The condensin II/TOP-2 axis silences transcription during germline specification in *C. elegans*

Short title: Genome silencing during germline development

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Abstract

In *C. elegans*, the germline is specified via a preformation mechanism that relies on the PIE-1 protein's ability to globally silence mRNA transcription in germline precursor cells, also known as the P-lineage. Recent work from our group has identified additional genome silencing events in *C. elegans*, during oogenesis and in starved L1 larvae, and these require the condensin II complex, topoisomerase II (TOP-2), and components of the H3K9me/heterochromatin pathway. Interestingly, silencing in oocytes also requires PIE-1, but this is not the case in starved L1s. Here, we ask if additional genome silencing components besides PIE-1 are required to repress gene expression in the P-lineage of early embryos, and we find that condensin II and TOP-2 are required and the H3K9me/heterochromatin pathway is not. We show that depletion of condensin II/TOP-2 activates the normally suppressed RNA polymerase II to inappropriately transcribe somatic genes in the P-lineage. We also present evidence that while both PIE-1 and condensin II/TOP-2 are required for genome silencing in the P-lineage, PIE-1 can silence transcription independently of condensin II/TOP-2 when misexpressed in somatic cells. Thus, in

27 oocytes all three genome silencing systems (TOP-2/condensin II, H3K9me, and PIE-1) are
28 operational while in both early embryos and starved L1s two of the three that are active (TOP-
29 2/condensin II and PIE-1 for early embryos, TOP-2/condensin II and H3K9me for starved L1s).
30 Our data show that multiple, redundantly acting genome silencing mechanisms act in a mix and
31 match manner to repress transcription at different developmental stages in the *C. elegans*
32 germline.

33 ## Introduction

34 Just like individual genes, the transcriptional output of entire genomes can be regulated
35 in a signal-mediated manner. In fact, genome repression and activation are recurring events
36 during germline development and gametogenesis in the roundworm *C. elegans*. In meiotic
37 prophase, for example, oocytes globally repress transcription as they prepare to divide (1,2).
38 The known transcriptional repressors, TOP-2, condensin II, the H3K9me heterochromatin
39 pathway, and PIE-1 are all involved in the silencing of oocytes during meiotic maturation (2,3).
40 After fertilization, transcription remains repressed, but repression is now mediated by the
41 OMA-1/2 proteins that bind to and inactivate the essential transcription factor TAF-4 (4). The
42 OMA system blocks transcription through the first two mitotic divisions and then these proteins
43 are rapidly degraded. Loss of OMA-1 and 2 activates transcription in three of the four cells in
44 the 4-cell embryo, with the holdout being the germline precursor P_2. P_2 remains
45 transcriptionally repressed via the action of PIE-1, and this is the case for the remainder of P-
46 lineage development (5-7).

47

48 Upon division of the P_4 cell, to form the Z2/Z3 primordial germ cells (PGCs), PIE-1 is
49 rapidly degraded, and the germline robustly activates transcription for the first time (5,8). Z2/Z3
50 are transcriptionally active for the remainder of embryogenesis, however if the embryo hatches
51 into a nutrient-free environment then the PGC genome is rapidly silenced, and this requires the
52 combined efforts of TOP-2/condensin II and the H3K9me heterochromatin pathway, working in
53 concert such that TOP-2/condensin II promotes H3K9me deposition, which is then acted upon

54 by H3K9me reader proteins like CEC-4 and HPL-2 to hyper-compact chromatin and silence
55 transcription (3). We named this system the Global Chromatin Compaction pathway to highlight
56 the linear arrangement of its components. Interestingly, in oocytes, despite the involvement of
57 both TOP-2/condensin II and the H3K9me heterochromatin pathway in genome silencing, there
58 is no requirement for TOP-2/condensin II in H3K9me deposition, and thus it appears that the
59 two chromatin compaction systems can work either together as a single pathway (Z2/Z3) or
60 separately as two redundant pathways (oocytes).

61

62 The findings discussed above highlight the plasticity in how the worm germline silences
63 transcription globally. Oocytes use TOP-2/condensin II, the H3K9me heterochromatin pathway,
64 and PIE-1, and loss of any one of these systems prompts unscheduled transcription. After
65 fertilization, PIE-1 becomes dispensable, even though it is still present, and OMA-1/2 take over
66 briefly to block transcription in P_0 and P_1. The requirement for PIE-1 returns with the birth of P_2,
67 and this lasts until PIE-1 is destroyed upon division of P_4. Later, in starved L1s, TOP-2/condensin
68 II and the H3K9me heterochromatin pathway are required once again, and this time they act in
69 a concerted manner. Thus, multiple systems are active in oocytes and Z2/Z3, however in the P-
70 lineage, to date, we only know of single systems – OMA-1/2 for P_0/P_1 and PIE-1 for P_2-P_4. In this
71 work we asked if TOP-2/condensin II and/or the H3K9me heterochromatin pathway are
72 required in the P-lineage for genome silencing and found that the H3K9me heterochromatin
73 pathway is totally dispensable while TOP-2/condensin II is required for P_2-P_4 but not for P_0-P_1.
74 We also find that TOP-2/condensin II require the germline progenitor state to silence the
75 genome, and that PIE-1 does not. These findings increase our understanding of how whole-

76 genome silencing occurs and highlight the importance of developmental fate in regulating the

77 ability of TOP-2/condensin II to perform this task.

78 # Materials and Methods

79 ## *C. elegans* strains

80 N2 (wild-type), WMM1 ([pie-1::gfp::pgl-1 + unc-119(+)]; [(pAA64)pie-1p::mCherry::his-
81 58 + unc-119(+)] IV), WMM2 (ltIs37 [(pAA64) pie-1p::mCherry::his-58 +unc-119(+)] IV; unc-
82 4(e120) top-2(it7ts) II), MT17463 (set-25(n5021) III), WM330 (pie-1(ne4301[pie-1::GFP]) III) and
83 1284 (rrrSi199[Pvet-4::NLS:gfp:gfp::vet-4 3'UTR; unc-119(+)] II; rrrSi198[Pvet-
84 4::NLS:gfp:gfp::vet-4 3'UTR; unc-119(+)] IV) strains were used in this study. Worms were
85 maintained on 60-mm plates containing nematode growth media (NGM) seeded with the *E. coli*
86 strain OP50 or HT115. Worms were grown at 20°C and propagated through egg preparation
87 (bleaching) every 72 hours.

88

89 ## Bacterial strains

90 OP50 bacteria served as the primary food source. It was grown in LB media containing
91 100 μg/ml streptomycin by shaking at 37°C overnight. 500 μl of the culture was seeded on Petri
92 dishes containing NGM + streptomycin. HT115 bacteria grown in LB media containing 100
93 μg/ml carbenicillin and 12.5 μg/ml tetracycline and seeded on NGM + carbenicillin +
94 tetracycline plates were also used as a source of food. Our RNAi strains were obtained from the
95 Ahringer library and verified by Sanger sequencing. Bacteria containing dsRNA were streaked
96 on LB-agar plates containing 100 μg/ml carbenicillin and 12.5 μg/ml tetracycline and incubated
97 at 37°C overnight. Single colonies were then picked and grown in 25 ml LB cultures with 100

98 μg/ml carbenicillin and 12.5 μg/ml tetracycline. 500 μl of this culture was seeded on 60-mm
99 Petri dishes containing 5mM IPTG.

100

101 **Egg preparation**

102 Bleach solution containing 3.675 ml H_2O, 1.2 NaOCl, and 0.125 ml 10N NaOH was
103 prepared. Adult worms were washed from plates with 5 ml of M9 minimal medium (22mM
104 KH_2PO_4, 22mM Na_2HPO_4, 85mM NaCl, and 2mM $MgSO_4$). Worms were centrifuged at 1.9 KRPM
105 for 1 minute and the excess medium was removed, then the bleach solution was added. Eggs
106 were extracted by vortexing for 30 seconds and shaking for 1 minute. This was done a total of 3
107 times and worms were vortexed one last time. Then the eggs were spun down at 1900 rpm for
108 1 minute and excess bleach solution was removed and the eggs were washed 3 times with M9
109 minimal medium.

110

111 **RNAi treatment**

112 RNAi containing NGM plates were prepared as described in the "Bacterial strains"
113 section. For double RNAi treatments, RNAi cultures were mixed at a 1:1 ratio by volume. HT115
114 cells transformed with an empty pL4440 vector was used as a negative control. RNAi conditions
115 used in this study and tests for their efficacy is described below:

116

117 *top-2* RNAi

118 L1 worms were plated on HT115 food plates for the first 24 hours and were then moved to
119 plates seeded with *top-2* RNAi for the remaining 48 hours. Embryonic lethality was observed at
120 >90%.

121 *capg-2* RNAi

122 Worms were grown on HT115 food plates for the first 24 hours and were moved to plates
123 containing *capg-2* RNAi for the remaining 48 hours. An embryonic lethality of 80%-100% was
124 seen with this RNAi treatment.

125 *top-2/capg-2* double RNAi

126 Worms were grown on HT115 food plates for the first 24 hours and were transferred to *top-*
127 *2/capg-2* double RNAi plates for the next 48 hours. Embryonic lethality ranged from 90%-100%
128 for this RNAi treatment.

129 *set-25* RNAi

130 Worms were grown on plates containing *set-25* RNAi for the entirety of their life cycle. Some of
131 the adult worms were bleached and an L1 chromatin compaction assay was performed on the
132 resulting larvae to test RNAi efficacy. Please see ref. 3 for details on the L1 compaction assay.

133 *pie-1* RNAi

134 Worms were grown on plates containing *pie-1* RNAi for the entirety of their life cycle. An
135 embryonic lethality of 100% was observed for this RNAi.

136 *mex-5/6* RNAi

137 Worms were grown on HT115 food plates for the first 48 hours of their life and were then
138 switched to mex-5/6 RNAi plates for the remaining 24 hours. An embryonic lethality of 100%
139 was observed for this RNAi treatment.

140

141 **Antibodies and dilutions**

142 RNAPIIpSer2: Rabbit antibody from Abcam (ab5095, Cambridge, Massachusetts) was
143 used at a dilution of 1:100. GFP: mouse Mab #3580, from EMD Millipore, was used at 1:500.
144 H3K9me3: Rabbit antibody from Abcam (ab176916, Cambridge, Massachusetts) was used at a
145 dilution of 1:1000. P-granules: mouse Mab K76, from the Developmental Studies Hybridoma
146 Bank, was used neat. Secondary antibodies: Alexa Fluor conjugated secondary antibodies from
147 Invitrogen (Thermofisher Scientific, Waltham, Massachusetts) were used at a dilution of 1:200.

148

149 **Immunofluorescence staining**

150 Adult worms were first washed off plates with 10 ml of M9 minimal medium and
151 washed 3 more times. Then, they were centrifuged at 1.9 KRPM and the excess medium was
152 removed. 20 µl of media containing about 50 worms were spotted on a coverslip and 3 µl of
153 anesthetic (20mM Sodium Azide and 0.8M Tetramisole hydrochloride) was added to immobilize
154 them. Worms were dissected using 25Gx5/8 needles (Sigma Aldrich, St. Louis, Missouri). To
155 release early embryos, worms were cut once midway through their length. The coverslip was
156 then mounted onto poly-L-lysine covered slides and let rest for 5 minutes. Slides were put on

157 dry ice for 30 minutes. Samples were then freeze-cracked by flicking the coverslips off for
158 permeabilization.

159

160 For RNAPIIpSer2 staining experiments, once samples are permeabilized, slides were put
161 in cold 100% methanol for 2 minutes and then fixing solution (0.08M HEPES pH 6.9, 1.6mM
162 $MgSO_4$, 0.8mM EGTA, 3.7% formaldehyde, 1X phosphate-buffered saline) for another 30
163 minutes. After fixing, slides were washed three times with TBS-T (TBS with 0.1% Tween-20) and
164 were blocked for 30 minutes with TNB (containing 100mM Tris-HCl, 200mM NaCl, and 1% BSA).
165 Primary antibodies were then applied at the dilutions described above in TNB and slides were
166 incubated at 4°C overnight.

167

168 For H3K9me3 and GFP staining experiments, permeabilized samples were put in cold
169 100% methanol for 10 seconds and then fixing solution (0.08M HEPES pH 6.9, 1.6mM $MgSO_4$,
170 0.8mM EGTA, 3.7% formaldehyde, 1X phosphate-buffered saline) for 10 minutes. After fixing,
171 slides were washed three times with TBS-T (TBS with 0.1% Tween-20) and were blocked for 2
172 hours with TNB (containing 100mM Tris-HCl, 200 mM NaCl, and 1% BSA) supplemented with
173 10% goat serum. Primary antibodies were then applied at the dilutions described above in TNB
174 and slides were incubated at 4°C overnight. On the next day, the slides were washed 3 times
175 with TBS and slides were incubated with secondary antibodies and Hoechst 33342 dye for 2
176 hours at room temperature. Slides were washed 3 times with TBS, mounting medium (50 %
177 glycerol in PBS), and coverslips were applied and sealed with Cytoseal XYL (Thermofisher).

178

179 **HCR (In Situ Hybridization Chain Reaction)**

180 A kit containing a DNA probe set, DNA hybridized chain reaction (HCR) amplifier
181 hairpins, and hybridization, wash, and amplification buffers were purchased from Molecular
182 Instruments (molecularinstruments.com). Genes that were examined were *vet-6* and *F58E6.6*.
183 DNA was visualized with Hoechst 33342 dye. Embryos were prepared by bleaching and were
184 immediately spotted on poly-L-lysine coated slides. Coverslips were applied and slides were
185 freeze-cracked to permeabilize samples. Immediately after freeze-cracking, 500 µl of 100% ice-
186 cold methanol was applied over the samples. Slides were dried off by tilting slides, 1 ml of 4%
187 paraformaldehyde (PFA) was added and samples were incubated in a humidity chamber for 10
188 minutes. Samples were then washed 3 times with 100 µl of PBS-T. A 1:1 solution of probe
189 hybridization buffer (PHB) and PBS-T was added to the samples, and they were incubated for 5
190 minutes at room temperature. Samples were then prehybridized with PHB for 30 minutes at
191 37°C and DNA probes (at a final concentration of 2 picomoles per 500 µl of PHB) were added to
192 the samples and were incubated overnight at 37°C. The next day, samples were washed 4 times
193 with probe wash buffer (PWB) at 37°C with 15 minutes of incubation for each wash. They were
194 then washed three more times with 5xSSCT at room temperature. Samples were pre-amplified
195 with Amplification buffer for 30 minutes at room temperature. Probe amplifier hairpins were
196 snap cooled by heating to 95°C for 90 seconds and putting in a dark drawer for 30 mins then
197 were added to the sample. Worms were incubated with the hairpins overnight in a dark
198 drawer. On the third day, samples were washed with 5xSSCT and incubated with Hoechst-

199 33342 (1:5000 dilution) for 15 minutes. Finally, samples were mounted on poly-L-lysine coated
200 slides and imaged.

201

202 Immunofluorescent imaging

203 All slides were imaged using an Olympus Fluoview FV1000 confocal microscope using
204 Fluoview Viewer software. A magnification of 600x (60x objective and 10x eyepiece
205 magnifications) was used. Laser intensity was controlled for experiments to achieve consistency
206 among samples.

207 # Results

208 **Loss of TOP-2 and condensin II function results in active RNA polymerase II**
209 **(RNAPII) in the P-lineage of early embryos.**

210 The goal of this study was to determine if the genome silencing systems TOP-
211 2/condensin II and/or the H3K9me heterochromatin pathway are required for transcriptional
212 repression in the P-lineage of early embryos. To do so, we stained the samples with an antibody
213 that detects a phospho-serine 2 epitope on the RNAPII carboxyl-terminal domain (CTD). The
214 presence of this RNAPIIpSer2 signal in nuclei is a marker for actively elongating RNAPII (9), and
215 we and others have used this approach extensively to identify transcriptionally active nuclei
216 within a variety of *C. elegans* tissues (1-4, 7, 10-11). The antibody that we are using, a rabbit
217 polyclonal that recognizes RNAPIIpSer2, has previously been validated by us for use in *C.*
218 *elegans* embryos (3). It is at the 4-cell stage that the somatic precursor cells ABa, ABp, and EMS
219 activate zygotic transcription (12). The remaining cell, P_2, remains transcriptionally repressed
220 due to the presence of PIE-1. We stained 4-cell embryos for RNAPIIpSer2 and in the control
221 samples the expected pattern emerged – signal could be seen in ABa, ABp, and EMS, but not P_2
222 (Figure 1). We next depleted either TOP-2 or the CAPG-2 subunit of condensin II. Interestingly,
223 in samples exposed to either *top-2* or *capg-2* RNAi, we could now detect RNAPIIpSer2 in P_2, and
224 the same was true of samples exposed to *pie-1* RNAi (Figure 1). We next examined the
225 remainder of the P-lineage (P_3 and P_4) and in both cases we could detect active transcription
226 after depletion of either TOP-2, CAPG-2, or PIE-1 (Figure 1). Interestingly, when 1- or 2-cell
227 embryos were examined after depletion of TOP-2, no RNAPIIpSer2 signal was observed in P_0 or

228 P_1 (Fig S1). Based on these data, we conclude that TOP-2 is dispensable for transcriptional
229 repression in P_0 and P_1, but for P_2, P_3, and P_4 it, along with condensin II, is required to silence
230 gene expression.

231

232 **Figure 1: Loss of TOP-2 or CAPG-2 allows for active RNAPII in the P-lineage of early embryos.**

233 Early embryos from N2 worms treated with control, *top-2*, *capg-2* or *pie-1* RNAi were fixed and
234 stained for P-granules (green), RNAPIIpSer2 (red) and DNA (blue). Shown on top are the entire
235 embryos and the P-cell is magnified below. Quantification of the data is shown at right.

236

237 A genome activation reporter gets misexpressed in the P-lineage when TOP-2 238 and condensin II are depleted.

239 RNAPIIpSer2 staining reveals that TOP-2/condensin II are required to suppress active
240 RNAPII in P cells. We next sought an independent readout for spurious transcription in these
241 cells and settled on a previously described reporter for "embryonic genome activation" (EGA),
242 which is GFP under control of the *vet-4* reporter (13-14). The *vet-4* transcript is first detected in
243 either 4- or 8-cell embryos and is expressed exclusively in somatic cells (6). Using the EGA-GFP
244 strain, we asked if the depletion of TOP-2 and CAPG-2 resulted in the misexpression of the GFP
245 reporter in P-lineage cells. In our hands, for control samples, no EGA-GFP signal was detected in
246 4- or 8-cell embryos at all, and this may reflect the time required to translate the *vet-4*
247 transcript. By the 28-51 cell stage, when P_4 is present, we could clearly detect GFP in numerous

248 somatic cells, but not P_4 (19 of 20 samples were negative for GFP; Figure 2). By contrast, when
249 TOP-2 and CAPG-2 where co-depleted, GFP signals emerged in P_4 in a substantial percentage of
250 the samples (14 of 20 were positive for GFP, Figure 2). These data provide additional evidence
251 for the derepression of transcription in the P-lineage when TOP-2 and condensin II are
252 depleted.

253

254 **Figure 2: EGA-GFP gets misexpressed in P_4 when TOP-2 and CAPG-2 are depleted.**

255 Early embryos from the 1284 strain were treated with either control or *top-2/capg-2* RNAi.
256 Samples were fixed and stained for P-granules (white), EGA-GFP (green) and DNA (blue). 20
257 samples were analyzed over 2 independently performed replicates. EGA-GFP signal was
258 aberrantly present in P_4 of embryos treated with *top-2/capg-2* RNAi. Quantification of the data
259 is shown below. Scale bar represents a length of 2 µm.

260

261 Aberrant expression of EMS-specific genes is observed in the P-lineage when
262 TOP-2 and condensin II are depleted.

263 Data presented thus far demonstrate aberrant RNAPII activity in P_2, P_3, and P_4 after
264 depletion of TOP-2 or CAPG-2 (Figure 1), as well as unscheduled gene expression (Figure 2). To
265 obtain additional evidence for the misregulation of gene expression in these cells, we
266 performed the *in-situ* hybridization chain reaction (HCR), also known as RNA-FISH (15), to
267 detect and localize mRNAs in early embryos. For this, we focused on 4-cell embryos, and we

268 started by looking at the expression pattern of the somatic gene *vet-6*, which has previously
269 been shown to be expressed exclusively in EMS at the 4-cell stage (6). In wild-type
270 embryos, *vet-6* expression was indeed localized to the EMS blastomere, with 100% of samples
271 showing *vet-6* transcript signals in EMS (Figure 3A). On the other hand, only 7.5% of wild-type
272 embryos had *vet-6* signal in P_2. Upon treatment with *top-2* RNAi, however, most of the samples
273 (57.5%) had *vet-6* transcripts in P_2 (Figure 3A). We next examined the expression pattern of an
274 additional transcript, F58E6.6. Similar to *vet-6*, F58E6.6 transcripts are seen predominantly in
275 EMS in wild-type embryos, with only 10-20% of the wild-type samples showing transcript
276 signals in ABa, ABp, or P_2 (Figure 3B, quantification of the total sample set is shown to the right
277 and quantification on a per embryo basis is shown in Figure S2A). After TOP-2 or CAPG-2
278 depletion, however, 50% and 82%, respectively, of the embryos expressed F58E6.6 in P_2 (Figure
279 3B and S2A). As a positive control, we depleted PIE-1 and found that both *vet-6* and F58E6.6
280 transcripts were expressed in P_2 in 85% and 100% of our samples (Figures 3A and S2B). In
281 Figures 3B and S2A we included dashed circles to highlight the nuclear boundaries, the
282 unmarked images are shown in Figure S2C. Based on these data, we conclude that depletion of
283 TOP-2 and condensin II results in the misexpression of EMS genes in the P-lineage of early
284 embryos.

285

286 **Figure 3: EMS specific genes are aberrantly expressed in the P_2 cell of 4-cell embryos after loss**
287 **of TOP-2 or CAPG-2.**

288 (A) 4-cell embryos from N2 animals treated with control, *top-2* or *pie-1* RNAi were fixed and
289 HCR was performed to probe for *vet-6* mRNA (white). DNA was stained using Hoechst-
290 33342 (blue). Representative images are shown and percentage of samples with *vet-6*
291 transcripts in P_2 are shown below (n=40).

292 (B) 4-cell embryos from N2 animals treated with control, *top-2* or *capg-2* RNAi were fixed
293 and HCR was performed to probe for F58E6.6 transcripts (white). DNA was visualized
294 using Hoechst-33342 dye (blue). P_2 nuclei are outlined with red dashed line while
295 somatic nuclei are outlined by white dashed line. Representative images are shown and
296 quantification of F58E6.6 signal in each blastomere is shown on the right. Scale bar
297 represents a length of 2 µm.

298

299 The H3K9 methyltransferase SET-25 is dispensable for genome silencing in the P-
300 lineage.

301 Data thus far have focused on the role of TOP-2/condensin II in transcriptional control
302 within the P-lineage. In starved L1s, TOP-2 and condensin II collaborate with the H3K9me
303 pathway to repress transcription, and in oocytes both pathways are required for genome
304 silencing, but they appear to function independently of one another (2,3). It was, therefore, of
305 interest to determine if H3K9me pathway components are required for P-lineage silencing. One
306 characteristic of the involvement of the H3K9me pathway in silencing is the massive
307 accumulation of H3K9me3 marks on chromatin, as this is observed in Z2/Z3 of starved L1s, and
308 in proximal oocytes (2,3). To determine if H3K9me marks also accumulate in transcriptionally

309 silenced cells of the P-lineage, we stained early embryos for H3K9me3. Figure 4A shows
310 representative images for both early and late zygotes. Importantly, we have previously shown
311 that the chromatin of pre-fertilization oocytes is heavily decorated with H3K9me3 marks, such
312 that the immunofluorescence signal for H3K9me3 overlaps entirely with the DNA-based DAPI
313 signal (2). This is not the case for nuclei in one-cell embryos, where H3K9me3 signal is present,
314 but is limited to small patches scattered along the chromatin. By contrast, H3K9me3 signals are
315 much stronger in polar bodies, where they overlap with much of the DAPI signal (Figure 4A).
316 These data suggest that, upon fertilization, H3K9me3 marks are rapidly erased on much of the
317 chromatin inside the female pronucleus, but not on the chromatin in polar bodies. We went on
318 to examine 4- and 8-cell embryos, comparing P_2 and P_3 to their sister cells EMS and C. All four
319 cell types showed a similar pattern of H3K9me3 foci scattered throughout the chromatin, and
320 quantification of these signals showed that P cells did not accumulate H3K9me3 marks to a
321 greater extent than their transcriptionally active sisters (Figure 4B). Consistent with a lack of
322 massive accumulation of H3K9me3 marks in the P-lineage we also observed that *set-25* mutant
323 embryos could readily silence transcription in the P-lineage, as assessed by RNAPIIpSer2
324 staining (Figure 4C), and the same is true in 1- and 2-cell embryos after *set-25* RNAi (Figure S1).
325 Thus, unlike in oocytes or starved L1s, where SET-25 plays a crucial role in genome silencing
326 (2,3), it is dispensable for silencing in the P-lineage.

327

328 **Figure 4: The H3K9me heterochromatin pathway is not required for transcriptional repression**
329 **in the P-lineage.**

330 (A) The indicated zygotes from N2 worms were fixed and stained for H3K9me3 (red) and
331 DNA (blue). Shown below the entire embryos are magnified views of the pronuclei and
332 polar bodies (PB). Note that the H3K9me3 signal overlaps with the Hoechst signal more
333 extensively on the polar bodies than within the pronuclei. Scale bar represents a length
334 of 2 µm.

335 (B) Same as (A) except four- and eight-cell embryos were examined. Quantification of
336 H3K9me3 foci for P_2 and P_3 (germ) or EMS and C (soma) is shown below. Scale bar
337 represents a length of 2 µm.

338 (C) Four-cell embryos, either wild type (N2) or *set-25* mutants (strain MT17463), were fixed
339 and stained for RNAPIIpSer2 (red) and P-granules (blue). 20 samples were analyzed over
340 2 independent experiments. Both wild-type and set-25 mutants had no H3K9me3 signal
341 in P_2. Scale bar represents a length of 2 µm.

342

343 **PIE-1 does not require TOP-2 in a compulsory manner to repress RNAPII.**

344 Previous data, together with work presented here, show that both the TOP-2/condensin
345 II axis and the PIE-1 protein are required for genome silencing in oocytes and in P_2, P_3, and P_4 of
346 early embryos. Whether TOP-2 and PIE-1 work together, or separately, to silence transcription
347 is not known, and this is a difficult question to answer given that standard genetic epistasis
348 tests are unavailable in this system due to an inability to generate *top-2* null embryos devoid of
349 maternally supplied protein. To get an indication, however, we reasoned that it was possible to
350 misexpress PIE-1 in early embryos, where it blocks transcription in all cells that contain it (6,16),

351 and then ask if TOP-2 was needed for ectopic PIE-1 to silence transcription. Previous work has
352 shown that depletion of the translational regulators MEX-5 and MEX-6 allow PIE-1 expression in
353 all cells of the 4-cell embryo (17). The loss of PIE-1 asymmetry in *mex-5/6* RNAi embryos is
354 accompanied by loss of somatic asymmetries as well, e.g. the SKN-1 protein that is normally
355 expressed only in the somatic EMS cell is also found in all four cells after *mex-5/6* RNAi (17).
356 Thus, it appears that depletion of MEX-5/6 results in blastomeres assuming a merged somatic
357 and germline precursor state. We first confirmed that TOP-2 is normally expressed in all cells at
358 the 4-cell stage, using a strain where TOP-2 contains a FLAG tag at the endogenous locus. FLAG-
359 TOP-2 retains TOP-2 function (18) and is indeed present in all 4 cells (Figure 5A). We next
360 confirmed the effect of *mex-5/*6 RNAi on PIE-1 localization using a PIE-1 with a GFP tag at the
361 endogenous locus (19,20). As shown in Figure S3, in control samples GFP-PIE-1 is expressed
362 solely in P_2, whereas loss of MEX-5/6 causes a redistribution to all four blastomeres, as
363 expected. Furthermore, when samples were stained for RNAPIIpSer2, control embryos
364 displayed signals in the three somatic cells, as expected, while the *mex-5/*6 RNAi samples did
365 not show active RNAPII in any cells, consistent with the misexpression of GFP-PIE-1. To examine
366 a role for TOP-2 in promoting transcriptional repression by ectopic PIE-1, we used a
367 temperature-sensitive allele of *top-2* (18) in combination with *mex-5/6* RNAi and examined
368 RNAIIpSer2 in 4-cell embryos at both the permissive temperature (15°C) and the non-
369 permissive temperature (24°C). For the control, we observed that inactivation of TOP-2 caused
370 the appearance of RNAIIpSer2 in P_2, as expected, whereas samples raised at the permissive
371 temperature were devoid of RNAIIpSer2 in P_2 (Figure 5B). Interestingly, after *mex-5/6* RNAi, PIE-
372 1 blocked transcription in all four blastomeres regardless of the activity state of TOP-2 (Figure

373 5B), and this show that PIE-1 does not depend on TOP-2 for genome silencing, at least in a
374 compulsory manner. These data also show that that the ability of TOP-2 to repress transcription
375 globally is tightly linked to the germline progenitor state, and when that state is perturbed (by
376 loss of MEX-5/6) then TOP-2 can no longer repress transcription. Ideas about why this is so are
377 presented in the Discussion, below.

378

379 **Figure 5: PIE-1 does not require TOP-2 to repress transcription in *C. elegans* early embryos.**

380 A. Wild type 4-cell embryos were fixed and stained for FLAG-TOP-2 (red) and DNA (blue).
381 TOP-2 is distributed across all 4 cells of the embryos. Scale bar represents a length of 2
382 µm.
383 B. 4-cell embryos from WMM2 (*top-2* TS) animals treated with either control or *mex-5/6*
384 RNAi were optionally shifted to the nonpermissive temperature of 24°C for 24 hours or
385 left at the permissive 15°C. Then samples were fixed and stained for P-granules (white),
386 RNAPIIpSer2 (red) and DNA (blue). 20 samples were analyzed over 2 independent
387 replicates. Attenuation of TOP-2 via temperature shift does not reverse the
388 transcriptional silencing by PIE-1 in *mex-5/6* RNAi embryos. Quantification of the data is
389 presented below. Scale bar represents a length of 2 µm.

390

391 ## Discussion

392 In this study we focused on the early embryonic P-lineage with the goal of determining
393 if the TOP-2/condensin II and/or H3K9me/heterochromatin genome silencing pathways are
394 required for transcriptional repression in these cells. Previous work has revealed that two
395 different genome silencers, OMA-1/2 and PIE-1, are active in the P-lineage, with OMA-1/2
396 responsible for P_0 and P_1 (as well as AB) and PIE-1 taking over for P_2-P_4 (Figure 6A). Our work
397 has added to this profile with the demonstration that TOP-2/condensin II are needed for
398 efficient silencing in P_2-P_4, but not P_0 or P_1, and that the H3K9me/heterochromatin pathway is
399 not required at all in the P-lineage. We base these conclusions on the following data. For TOP-
400 2/condensin II we show that depletion of either TOP-2, the condensin II subunit CAPG-2, or co-
401 depletion of both results in the appearance of RNAPIIpSer2 signals in P_2, P_3, and P_4 (Figure 1),
402 activation of the *vet-4* promoter in P_4 (Figure 2), and inappropriate transcription of the somatic
403 *vet-6* and F58E6.6 genes in P_2 (Figure 3). We note that the impact of TOP-2 and/or CAPG-2
404 depletion on transcription in the P-lineage was not as profound as depletion of PIE-1 (Figures 1-
405 3), and this is likely due to the fact that we use rather mild RNAi conditions for TOP-2/CAPG-2,
406 relative to PIE-1, as stronger conditions for TOP-2/CAPG-2 result in torn, cut, and malformed
407 nuclei, as has been reported previously (21-23). Although we tested just one condensin II
408 subunit in this work, our previous work had shown that depletion of KLE-2, in addition to CAPG-
409 2, disrupts silencing in starved Z2/Z3 (3), and thus it seems likely that the *capg-2* phenotypes
410 reported here represent a loss of condensin II function and not a condensin II independent
411 function of CAPG-2.

412

413 For the H3K9me/heterochromatin pathway we focused on the SET-25
414 methyltransferase, which we have previously shown to be required for genome silencing in
415 both oocytes and in starved Z2/Z3 (2,3). We found no role for SET-25 in P-lineage silencing
416 (Figures 4 and S1). Furthermore, unlike oocytes and starved Z2/Z3, we did not observe a hyper-
417 accumulation of H3K9me3 marks in P-lineage nuclei, adding further support to the conclusion
418 that the H3K9me/heterochromatin pathway is dispensable for genome silencing in early
419 embryos.

420

421 Figure 6 summarizes current knowledge of how genome silencing occurs in the
422 nematode germline. In oocytes, which are named based on their position relative to the
423 spermatheca in gonadal tube (-1 is closest, -2 one position more distal, etc), our previous work
424 showed that three of the four known genome silencing pathways are required (TOP-
425 2/condensin II, H3K9me/heterochromatin pathway, and PIE-1; Belew et al, 2023). Interestingly,
426 once the oocyte is fertilized, then all three pathways become dispensable – despite their
427 physical presence in the zygote – and only OMA-1/2 can repress transcription (ref. 4 and this
428 work). In addition, we found that the H3K9me3 marks that prominently decorate oocyte
429 chromatin (2), are rapidly erased from the maternal pronucleus in the zygote but not from
430 polar bodies (Figure 4). Thus, it is clear that while transcriptional repression continues during
431 and after the egg-to-zygote transition the mechanisms are fundamentally different. Another
432 handoff occurs when P_1 divides to form P_2 and OMA-1/2 are abruptly degraded. At this

433 juncture, both PIE-1 and TOP-2/condensin II are once again required for silencing (Figures 1-3).
434 Thus, a fascinating question becomes how PIE-1 and TOP-2/condensin II are regulated such that
435 they can repress transcription in P_2-P_4 but not in P_0-P_1 despite being present in these cells. A
436 related and equally fascinating question is how TOP-2/condensin II is regulated such that the
437 system silences gene expression in P_2-P_4 but not in early embryonic somatic cells, again despite
438 being present in these cells. The final genome silencing event that we know of occurs in the
439 Z2/Z3 PGCs during L1 starvation. PIE-1 is not present in these cells, and silencing occurs via a
440 concerted effort between the TOP-2/condensin II and H3K9me/heterochromatin pathways (3).
441 All told, these findings make it clear that while genome silencing is a predominant feature of
442 germline development, in the worm it occurs in a "mix and match" manner where different
443 silencing systems are employed dependent on the developmental context.

444

445 **Figure 6: Summary of genome silencing during germline development.**

446 (A) Cartoon summarizes current knowledge of factors required for genome silencing at
447 different developmental stages in the germline.

448 (B) Summary of experimental data presented in Figure 5.

449

450 Other important questions raised by this work include how do these different silencing
451 pathways work, mechanistically, and do the different systems interact or do they work
452 independently of one another? Regarding mechanism, how PIE-1 inhibits transcription is still

453 poorly understood but has been linked to the phosphorylation of RNAPII on serines 2 and 5
454 within its CTD (24). The RNAPII CTD contains numerous copies of the heptapeptide repeat
455 sequence $Y_1S_2P_3T_4S_5P_6S_7$, with humans containing 52 copies and nematodes 42 copies (25).
456 During the transcription cycle, RNAPII arrives at the promoter in an unphosphorylated state and
457 is then phosphorylated on serine 5 to allow promoter clearance. Phosphorylation on serine 2
458 follows, and this has been linked to an active, elongating state for RNAPII (26). PIE-1 blocks both
459 serine 5 and serine 2 phosphorylation, and genetic experiments using separation-of-function
460 mutants have shown that the ability to block serine 5 phosphorylation is critical to suppressing
461 transcription in the P-lineage, whereas the ability to block serine 2 phosphorylation is
462 dispensable (27). More recent work has shown that the phosphorylation of serine 2 on RNAPII
463 CTD is dispensable for embryonic development (28). This softens the link between active
464 transcription and RNAPII serine 2 phosphorylation and supports the idea that PIE-1 represses
465 transcription by acting on serine 5 phosphorylation. How is PIE-1 connected to TOP-
466 2/condensin II? In oocytes, there may be an indirect connection as we have previously observed
467 that depletion of TOP-2/condensin II delays nucleolar dissolution and PIE-1 is primarily localized
468 to the nucleolus prior to dissolution (2). Thus, it may be that PIE-1 is held in an inactive state via
469 nucleolar residency and TOP-2/condensin II are needed to release PIE-1. In the P-lineage PIE-1
470 is not a nucleolar protein, and thus if TOP-2/condensin II controls PIE-1 it must be via a different
471 mechanism. To address this, in this work, we used *mex-5/6* RNAi to distribute PIE-1 evenly in all
472 blastomeres of a 4-cell embryo and observed, as expected, that transcription was repressed in
473 all four cells. We then asked if PIE-1 required TOP-2 for this silencing and found that it did not
474 (Figures 5 and 6B). This result makes two important points. One, PIE-1 is not obligated to TOP-2

475 to repress transcription, it can do so independently. This does not mean that the situation is the
476 same in a proper P-cell, as the composition of a 4-cell *mex-5/6* RNAi embryo is very different
477 from a wild-type embryo. Second, the data show that TOP-2 (and presumably condensin II as
478 well) requires the germline progenitor identity to block transcription, as when that identity is
479 abolished by *mex-5/6* RNAi then TOP-2 can no longer silence the genome. How might this work
480 at a molecular level? It may simply be that a co-factor required by TOP-2 for repression is
481 asymmetrically localized to P-cells, and after *mex-5/6* RNAi this factor becomes diluted across
482 all four blastomeres and can no longer perform its function. It is fair to say that the work
483 presented here opens the door to many important and exciting lines of future research.

484

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488

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551 ## Supporting information captions

552 **Figure S1: RNAPII-mediated transcription is still repressed in one- and two-cell embryos after**
553 **loss of TOP-2 or SET-25.**

554 One- and two-cell embryos from N2 worms treated with the indicated RNAi were fixed and
555 stained for P-granules (white), RNAPIIpSer2 (red) and DNA (blue). No active RNAPII was
556 observed in the more than 20 samples we examined for both one- and two-cell embryos over 2
557 replicates. Note the RNAPIIpSer2 signals in the adjacent, older embryo in the upper right panel
558 – this shows that the staining worked efficiently. Scale bar represents a length of 2 µm.

559

560 **Figure S2: Control experiments for somatic gene expression in 4-cell embryos.**

561 (A) Samples were prepared in the same way as Figure 3B except RNAi against *pie-1* was
562 utilized. Scale bar represents a length of 2 µm.

563 (B) The same panels as those shown in Figure 3B are displayed without the dashed circles.
564 Scale bar represents a length of 2 µm.

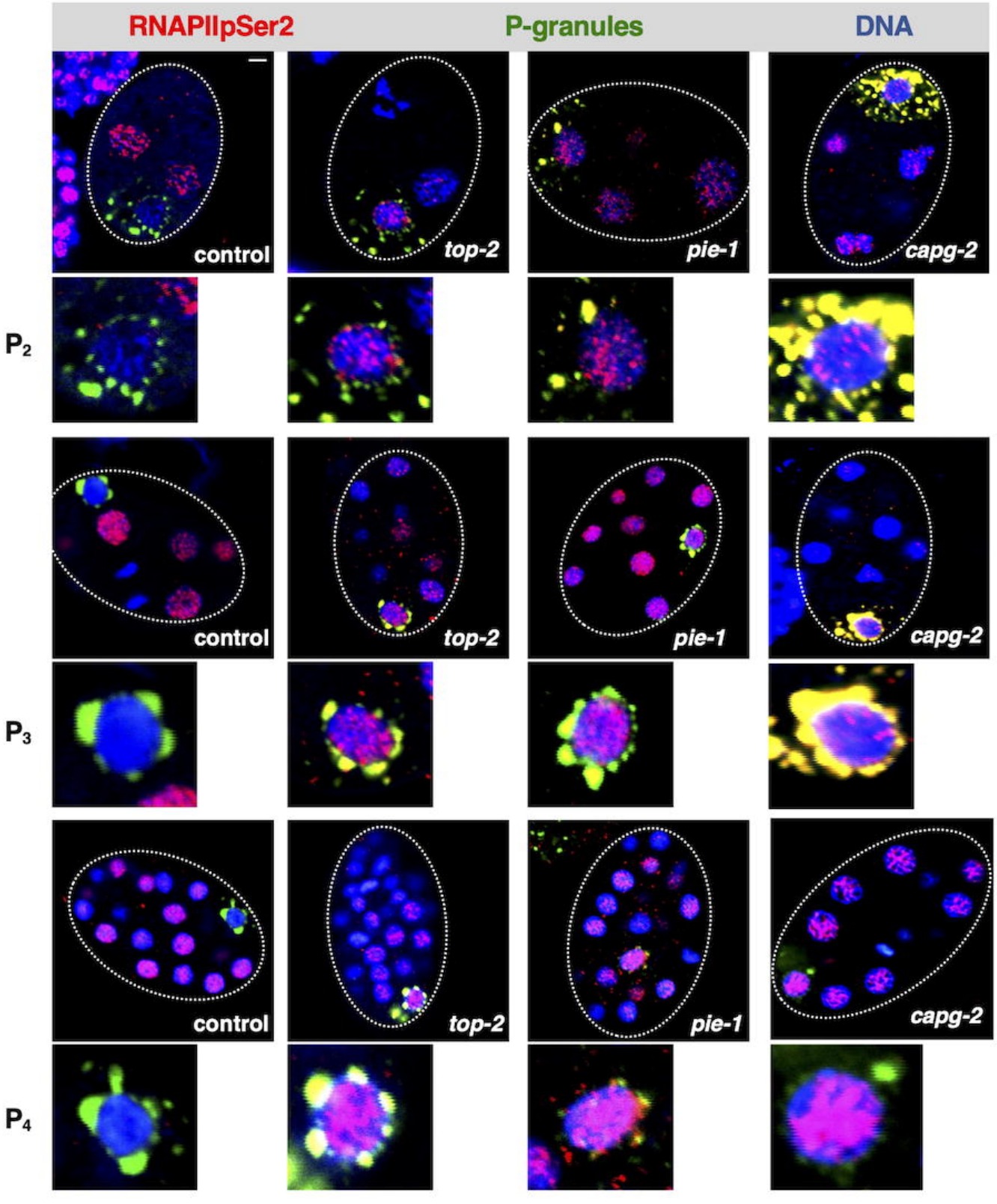
565 (C) A summary of the data shown in Figures 3B and S2A.

566

567 **Figure S3: Depletion of MEX-5 and MEX-6 results in PIE-1 presence in all blastomeres of early**
568 **embryos.**

569 4-cell embryos from WM330 animals optionally treated with either control or *mex-5/6* RNAi
570 were fixed and stained for P-granules (white), PIE-1::GFP (green), RNAPIIpSer2 (red) and DNA
571 (blue). Images of the same embryo stained for PIE-1::GFP and RNAPIIpSer2 are shown side by
572 side. 20 samples were analyzed over 2 independent replicates. Depletion of *mex-5/6* results in
573 the loss of asymmetric distribution of PIE-1 to the germline. PIE-1 is present in all blastomeres
574 and that is sufficient to repress transcription in the whole embryo as shown by low RNAPIIpSer2
575 levels. Scale bar represents a length of 2 µm.

Figure 1



pSer2-postive P_2 after given RNAi:

vector	0/62	0%
top-2	23/41	56.1%
pie-1	9/13	69.2%
capg-2	5/18	27.8%

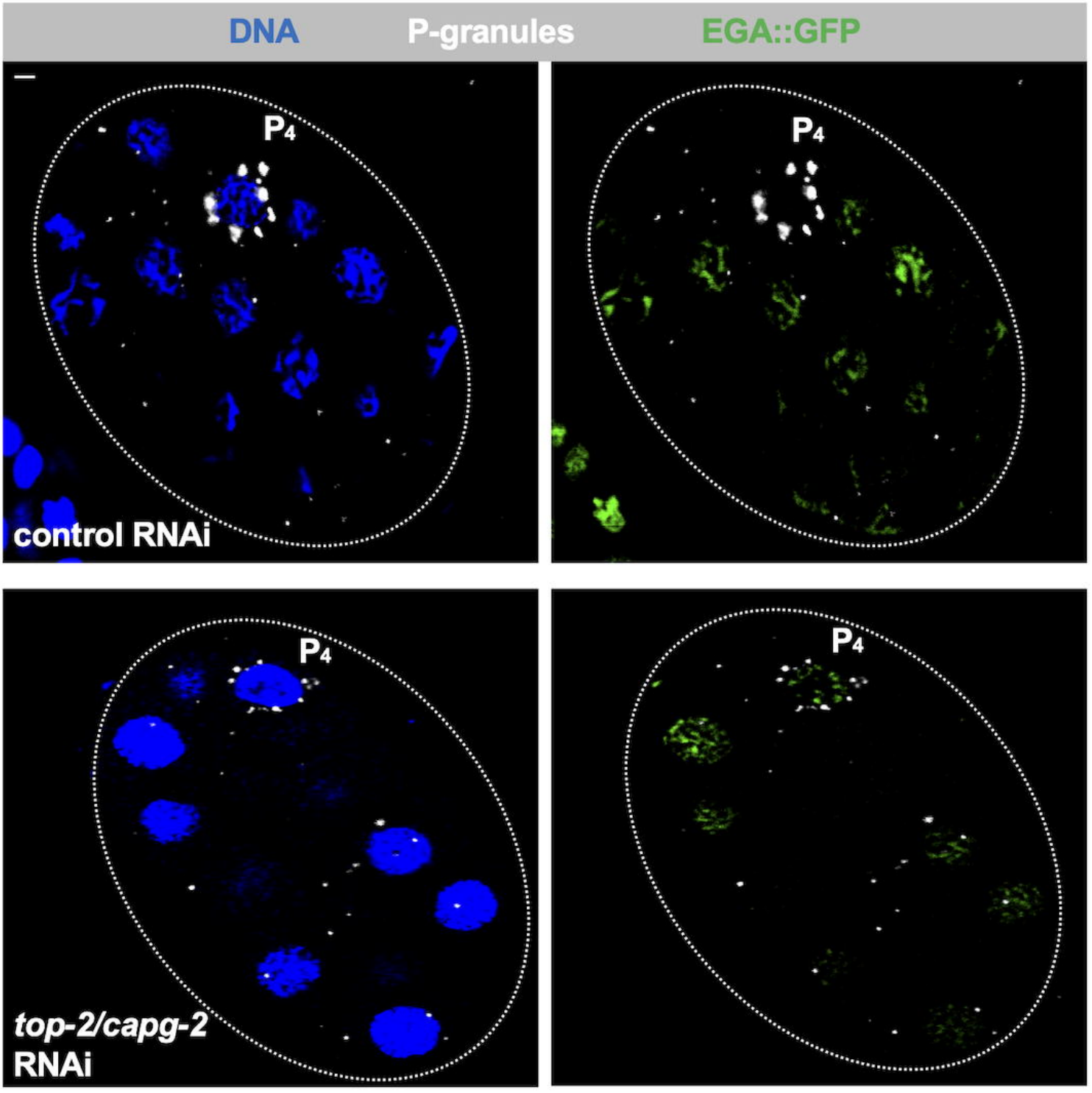
pSer2-postive P_3 after given RNAi:

vector	1/87	1.1%
top-2	26/52	50%
pie-1	17/18	94.4%
capg-2	16/40	40%

pSer2-postive P_4 after given RNAi:

vector	6/89	6.7%
top-2	26/45	57.8%
pie-1	14/15	93.3%
capg-2	22/42	52.4%

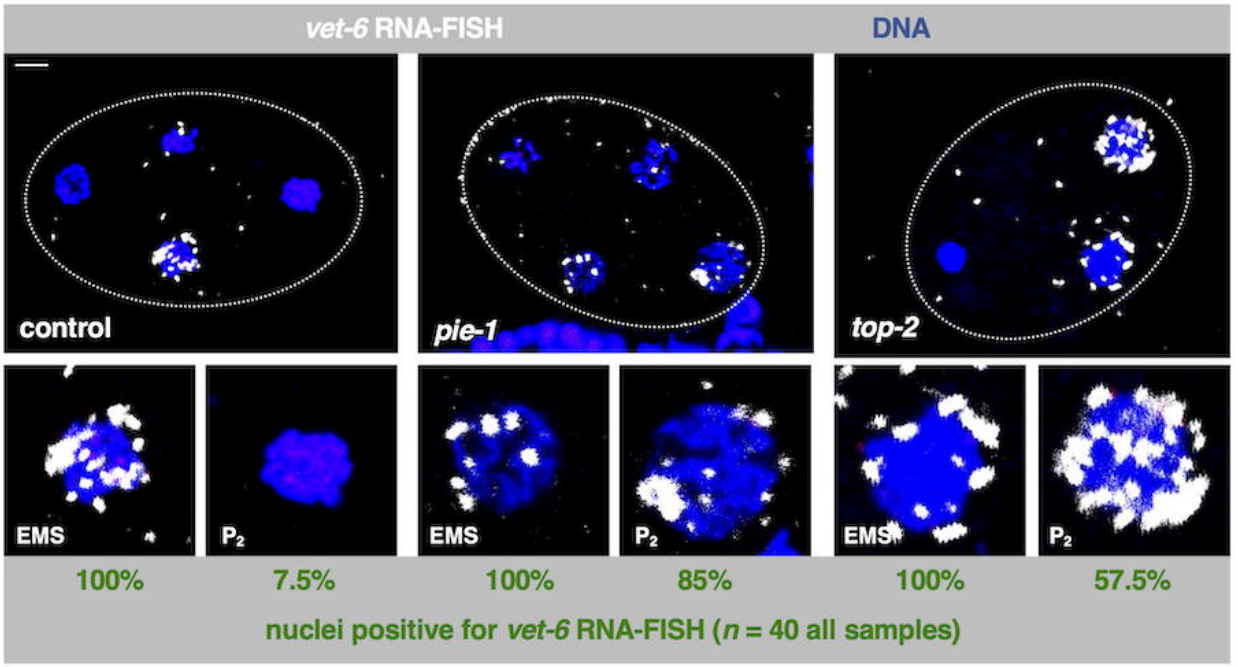
Figure 2



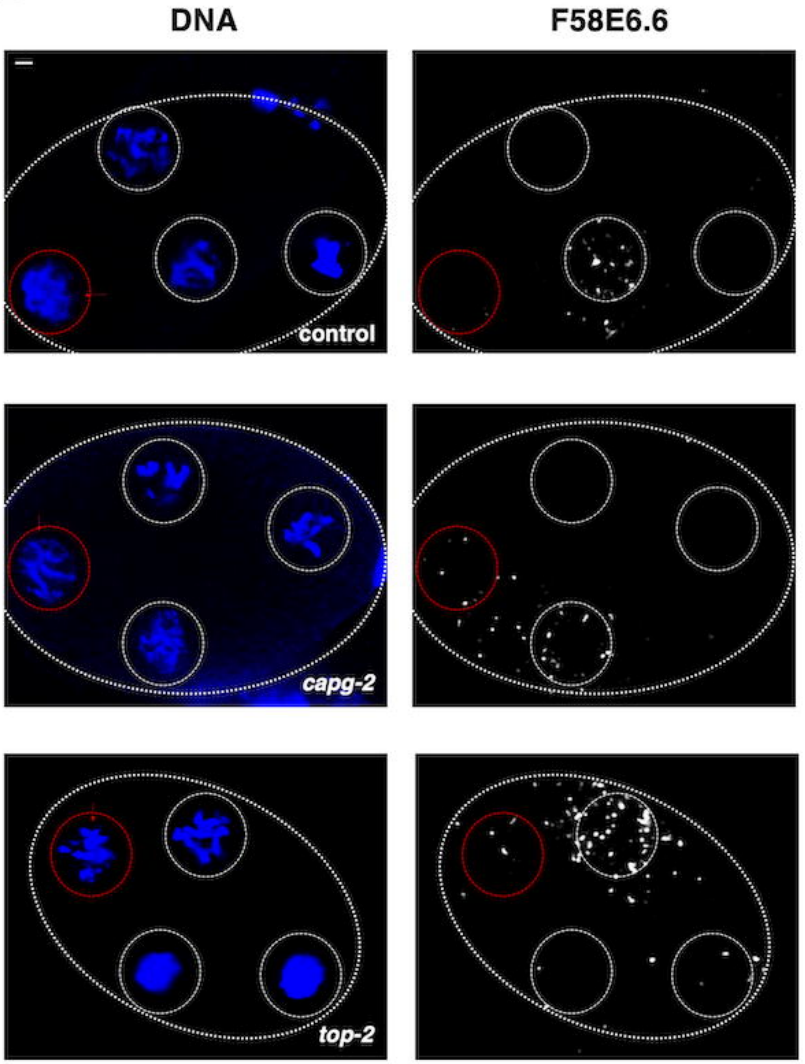
Treatment	Number of EGA::GFP positive P_4
Control RNAi	1/20
top-2/capg-2 RNAi	14/20

Figure 3

A



B



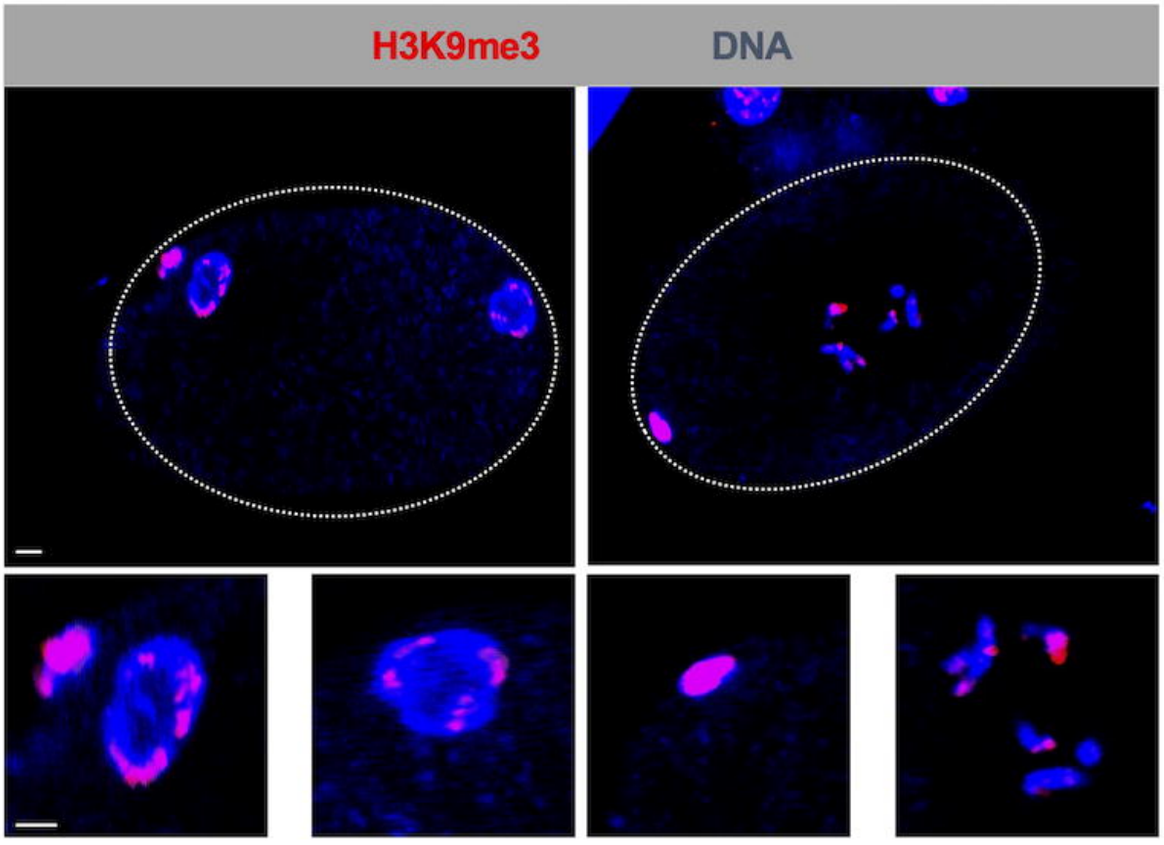
C

% of cells expressing F58E6.6 transcript

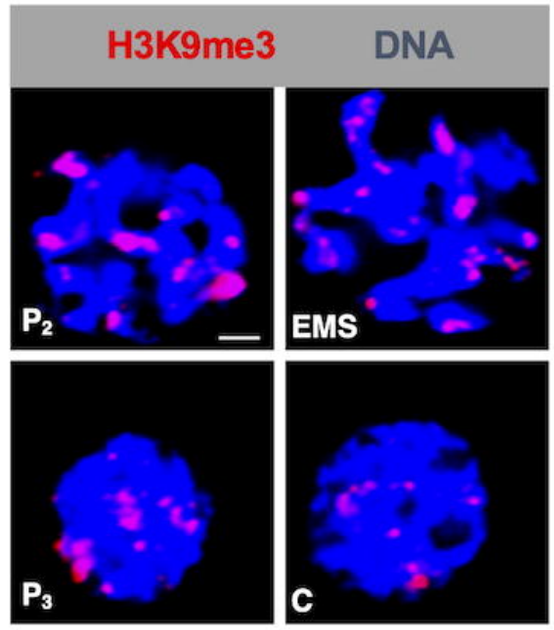
	P_2	EMS	ABa	ABb	*n*
control	20	100	15	10	20
capg-2	82	100	0	18	11
top-2	50	100	40	40	10

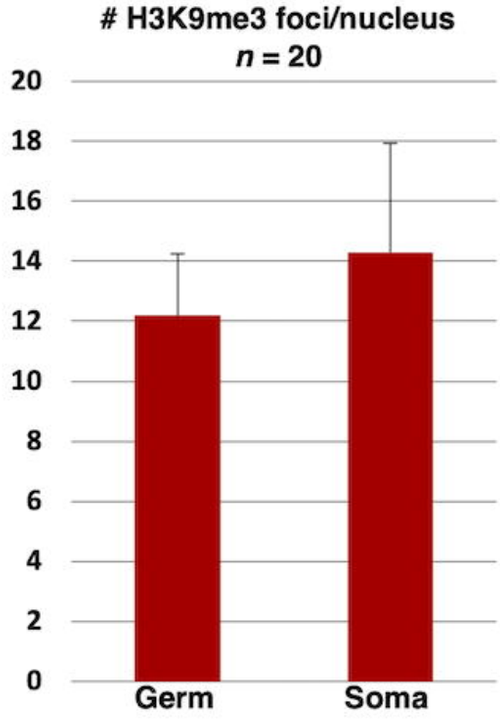
Figure 4

A

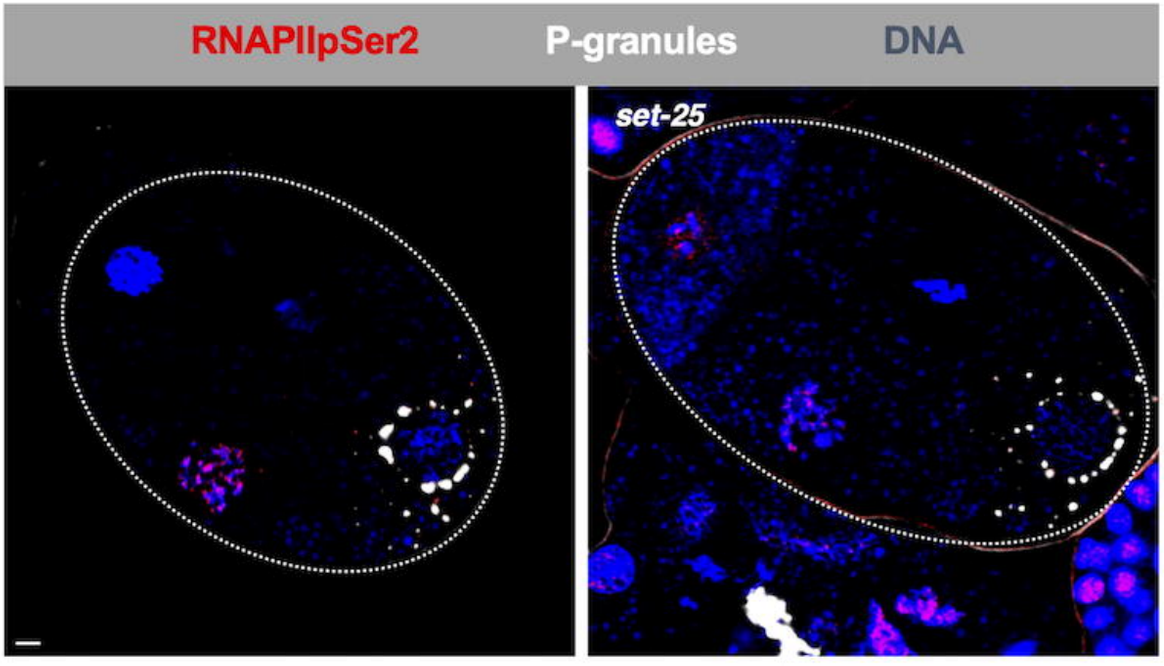


B





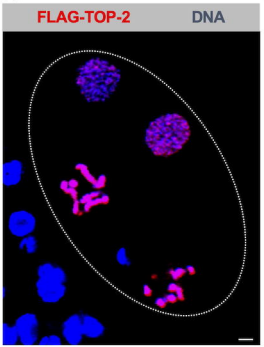
C



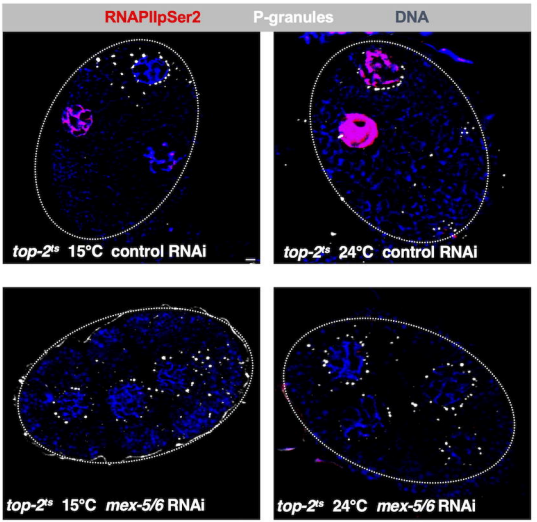
0/10 RNAPIIpSer2+ 0/10 RNAPIIpSer2+

Figure 5

A

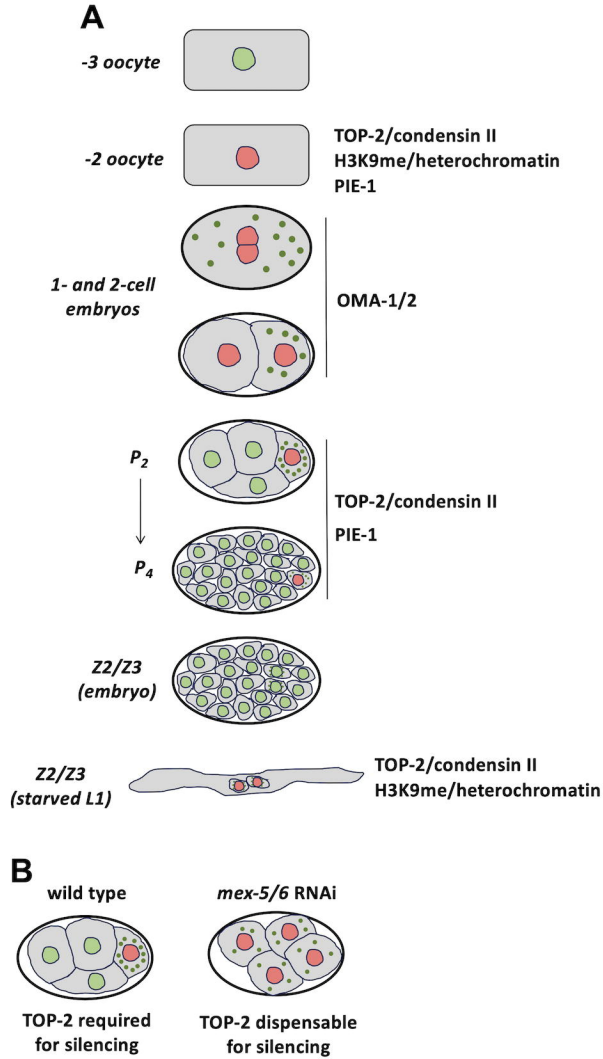


B



	pSer2-postive cell after given condition		
	P_2	soma	mixed
control RNAi at 15°C	1/20	18/20	na
control RNAi at 24°C	7/20	17/20	na
mex-5/6 RNAi at 15°C	na	na	2/40
mex-5/6 RNAi at 24°C	na	na	1/40

Figure 6


A
-3 oocyte
-2 oocyte
TOP-2/condensin II
H3K9me/heterochromatin
PIE-1
1- and 2-cell embryos
OMA-1/2
P2
P4
TOP-2/condensin II
PIE-1
Z2/Z3 (embryo)
Z2/Z3 (starved L1)
TOP-2/condensin II
H3K9me/heterochromatin
B
wild type
mex-5/6 RNAi
TOP-2 required for silencing
TOP-2 dispensable for silencing