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

34 ## Introduction

35 Just like individual genes, the transcriptional output of entire genomes can be regulated
36 in a signal-mediated manner. This is particularly true during germline development in *C. elegans,*
37 where multiple cycles of whole genome activation and silencing occur (summarized in Fig 1).
38 Starting with the gametes, we and others have shown that for both oocytes and spermatocytes
39 transcription is globally silenced as these cells enter meiotic prophase (1-3). Silencing during
40 gametogenesis requires three distinct pathways. One is comprised of topoisomerase II (TOP-2 in
41 worms) and condensin II, which work together to compact chromatin and to silence gene
42 expression (2,3). The second is comprised of components of the H3K9me/heterochromatin
43 pathway, for example the methyltransferases SET-25 and MET-2, and is characterized by a large-
44 scale buildup of H3K9me marks on chromatin as the genome is silenced (2). Lastly, the PIE-1
45 protein is also required for genome silencing during oogenesis (2). Upon fertilization,
46 transcription continues to be repressed in the newly formed zygote, via the activities of the OMA-
47 1/2 proteins, and this continues through first mitosis into the 2-cell embryo (4). After the second
48 mitosis, which forms the 4-cell embryo, OMA-1/2 are abruptly degraded and this allows
49 transcriptional activation in the somatic cells ABa, ABp, and EMS. The germline precursor, $P_2$,
50 remains transcriptionally repressed due to the activity of PIE-1 (5,6). PIE-1 continues to repress
51 transcription during germline specification in the so-called P lineage ($P_2$, $P_3$, and $P_4$) until $P_4$
52 divides to form the primordial germ cells Z2 and Z3. Upon division of $P_4$ the PIE-1 protein is
53 instantly degraded (5,7), and transcription is activated in the germline for the first time. Recent
54 work for our group has shown that transcription continues in Z2/Z3 as the embryo hatches to
55 form an L1 larva, but only if nutrients are present (8). If embryos hatch into a nutrient-free

56 environment, then the energy sensor AMPK is activated and this promotes chromatin
57 compaction and genome silencing in a manner dependent on the TOP-2/condensin II and
58 H3K9me pathways (8). In this condition PIE-1 is dispensable for genome silencing as there is no
59 detectable PIE-1 protein present in starved Z2/Z3 (E.C. and W.M.M., unpublished observation).
60 Thus, for the germline, transcription is globally repressed during gametogenesis, as well as in 1-
61 and 2-cell embryos. Transcription remains repressed in the germline progenitor P cells prior to
62 activation in Z2/Z3, and can then be silenced again during L1 starvation and reactivated after
63 feeding.

64

65 The findings discussed above highlight the mechanistic plasticity in how germline
66 transcription is controlled globally. Oocytes use TOP-2/condensin II, the H3K9me pathway, and
67 PIE-1, and loss of any one of these pathways prompts unscheduled transcription. After
68 fertilization, OMA-1/2 become essential for silencing in $P_0$ and $P_1$. The requirement for PIE-1
69 returns with the birth of $P_2$, and this lasts until PIE-1 is destroyed upon division of $P_4$. Later, in
70 starved L1s, TOP-2/condensin II and the H3K9me heterochromatin pathway are required once
71 again, and this time they act in a concerted manner. Thus, multiple systems are active in oocytes
72 and Z2/Z3, however in the P-lineage, to date, we only know of single systems – OMA-1/2 for $P_0$/$P_1$
73 and PIE-1 for $P_2$-$P_4$. In this work we asked if the TOP-2/condensin II and/or the H3K9me
74 heterochromatin pathways are required in the P-lineage for genome silencing, and found that
75 the H3K9me heterochromatin pathway is dispensable while TOP-2/condensin II is required for
76 $P_2$-$P_4$, but not for $P_0$-$P_1$. We also find that TOP-2/condensin II requires the germline progenitor
77 state to silence the genome, and that PIE-1 does not. These findings increase our understanding

78 of how whole-genome silencing occurs and highlight the importance of developmental context

79 in regulating the ability of TOP-2/condensin II to perform this task.

80 # Materials and Methods

81 ## *C. elegans* strains

82 N2 (wild-type), WMM2 *(ltls37 [(pAA64) pie-1p::mCherry::his-58 +unc-119(+)] IV; unc-*
83 *4(e120) top-2(it7ts) II)*, MT17463 *(set-25(n5021) III)*, WM330 *(pie-1(ne4301[pie-1::GFP]) III)*,
84 AG275 *(top-2(av64)[TOP-2::3XFLAG] II)* and EG5175 *(pie-1(ne4301[pie-1::gfp]) III; mex-*
85 *5(egx1[F294N & F339N]) IV)* strains were used in this study. Worms were maintained on 60-mm
86 plates containing nematode growth media (NGM) seeded with the *E. coli* strain OP50 or HT115.
87 Worms were grown at 20°C and propagated through egg preparation (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 μg/ml
93 carbenicillin and 12.5 μg/ml tetracycline and seeded on NGM + carbenicillin + tetracycline plates
94 were also used as a source of food. Our RNAi strains were obtained from the Ahringer library and
95 verified by Sanger sequencing. Bacteria containing dsRNA were streaked on LB-agar plates
96 containing 100 μg/ml carbenicillin and 12.5 μg/ml tetracycline and incubated at 37°C overnight.
97 Single colonies were then picked and grown in 25 ml LB cultures with 100 μg/ml carbenicillin and
98 12.5 μg/ml tetracycline. 500 μl of this culture was seeded on 60-mm Petri dishes containing 5
99 mM 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 1
108 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" section.
113 For double RNAi treatments, RNAi cultures were mixed at a 1:1 ratio by volume. HT115 cells
114 transformed with an empty pL4440 vector was used as a negative control. RNAi conditions used
115 in this study and tests for their efficacy is described below:

116

117 <u>*top-2* RNAi</u>

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

120 *capg-2* RNAi

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

124 *pie-1* RNAi

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

127 *mex-5/6* RNAi

128 Worms were grown on HT115 food plates for the first 48 hours of their life and were then
129 switched to *mex-5/6* RNAi plates for the remaining 24 hours. An embryonic lethality of 90%-100%
130 was observed for this RNAi treatment.

131 *mex-6*/control RNAi

132 Worms were grown on *mex-6*/control RNAi plates for the entirety of their life cycle. An embryonic
133 lethality of 90%-100% was observed.

134 *mex-6/pie-1* RNAi

135 Worms were grown on *mex-6/pie-1* RNAi plates for the entirety of their life cycle. Am embryonic
136 lethality of 90%-100% was observed.

137

138 **Antibodies and dilutions**

139 RNAPIIpSer2: Rabbit antibody from Abcam (ab5095, Cambridge, Massachusetts) was
140 used at a dilution of 1:100. GFP: mouse Mab #3580, from EMD Millipore, was used at 1:500.
141 FLAG: Mouse antibody F1804 from EMD Millipore was used at 1:1000. H3K9me3: Rabbit antibody
142 from Abcam (ab176916, Cambridge, Massachusetts) was used at a dilution of 1:1000. P-granules:
143 mouse Mab OIC1D4 (Fig 1A) or mouse Mab K76 (remainder of study), both from the
144 Developmental Studies Hybridoma Bank, were used at a dilution of 1:10 or 1:20, respectively.
145 Secondary antibodies: Alexa Fluor conjugated secondary antibodies from Invitrogen
146 (Thermofisher Scientific, Waltham, Massachusetts) were used at a dilution of 1:200.

147

148 **Immunofluorescence staining**

149 Adult worms were first washed off plates with 10 ml of M9 minimal medium and washed
150 3 more times. Then, they were centrifuged at 1.9 KRPM and the excess medium was removed.
151 20 µl of media containing about 50 worms were spotted on a coverslip and 3 µl of anesthetic
152 (20mM Sodium Azide and 0.8M Tetramisole hydrochloride) was added to immobilize them.
153 Worms were dissected using 25Gx5/8 needles (Sigma Aldrich, St. Louis, Missouri). To release
154 early embryos, worms were cut once midway through their length. To release gonads for oocyte
155 staining, worms were cut twice, once near the head and once near the tail. The coverslip was
156 then mounted onto poly-L-lysine covered slides and let rest for 5 minutes. Slides were put on dry
157 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 10%
173 goat serum. Primary antibodies were then applied at the dilutions described above in TNB and
174 slides were incubated at 4°C overnight. On the next day, the slides were washed 3 times with TBS
175 and slides were incubated with secondary antibodies and Hoechst 33342 dye for 2 hours at room
176 temperature. Slides were washed 3 times with TBS, mounting medium (50 % glycerol in PBS), and
177 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 hairpins,
181 and hybridization, wash, and amplification buffers were purchased from Molecular Instruments
182 (molecularinstruments.com). Genes that were examined were *vet-6* and *F58E6.6*. DNA was
183 visualized with Hoechst 33342 dye. Embryos were prepared by bleaching and were immediately
184 spotted on poly-L-lysine coated slides. Coverslips were applied and slides were freeze-cracked to
185 permeabilize samples. Immediately after freeze-cracking, 500 µl of 100% ice-cold methanol was
186 applied over the samples. Slides were dried off by tilting slides, 1 ml of 4% paraformaldehyde
187 (PFA) was added and samples were incubated in a humidity chamber for 10 minutes. Samples
188 were then washed 3 times with 100 µl of PBS-T. A 1:1 solution of probe hybridization buffer (PHB)
189 and PBS-T was added to the samples, and they were incubated for 5 minutes at room
190 temperature. Samples were then prehybridized with PHB for 30 minutes at 37°C and DNA probes
191 (at a final concentration of 2 picomoles per 500 µl of PHB) were added to the samples and were
192 incubated overnight at 37°C. The next day, samples were washed 4 times with probe wash buffer
193 (PWB) at 37°C with 15 minutes of incubation for each wash. They were then washed three more
194 times with 5xSSCT at room temperature. Samples were pre-amplified with Amplification buffer
195 for 30 minutes at room temperature. Probe amplifier hairpins were snap cooled by heating to
196 95°C for 90 seconds and putting in a dark drawer for 30 mins then were added to the sample.
197 Worms were incubated with the hairpins overnight in a dark drawer. On the third day, samples
198 were washed with 5xSSCT and incubated with Hoechst-33342 (1:5000 dilution) for 15 minutes.
199 Finally, samples were mounted on poly-L-lysine coated slides and imaged.

200

201 **Immunofluorescent imaging**

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

205 # Results

206 ## One- and two-cell embryos do not require either SET-25 or TOP-2 for genome
207 silencing.

208 We first examined one- and two-cell embryos, which previous work has shown to be
209 reliant on OMA-1/2 for transcriptional repression (4). One hallmark of silencing in both oocytes
210 and starved Z2/Z3 is a large accumulation of H3K9me3 marks on chromatin, as inferred by
211 immunofluorescence microscopy (2,8). In *C. elegans*, oocytes are named by their position relative
212 to the spermatheca in the tube-shaped gonad, where -1 is closest. Starting at the -5 position,
213 H3K9me3 marks begin to accumulate on chromatin such that at the -2 and -1 positions H3K9me3
214 signals overlap with much of the DAPI-based DNA signal (ref 2, see Fig 2A). By contrast, in zygotes,
215 the maternal pronucleus (as well as its paternal counterpart) showed greatly reduced H3K9me3
216 signal intensity, manifesting as small patches on otherwise barren chromatin (Fig 2B). This was
217 true of both newly fertilized zygotes and those that were nearing mitosis (Fig 2B). Interestingly,
218 the polar bodies retained strong H3K9me3 signals (Fig 2B). These data suggest that, upon
219 fertilization, H3K9me3 marks are rapidly erased from maternal chromatin, but this does not occur
220 on polar bodies as they have been ejected from the zygote's cytoplasm and thereby escape
221 erasure.

222

223 Our previous work has shown that the SET-25 methyltransferase is responsible for the
224 accumulation of H3K9me3 marks on both oocyte and starved Z2/Z3 chromatin, and this enzyme

225 is also required for transcriptional repression in these cells (2,8). To ask if SET-25 plays a role in
226 transcriptional silencing in one- or two-cell embryos we monitored *set-25*$^{n5021}$ mutant embryos
227 for active RNA polymerase II (RNAPII). For this we used immunofluorescence staining with an
228 antibody that detects a phospho-serine 2 epitope on the RNAPII carboxyl-terminal domain (CTD).
229 The presence of this RNAPIIpSer2 signal in nuclei is a marker for actively elongating RNAPII (9),
230 and we and others have used this approach extensively to identify transcriptionally active nuclei
231 within a variety of *C. elegans* tissues (1-4, 10-16). The antibody that we are using, a rabbit
232 polyclonal that recognizes RNAPIIpSer2, has previously been validated by us for use in *C. elegans*
233 embryos (8). The samples were also stained with an antibody recognizing P-granules, as these
234 structures are specific to P-cells and can therefore be used to distinguish them from somatic cells.
235 As shown in Fig 2C, *set-25* mutants were still fully capable of repressing transcription in both one-
236 and two-cell embryos. Next, we used RNAi to deplete TOP-2. Our previous work, with these same
237 RNAi conditions, has shown that loss of TOP-2 promotes unscheduled transcription in oocytes,
238 spermatocytes, and starved Z2/Z3 (2,3,8). However, in one- or two-cell embryos, TOP-2 depletion
239 had no impact on transcriptional repression (Fig 2C). We conclude that the H3K9me pathway is
240 not required for transcriptional silencing in one- or two-cell embryos, and that the same is true
241 for TOP-2.

242

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

245 We next examined $P_2$ and $P_3$ for the presence of H3K9me3 as well as a requirement for
246 SET-25 in transcriptional repression, with the idea that the H3K9me pathway might become
247 required for silencing upon loss of OMA-1/2. This was not the case, however, as there were no
248 detectable differences in the amount of H3K9me3 marks on $P_2$ or $P_3$ relative to their somatic
249 sister cells (EMS and C, respectively, see Fig S1A). In addition, transcription was not derepressed
250 in $P_2$ in *set-25*$^{n5021}$ mutant embryos (Fig S1B). We thus turned our attention to the possibility that
251 the TOP-2/condensin II pathway might play a role in genome silencing in $P_2$, $P_3$, and $P_4$. For this
252 we again stained embryos for RNAPIIpSer2 and P-granules, as we did in Fig 1C. In control 4-cell
253 embryos the expected pattern emerged – signal could be seen in the somatic ABa, ABp, and EMS
254 cells, but not $P_2$ (Fig 3A). We next depleted either TOP-2 or the CAPG-2 subunit of condensin II.
255 Interestingly, in samples exposed to either *top-2* or *capg-2* RNAi, we could now detect
256 RNAPIIpSer2 in $P_2$, and the same was true of samples exposed to *pie-1* RNAi (Fig 3A). We also
257 examined the remainder of the P-lineage ($P_3$ and $P_4$) and in both cases we could detect active
258 transcription after depletion of either TOP-2, CAPG-2, or PIE-1 (Fig 3A). Based on these data, we
259 conclude that TOP-2/condensin II is dispensable for transcriptional repression in $P_0$ and $P_1$, but
260 for $P_2$, $P_3$, and $P_4$ the pathway is required to silence gene expression. We note that in Fig 3A the
261 P-granules would occasionally manifest as a yellow color, instead of green as would be expected
262 by the combination of primary and secondary antibodies used to detect them. We believe this is
263 due to occasional cross-reactivity between the secondary antibody used to detect RNAPIIpSer2
264 (Alexa 555 goat anti-rabbit, ThermoFisher #A21428) and the primary antibody used to detect P-
265 granules (Mab OIC1D4, from ATCC). This was not an issue when we used Mab K76 to detect P-
266 granules (see Figs 2C and S1B) and because P-granules are localized outside of the nucleus the

occasional cross-reactivity between Alexa 555 goat anti-rabbit and Mab OIC1D4 did not impact data collection or interpretation.

It was important to check if *top-2* or *capg-2* depletion reduced PIE-1 expression in $P_2$-$P_4$, as this would explain the appearance of RNAPIIpSer2 in these cells. To do so we used a strain harboring PIE-1 tagged with GFP at the endogenous locus (referred to as PIE-1::GFP, refs 17,18). PIE-1::GFP is expressed normally and retains PIE-1 function (17,18). We measured GFP signal intensity in P-cells after control, *top-2*, or *capg-2* RNAi and, as shown in Fig 3B, PIE-1::GFP expression in $P_2$ was not noticeably altered after depletion of TOP-2 or CAPG-2. Thus, the mechanism by which TOP-2/condensin II silences gene expression in the P-lineage does not involve the control of PIE-1 expression levels.

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

Data presented thus far demonstrate aberrant RNAPII activity in the P-lineage after depletion of TOP-2 or CAPG-2 (Fig 3A). To obtain additional evidence for the misregulation of gene expression in these cells, we performed the *in-situ* hybridization chain reaction (HCR), also known as RNA-FISH (19), to detect and localize mRNAs in early embryos. We identified a transcript, F58E6.6, that is found predominantly in EMS under normal conditions, with only 10-20% of the wild-type samples showing transcript signals in ABa, ABp, or $P_2$ (Fig 4A, quantification

287 is shown to the right in Fig 4B on a per embryo basis). After TOP-2 or CAPG-2 depletion, however,
288 50% and 82%, respectively, of the embryos expressed F58E6.6 in $P_2$ (Fig 4A&B). As a positive
289 control, we depleted PIE-1 and found that F58E6.6 was expressed in $P_2$ in 5 out of 5 samples (Fig
290 4A&B). These data show that loss of either TOP-2/condensin II or PIE-1 allows unscheduled
291 transcription of F58E6.6 in $P_2$. We also examined another EMS-specific transcript, the previously
292 reported *vet-6* (6). We observed that *vet-6* was also misexpressed in $P_2$ upon depletion of TOP-
293 2, CAPG-2, or PIE-1 (Fig S2), however the effect of CAPG-2 depletion was not as pronounced as
294 that seen for the F58E6.6 transcripts in Fig 4. Based on these data, we conclude that depletion of
295 TOP-2 and condensin II results in the misexpression of EMS genes in the P-lineage of early
296 embryos.

297

298 **PIE-1 can repress transcription outside of the P-lineage whereas TOP-2 cannot.**

299 Our work has shown that genome silencing in the $P_2$, $P_3$, and $P_4$ cells requires both PIE-1
300 and the TOP-2/condensin II pathway. PIE-1 is expressed exclusively in the P-lineage, however
301 TOP-2 and condensin II are likely expressed in all blastomeres of the early embryo. To confirm
302 this for TOP-2 we used a strain where TOP-2 contains a FLAG tag at the endogenous locus
303 (referred to as TOP-2::FLAG). TOP-2::FLAG retains TOP-2 function (20) and is indeed present in all
304 4 cells of the 4-cell embryo (Fig S3A). This raises the question of why TOP-2 can repress
305 transcription in P-cells but not somatic cells. One possibility is that TOP-2 requires the presence
306 of PIE-1 to silence the genome. We have previously shown that this is true in oocytes, where loss
307 of either PIE-1 or TOP-2 prevents transcriptional repression (2). By contrast, PIE-1 is not

308 expressed in the Z2/Z3 PGCs and TOP-2 is nonetheless required for genome silencing during L1
309 starvation (8). These findings show that TOP-2 can repress transcription through both PIE-1
310 dependent and independent mechanisms.

311

312 To discern the relationship between PIE-1 and TOP-2 in early embryos we sought a means
313 of misexpressing PIE-1 in the early embryo. Previous work has shown that depletion of the
314 translational regulators MEX-5 and MEX-6 allows PIE-1 expression in all cells of the 4-cell embryo
315 (21). The loss of PIE-1 asymmetry in *mex-5/6* RNAi embryos is accompanied by loss of somatic
316 asymmetries as well, e.g. the SKN-1 protein that is normally expressed only in the somatic EMS
317 cell is also found in all four cells after *mex-5/6* RNAi (21). Thus, depletion of MEX-5/6 results in
318 blastomeres assuming a merged somatic and germline precursor state. We confirmed the effect
319 of *mex-5/*6 RNAi on PIE-1 localization by using the strain expressing PIE-1::GFP. As shown in Fig
320 S3B, in control samples PIE-1::GFP is expressed solely in $P_2$, whereas loss of MEX-5/6 causes a
321 redistribution to all four blastomeres, as expected. Furthermore, when samples were stained for
322 RNAPIIpSer2, control embryos displayed signals in EMS but not $P_2$, as expected, while the *mex-*
323 *5/*6 RNAi samples did not show active RNAPII in any cells, consistent with the misexpression of
324 PIE-1::GFP. Thus, loss of MEX-5/6 causes misexpression of PIE-1::GFP in all blastomeres, and also
325 represses transcription in all blastomeres. To determine if misexpressed PIE-1::GFP is the reason
326 that *mex-5/6* embryos are blocked for transcription we used a strain expressing both an RNA-
327 binding deficient form of MEX-5 (*mex-5*$^{egx1}$) and PIE-1::GFP (18). This strain is disabled for MEX-5
328 function but remains viable through MEX-6 activity (18). We treated this strain with *mex-6* RNAi
329 together with either control or *pie-1* RNAi to create conditions where animals were depleted of

330 either MEX-5/6 activity alone or MEX-5/6 and PIE-1::GFP activity. Transcriptional activity in four-
331 cell embryos was then assessed via RNAPIIpSer2 staining. As shown in Fig S3C, in samples treated
332 with *mex-6* plus control RNAi, PIE-1::GFP was expressed in all four blastomeres and RNAPIIpSer2
333 was attenuated. By contrast, in samples exposed to RNAi against both MEX-5 and GFP-PIE-1, GFP
334 signals were reduced and RNAPIIpSer2signals were easily detected in all four blastomeres. We
335 conclude that the pan-embryo transcriptional repression observed after loss of MEX-5/6 activity
336 is due to misexpression of PIE-1::GFP.

337

338 To examine a role for TOP-2 in promoting transcriptional repression by misexpressed PIE-
339 1, we used a temperature-sensitive allele of *top-2* (20) in combination with *mex-5/6* RNAi and
340 examined RNAIIpSer2 in 4-cell embryos at both the permissive temperature (15°C) and the non-
341 permissive temperature (24°C). For control RNAi, we observed that inactivation of TOP-2 caused
342 the appearance of RNAIIpSer2 in $P_2$, as expected, whereas samples raised at the permissive
343 temperature were devoid of RNAIIpSer2 in $P_2$ (Fig 5A upper panels). Interestingly, after *mex-5/6*
344 RNAi, transcription was attenuated in all four blastomeres, regardless of the activity state of TOP-
345 2 (Fig 5A, bottom panels). Taken together, our data show that inactivation of PIE-1 in a MEX-5/6
346 deficient condition prevents pan-embryo transcriptional repression (Fig S3C), whereas
347 inactivation of TOP-2 in MEX-5/6 deficient samples does not (Fig 5A). These findings, in turn,
348 suggest that TOP-2 requires the germline progenitor state for genome silencing in early embryos
349 and that when this state is perturbed then TOP-2 loses its ability to repress transcription
350 (summarized in Fig 5B).

351 ## Discussion

352 In this study we focused on the early embryonic P-lineage with the goal of determining if
353 the TOP-2/condensin II and/or H3K9me/heterochromatin genome silencing pathways are
354 required for transcriptional repression in these cells. Previous work has revealed that two
355 different genome silencers, OMA-1/2 and PIE-1, are active in the P-lineage, with OMA-1/2
356 responsible for $P_0$ and $P_1$ (as well as AB) and PIE-1 taking over for $P_2$-$P_4$ (Fig 1). Our work has added
357 to this profile with the demonstration that TOP-2 and condensin II are needed for efficient
358 silencing in $P_2$-$P_4$, but not $P_0$ or $P_1$, and that the H3K9me/heterochromatin pathway is not
359 required at all in the P-lineage. We base these conclusions on the following data. For TOP-
360 2/condensin II we show that depletion of either TOP-2 or the condensin II subunit CAPG-2 results
361 in the appearance of RNAPIIpSer2 signals in $P_2$, $P_3$, and $P_4$ (Fig 3A) and inappropriate transcription
362 of the somatic F58E6.6 and *vet-6* genes in $P_2$ (Figs 4 and S2). We note that the impact of TOP-2
363 or CAPG-2 depletion on transcription in the P-lineage was not as profound as depletion of PIE-1
364 (Figs 3,4, and S2), and this is likely due to the fact that we use mild RNAi conditions for TOP-
365 2/CAPG-2, relative to PIE-1, as stronger conditions for TOP-2/CAPG-2 result in torn, cut, and
366 malformed nuclei, as has been reported previously (22-24). Although we tested just one
367 condensin II subunit in this work, our previous work had shown that depletion of KLE-2, in
368 addition to CAPG-2, disrupts silencing in starved Z2/Z3 (8), and thus it seems likely that the *capg-*
369 *2* phenotypes reported here represent a loss of condensin II function and not a condensin II
370 independent function of CAPG-2.

371

372 For the H3K9me/heterochromatin pathway we focused on the SET-25 methyltransferase,
373 which we have previously shown to be required for genome silencing in both oocytes and in
374 starved Z2/Z3 (2,8). We could not reveal a role for SET-25 in P-lineage silencing (Figs 2 and S1).
375 Furthermore, unlike oocytes and starved Z2/Z3, we did not observe a hyper-accumulation of
376 H3K9me3 marks in P-lineage nuclei (Figs 2 and S1), adding further support to the conclusion that
377 the H3K9me/heterochromatin pathway is dispensable for genome silencing in early embryos.

378

379 Our work, presented here and elsewhere, has revealed a role for the TOP-2/condensin II
380 pathway in transcriptional repression in three different contexts – oocytes and spermatocytes
381 (refs 2-3 and Fig 6A ), starved Z2/Z3 PGCs (ref 8 and Fig 6B) and in the $P_2$, $P_3$, and $P_4$ cells of early
382 embryos (this work and Fig 6C). Mechanistically, available data suggest that TOP-2/condensin II
383 silences transcription in oocytes via chromatin compaction, which could block access of the
384 transcriptional machinery to gene promoters ("promoter occlusion" in Fig 6A). The same
385 mechanism also applies to starved Z2/Z3, as we have shown that starvation triggers extreme
386 chromatin compaction and that when compaction fails then nuclei remain transcriptionally active
387 (ref 8 and Fig 6B). How the TOP-2/condensin II pathway silences transcription in $P_2$-$P_4$ is not clear
388 as the requirement for DNA replication during cell division seemingly precludes the
389 establishment of a stably compacted chromatin state. Another important question raised by our
390 findings is why, in the early embryo, TOP-2/condensin II is only active for transcriptional
391 repression in P-cells, despite being ubiquitously expressed. Indeed, we have shown here that
392 when P-cell identity is lost, then PIE-1 no longer requires the presence of TOP-2 to block
393 transcription, as it does in both the P-lineage and in oocytes (this work and ref 2). Thus, it appears

394 that the there is something about the germline or germline progenitor state that transforms TOP-
395 2/condensin II into a genome silencing system, and further work is needed to understand how
396 TOP-2/condensin II is differentially regulated in germline versus soma.

397

398 Lastly, our work has also revealed an intriguing connection between PIE-1 and the TOP-
399 2/condensin II pathway during germline genome silencing. We found that loss of either
400 pathway prevents silencing in both oocytes and in $P_2$-$P_4$, suggesting that the two systems are
401 dependent on one another as opposed to acting redundantly. The mechanism by which PIE-1
402 blocks transcription is unclear. Early work suggested that PIE-1 competes with the RNAPII CTD
403 for binding to the cyclin T component of the CDK9-cyclin T complex that is required for
404 phosphorylation and activation of RNAPII (25,26). PIE-1 has been proposed to titrate cyclin T
405 away from RNAPII, thereby suppressing the CTD phosphorylations that are needed to trigger
406 RNAPII elongation. More recent work, however, has shown that PIE-1 in the adult germline
407 controls SUMOylation of chromatin remodeling factors, such as the NuRD complex component
408 and the histone deacetylase HDA-1 (27). Thus it may be that PIE-1 connects to the TOP-
409 2/condensin II pathway through chromatin as opposed to direct regulation of RNAPII.

410 **Acknowledgements**

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414


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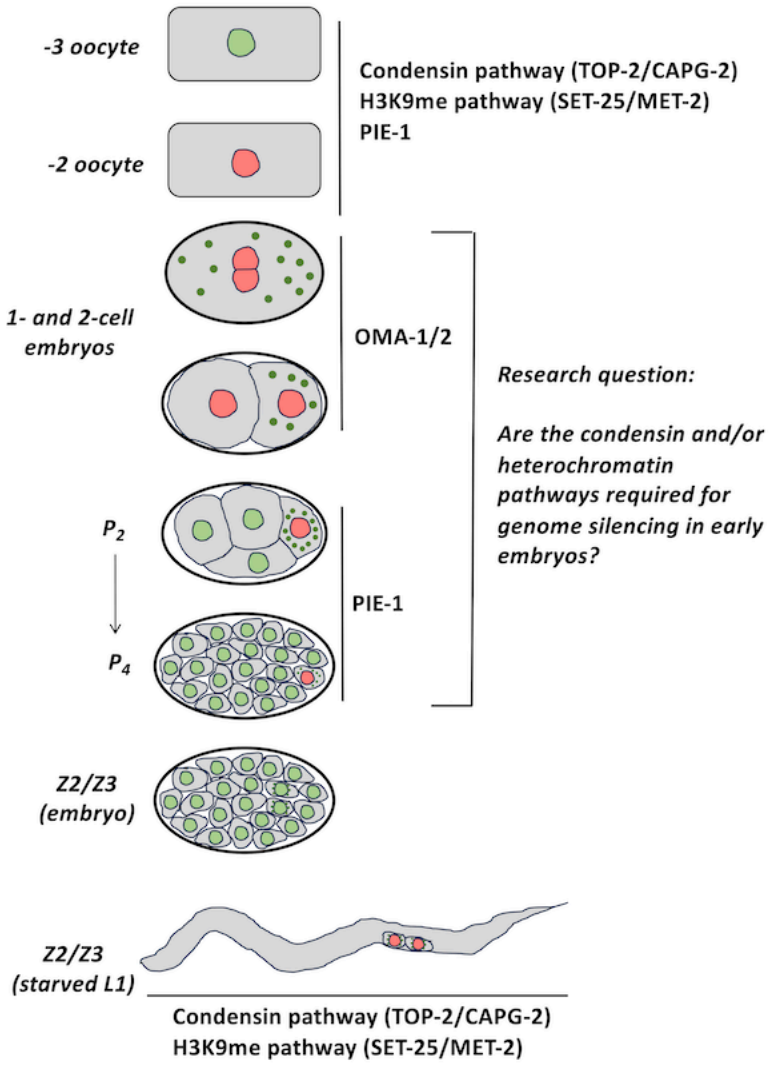
475 **Figures and Legends**



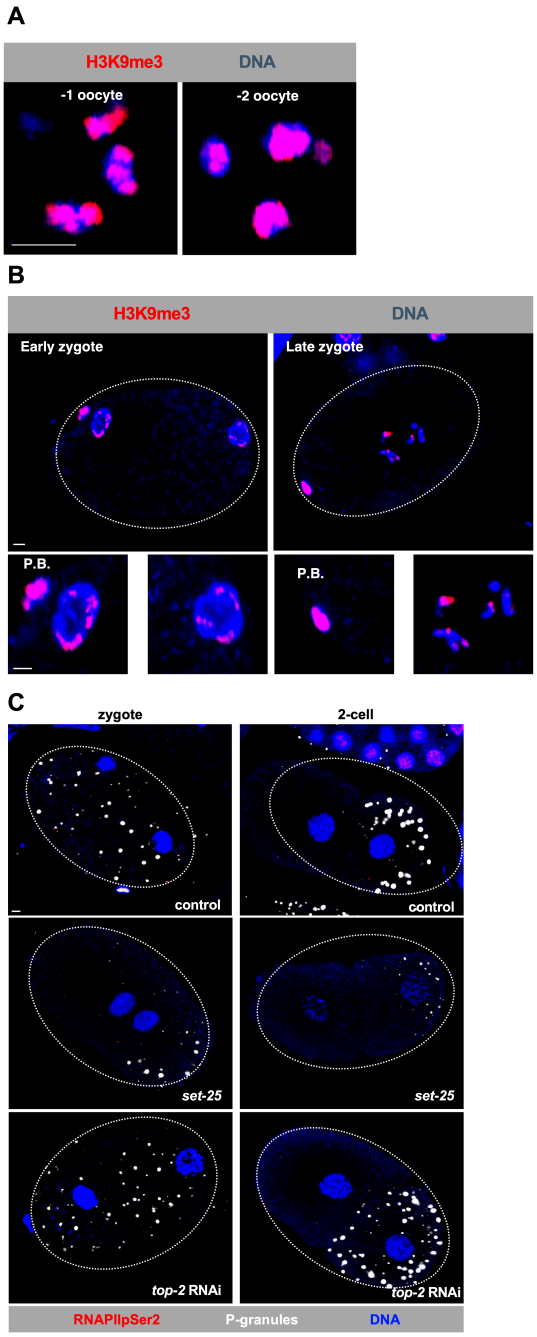

476

477 **Figure 1: Schematic illustrating known genome silencing components during *C. elegans***

478 **germline development.**

479 Please see text for details.

480




481

482 **Figure 2: No role for either the H3K9me heterochromatin pathway or the TOP-2/condensin II**

483 **pathway in transcriptional repression in zygotes or two-cell embryos.**

484 (A) Wild-type oocytes were fixed and stained for H3K9me3 (red) and DNA (blue). Shown are

485 magnified views of the -1 and -2 oocytes, which are the oocytes most proximal and

486 second-most proximal to the spermatheca, respectively. The H3K9me3 signal overlaps
487 with the Hoechst signal extensively. Scale bar represents a length of 2 µm.
488 (B) The indicated zygotes from N2 worms were fixed and stained for H3K9me3 (red) and DNA
489 (blue). Shown below the entire embryos are magnified views of the pronuclei and polar
490 bodies (PB). Note that the H3K9me3 signal overlaps with the Hoechst signal more
491 extensively on the polar bodies than within the pronuclei. Scale bar represents a length
492 of 2 µm.
493 (C) Zygotes and two-cell embryos were fixed and stained for RNAPIIpSer2 (red), DNA (blue),
494 and P-granules (white). No active RNAPII was observed in the more than 20 samples we
495 examined for both one- and two-cell embryos over 2 replicates. Note the RNAPIIpSer2
496 signals in the adjacent, older embryo in the upper right panel – this shows that the
497 staining worked efficiently. Scale bar represents a length of 2 µm.

498

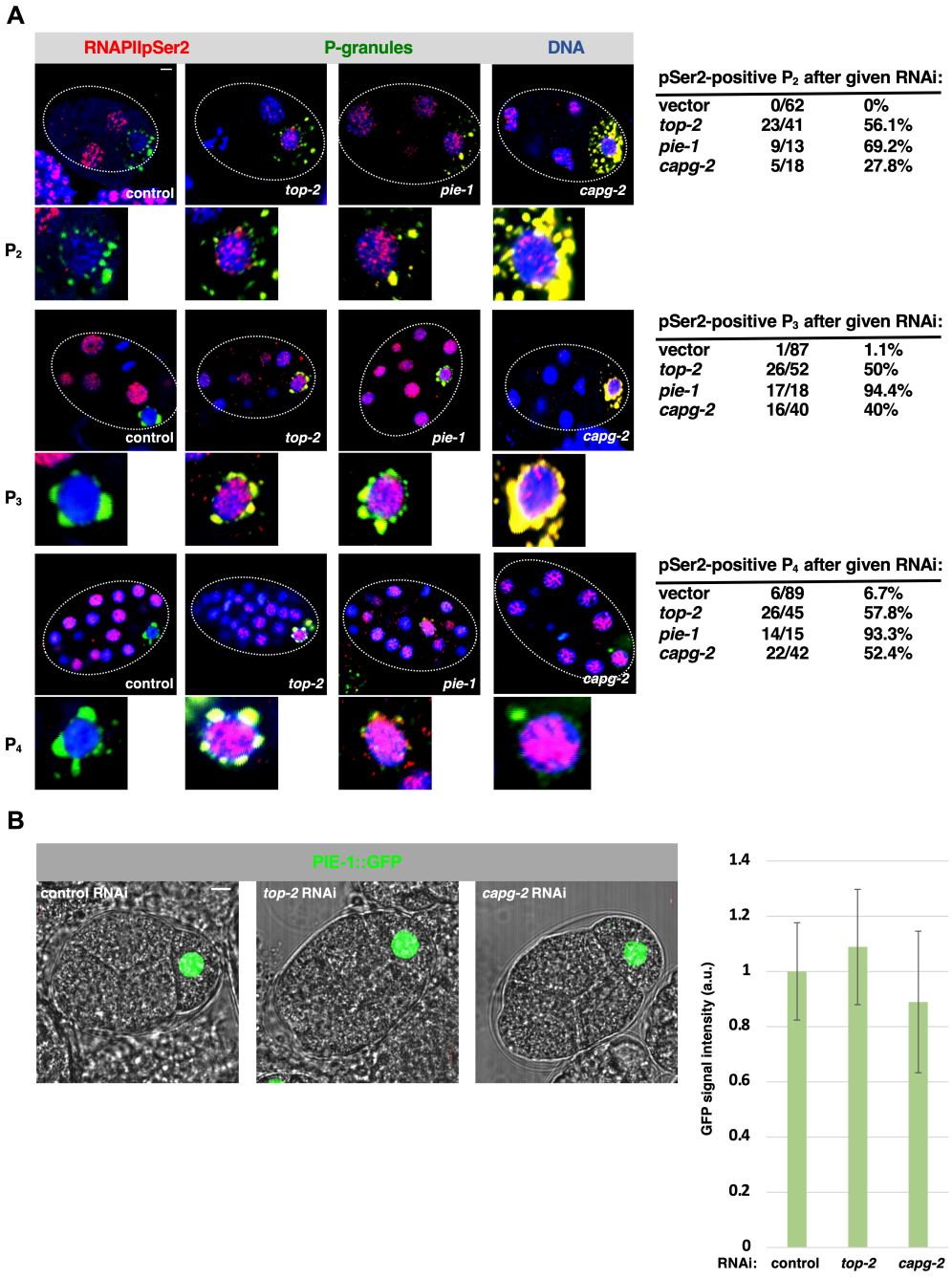



**Figure 3: Loss of TOP-2 or CAPG-2 allows for active RNAPII in the P-lineage of early embryos.**

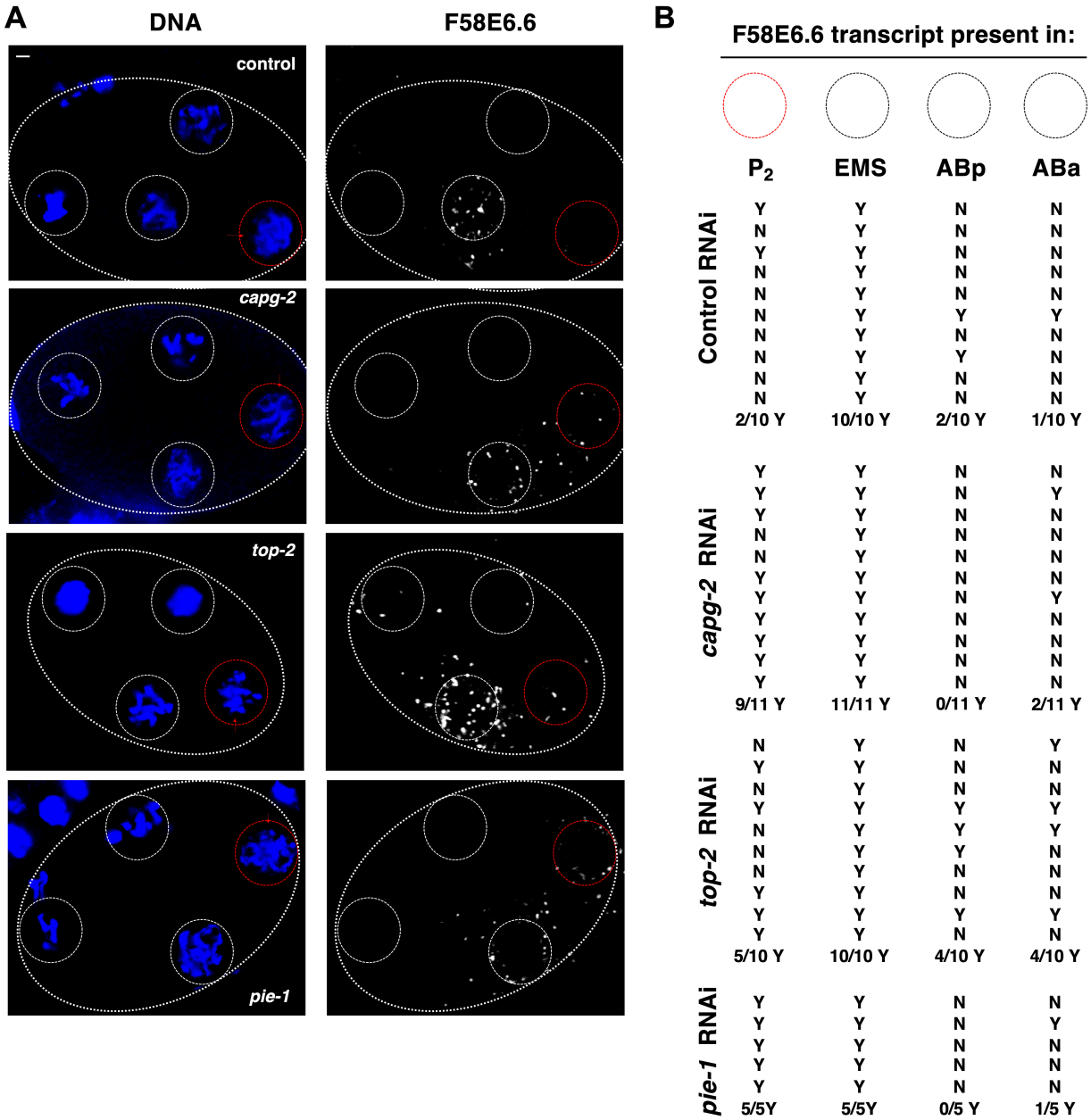
(A) Early embryos from N2 worms treated with control, *top-2*, *capg-2,* or *pie-1* RNAi were fixed and stained for RNAPIIpSer2 (red), DNA (blue) and P-granules (green). Shown on top are the entire embryos and the P-cell is magnified below. Quantification of the data is

504 shown at right. We note that the P-granules in this data set would occasionally manifest
505 as a yellow signal, as opposed to the green signal expected, from the combination of
506 primary and secondary antibodies used to detect them. Please see Results for an
507 explanation for this.

508 (B) Fluorescence and phase-contrast microscopy was used to image four-cell embryos
509 expressing PIE-1::GFP after treatment with the indicated RNAi. The graph shows GFP
510 signal quantification across the three samples. For signal quantification, confocal slices
511 corresponding to maximal signal intensity were analyzed using ImageJ software to
512 measure pixel density. GFP signal intensity was normalized to control. Scale bar
513 represents a length of 5 µm.

514

515




516

517 **Figure 4: EMS-specific gene is aberrantly expressed in the $P_2$ cell of 4-cell embryos after loss of**

518 **TOP-2 or CAPG-2.**

519 (A) Four-cell embryos from N2 animals treated with control, *capg-2*, *top-2,* or *pie-1* RNAi were

520 fixed and HCR was performed to probe for *F58E6.6* mRNA (white). DNA was stained using

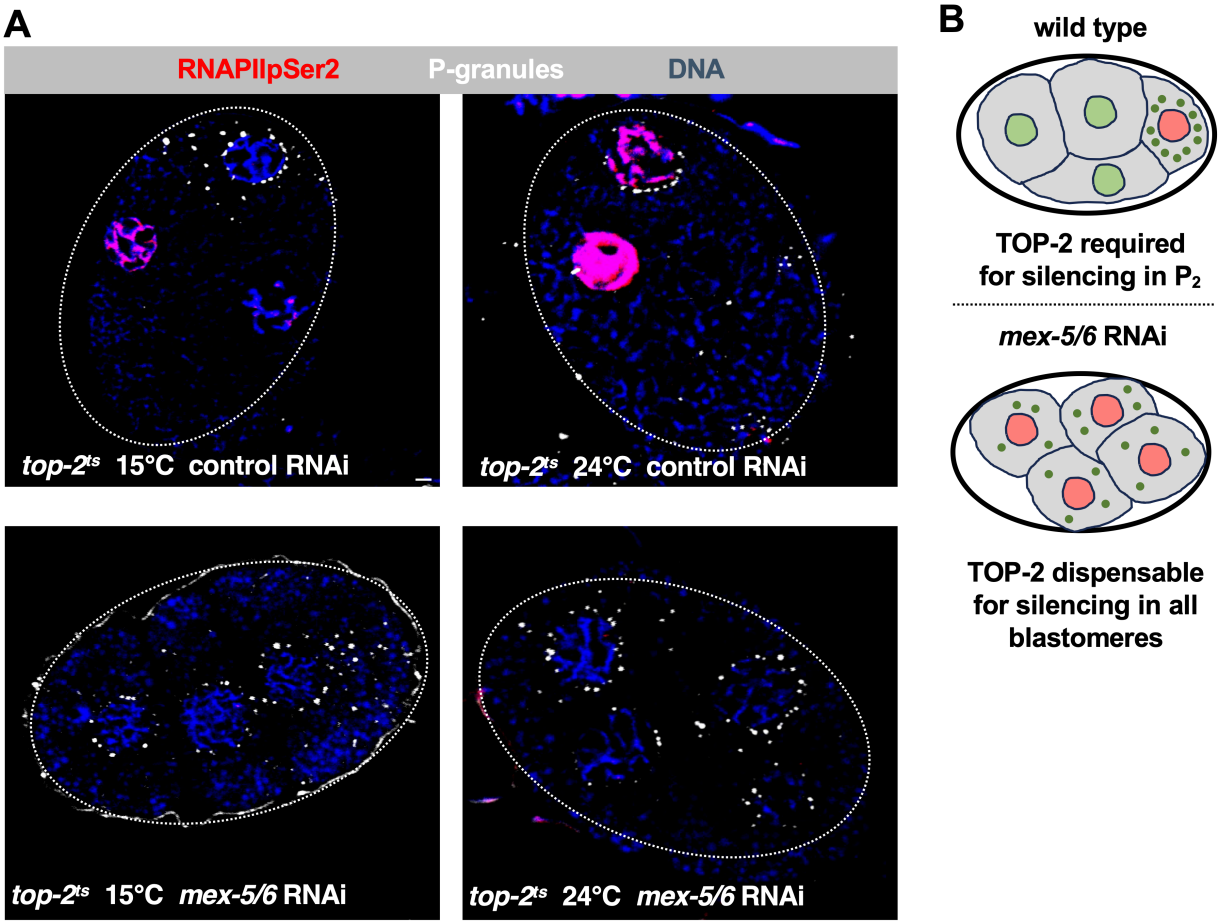
521 Hoechst-33342 (blue). Representative images are shown. White dashed circles represent

522 the EMS cell and the red dashed circles represent $P_2$.

523 (B) Percentage of samples with *F58E6.6* transcripts in EMS $P_2$ (n=40).

524

525




| | pSer2-positive 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 5: PIE-1 does not require TOP-2 to repress transcription in *C. elegans* early embryos.**

(A) Four-cell embryos from WMM2 (*top-2*$^{ts}$) animals treated with either control or *mex-5/6* RNAi were optionally shifted to the nonpermissive temperature of 24°C for 24 hours or left at the permissive 15°C. Then samples were fixed and stained for RNAPIIpSer2 (red),

531 DNA (blue), and P-granules (white). 20 samples were analyzed over 2 independent
532 replicates. Attenuation of TOP-2 via temperature shift does not reverse the
533 transcriptional silencing by PIE-1 in *mex-5/6* RNAi embryos. Quantification of the data is
534 presented below, where "soma" indicates the EMS cell in control RNAi samples. Scale bar
535 represents a length of 2 µm.

536 (B) Cartoon of TOP-2 requirement for transcriptional silencing at the 4-cell embryonic stage.
537 Nuclei colored in red indicate transcriptionally silenced cells.

538

539

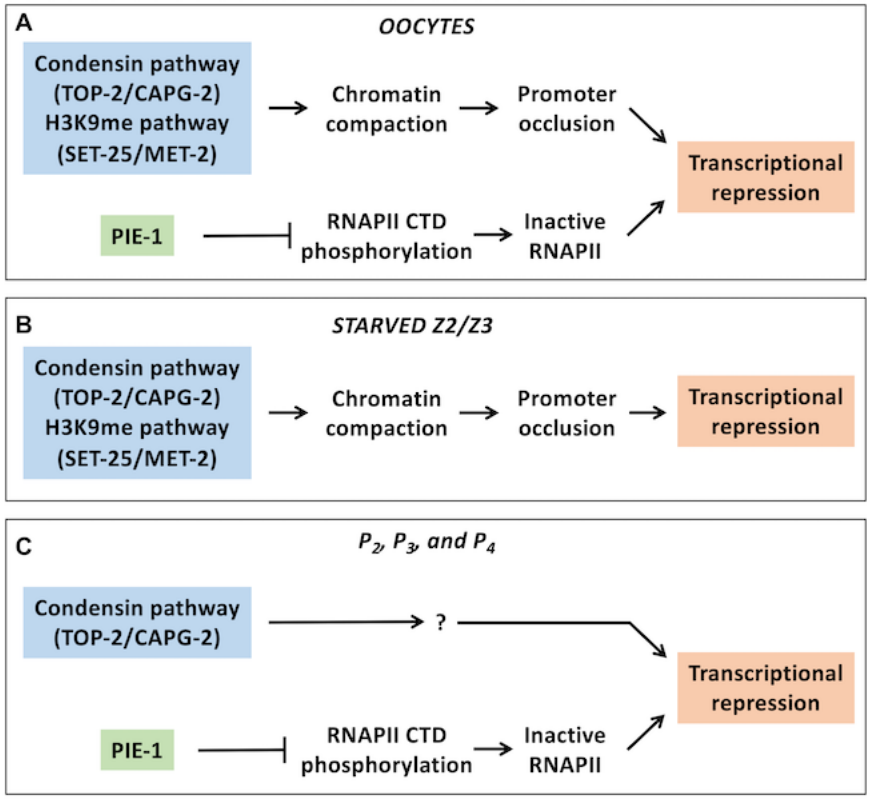


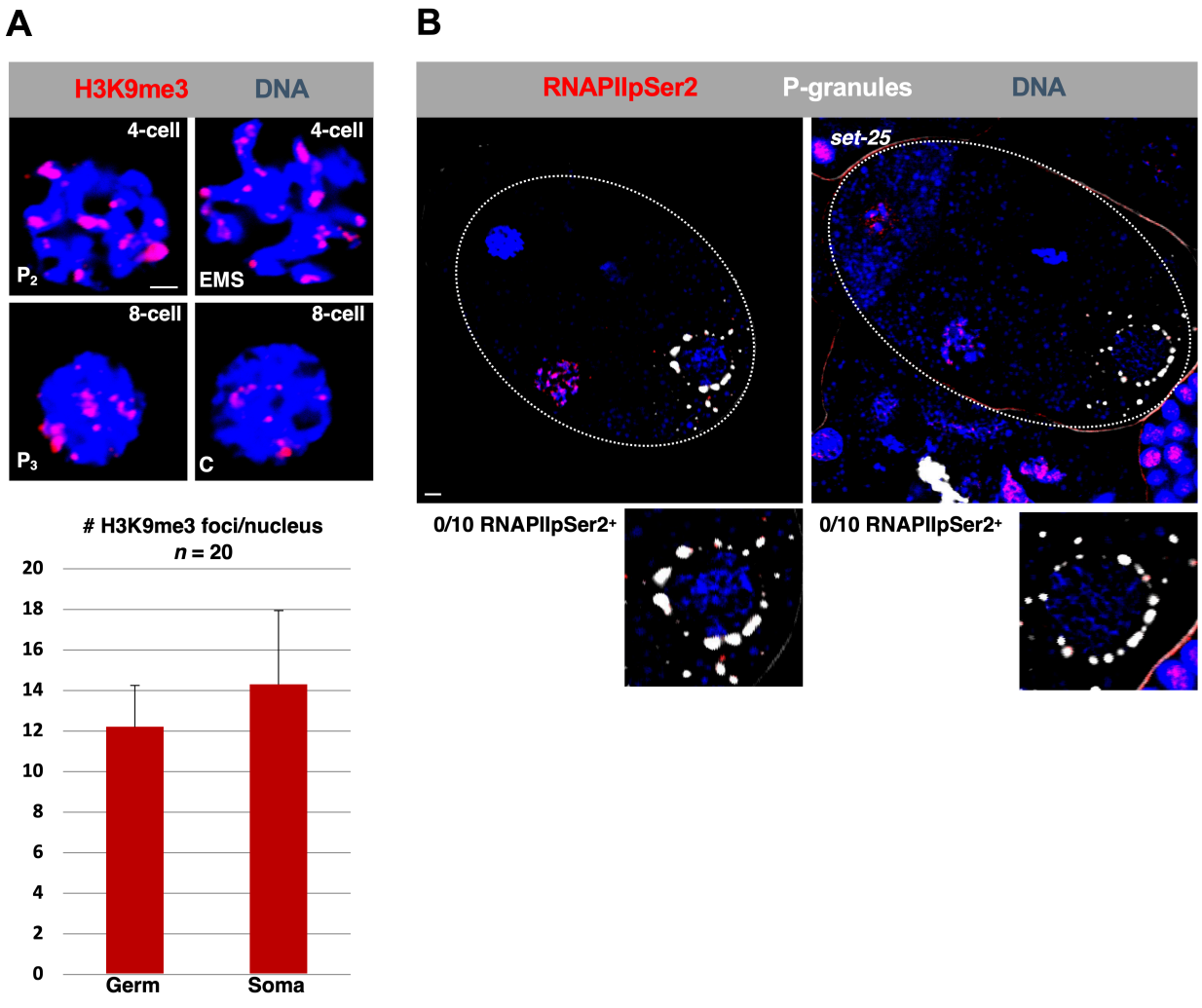

**Figure 6: Summary of genome silencing components at different developmental stages in the germline.**

(A) In oocytes, TOP-2/CAPG-2 and the H3K9me pathway repress transcription via chromatin compaction while PIE-1 may work through control of RNAPII CTD phosphorylation.

(B) In starved L1 larvae, TOP-2/CAPG-2 and the H3K9me pathway are required for transcriptional repression of Z2/Z3.

(C) In P-lineage cells, TOP-2/CAPG-2 works independently of PIE-1 to promote transcriptional repression, although the precise mechanism by which TOP-2/CAPG-2 works is not yet known.

551 # Supporting information figures and legends




552

553 **Figure S1: RNAPII-mediated transcription is still repressed in $P_2$ after loss of SET-25.**

554 (A) Four- and eight-cell embryos were examined were fixed and stained for H3K9me3 (red)

555 and DNA (blue). Quantification of H3K9me3 foci for $P_2$ and $P_3$ (Germ) or EMS and C (Soma)

556 is shown below. Scale bar represents a length of 2 µm.

557 (B) Four-cell embryos, either wild type (N2) or *set-25* mutants (strain MT17463), were fixed

558 and stained for RNAPIIpSer2 (red) and P-granules (blue). 10 samples were analyzed. Both

559 wild-type and *set-25* mutants lacked RNAPIIpSer2 signal in $P_2$. Scale bar represents a

560 length of 2 µm.

561

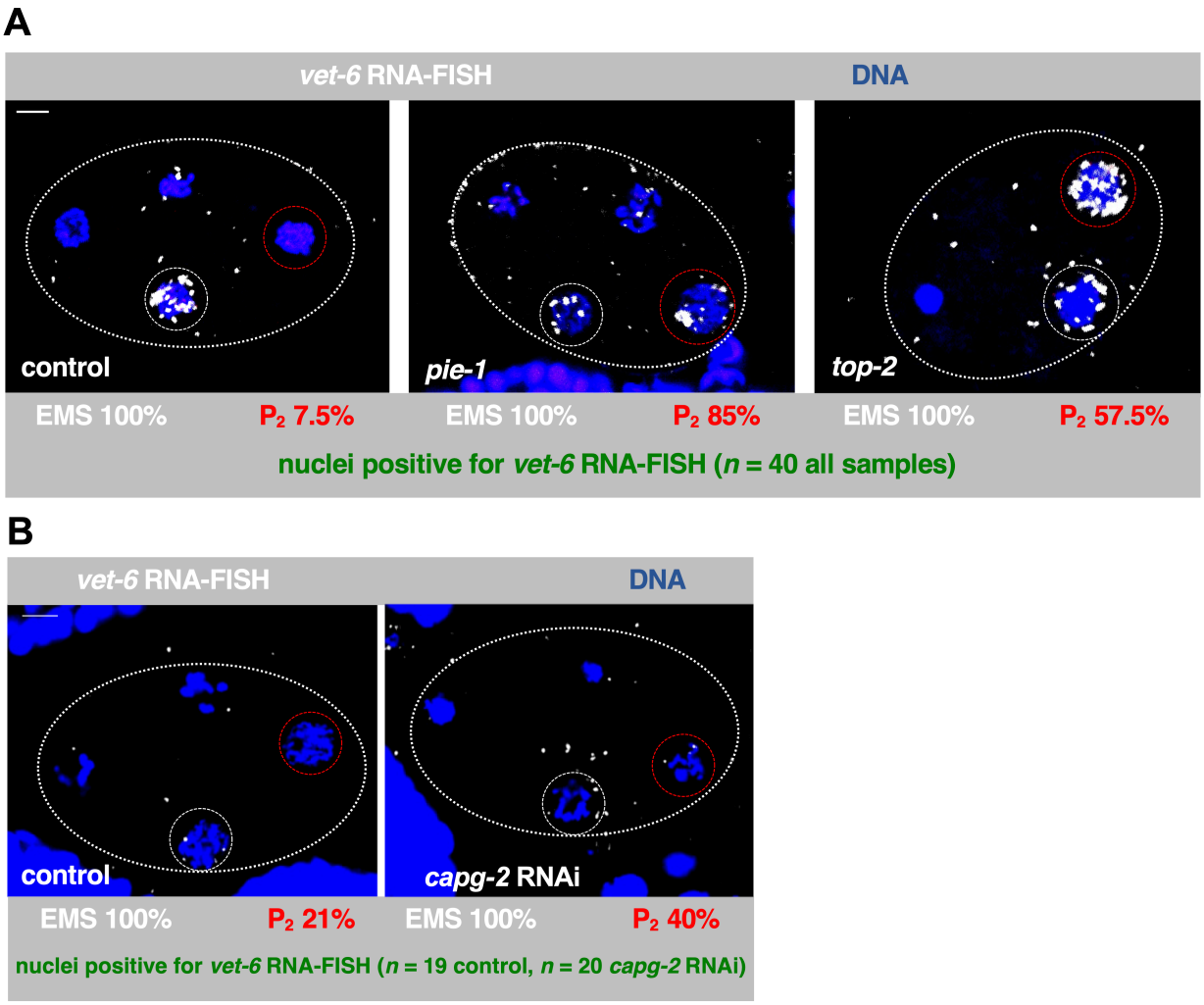
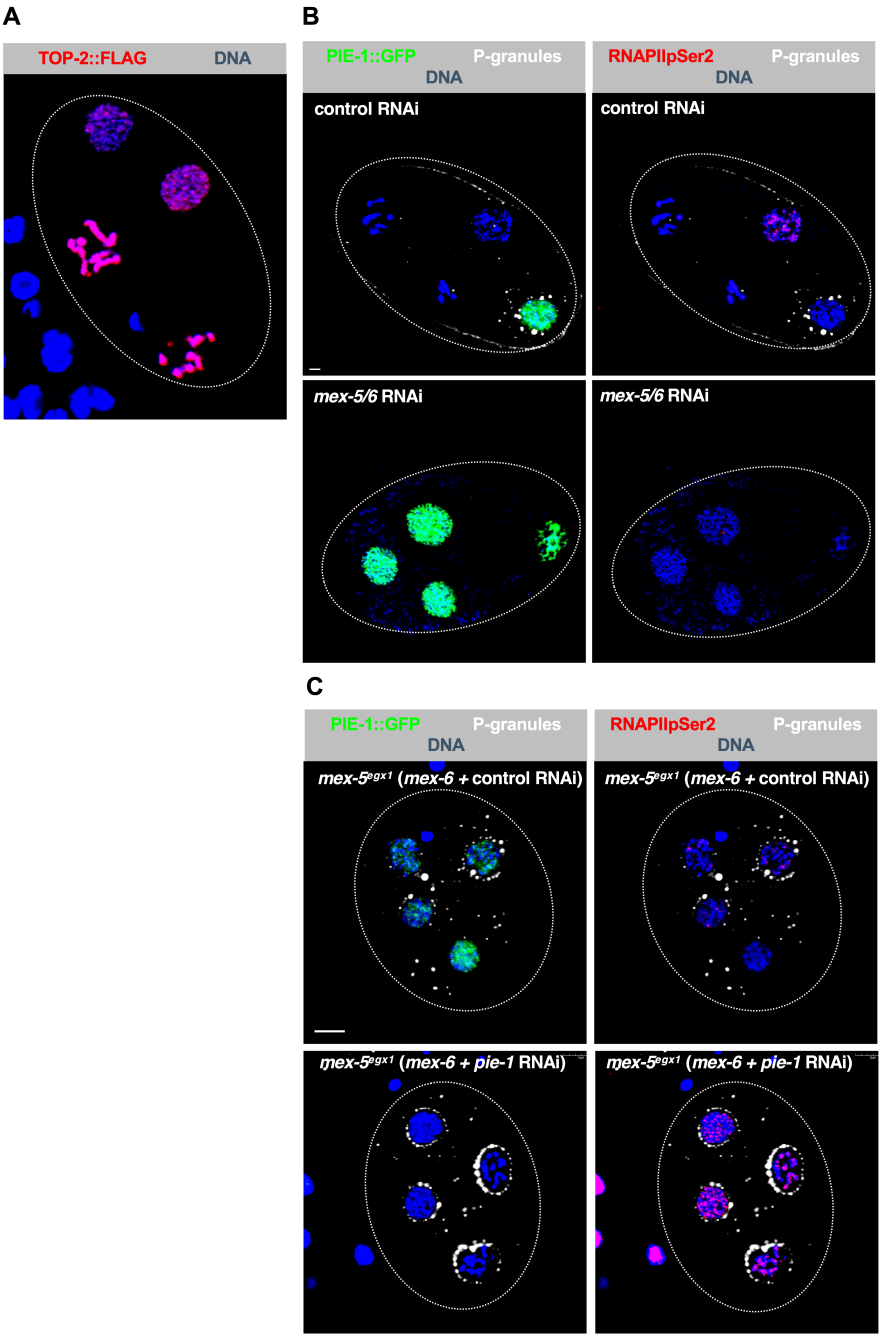



**Figure S2: EMS specific gene is aberrantly expressed in the $P_2$ cell of 4-cell embryos after loss of TOP-2 but not CAPG-2.**

(A) HCR was performed using 4-cell embryos from N2 animals treated with control, *pie-1*, or *top-2* RNAi to probe for *vet-6* mRNA (white). DNA was stained using Hoechst-33342 (blue). $P_2$-associated mRNA signal appears after RNAi depletion of *pie-1* and *top-2*. White dashed circles represent the EMS cell and the red dashed circles represent $P_2$. Scale bar represents a length of 5 µm.

570 (B) HCR was performed using 4-cell embryos treated with *capg-2* RNAi to probe for *vet-6*
571 mRNA (white). DNA was stained using Hoechst-33342 (blue). White dashed circles
572 represent the EMS cell and the red dashed circles represent $P_2$. Scale bar represents a
573 length of 5 µm.

574




575

576 **Figure S3: Depletion of MEX-5 and MEX-6 results in PIE-1 expression in all blastomeres of early**

577 **embryos.**

578 (A) Four-cell AG275 embryo fixed and stained for TOP-2::FLAG (red) and DNA (blue). TOP-2 is

579 distributed across all 4 cells of the embryos. Scale bar represents a length of 2 µm.

580 (B) Four-cell embryos from WM330 animals optionally treated with either control or *mex-*
581 *5/6* RNAi were fixed and stained for PIE-1::GFP (green), RNAPIIpSer2 (red), DNA (blue),
582 and P-granules (white). Images of the same embryo stained for PIE-1::GFP and
583 RNAPIIpSer2 are shown side by side. Depletion of *mex-5/6* results in the loss of
584 asymmetric distribution of PIE-1 to $P_2$. Scale bar represents a length of 2 µm.
585 (C) Four-cell embryos from EGD175 animals were treated with a combination of either *mex-*
586 *6*/control or *mex-6/pie-1* RNAi, then fixed and stained for PIE-1::GFP (green), RNAPIIpSer2
587 (red), DNA (blue), and P-granules (white). *mex-5* mutant embryos depleted of *mex-6* and
588 *pie-1* produce aberrant RNAPIIpSer2 signal in all blastomeres. Scale bar represents a
589 length of 5 µm.

590

591