DIV-1/PolA2 Promotes GLP-1/Notch-Mediated Cellular Events in *Caenorhabditis elegans*

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ABSTRACT

Notch signaling is a highly conserved cell signaling system in most multicellular organisms and plays a critical role in animal development. In various tumor cells, Notch signaling is elevated and has been considered as an important target in cancer treatments. In *C. elegans*, GLP-1 (one of two *C. elegans* Notch receptors) activity is required for cell fate specification in germline and somatic tissues. In this study, we have identified *div-1* gene as a positive regulator for GLP-1/Notch-mediated cellular events. *C. elegans div-1* encodes the B subunit of the DNA polymerase alpha-primase complex and is highly expressed in proliferative germ cells. Functional analyses demonstrated that *i*) DIV-1 is required for the robust proliferation typical of the germline, *ii*) loss of DIV-1 enhances and suppresses specific phenotypes that are associated with reduced and elevated GLP-1/Notch activity in germline and somatic tissues, and *iii*) DIV-1 works together with FBF/PUF proteins, downstream regulators of GLP-1/Notch signaling, to promote germline stem cell (GSC) maintenance and germline proliferation. To maintain GSCs and proliferative cell fate, GLP-1/Notch activity must remain above a threshold for proliferation/differentiation decision. Our results propose that DIV-1 may control the level of threshold for GLP-1/Notch-mediated germline proliferation. PolA2, a mammalian homolog of the *C. elegans* DIV-1, has been emerged as a therapeutic target for non-small cell lung cancer (NSCLC). Notably, Notch signaling is altered in approximately one third of NSCLCs. Therefore, the discovery of the DIV-1 effect on GLP-1/Notch-mediated cellular events has implications for our understanding of vertebrate PolA2 protein and its influence on stem cell maintenance and tumorigenesis.

INTRODUCTION

Germline stem cells (GSCs) are characterized by their ability to produce themselves (known as "self-renewal") and to generate gametes – sperm or eggs (known as "differentiation"). A balance between self-renewal and differentiation of GSCs is strictly regulated by a systematic regulatory network, including extrinsic cues and intrinsic regulators (Wong et al., 2005). Therefore, aberrant regulation of this network can result in either loss of a specific germ cell type or over-proliferation of undifferentiated germ cells, which is associated with germline tumors (Wong et al., 2005). One of the key extrinsic cues is Notch signaling (Liu et al., 2010). This signaling plays varied and essential roles in regulating many types of stem cells (Liu et al., 2010). In *C. elegans* germline, GLP-1 (one of two *C. elegans* Notch receptors) signaling promotes mitotic proliferation of germ cells and maintenance of GSCs (Kimble and Crittenden, 2007) (Fig. 1A). Briefly, Notch ligand, LAG-2, is expressed in stem cell niche (called by "distal tip cell [DTC]" in *C. elegans*) (Henderson et al., 1994) and interacts with the GLP-1/Notch receptor, following by proteolytic cleavage of the GLP-1/Notch receptor. GLP-1/Notch intracellular domain (NICD) is translocated from membrane into nucleus. In nucleus, the NICD forms ternary complex with LAG-1/CSL DNA binding protein and LAG-3/SEL-8/Mastermind transcription coactivator to activate the expression of target genes (Fig. 1B and 1C). Those include *fbf-2* (a member of the PUF (Pumilio/FBF) RNA-binding protein family) (Lamont et al., 2004), *lip-1* (a homolog of the dual-specificity phosphatase) (Lee et al., 2006), *lst-1* (lateral signaling target-1, unknown protein) (Kershner et al., 2014), and *sygl-1* (synthetic Glp-1, unknown protein) (Kershner et al., 2014). These genes function redundantly to maintain GSCs in *C. elegans* germline (Kershner et al., 2014). For example, *lst-1* and *sygl-1* single mutants possessed germlines comparable in size and organization to wild-type, and they were self-fertile (Kershner et al., 2014). However, most *lst-1 sygl-1* double mutants displayed a premature meiotic entry (called Glp [GermLine proliferation defect], no mitotic cells, few germ cells, and all sperm) phenotype and they were thus sterile (Kershner et al., 2014). In addition, FBF-2 and LIP-1 proteins promote germline proliferation by inhibiting the expression or activity of the meiosis-promoting regulators (e.g., GLD-1/Quaking and MPK-1/ERK) (Lee et al., 2006; Kimble and Crittenden, 2007) and cell cycle regulators

74   (e.g., CKI-2, a Cyclin E/CDK2 inhibitor (Kalchhauser et al., 2011)) in the *C. elegans*
75   germline. Notably, Cyclin E has been identified as a positive regulator for GSC
76   maintenance and germline proliferation in *C. elegans* (Fox et al., 2011) and *Drosophila*
77   (Ables and Drummond-Barbosa, 2013). Therefore, elevated GLP-1/Notch activity
78   promotes germline proliferation and inhibits meiotic entry, resulting in germline tumors
79   (Berry et al., 1997). However, reduced GLP-1/Notch activity causes all GSCs to cease
80   self-renewal/germline proliferation and differentiate as sperm (Glp phenotype) (Fig. 1D
81   and 1E).

82   Cell cycle control is a critical step to generate specific cells and tissues during
83   developments and maintain cellular homeostasis in adult (Besson et al., 2008). The cell
84   cycle is tightly regulated by complexes containing Cyclins and Cyclin-dependent
85   kinases (CDKs) (Johnson and Walker, 1999; Murray, 2004). Dysregulation of this
86   process can result in either loss of specific cell types or over-proliferation, which could
87   lead to a number of human diseases, including cancers (Vermeulen et al., 2003). In
88   general, cell cycle process can be divided in two stages: interphase (accumulating
89   nutrients and duplicating DNA) and mitosis (dividing process). The interphase includes
90   G1, S, and G2 phases. During the G1 phase, cells prepare for the process of DNA
91   replication and make a decision to enter S phase. Cyclin E associates with CDK2, and
92   pushes the cell from G1 to S phase (Ohtsubo et al., 1995). Replication of DNA occurs in
93   S phase through interaction with many replication factors. The second gap phase, G2,
94   is required for preparing cell division. To proceed from G2 to M phase, G2/M checkpoint
95   needs to be passed by Cyclin A/B/CDK-1 complex (Girard et al., 1991). During mitosis,
96   replicated chromosomes are segregated into separate nuclei followed by cytokinesis to
97   form two daughter cells. Since cell division and development are tightly coordinated, it is
98   plausible that cell cycle and DNA replication factors are critical for the process of
99   development. Notably, mammalian stem cells have short G1 and greater than 70% of
100   the stem cell population are in S phase (Savatier et al., 1996). However, the function of
101   S phase in stem cell maintenance remains poorly understood. Moreover, key S phase
102   regulators that are associated with stem cell maintenance have not yet been identified
103   in any other model systems.

104   In this study, we explored the role of S phase factors in *C. elegans* GLP-1/Notch-

105   mediated cellular events. We employed previously well-characterized three mutant

106   alleles that have reduced or elevated GLP-1/Notch activities: *glp-1(bn18)*, *glp-1(ar202)*,

107   and *glp-1(q35)* mutants (Fig. 1D-1G, Supplementary material Table S1). A focused

108   RNAi screen using these mutants has identified *div-1* (a homolog of the human PolA2,

109   the B subunit of the DNA polymerase alpha-primase complex) as a positive regulator of

110   GLP-1/Notch signaling. Specifically, depletion of DIV-1 enhanced the Glp phenotype

111   that is caused by reduced GLP-1/Notch activity. It also suppressed the formation of

112   germline tumors and somatic multivulva that are caused by elevated GLP-1/Notch

113   activity. Notch signaling and cell cycle regulators are highly conserved in *C. elegans*.

114   Therefore, our findings may provide a powerful organism model system to explore the

115   connection between Notch signaling and cell cycle progression, as well as have

116   important implications for development and treatment of Notch signaling-associated

117   human diseases, including cancer.

118

119   **RESULTS**

120   **S phase arrest by chemical promotes a Glp phenotype in *glp-1(bn18)* mutants**

121   To explore whether S phase progression is functionally linked with GLP-1/Notch-

122   mediated germline proliferation in *C. elegans*, we utilized temperature sensitive (ts) *glp-*

123   *1(bn18)* loss-of-function mutants (Fig. 1D), which provide a sensitized genetic

124   background to determine the genetic connection with GLP-1/Notch signaling pathway

125   (Fox et al., 2011). The *glp-1(bn18)* mutants are nearly wild-type germline proliferation

126   albeit with reduced germ cell number (~50% of wild-type at young adult stage) and self-

127   fertile at permissive temperature (20°C) (Qiao et al., 1995; Maine et al., 2004). However,

128   most *glp-1(bn18)* mutants display a Glp phenotype at restrictive temperature (25°C)

129   (Kodoyianni et al., 1992) (Fig. 1E). First, to investigate the effect of S phase on GLP-

130   1/Notch-mediated germline proliferation, we treated wild-type and *glp-1(bn18)* mutants

131   from L1 larval stage with 40 mM hydroxyurea (HU, a DNA synthesis inhibitor, (Fig. 2A))

132   at 20°C. Three days later (adult stage), germline phenotype was determined by staining

133   whole worms with DAPI (4',6-diamidino-2-phenylindole). Wild-type adult worms did not

134   display a Glp phenotype in both absence (-) and presence (+) of HU, as previously

135 reported (Fox et al., 2011) (Fig. 2B). However, forced S phase arrest by HU treatment

136 dramatically enhanced a Glp phenotype up to 93% in *glp-1(bn18)* mutants, while only

137 2% of *glp-1(bn18)* (HU-) mutants showed the Glp phenotype at the same condition (Fig.

138 2B). DAPI staining showed that the *glp-1(bn18)* (HU-) mutants were fertile albeit with

139 smaller gonads (Fig. 2C), but most *glp-1(bn18)* (HU+) mutants had a typical Glp

140 phenotype, composed solely of a few mature sperm (Fig. 2D). Notably, Fox *et al*.

141 previously reported the treatment of *glp-1(bn18)* mutants from L4/young adult stages

142 with HU did not induce a Glp phenotype. These results propose that S phase

143 progression may be functionally linked with GLP-1/Notch-mediated GSC maintenance

144 and germline proliferation during early larval stages rather than adulthood because S

145 phase arrest by HU treatment at only early larval stages promoted the Glp phenotype.

146

147 **DIV-1 is required for GLP-1/Notch-mediated germline proliferation**

148 To further explore the requirement of S phase progression in GLP-1/Notch-

149 mediated early germline proliferation, we depleted the expression of genes, encoding

150 Cyclins (Group 1), DNA replication processing proteins (Group 2), and DNA replication

151 licensing proteins (Group 3) by feeding RNAi of *glp-1(bn18)* mutants (Fig. 2E-2G).

152 Specifically, gravid adult *glp-1(bn18)* hermaphrodites were transferred to each feeding

153 RNAi plate, and the germline phenotypes of progeny were examined by DAPI staining

154 when they became adults (3 days after L1) (Fig. 2E). Previously, Fox *et al*., showed that

155 GLP-1/Notch-mediated germline proliferation is required for CYE-1, but not CYA-1 and

156 CYB-1 (Fox et al., 2011). Thus, *cye-1(RNAi)* was used as a positive control. *cya-

157 1(RNAi)* and *cyb-1(RNAi)* were used as negative controls for focused RNAi screening

158 (Fig. 2F). As previously reported, *cye-1(RNAi)* dramatically enhanced a Glp phenotype

159 in *glp-1(bn18)* mutants even at 20°C, but *cya-1(RNAi)* or *cyb-1(RNAi)* did not (Fox et al.,

160 2011) (Fig. 2F). Based on these results, we examined the effects of DNA replication

161 regulators in Group 2 and 3 on GLP-1/Notch-mediated germline proliferation (Fig. 2F).

162 We selected key 11 DNA replication-related genes based on Gene Ontology (GO)

163 database (GO:0006260), previous publications, and RNAi accessibility in our library.

164 Notably, depletion of DNA replication-related genes by RNAi enhanced the Glp

165 phenotype in *glp-1(bn18)* mutants with a wide range of penetrance (Fig. 2F). Among

them, *div-1(RNAi)* and *chk-1(RNAi)* dramatically promoted the Glp phenotype in *glp-1(bn18)* mutants even at 20°C, as seen in *cye-1(RNAi)* (Fig. 2F and 2H). We also performed RNAi experiments at the same condition in *eri-1(mg366)* mutants that is hypersensitive to RNAi (Kennedy et al., 2004) (Fig. 2G). RNAi of *cye-1* and *chk-1* genes also showed the Glp phenotype (14% and 24%, respectively) in *eri-1* mutants (Fig. 2G), but *div-1(RNAi)* did not (Fig. 2G and 2I). These results suggest that CYE-1, CHK-1, and DIV-1 play an important role in GLP-1/Notch-mediated germline proliferation. In particular, the effects of DIV-1 on germline proliferation more likely depends on GLP-1/Notch activity because *div-1(RNAi)* enhanced a Glp phenotype in *glp-1(bn18)* sensitize mutants, but not in *eri-1* mutants. Next, to ask whether *div-1* is also required for GLP-1/Notch-mediated GSC maintenance during adulthood, young adult (2.5 days after L1) staged *glp-1(bn18)* mutants were placed on the RNAi plates of *div-1*, *vector* (negative control), and *cye-1* (positive control) for 72 hours at 20°C (Fig. 2J). Their germline phenotypes were determined by staining dissected gonads with cell fate specific markers such as nucleoplasmic REC-8 for proliferative germ cells (Hansen et al., 2004b) and HIM-3 for meiotic prophase germ cells (Zetka et al., 1999) (Fig. 2L-2Q). About 5% of *vector(RNAi); glp-1(bn18)* animals displayed a Glp phenotype (Fig 2K-2M), but most *cye-1(RNAi); glp-1(bn18)* mutants had a Glp phenotype (Fig. 2K, 2N, and 2O), as previously reported (Fox et al., 2011). Interestingly, *div-1(RNAi)* at early larval stages significantly induced a Glp phenotype in *glp-1(bn18)* mutants (90%) (Fig. 2F), but *div-1(RNAi)* at adult stages slightly induced the Glp phenotype in *glp-1(bn18)* mutants (17%) (Fig. 2K, 2P, and 2Q). These results suggest that DIV-1 is required more for GLP-1/Notch-mediated early germline proliferation than for GSC maintenance during adulthood, which is consistent with the results of HU treatment in early larval stage (Fig. 2B) and adult stages (Fox et al., 2011).

DIV-1 controls the extent of germline proliferation

To investigate the function of *div-1* in germline proliferation, we utilized a temperature sensitive (ts) *div-1(or148)* loss-of-function mutant (Encalada et al., 2000). Their germline phenotypes were analyzed by staining dissected gonads with cell fate-specific markers: anti-REC-8 (Hansen et al., 2004b) and anti-HIM-3 (Zetka et al., 1999)

197 antibodies. The mitotic zone of the wild-type hermaphrodite gonad has two pools with

198 distinct properties. Germ cells in the distal pool are maintained in a stem cell-like state

199 (weak REC-8(+) signals in the nucleoplasm of mitotic nuclei, state(I)); germ cells in the

200 proximal pool in mitotic zone are maturing from the stem cell-like state to mitotically

201 cycling and early differentiating state (strong REC-8(+) signals on chromatin of mitotic

202 nuclei, state(II)) (Hubbard, 2007; Fox and Schedl, 2015) (Fig. 3A). Once germ cells

203 leave mitotic cell cycle, they enter meiotic prophase (REC-8(-)/HIM-3(+)) (Fig. 3C).

204 Therefore, mitotic cells and meiotic cells can be distinguished using REC-8 and HIM-3

205 antibodies (Hubbard, 2007; Fox and Schedl, 2015). The *div-1(or148)* mutants displayed

206 embryonic lethal phenotype due to delayed embryonic cell division at 25°C (Encalada et

207 al., 2000). To assess the germline phenotype, we thus cultured synchronized *div-*

208 *1(or148ts)* mutants until L1 at 20°C and upshift them to 25°C. Three days later (adult

209 stage), germline phenotypes were determined by staining dissected gonads with cell

210 fate-specific markers. Wild-type and *div-1(or148ts)* germlines grown at 25°C maintain

211 stem cell-like state (state(I)) as well as mitotically cycling and early differentiating state

212 (state(II)) (Fig. 3A). However, most germlines of *div-1(or148ts)* mutants grown at 25°C

213 had no or less stem cell-like state (state(I)) (~72%, n=42) (Fig. 3B), indicating that DIV-1

214 may be required for the maintenance of stem cell-like state. In addition, the germline

215 size of *div-1(or148ts)* worms at 25°C was smaller than that of wild-type and *div-*

216 *1(or148ts)* worms grown at 20°C (Fig. 3B and 3D). We then scored the number of

217 mitotic germ cells in wild-type and *div-1(or148ts)* germlines at adult stage (three days

218 after L1). Wild-type possess ~225 mitotic germ cells (REC-8(+)/HIM-3(-)) (Fig. 3E).

219 Similarly, most *div-1(or148ts)* mutants grown at 20°C are fertile and possess ~218

220 mitotic germ cells (range: 200-230) (Fig. 3E). However, the average number of mitotic

221 germ cells in the *div-1(or148ts)* mutants grown at 25°C was 109 (range: 86-140) (Fig.

222 3E). We next determined the S phase index (S-index) by counting number of EdU-

223 positive nuclei in a 30-min pulse/total number of REC-8-positive nuclei, as previously

224 reported (Crittenden et al., 2006; Fox et al., 2011). The *div-1(or148ts)* mutants grown at

225 25°C had longer S phase index than that grown at 20°C (60% at 20°C vs 72% at 25°C)

226 (Fig. 3F and 3G). Moreover, adult *div-1(or148ts)* mutants grown at 25°C from L1

227   displayed an increased number of partially incorporated EdU(+) cells probably due to
228   impaired DNA replication in the most distal germline (Fig. 3F and 3G). These results
229   suggest that DIV-1 may be required for the maintenance of stem cell-like state and the
230   normal extent of germline proliferation probably through a normal cell cycle progression.

231   We next assessed the localization of DIV-1 in *C. elegans* germline. No DIV-1-
232   specific antibody is available. Thus, the subcellular location of DIV-1 was analyzed
233   using a transgenic worm expressing both *pie-1 (promoter)::GFP::div-1::pie-1 3'UTR* and
234   *pie-1 (promoter)::mCherry::his-58::pie-1 3'UTR* (an internal control) transgenes (Fig.
235   3H). GFP::DIV-1 was detected in the nuclei of germ cells during germline development
236   (Supplementary material Fig. S1). Notably, GFP::DIV-1 was highly accumulated in the
237   proximal mitotic region (state(II)) that contains a mixture of mitotically cycling cells and
238   early differentiating cells in adult germlines (Fig. 3I-3K), compared to the expression of
239   mCherry::HIS-58). This result suggests that DIV-1 may be required for the robust
240   proliferation typical of the mitotic germ cells during development. Next, we asked
241   whether DIV-1 controls germline proliferation by affecting the expression of Notch
242   signaling genes (*lag-2*, Notch ligand; *glp-1*, Notch receptor) and *gld-1* gene (a key
243   regulator for germ cell differentiation). To answer the question, we performed *div-
244   1(RNAi)* in wild-type worms and two transgenic worms expressing *lag-2::GFP* and *gld-
245   1::GFP* transgenes from L1 stage and stained dissected gonads with anti-GLP-1 and
246   anti-GFP antibodies. The staining showed that depletion of DIV-1 did not affect the
247   expression of LAG-2, GLP-1, and GLD-1 (Supplementary material Fig. S2). This result
248   indicates that DIV-1 may not directly influence the expression of well-known key
249   proliferation and differentiation regulators, LAG-2, GLP-1, and GLD-1. How then does
250   DIV-1 affect germline proliferation? One possible mechanism is that DIV-1 may promote
251   GSC maintenance and germline proliferation by maintaining a threshold for germline
252   proliferation/differentiation decision below GLP-1/Notch activity (see Fig. 7B and
253   Supplementary material Fig. S5). Recent studies from Schedl's group proposed that
254   germline proliferation/differentiation decision is controlled by a threshold for GLP-
255   1/Notch activity; GLP-1/Notch activity must be above a threshold for germline
256   proliferation and its activity must be fall below a threshold for differentiation (Fox and
257   Schedl, 2015). Based on this report, we suggest that loss of DIV-1 may rise a threshold

258 for germline proliferation/differentiation decision (see Fig. 7B, Supplementary material
259 Fig. S5).

260

261 **Depletion of DIV-1 partially suppresses elevated GLP-1/Notch activity-mediated**
262 **germline tumor formation**

263 Next, the role of DIV-1 in the formation of germline tumors was examined using a
264 *glp-1(oz112)* gain-of-function (gf) mutant (see Fig. 1D and 1F). In *C. elegans*, elevated
265 GLP-1/Notch activity promotes the formation of germline tumors (Berry et al., 1997). To
266 explore the role of DIV-1 in the elevated GLP-1/Notch activity-mediated germline tumor
267 formation, we depleted the expression of *div-1* gene by RNAi in *glp-1(oz112gf)* mutants
268 (Berry et al., 1997) (Fig. 4A). While 82% of *glp-1(oz112gf)* mutants generate germline
269 tumors at 20°C (Berry et al., 1997) (Fig. 4A and 4B), *div-1(RNAi)* partially suppressed
270 the germline tumor formation of *glp-1(oz112gf)* mutants (~28% reduction) (Fig. 4A and
271 4C). The role of DIV-1 in *glp-1(oz112gf)*-mediated germline tumor formation was
272 confirmed in temperature-sensitive *glp-1(ar202gf)* mutants. The *glp-1(ar202gf)* mutants
273 are typically normal at 15°C, but most of them generate proximal germline tumors at
274 restrictive temperature (25°C) (Pepper et al., 2003). *div-1(RNAi)* also showed ~20% and
275 ~10% reduction of *glp-1(ar202gf)* germline tumor formation in compared to *vector*
276 *(RNAi)* at 23.5°C and 25°C, respectively (Fig. 4A). Why did the effect of *div-1(RNAi)* on
277 *glp-1(oz112gf)* or *glp-1(ar202gf)* mutants (Fig. 4A) was much weaker than that of *div-*
278 *1(RNAi)* on *glp-1(bn18)* mutants (Fig. 2E) at 20°C. One possible idea is that the
279 phenotype of *glp-1(oz112)* or *glp-1(ar202)* mutants may be stronger than that of *glp-*
280 *1(bn18)* mutants. However, we do not think because RNAi of genes that are associated
281 with core GLP-1/Notch components (*glp-1* or *lag-3*) sufficiently repressed the formation
282 of *glp-1(oz112gf)* germline tumors (Supplementary material Fig. S3). Moreover, RNAi of
283 *rpa-1*, *cya-1*, and *chk-1* genes also suppressed the formation of *glp-1(ar202)* germline
284 tumors, although RNAi of *rpa-1* and *cya-1* did not enhance a Glp phenotype in the *glp-*
285 *1(bn18)* mutants (Fig. 2E, Supplementary material Fig. S3). These results also indicate
286 that GLP-1/Notch-mediated germline proliferation and tumor formation may require
287 different cell cycle regulators. Similarly, Nusser-Stein *et al.* recently reported that Notch
288 signaling relies on different cell cycle regulators for the formation of a stable cell fate

289 pattern during *C. elegans* vulva development (Nusser-Stein et al., 2012). Another
290 possible idea is that DIV-1 is required more for germline proliferation in early larval
291 stages than for germline proliferation during adulthood. Two lines of evidence support
292 the latter idea: 1) the germline tumors that are caused by elevated GLP-1/Notch activity
293 are initiated in the mid L4 stage (Pepper et al., 2003). 2) *div-1(RNAi)* from L4 staged
294 larvae partially enhanced a Glp phenotype in adult *glp-1(bn18)* mutant germlines (Fig.
295 2J and 2K). Therefore, we suggest that DIV-1 is required for GLP-1/Notch-mediated
296 GSC maintenance and germline proliferation during early larval development (Fig. 2)
297 and also is partially necessary for the formation of germline tumors during L4 and later
298 stages (Fig. 4).

299   Next, to ask whether the effect of DIV-1 on germline proliferation is specific to
300 GLP-1/Notch signaling, we depleted the expression of *div-1* by RNAi in *gld-3(q730) nos-*
301 *3(q650)* mutants with synthetic germline tumors. GLD-3 (a member of the Bicaudal-C
302 family of RNA-binding proteins) and NOS-3 (a member of the Nanos family of RNA-
303 binding proteins) promote entry into a program of differentiation (Eckmann et al., 2004).
304 The *gld-3 nos-3* germline tumor is independent on GLP-1/Notch activity (Eckmann et al.,
305 2004). Result showed that *div-1(RNAi)* failed to suppress *gld-3 nos-3* germline tumors
306 (Fig. 4A). This suggests that DIV-1 may act upstream of GLD-3 and NOS-3 pathways
307 (see Fig. 7A). To confirm this result, we also performed *div-1(RNAi)* in *glp-1(q224); gld-*
308 *3 nos-3* triple mutants at 25°C. *glp-1(q224)* is a temperature-sensitive and loss-of-
309 function mutant like *glp-1(bn18)* mutants. Most of the *glp-1(q224ts)* mutants are typically
310 fertile, but they are completely sterile due to a Glp phenotype at 25°C (Austin and
311 Kimble, 1987). However, homozygotes for *glp-1(q224ts); gld-3 nos-3* have germline
312 tumors even at 25°C because the redundant GLD-3 and NOS-3 pathways act
313 downstream of GLP-1 to promote meiotic entry (Austin and Kimble, 1987; Kadyk and
314 Kimble, 1998; Hansen et al., 2004a) (Fig. 4A and 4D). Interestingly, *div-1(RNAi)* failed
315 to suppress the formation of germline tumors in *glp-1(q224ts); gld-3 nos-3* mutants at
316 25°C (Fig. 4A and 4E). These results suggest that DIV-1 acts upstream of GLD-3 and
317 NOS-3 pathways to GLP-1/Notch-mediated germline proliferation (see Fig. 7A).

318

319 **DIV-1 works with FBF/PUF to control mitotic cell cycle**

320   In addition to GLP-1/Notch signaling, a battery of RNA regulators also control a

321   balance between proliferative and differentiation. One of well conserved RNA regulators

322   is PUF (Pumilio/FBF) RNA-binding protein. PUF proteins control various physiological

323   processes such as stem cell maintenance and cell fate specification by interacting with

324   3' untranslated regions (3'UTRs) and modulating mRNA expression in a wide variety of

325   eukaryotes (Wickens et al., 2002). *C. elegans* has multiple PUF genes with special roles.

326   In particular, FBF-1 and FBF-2 (96% identical, henceforth called FBF) proteins have an

327   essential role in *C. elegans* GSC maintenance and germline proliferation (Crittenden et

328   al., 2002). These two nearly identical FBF proteins are largely redundant: *fbf-1* and *fbf-2*

329   single mutants are both self-fertile with germlines organized as in wild-type. By contrast,

330   in *fbf-1 fbf-2* double mutants, GSCs are maintained until the L4 stage, but most GSCs

331   leave mitotic cell cycle, enter meiosis, and eventually differentiated into sperm (Glp

332   phenotype) (Crittenden et al., 2002). Interestingly, we found that the Glp phenotype of

333   *fbf-1 fbf-2* mutants was sufficiently suppressed by additional removal of PUF-8 (another

334   PUF protein) (Fig. 5A and 5B), a key regulator for *C. elegans* germline development

335   (Subramaniam and Seydoux, 2003; Ariz et al., 2009; Cha et al., 2012; Racher and

336   Hansen, 2012; Pushpa et al., 2013; Vaid et al., 2013; Datla et al., 2014; Sorokin et al.,

337   2014; Priti and Subramaniam, 2015). The *puf-8 fbf-1 fbf-2* triple mutants displayed

338   mitotically dividing cells in the germline (Fig. 5B). This result indicates that PUF-8

339   inhibits FBF-mediated GSC maintenance and germline proliferation. Intriguingly, *div-*

340   *1(RNAi)* inhibited the restoration of mitotically dividing cells in *fbf-1 fbf-2 puf-8* triple

341   mutant germlines (Fig. 5B). Mitotically dividing germ cells were detected by staining

342   dissected gonads with mitotic/proliferative cell markers using the Click-iT EdU Alexa

343   Fluor 488 Imaging Kit. 76% of *puf-8 fbf-1 fbf-2* germlines were positive for EdU-labeling

344   in the germline (Fig. 5D-5G). However, only 29% of *div-1(RNAi); puf-8 fbf-1 fbf-2*

345   germlines were positive for EdU-labeling (Fig. 5H-5K). This result suggests that DIV-1

346   may be required for GSC maintenance and germline proliferation in *puf-8 fbf-1 fbf-2*

347   mutant germlines.

348   It was previously reported that PUF-8 also represses GLP-1/Notch-mediated

349   germline proliferation (Racher and Hansen, 2012; Datla et al., 2014) (Fig. 5A): while *glp-*

350   *1(ar202gf)* mutants produce both sperm and oocyte, which are self-fertile at 20°C, the

351 *puf-8* mutation strongly enhances the germline tumor phenotype of the *glp-1(ar202gf)*

352 mutant even at 20°C (Racher and Hansen, 2012) (Fig. 5C). This suggests that PUF-8

353 inhibits proliferative fate through negative regulating GLP-1/Notch signaling or by

354 functioning parallel to it (Racher and Hansen, 2012). We also tested if *div-1(RNAi)*

355 suppresses the formation of germline tumors in *puf-8(q725); glp-1(ar202gf)* double

356 mutants at 20°C. Interestingly, *div-1(RNAi)* failed to suppress the formation of germline

357 tumors in *puf-8(q725); glp-1(ar202gf)* mutants (Fig. 5C). How does *div-1(RNAi)*

358 suppress the formation of germline tumors of *glp-1(ar202gf)*, but not that of *puf-8; glp-*

359 *1(ar202)*? One possible idea is that DIV-1 may fall the threshold for germline

360 proliferation/differentiation decision, resulting in partially suppression of germline tumor

361 phenotype (Fig. 7B, Supplementary material Fig. S5). However, *div-1(RNAi)* may be not

362 sufficient to fall below the threshold that was raised by loss of PUF-8 (Supplementary

363 material Fig. S5). These results suggest that DIV-1 and PUF-8 may have opposite roles

364 in altering the threshold for GLP-1/Notch-mediated germline proliferation.

365

366 **DIV-1 is required for *glp-1(q35)* multi-vulva (Muv) formation**

367 We showed here that DIV-1 promotes GLP-1/Notch-mediated germline

368 proliferation. However, it still has a possibility that DIV-1 may influence germline

369 proliferation through general cell cycle control, regardless of GLP-1/Notch signaling

370 pathway. To test this possibility, we employed *glp-1(q35)* mutant. The *glp-1(q35)* has a

371 premature stop codon, which results in a truncated GLP-1/Notch protein that lacks a

372 negative regulatory domain (ProGluSerThr [PEST]) (Fig. 1D) (Mango et al., 1991). The

373 PEST domain is associated with a shorter GLP-1(intra) half-life and more rapid

374 degradation. The *glp-1(q35)* mRNA with a premature stop codon is degraded by

375 nonsense-mediated decay (NMD) (Mango et al., 1991). Mutation of *smg-1*, which is

376 required for NMD in *C. elegans*, stabilized *glp-1(q35)* mRNA, and thereby, suppressed

377 the Glp phenotype of *glp-1(q35)* mutant. Wild-type adult hermaphrodites have normally

378 one vulva (Fig. 6A and 6D). However, *smg-1; glp-1(q35)* double mutants have a

379 multivulva phenotype similar to that caused by dominant *lin-12* (one of two *C. elegans*

380 Notch receptors) mutations (Mango et al., 1991) (Fig. 1G, 6B and 6E). Next, to test

381 whether DIV-1 is necessary for the *smg-1; glp-1(q35)* Muv phenotype, we performed

382   RNAi of *div-1* and *vector* (negative control) into *smg-1; glp-1(q35)* double mutants at

383   20°C, and scored the number of extra vulva under differential interference contrast

384   microscopy. Adult wild-type hermaphrodites typically have one vulva (Fig. 6A and 6D),

385   but *vector(RNAi); smg-1; glp-1(q35)* mutants displayed a semidominant multivulva

386   phenotype as well as the recessive, loss-of-function GLP phenotype (data not shown)

387   (Mango et al., 1991). However, *div-1(RNAi)* significantly reduced the number of extra

388   vulvae (Fig. 6C and 6F) and promote the Glp phenotype (data not shown) in the *smg-1;*

389   *glp-1(q35)* mutants. We also examined the effects of other cell cycle regulators on *glp-*

390   *1(q35)*-mediated muv phenotype. Intriguingly, depletion of CYE-1 and CYA-1 that

391   showed an important role in GLP-1/Notch-mediated germline proliferation and germline

392   tumor formation, respectively (Supplementary material Fig. S3) did not influence *glp-*

393   *1(q35)*-mediated Muv formation (Supplementary material Fig. S4). By contrast, PRI-1 (a

394   homolog of the DNA polymerase alpha-primase subunit B) appears to have a critical

395   role in *glp-1(q35)*-mediated Muv formation (Supplementary material Fig. S4), although it

396   did not play an important role in GLP-1/Notch-mediated germline proliferation and

397   tumorigenesis, as previously reported (Fox et al., 2011) (Fig. 2E, Supplementary

398   material Fig. S3). This result suggests that unique cell cycle length and structure may

399   specify cell fate differently in germline and soma. All together, we propose that DIV-1

400   plays an important role in GLP-1/Notch signaling to control cellular events in the *C.*

401   *elegans* germline and soma.

402

403   **DISCUSSION**

404   In eukaryotes, intercellular signaling through Notch receptors regulates growth

405   and differentiation during animal development (Lai, 2004). Moreover, aberrant

406   regulation of the Notch signaling is highly associated with human diseases, including

407   cancers (Allenspach et al., 2002; Rizzo et al., 2008; Yuan et al., 2015). In *C. elegans*,

408   the mechanisms of GSC maintenance and germline proliferation largely rely on GLP-

409   1/Notch signaling (Austin and Kimble, 1987) and a battery of RNA regulators (e.g., FBF

410   (Kimble and Crittenden, 2007)). In this study, we found new tenable links between DIV-

411   1 and GLP-1/Notch signaling in GSC maintenance, and germline proliferation.

412   Importantly, stem cells have a unique cell cycle structure (short or absent G1 and long S

413   phase), suggesting the potential role of G1/S and S phase in stem cell regulation. We

414   specifically demonstrated for the first time that DIV-1 works together with GLP-1/Notch

415   signaling and its downstream RNA regulators (e.g., FBF) for GSC maintenance and

416   germline proliferation in *C. elegans*. All regulators that we studied here are highly

417   conserved in other multicellular organisms, including humans. Therefore, our findings

418   may provide insights into the cell cycle control of Notch-mediated cellular events in

419   other model systems.

420

421   **Notch signaling and cell cycle control in stem cells and cancer cells**

422   Notch signaling plays varied and critical roles in regulating many types of stem

423   cells by receiving signals from their microenvironment (also known as "niche"). Notch

424   signaling is critical in stem cells, not only for appropriately balancing self-renewal and

425   differentiation, but also for regulating cell cycle progression to protect from

426   tumorigenesis. In addition to Notch signaling, cell cycle control is a critical step in

427   generating specific cells and tissues during development. The association of Notch

428   signaling and cell cycle progression with cancer development has also been studied in

429   various animal systems. For example, Notch signaling activates Cyclin D1 transcription

430   and CDK2 activity, promoting S phase entry (Ronchini and Capobianco, 2001). In the

431   pancreatic cancer cells, Notch signaling regulates the expression levels of Cyclin D1,

432   Cyclin A, and cell cycle inhibitors (e.g., p21^{CIP} and p27^{KIP1}, and CDK inhibitors) (Wang

433   et al., 2006). In addition, Notch signaling mediates G1/S cell cycle progression through

434   overexpression of Cyclin D3 and CDK4 in T cells (Joshi et al., 2009) and through

435   reduction of p27^{KIP1}, leading to more rapid cell cycle progression in T-cell acute

436   lymphoblastic leukemia cells (Dohda et al., 2007). To date, many reports support the

437   notion that Notch signaling affects cell cycle progression (Hori et al., 2013). However, it

438   is poorly understood how cell cycle regulators work with Notch signaling to regulate

439   cellular events in a whole animal. Recently, it was reported that Notch signaling is tightly

440   linked to cell cycle progression during *C. elegans* vulval development (Nusser-Stein et

441   al., 2012). Specifically, CYD-1/Cyclin D and CYE-1/Cyclin E stabilize LIN-12/Notch

442   receptor in vulva precursor cells (VPCs) (Nusser-Stein et al., 2012). In *C. elegans*

443   germline, CYE-1 is critical for GLP-1-mediated germline proliferation (Fox et al., 2011),

444   but CYD-1 is not (Fox et al., 2011). Moreover, *C. elegans* CYD-1 and CYE-1 induce

445   distinct cell cycle re-entry programs in differentiated muscle cells (Korzelius et al., 2011).

446   These reports and our findings suggest that cell fate determination in different tissues

447   (e.g., germline and soma) and/or developmental conditions (e.g., early/late development,

448   early/terminal differentiation) may be regulated by different cell cycle progressions and

449   quiescence. How does cell cycle regulate cell fate differently in germline and soma? We

450   still do not know yet, but suggest that distinct cell cycle length and structure may

451   contribute to cell fate specification differently in germline and soma. Similar features

452   were observed in other systems, including mouse embryonic stem cells: greater than

453   70% of the cell population are in S phase (Savatier et al., 1996; Seidel and Kimble,

454   2015), suggesting that potential role of S phase in stem cell regulation.

455

456   **Role of DIV-1/PolA2 in GLP-1/Notch-mediated cellular events**

457   The initiation of DNA replication during S phase relies on the DNA polymerase

458   alpha-primase complex, including the catalytic subunit PolA1, the regulatory subunit

459   PolA2, and the small and the large primase subunits "Prim1 and Prim2", respectively.

460   However, this complex seems to play additional roles in other cellular processes, such

461   as DNA damage response, telomere maintenance, and the epigenetic control (Muzi-

462   Falconi et al., 2003). This complex is well conserved in *C. elegans*: Y47D3A.29/PolA1,

463   DIV-1/PolA2, PRI-1/Prim1, and PRI-2/Prim2. Our study showed that depletion of DIV-

464   1/PolA2 affected GLP-1/Notch-mediated cellular events in the *C. elegans* germline and

465   soma. How does DIV-1 control GLP-1/Notch-mediated GSC maintenance? We still do

466   not know yet, but we suggest two mechanisms: *First*, DIV-1 may control GSC

467   asymmetric division through cell division timing. A defining characteristic of stem cells is

468   their ability to divide asymmetrically (Morrison and Kimble, 2006). Recently advanced

469   studies provide ample evidence that many stem cells can still divide symmetrically,

470   particularly when they are expanding in number during development or after injury

471   (Morrison and Kimble, 2006). Thus, it is generally accepted that asymmetric division is a

472   tool that stem cells can use to maintain stem cell homeostasis and appropriate numbers

473   of progeny. Notably, *C. elegans div-1* was first identified as a key regulator for

474   asymmetric cell division by controlling the proper timing of early embryonic cell division

475 (Encalada et al., 2000). *div-1* mutants exhibit loss of asymmetry during early embryonic
476 cleavages and result in the lacking of the endodermal and mesodermal cell fates
477 (Encalada et al., 2000). This result speculates a possibility that *div-1* may also be
478 involved in both asymmetric and symmetric division by controlling the proper timing of
479 germ cell division. This idea can be determined by *in vivo* tracking of single cell behavior
480 in the live *C. elegans* germline or by monitoring cell division in germ cell culture system.
481 However, single germ cell tracking and germ cell culture systems have not yet been
482 developed in *C. elegans*. Studying the role of *div-1* in the asymmetric/symmetric division
483 of stem cells remains a challenge for the future. *Second*, depletion of *div-1* by RNAi may
484 alter a threshold for germline proliferation/differentiation decision probably through cell
485 division timing. As described in Fig. 7B, DIV-1/FBF and GLDs/NOS-3 may control the
486 threshold for germline proliferation/differentiation decision. In addition, PUF-
487 8/GLDs/NOS-3 may inhibit GLP-1 activity. Notch signaling controls cell division timing
488 (Hunter et al., 2016). Therefore, Notch signaling, downstream regulator (PUF proteins),
489 and DIV-1 may work together to control the threshold for germline
490 proliferation/differentiation decision probably through cell division timing. This is
491 consistent with the known role of DIV-1 in embryonic cell division timing (Encalada et al.,
492 2000). However, understanding the mechanism of how aberrant cell division timing
493 controls the threshold for germline proliferation/differentiation decision remains a major
494 challenge for the future.

495

496 **DIV-1/PolA2 as a potential target for cancer treatments**

497 PolA2 (a regulatory subunit of the DNA polymerase) plays an essential role in the
498 initiation of DNA replication. During the S phase of the cell cycle, the PolA2 is recruited
499 to DNA at the replicative forks. PolA2 (G583R) mutation led to localize in the cytoplasm
500 instead of the nucleus, which inhibits DNA replication in cancer cells (e.g., non-small
501 cell lung cancer (NSCLC) and makes them sensitive to chemotherapy (e.g.,
502 Gemcitabine) (Mah et al., 2014). In addition, the synthetic retinoid 6-[3-(1-adamantyl)-4-
503 hydroxyphenyl]-2-naphthalene carboxylic acid (CD437) is an inhibitor of PolA1 and
504 selectively induces apoptosis in human lung cancer cells (Sun et al., 2002). Cancer
505 cells have a different response to inhibition of PolA1 by CD437 as compared to

506 aphidicolin. CD437-mediated inhibition of PolA1 leads to cell death in cancer cells, but it
507 induces cell cycle arrest in normal epithelial cells (Sun et al., 2002). Notably, Notch
508 signaling is altered in approximately one third of NSCLCs, which are the leading cause
509 of cancer-related deaths. Although the role of PolA1/2 in elevated Notch activity-
510 mediated NSCLCs has not yet been studied, a fundamental mechanism in stem cell
511 maintenance and tumorigenesis may be conserved. Therefore, understanding a
512 mechanism underlying Notch signaling and PolA1/2 may hold promise for molecular
513 and cellular therapies in these tumors.

514

515 **MATERIALS AND METHODS**

516 **Nematode strains**

517 All strains were derived from Bristol strain N2 and maintained at 20°C as
518 described unless otherwise noticed (Brenner, 1974). Mutants and transgenic worms are
519 listed in Supplementary Table S1.

520

521 **Hydroxyurea (HU) treatment and whole worm DAPI staining**

522 L2/3 stage of wild-type and *glp-1(bn18)* worms were transferred to NGM
523 (Nematode Growth Media) agar plates containing 40 mM Hydroxyurea (HU) and
524 incubated at 20°C. After 18 hours with HU plate incubation, worms were transferred to a
525 normal NGM plate and let them grow up to adults. For DAPI staining, whole worms
526 were collected using M9 buffer from NGM agar plates and transferred into a 2 ml
527 Eppendorf tube. After washing two times with M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g
528 NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 litre), whole worms were fixed with 3%
529 paraformaldehyde/0.1M K_2HPO_4 (pH 7.2) solution for 20 min, and then post-fixed with
530 cold 100% methanol for 5 min at –20°C (alternatively, fixed worms can be stored in cold
531 methanol at -20°C for a few days). After washing with 1x PTW (1x PBS and 0.1%
532 Tween 20), add DAPI (100 ng/mL) and incubate the fixed gonads for 10 min at room
533 temperature, following by washing them with 1x PTW three times. DAPI staining was
534 observed using a fluorescence microscopy, as previously described (Yoon et al., 2016).

535

536 **RNA interference (RNAi)**

537   RNAi constructs were obtained from the *C. elegans* RNAi Library (Thermo
538   Scientific) or kindly provided by Kimble's lab (University of Wisconsin-Madison). RNAi
539   was performed by feeding adult staged worms (P0) bacterial expressing double
540   stranded RNAs (dsRNAs) corresponding to the gene of interest as previously described
541   (Kamath et al., 2001; Ashrafi et al., 2003) (see Fig. 2E). The germline phenotypes were
542   analyzed when F1 progeny reach to adult stage (3 days later from L1). The effect of *div-*
543   *1* and *cye-1* (positive control) on GSC maintenance and germline proliferation in adult
544   *glp-1(bn18)* mutants was tested by RNAi from young adult animals (see Fig. 2J). 3 days
545   (72 h) later, the germline phenotypes were analyzed by staining dissected gonads with
546   anti-REC-8 and anti-HIM-3 antibodies.

547

548   **Germline antibody staining**

549   Germline antibody staining was performed as described in (Yoon et al., 2016).
550   Briefly, dissected gonads were fixed in 3% paraformaldehyde/0.1M K_2HPO_4 (pH 7.2)
551   solution for 10-20 min, and then post-fixed with cold 100% methanol for 5 min
552   (Alternatively, fixed worms can be stored in cold methanol at -20°C for a few days).
553   After 30 min blocking with 1x PTW/0.5% BSA (Bovine Serum Albumin) solution, add
554   primary antibody and incubate for 2 hours at room temperature or overnight at 4°C. The
555   dissected gonads were washed three times for at least 30 min (10-min interval) with 1x
556   PTW/0.5%BSA solution and incubated the dissected gonads in the 1x PTW/0.5% BSA
557   solution containing the fluorescence-conjugated secondary antibodies for 1-2 hours at
558   room temperature. After washing three times using 1x PTW/0.5% BSA solution for at
559   least 30 min (10-min interval), the dissected gonads were stained with DAPI solution
560   (100 ng/mL) for 10 min at room temperature and were then washed with 1x PTW/0.5%
561   BSA solution three times. The antibody staining was observed using a fluorescence
562   microscopy. See Supplementary Table 2 for a list and working conditions of primary
563   antibodies that we used in this study.

564

565   **EdU labeling**

566   For EdU labeling, animals were incubated with rocking in M9/0.1% Tween 20/1
567   mM EdU for 30 min at room temperature. Gonads were dissected as for germline

568   antibody staining and fixed in 3% paraformaldehyde/0.1M K_2HPO_4 (pH 7.2) solution for

569   10-20 min, followed by -20°C methanol fixation for 10 min. Dissected gonads were

570   blocked in 1xPTW/0.5% BSA solution for 30 min at room temperature. EdU labeling was

571   detected using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen, CA, #C10337),

572   according to the manufacturer's instructions. After washing three times with 1x

573   PTW/0.5% BSA solution for at least 30 min (10-min interval), the dissected gonads were

574   stained with DAPI solution (100 ng/mL) for 10 min at room temperature and were

575   washed with 1x PTW/0.5% BSA solution three times. The EdU labeling was observed

576   using a fluorescence microscopy.

577

578   **S-phase index**

579   S-phase index was determined by pulsing animals with EdU for 30 min and

580   counting total cells of REC-8-positve cells, as previously described (Crittenden et al.,

581   2006; Fox et al., 2011).

582

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593

594   **AUTHOR CONTRIBUTIONS**

595   DSY, DSC, and MHL conceived and designed the experiments, analyzed the data, and

596   wrote the paper. DSY, DSC, and MHL performed the experiments.

597

598   **CONFLICT OF INTEREST**

599   The authors declare that there is no conflict of interest.

600

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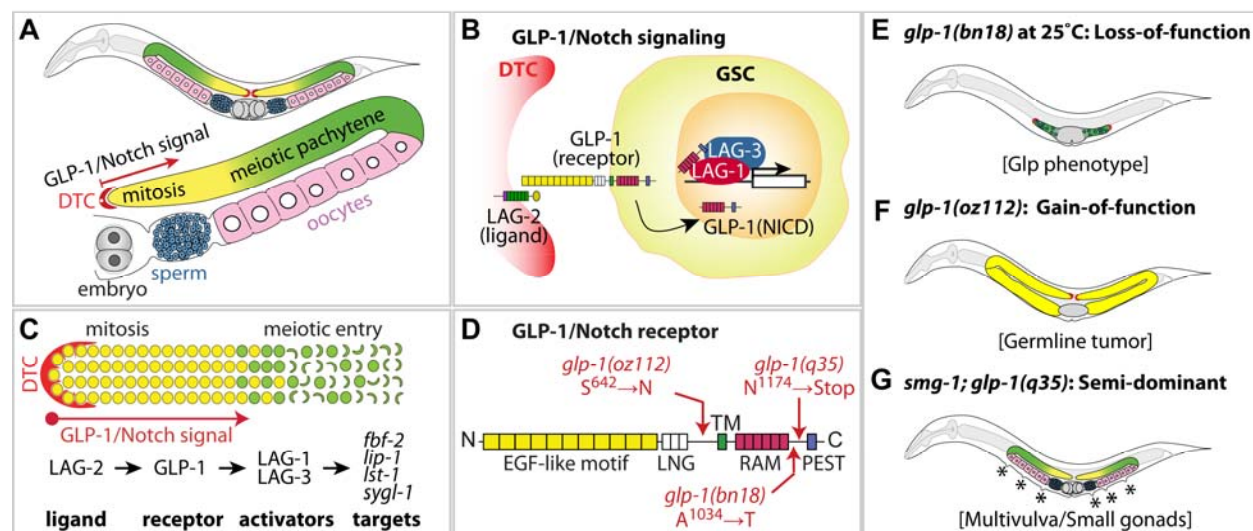787

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789   **FIGURE LEGENDS**

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792   **Figure 1. *C. elegans* germline and GLP-1/Notch signaling pathway.** (A) Schematic

793   of adult hermaphrodite gonad. Germ cells at the distal end of the germline, including

794   germline stem cells (GSCs), divide mitotically (yellow). As germ cells move proximally,

795   they enter meiosis (green) and differentiate into either sperm (blue) or oocytes (pink).

796   GLP-1/Notch signaling is activated in the distal mitotic germline. Slightly modified from

797   (Kobet et al., 2014). (B) *C. elegans* GLP-1/Notch signaling pathway. The LAG-2 ligand,

798   localized to the DTC, signals to GLP-1/Notch receptor in GSCs and mitotically dividing

799   germ cells. Upon GLP-1 activation, the GLP-1 intracellular domain (ICD), LAG-1 and

800   LAG-3 form a ternary complex in the nucleus and activate transcription of target genes.

801   Slightly modified from (Kobet et al., 2014). (C) GLP-1/Notch signaling pathway in the *C.*

802   *elegans* distal germline. Red, DTC; yellow circles, germ cells in mitotic cell cycle; green

803   circles, germ cells in meiotic S phase; green crescents, germ cells in early meiotic

804   prophase. Bottom shows the core regulators of GLP-1/Notch signaling pathway and its

805   target genes. (D) The GLP-1 domains and mutations. *glp-1(bn18)* is a temperature-

806   sensitive, loss-of-function mutation (A1034→T) (Kodoyianni et al., 1992), *glp-1(oz112)*

807   is a gain-of-function mutation (S642→N) (Berry et al., 1997), and *glp-1(q35)* is a

808   nonsense allele with both gain-of-function in somatic vulva precursor cells and loss-of-

809   function in germline (Mango et al., 1991). EGF, epidermal growth factor; LNG, LIN-

810   12/Notch/GLP-1; TM, transmembrane; RAM, Rbp-associated molecule domain; PEST,

811   Proline (P) Glutamic acid (E), Serine (S), and Threonine (T). (E-G) Schematics of

812   germline phenotypes in adult hermaphrodites that are associated with *glp-1(bn18)* loss-

813   of-function (E), *glp-1(oz112)* gain-of-function (F), and *smg-1; glp-1(q35)* semi-dominant
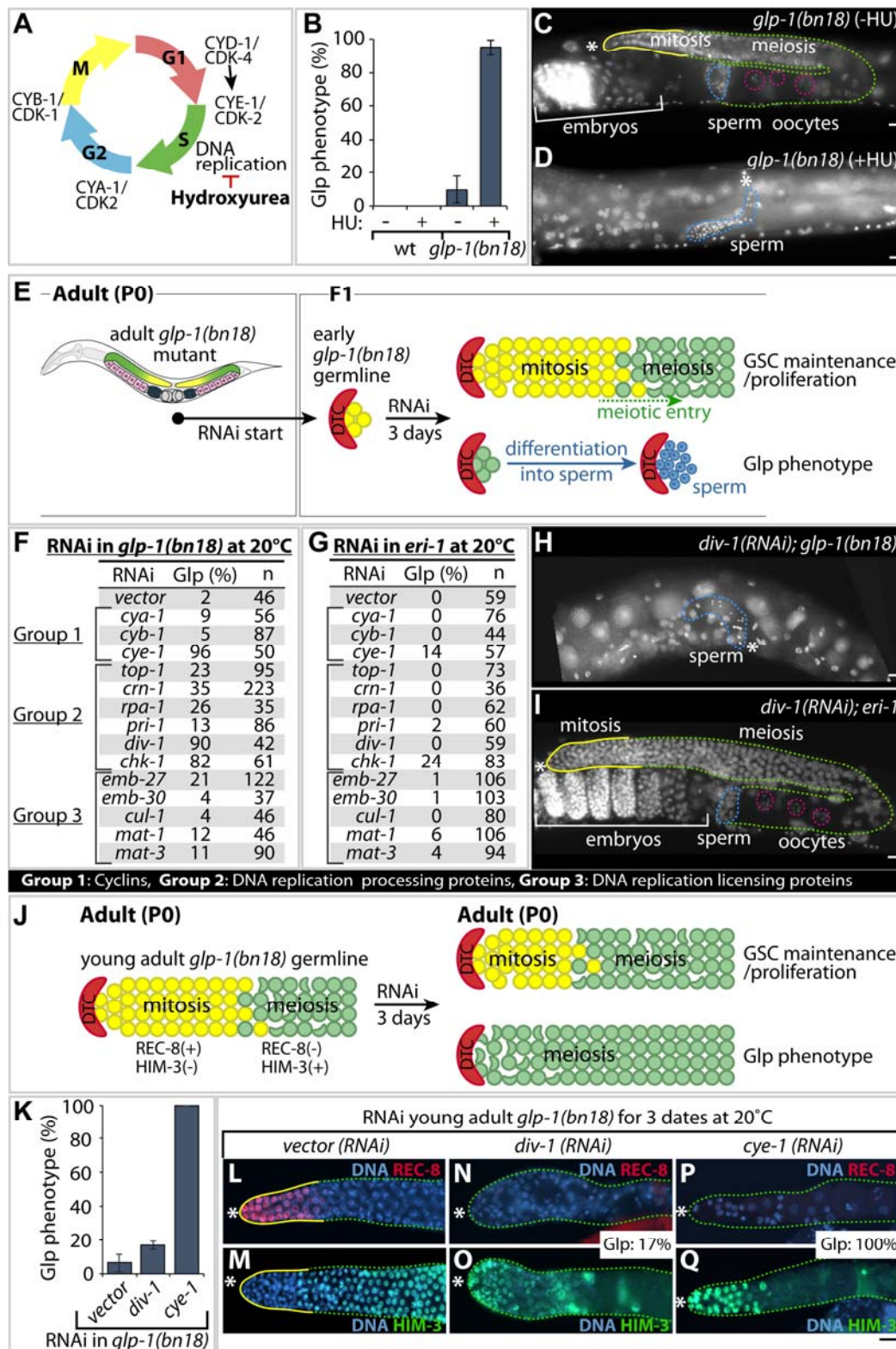
814   mutations (G).

815

Figure 2

F	RNAi in glp-1(bn18) at 20°C			G	RNAi in eri-1 at 20°C		
	RNAi	Glp (%)	n		RNAi	Glp (%)	n
	vector	2	46		vector	0	59
Group 1	cya-1	9	56		cya-1	0	76
	cyb-1	5	87		cyb-1	0	44
	cye-1	96	50		cye-1	14	57
Group 2	top-1	23	95		top-1	0	73
	crn-1	35	223		crn-1	0	36
	rpa-1	26	35		rpa-1	0	62
	pri-1	13	86		pri-1	2	60
	div-1	90	42		div-1	0	59
	chk-1	82	61		chk-1	24	83
Group 3	emb-27	21	122		emb-27	1	106
	emb-30	4	37		emb-30	1	103
	cul-1	4	46		cul-1	0	80
	mat-1	12	46		mat-1	6	106
	mat-3	11	90		mat-3	4	94

Group 1: Cyclins, Group 2: DNA replication processing proteins, Group 3: DNA replication licensing proteins

817 **Figure 2. S phase progression is required for GLP-1/Notch-mediated germline**

818 **proliferation.** (A) Cell cycle progression and key regulators. Hydroxyurea arrests DNA

819 replication. (B) The percentage of worms scored a Glp (premature meiotic entry)

820 phenotype at 20°C. Standard deviation bars were calculated from three independent

821 experiments. (C, D) DAPI-stained germlines. Solid yellow lines, mitotic germ cells;

822 broken green lines, meiotic germ cells and differentiated gametes (broken pink circles,

823 oocyte nuclei; broken blue line, sperm). *, distal end. (E) Schematic of RNAi experiment

824 to test the effect of cell cycle genes on early GSC maintenance/proliferation and Glp

825 phenotype. RNAi was performed by feeding adult staged worms (P0) bacterial

826 expressing double stranded RNAs (dsRNAs) corresponding to the gene of interest.

827 Phenotypes were analyzed when F1 progeny reach to adult stage (four days later). DTC,

828 distal tip cells. (F, G) The percentage of worms scored a Glp phenotype by RNAi of cell

829 cycle regulator genes in *glp-1(bn18)* and *eri-1(mg366)* mutants at 20°C. Group 1: RNAi

830 of genes encoding cyclins. Group 2: RNAi of genes encoding DNA replication

831 processing proteins. Group 3: RNAi of genes encoding DNA replication licensing

832 proteins. The Glp phenotype was strictly defined as no mitotic cells and only sperm by

833 DAPI staining. (H, I) DAPI-stained germlines. Solid yellow lines, mitotic germ cells;

834 broken green lines, meiotic germ cells and differentiated gametes (broken pink circles,

835 oocyte nuclei; broken blue line, sperm). *div-1(RNAi)* dramatically induced the Glp

836 phenotype in *glp-1(bn18)* mutant, but not in *eri-1(mg366)* mutant. *, distal end. (J)

837 Schematics of RNAi experiment to test the effect of *div-1*, *vector* (negative control), and

838 *cye-1* (positive control) on adult germline proliferation. RNAi was performed by feeding

839 young adult *glp-1 (bn18)* mutants and 3 days (72 h) later, germline phenotypes were

840 determined by staining dissected gonads with anti-REC-8 and anti-HIM-3 antibodies.

841 (K) The percentage of worms scored a Glp phenotype at 20°C. Standard deviation bars

842 were calculated from three independent experiments. (L, N, P) Germline staining with

843 anti-REC-8 antibody (proliferation marker). (M, O, Q) Germline staining with anti-HIM-3

844 antibody (differentiation marker). *, distal end. Scale bars: 10 µM.
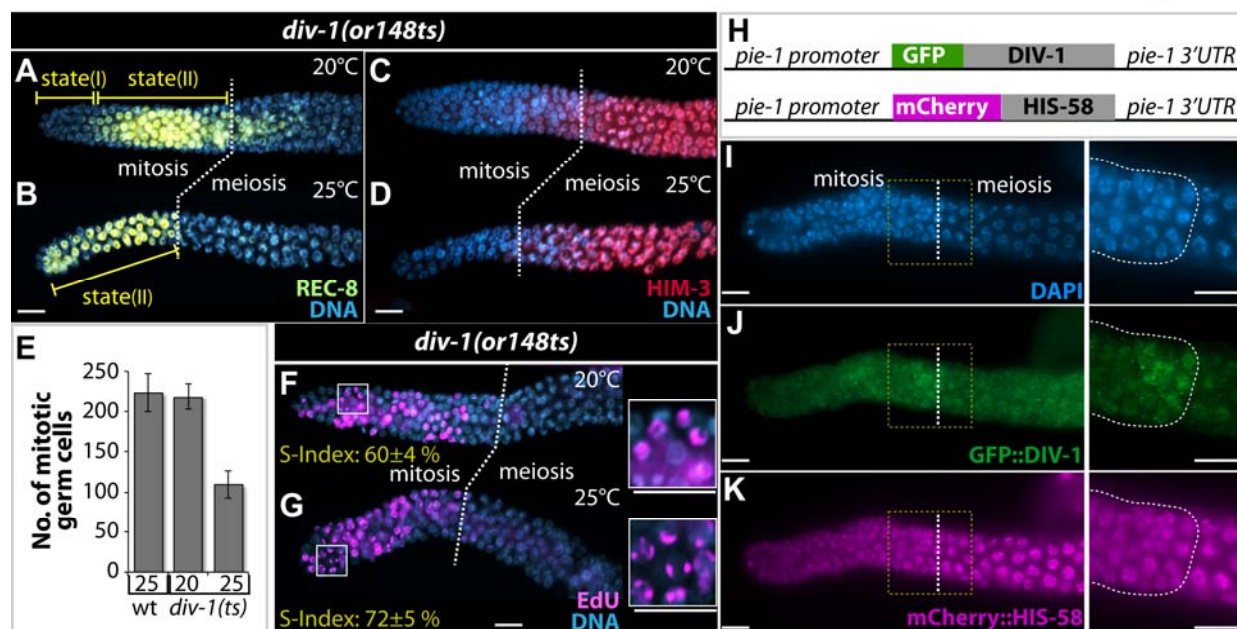
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846

Figure 3. DIV-1 promotes germline proliferation. (A-D) Adult hermaphrodite germlines of *div-1(or148)* mutants grown at 20°C and 25°C were extruded and stained with REC-8 (mitotic/proliferative germ cell marker) (A and B) HIM-3 (meiotic prophase marker) (C and D). Broken lines, boundary between mitosis and meiosis. (E) Number of mitotic germ cells [REC-8(+)/HIM-3(-)]. Standard deviation bars were calculated from three independent experiments. (F, G) Adult hermaphrodite germlines of *div-1(or148)* mutants grown at 20°C and 25°C were extruded and stained with the Click-iT EdU Alexa Fluor 488 Imaging Kit (S phase maker). Broken lines, boundary between mitosis and meiosis. Magnified pictures of inset in F and G. (H) The design of the *GFP::div-1* and *mCherry::his-58* (internal control) transgenes. The *pie-1* promoter and 3'UTR are permissive for expression in all germ cells. (I-K) Expression of GFP::DIV-1 and mCherry::HIS-58 in the same germline. mCherry::HIS-58 was detected in all germ cell chromosome. GFP::DIV-1 was highly accumulated in the proximal mitotic zone containing actively dividing germ cells. Magnified pictures of inset in I-K. Broken lines, boundary between mitotic zone and meiotic prophase. Expression level of GFP::DIV-1 was dramatically decreased as germ cells enter meiotic prophase. Scale bars: 10 µM.
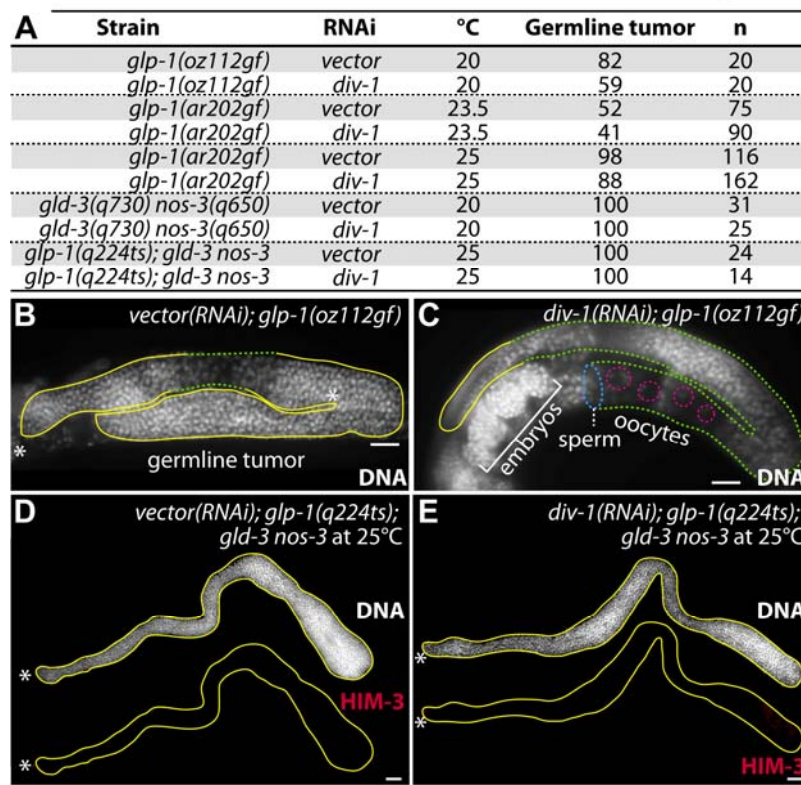
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Figure 4

A

Strain	RNAi	°C	Germline tumor	n
glp-1(oz112gf)	vector	20	82	20
glp-1(oz112gf)	div-1	20	59	20
glp-1(ar202gf)	vector	23.5	52	75
glp-1(ar202gf)	div-1	23.5	41	90
glp-1(ar202gf)	vector	25	98	116
glp-1(ar202gf)	div-1	25	88	162
gld-3(q730) nos-3(q650)	vector	20	100	31
gld-3(q730) nos-3(q650)	div-1	20	100	25
glp-1(q224ts); gld-3 nos-3	vector	25	100	24
glp-1(q224ts); gld-3 nos-3	div-1	25	100	14

B vector(RNAi); glp-1(oz112gf) — germline tumor — DNA

C div-1(RNAi); glp-1(oz112gf) — embryos, sperm, oocytes — DNA

D vector(RNAi); glp-1(q224ts); gld-3 nos-3 at 25°C — DNA — HIM-3

E div-1(RNAi); glp-1(q224ts); gld-3 nos-3 at 25°C — DNA — HIM-3

Figure 4. Depletion of DIV-1 partially suppresses the formation of germline tumors caused by elevated GLP-1/Notch activity. (A) The percentage of worms scored germline tumors. The germline tumors were defined as the presence of mitotically dividing cells (REC-8(+)/HIM-3(-)) in the proximal germlines. The *glp-1(oz112)* and *glp-1(ar202)* are gain-of-function mutants. The *gld-3 nos-3* mutants generate synthetic germline tumors independently with GLP-1/Notch activity. (B, C) Germline staining with DAPI. *div-1(RNAi)* partially suppressed the formation of germline tumors in *glp-1(oz112)* mutants. Solid yellow lines, mitotic germ cells; broken green lines, meiotic germ cells and differentiated gametes; (broken pink circles, oocyte nuclei; broken blue line, sperm). (D, E) Germline staining with anti-HIM-3 antibody. *div-1(RNAi)* did not promote germline differentiation in *glp-1(q224); gld-3 nos-3* mutants at 25°C. *, distal end. Scale bars: 20 μM.
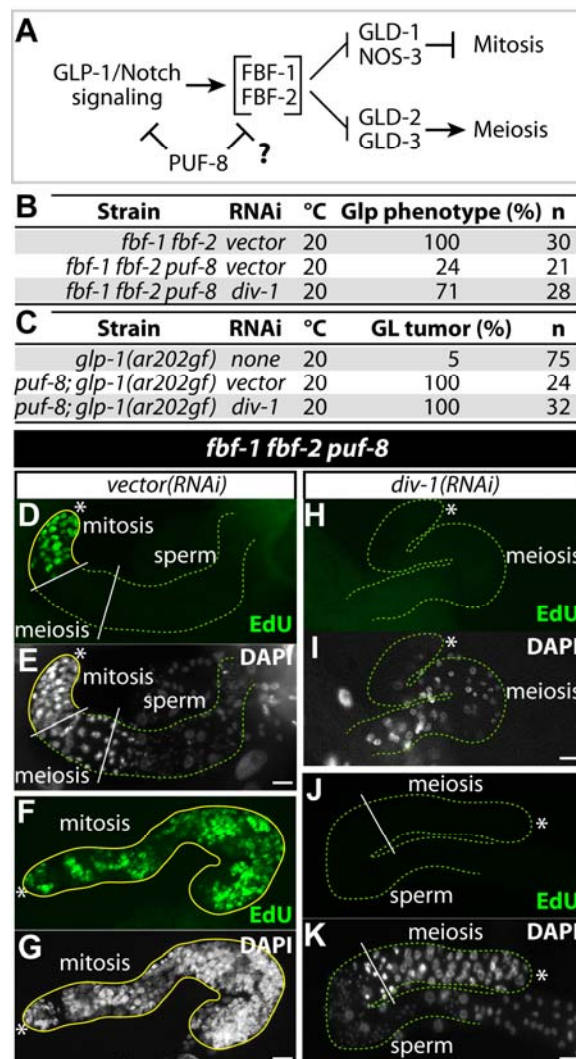
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Figure 5

Figure 5. DIV-1 works together with FBF to promote GSC maintenance and germline proliferation. (A) A simplified network controlling the mitosis/meiosis decision. GLP-1/Notch signaling promotes GSC maintenance and germline proliferation, in part by transcriptional activation of the *fbf-2* gene (Lamont et al., 2004). FBF-1 and FBF-2 maintain GSCs by repressing the activity of the GLD-1/NOS-3 and GLD-2/GLD-3 pathways. Specifically, GLD-1/NOS-3 represses mitosis-promoting mRNAs and GLD-2/GLD-3 activates meiosis-promoting mRNAs. PUF-8 protein represses the proliferative fate through inhibiting GLP-1/Notch signaling (Racher and Hansen, 2012) and FBF (this study). (B) The percentage of the Glp phenotype. Glp phenotype was defined as

892 germlines with no EdU-positive cells (see H-K). (C) The percentage of germline (GL)

893 tumor phenotype. (D-G) EdU staining of dissected adult hermaphrodite germlines.

894 *vector(RNAi); fbf-1 fbf-2 puf-8* mutants had mitotically dividing germ cells in the distal

895 gonads (D and E) or throughout the germlines (F and G). (H-K) About 70% of *div-*

896 *1(RNAi); fbf-1 fbf-2 puf-8* mutants lost mitotically dividing germ cells and displayed a Glp

897 phenotype. EdU(-) germlines had two phenotypes; Glp germlines with only a few of

898 meiotic germ cells (H and I) and with a few of meiotic germ cells and sperm (J and K).

899 Solid yellow lines, a region with EdU(+) cells. Broken green lines, a region differentiating

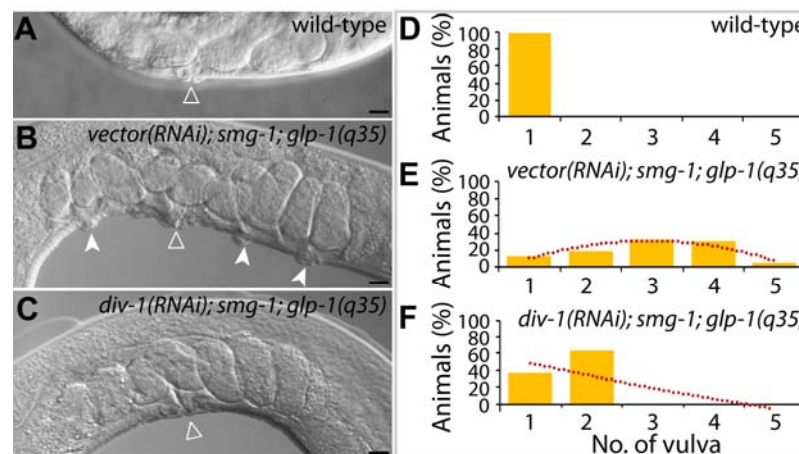900 cells (meiotic cells and sperm). *, distal end. Scale bars: 10 µM.

901

Figure 6. Depletion of DIV-1 suppresses the formation of multi-vulva caused by GLP-1(q35) activity. (A) Adult wild-type, Nomarski micrograph. A single vulva is induced (open triangle). (B) Adult *vector(RNAi); smg-1; glp-1(q35)* mutant, Nomarski micrograph. Ectopic vulvae (filled arrowhead) are induced in addition to the main vulva (open triangle). (C) Adult *div-1(RNAi); smg-1; glp-1(q35)* mutant, Nomarski micrograph. *div-1(RNAi)* suppress multi-vulva phenotype. (D-F) Graph showing the percentage of multi-vulva in adult animals. The number of vulva was determined by Nomarski DIC optics. Dotted red lines indicate trendline. See Supplementary material Fig. S4 for the effects of other cell cycle regulators on *glp-1(q35)*-mediated multi-vulva phenotype. Scale bars: 10 μM.
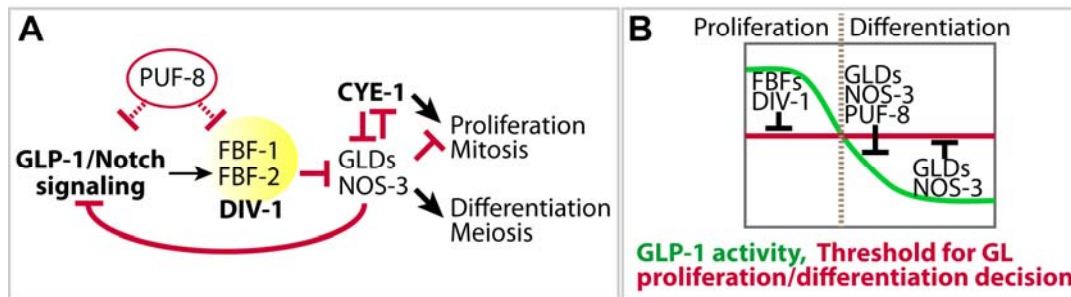
Figure 7. Working model. (A) DIV-1 works together with FBFs to promote germline proliferation and inhibit differentiation. PUF-8 inhibits the function of GLP-1/Notch signaling and its downstream regulators (FBFs and probably DIV-1). (B) The potential roles of DIV-1 and other key regulators in controlling a threshold for germline proliferation/differentiation decision. See Supplementary material Fig. S5 for the effects of other key regulators on controlling a threshold for germline proliferation/differentiation decision.