklin, G., Kershner, A., Fritz, H., Kimble, J., Fire, A., and  
506 Kennedy, S. (2012). A nuclear Argonaute promotes multigenerational epigenetic inheritance and  
507 germline immortality. *Nature* **489**, 447–451.

508 Burkhart, K.B., Guang, S., Buckley, B.A., Wong, L., Bochner, A.F., and Kennedy, S. (2011). A pre-  
509 mRNA-associating factor links endogenous siRNAs to chromatin regulation. *PLoS Genet.* **7**,  
510 e1002249.

511 Burton, N.O., Burkhart, K.B., and Kennedy, S. (2011). Nuclear RNAi maintains heritable gene silencing



- 512 in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U. S. A.* *108*, 19683–19688.
- 513 Devanapally, S., Ravikumar, S., and Jose, A.M. (2015). Double-stranded RNA made in *C. elegans*  
514 neurons can enter the germline and cause transgenerational gene silencing. *Proc. Natl. Acad. Sci. U.*  
515 *S. A.* *112*, 2133–2138.
- 516 Farooq, Z., Banday, S., Pandita, T.K., and Altaf, M. (2016). The many faces of histone H3K79  
517 methylation. *Mutat. Res. Mutat. Res.* *768*, 46–52.
- 518 Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S., and Mello, C.C. (1998). Potent and specific  
519 genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* *391*, 806–811.
- 520 Friedland, A.E., Tzur, Y.B., Esvelt, K.M., Colaiácovo, M.P., Church, G.M., and Calarco, J.A. (2013).  
521 Heritable genome editing in *C. elegans* via a CRISPR-Cas9 system. *Nat. Methods* *10*, 741–743.
- 522 Greer, E.L., Maures, T.J., Ucar, D., Hauswirth, A.G., Mancini, E., Lim, J.P., Benayoun, B.A., Shi, Y., and  
523 Brunet, A. (2011). Transgenerational epigenetic inheritance of longevity in *Caenorhabditis elegans*.  
524 *Nature* *479*, 365–371.
- 525 Greer, E.L., Beese-Sims, S.E., Brookes, E., Spadafora, R., Zhu, Y., Rothbart, S.B., Aristizábal-Corrales,  
526 D., Chen, S., Badeaux, A.I., Jin, Q., et al. (2014). A histone methylation network regulates  
527 transgenerational epigenetic memory in *C.elegans*. *Cell Rep.* *7*, 113–126.
- 528 Grishok, A., Tabara, H., and Mello, C.C. (2000). Genetic requirements for inheritance of RNAi in *C.*  
529 *elegans*. *Science* *287*, 2494–2497.
- 530 Gu, S.G., Pak, J., Guang, S., Maniar, J.M., Kennedy, S., and Fire, A. (2012). Amplification of siRNA in  
531 *Caenorhabditis elegans* generates a transgenerational sequence-targeted histone H3 lysine 9  
532 methylation footprint. *Nat. Genet.* *44*, 157–164.
- 533 Guang, S., Bochner, A.F., Burkhart, K.B., Burton, N., Pavelec, D.M., and Kennedy, S. (2010). Small  
534 regulatory RNAs inhibit RNA polymerase II during the elongation phase of transcription. *Nature* *465*,  
535 1097–1101.
- 536 Houri-Ze’evi, L., Korem, Y., Sheftel, H., Faigenbloom, L., Toker, I.A., Dagan, Y., Awad, L., Degani, L.,  
537 Alon, U., and Rechavi, O. (2016). A Tunable Mechanism Determines the Duration of the  
538 Transgenerational Small RNA Inheritance in *C. elegans*. *Cell* *165*, 88–99.
- 539 Hughes, C.S., Foehr, S., Garfield, D.A., Furlong, E.E., Steinmetz, L.M., and Krijgsveld, J. (2014).  
540 Ultrasensitive proteome analysis using paramagnetic bead technology. *Mol. Syst. Biol.* *10*, 757.

- 541 Kalinava, N., Ni, J.Z., Peterman, K., Chen, E., and Gu, S.G. (2017). Decoupling the downstream effects  
542 of germline nuclear RNAi reveals that H3K9me3 is dispensable for heritable RNAi and the  
543 maintenance of endogenous siRNA-mediated transcriptional silencing in *Caenorhabditis elegans*.  
544 *Epigenetics Chromatin* 10.
- 545 Kamath, R.S., Martinez-Campos, M., Zipperlen, P., Fraser, A.G., and Ahringer, J. (2001). Effectiveness  
546 of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis*  
547 *elegans*. *Genome Biol.* 2, 2.1-2.10.
- 548 Klosin, A., Casas, E., Hidalgo-Carcedo, C., Vavouri, T., and Lehner, B. (2017). Transgenerational  
549 transmission of environmental information in *C. elegans*. *Science* (80-. ). 356.
- 550 Lev, I., Seroussi, U., Gingold, H., Bril, R., Anava, S., and Rechavi, O. (2017). MET-2-Dependent H3K9  
551 Methylation Suppresses Transgenerational Small RNA Inheritance. *Curr. Biol.* 27, 1138–1147.
- 552 Maklakov, A.A., and Immler, S. (2016). The Expensive Germline and the Evolution of Ageing. *Curr.*  
553 *Biol.* 26, R577–R586.
- 554 Mao, H., Zhu, C., Zong, D., Weng, C., Yang, X., Huang, H., Liu, D., Feng, X., and Guang, S. (2015). The  
555 Nrde Pathway Mediates Small-RNA-Directed Histone H3 Lysine 27 Trimethylation in *Caenorhabditis*  
556 *elegans*. *Curr. Biol.* 25, 2398–2403.
- 557 McMurchy, A.N., Stempor, P., Gaarenstroom, T., Wysolmerski, B., Dong, Y., Aussanikava, D., Appert,  
558 A., Huang, N., Kolasinska-Zwierz, P., Sapetschnig, A., et al. (2017). A team of heterochromatin factors  
559 collaborates with small RNA pathways to combat repetitive elements and germline stress. *Elife* 6,  
560 e21666.
- 561 Meister, P., and Taddei, A. (2013). Building silent compartments at the nuclear periphery: a  
562 recurrent theme. *Curr. Opin. Genet. Dev.* 23, 96–103.
- 563 Minkina, O., and Hunter, C.P. (2017). Stable Heritable Germline Silencing Directs Somatic Silencing at  
564 an Endogenous Locus. *Mol. Cell* 65, 659–670.e5.
- 565 Miska, E.A., and Ferguson-Smith, A.C. (2016). Transgenerational inheritance: Models and  
566 mechanisms of non-DNA sequence-based inheritance. *Science* 354, 59–63.
- 567 Nishimura, H., Tajima, T., Comstra, H.S., Gleason, E.J., and L'Hernault, S.W. (2015). The  
568 Immunoglobulin-like Gene *spe-45* Acts during Fertilization in *Caenorhabditis elegans* like the Mouse  
569 *Izumo1* Gene. *Curr. Biol.* 25, 3225–3231.

- 570 Norris, A.D., Kim, H.-M., Colaiácovo, M.P., and Calarco, J.A. (2015). Efficient Genome Editing in  
571 *Caenorhabditis elegans* with a Toolkit of Dual-Marker Selection Cassettes. *Genetics* 201, 449–458.
- 572 Pak, J., and Fire, A. (2007). Distinct populations of primary and secondary effectors during RNAi in *C.*  
573 *elegans*. *Science* 315, 241–244.
- 574 Partridge, L., Gems, D., and Withers, D.J. (2005). Sex and Death: What Is the Connection? *Cell* 120,  
575 461–472.
- 576 Rechavi, O., Minevich, G., and Hobert, O. (2011). Transgenerational inheritance of an acquired small  
577 RNA-based antiviral response in *C. elegans*. *Cell* 147, 1248–1256.
- 578 Sapetschnig, A., Sarkies, P., Lehrbach, N.J., and Miska, E.A. (2015). Tertiary siRNAs Mediate  
579 Paramutation in *C. elegans*. *PLoS Genet.* 11, e1005078.
- 580 Shirayama, M., Seth, M., Lee, H.C., Gu, W., Ishidate, T., Conte, D., and Mello, C.C. (2012). PiRNAs  
581 initiate an epigenetic memory of nonself RNA in the *C. elegans* germline. *Cell* 150, 65–77.
- 582 Sijen, T., Fleenor, J., Simmer, F., Thijssen, K.L., Parrish, S., Timmons, L., Plasterk, R.H., and Fire, A.  
583 (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* 107, 465–476.
- 584 Sijen, T., Steiner, F.A., Thijssen, K.L., and Plasterk, R.H.A. (2007). Secondary siRNAs result from  
585 unprimed RNA synthesis and form a distinct class. *Science* 315, 244–247.
- 586 Snyder, M.J., Lau, A.C., Brouhard, E.A., Davis, M.B., Jiang, J., Sifuentes, M.H., and Csankovszki, G.  
587 (2016). Anchoring of Heterochromatin to the Nuclear Lamina Reinforces Dosage Compensation-  
588 Mediated Gene Repression. *PLOS Genet.* 12, e1006341.
- 589 Spracklin, G., Fields, B., Wan, G., Becker, D., Wallig, A., Shukla, A., and Kennedy, S. (2017). The RNAi  
590 Inheritance Machinery of *Caenorhabditis elegans*. *Genetics* 206, 1403–1416.
- 591 Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., and Mello, C.C.  
592 (1999). The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* 99, 123–132.
- 593 Towbin, B.D., González-Aguilera, C., Sack, R., Gaidatzis, D., Kalck, V., Meister, P., Askjaer, P., and  
594 Gasser, S.M. (2012). Step-wise methylation of histone H3K9 positions heterochromatin at the  
595 nuclear periphery. *Cell* 150, 934–947.
- 596 Vastenhouw, N.L., Brunschwig, K., Okihara, K.L., Müller, F., Tijsterman, M., and Plasterk, R.H.A.  
597 (2006). Gene expression: long-term gene silencing by RNAi. *Nature* 442, 882.

- 598 Ward, S., and Carrel, J.S. (1979). Fertilization and sperm competition in the nematode  
599 *Caenorhabditis elegans*. *Dev. Biol.* *73*, 304–321.
- 600 Watson, M., Schnettler, E., and Kohl, A. (2013). viRome: an R package for the visualization and  
601 analysis of viral small RNA sequence datasets. *Bioinformatics* *29*, 1902–1903.
- 602 Weiser, N.E., Yang, D.X., Feng, S., Kalinava, N., Brown, K.C., Khanikar, J., Freeberg, M.A., Snyder, M.J.,  
603 Csankovszki, G., Chan, R.C., et al. (2017). MORC-1 Integrates Nuclear RNAi and Transgenerational  
604 Chromatin Architecture to Promote Germline Immortality. *Dev. Cell* *41*, 408–423.e7.
- 605 Yigit, E., Batista, P.J., Bei, Y., Pang, K.M., Chen, C.-C.G., Tolia, N.H., Joshua-Tor, L., Mitani, S., Simard,  
606 M.J., and Mello, C.C. (2006). Analysis of the *C. elegans* Argonaute Family Reveals that Distinct  
607 Argonautes Act Sequentially during RNAi. *Cell* *127*, 747–757.
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610 **Figure Titles and Legends**

611 **Figure 1. *set-25* and *set-32* are required for transgenerational epigenetic inheritance to the F1**

612 **generation. A.** The percentage of GFP-silenced animals following exposure to GFP RNAi. P0 animals  
613 were exposed to GFP RNAi. F1-F3 animals were not exposed to the RNAi trigger, and were created  
614 by selecting silenced individuals and allowing them to self-reproduce. Data are mean  $\pm$ SEM, n>100.  
615 Comparisons were performed by two-way ANOVA with Tukey's post hoc. Statistics shown are  
616 mutant F1 compared to sensor strain F1, \*\*\*\* p<0.0001 **B.** Scheme of experiment to determine the  
617 requirement for *set-25* and *set-32* in the P0 or F1 generation for heritable silencing. *set-32* or *set-25*  
618 (*set-x*) heterozygous individuals were exposed to GFP RNAi. Their unexposed F1 progeny were  
619 scored for silencing inheritance, where an increased proportion of GFP positive worms compared  
620 with wild-type would indicate a requirement for SET-32 or SET-25 in the F1 generation. GFP positive  
621 worms were also collected and genotyped. **C.** The percentage of GFP-silenced animals produced by  
622 the assay in B. Data are mean  $\pm$ SEM, n>100. Comparisons were performed by two-way ANOVA with  
623 Tukey's post hoc. Statistics shown are mutant F1 compared to sensor strain F1, \*\*\*\* p<0.0001 **D.**  
624 The observed number of wild-type (+/+), heterozygous (+/-) and homozygous mutant (-/-) GFP  
625 positive F1 animals produced by the assay in B, and the expected number according to Mendel's  
626 ratio. p values calculated by chi-square tests. **E.** Proposed three-step model of TEI.

627 **Figure 2. Small RNA analysis in the context of transgenerational epigenetic inheritance. A.** The

628 number of reads mapping to the GFP transgene in the sense (i) and antisense (ii) orientation for six  
629 different strains is shown. Points indicate the replicate while the line indicates the mean. Strains are  
630 identified by colour. **B.** Antisense *gfp*-mapping siRNAs (2° siRNAs) are shown for the P0 and F1  
631 generations in various mutant strains as indicated. F1 animals were split into GFP silenced ('OFF')  
632 and GFP expressing ('ON') pools before library preparation. **C.** 2° siRNAs for the F1 generation only.  
633 **D.** Fold change of GFP mapping 2° siRNAs in GFP silenced ('OFF') vs GFP expressing ('ON') animals.  
634 Dashed line indicates equal expression between silenced and expressing animals. **E-I.** Small RNAs in

635 reads per million (rpm) with the indicated biotype. Strains are indicated: wt is wild-type, *s25* is *set-*  
636 *25(n5021)*, *s32<sup>CR</sup>* is *set-32(smb11)*, *s32<sup>Del</sup>* is *set-32(ok1457)*, *nrde* is *nrde-2(mj168)*, *hrde* is *hrde-*  
637 *1(tm1200)*. The top panel shows P0 control and RNAi-treated combined (P0) and F1 GFP-expressing  
638 and GFP-silenced animals combined (F1). Two-way ANOVA was performed and results for variance  
639 at the genotype or generation level is shown. The bottom panel shows P0 and F1 individually,  
640 normalised to the relevant control (dashed black line). Comparisons were performed by two-way  
641 ANOVA with Tukey's post hoc. ^ 0.05<p<0.1 \* p<0.05 \*\* p<0.01 \*\*\*\* p<0.0001

642 **Figure 3. SET-32 and SET-25 expression patterns and H3K9 methylation analysis. A.** One germline of  
643 a representative L4 mCherry::SET-25 expressing animal. Germline is outlined in white. **B.** Nuclear  
644 localisation of mCherry::SET-25 in the mitotic zone of an adult *C. elegans*. **C.** Embryo expressing  
645 mCherry::SET-25 (left). Chromatin is marked by HIS-58::GFP (middle). Arrows indicate dividing cells  
646 with condensed chromatin. **D.** The entire germline of a wild-type adult (top), and SET-32::mCherry  
647 expressing adult (middle) and L4 (bottom) animals. Germline is outlined in white. **E.** Fluorescence  
648 intensity in the entire germline of SET-32::mCherry (n=15) and wild-type (n=11) animals was  
649 quantified using ImageJ. Comparison was performed by t-test. \*\*\*\* p<0.0001. **F.** Representative  
650 single confocal plane images of dissected one-day-old adult gonads of the indicated strain. The  
651 mitotic zone is on the left and the pachytene zone is on the right. Panels show a portion of the  
652 mitotic zone (enlarged from the region indicated by the dashed white box), anti-H3K9me3 staining  
653 (yellow), DAPI staining (blue), and an overlay of H3K9me3 and DAPI staining, as indicated. Strains  
654 were imaged under identical parameters within staining conditions. All scale bars in **A-F** represent  
655 20  $\mu$ m. **G.** Quantification of fluorescence intensity of H3K9me3 staining in the mitotic and pachytene  
656 zones. ImageJ was used to generate an intensity threshold-based mask for nuclei using the DAPI  
657 signal, then the average intensity of H3K9me3 staining in nuclei was measured. Data are mean  
658  $\pm$ SEM, n=25-39. Comparisons were performed by two-way ANOVA with Tukey's post hoc. **H.** Ratio of  
659 fluorescence intensity in mitotic:pachytene zones. Data are mean  $\pm$ SEM, n=25-39. Comparison was  
660 performed by t-test. \*\*\*\* p<0.0001 **I.** Quantification of H3K9, H3K23 and H3K27 levels in the

661 indicated mutant strains by mass spectrometry. For each modification, levels were normalised to a  
662 relevant control peptide and are displayed as % of wild-type (indicated by dotted line). Data are  
663 mean  $\pm$ SEM, n=3 replicates for each mutant strain. Comparisons were performed by two-way  
664 ANOVA with Dunnett's post hoc.  $\wedge$  p=0.058, \* p<0.05, \*\*\*\* p<0.0001

665 **Figure 4. *set-32* mutants display a mortal germline phenotype, defective sperm, and extended**  
666 **lifespan. A.** Animals were shifted to 25°C at L3 and the brood size of successive generations created  
667 by passaging individual worms was counted. Data are mean  $\pm$ SEM, n=20 lines. **B-C.** The total number  
668 of **B.** live progeny and **C.** unfertilised eggs per animal was counted. Data are mean  $\pm$ SEM, n=18-20.  
669 Comparisons were performed by one-way ANOVA with Dunnett's post hoc and Tukey's post hoc  
670 respectively. \* p<0.05 \*\*\* p<0.001 \*\*\*\* p<0.0001 **D.** Brood sizes of wild-type or *set-32(ok1457)*  
671 hermaphrodites mated to wild-type males were counted. Data are mean  $\pm$ SEM, n=19. Comparison  
672 was performed by t-test. **E.** Sperm nuclei were visualised by DAPI and the number of sperm in one  
673 spermatheca per animal was counted 12 hours (n=6) and 24 hours (n=10-12) after L4. Data are mean  
674  $\pm$ SEM. Comparisons were performed by two-way ANOVA with Sidak's post hoc. \*\*\*\* p<0.0001 **F.**  
675 Representative maximum intensity Z-projection of a spermatheca in DAPI-stained animals, 12 hours  
676 and 24 hours after L4. **G.** Representative images of DAPI-stained animals 24 hours after L4. In *set-*  
677 *32(ok1457)* animals, sperm can be detected throughout the uterus, indicated by red arrowheads. In  
678 **F-G** scale bars represent 50  $\mu$ m and the position of the spermatheca is indicated by dashed yellow  
679 line. **H.** Survival curves for animals of the indicated genotype in the absence of FUDR. p<0.0001 for  
680 *set-32(smb11)* vs. wild-type and *set-32(ok1457)* vs. wild-type, ns for *set-25 (n5021)* vs. wild-type.  
681 n=110 at Day 0, comparisons were performed by Log-rank test.

## 682 **Methods**

### 683 Strain list

684 Wild-type Bristol N2, SX461 *mjls31[ppie-1::gfp::h2b]* II, AKA33 *set-32(smb11)* I; *mjls31[ppie-*  
685 *1::gfp::h2b]* II, AKA35 *mjls31[ppie-1::gfp::h2b]* II; *set-25(n5021)* III (outcrossed 6x from MT17463),  
686 AKA36 *set-32(ok1457)* I; *mjls31[ppie-1::gfp::h2b]* II (outcrossed 6x from VC967), AKA37 *set-*  
687 *32(ok1457)* I; *mjls31[ppie-1::gfp::h2b]* II; *set-25(n5021)* III, SX1442 *mjls31[ppie-1::gfp::h2b]* II; *nrde-*  
688 *2(mj168)* II, SX2127 *mjls31[ppie-1::gfp::h2b]* II; *hrde-1(tm1200)* III, AKA48 *mjls31[ppie-1::gfp::h2b]* II;  
689 *mCherry::set-25+loxP(smb16)* III, PHX229 *set-32(syb229)* I, AKA59 *mjls31[ppie-1::gfp::h2b]* II;  
690 *rhIs4[glr-1::gfp]* III; *him-8(e1489)* IV, AKA60 *set-32(ok1457)* I; *mjls31[ppie-1::gfp::h2b]* II, *rhIs4[glr-*  
691 *1::gfp]* III; *him-8(e1489)* IV.

692 In all experiments strains were in *mjls31[ppie-1::gfp::h2b]* background and SX461 was used as the  
693 wild-type, except in expression experiments (Figure 3A-D and Supplementary Figure S3A) where N2  
694 was used as the wild-type.

#### 695 Cultivation and maintenance of *C. elegans*

696 Animals were cultured according to standard procedures (Brenner, 1974). Unless otherwise  
697 indicated, animals were grown on Nematode Growth Medium (NGM) (2% (w/v) agar, 50 mM NaCl,  
698 0.25% (w/v) peptone, 1 mM CaCl<sub>2</sub>, 5 µg/ml cholesterol, 25 mM K<sub>3</sub>PO<sub>4</sub> and 1 mM MgSO<sub>4</sub> in H<sub>2</sub>O)  
699 plates seeded with OP50 *E. coli* bacteria, and experiments were performed at 20°C.

#### 700 *C. elegans* synchronisation

701 To obtain synchronised adults for extraction of small RNAs, gravid adults were bleached and the  
702 resulting staged embryos were plated and grown for 4 days. To obtain synchronised animals for  
703 other experiments, young adults laid embryos for 2 hours before being removed from plates.  
704 Resulting staged embryos were grown for ~60 hours to produce larval stage 4 (L4) animals.

#### 705 CRISPR/Cas9



706 All plasmid sequences were confirmed by Sanger sequencing and purified with DNA Clean and  
707 Concentrator-5 (Zymo Research). sgRNA target sequences were designed and incorporated into a  
708 *pU6::klp-12* sgRNA expression vector by PCR as previously described (Friedland et al., 2013; Norris et  
709 al., 2015). To create the *set-32(smb11)* deletion, an injection mix was injected into gonads of young  
710 adult animals consisting of sgRNA expression vector (150 ng/μL), Cas9 expression vector (*peft-*  
711 *3::cas9::tbb-2*) (150 ng/μL) (a kind gift from the de Bono Lab), pCFJ90 (*pmyo-2::mCherry::unc-54*) (5  
712 ng/μL) and pCFJ104 (*pmyo-3::mCherry::unc-54*) (5 ng/μL). PCR was performed on the genomic DNA  
713 of mCherry positive animals to identify deletions. To create the *mCherry::set-25* strain (AKA48) we  
714 used the strategy outlined in Norris et al. (2015). Briefly, a repair template was constructed from  
715 approximately 950 base pair homology arms cloned into a loxP\_myo2\_neoR\_mCherry\_intron repair  
716 template vector also containing a neomycin resistance gene and *pmyo-2::GFP* (Norris et al., 2015).  
717 Homology arms contained synonymous mutations at the sgRNA target site. An injection mix was  
718 injected into gonads of young adult animals consisting of the sgRNA expression vector (200 ng/μL),  
719 repair template (70 ng/μL), Cas9 expression vector (45 ng/μL), pCFJ90 (5 ng/μL) and pCFJ104  
720 (7 ng/μL). Transgenic animals were identified by survival after addition of G418 (500 μL of 25 mg/mL  
721 solution added to standard 6 cm plates). Once integrated lines were confirmed, animals were  
722 injected with pDD104 (*peft-3::Cre*) (50 ng/μL) and pCFJ90 (5 ng/μL). Successful Cre recombination  
723 was detected by the loss of pharyngeal GFP. Correct integration and Cre recombination was  
724 confirmed by Sanger sequencing across the homology arms. The *set-32::mCherry* strain was  
725 generated by SunyBiotech (China) and correct integration confirmed by sequencing.

#### 726 RNAi inheritance assays

727 RNAi was performed by feeding as previously described (Kamath et al., 2001). HT115(DE3) bacteria  
728 carrying IPTG-inducible L4440 (empty vector) or L4440-*gfp* plasmids was grown at 37°C for 7-8 hours  
729 with 100 μg/mL ampicillin. Cultures were seeded on NGM plates containing 25 μg/mL carbenicillin  
730 and 1 mM IPTG grown overnight at room temperature. Young adults were plated onto RNAi bacteria

731 and their progeny scored for the presence of GFP as adults 4 days later. Silenced adults were  
732 transferred to OP50 plates to produce subsequent generations.

733 To test for a requirement in the P0 or F1 generation, *set-32(ok1457)* or *set-25(n5021)* L4  
734 hermaphrodites were crossed to wild-type males on OP50 plates. 24 hours later, fertilised adult  
735 hermaphrodites were plated onto RNAi food plates prepared as above. 3 days later, L4  
736 hermaphrodite progeny (the P0 generation) were moved to new RNAi plates to prevent mating with  
737 male progeny. 24 hours later, adult hermaphrodites were transferred to OP50 plates to produce the  
738 F1 generation. The P0 adults were then lysed and genotyped to confirm heterozygosity, and GFP-  
739 positive F1 offspring were lysed and genotyped.

740 Each experiment was performed in triplicate (5 independent plates per replicate), with ~100 animals  
741 per generation scored in each replicate. Scoring was performed blind to the genotype of the strains.

#### 742 Small RNA sequencing

743 The RNAi inheritance assay was performed as above, with the exception that populations were  
744 created by plating bleached embryos. RNAi-treated P0 animals (~200 per replicate), and F1 animals  
745 (~20 per replicate) sorted into GFP-expressing or -silenced groups, were collected in 1 mL TriSure  
746 (Bioline). Animals were cracked by five freeze/thaw cycles in liquid nitrogen, and RNA extracted  
747 using the manufacturer's instructions with the exception that 1  $\mu$ L Glycogen (20 mg/mL, Roche) was  
748 added as a carrier during the precipitation step, which was performed overnight at 20 °C. 250 – 500  
749 ng of RNA was treated with 5' polyphosphatase (Epicentre) following the manufacturer's instructions  
750 after which small RNA libraries were prepared using the NEBNext Multiplex Small RNA Library Prep  
751 Set essentially as described in the manual. Sequencing of libraries was performed by AGRF on an  
752 Illumina HiSeq2500. The experiment was performed in duplicate except for strains AKA33 and  
753 SX2127 for which the P0 generation was performed in triplicate.

#### 754 Bioinformatic analysis

755 All initial QC, trimming and mapping analysis was performed using CLC Genomics (Qiagen). Libraries  
756 were trimmed to remove adapters and filtered for a quality score of >30. Reads were mapped to  
757 WormBase release WS260 and the GFP transgene with a maximum of one mismatch. For the GFP-  
758 mapping siRNA analysis, BAM files were exported from CLC Genomics and analysed in R using a  
759 combination of the viRome package (Watson et al., 2013) and custom scripts. For endo siRNA  
760 analysis, the number of reads mapping to particular biotypes was determined in CLC Genomics using  
761 WormBase WS260 annotations and extracted. Data analysis and visualisation was performed using  
762 Excel and GraphPad Prism. Sequence data has been submitted to the SRA at NCBI (BioProject ID  
763 PRJNA431114).

#### 764 Microscopy and analysis

765 Animals were scored for the presence or absence of GFP with a Nikon SMZ18 Microscope.

766 For Figure 3A-E and Figure S3A, animals were immobilised in M9 with 0.2% Tetramizol and mounted  
767 on glass slides. DIC and fluorescent imaging was performed using standard methods using a Leica  
768 Sp5 Multiphoton confocal microscope (SET-32::mCherry and mCherry::SET-25) and a Nikon Ti-E  
769 spinning disk microscope (mCherry::SET-25). For SET-32::mCherry analysis, all images of the tagged  
770 strain and N2 control animals were taken under identical conditions. Quantification was performed  
771 using ImageJ and statistical tests performed in Excel.

772 For immunofluorescence experiments in Figure 3F-H and Figure S3B-D, germlines were imaged using  
773 a Nikon C2 Basic Confocal microscope. Within conditions (DAPI, H3K9me2, and H3K9me3 staining) all  
774 strains were imaged under identical parameters. Average fluorescence intensity was quantified  
775 using ImageJ (n=25-39). An intensity threshold-based mask for nuclei was generated using the DAPI  
776 signal then used to determine the average intensity of H3K9me3 and H3K9me2 staining in nuclei, to  
777 control for variable space between nuclei in different germlines. In all strains we observed lower  
778 average intensity of H3K9me3 in the mitotic zone of the germline compared with the pachytene

779 zone and so quantified intensity for each separately: average intensity was measured for the mitotic  
780 and pachytene zones of the germline separately by drawing a region around the whole mitotic zone,  
781 and around the beginning of the pachytene zone of equal size (by number of cells) to the mitotic  
782 region (n=24-39). The ratio of intensity in the mitotic:pachytene zones was calculated by dividing the  
783 mitotic intensity by the pachytene intensity for each germline. H3K9me2 staining was consistent  
784 throughout the germline, so the mitotic region was used for quantification. Intensity in individual  
785 nuclei was quantified by drawing individual regions around the 20 most distal germline nuclei in a  
786 single plane in 4 representative animals per strain.

787 For Figure 4E-G, synchronised L4 hermaphrodites were incubated for 12 or 24 hours, then fixed as  
788 previously described (Nishimura et al., 2015). DNA was visualised with DAPI. Imaging was performed  
789 using an Olympus FluoView FV1000 Confocal microscope, and processed using Olympus FluoView  
790 software. A Z-stack of images from one spermatheca per hermaphrodite was collected and flattened  
791 to create a maximum intensity projection image using ImageJ. Sperm from one spermatheca per  
792 hermaphrodite were counted as previously described (Nishimura et al., 2015) (n=6 for 12 hours,  
793 n=10-11 for 24 hours).

#### 794 Germline immunofluorescence

795 One-day-old adult hermaphrodites were dissected in M9 with 0.05% Tetramizol to release gonads  
796 onto poly-L-lysine coated slides. Germlines were cracked by freeze/thawing in liquid nitrogen, then  
797 fixed in -20°C methanol for 1 min followed by 3.7% paraformaldehyde, 1xPBS, 0.08 M HEPES (pH  
798 6.9), 1.6 mM MgSO<sub>4</sub>, 0.8 mM EGTA for 30 min. Primary antibodies used were rabbit polyclonal to  
799 Histone H3 (tri methyl K9) (Ab8898, Abcam) and mouse monoclonal to Histone H3 (di methyl K9)  
800 (Ab1220, Abcam), diluted 1:300 in 30% normal goat serum in PBS. Secondary antibodies used were  
801 goat anti-rabbit Alexa Fluor 488 and rabbit anti-mouse Alexa Fluor 555 (Invitrogen), diluted 1:1000 in  
802 30% normal goat serum in PBS. DNA was visualised with DAPI.

### 803 Protein Subcellular Fractionation

804 Large-scale populations of animals were grown on enriched peptone plates (20 mM NaCl, 20 g/L  
805 peptone, 25 g/L agar, 5 µg/mL cholesterol, 1 mM MgSO<sub>4</sub>, 25 mM K<sub>3</sub>PO<sub>4</sub>) seeded with NA22 *E. coli*  
806 bacteria. Animals were washed several times in M9 buffer before homogenisation. Approximately  
807 20,000 whole worms per strain were fractionated using a detergent solubility-based kit designed for  
808 tissue separations (Pierce Tissue Subcellular Fractionation Kit, Thermo). Briefly, whole worms were  
809 resuspended in 1 mL of cytosol extraction buffer containing protease inhibitors, combined with an  
810 equal volume of 0.7 mm zirconia beads in a 2 mL screw-cap tube, and bead-beated for 5 seconds at  
811 4°C using in a BioSpec Products MiniBeadBeater-24. This extract was fractionated according to  
812 manufacturer's instructions for the Pierce Tissue Subcellular Fractionation Kit (Thermo). The protein  
813 content in each fraction was quantified by a BCA protein assay (Thermo).

### 814 Protein digestion, peptide clean-up and quantitation

815 Proteins from the chromatin fraction (50 µg) were denatured and reduced in 2% SDS and 10 mM  
816 tris(carboxyethyl)phosphine (TCEP), and 20 mM sodium phosphate buffer pH 6.0 and 150 mM NaCl.  
817 The protein samples were heated to 65°C in a ThermoMixer-C (Eppendorf) for 10 min at 1000 rpm.  
818 Once cooled to room temperature, N-ethylmaleimide (NEM) was added to the fractions at a final  
819 concentration of 20 mM and allowed to incubate for 30 min at room temperature. The fractions  
820 were buffer exchanged and trypsin digested using the SP3 method described previously (Hughes et  
821 al., 2014).

### 822 LC-MS/MS and analysis of spectra

823 Using a Thermo Fisher Scientific EasyLC 1200 UHPLC, peptides in 4% (vol/vol) formic acid (injection  
824 volume 3 µL, approximately 500 ng peptides) were directly injected onto a 50 cm × 75 µm reverse  
825 phase C18 column with 1.9 µm particles (Dr. Maisch GmbH) with integrated emitter. Peptides were  
826 separated over a gradient from 4% acetonitrile to 32% acetonitrile over 30 min with a flow rate of

827 300 nL min<sup>-1</sup>. The peptides were ionized by electrospray ionization at +2.3 kV. Tandem mass  
828 spectrometry analysis was carried out on a Q-Exactive HF mass spectrometer (Thermo Fisher  
829 Scientific) using HCD fragmentation. The data-dependent acquisition method used acquired MS/MS  
830 spectra on the top 5 most abundant ions at any one point during the gradient. All the RAW MS data  
831 have been deposited to the ProteomeXchange Consortium  
832 (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository with the dataset  
833 identifier PXD008754, username: reviewer17814@ebi.ac.uk, password: 9GD5YNDU. The RAW data  
834 produced by the mass spectrometer were analysed using Proteome Discoverer 2.2 (Thermo) and the  
835 Byonic Search Engine (Protein Metrics). Peptide and protein level identification were both set to a  
836 false discovery rate of 1% using a target-decoy based strategy. The database supplied to the search  
837 engine for peptide identifications was a combined *C. elegans* and *E. coli* Swissprot database  
838 downloaded on the 11th April 2017. The mass tolerance was set to 3 ppm for precursor ions and  
839 MS/MS mass tolerance was set at 10 ppm. Enzyme was set to trypsin (cleavage C-terminal to R/K)  
840 with up to 3 missed cleavages. Deamidation of N/Q, oxidation of M were set as common variable  
841 modifications of which only 1 was allowed. N-terminal pyro-E/Q, protein N-terminal acetylation,  
842 acetylation of K, methylation of K/R, dimethylation of K/R, trimethylation of K were set as rare  
843 variable modifications of which only 2 were allowed. N-ethylmaleimide on C was searched as a fixed  
844 modification. The output from the Byonic search has also been uploaded to the ProteomeXchange  
845 Consortium under the same identifier given above.

#### 846 Histone Peptide Quantitation

847 The ratio of each modified peptide to a control peptide (either the cognate unmodified peptide, or  
848 an unmodified peptide from elsewhere in the protein) was calculated from extracted ion  
849 chromatograms of each, across all samples. The area under each peak was integrated and ratios  
850 calculated. Significance was calculated using two-way ANOVA in GraphPad Prism.

#### 851 Mortal germline assays

852 Animals were maintained at 20°C for at least 5 generations before being shifted to 25°C at L3 stage.  
853 20 replicate lines were created from individual animals and maintained as separate populations  
854 throughout the experiment. A single L3 animal per line was picked to a new plate to create each  
855 subsequent generation. In the regular mortal germline assay, the mean brood size per strain was  
856 calculated at each generation by averaging the number of progeny between lines. When a sterile  
857 individual arose resulting in no progeny to produce the next generation, the line was discarded. For  
858 the purpose of data analysis discarded lines were recorded as having zero progeny for all subsequent  
859 generations. In the cumulative sterility assay, the number of times sterility arose was counted. When  
860 a sterile individual arose, that individual was replaced with an animal from a backup population  
861 which had been maintained at 25°C for the same number of generations in order to retain a  
862 consistent number of lines. For both assays, scoring was performed blind to the strain genotype.

#### 863 Brood size assays

864 To measure regular brood size, L4 hermaphrodites were plated onto growth plates and transferred  
865 every 12 hours for the first three days, then every 24 hours until they had stopped laying or died.  
866 Plates from which animals had been transferred were incubated for 48 hours, then the numbers of  
867 live progeny and unfertilised eggs scored.

868 To test for a male germline defect, *set-32(ok1457)* or wild-type control L4 hermaphrodites were  
869 mated to wild-type L4 males for 24 hours. Males were then removed, and hermaphrodites  
870 transferred every 12 hours for the first three days, then every 24 hours until they had stopped laying  
871 or died. Live progeny were scored as above. The percentage of male progeny was monitored, and  
872 only the progeny of successfully-mated hermaphrodites were included in analysis, indicated by the  
873 presence of ~50% males.

874 To test for a defect in male sperm, wild-type L4 hermaphrodites were mated to control *him-8(e1489)*  
875 L4 males or mutant *set-32(ok1457); him-8(e1489)* L4 males carrying an integrated *glr-1::gfp*

876 neuronal reporter transgene for 24 hours. Males were then removed, and hermaphrodites  
877 transferred every 24 hours until they had stopped laying or died. Numbers of GFP-positive (cross-  
878 progeny) and -negative (self-progeny) live offspring were scored.  
879 Each experiment was performed in duplicate with n=10 animals at Day 0 per replicate. Animals  
880 which died or were lost within the first 24 hours of adulthood were excluded from analysis. Scoring  
881 was performed blind to the strain genotype.

#### 882 Lifespan assays

883 Synchronised L4 animals were plated on growth plates in the absence (n=110) or presence (n=100)  
884 of FUDR (100  $\mu$ M). In the absence of FUDR, animals were transferred to new plates every day for the  
885 first 8 days to separate adults from progeny, and then once per week until death. In the presence of  
886 FUDR, animals were transferred once after 10 days. Animals were scored daily and considered dead  
887 when they did not respond to gentle touch with a platinum wire. Animals that displayed vulval  
888 rupture or progeny hatching within the parent were removed from plates and censored from  
889 analysis. Scoring was performed blind to the strain genotype.

#### 890 **Supplemental Information Titles and Legends**

891 **Figure S1. The *set-25* and *set-32* loci and mutant alleles. A.** Schematic representing the *set-25* and  
892 *set-32* transcripts. Exons are represented by black boxes, introns by connecting lines, and  
893 untranslated regions by white boxes. The sequence encoding the SET domain is indicated in yellow.  
894 Brackets indicate deleted sequence in mutant alleles. **B.** Predicted protein for *set-25*, *set-32* and  
895 mutants. The location of the SET domain is indicated in yellow. aa denotes length in amino acids.

896 **Figure S2. Extended small RNA analysis. A.** Small RNAs (reads per million) in the wild-type strain  
897 that map to the indicated biotype. **B-D.** Small RNAs (reads per million) with the indicated biotype.  
898 Strains are indicated: wt is wild-type, *s25* is *set-25(n5021)*, *s32<sup>CR</sup>* is *set-32(smb11)*, *s32<sup>Del</sup>* is *set-*  
899 *32(ok1457)*, *nrde* is *nrde-2(mj168)*, *hrde* is *hrde-1(tm1200)*. The top panel shows P0 control and

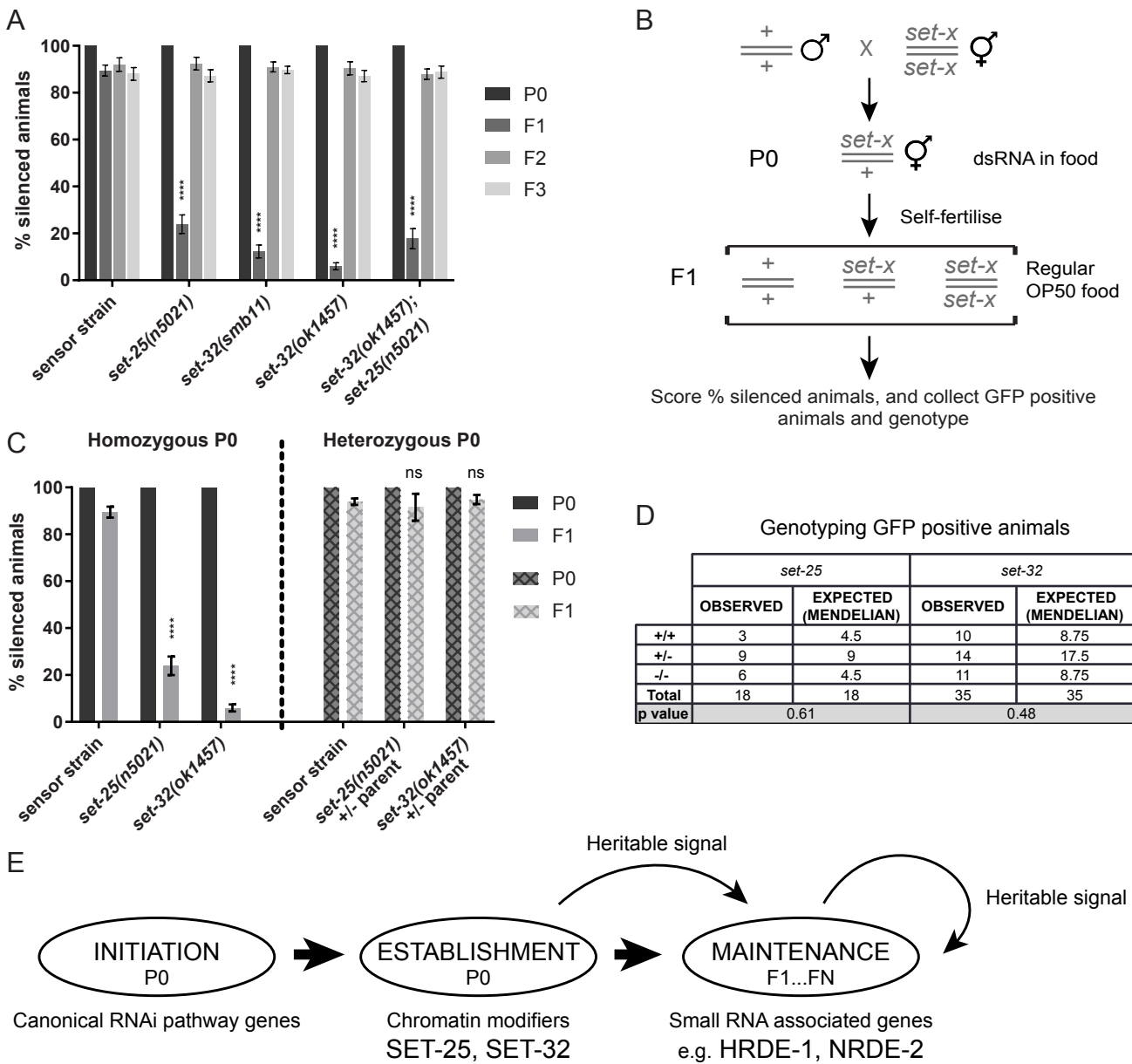


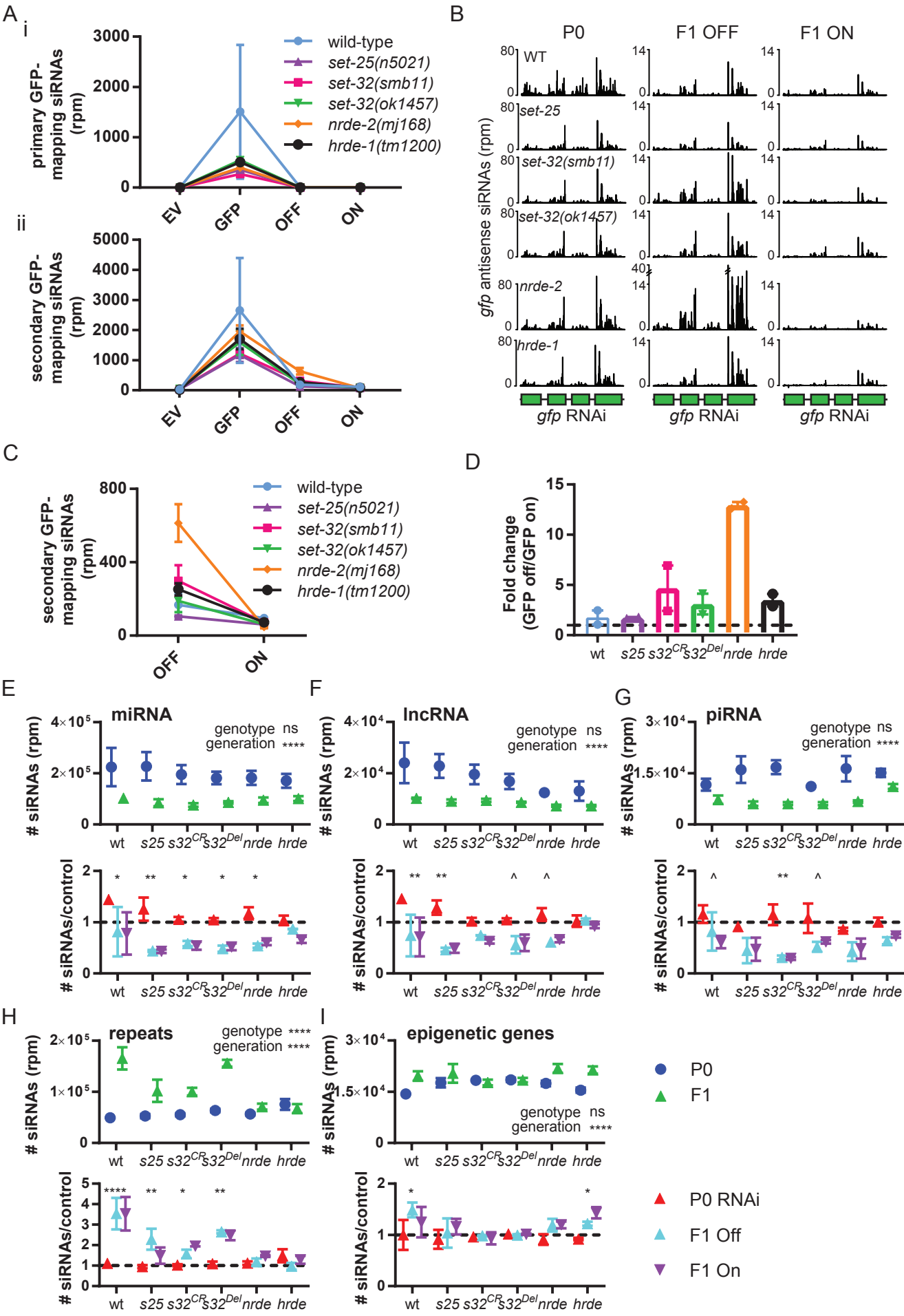
900 RNAi-treated combined (P0) and F1 GFP-expressing and GFP-silenced animals combined (F1). Two-  
901 way ANOVA was performed and results for variance at the genotype or generation level is shown.  
902 The bottom panel shows P0 and F1 individually, normalised to the relevant control (dashed black  
903 line). Comparisons were performed by two-way ANOVA with Tukey's post hoc. \*  $p < 0.05$  \*\*  $p < 0.01$   
904 \*\*\*\*  $p < 0.0001$

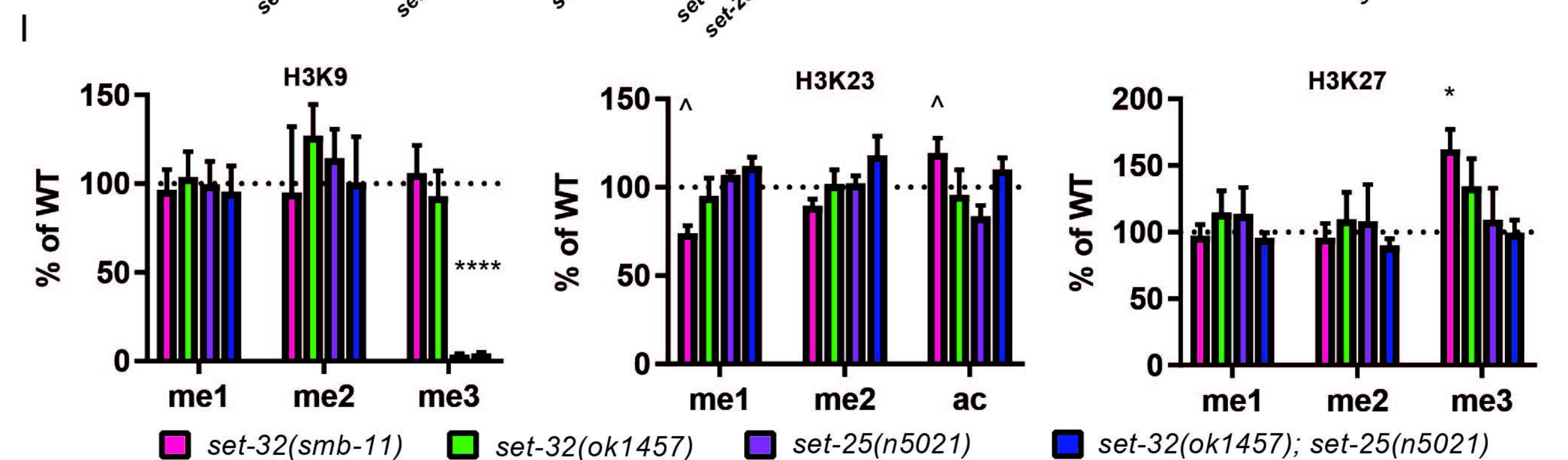
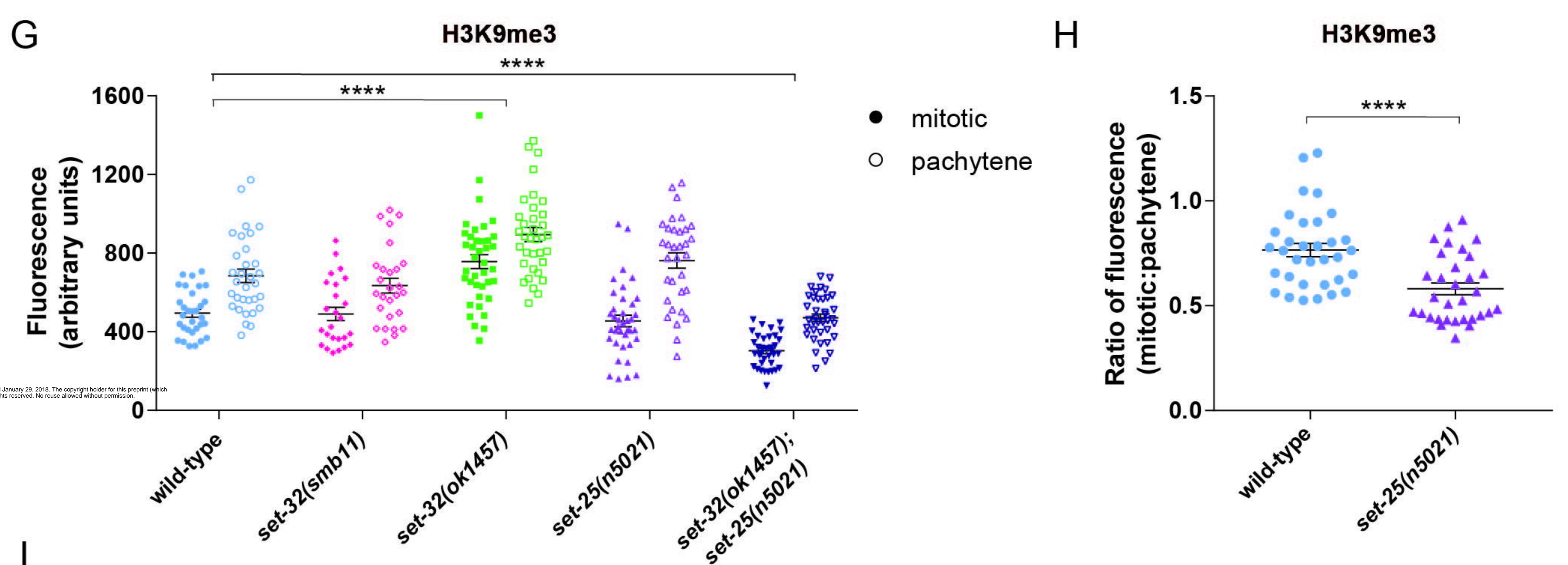
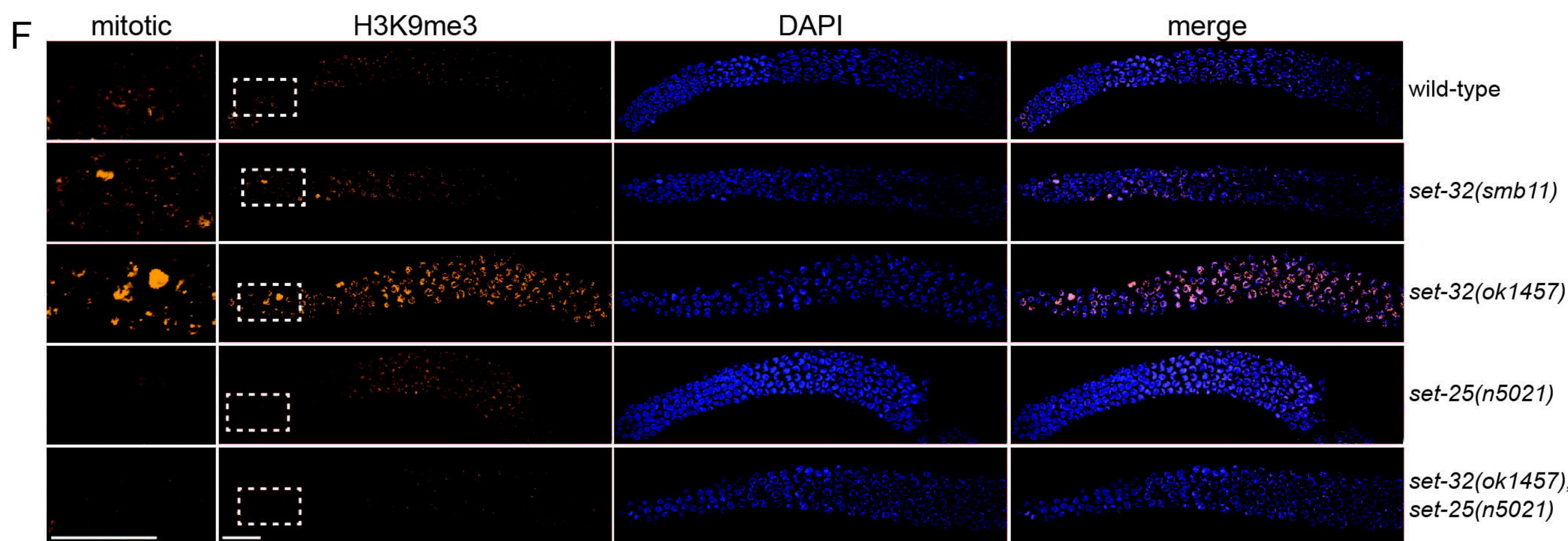
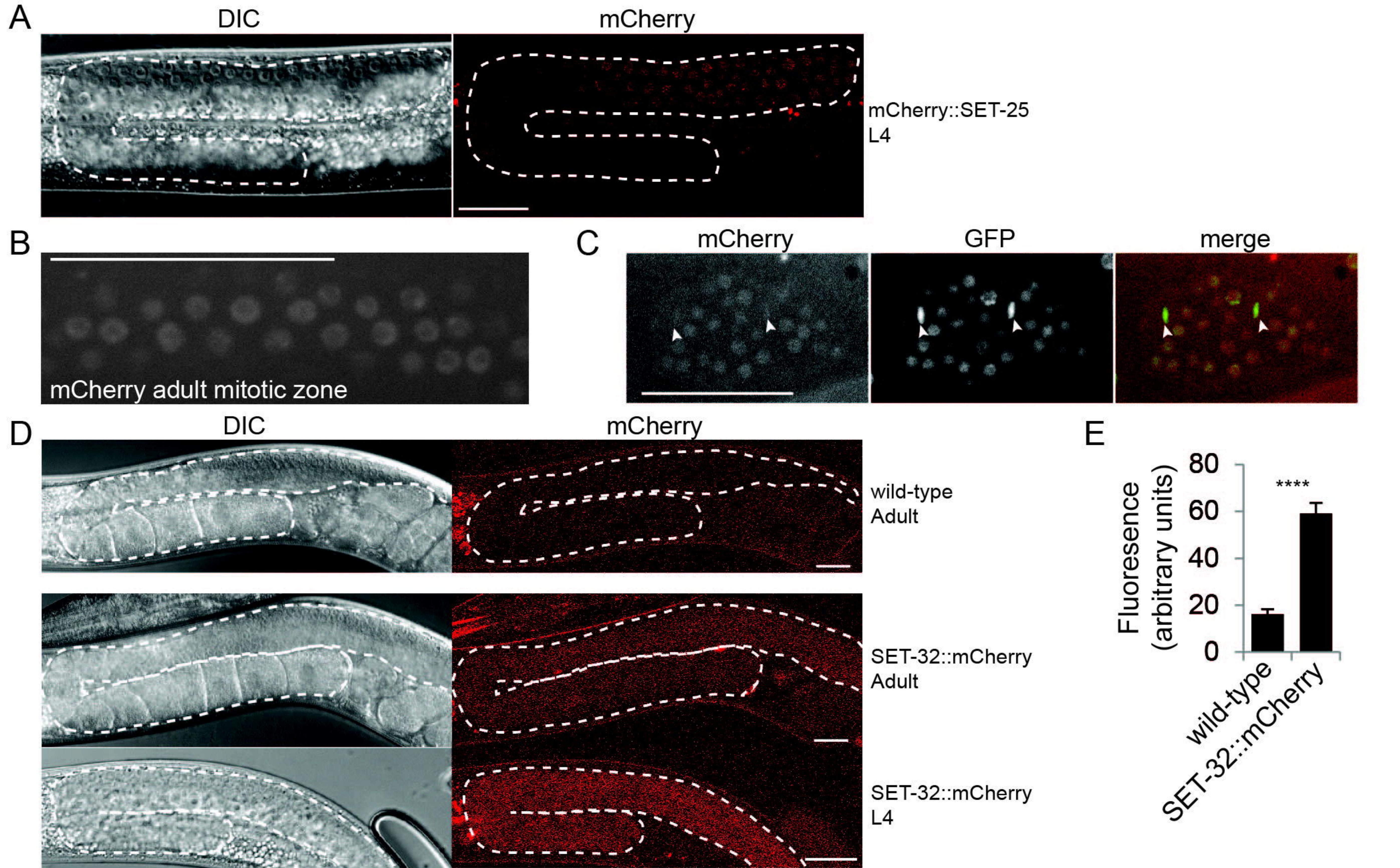
905 **Figure S3. SET-32 expression patterns and H3K9 methylation analysis. A.** The entire germline of  
906 representative SET-32::mCherry expressing animals and wild-type animals of the indicated age.  
907 Germline is outlined in white. **B.** Representative single confocal plane images of the mitotic zone of  
908 dissected adult gonads of the indicated strain. Panels show anti-H3K9me2 staining (yellow), DAPI  
909 staining (blue), and an overlay of H3K9me2 and DAPI staining, as indicated. Strains were imaged  
910 under identical parameters within staining conditions. Scale bars in **A-B** represent 20  $\mu\text{m}$ . **C.**  
911 Quantification of fluorescence intensity of H3K9me2 staining in the mitotic zone. ImageJ was used to  
912 generate an intensity threshold-based mask for nuclei using the DAPI signal, then the average  
913 intensity of H3K9me2 staining in nuclei was measured. Data are mean  $\pm$ SEM,  $n=24-29$ . Comparisons  
914 were performed by one-way ANOVA with Tukey's post hoc. \*\*\*\*  $p < 0.0001$  **D.** Quantification of  
915 fluorescence intensity of H3K9me3 staining in individual nuclei. ImageJ was used to generate an  
916 intensity threshold-based mask for nuclei using the DAPI signal, then the average intensity of  
917 H3K9me3 staining in individual nuclei was measured. Data are mean  $\pm$ SEM,  $n=80$  nuclei from 4  
918 germlines. **E.** Quantification of H3K79 levels in the indicated mutant strains by mass spectrometry.  
919 For each modification, levels were normalised to a relevant control peptide and are displayed as %  
920 of wild-type (indicated by dotted line). Data are mean  $\pm$ SEM,  $n=3$  replicates for each mutant strain.  
921 Comparisons were performed by two-way ANOVA with Dunnett's post hoc. \*  $p < 0.05$

922 **Figure S4. set-32 mutants display a mortal germline phenotype, defective sperm, and extended**  
923 **lifespan. A.** The number of times a line went sterile during maintenance at 25°C was counted, and  
924 the sterile line replaced by an animal from a backup population. Data are mean  $\pm$ SEM,  $n=20$  lines. **B-**

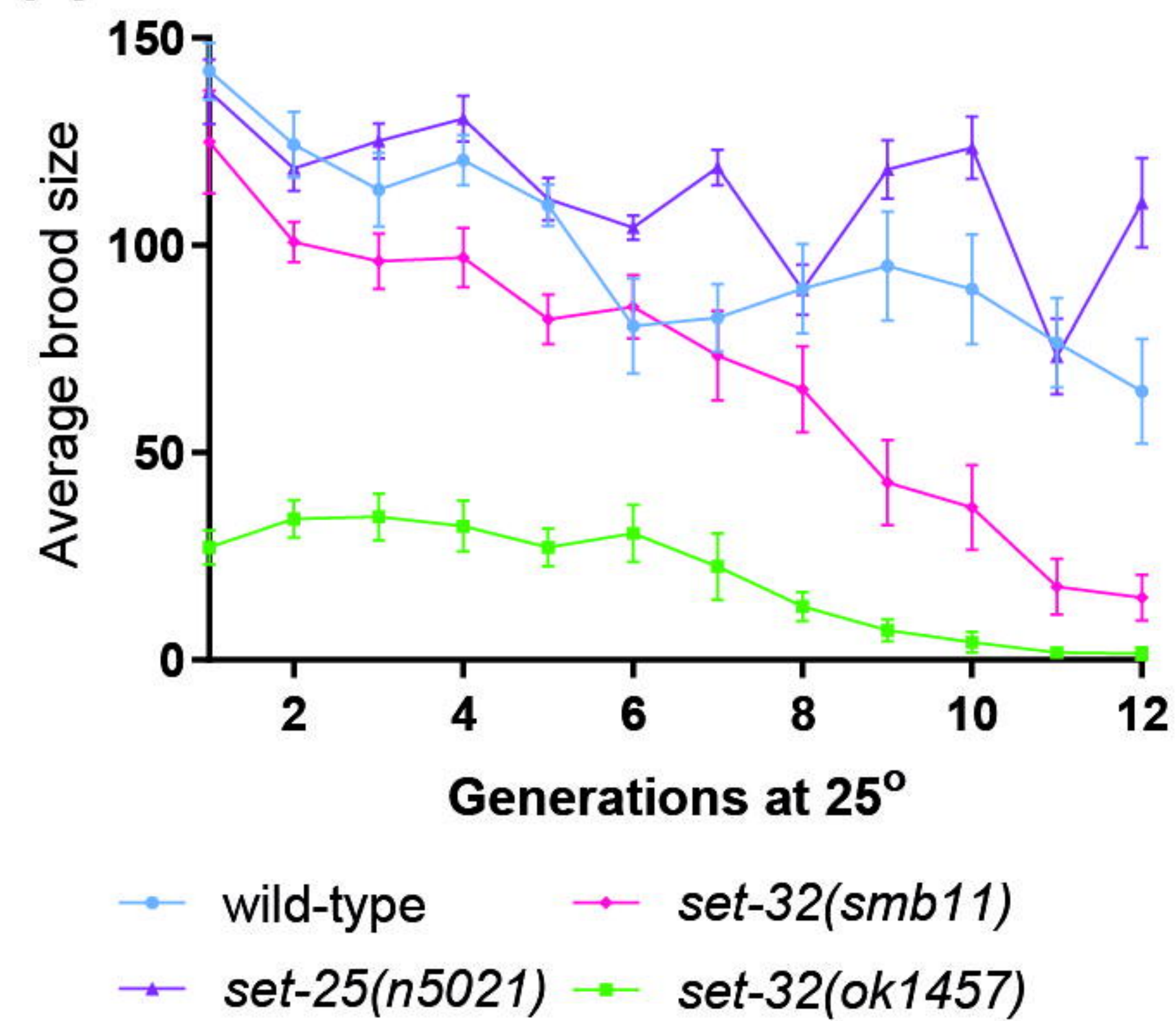
925 **C.** The average number of **B.** live progeny and **C.** unfertilised eggs produced per animal per day. Data  
926 are mean  $\pm$ SEM per day, n=18-20. **D.** Brood sizes of wild-type or *set-32(ok1457)* hermaphrodites  
927 mated to wild-type males. Data are mean  $\pm$ SEM per day, n=19. **E.** Wild-type hermaphrodites were  
928 crossed to males of the indicated genotype carrying a neuronal GFP transgene. Cross-progeny  
929 (expressing the GFP transgene) and self-progeny (not expressing GFP) were counted. Data are mean  
930  $\pm$ SEM, n=20-26. Comparisons were performed by two-way ANOVA with Sidak's post hoc. \*\*\*\*  
931  $p < 0.0001$  **F.** Replicate of assay in Figure 4H; lifespan assay performed in the absence of FUDR, n=110  
932 at Day 0. **G-I.** Independent replicate lifespan assays performed in the presence of FUDR, n=100 at  
933 Day 0. **J.** Median lifespan of each strain and p value of the survival curves of each mutant strain  
934 compared with wild-type. Comparisons were performed by Log-rank test. \*  $p < 0.05$  \*\*  $p < 0.01$  \*\*\*  
935  $p < 0.001$  \*\*\*\*  $p < 0.0001$



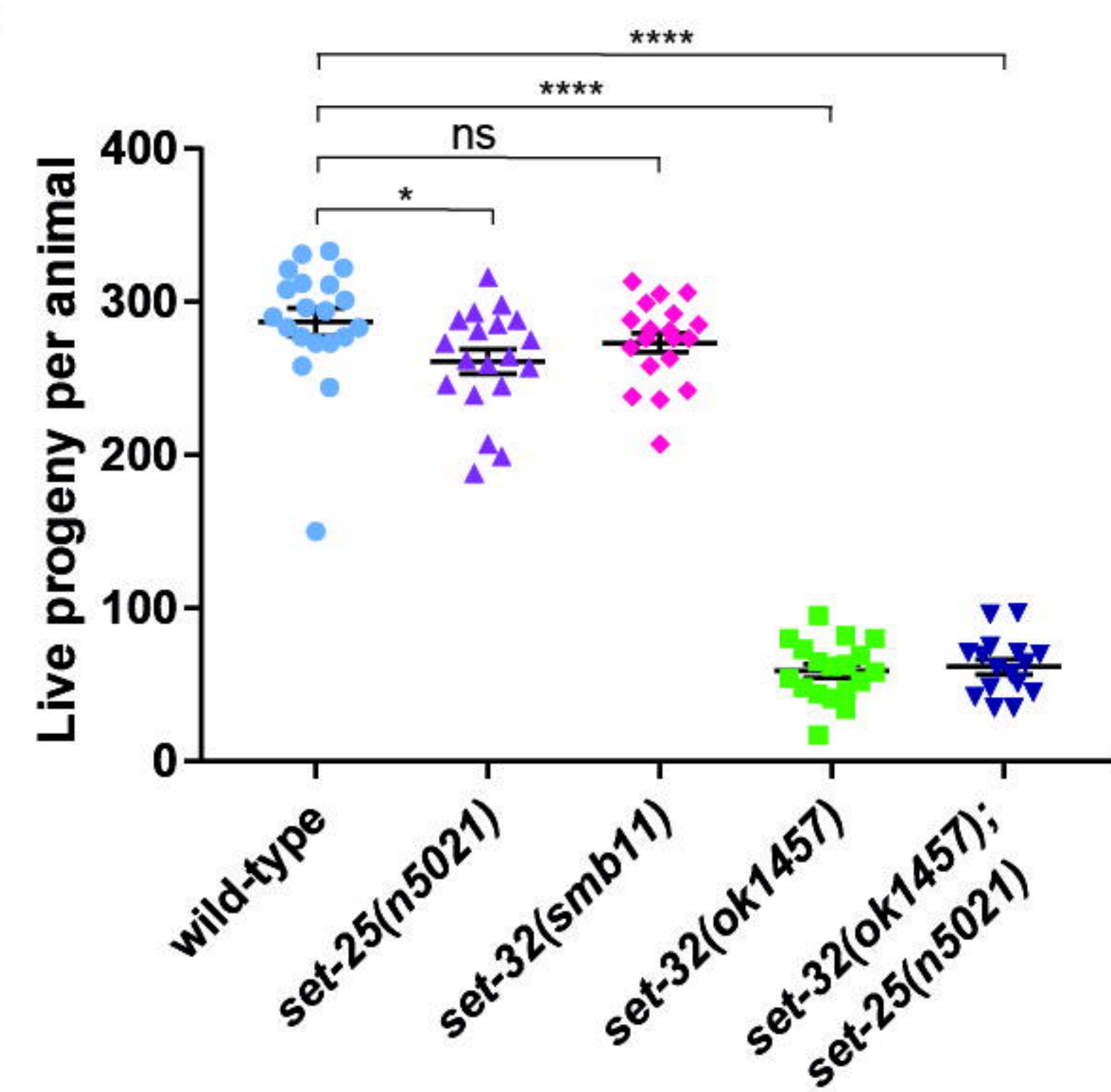




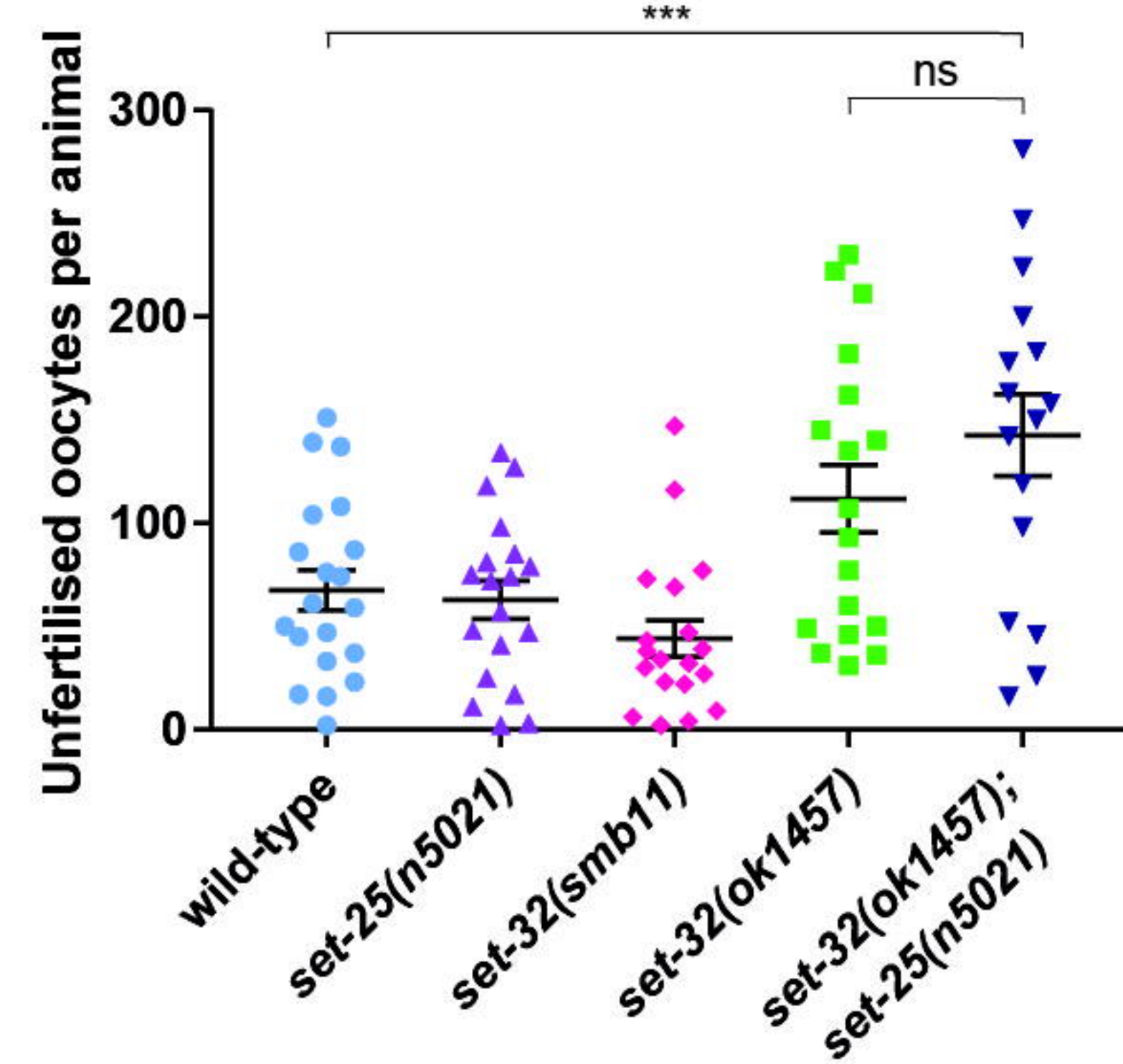
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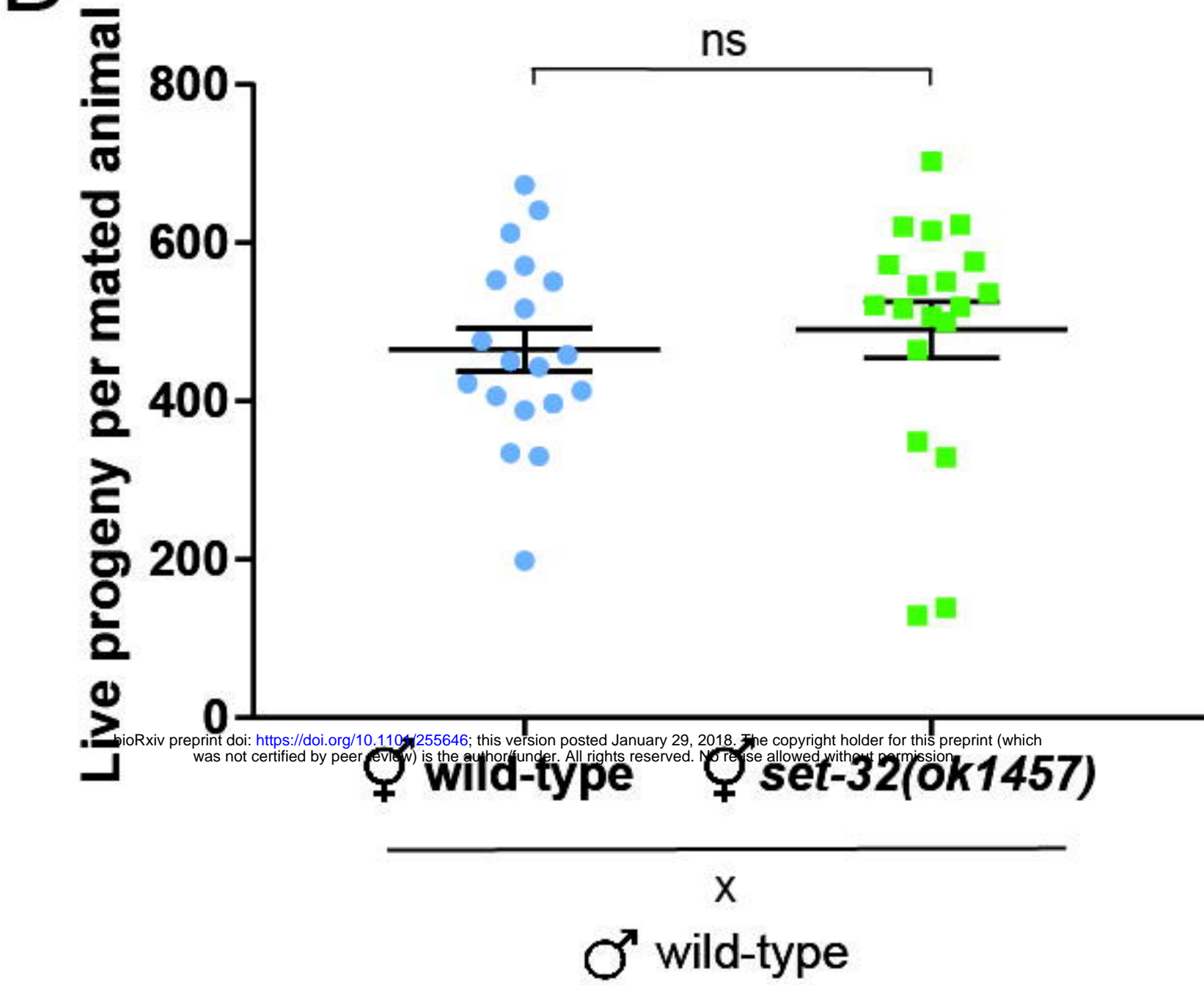
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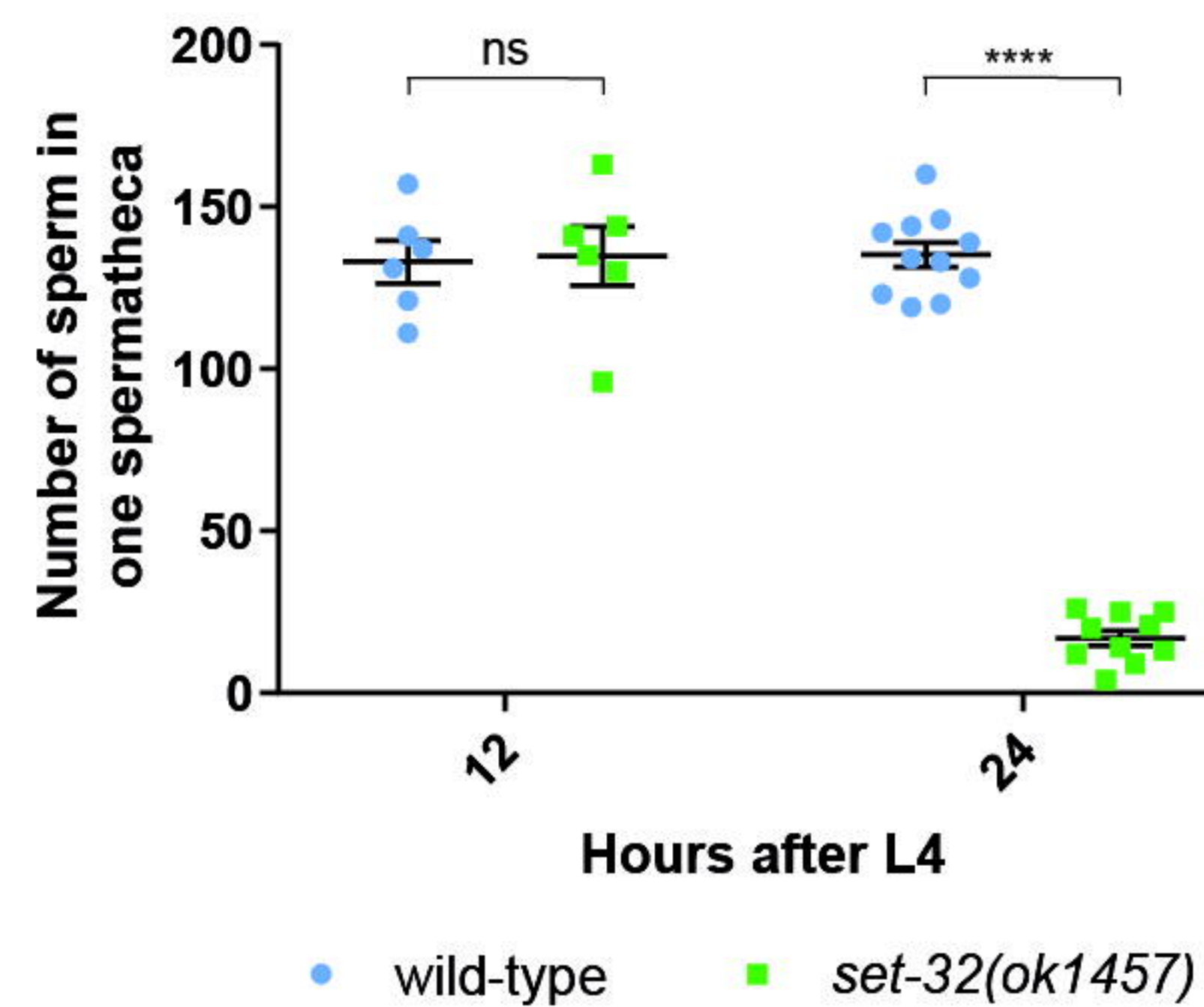
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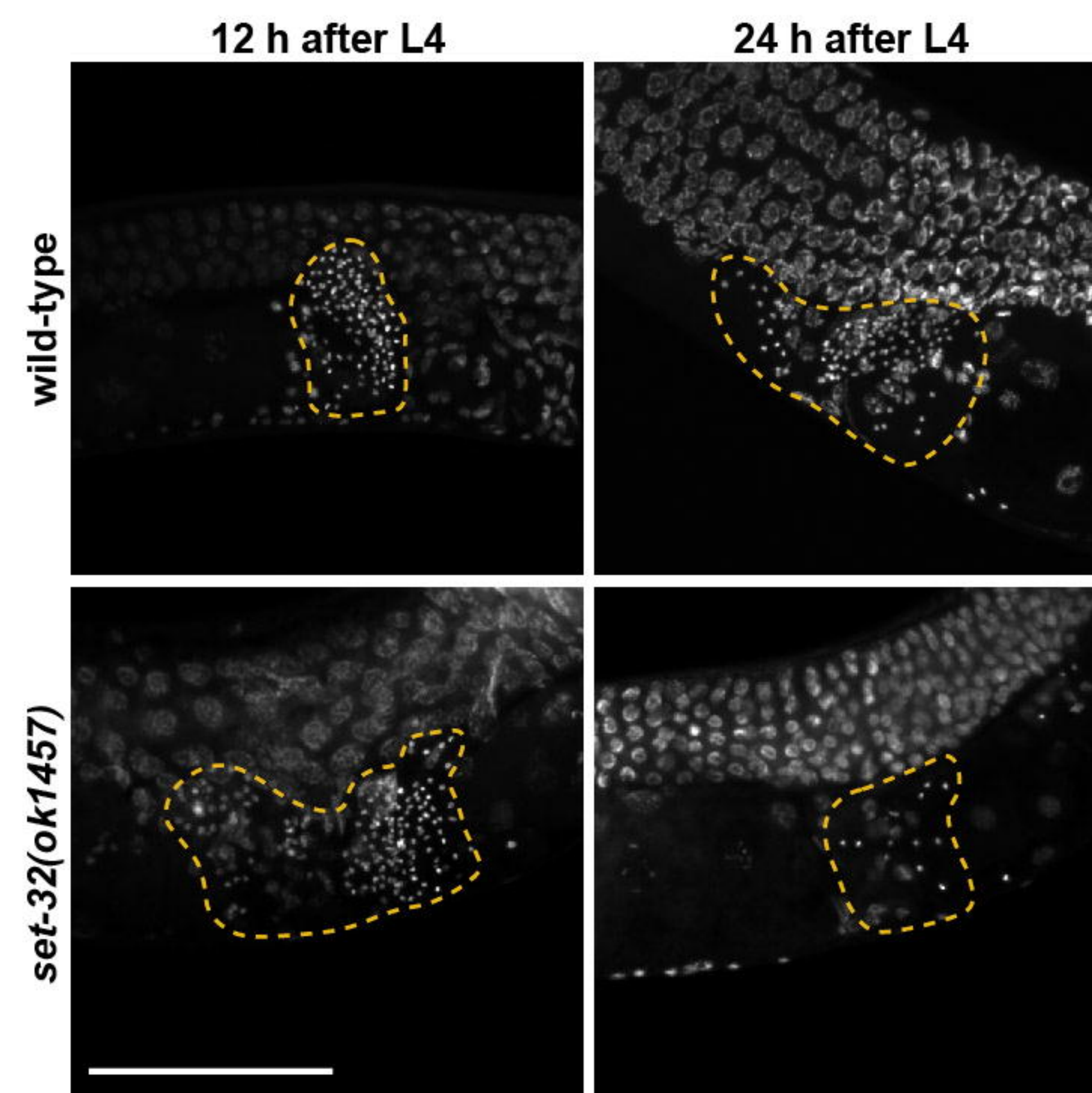
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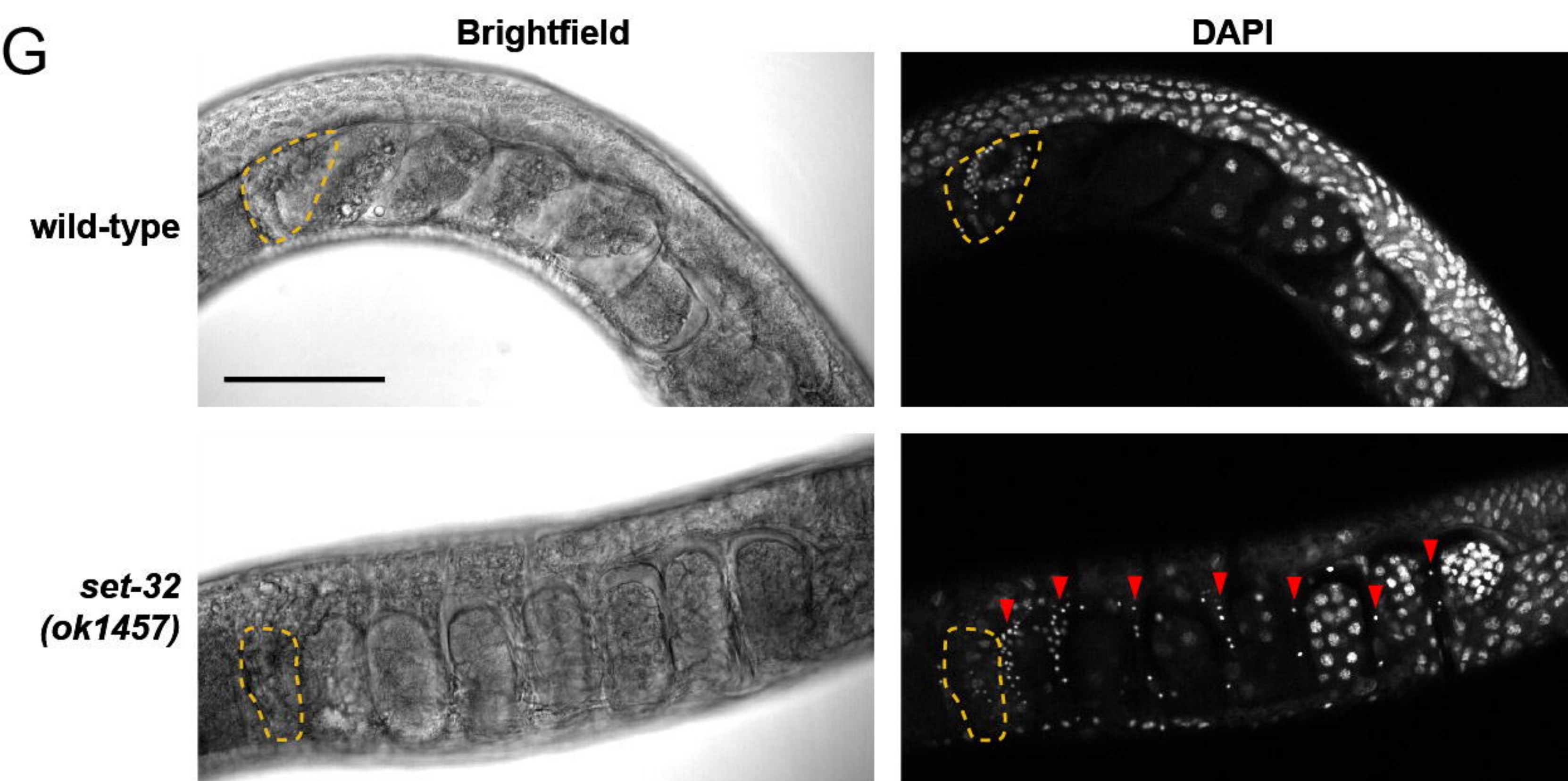
E



F



G



H

