Subunits of the DNA polymerase alpha-primase complex promote Notch-mediated proliferation with discrete and shared functions in *C. elegans* germline

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Abbreviations: *C. elegans*, *Caenorhabditis elegans*; DNA pol α-primases, DNA polymerase α-primases; GLP, Germline proliferation defect; PUF, PUMILIO/FBF; GSC, Germline stem cell; NICD, Notch intracellular domain; HU, Hydroxyl urea; RNAi, RNA interference; GFP, Green fluorescent protein; DTC, Distal tip cell; 3'UTR, 3' untranslated region; EdU, 5-ethynyl-2'-deoxyuridine

ABSTRACT

Notch receptor signaling is a highly conserved cell communication system in most multicellular organisms and plays a critical role at several junctures in animal development. In *C. elegans,* GLP-1/Notch signaling is essential for both germline stem cell maintenance and germ cell proliferation during gonad development. Here, we show that subunits (POLA-1, DIV-1, PRI-1, and PRI-2) of the DNA polymerase alpha-primase complex are required for germ cell proliferation in response to GLP-1/Notch signaling in different tissues at different developmental stages. Specifically, genetic and functional analyses demonstrated that 1) maternally contributed DIV-1 (regulatory subunit) is indispensable non-cell autonomously for GLP-1/Notch-mediated germ cell proliferation during early larval development, whereas POLA-1 (catalytic subunit) and two primase subunits, PRI-1 and PRI-2, do not appear to be essential; 2) germline POLA-1, PRI-1 and PRI-2 play a crucial role in GLP-1/Notch-mediated maintenance of proliferative cell fate during adulthood, while DIV-1 is dispensable; and 3) germline POLA-1, DIV-1, PRI-1, and PRI-2 function in tandem with PUF (Pumilio/FBF) RNA-binding proteins to maintain germline stem cells in the adult gonad. These findings suggest that the subunits of the DNA polymerase alpha-primase complex exhibit both discrete and shared functions in GLP-1/Notch or PUF-mediated germ cell dynamics in *C. elegans*. These findings link the biological functions of DNA replication machineries to signals that maintain a stem cell population, and may have further implications for Notch-dependent tumors.

INTRODUCTION

Germline stem cells (GSCs) are characterized by their ability to self-renew and to give rise to either sperm or eggs (differentiation to gametes). A balance between self-renewal and differentiation of GSCs is strictly controlled by an intricate network encompassing both extrinsic pathways and intrinsic regulators [1]. Aberrant regulation of this network has impacts ranging from loss of the stem cell pool or a specific germ cell type to over-proliferation of undifferentiated germ cells. The latter has been implicated in germline tumors [1].

One of the key extrinsic cues for stem cell maintenance is Notch signaling [2, 3]. The resulting intercellular signal plays essential and varied roles in the regulation of many types of stem cells [2, 3]. In *C. elegans*, GLP-1/Notch signaling is required for GSC maintenance and germ cell proliferation during development [4] (Fig. 1A). Briefly, the Notch ligand, LAG-2, is expressed in stem cell niche (a function performed by "distal tip cells" [DTCs] in *C. elegans*) [5]. LAG-2 interacts with the GLP-1/Notch receptor, leading to its proteolytic cleavage. This is followed by the translocation of GLP-1/Notch intracellular domain (NICD) from the cell membrane to the nucleus, where it forms a ternary complex with LAG-1 (DNA-binding protein) and LAG-3/SEL-8 (transcription coactivator) to stimulate the expression of target genes [6, 7] (Fig. 1B). Among the target genes are *fbf-2* [a member of the PUF (Pumilio/FBF) RNA-binding protein family] [8], *lip-1* [a dual specificity phosphatase] [9, 10], *lst-1* [lateral signaling target-1, no known homolog] [11, 12], *sygl-1* [synthetic germline proliferation defect, no known homolog] [11]. Among them, *lst-1* and *sygl-1* function redundantly to promote germ cell proliferation and maintain GSCs in *C. elegans* [11]. For example, *lst-1* and *sygl-1* single mutant worms have self-fertile hermaphrodite germlines comparable in size and organization to wild-type worms [11]. In contrast, most *lst-1 sygl-1* double mutant worms displayed a premature meiotic entry (defined as Glp [Germline proliferation defect]) phenotype [11]. Additionally, FBF-2 and LIP-1 proteins promote germ cell proliferation by inhibiting meiosis-promoting regulators such as GLD-1/Quaking and MPK-1/ERK [4, 9, 13] and cell cycle regulators like the cyclin E/CDK2 inhibitor, CKI-2 [14]). Therefore, reduced

GLP-1/Notch activity inhibits germ cell proliferation. As an alternative fate, these cells differentiate into mature sperm [15]. Conversely, elevated GLP-1/Notch activity promotes germ cell proliferation and simultaneously inhibits their differentiation, resulting in germline tumors [16].

In this study, we explored the role of the DNA polymerase alpha-primase (henceforth termed DNA pol α-primase) complex in *C. elegans* GLP-1/Notch-mediated germ cell proliferation. Using a temperature sensitive *glp-1(bn18)* loss-of-function mutant worm, our focused RNAi screen has identified *div-1* (a homolog of the human POLA2 regulatory subunit) as a positive regulator of GLP-1/Notch-mediated germ cell proliferation. Interestingly, DIV-1 is required non-cell autonomously for germ cell proliferation during early larval development. Conversely, other DNA pol α-primase subunits, POLA-1 (a homolog of mammalian POLA1 catalytic subunit), PRI-1 (a homolog of mammalian PRIM1 primase) and PRI-2 (a homolog of mammalian PRIM2 primase), control germ cell proliferation cell autonomously in the adult gonad. These findings suggest that each subunit may respond independently to GLP-1/Notch signaling to mediate germ cell proliferation at different developmental stages. The regulatory function of these subunits appears to be distinct from their initiation role in DNA replication. Furthermore, all four subunits have a shared cell autonomous function in PUF-mediated GSC maintenance. Therefore, our findings may provide a powerful model organism to investigate the connection between Notch signaling and DNA replication machineries during animal reproductive development.

RESULTS

S phase arrest by HU enhances a Glp phenotype in *glp-1(bn18)* mutant worms

The cell cycle state of stem cells determines cell fate [17, 18]. Recent studies show that cyclin E and CDK1 (S phase entry factors) are critical for the maintenance of germline stem cells (GSCs) and proliferative cell fate in flies and worms [19-21]. Since GSCs have a short or absent G1 and a long S phase [20], it seems likely that events in early S phase involving this kinase complex

might be essential to maintain their undifferentiated state. First, we asked whether S phase progression is functionally linked to GLP-1/Notch signaling that is essential for germ cell proliferation. We used a temperature sensitive *glp-1(bn18)* loss-of-function mutant worm that exhibits a sensitized background to determine the genetic connection of S phase progression to GLP-1/Notch signaling *in vivo* [20]. Proliferating germ cells in the *glp-1(bn18)* mutant worms resemble those of wild-type, albeit with a reduced germ cell number (~50% of wild-type at young adult stage) at permissive temperature (20°C) (Fig. 1C, D) [22, 23]. At the restrictive temperature (25°C), however, a premature meiotic entry causes most *glp-1(bn18)* mutant worms to display the Glp (germline proliferation defect) phenotype, which produces just a few mature sperm cells and no oocytes (Fig. 1E, F) [15]. To determine the role of molecular events during S phase in GLP-1/Notch-mediated germ cell proliferation during early larval development, we treated wild-type and *glp-1(bn18)* mutants at L1 stage with 40 mM hydroxyurea (HU, a DNA synthesis inhibitor) at 20°C. After incubation for 18 hours in HU plate, worms were transferred to a normal NGM plate, and were allowed to grow to adults. The Glp phenotype was scored by staining whole worms with DAPI. Wild-type germlines did not show a Glp phenotype in the absence or presence of HU (Fig. 2A) and most *glp-1(bn18)* (-HU) mutants were normal and self-fertile (Fig. 2A, B). However, HU-treated *glp-1(bn18)* mutants exhibited a substantial increase in the Glp phenotype up to 93% (Fig. 2A, C). These results suggest that S phase arrest by HU impairs GLP-1/Notch-mediated germline proliferation during early larval development.

DIV-1/POLA2 promotes GLP-1/Notch-mediated germ cell proliferation during early larval development

To further elucidate the role of molecular events during S phase in GLP-1/Notch-mediated germ cell proliferation during early larval development, we knocked down the expression of key regulator genes encoding Cyclins (Group 1), DNA replication processing proteins (Group 2), and DNA replication licensing proteins (Group 3), by feeding RNAi to *glp-1(bn18)* mutant worms at

20°C (Table 1). Specifically, young adult (yAd) *glp-1(bn18)* hermaphrodites (P0) were transferred to respective feeding RNAi plates to deplete the expression of both maternal and zygotic mRNAs (Fig. 2D). RNAi of these genes showed substantial embryonic lethality as previously reported by others [24, 25]. To bypass the requirement for GLP-1/Notch during embryogenesis and enable the monitoring of GLP-1/Notch-dependence during GSC expansion in larvae, RNAi knockdowns were performed in *glp-1(bn18)* mutant worms that have been upshifted to 20°C in early larval development. The germline phenotypes of the surviving F1 progeny were scored by DAPI staining fixed adult worms (3 days post L1, 5 days RNAi) (Fig. 2D). Consistent with the results of others [20], *cye-1*(RNAi) significantly enhanced the Glp phenotype of *glp-1(bn18)* mutant worms even at 20°C (Fig. 2E). Under the same conditions, we also examined the effects of S phase regulators on GLP-1/Notch-mediated germ cell proliferation. Interestingly, RNAi of some S phase genes, but not all, enhanced the Glp phenotype of *glp-1(bn18)* mutant worms with a wide range of penetrance (Fig. 2E, yellow bars). In particular, the Glp phenotype was considerably enhanced by inhibition of the *div-1*, *chk-1*, or *orc-2* genes at 20°C (Fig. 2E, F). *C. elegans div-1* (a homolog of the mammalian POLA2) encodes the regulatory subunit of the DNA pol α-primase complex and has an essential role in early embryogenesis [24]. The DIV-1 protein forms a complex with POLA-1 (catalytic subunit) and two primases, PRI-1 and PRI-2, to initiate DNA replication in proliferating cells. To test if other DNA pol α-primase complex subunits are required for GLP-1/Notch-mediated germ cell proliferation, we also depleted the expression of *pola-1*, *pri-1*, or *pri-2* genes by RNAi in *glp-1(bn18)* mutant worms at 20°C. Intriguingly, RNAi of *pola-1*, *pri-1*, or *pri-2* genes very slightly enhanced the Glp phenotype, suggesting a non-complex function for DNA pol α-primase (Fig. 2E, G-I). We also performed RNAi experiments in the same conditions on *eri-1(mg366)* mutant worms, which are hypersensitive to RNAi [26] (Fig. 2E, blue bars). Loss of *cyb-3*, *pola-1*, *chk-1*, *mcm-6*, or *orc-2* expression partially enhanced the Glp phenotype in *eri-1(mg366)* mutant worms but at lower efficiencies, whereas no effect was seen with RNAi of *div-1*, *pri-1*, and *pri-2* (Fig. 2E, blue

bars). These results suggest that DIV-1 may have a specialized function in GLP-1/Notch-mediated germ cell proliferation during early larval development.

Non-cell autonomous function of DIV-1 in GLP-1/Notch-mediated germ cell proliferation

The *div-1(or148)* loss-of-function mutant strain of worms displays embryonic lethality due to delayed embryonic cell division [24]. To avoid the lethality and characterize the function of DIV-1 in germ cell proliferation, we used a less penetrant approach instead of the *div-1(or148)* mutant worms. First, to test whether DIV-1 is necessary cell autonomously for early germ cell proliferation, we used an *rrf-1(pk1417); glp-1(bn18)* double mutant worm. The *rrf-1(pk1417)* mutation prevents RNAi effectiveness in the soma, but not in the germline [27]. Resulting phenotypes must therefore be due to gene function only in the germ cells themselves. We depleted both maternal and zygotic pools of *div-1* and *cye-1* mRNAs by RNAi in *glp-1(bn18)* and *rrf-1; glp-1(bn18)* mutant worms starting at the young adult (yAd) P0 stage, and subsequently analyzed their germline phenotypes in F1 progeny as worms reached adult stage (Fig. 3A). Because the expression of zygotic/somatic target genes is preserved in *rrf-1; glp-1(bn18)* mutant worms (Fig. 3B), reversion of phenotypes in this strain indicate that the target gene is affecting germ cells only through expression in neighboring somatic cells. Results showed that *cye-1*(RNAi) considerably enhanced the Glp phenotype of both *glp-1(bn18)* and *rrf-1; glp-1(bn18)* mutant worms even at 20°C (Fig. 3C). In contrast, *div-1*(RNAi) promoted the Glp phenotype in *glp-1(bn18)* mutant worms, but not in *rrf-1; glp-1(bn18)* double mutant worms at 20°C (Fig. 3C). Depletion of germline DIV-1 by *div-1*(RNAi) was confirmed by immunohistochemistry and western blot as well as by assessing the embryonic lethality of progeny (Fig. S1). Therefore, CYE-1 functions within germ cells to promote proliferation, as one might expect. Unexpectedly, however, the DIV-1 functions in somatic cells to drive neighboring germ cells into proliferation. To confirm this result, we used a germline-RNAi incompetent *ppw-1(pk1425); glp-1(bn18)* double mutant. PPW-1 (a PAZ/PIWI domain-containing protein) belongs to the Argonaute family of proteins [28]. The *ppw-1(pk1425)* mutation prevents

RNAi effectiveness in the germline, but not in the soma [28]. Resulting phenotypes must therefore be due to gene function only in the somatic cells themselves. Notably, *div-1*(RNAi) significantly enhanced the Glp phenotype in *ppw-1; glp-1(bn18)* double mutant worms at 20°C (Fig. 3C). These observations suggest that DIV-1 is required non-cell autonomously for GLP-1/Notch-mediated germ cell proliferation during early larval development. Because depletion of DIV-1 selectively from the soma (ppw-1 background) enhances Glp phenotype, but its selective loss in germ cells (rrf-1 background) has no effect on proliferation, DIV-1 must be acting from the somatic sheath cells to alter the fate of underlying germ cells. The path by which this occurs is not known.

We then assessed the pattern of DIV-1 expression using a transgenic worm that expresses an extrachromosomal *div-1(promoter)::div-1(exon 1 and 2)::GFP* (henceforth referred to as *div-1p::GFP*) transgene (Fig. 3D). The zygotic *div-1p::GFP* was first detected at about the 80-cell stage in embryos and remained strongly expressed in dividing cells throughout the rest of embryogenesis (Fig. 3E). *div-1p::GFP* was also highly expressed in the pharynx and DTCs in early larval stages (Fig. 3F), in vulva precursor cells in L3, and in vulva precursor cells in L4 (Fig. 3G). Most strikingly, *div-1p::GFP* was strongly expressed in the somatic gonadal tissues, including gonadal sheath cells, spermatheca, and the vulva (Fig. 3H). It was previously reported that somatic sheath/spermatheca tissues interact with germ cells by induction events to control their proliferation and differentiation [29]. Therefore, we suggest that DIV-1 may function non-cell autonomously in somatic gonadal tissues to promote the GLP-1/Notch signaling that drives early germ cells to proliferate (see Fig. 6B).

Next, we addressed what portion of soma-germ cell communication might require DIV-1. To do so, we tested whether *div-1* knockdown disrupts somatic gonad development itself, or if it may affect the expression of LAG-2 and GLP-1, which are the ligand-receptor pair that signal maintenance of mitosis for germ cell proliferation. We performed *div-1*(RNAi) in *lag-2(promoter)*::GFP transgenic and wild-type worms, and then immunostained dissected gonads with anti-GFP and anti-GLP-1 antibodies. Result showed that *div-1*(RNAi) did not affect somatic

gonad development (data not shown) as the formation and morphology of the sheath was normal. Loss of DIV-1 did not reduce the expression of *lag-2p::GFP* in the DTC (Fig. 3I, J), nor was there any substantial change in GLP-1 abundance in the membrane of germ cells (Fig. 3K, L). Thus, the primary signal for sustained mitosis of early germ cells is unperturbed by depletion of *div-1*. While it is still unclear how DIV-1 functions non-cell autonomously in somatic cells to influence germ cell proliferation, it is likely to modulate GLP-1/Notch signaling indirectly or its non-cell autonomous function may be independent of GLP-1/Notch signaling. Our findings do not rule out that *div-1* knockdown may delay somatic development and affect soma-germline interaction by other means. Studying the function of DIV-1 in the somatic development remains a challenge for the future.

Once signaled, the maintenance of proliferative cell fate requires POLA-1, PRI-1, and PRI-2, but not DIV-1 during adulthood

During adulthood, GLP-1/Notch signaling also maintains proliferative cell fate in the germline following the initial mitotic expansion in larvae. To examine whether *div-1* is also required for maintenance of proliferative cell fate during adulthood, L4 staged *glp-1(bn18)* and *rrf-1; glp-1(bn18)* mutant worms were depleted of *vector* (negative control), *cye-1* (positive control), *pola-1*, *div-1*, *pri-1*, or *pri-2* by RNAi for 4 days at 20°C (Fig. 4A). Germline phenotypes were determined by staining dissected gonads with anti-REC-8 antibody, a marker of mitotic germ cells [30], and anti-HIM-3 antibody, a marker of meiotic germ cells [31]. In both *glp-1(bn18)* and *rrf-1; glp-1(bn18)* mutant worms, *div-1* was not required for germ cell proliferation, whereas *cye-1*, *pola-1*, *pri-1,* and *pri-2* were all found to be essential (Fig. 4B). Similar to wild-type, *glp-1(bn18)* mutant germlines maintain mitotically dividing cells, as evidenced by the REC-8(+)/HIM-3(-) region (yellow outlines) and transition to meiotic cells (REC-8(-)/HIM-3(+); broken green outlines) at 20°C (Fig. 4C). As previously described by others [20], *cye-1*(RNAi) dramatically enhanced the Glp phenotype of both *glp-1(bn18)* single and *rrf-1; glp-1(bn18)* double mutant worms, such that no mitotic germ

cells are evident (Fig. 4B, D). RNAi of *pola-1*, *pri-1*, or *pri-2* also enhanced the Glp phenotype of both mutant worms (Fig. 4B, E, G, H). However, *div-1*(RNAi) failed to enhance the Glp phenotype in both mutant worms (Fig. 4B, F). Since all these regulators are expressed in the *C. elegans* germline, we suggest that GLP-1/Notch-mediated maintenance of proliferative cell fate requires germline POLA-1, PRI-1, and PRI-2, but not DIV-1 during adulthood. To confirm this result, we performed somatic RNAi in *ppw-1; glp-1(bn18)* double mutant worms, in which RNAi is largely defective in the germline [28]. Their germline phenotypes were determined by staining dissected gonads with anti-REC-8 and anti-HIM-3 antibodies (Fig. 4I-N). Although the percentage of Glp phenotype by *cye-1*(RNAi) was slightly decreased in the *ppw-1; glp-1(bn18)* mutant worms in comparison to *rrf-1; glp-1(bn18)* mutant worms, *cye-1*(RNAi) significantly enhanced the Glp phenotype in both *rrf-1; glp-1(bn18)* and *ppw-1; glp-1(bn18)* mutant worms (Fig. 4B, J). This result indicates that CYE-1 appears to be critical for GLP-1/Notch-mediated germ cell proliferation in both somatic and germline tissues. However, the percentages of Glp phenotype by RNAi of *pola-1*, *pri-1*, or *pri-2* were significantly decreased in the *ppw-1; glp-1(bn18)* mutant worms (Fig. 4B, K-N). These results suggest that GLP-1/Notch-mediated maintenance of proliferative cell fate requires cell autonomously POLA-1, PRI-1, and PRI-2, but not DIV-1 during adulthood.

Fox et al. previously reported that a 2-day *pri-1*(RNAi) silencing failed to enhance the Glp phenotype of *rrf-1; glp-1(bn18)* mutant worms (REF). However, our study shows that 4-day RNAi silencing of *pola-1*, *pri-1* or *pri-2* dramatically enhances the Glp phenotype of *rrf-1; glp-1(bn18)* mutant worms (Fig. 4B). We therefore reevaluated the roles of both primase (PRI-1 and PRI-2) on GLP-1/Notch-mediated maintenance of proliferative cell fate. In this study, we did not test the effect of POLA-1 potential redundancy with PRI-1 and PRI-2 due to genetic complexity/efficacy of such triple depletions. As previously observed by others [20], most *pri-1*(RNAi) and *pri-2*(RNAi) germlines expressed REC-8 after a 2-day depletion. However after 4-days of silencing, adult germlines exhibited a pronounced Glp phenotype, indicated by a lack of REC-8 and positive HIM-3 staining beginning at the DTC (presumptive niche;Fig. 4B, G, H). This result suggested that

germlines with a larval mitotic germ cell population progressively lost all those proliferative cells after 4-days of *pri-1*(RNAi) or *pri-2*(RNAi) silencing. To test whether the delay in Glp phenotype development was due to redundancy, we performed double RNAi of *pri-1* and *pri-2* in *rrf-1; glp-1(bn18)* for 2 days at 20°C and scored the percentage of worms with the Glp phenotype, again by staining gonads with anti-REC-8 and anti-HIM-3 antibodies. Interestingly, the percentage of *pri-1/-2*(RNAi) worms with the Glp phenotype was significantly increased (52% ± 3, n=98) even at just 2 days. These results suggest that the two primase subunits (PRI-1 and PRI-2) of the DNA pol α-primase complex must be present in adult germ cells for the GLP-1/Notch-mediated signal that maintains a proliferating population (see Fig. 6C).

DIV-1 may work with FBF/PUF RNA-binding proteins to maintain GSCs

In addition to GLP-1/Notch signaling, a battery of mRNA regulators function to maintain the balance between proliferation and differentiation (Fig. 5A). A well-conserved mRNA translational regulator is the PUF (Pumilio/FBF) RNA-binding protein. PUF proteins control various physiological processes, including GSC maintenance and cell fate specification, by interacting with 3' untranslated regions (3'UTRs) and modulating mRNA translation in a wide variety of eukaryotes [32]. Translational control is prevalent in the germlines of all organisms in order to promote gamete development and circumvent meiotic suppression of gene transcription [33-35]. *C. elegans* has multiple PUF genes with individualized roles. In particular, FBF-1 and FBF-2 proteins play an essential role in GSC maintenance and germ cell proliferation [36]. FBF-1 and FBF-2 proteins are 96% identical (henceforth called FBF), and are largely redundant: *fbf-1* and *fbf-2* single mutant worms are both self-fertile with germlines morphologically identical to wild-type [36]. In contrast, *fbf-1 fbf-2* double mutant worms maintain their GSCs until the L4 stage, but then most GSCs leave mitotic cell cycle, enter meiosis, and the entire (small) population eventually differentiates into sperm at 20°C, leaving no GSC population (Glp phenotype) [36] (Fig. 5B, C). Remarkably, the Glp phenotype of *fbf-1 fbf-2* mutant worms was suppressed by loss of

PUF-8 as evidenced by the restoration of mitotically dividing cells (EdU-positive) in the germlines of *puf-8 fbf-1 fbf-2* triple mutant worms at 20°C (Fig. 5B, D). These results indicate that PUF-8 antagonizes FBF-mediated GSC maintenance. Finally, we sought to establish whether the DNA pol α-primase subunits are required to act in germ cells for PUF-mediated GCS maintenance. We again performed RNAi of *pola-1*, *div-1*, *pri-1*, or *pri-2* this time in *rrf-1; puf-8 fbf-1 fbf-2* quadruple mutant worms at 20°C. Again, the *rrf-1(pk1417)* mutation abrogates RNAi in the soma, but not in the germline [27]. Most *vector*(RNAi)*; rrf-1; puf-8 fbf-1 fbf-2* germlines were positive for EdU-labeling at 20°C (Fig. 5E, F). However, the RNAi of *pola-1*, *pri-1*, *pri-2*, or *div-1* genes dramatically subverted the restoration of mitotically dividing germ cells in *rrf-1; puf-8 fbf-1 fbf-2* mutant worms, which were all negative for EdU-labeling (Fig. 5E, G-J). These results suggest that germline POLA-1, DIV-1, PRI-1, and PRI-2 may have a shared function in the PUF-mediated contribution to GSC maintenance and mitotic cell cycle progression that is germ cell autonomous (see Fig. 6D).

DISCUSSION

In eukaryotes, intercellular signaling through Notch receptors regulates growth and differentiation in several contexts during animal development [37]. Moreover, aberrant regulation of the Notch signaling pathway is highly correlated to human disease, including cancers [38-40]. In *C. elegans*, the mechanisms of GSC maintenance and proliferation largely rely on GLP-1/Notch signaling [41] and a battery of mRNA regulators (e.g., PUF proteins) [4]. Here, we report on new, tenable links between DNA pol α-primase subunits, GLP-1/Notch signaling, and PUF proteins for those germ cell proliferation and maintenance functions (Fig. 6A-C). Specifically, DIV-1 is required non-cell autonomously for GLP-1/Notch-mediated germ cell proliferation during early larval development, whereas other subunits do not appear to be essential (Fig. 6B). During adulthood, GLP-1/Notch-mediated maintenance of proliferative cell fate requires POLA-1, PRI-1, and PRI-2,

but not DIV-1 (Fig. 6C). However, all these subunits work with FBF/PUF RNA-binding proteins to maintain GSCs (Fig. 6D). How is it that DIV-1 is not required for GSC maintenance/proliferation in the germline during adulthood, but becomes important when PUF proteins are missing? While it still eludes us, we speculate that DIV-1 may regulate GSC proliferation within the germline, but it is largely compensated when PUF proteins are present. Understanding how this works at the molecular level will be an important challenge for the future.

The major role of the DNA pol α-primase complex is in the initiation of DNA replication at chromosomal origins. However, this complex has also been implicated in a variety of cellular processes including genome integrity, DNA metabolism, and telomere maintenance. For example, mutations in the subunits of the DNA pol α-primase complex have been demonstrated to highly influence the checkpoint pathway. In *Saccharomyces cerevisiae*, the *pri1-M4* mutation in the gene coding for the p48 primase subunit was associated with an accelerated progression of the S phase as well as hypersensitivity of DNA to damage [42]. Also, a missense mutation in the small subunit of DNA primase (Prim1) led to a profound apoptotic activity in retinal neurons in zebrafish [43]. Interestingly, apoptosis induced by the *Prim1* mutation was also dependent on activation of ataxia telangiectasia mutated (ATM), Chk2, and p53, pointing at a possible role of Prim1 in genomic surveillance and response to DNA damage [43]. Similarly, differences in the requirement for phosphorylation by kinases provided supportive evidence for the interplay between replication proteins and the checkpoint pathway. Pellicioli *et al.* reported that Rad53, the *S. cereveisiae* homolog of Chk2, regulates phosphorylation and activity of the DNA pol α-prim B subunit under DNA stress [44]. Several studies have demonstrated the role played by the DNA pol α-primase complex in the maintenance of chromosomal stability. Cdc13, a telomere-binding protein, was shown to interact with DNA pol α and the Cdc13-Stn1-Ten1 (CST) complex, required for telomere protection, was proposed to regulate DNA pol α-primase activity [45, 46]. Moreover, increasing evidence suggests that DNA pol α is also involved in the epigenetic control of

chromatin assembly and gene expression. Nakayama *et al.* demonstrated that a mutated *swi7* gene, which encodes the catalytic subunit of DNA pol α-primase complex, results in altered localization of the chromodomain protein Swi6, which also directly interacts with DNA pol α *in vitro* [47].

During S phase POLA2 is recruited to DNA at the replicative forks. A POLA2 (G583R) mutation leads to mislocalization of the subunit to the cytoplasm instead of the nucleus. This not only inhibits DNA replication in cancer cells [e.g., non-small cell lung cancer (NSCLC)], it also sensitizes them to chemotherapy (e.g., Gemcitabine) [48]. In addition, the synthetic retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437) is a potent inhibitor of POLA1 and was shown to selectively induce apoptosis in human lung cancer cells [49]. Inhibition of DNA replication by CD437 elicits different responses in cancer cells. Whereas CD437-mediated inhibition of POLA1 leads to cell death in cancer cells, it merely induces cell cycle arrest in normal epithelial cells [49]. Notably, Notch signaling is altered in approximately one third of NSCLCs, which are the leading cause of cancer-related deaths [50]. Although the role of POLA1/2 in NSCLCs with elevated Notch activity has not yet been studied, the fundamental mechanism that we observe in GSC maintenance may be conserved with that in tumorigenesis. At the molecular level, it is intriguing that DNA primases play a key role in the initiation of DNA replication and in the synthesis of Okazaki fragments on the lagging strand. Primases are also involved in checkpoint pathways, coupling DNA replication to DNA repair [43, 51, 52]. Importantly, mammalian DNA primases, PRIM-1 and PRIM-2 are highly associated with human malignancies [51, 53, 54]. In this study, we also found that the subunits of the DNA pol α-primase complex promote GLP-1/Notch-mediated proliferation with discrete and shared function in *C. elegans* germline. To date, this novel role has not yet been reported in any other organisms. Therefore, understanding the physiological processes that link Notch signaling to the role of the DNA pol α-primase subunits may hold promise for the development of antineoplastic molecular and cellular therapies.

Recently, it was reported that Notch signaling is tightly linked to cell cycle progression during vulval development in *C. elegans* [55]. Specifically, CYD-1/Cyclin D and CYE-1/Cyclin E stabilize LIN-12/Notch receptor in vulva precursor cells (VPCs) [55]. In *C. elegans* germline, CYE-1, but not CYD-1, is essential for GLP-1/Notch-mediated proliferation [20]. Also, *C. elegans* CYD-1 and CYE-1 induce distinct cell cycle re-entry programs in differentiated muscle cells [56]. These studies suggest that cell fate determination in very varied tissues (e.g., germline and soma) and at disparate times in development (e.g., early lineage establishment and late terminal differentiation), may be regulated by different cell cycle progression events and regulators, including Notch. In this study, we also showed that depletion of individual DNA pol α-primase subunits (POLA-1, DIV-1, PRI-1, or PRI-2) affected GLP-1/Notch-mediated germ cell proliferation differently from somatic or germline sources at different developmental stages (see Fig. 6A-D). Specifically, somatic DIV-1 promotes Notch-mediated germ cell proliferation non-cell autonomously during early larval development. Although it remains elusive how cell cycle signature or regulators in somatic tissues influence the proliferation of their neighboring germ cells, previous studies showed that somatic gonadal tissues such as DTC and sheath cells establish the both germ cell proliferation and differentiation patterns [57]. Our study points to a somatic molecular pathway that may be responsible for such induction events. Understanding the mechanism of how a somatic protein with a DNA replication function, DIV-1, influences Notch signaling for germ cell proliferation non-cell autonomously is still a major challenge for future studies.

MATERIALS AND METHODS

Nematode Strains

All strains were derived from Bristol strain N2 and maintained at 20°C as described unless otherwise noted [58]. Mutations and balancers used in this work include: ***LG I***: *rrf-1(pk1417)*, *ppw-1(pk1426)*; ***LG II***: *fbf-1(ok91)*, *fbf-2(q738)*, *puf-8(q725)*; *mIn1[mIs14 dpy-10(e128)]*; ***LG III***: *glp-*

1(bn18). MHL52 [div-1(promoter)::div-1(exon1 and 2)::GFP], *JK2868 [lag-2p::GFP+unc-119(+)]*, *TG1755 [unc-119(ed3); ltIs37 ((pAA64) pie-1p::mCherry::his-58 + unc-119(+); gtIs66 (pie-1p::GFP(lap)::div-1 + unc-119(+)].*

Hydroxyurea (HU) Treatment

L1 staged wild-type and *glp-1(bn18)* mutant worms were transferred to NGM (Nematode Growth Media) agar plates containing 40 mM HU (Sigma-Aldrich, St. Louis, MO) and incubated at 20°C. After 18 h with HU plate incubation, worms were transferred to a normal NGM plate and allowed to grow to adults.

***pola-1::L4440* feeding RNAi vector**

To construct a *pola-1::L4440* feeding RNAi vector, *pola-1* genomic fragments (958 nt) were amplified from wild-type (N2) DNA by PCR using two primers [pola1(F): 5'-CCAGCGGTAACGCCTGGGAG-3' and pola1(R): 5'-GCTGTGAGCTCCCATTGGCC-3']. PCR products were purified by QIAquick PCR purification kit (QIAGEN, Chatsworth, CA; Cat # 28106). Purified *pola-1* PCR products and feeding RNAi vector (L4440) (Addgene, Plasmid #1654) were double digested by *Xba*1 and *Pst*1 (New England Biolabs, Ipswich, MA, USA) for 4 hours at 37°C. The digested DNA fragments were eluted by QIAquick Gel Extraction kit (QIAGEN, Cat # 28704). Eluted *pola-1* DNA fragments were introduced into the eluted feeding RNAi vector *L4440* by a ligation enzyme reaction. Finally, cloned *pola-1::L4440* plasmids were transformed into HT115 (DE3), an RNase III-deficient *E. coli* strain with IPTG-inducible T7 polymerase activity.

Feeding RNAi

RNAi constructs were obtained from the *C. elegans* ORF RNAi Library (Thermo Fisher Scientific, San Jose, CA, USA). For yAd(P0)→Ad(F1) RNAi (see Fig. 2D, 3A), young adult (yAd) staged worms (P0) were placed on feeding RNAi plates seeded bacteria expressing double

stranded RNAs (dsRNAs) corresponding to the gene of interest as previously described [59-61]. The germline phenotypes were analyzed by DAPI staining when F1 progeny reached adult stage at 20°C (3 days from L1). For yAd(P0)→Ad(P0) RNAi (see Fig. 4A), synchronized L4 staged worms were placed on feeding RNAi plates and incubated at 20°C and the germline phenotypes were analyzed by DAPI staining 4 days later.

Germline Antibody Staining

Germline antibody staining was performed as previously described [62]. Briefly, dissected gonads were fixed in 3% paraformaldehyde/0.1M K_2HPO_4 (pH 7.2) solution for 10-20 min, and then post-fixed with cold 100% methanol for 5 min. After blocking for 30 min in 1x PTW (1XPBS + 0.1% Tween20)/0.5% BSA (Bovine Serum Albumin) solution, primary antibody was added, followed by incubation for 2 h at 20°C. The dissected gonads were washed three times for at least 30 min with 1x PTW/0.5% BSA solution and incubated in the same solution containing the fluorescence-conjugated secondary antibodies for 1-2 h at 20°C. After washing three times in 1x PTW/0.5% BSA solution for at least 30 min, the dissected gonads were stained with 100 ng/mL DAPI solution for 10 min at 20°C and were again washed in 1x PTW/0.5% BSA solution three times. The antibody staining was observed using fluorescence microscopy. Antibodies used in this study include anti-REC-8 (1:200 dilution, kindly provided by Dr. Josef Loidl's lab, University of Vienna, Austria), anti-HIM-3 (1:400 dilution, Novus Biologicals, Littleton, CO, USA), anti-GFP (1:400 dilution, Abcam, Cambridge, MA, USA), and anti-GLP-1 (1:200 dilution, kindly provided by Dr. Judith Kimble's lab, University of Wisconsin-Madison, WI, USA).

Generation of *div-1p::GFP* Transgenic Worms

To make a *div-1p::GFP* transgene, the 5' upstream sequences (1,940 bp), exon1, intron 1, and exon 2 of the *div-1* gene were amplified from wild-type (N2) genomic DNA using two primers

[div-1(Sph1)F1: 5'-AGGTTAGCATGCATCCGGAATCACCCACACTTTAC-3' and div-1(BamHI)R1: 5'-CGCGGATCCGGTTTTTTCACAGATCTCGGCGTG-3']. Purified PCR products were digested by *Sph*1 and *BamH*1 (New England Biolabs, Ipswich, MA, USA), and inserted into the pPD95.67 vector upstream and in frame with a *C. elegans* optimized gfp gene. Wild-type worms were injected with the *div-1p::GFP* transgene (50 ng/ul) and a pCFJ90 co-injection marker (*myo-2p::mCherry*). A total of three transgenic lines expressing both *div-1p::GFP* and *myo-2p::mCherry* were generated. pPD95.67 and pCFJ90 were gifts from Andrew Fire (Stanford University; Addgene plasmid #1490) and Erik Jorgensen (University of Utah; Addgene, plasmid #19327), respectively.

EdU (5-ethynyl-2'-deoxyuridine) Labeling

For metabolic labeling of DNA synthesis, animals were incubated with rocking in M9/0.1% Tween 20/1 mM EdU for 30 min at 20°C. Gonads were dissected and fixed in 3% paraformaldehyde/0.1M K_2HPO_4 (pH 7.2) solution for 10-20 min, followed by -20°C methanol fixation for 10 min. Dissected gonads were blocked in 1x PTW, 0.5% BSA solution for 30 min at 20°C. EdU labeling was detected using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen, San Jose, CA, USA, #C10337), according to the manufacturer's instructions. After washing three times with 1x PTW/0.5% BSA solution for at least 30 min at 10-min intervals, the dissected gonads were stained with DAPI solution (100 ng/mL) for 10 min at 20°C and were finally washed with 1x PTW, 0.5% BSA solution three times. The EdU labeling was observed by fluorescence microscopy.

Western Blot

For the preparation of whole protein samples, collected *C. elegans* worms were lysed in Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) by boiling for 10 minutes. The lysed proteins analyzed by 10% sodium-dodecyl sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE) (Bio-Rad Laboratories, Inc.). Transferred membranes were blocked with 5% nonfat dry milk (Biotium, Inc., Fremont, CA, USA) in Tris-buffered saline (TBS) with 0.5% Tween 20 (TBST) and anti-GFP antibodies (1:1000 dilution, Abcam) at 4°C. The blot was probed with HRP-conjugated anti-rabbit secondary antibodies in 5% (w/v) nonfat dry milk in TBST for 1 hour at room temperature. Western blot images were obtained using the LI-COR C-DiGit Chemiluminescence Western Blot Scanner and Image software (LI-COR, Lincoln, Lebraska, USA).

Data Analysis

Statistical significance was analyzed using the two-tailed student's t-test. The error bars reflect respective standard deviation values.

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AUTHOR CONTRIBUTIONS

DSY, DSC, MAA, and MHL conceived and designed the experiments. DSY, DSC, MAA, BDK and MHL analyzed the data, and wrote the paper. DSY, DSC, and MHL performed the experiments.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

Table 1. Summary of RNAi genes

Gene	Molecular Identity	Mammalian Homolog
Group 1 (Genes encoding cyclins)		
cya-1	A-type cyclin	Cyclin A
cyb-1	B-type cyclin	Cyclin B1
cyb-2.1	B-type cyclin	Cyclin B1
cyb-2.2	B-type cyclin	Cyclin B1
cyb-3	B-type cyclin	Cyclin B3
cyd-1	D-type cyclin	Cyclin D
cye-1	E-type cyclin	Cyclin E
Group 2 (Genes encoding DNA replication processing proteins)		
top-1	Type 1 Topoisomerase	TOP-1
crn-1	5'3' exonuclease	Flap endonuclease 1
rpa-1	Replication protein A	RPA
pola-1	DNA Polα catalytic subunit A	POLA1
div-1	DNA Polα regulatory subunit B	POLA2
pri-1	DNA primase subunit D	PRIM1
pri-2	DNA primase subunit C	PRIM2
chk-1	Serine Threonine protein kinase	CHK1
Group 3 (Genes encoding DNA replication licensing proteins)		
mcm-2	DNA replication licensing factor	MCM2
mcm-3	DNA replication licensing factor	MCM2/3/5
mcm-5	DNA replication licensing factor	MCM5
mcm-6	DNA replication licensing factor	MCM6
mcm-7	DNA replication licensing factor	MCM7
cdt-1	DNA replication licensing factor	CDT1
orc-2	Origin recognition complex subunit	ORC2
emb-27	Anaphase-promoting complex (APC) subunit	CDC16
emb-30	APC/cyclosome component	ANAPC4
cul-1	Cullin	CUL1
mat-1	APC/cyclosome subunit	CDC27/APC3
mat-3	APC subunit	CDC23/APC8

FIGURES and FIGURE LEGENDS

Figure 1. *C. elegans* germline and GLP-1/Notch signaling pathway. (A) Schematic of *C. elegans* germline development. GLP-1/Notch signaling pathway is required for germ cell proliferation during larval development. In coordination with PUF (Pumilio/FBF) proteins, GLP-1/Notch signaling is crucial for GSCs maintenance during adulthood. In the distal end, the somatic gonadal cells, called DTCs (distal tip cells), act as a GSC niche which is essential for GSC maintenance and germ cell proliferation. Germ cells (yellow) at the distal end of the germline divide mitotically. As germ cells move proximally, they enter meiosis (green) and differentiate into either sperm (blue) or oocytes (pink). GLP-1/Notch signaling is activated in the distal mitotic germline. (B) *C. elegans* GLP-1/Notch signaling pathway. The LAG-2 ligand, localized in the DTC, signals to both GLP-1/Notch receptor in GSCs and in mitotically dividing germ cells. Upon GLP-1 activation, the GLP-1/Notch intracellular domain (NICD), LAG-1 and LAG-3 form a ternary complex in the nucleus and activate the transcription of target genes. (C and D) Schematics of normal germ cell proliferation and the germline phenotype of *glp-1(bn18)* mutant at 20°C. In a normal germline, GLP-1/Notch signaling promotes GSC maintenance and germ cell proliferation. Once germ cells (yellow) move proximally, they enter meiosis (green). (E and F) Schematic of premature meiotic entry (also called Glp) phenotype and the germline phenotype of *glp-1(bn18)* mutant at 25°C. Germline with a Glp phenotype, occurring very early in larval development, has a complete depletion of proliferative cells and only ~16 sperm being formed per gonad arm.

Figure 2. DIV-1 is required for GLP-1/Notch-mediated early germ cell proliferation. (A) The percentage of animals scored with a Glp phenotype following hydroxyl urea (HU) treatment. The Glp phenotype was strictly defined as no mitotic cells and only sperm by DAPI staining. Standard deviation bars were calculated from three independent experiments. n.s., not statistically significant; *** $p<0.001$. (B and C) DAPI-stained adult hermaphrodite germlines. (D) Schematic of

RNAi experiment. (E) The percentage of animals scored with a Glp phenotype by RNAi of cell cycle regulator genes in *glp-1(bn18)* and *eri-1(mg366)* mutant worms at 20°C. The dotted red box contains genes that encode the subunits of the DNA pol α-primase complex. (F-I) DAPI-stained adult hermaphrodite germlines. (*) distal end; yellow lines, mitotic cells; broken green lines, meiotic cells; broken pink circles, oocyte nuclei; broken blue lines, sperm; broken white lines, embryos; broken dark green line in G, arrested mitotic cells. Scale bars: 10 μm.

Figure 3. Somatic DIV-1 promotes GLP-1/Notch-mediated germ cell proliferation during early larval development. (A) Schematic of RNAi experiment. (B) RNAi in *glp-1(bn18)*, *rrf-1(pk1417); glp-1(bn18)*, and *ppw-1(pk1425); glp-1(bn18)* mutant worms. The expression of zygotic/somatic target genes is protected from RNAi in *rrf-1(pk1417)* and *ppw-1(pk1425)* mutant background. (C) The percentage of animals scored with a Glp phenotype. Standard deviation bars were calculated from three independent experiments. n.s., not statistically significant; ** $p<0.01$; *** $p<0.001$. (D) Design of *div-1p::GFP* transgene. (E-H) The expression of *div-1p::GFP* expression in embryos (E), L1 (F), L3/L4 (G), and young adult (H). (I-L) The expression of LAG-2::GFP and GLP-1. Young adult staged *lag-2(promoter)::GFP* transgenic worms and wild-type worms were plated on *div-1*(RNAi) plates, and 5 days later, the expression levels of *lag-2p::GFP* and GLP-1 were determined by staining dissected gonads with anti-GFP and anti-GLP-1 antibodies. Broken lines indicate the boundary between mitosis and meiosis. (*) distal end, Scale bars: 10 μm.

Figure 4. POLA-1, PRI-1 and PRI-2 are critical cell autonomously for GLP-1/Notch-mediated maintenance of proliferative cell fate during adulthood. (A) Schematic of RNAi experiment. (B) The percentage of worms exhibiting a Glp phenotype at 20°C. Standard deviation bars were calculated from three independent experiments. n.s., not statistically significant; * $p<0.05$; **

p<0.01; *** p<0.001. (C-N) Germline staining with anti-REC-8 and anti-HIM-3 antibodies. Solid yellow lines, mitotic cells (REC-8(+)/HIM-3(-)); broken green lines, meiotic cells (REC-8(-)//HIM-3(+)). (*) distal end. Scale bars: 10 μm.

Figure 5. POLA-1, DIV-1, PRI-1 and PRI-2 are necessary cell autonomously for PUF-mediated GSC maintenance. (A) A simplified network controlling the germ cell proliferation/differentiation decision. (B) The percentage of worms with EdU-positive germ cells. Standard deviation bars were calculated from three independent experiments. *** p<0.001. (C) DAPI staining of dissected adult *fbf-1 fbf-2* gonads. (D) EdU staining of dissected adult *puf-8 fbf-1 fbf-2* mutant gonads. (E) The percentage of worms with EdU-positive germ cells. Standard deviation bars were calculated from three independent experiments. *** p<0.001. (F-I) EdU staining of dissected adult gonads. Solid yellow lines, a region with EdU(+) cells; broken green lines, a region differentiating cells (meiotic cells and sperm); (*) distal end. Scale bars: 10 μm.

Figure 6. Working Model. (A) Germline development during early larval stage and adulthood. (B and C) The discrete function of POLA-1, DIV-1, PRI-1, and PRI-2 in GLP-1/Notch-mediated germ cell proliferation either non-cell autonomously or cell autonomously during early larval development and adulthood, respectively. (D) The shared function of POLA-1, DIV-1, PRI-1, and PRI-2 in PUF-mediated GSC maintenance.

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