GSK-3 promotes S phase entry and progression in \textit{C. elegans} germline stem cells to maintain tissue output

Tokiko Furuta\textsuperscript{#1}, Hyoe-Jin Joo\textsuperscript{#1}, Kenneth A. Trimmer\textsuperscript{#1,2}, Shin-Yu Chen\textsuperscript{1}, and Swathi Arur\textsuperscript{1,2*}.

1: Department of Genetics, U.T. MD Anderson Cancer Center, Houston, TX, 77030, USA
2: Genes and Development Graduate Program, MD Anderson Cancer Center UT Health Graduate School of Biomedical Sciences, Houston, TX, 77030, USA

#: Contributed equally to this work

*Address correspondence to:
Swathi Arur, Ph.D
Department of Genetics
UT MD Anderson Cancer Center
1515 Holcombe Blvd
Houston, 77030
Phone: 713-745-8424
Email: sarur@mdanderson.org

Keywords: GSK3β, cell cycle, germ cells, stem cell proliferation
Summary Statement: GSK-3 promotes CDK-2 transcription, which in turn maintains germline stem cell proliferation by establishing a short G1 and efficient S phase, allowing for normal germline tissue output.

ABSTRACT

Adult *C. elegans* germline stem cells (GSCs) and mouse embryonic stem cells (mESCs) exhibit a non-canonical cell cycle structure with an abbreviated G1 phase and phase-independent expression of Cdk2 and Cyclin E. Mechanisms that promote the abbreviated cell cycle remain unknown, as do the consequences of not maintaining an abbreviated cell cycle in these tissues. In GSCs, we discovered that loss of *gsk-3* results in reduced GSC proliferation without changes in differentiation or responsiveness to GLP-1/Notch signaling. We find that DPL-1 transcriptional activity inhibits CDK-2 mRNA accumulation in GSCs, which leads to slower S phase entry and progression. Inhibition of *dpl-1* or transgenic expression of CDK-2 via a heterologous germline promoter rescues the S phase entry and progression defects of the *gsk-3* mutants, demonstrating that transcriptional regulation rather than post-translational control of CDK-2 establishes the abbreviated cell cycle structure in GSCs. This highlights an inhibitory cascade wherein GSK-3 inhibits DPL-1 and DPL-1 inhibits *cdk-2* transcription. Constitutive GSK-3 activity through this cascade maintains an abbreviated cell cycle structure to permit the efficient proliferation of GSCs necessary for continuous tissue output.
INTRODUCTION

Stem cells sustain tissue development and growth, and maintain tissue homeostasis by balancing the rates of proliferation and differentiation (Morrison and Spradling, 2008). *Caenorhabditis elegans* adult germline stem and progenitor cells (stem cells and their proliferative progeny, henceforth referred to collectively as GSCs) support gamete production and sustain germline development by maintaining this balance (Hansen and Schedl, 2013). GSCs exhibit two intrinsic properties that help sustain the growth of the germline: they undergo constant self-renewal in a Notch signaling pathway-dependent manner (Austin and Kimble, 1987; Berry et al., 1997), and they display a cell cycle structure with a very short G1 phase (henceforth referred to as an “abbreviated” cell cycle) (Fox et al., 2011). Mechanisms that promote the abbreviated cell cycle remain unknown, as do the consequences of not maintaining an abbreviated cell cycle in this tissue.

While GSCs represent an adult stem cell population, they are more similar to mouse embryonic stem cells (mESCs) with respect to cell cycle structure and regulation (Fox et al., 2011; White and Dalton, 2005). This supports the idea that the cell cycle characteristics of stem cells reflect the demands of the tissues they support rather than the stage of the organism from which the cells are derived. For example, the adult mammalian satellite cells (muscle stem cells) and bulge stem cells (hair follicle stem cells) are required for tissue regeneration and thus remain quiescent (G0) for most of their adult life. However, when their host tissue is stressed or damaged, they re-enter the cell cycle and undergo G1, S, G2, and M phases to repopulate the tissue; after which they re-enter quiescence, effectively meeting the demands of the tissue (Cotsarelis et al., 1990; Schultz, 1974, 1985; Snow, 1977). In contrast, early embryonic cells from *C. elegans, D. melanogaster* and *X. laevis* require rapid expansion, and thus abbreviate both gap (G1 and G2) phases which, when
coupled with rapid DNA replication, results in an exceedingly fast cell cycle which is necessary to generate the requisite number of cells for the onset of early gastrulation events (Edgar and McGhee, 1988; Graham, 1966a, b; Kermi et al., 2017; Takada and Cha, 2011). While mESCs also display rapid expansion in culture, they maintain a G2 phase and S-phase length similar to that of differentiated mouse somatic cells (Stead et al., 2002). Instead, their rapid expansion is due to an abbreviated G1 phase allowing these cells to cycle rapidly while protecting their DNA through the intra-S and G2 checkpoints (Chuykin et al., 2008; Stead et al., 2002; White and Dalton, 2005). Likewise, GSCs abbreviate the G1 phase (Fox et al., 2011) while retaining the G2 checkpoints (Garcia-Muse and Boulton, 2005; Seidel and Kimble, 2015). Since the C. elegans germline continuously produces oocytes while sperm is available (Jaramillo-Lambert et al., 2007), the GSCs likely meet the constant demand for gametes by shortening their G1 and abbreviating their cell cycle to increase the rate of proliferation. This abbreviated cell cycle is seemingly regulated differently than the canonical somatic cell cycle.

Unlike somatic cells where the G1 phase is marked by oscillating cyclin expression (Aleem et al., 2005; Guevara et al., 1999), G1 in the abbreviated cell cycle structure of both GSCs and mESCs is seemingly absent with stem cells displaying a phase independent expression of the G1/S regulators CDK2 and Cyclin E (Fox et al., 2011; White and Dalton, 2005). However, a mechanism for sustaining an abbreviated cell cycle structure with an abbreviated G1 remains unresolved. Here, we describe the consequences of abnormal S phase entry and progression and the mechanism through which constitutive GSK-3 activity (Glycogen synthase kinase 3 beta or GSK3β in mammals) promotes G1/S progression in GSCs to maintain constant growth in the tissue.
GSK3β functions in several signaling pathways, such as the insulin, TOR and Wnt pathways, to regulate proliferation, differentiation, and apoptosis (Bouskila et al., 2008; Campbell et al., 2012; McManus et al., 2005; Parisi et al., 2011), in addition to its original role of inhibiting glycogen synthase (Larner et al., 1968; Rylatt et al., 1980). While GSK3β is known to promote differentiation of mESCs via the inhibition of pluripotency factors through beta-catenin (Ying et al., 2008), we describe here for the first time its role in promoting a unique cell cycle structure shared by C. elegans GSCs, mESCs and possibly other tissues. We discovered that GSCs maintain an abbreviated G1 due to continuously high Cdk2 (C. elegans CDK-2) mRNA expression, such that in wild type worms GSK-3 is constitutively active, CDK-2 is constantly high and GSCs are continuously poised to enter and progress through the S phase. We show that GSK-3 inhibits DPL-1 transcription factor, which in turn inhibits cdk-2 mRNA accumulation, thus, under constitutively high activity of GSK-3, DPL-1 is inhibited and CDK-2 levels are persistently high, revealing a mechanism driving the unique cell cycle structure of GSCs. Loss of gsk-3 results in a reduction of CDK-2 in GSCs, leading to slower entry and progression of S phase and reducing tissue output. Thus, GSK-3 promotes an abbreviated cell cycle structure to permit the continuous proliferation of GSCs for gamete production.

RESULTS

GSK-3 promotes GSC proliferation in a germline autonomous and kinase-dependent manner during larval and adult development

The adult wild-type C. elegans germline harbors a population of ~200-250 GSCs (stem cells and their proliferative progeny, henceforth referred to collectively as GSCs), which are maintained by Notch signaling (Berry et al., 1997; Crittenden et al., 2003; Fox and Schedl, 2015; Fox et al., 2011;
Kimble and Crittenden, 2005) and can be assayed by labeling with REC-8 (stem cell specific marker (Hansen et al., 2004)), or by absence of HIM-3 (a meiotic specific marker (Zetka et al., 1999)) (Figure 1A-B, S1). As the GSCs move away from the distal tip cell (DTC), (Figure 1A, S1) they differentiate and enter meiosis, at ~20-22 cell diameters, in both L4 and adult germlines. Characterization of two strong loss-of-function alleles of gsk-3 (nr2047 and tm2223) (Supplementary Information), revealed that adult gsk-3 mutant germlines contained ~90 GSCs, compared to the ~200-250 found in wild-type (Figure 1B, 1C, S1). However, the GSCs entered meiosis ~20-22 cell rows away from the DTC in the gsk-3 mutants, similar to wild-type (Figure 1B, 1D, S1). To determine whether the defect in GSC accumulation in gsk-3 mutant animals occurred throughout development, or if it occurred only in adults, we analyzed earlier developmental time-points. We found that the number of germ cells in the two gsk-3 mutant alleles was significantly reduced as early as L3, with ~14 germ cells compared to the wild-type ~34 germ cells (Table S1). At the L4 stage and into adulthood, the GSCs in gsk-3 mutants remained at ~90 (Figure 1C). This failure to expand the progenitor population suggested that the germline output would also be reduced. To determine whether the lower number of GSCs in the gsk-3 mutant resulted in a decrease of tissue output, we assayed the number of oocytes produced in the mutant by counting the number of oocytes marked by RME-2 labeling, a yolk receptor. We found that gsk-3 mutants contain fewer developing oocytes throughout adulthood when compared to wild-type (Figure 1E, F). Taken together, the GSCs fail to expand in number in the gsk-3 mutants from early larval stages on into adulthood, possibly leading to a decrease in germ cell output.
To test whether the GSC defect was due to a germline autonomous function of \textit{gsk-3}, we generated two germline-specific GFP::GSK-3 transgenes, driven by either the \textit{pie-1} or the \textit{mex-5} promoter, referred to here as GFP::GSK-3 (WT) (Supplementary Information, S2A-S2B). Expression of either germline specific GFP::GSK-3 (WT) transgene rescued the GSC defect in \textit{gsk-3} mutants (Figure 1G-1I), suggesting that the GSC defect is due to an autonomous function of GSK-3 in the germline.

To determine whether GSK-3 kinase activity was necessary for GSC proliferation, we generated a kinase dead transgene by mutating the kinase core residues (K65, E77, D161 and D180) to Alanine (Doble and Woodgett, 2003) (Supplementary Information). The resulting transgene, GFP::GSK-3 (K65A, E77A, D161A and D180A), referred to here as GFP::GSK-3 (KD), is driven by the \textit{mex-5} germline specific promoter and is inserted at the identical chromosomal location as GFP::GSK-3 (WT) using MosSci integration system on Chromosome II (Supplementary Information). While GFP::GSK-3 (WT) and GFP::GSK-3 (KD) are expressed at the same level, and in similar cellular compartments in the progenitor zone (Figure S2C), we find that, unlike the wild-type transgene, GFP::GSK-3 (KD) did not rescue the GSC defects (Figure 1H-J). These data together demonstrate that \textit{gsk-3} functions germline-autonomously in a kinase-dependent manner to promote GSC proliferation in the germline.
We next addressed the question of whether the defects in GSCs occur as a consequence of increased differentiation, loss of self-renewal, or a cell cycle defect. The GSC defect is likely not due to increased differentiation, as meiotic entry was maintained at 20-22 cell diameters from the DTC in both wild-type and gsk-3 mutants (Figure 1D, 1I and S1). Therefore, we next investigated whether the gsk-3 mutant GSCs remained responsive to Notch signaling for self-renewal.

**gsk-3 mutant GSCs remain responsive to Notch signaling**

To determine whether the gsk-3 mutant GSCs respond to Notch signaling, we assayed gsk-3 mutant GSCs in conditions with decreased or increased Notch receptor (GLP-1) activity by utilizing the *glp-1* temperature sensitive alleles *bn18ts* (reduction-of-function) and *ar202gf* (gain-of-function). To determine the effect of loss of *glp-1* signaling on gsk-3 mutant GSC’s we used *glp-1(bn18ts)* with *gsk-3(nr2047)*. At the permissive temperature of 15°C, *glp-1(bn18ts)* as well as *gsk-3(nr2047);glp-1(bn18ts)* germlines produce GSCs as well as meiotic cells, as assayed by labeling with HIM-3 to mark the meiotic cells (Figure 2A, 2C, 2E, 2G). However, shifting *glp-1(bn18ts)* or *gsk-3(nr2047);glp-1(bn18ts)* mutants as embryos to the restrictive temperature of 25°C results in loss of the GSC population with only sperm being produced in 100% of adult germlines (Figure 2F, 2H). Wild-type or *gsk-3(nr2047)* mutants at 25°C are indistinguishable from their 15°C counterparts. These data suggest that the gsk-3 mutant GSCs require *glp-1* activity to self-renew.
To determine the impact of increased glp-1 Notch signaling on gsk-3 mutant GSCs, we assayed glp-1(ar202gf) with gsk-3(nr2047). Adult glp-1(ar202gf) mutant and gsk-3(nr2047);glp-1(ar202gf) mutant germlines at the permissive temperature of 15°C have GSCs and meiotic cells (Figure 2I and 2K). In contrast, shifting glp-1(ar202gf) or gsk-3(nr2047);glp-1(ar202gf) mutants as embryos to the restrictive temperature of 25°C results in adult tumorous germlines (Figure 2J, 2L). Although, the gsk-3(nr2047);glp-1(ar202gf) mutant tumors appear “skinnier” (with respect to the size of the gonads) relative to glp-1(ar202gf) single mutant tumors (Figure 2J, 2L). These data indicate that glp-1 activity is sufficient to drive self-renewal at the expense of differentiation in gsk-3 mutants. Together, these data demonstrate that gsk-3 mutant GSCs remain responsive to glp-1 signaling. As neither differentiation, nor self-renewal was affected in gsk-3 mutant GSCs, we investigated their cell cycle parameters.

gsk-3 mutant GSCs enter and progress through S phase inefficiently

To determine the cell cycle parameters of the gsk-3 mutant GSCs, we analyzed the cell cycle phases via immunofluorescence analysis of phospho-histone H3 (pH3) labeling to mark M phase and EdU incorporation to mark S phase (Materials and Methods). GSCs from both gsk-3 mutant and wild-type adult and mid-L4 germlines contained pH3 positive cells (Figure 3A-3C). The number of pH3 positive nuclei in wild-type gonads ranged from 4-13 and gsk-3 mutant gonads ranged from 0-9 with no statistically significant difference in the average mitotic index of gsk-3 mutant GSCs compared to wild-type (Figure 3C, S1). These data indicate that GSCs in gsk-3 mutants enter productive M phase. In contrast, S phase appeared markedly altered in gsk-3 mutants. While 100% of wild-type germlines incorporate EdU and display an S phase index of ~55% upon 30 min of feeding the EdU bacteria (Figure 3A, S3), over 90% of the gsk-3 mutant
germlines failed to incorporate detectable EdU as either adults (Figure 3A) or mid-L4s (Figure 3B). Of those germlines that did incorporate EdU, the levels of EdU were very low per germ cell, based on the label intensity. The S phase index of those germlines with low EdU incorporating cells was ~6% (Figure 3D). The rate of detectable EdU incorporation did not change in the gsk-3 mutant even upon longer EdU feeding of 1 hour, 3 hours or 5 hours (Figures S3). Because, the standard method in the field uses feeding of EdU bacteria as a tool for EdU incorporation, it is possible that the feeding rate of gsk-3 mutants affected this analysis. To investigate this possibility, we used eat-2 mutants, which have a lower feeding rate, together with gsk-3 mutants to assay whether feeding rate affects EdU incorporation. While eat-2 mutants have a reduced pharyngeal pumping rate of only ~77 pumps/min compared to wild-type rate of ~274 pumps per minute, gsk-3 mutant displayed a rate of ~204 pumps/min (Figure S4). However, despite a 72% reduction in pharyngeal pumping rate in eat-2 mutant animals, 100% of the eat-2 mutant germlines incorporated the robust EdU label on “feeding” (Figure S4), suggesting that feeding rate is not affecting EdU incorporation in the gsk-3 mutant. In addition, we developed an assay that did not involve eating, and instead soaked the wild-type and mutant gsk-3 animals in an EdU solution for 10 minutes (Materials and Methods). As with feeding of EdU, 100% of wild-type animals displayed robust EdU incorporation with an S phase index of ~60% while gsk-3 mutant germlines continued to display low / no EdU incorporation. We use the soaking method of EdU detection for the remainder of this manuscript (Figures 4-6, Supplement).
The failure of a majority of *gsk-3* mutant germlines to incorporate detectable EdU suggests that GSCs in *gsk-3* mutants incorporate fewer molecules of EdU per GSC compared to wild-type GSCs, possibly because they are either entering or progressing through S phase more slowly. Additionally, some of the GSCs in the *gsk-3* mutant germlines displayed variable cell size, a few of which are reminiscent of GSC arrest (Figure S5). These data suggest that the cell cycle defects in *gsk-3* mutant animals are a combination of slow entry, and progression through the S phase, with some GSCs that may be arrested in G1. However, because the *gsk-3* mutant germline continued to produce sperm (during L4, Figure 2), oocytes (during adulthood, Figure 2) and embryos (Shirayama et al., 2006) we hypothesize that at least some of the *gsk-3* mutant GSCs continue to progress through the cell cycle.

To determine whether the GSCs in *gsk-3* mutant animals were arrested / delayed in G1, leading to inefficient S phase entry, we assayed for subcellular GFP::MCM-3 (*gtIs64*) localization (Sonneville et al., 2012). MCM3 is a component of the pre-replication complex that accumulates in the nucleus in early-mid G1 in vertebrate cultured cells, and is phosphorylated by CDK2 and re-localized to the cytoplasm in late G1 or early S to prevent re-replication (Li et al., 2011); thus, nuclear localization of MCM3 indicates nuclei in G1 (Blow, 1993; Chong and Blow, 1996). To test whether GFP::MCM-3 had cellular localization dynamics in *C. elegans* GSCs similar to vertebrate cultured cells, we depleted *cdk-2* in GFP::MCM-3 animals and determined its localization. As previously described (Fox et al., 2011), depletion of *cdk-2* caused G1 cell cycle arrest wherein all cells were negative for EdU (S phase) and pH3 (M phase) (Figure S6). GFP::MCM-3 localized to the GSC nuclei upon depletion of *cdk-2* (Figure 3F, F’) indicating that
GFP::MCM-3 changed cellular localization in GSCs in response to loss of *cdk-2*, and by extension G1 arrest, as would be predicted from the vertebrate system. Additionally, GFP::MCM-3 was excluded from meiotic germ cell nuclei in animals treated with *cdk-2* RNAi (Figure 3F, F”, S6), indicating that the reporter changed localization based on the phase of the mitotic cell cycle. Consistent with previous reports that the GSCs display a very short (seemingly absent) G1, wild-type germlines exhibit cytoplasmic GFP::MCM-3 in both GSCs and meiotic cells (Figure 3E, E’, E”). In contrast, GFP::MCM-3 was nuclear in *gsk-3* mutant GSCs (Figure 3G, G’) in 56% ± 3% of the cells (calculated from over 20 germlines), and was cytoplasmic when the cells entered meiosis (Figure 3G, G”). Taken together, these data suggest that *gsk-3* mutant GSCs are either arrested in G1 phase of the cell cycle or lingering in G1 for a longer period relative to wild-type due to inefficient entry into S phase. Additionally, increased nuclear GFP::MCM-3 suggests that CDK-2 activity may be affected in *gsk-3* mutants, which could result in the observed proliferation defects.

**GSK-3 promotes *cdk-2* transcription to regulate rapid S phase entry and progression**

CDK-2 and cyclin E (CYE-1) are expressed and active in all wild-type GSCs (Fox et al., 2011). Thus, to determine whether CDK-2 function was reduced in *gsk-3* mutant germlines we tested the expression of CYE-1 and CDK-2. We found that the protein expression pattern of CYE-1 in *gsk-3* mutant germlines was similar to wild-type even though the levels appeared slightly lower in *gsk-3* mutant germlines (not significant) (Figure S7). Since no CDK-2 antibody exists, we obtained a transgenic YFP::CDK-2 (Cowan and Hyman, 2006) driven by the germline specific *pie-1* promoter and expressed it in the *gsk-3* mutant background to assess its localization. Surprisingly, the transgenic expression of YFP::CDK-2 rescued GSC numbers (Figure 4A-4E) and S phase onset
and progression in both L4 (Figure S8) and adult germlines in *gsk-3* mutants (Figure 4A-4D, 4F). Additionally, CDK-2 is expressed throughout the GSCs in both wild-type and *gsk-3* mutant cells in this context (Figure 4G-4H). Because *pie-1* driven YFP::CDK-2 rescued the *gsk-3* mutant GSC defects and localized to the *gsk-3* mutant GSCs in a manner similar to wild-type, it suggested that the defects in *gsk-3* mutants are driven by abnormal CDK-2 function and that the relevant regulation of CDK-2 via GSK-3 was not through post-translational mechanisms, such as regulating CDK-2 protein degradation or activity through phosphorylation. Together, these data demonstrate that *gsk-3* mutant GSCs enter and progress through S phase abnormally due to lower CDK-2 accumulation, likely at the transcriptional level.

To determine whether GSK-3 regulates CDK-2 levels transcriptionally in the GSCs, we assayed CDK-2 mRNA in dissected germlines from *gsk-3* and wild-type animals, using qRT-PCR (Materials and Methods). CDK-2 mRNA levels were lower by 10-fold in *gsk-3* mutant germlines compared to wild-type (Figure 4I), and restored in *gsk-3* mutant animals carrying the transgenic YFP::CDK-2 (Figure 4I). To determine whether the lower transcript level of CDK-2 in *gsk-3* mutant germlines was specific to the progenitor zone, we performed single molecule hairpin chain reaction FISH (Shah et al., 2016) using CDK-2 mRNA probes in wild-type and *gsk-3* mutant germlines. CDK-2 mRNA accumulates throughout the progenitor zone, predominantly in the cytoplasm, and is specific to *cdk-2* (Figure 4J, 4L). However, in *gsk-3* mutant germlines *cdk-2* mRNA levels are much lower relative to wild-type germlines (Figure 4K). These data demonstrate that GSK-3 promotes *cdk-2* mRNA levels in the wild-type progenitor zone.
To directly assess whether GSK-3 regulates the transcription of CDK-2, we designed a transcriptional reporter for CDK-2. Perusal of the CDK-2 gene structure on Wormbase (www.wormbase.org, ver WS258), however revealed that intron 1 (Figure 5A), rather than the promoter annotated by Wormbase, contained multiple transcription factor and RNA polymerase II binding sites as well as an SL1 splice site (Wormbase WS258) (Figure S9A). These observations suggested that intron 1, rather than the promoter, may drive CDK-2 expression. Thus, we generated two distinct transgenes: one with the Wormbase predicted 2Kb promoter driving GFP (cdk-2[Pr::GFP]), and one containing the intron 1 driving GFP (cdk-2[In1]::GFP) (Figure 5A, Supplementary Information). As hypothesized, we observed that cdk-2[Pr::GFP] did not express in the germline, suggesting that the predicted promoter does not drive cdk-2 expression (vizSi34, data not shown, Wormbase WS258). Instead, cdk-2[In1]::GFP was expressed throughout the germline (Figure 5B), suggesting that intron 1 drives cdk-2 expression in the germline. In comparison to wild-type, expression of cdk-2[In1]::GFP in gsk-3 mutants resulted in lower GFP accumulation of the reporter in the distal region, but not in oocytes (Figure 5C, 5D). These data demonstrate that GSK-3 regulates CDK-2 transcription in the GSCs.

DPL-1 represses CDK-2 expression and S phase entry and progression in GSCs and is inhibited by GSK-3

To determine the factors that regulate CDK-2 transcription downstream of GSK-3, we perused the Chromatin Immunoprecipitation (ChIP) data for the CDK-2 intron 1 using Wormbase and ModEncode (Elsner and Mak, 2011; Muers, 2011). This analysis identified several transcription factors that bind to Intron 1 of CDK-2, most notably LIN-35, EFL-1 and DPL-1 (Figure S9A), all
of which promote S phase entry in vertebrate systems (Almasan et al., 1995; Muller et al., 1997). Because loss of lin-35, efl-1 or dpl-1 individually does not inhibit S phase in C. elegans (Ceol and Horvitz, 2001; Chi and Reinke, 2006, 2009), it suggested to us that they do not promote S phase in C. elegans. Thus, we wondered whether each of these transcription factors represses rather than promotes S phase in the context of GSK-3. To determine whether these factors regulate cdk-2 mRNA and thus S phase entry and progression downstream to gsk-3, we assayed them via RNAi-mediated depletion in the gsk-3 background. Depletion of dpl-1 in L4 animals from wild-type or gsk-3 heterozygous animals resulted in strong embryonic lethality in F1 progeny, as did the double mutants between dpl-1 and gsk-3 (not shown). Thus, we depleted dpl-1 starting at L4 in wild-type and gsk-3 mutant animals for 48 hours and assayed for EdU incorporation in the germlines using the EdU soaking method (Figure S9B). Wild-type germlines from control (luciferase) RNAi and dpl-1 RNAi exhibited normal EdU incorporation with S phase indices of ~55% and ~67% respectively (Figure 6A, 6C, S9C), as well as endomitotic oocytes in the proximal germlines, as described previously (Chi and Reinke, 2009). dpl-1 RNAi in gsk-3 mutant animals restored EdU incorporation in all germlines with an S phase index of ~35% (Figure 6B, 6D, S9C), partially rescuing the S phase progression defect. These data suggest that gsk-3 normally inhibits dpl-1 in the GSCs to promote S phase progression likely through cdk-2 transcriptional regulation.

To determine whether dpl-1 regulated cdk-2 mRNA levels, we performed FISH analysis on gsk-3(nr2047);dpl-1(RNAi) dissected germlines. We found that the cdk-2 mRNA was restored in the gsk-3(nr2047);dpl-1(RNAi) double mutant germlines and was equivalent to dpl-1 RNAi alone (Figure 6E-6H). In summary, these data demonstrate that GSK-3 inhibits DPL-1 to maintain
persistent high levels (and thus activity) of CDK-2 mRNA expression in wild-type GSCs, resulting in rapid S phase entry and progression.

DISCUSSION

We report the mechanism through which *C. elegans* germline stem cells maintain their unique cell cycle structure with an abbreviated G1 phase. We show that consistent high and phase independent expression of *cdk-2* mRNA is regulated by constitutive GSK-3 activity in the GSCs such that when the cells reach the end of mitotic M phase they can transition into S phase with minimal time spent in G1. Abbreviating the G1 phase of the cell cycle allows the cells to proliferate rapidly to meet the tissue demands imposed by continuous production of embryos. Our results also highlight that CDK-2 transcriptional control rather than post-translational control plays a central role in cell cycle phase transition from mitotic G1 to S in the *C. elegans* GSCs, thus uncovering a novel layer of cell cycle regulation.

We identified a role for GSK-3 in maintaining the abbreviated cell cycle structure via transcriptionally regulating *cdk-2* mRNA through inhibition of DPL-1. Loss of *gsk-3* function results in GSCs that progress through the cell cycle inefficiently, due to a longer time spent in G1, either because of slow progression or arrest in G1, coupled with slow replication and transition through the S phase. These defects result in a germline with fewer germ cells that is also shorter in length (Figure 2), thus a “skinny” germline, with fewer embryos produced. GSK-3 was originally described as a key metabolic regulator. However, in the context of the GSCs, our data suggests that GSK-3 is constitutively active and not regulated. In vertebrates, GSK3 is inactivated by phosphorylation at Serine 9 downstream to Insulin signaling (Sutherland et al., 1993). However,
we find that in *C. elegans* GSK-3 Serine 9 is absent suggesting that GSK-3 may be refractory to inactivation by Insulin signaling. In addition, we find that *gsk-3* mutant GSCs respond to nutritional deprivation similar to the wild-type mutant GSCs and arrest in G2 (Figure S10, (Seidel and Kimble, 2015)). These observations suggest that the GSK-3 does not respond to the nutritional cues, and that the cell cycle arrest in *gsk-3* mutant GSCs is not at G2. In sum, our data suggests that GSK-3 may not be modified to regulate the cell cycle, but rather is constitutively high to ensure the rapid progression through G1 phase of the cell cycle, both under conditions of constitutive growth and when transitioning from poor environmental conditions to optimal ones. It is likely that the regulation of cell cycle in germline stem cells occurs predominantly at G2, such that the cell cycle structure is set up to maintain an abbreviated G1 under all conditions, and constitutive GSK-3 activity promotes CDK-2/Cyclin E activity to facilitate this process (Figure 7).

**How does constitutively high expression of CDK-2 regulate G1/S switch?**

We find that the *cdk-2* mRNA maintains a constitutively high level of expression in the GSCs, which is necessary for the abbreviated cell cycle structure. The expression of CDK-2 also reflects its activity, as shown previously by phospho-CDC-6 expression, and functional analysis (Fox et al., 2011) (this study). Using the nuclear to cytoplasmic shuttling of MCM-3 as a dynamic read out of CDK-2 function, we show that MCM-3 is nuclear when CDK-2 function is lower and cytoplasmic when CDK-2 function (or expression) is high. Interestingly, in *gsk-3* mutant GSCs, MCM-3 is nuclear in about 56% of the cells, the levels of *cdk-2* mRNA are significantly reduced, and the cells incorporate EdU very inefficiently. Further, over-expression of CDK-2 in the *gsk-3* mutants completely rescues the GSC defects. Together, these data suggest that in GSCs, the cell cycle at the G1/S boundary is largely regulated by the accumulation of CDK-2. Consistent with
this is the observation that depletion (via RNAi) and reduction of cdk-2 mRNA result in two distinct phenotypes: cell cycle arrest in the former (Fox et al., 2011) and slow S phase entry and progression in the latter (this study).

That CDK-2 expression levels regulate the G1/S switch may be unique to cells that have a short G1. To put this in the context of canonical mammalian cell cycle progression which is regulated via low Cdk2 activity in G1 to enable pre-replication complexes to assemble at origins (Blow and Hodgson, 2002), we propose the following model. Canonically, inactive Cdk2 enables the loading of the pre-replication complex into the nucleus at the end of G1, and active Cdk2 then initiates S phase, both of which are regulated through post-translational mechanisms such as phosphorylation. In the context of GSCs, it is likely that different thresholds form distinct complexes of CDK-2 that mediate its differing roles in G1 and S. For example, it is likely that the pre-replication complex can form at a lower threshold of CDK-2 (mimicking an ‘inactive” pool of Cdk2) but not at a threshold of CDK-2 that drives entry and progression through S phase. Observations from mESCs support this model. In mESCs despite continuous high expression of Cdk2 and cyclin E, a subset of Cdk2 complexes are in fact “inactivated” at G1, allowing pre-replication complexes to assemble transiently and allow S phase entry (Ohtsuka and Dalton, 2008). Additionally, these complexes are thought to be undetectable above the elevated Cdk2 expression (Ohtsuka and Dalton, 2008). Therefore, one possible hypothesis is that much like in mouse ES cells, different complexes of CDK-2 exist, and some of these are inactivated at late G1 (despite the high expression of CDK-2) in GSCs coupled with a very transient loading of the pre-replication complex. This notion of a very transient loading of the licensing complex may account for the inability to visualize the nuclear pre-replication complex in wild-type GSCs. Together, this leads to the model that a pool
of CDK-2 is inactive, which enables loading of the pre-replication complex, but that high CDK-2/CYE-1 levels throughout GSCs result in the loading being transient thus facilitating an accelerated entry into S phase, effectively coupling the high expression of CDK-2 with the G1/S switch, and abbreviation of G1.

**DPL-1 as a transcriptional repressor of CDK-2 to regulate G1 and S phase progression.**

DP1/E2F proteins are canonical G1/S phase regulators across multiple systems, where they promote G1/S phase progression through activation of S phase target genes. However, in *C. elegans* loss of dpl-1 had not previously revealed any loss of G1/S phase in the proliferative cells either in the soma (Ceol and Horvitz, 2001; Chi and Reinke, 2006; Reddien et al., 2007) or the germline (this study). These data suggested that DP1/E2F may not function canonically to promote proliferative S phase in *C. elegans*. Instead, in this study, we discovered that genetically DPL-1 repressed cdk-2 mRNA accumulation, and together with the modEncode data, we infer that DPL-1 by binding to the CDK-2 intron 1 represses CDK-2 mRNA transcription and thus S phase progression. Together with our observation that DPL-1 functions downstream to GSK-3 to control CDK-2 mRNA, we propose that under normal conditions, DPL-1 is inactivated by continuous expression of GSK-3, resulting in continuous high levels of CDK-2. EFL-1, the partner of DPL-1, is closest in homology to E2F4 family of E2F transcription factors (Smith et al., 1996), and in vertebrates E2F4 family members have been implicated in repression of S phase target genes rather than activation (Dominguez-Brauer et al., 2009; Pilkinton et al., 2007; Popov et al., 2005). Thus, it is possible, that in *C. elegans*, the DPL-1/EFL-1 complex function more like the DP1/E2F4 complex in vertebrates and repress G1/S transition. Together, these data reveal a novel mode of cell cycle regulation via transcriptional control of cyclin dependent kinases.
MATERIALS AND METHODS

C. elegans strains and culture

Standard culture conditions were used with maintenance at 20°C unless otherwise noted (Brenner, 1974). Temperature sensitive strains containing glp-1(bn18ts) or glp-1(ar202gf) were maintained at 15°C and were shifted to 25°C as embryos along with controls for experiments. Complete strain list can be found in the supplemental materials and methods.

Larval germ cell counts

Germ cell counts were performed using whole mount visualization of zuIs252[PGL-1::mRFP] and DAPI. Counts were performed at L1, early L2, early L3 and early L4 in both wild-type and two gsk-3 alleles (tm2223 and nr2047).

EdU labeling

EdU feeding experiments were performed as described previously (Fox et al., 2011). Soaking EdU experiments were performed with worms grown on nematode growth medium (NGM) plates with E. coli OP50 bacteria as required, washed thrice with M9T (M9 buffer, 0.1% Tween 20), and transferred to a flat-bottom 48-well plate, followed by incubation with 200 µM of EdU solution for 10 minutes at room temperature in the dark. The animals were then dissected and germlines processed using the Click-iT® Plus EdU Alexa Fluor® 594 Imaging Kit (ThermoFisher Scientific, cat# C10639) per the manufacturer’s recommendations, with a minor modification. Instead of the copper protectant provided with the kit (Component E), 2 mM CuSO₄ was used.
**Antibodies**

The following antibodies were used: Anti-HIM-3 (Sdix); Anti-REC-8 (Novus); Anti-phospho-Histone H3 (Ser10) (Millipore); Anti-GFP (Novus Biologicals); Anti-CYE-1 was obtained from Dr. Edward T. Kipreos (University of Georgia); Gp-anti-Lamin was obtained from Dr. Kelly Liu (Cornell University); Anti-beta Tubulin (Sigma); Donkey anti-mouse Alexa 594, goat anti-mouse Alexa Cy5, goat anti-mouse Alexa 488, goat anti-rabbit Alexa 488, goat anti-guinea pig Alexa 594, Anti-Rabbit HRP, and Anti-Mouse HRP secondary antibodies (Molecular Probes).

**Germline dissection and staining**

All animals were dissected as adults at 24 hours past L4 stage of development, unless otherwise mentioned. Germlines were dissected and stained as described previously (Arur et al., 2011; Arur et al., 2009; Drake et al., 2014; Suen et al., 2013).

**PLP mounting for GFP visualization**

To assay the transcriptional reporter GFP worms, \textit{vizSi32[Intron cdk-2::NLS::GFP::tbb-2 3’UTR]}, \textit{wt} and \textit{gsk-3(nr2047)} were dissected in the same dish 24 hours past L4 in PBST and fixed for 3 min using 4% PLP (Periodate/Lysine/Paraformaldehyde) (Hixson et al., 1981) with 4μg/ml DAPI. After washing the fixed germlines three times, germlines were mounted using 2% agarose pad and observed immediately. The pictures were taken on the same slide with identical exposure and gain for the GFP channel.
Image acquisition and processing on Zeiss Axio imager

Each image was captured with overlapping cell (Arur et al., 2009) boundaries at 40x or 63x objectives, to make the montage. The focal plane was maintained throughout the experiment. All images were taken on a Zeiss Axio Imager upright microscope by using AxioVs40 V4.8.2.0 SP1 micro-imaging software and a Zeiss Axio MRm camera. The montages were then assembled in Adobe Photoshop CS5.1 and processed identically.

Quantitative real-time -PCR (qRT-PCR)

Total RNA was isolated from at least 100 dissected germlines using the miRNeasy Mini Kit (Qiagen). cDNA was synthesized from 500 ng of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). cDNA was amplified for qRT-PCR using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer’s instructions. Amplified cDNA was monitored after each cycle and measured ΔCt in the CFX96 Real time system (Bio-Rad Laboratories). The relative expression rate was determined using the ΔCt method as described in the manufacturer’s instructions (Bio-Rad Laboratories). Average expression of the reference gene act-1 was used to control for template levels.

RNA interference (RNAi) analysis

RNAi was performed by feeding as described previously (Arur et al., 2009). cdk-2, cye-1, dpl-1, efl-1 and lin-35 RNAi clones (Vidal ORFeome Library, or Ahringer RNAi library) were sequence verified and grown overnight on solid LB agar plates containing 100μg / ml of Ampicillin and 50μg / ml of Tetracycline at 37°C. Single colonies were then inoculated in LB liquid cultures
containing 100μg/ml of Ampicillin and 50μg/ml of Tetracycline and grown to necessary densities as described previously (Arur et al., 2009). The cultures were then seeded onto the standard NGM agar plates supplemented with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and containing 100μg/ml of Ampicillin and 50μg/ml of Tetracycline. For P0 RNAi, L4 stage wild-type or gsk-3 homozygous mutants were transferred to RNAi plates, and dissected for analysis after 48 hrs. For F1 RNAi, L4 stages of wild-type or gsk-3 heterozygous animals were allowed to lay progeny on the RNAi plates for 24 hours, and transferred to a fresh RNAi plate every 24-hours for an additional three days. Wild-type and gsk-3 homozygous F1 progeny from these plates were then synchronized at mid-L4 stage and dissected for analysis at 48 hours past mid-L4.

**Western Blot analysis**

Wild-type (N2), and GFP::GSK-3 L4 hermaphrodites were hand-picked (250 for each lane), grown for 24 hours and then harvested for western analysis as previously described (Arur et al., 2011; Arur et al., 2009). The extracts were resolved on 10% SDS-PAGE, transferred to PVDF membrane, and probed with antibodies to GFP (Novus Biologicals) and beta tubulin (1:1000). Western blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL), on Kodak BioMax MS films.

**Hairpin Chain Reaction based in situ mRNA hybridization**

cdk-2 mRNA FISH was performed using hairpin chain reaction as described (Huang et al., 2016; Wei et al., 2016; Xuan and Hsing, 2014) except that the analysis in the present study was conducted on dissected germlines that were fixed in 3% Paraformaldehyde, 0.25% glutaraldehyde solution at
room temperature for 2 hours. The probes were obtained from Molecular Instruments. Inc (Berkley, CA) and manufacturer’s instructions followed.

**Confocal analysis of nuclei sizes**

Wild-type and *gsk-3(nr2047)* germlines were dissected and stained with DAPI and Gp-anti-Lamin (1:200). Germlines were mounted in Vectashield and let settle overnight at 4°C in dark. Z-stack images of the entire mitotic zone were acquired using a Nikon A1 laser scanning confocal microscope with a 60x oil objective (z step=0.5μm). Images were deconvolved using AutoQuant X3 (20 iteration) and cell sizes were analyzed using ImageJ. The middle planes of the nuclei were selected and nuclei sizes were quantified by measuring the circumference of each nucleus based on Lamin staining. A total of 471 (from 5 animals) and 404 nuclei (from 7 animals) were analyzed for wild-type and *gsk-3(nr2047)*, respectively.

**Measuring progenitor zone length, M phase and S phase indices**

Progenitor zone length was measured spatially as the distance from the distal tip until the onset of the first HIM-3 positive nucleus. Each nucleus was visualized by DAPI. M phase index was calculated as percent of number of pH3 positive cells in the progenitor zone over the total number of cells in the progenitor zone. S-phase index was calculated as a percent of EdU positive cells in the progenitor zone out of the total number of cells in the progenitor zone. The criterion used for distinguishing the progenitors from meiotic cells was the HIM-3 boundary.
AUTHOR CONTRIBUTIONS
TF, HJJ, and KAT designed the study, performed experiments, analyzed data and wrote the manuscript. First author listing is alphabetical. SYC performed experiments and analyzed data. SA designed the study, analyzed data and wrote the manuscript.

ACKNOWLEDGEMENTS
We thank Dr. Jane Hubbard and members of the Arur Lab for critical comments on the manuscript and helpful discussions. We thank Jessica Chen for technical help with Figure S5A. This work is funded by NIH GM98200, ACS RSG014044DDC, CPRIT RP160023 and Anna Fuller Funds to SA, NIH GM98200S1 to KAT. SA is an Andrew Sabin Family Foundation Fellow at the University of Texas MD Anderson Cancer Center. Worm strains were obtained from C. elegans Genetics stock center at University of Minnesota funded by NIH (P40 OD010440), and Dr. Shohei Mitani (NBRP, Japan). We thank Dr. Hyman for the CDK-2 transgenic line, ddIs30.
REFERENCES


Figures
Figure 1: GSK-3 regulates accumulation of germline stem cells.

(A) Schematic representation of the progenitor zone of an adult hermaphroditic germline. * marks the DTC (distal tip cell). Each circle, and black bar, is a GSC. Progenitor zone, 20-22 cell diameters, harbors a population of 200-250 GSCs. Black bars represent GSCs undergoing mitosis. GSCs enter meiosis (half-moon) and differentiate 20-22 cell diameters away from the DTC. (B) Dissected germlines from adult (24 hours past L4) wild-type (top) and gsk-3(nr2047) (bottom) labeled with REC-8 (green, GSCs) and DAPI (white, DNA). (C) Quantitation of total number of GSCs (positive REC-8 staining) in gsk-3(nr2047) and wild-type germlines from mid-L4 until 60 hours after mid-L4. (D) Quantitation of the progenitor zone length from wild-type and gsk-3(nr2047) at mid-L4 and adult stages of development. (E) Proximal region of dissected germlines with the end of pachytene on the left, and the -1 oocyte on the right, labeled with RME-2 (green, oocytes), and DAPI (white, DNA). (F) Time course analysis of the number of RME-2 positive cells (oocytes) in the germline from 24 hours past L4 to 60 hours past L4. (G) Transgenic expression of a wild-type GFP::GSK-3 driven via the pie-1 promoter (vizIs27[Ppie-1::GFP::GSK-3(WT)]) rescues GSC defects of gsk-3 mutant animals. (H) Quantitation of GSCs from gsk-3 mutant animals with and without the wild-type and kinase dead GFP::GSK-3 transgenes. (I) Quantitation of the progenitor zone length from gsk-3 mutant animals with and without the wild-type and kinase dead GFP::GSK-3 transgenes. (J) Transgenic single copy GFP::GSK-3 (vizSi44[Pmex-5::GFP::GSK-3 [WT]]) rescues gsk-3 mutant GSC defects, while GFP::GSK-3 (KD) (vizSi20[Pmex-5::GFP::GSK-3 [K65A,E77A,D180A,D161A]) does not. The somatic phenotypes (Gleason et al., 2006; Maduro et al., 2001) of gsk-3 mutants are not rescued with either transgene, likely due to lack of promoter activity in these tissues (not shown). X-axes are identical.
for H, I and so are listed on the lower graph. Each experiment was performed at least three times. For panels C, D and F, 30 germlines were assayed each time. Error bars indicate standard deviation.

End of each progenitor zone is labeled with a solid line. Scale bars: 40µm
Figure 2: *gsk-3* mutant GSCs respond to GLP-1/Notch signaling.

Dissected germlines from adult (24 hours past L4) animals of indicated genotypes labeled with DAPI (white, DNA) and HIM-3 (green, meiotic cells) are oriented from left (progenitor zone) to right (oocytes). The left panel shows micrographs of germlines from animals maintained at
permisive temperature of 15°C; the right panel displays adult germlines from animals shifted to the restrictive temperature of 25°C as embryos. (A-B) Wild-type germlines contain GSCs and meiotic cells both at 15°C and 25°C. (C-D) gsk-3(nr2047) mutant germlines contain GSCs and meiotic cells at 15° and 25°C. The gsk-3 mutant germlines are smaller compared to wild-type. (E-F) glp-1(bn18ts) mutant germlines harbor GSCs and meiotic cells at 15°C but have only sperm at 25°C. (G-H) gsk-3(nr2047);glp-1(bn18ts) double mutant germlines harbor both GSCs and meiotic cells at 15°C but only sperm at 25°C. (I-J) glp-1(ar202gf) mutant germlines harbor both GSCs and meiotic cells at 15°C, but generate adult tumorous germlines at 25°C. (K-L) gsk-3(nr2047);glp-1(ar202gf) double mutants have GSCs and meiotic cells at 15°C and generate tumors at 25°C. Each experiment was performed three times, and each time an N of 30 germlines was assayed. Germlines are outlined with a dashed line. End of each progenitor zone is labeled with a solid line. Scale bars: (F,H): 20µm, (A-E,G,I-L): 50µm.
Figure 3: *gsk-3* regulates entry into and progression through S phase in GSCs.

Dissected germlines displaying the DTC (*) on the left. (A-B) *gsk-3* mutant and wild-type GSCs from adult (24 hours past L4) and mid-L4 germlines labeled for M phase (pH3, red), and S phase (EdU, green). Germlines for mutants and wild-type were processed in the same tube, and pictures taken at constant exposure and gain, to compare the level of EdU incorporation. (C) Quantitation of the mitotic index from wild-type and *gsk-3* mutant germlines. Number of M phase nuclei in wild-type gonads range from 4-13 and *gsk-3* mutant gonads ranged from 0-9. M phase index between the wild-type and *gsk-3* mutant gonads is not significantly different. 40 germlines were analyzed for each time point. (D) *gsk-3* mutant GSCs with very low but detectable EdU incorporation exhibit an S phase index of ~6% versus ~55% strong EdU incorporation in wild-
type (adult) and ~67% (mid-L4) germlines. (E-G) GFP::MCM-3(gts64) (green) localizes to the cytoplasm in wild-type GSCs. GFP::MCM-3 is localized to the nucleus in cdk-2 RNAi and gsk-3 mutant GSCs, but is cytoplasmic in the meiotic cells from these genotypes. Each experiment in panels A-D was performed five times, and at least 30 germlines were assayed each time. Experiments in panels E-G were performed three times, and 25 germlines were assayed each time. Error bars represent the standard deviation. End of each progenitor zone is labeled with a solid line. Scale bar: 40μm.
Figure 4: CDK-2 transgenic expression via a germline promoter, rescues the gsk-3 germline stem cell proliferation defect.

Dissected germlines displaying the DTC(*) to the left. (A-D) EdU incorporation (green), via soaking method, in germlines from wild-type or gsk-3 mutant animals with transgenic YFP::CDK-2 (ddIs30, via Ppie-1 promoter). (E) Quantitation of the total number of GSCs in germlines from wild-type and gsk-3 mutants with transgenic YFP::CDK-2 expression. (F) Quantitation of the S phase index from wild-type and gsk-3 mutant GSCs with transgenic YFP::CDK-2 expression. X-axis labels are the same for both graphs. The # signifies that the EdU signal was below detection limit for a majority of the germlines. (G-H) Expression of YFP::CDK-2 in the GSCs of wild-type
and gsk-3 mutant animals visualized with anti-GFP antibody staining. (I) qRT-PCR of cdk-2 mRNA on gsk-3 mutant germline with or without YFP::CDK-2 transgene relative to wild-type. YFP::CDK-2 transgene in wild-type background has higher cdk-2 mRNA compared to wild-type, revealing over-expression of CDK-2 in the transgene. (J-L) cdk-2 FISH analysis on dissected germlines from wild-type (J), cdk-2 depleted (L) and gsk-3 mutant germlines (K). A-H the experiments were performed four times, each time 30-35 germlines analyzed. Panel I the experiment was performed three times and each time 100 germlines were dissected and assayed by qRT-PCR. J-L the experiment was performed three times, each time 12-15 germlines were assayed. Error bars indicate standard deviation. All experiments were performed on adult animals, at 24 hours past L4 stage of development. End of each progenitor zone is labeled with a solid line. Scale bar: 40μm.
**Figure 5: CDK-2 is transcriptionally regulated in a gsk-3 dependent manner in the germline.**

(A) Gene structure of *cdk-2* on Wormbase Ver WS258. (B) Differential interference contrast (DIC) and GFP live image of the transcriptional reporter driven by Intron 1 of *cdk-2* in the germline and embryos. Dashed line marks outline of distal germline. (C-D) * marks the DTC. Dissected germlines from adult (24 hours past L4) wild-type and *gsk-3* mutants harboring the *cdk-2* transcriptional reporter driven by Intron 1 reporter, mounted in PLP media (no GFP staining, Materials and Methods) on the same slide, with oocytes on the right (solid lines). Intron 1 of *cdk-2* drives GFP expression in the distal germline of wild-type (C) but less so in the *gsk-3* mutant (D). Scale bar: 50μm.
Figure 6: GSK-3 inhibits DPL-1 to regulate cdk-2 transcription and S phase progression in *C. elegans* GSCs.

Dissected adult (24 hours past L4) germlines oriented with the DTC (*) to the left. (A-D) DNA is labeled in white (DAPI), and EdU (green) labels S phase. EdU incorporation in germlines from *gsk-3* mutant animals with RNAi-mediated depletion of *dpl-1* (D) and luciferase (as control) (B). (E-H) DNA is labeled in white (DAPI), and cdk-2 mRNA is labeled in green. cdk-2 mRNA FISH analysis from *gsk-3* mutant animals with RNAi-mediated depletion of *dpl-1* and luciferase (control). The photographs for *cdk-2* FISH were captured at 220ms each. The RNAi experiments were performed five times, and for A-D 20-22 germlines were imaged each time; D-H, 10-12 germlines were imaged each time. Scale bar: 40μm.
Figure 7: Model

GSK-3 inhibits DPL-1 transcriptional repression to maintain persistent high levels of CDK-2 and promote a short G1 coupled with rapid S phase entry and progression in *C. elegans* germline stem cells. (Left) Inhibitory cascade depicting the double negative pathway, with *gsk-3* inhibiting *dpl-1*, which in turn inhibits *cdk-2* transcription. (Middle) CDK-2 accumulation axis, highest being at the top. Circles represent germ cells. Green intensity represents CDK-2 accumulation, with darker green being higher accumulation. (Right) Representative germline drawings for wild-type and *gsk-3* mutants. Intensity of the green inside each nucleus represents the amount of CDK-2 accumulation in those nuclei. (Bottom) Cell cycle structures at different CDK-2 accumulation levels. In wild-type (dark green), GSK-3 inhibits *dpl-1* so that CDK-2 is produced at levels far above the threshold for S phase entry and progression in all cells. This allows all the germline stem cells to enter and progress efficiently (dark green circles). In *gsk-3* mutants, DPL-1 inhibits CDK-2 transcription, so that CDK-2 is reduced to or below the threshold for S phase entry and
progression. Fluctuating levels of CDK-2 accumulation may lead to some nuclei (green and light green circles) progressing slowly, with an aberrant S phase or long G1, while others (white circles) may arrest in G1, thus resulting in a germline with low tissue output.
**SUPPLEMENTAL INFORMATION**

**Supplemental Materials and Methods**

**Strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>Wild-type</td>
</tr>
<tr>
<td>EG4322</td>
<td>ttTi5605 II; unc-119(ed3) III</td>
</tr>
<tr>
<td>EG8082</td>
<td>unc-119(ed3) III; oxTi365 V</td>
</tr>
<tr>
<td>AUM1012</td>
<td>gsk-3(nr2047)/hT2[bli-4(e937)let-4(q782) qIs48] I; unc-119(ed3)III/ hT2 [bli-4(e937)let-4(q782) qIs48] (I; III)</td>
</tr>
<tr>
<td>AUM1054</td>
<td>gsk-3(tm2223) II/ hT2 [bli-4(e937)let-4(q782) qIs48] (I; III)</td>
</tr>
<tr>
<td>BS121</td>
<td>glp-1(bn18ts) III</td>
</tr>
<tr>
<td>BS3148</td>
<td>glp-1(ar202ts) III</td>
</tr>
<tr>
<td>AUM1081</td>
<td>gsk-3(tm2223) II/ hT2[bli-4(e937)let-4(q782)qIs48] (I;III). Line 1</td>
</tr>
<tr>
<td>AUM1082</td>
<td>gsk-3(tm2223) II/ hT2[bli-4(e937)let-4(q782)qIs48] (I;III). Line 2</td>
</tr>
<tr>
<td>AUM1260</td>
<td>gsk-3(nr2047)/oxTi398 I</td>
</tr>
<tr>
<td>AUM1262</td>
<td>gsk-3(nr2047)/oxTi398 I; gts64[mcm-3::GFP]; odls57[Ppie-1::mCherry::H2B]. Line 1</td>
</tr>
<tr>
<td>AUM1266</td>
<td>gsk-3(nr2047)/oxTi398 I; gts64[mcm-3::GFP]; odls57[Ppie-1::mCherry::H2B]. Line 2</td>
</tr>
<tr>
<td>AUM1279</td>
<td>gsk-3(nr2047)/oxTi398 I; dds30[Ppie-1::YFP::CDK-2]</td>
</tr>
<tr>
<td>AUM2026</td>
<td>gsk-3(nr2047)I;unc-119(ed3) III; vizIs26[Ppie-1::GFP::GSK-3(WT)::pie-1 3’UTR unc-119(+)]</td>
</tr>
<tr>
<td>AUM2027</td>
<td>gsk-3(nr2047)I;unc-119(ed3) III; vizIs27[Ppie-1::GFP::GSK-3(WT)::pie-1 3’UTR unc-119(+)]</td>
</tr>
<tr>
<td>AUM2028</td>
<td>gsk-3(nr2047)I;unc-119(ed3) III; vizIs28[Ppie-1::GFP::GSK-3(WT)::pie-1 3’UTR unc-119(+)]</td>
</tr>
<tr>
<td>AUM2029</td>
<td>gsk-3(nr2047)I;unc-119(ed3) III; visIs29[Ppie-1::GFP::GSK-3(WT)::pie-1 3’UTR unc-119(+)]</td>
</tr>
<tr>
<td>AUM2083</td>
<td>vizSi44 [Pmex-5::GFP::GSK-3(WT)::ttb-2 3’UTR unc-119(+)] *ttTi5605 II; unc-119(ed3) III</td>
</tr>
<tr>
<td>AUM2085</td>
<td>vizSi20[Pmex-5::GFP::GSK-3 [K65A,E77A,D180A,D161A];gsk-3 3’UTR] *ttTi5605 II; unc-119(ed3) III</td>
</tr>
<tr>
<td>AUM1295</td>
<td>gsk-3(nr2047)/hT2 [bli-4(e937)let-4(q782) qIs48] I; vizSi20[Pmex-5::GFP::GSK-3[K65A,E77A,D180A,D161A];gsk-3 3’UTR] *ttTi5605 II; unc-119(ed3)/hT2 [bli-4(e937)let-4(q782) qIs48] III</td>
</tr>
<tr>
<td>TG1753</td>
<td>unc-119(ed3) III; gts64 [pie-1p::GFP(lap)::mcm-3 + unc-119(+)] ltsIs7 [pie-1p::mCherry::hIs-58 + unc-119(+)]</td>
</tr>
<tr>
<td>TH98</td>
<td>unc-119(ed3) III; dds30 [Ppie-1::YFP::CDK-2 (K03e5.3a) unc-119(+)] II</td>
</tr>
<tr>
<td>WM69</td>
<td>gsk-3(nr2047) I/ hT2 [bli-4(e937)let-4(q782) qIs48] (I; III)</td>
</tr>
<tr>
<td>AUM1294</td>
<td>gsk-3(nr2047) I/ hT2 [bli-4(e937)let-4(q782) qIs48] (I; III);glp-1(ar202ts)III/hT2 [bli-4(e937)let-4(q782) qIs48] (I; III)</td>
</tr>
<tr>
<td>AUM1293</td>
<td>gsk-3(nr2047) I/ hT2 [bli-4(e937)let-4(q782) qIs48] (I; III); glp-1(bn18ts) III/ hT2 [bli-4(e937)let-4(q782) qIs48] (I; III);</td>
</tr>
<tr>
<td>EG7843</td>
<td>ttTi398 [Peft-3;:tdTomato::H2B::unc-54 3'UTR Cbr-unc-119(+)]</td>
</tr>
<tr>
<td>EG7844</td>
<td>ttTi413 [Peft-3;:tdTomato::H2B::unc-54 3’UTR Cbr-unc-119(+)]</td>
</tr>
<tr>
<td>AUM2073</td>
<td>vizSi34 [cdk-2[Promoter];:NLS::GFP::ttb-2 3’UTR] *ttTi5605 II; unc-119(ed3) III</td>
</tr>
<tr>
<td>AUM2071</td>
<td>unc-119(ed3) III; vizSi32[cdk-2[Intron1];:NLS::GFP::ttb-2 3’UTR] *oxTi365 V</td>
</tr>
<tr>
<td>AUM1339</td>
<td>gsk-3(nr2047) I/ hT2 [bli-4(e937)let-4(q782) qIs48] (I; III);vizSi32 [cdk-2[Intron1]] ::NLS::GFP::ttb-2 3’UTR] *oxTi365 V</td>
</tr>
</tbody>
</table>
Characterization of gsk-3 deletions

PCR product using primers, \textit{nr2047} MuF (CTACATGGCTGATAGCTTGG), \textit{nr2047} MuB1 (CCGGGAATGGCTGATCTA), and \textit{nr2047} midsequence B1 (GAACGGAGAAGCTGATACATG) was obtained using the template \textit{nr2047/nr2047} worms. This primer set yields a 2053 bp and 351 bp DNA fragment in wild-type animals and truncated 524 bp DNA fragment in \textit{gsk-3(nr2047)} homozygote, because of primer Mid B1 lies inside of the deletion, MuF/Mid B1 detects only WT. The PCR product of the \textit{gsk-3} deletion was sequenced. Sequencing results confirm that \textit{nr2047} is a 1529bp deletion with a 7bp insertion (AAAATTT). This mutation deletes the entire exon 3 and adjacent part of introns. cDNA amplification and sequencing from \textit{nr2047} suggests only exon 3 is deleted in the transcript with a frame shift in exon 4 that causes read through to the partial 3’UTR region, adding a novel 76 amino acid protein fragment to the truncated GSK-3 protein.

The \textit{gsk-3} mutant animals produce embryos at a slower rate than the wild-type, however, brood size analysis of these animals is not possible since the embryos do not progress to gastrulation and fail to undergo morphogenesis.

Transgenic construction of GFP::GSK-3

To generate a GFP::GSK-3 construct, the \textit{gsk-3} coding region from the start codon to the translation stop was amplified as one PCR product of 5.5 Kb with KOD polymerase (Novagen, Madison, WI), using primers:

\begin{verbatim}
attb1 GSK-3 ggggacagttttgaacaaaaagcagctgtggATGAGTAAGAGCTACATTGCTG
attb2 GSK-3 ggggacagttttgacaaagaagctggtgTTAAGCGGATGGGCCAGCCAC
\end{verbatim}

from N2 cDNA and cloned into the pDONR221 vector of the gateway system using BP reaction. The pDONR plasmid was then sequenced to confirm integrity and recombined into the pID3.02 \textit{pie-1} GFP plasmid (pENTR) containing the \textit{unc-119} transformation marker using the LR reaction to generate pSYC003[\textit{pie-1::GFP::GSK-3::pie-1} 3’UTR]. Microparticle biolistic transformation
(Praitis, 2006) was used to create low-copy integrated transgenic lines in \textit{unc-119}(ed3) animals as described earlier. Wild-type animals were individually cloned and assayed for integration of the transgene. Six integrated lines were obtained and named \textit{vizIs26, vizIs27, vizIs28, vizIs29, vizIs30} and \textit{vizIs31}.

To generate \textit{mex-5} driven GSK-3 transgenes, full-length coding region of \textit{gsk-3} was amplified from \textit{N2} cDNA and cloned via Gateway system into pDONR221. The clones were verified for sequence integrity, and combined with \textit{Pmex-5::GFP} (pJA245) and the \textit{tbb-23} 3’UTR using (pCM1.36). The final clone form \textit{Pmex-5::GFP::GSK-3::tbb-2} 3’UTR was cloned into the destination vector pCFJ150 via LR clonase reaction. pSYC001 clones obtained from the LR reaction were sequence verified for integrity.

Kinase dead GSK-3 was generated by modifying pSYC001 with a series of site-directed mutagenesis using the following primers:

- \textit{GSK3\_K65A\_FP}: AAATGAAAATGGTTGCAATCgctAAAGTTCTTCA,
- \textit{GSK3\_K65A\_RP}: GTTTGGCTCCTGAAGAACTTTagcGATTGCAACCA,
- \textit{GSK3\_E77A\_FP}: CAAACGATTTCAAGAATCGTgctCTACAGATTAT
- \textit{GSK3\_E77A\_RP}: ATTTTCGCATAATCTGTAGagcACGATTCTTG
- \textit{GSK3\_D161A\_FP}: CATTGGAATCTGTCACCGTgctATTAAGCCTCA
- \textit{GSK3\_D161A\_RP}: GCAAATTCTGAGGCTTAATagcACGGTGACAGA
- \textit{GSK3\_D180A\_FP}: CGGAGTGCTTAAGCTCTGTgctTTTGGATCTGC
- \textit{GSK3\_D180A\_RP}: AATATTTGGCAGATCCAAAagcACAGAGCTTAA.

The final GSK-3(KD) pENTR clone pSYC099 was sequenced to verify all the modifications. To generate \textit{mex-5} driven GSK-3 transgenes, pSYC001(GSK-3(wt)), or pSYC099(GSK-3(KD K65A, E77A, D161A, D180A)) was combined with \textit{Pmex-5::GFP} (pJA245), \textit{tbb-2} 3’UTR (pCM1.36), and destination vector pCFJ150 using multi fragment gateway system. The final expression constructs pHJJ002(\textit{Pmex-5::GFP::GSK-3(wt)::tbb-2} 3’UTR) or pSYC100 (\textit{Pmex-5::GFP::GSK-3(KD)::tbb-2} 3’UTR) were sequenced for the junctions. These constructs were injected into EG4322 worms for Chromosome II integration, and selected as described previously (Drake et al., 2014; Zeiser et al., 2011).

**Allelic detection PCR**

The following primers were used for detecting specific alleles:

- \textit{oxTi398 detection}
  - TF001: GGTGGTTCGACAGTCAAGGT
  - TF003: GGAATAGCGCTGAGACACAG
  - TF004: GGACTCCGAATGGATTCATC
  - Detecting 471bp PCR product in WT and 298bp in \textit{oxTi398}

- \textit{ddIs30}[YFP::CDK-2] detection
  - TF010: AAAATGTTCACTGATAAGCACAC
  - GFP676F: GATGGAAGCGTTCAACTGACAGAC
  - Detects 275bp in \textit{ddIs30}
CDK-2 transcriptional reporter construction
2kb upstream of cdk-2 exon 1 (Wormbase Ver 258) was amplified for the “promoter” construct using primers: GGGGACAACCTTTGTATAGAAAAAGTTGATcgcgggaagataagtggagagggag
GGGGACTGCTTTTTTTGTACAAACTTGTTtttccaccttttaaccagcattttt. 1880bp (Wormbase Ver 258) of Intron 1 was amplified using primers:
GGGGGACAACTTTGTATAGAAAAGTTGATgtgttacaagttctttgtgcaag
GGGGACTGCTTTTTTTGTACAAACTTGTCggagaattttt. The amplified fragments were cloned into pDONR P4P1R vector using BP reaction to make pENTR clones, pSYC122, and pSYC111. SV40NLS::GFP with synthetic introns was amplified from pPD95.67 using primers GgggacaagtttgtacaaaaaagcaggcttgATGACTGCTCCAAAGAAGAAGCG,
ggggaccactttgtacaagaagctgggtCTATTTGTATAGTTCCATCCATGCC
and cloned into pDONR221 vector. The plasmids were assembled with the tbb-2 3’UTR, pCM1.36 and pCFJ150 for MosSCI recombination site using Gateway LR reaction. Final expression plasmids were pSYC112 (Intron cdk-2::SV40NLS::GFP::tbb-2 3’UTR MosSCI site ttTi5605) and pSYC123 (promoter cdk-2::SV40NLS::GFP::tbb-2 3’UTR MosSCI site ttTi5605), sequenced for integrity.
Supplemental Tables, Figures and Figure Legends

Table S1: gsk-3 promotes developmental germ cell expansion

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>PGL-1::mCherry Positive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT gsk-3(nr2047) gsk-3(tm2223)</td>
</tr>
<tr>
<td>L1</td>
<td>2 2 2</td>
</tr>
<tr>
<td>Early L2</td>
<td>12 10±2 9±2</td>
</tr>
<tr>
<td>Early L3</td>
<td>34±1 14±2 13±2</td>
</tr>
<tr>
<td>Early L4</td>
<td>80±2 60±2 53±2</td>
</tr>
</tbody>
</table>

Whole mount germ cell counts of PGL-1::mCherry transgene from individual gonad arms at larval stages early L2-early L4 from wild-type and two gsk-3 mutant alleles (nr2047 and tm2223). Germ cell counts for L1 reflect total germ cell counts, since individual gonad arms have not developed at at this developmental stage. gsk-3 mutants (nr2047 and tm2223) contain fewer germ cells than wild-type throughout development. N=30 animals for each stage and genotype. Counts are presented as mean ± SD.
Figure S1: gsk-3 regulates GSC proliferation.

The progenitor zone of a dissected hermaphrodite germline with distal tip cell (*) on the left. (A) Two distinct loss-of-function alleles of gsk-3 display lower GSC number. DAPI (white) marks DNA, HIM-3 (green) marks meiotic cells and pH3 (red) marks mitotic cells. GSCs enter meiosis at the same distance in wild-type and gsk-3 mutants, also represented as a graph in C. End of each progenitor zone is labeled with a solid line. Scale bar: 20 μm. (B) Quantification of the number of GSCs in wild-type and gsk-3 mutants at 24 hours past L4 stage of development. (C) Quantification of the progenitor zone length from the DTC in wild-type and gsk-3 mutant germlines. In B and C, error bars indicate mean ± SD. **P<0.001, and n.s., not significant. n= number of animals counted.
Figure S2: Expression and western analysis of germline specific GFP::GSK-3 transgenes.

(A) Dissected germline from a gsk-3(nr2047);[Ppie-1::GFP::GSK-3(WT)] worm. DAPI (White) labels DNA, GFP (Green) labels the GFP::GSK-3(WT) transgene using the mex-5 Promoter. The transgene is expressed throughout the germline. -1, -2, represents the birth order of the oocytes. Both pie-1 driven and mex-5 driven GFP::GSK-3 transgenes express throughout the germline. (B) Western Blot analysis was performed using anti-GFP antibody on WT and gsk-3(nr2047);vizIs27[Ppie-1::GFP::GSK-3(WT)] worm extracts. A 75kDa band corresponding to GFP::GSK-3 was detected in the gsk-3(nr2047);[Ppie-1::GFP::GSK-3(WT)] worms, but not in wild-type (N2). Tubulin was used for internal control. (C) Confocal microscopy based zoomed view of the distal region to assess the subcellular localization of GFP::GSK-3 transgene (vizSi44[Pmex-5::GFP::GSK-3]) and a kinase dead GFP::GSK-3 transgene (vizSi20[Pmex-5::GFP::GSK-3(KD)]) in the progenitor region. This analysis reveals GSK-3 localization to be nuclear and cytoplasmic compartment in the progenitor zone. Scale bar: 40μm.
Figure S3: *gsk-3* GSCs do not efficiently incorporate EdU with a long EDU feeding pulse.

Wild-type and *gsk-3(nr2047)* worms were fed with EdU bacteria for 1 hour, 3 hours or 5 hours, and processed for detection of EdU incorporation. DAPI (white) labels DNA, and EdU (green) labels actively replicating S phase cells. Wild-type but not *gsk-3* mutant GSCs incorporate EdU label robustly after 1 hour of feeding with EdU bacteria. Loss of *gsk-3* results in extremely poor EdU incorporation despite continuous EdU pulse, suggesting a defect in S phase entry or replication rate. Asterisk marks the distal tip, and the end of each progenitor zone is labeled with a solid line. Scale bar: 40 μm.
Figure S4: Lack of EdU incorporation in gsk-3 mutant GSCs, upon a pulse of feeding is not due to poor pharyngeal pumping rates.

(A) Number of pumps per minute (Y axis) for each of the genotype indicated (X axis). gsk-3 mutants display pharyngeal pumping rate lower than wild-type animals, but significantly higher than eat-2 mutant. Error bars indicate mean ± standard deviation. *P<0.05, **P<0.001. n= number of animals counted. (B) The microscopy image in the figure represents the progenitor region of a dissected hermaphrodite adult germline with DTC (*) on the left of the photograph. DAPI (white) marks DNA, and EdU (green) labels S phase cells. EdU incorporation by feeding in eat-2 mutant animals, vs gsk-3 mutants and wild-type animals. Loss of eat-2 does not affect EdU incorporation in GSCs. Loss of gsk-3 results in barely visible (arrows) EdU labeling. Scale bar: 40μm.
Figure S5: GSCs in gsk-3 mutant animals display a variable nuclear size.

(A) Confocal slice of germlines labeled with DAPI (white) to visualize DNA and LMN-1 (lamin) (red) to visualize the nuclear envelope. (B) Magnified view of the designated region (in white boxes from panel A) of wild-type and gsk-3 mutant. (C) The nuclear size was calculated via measurement of circumference of nuclear envelope using Image J software based on the lamin-staining. 471 germ cells from 5 wild-type worms and 404 germ cells from seven gsk-3 mutants were used for this analysis. P value was derived using the F-test in Excel. Scale bar: 50μm
**Figure S6: cdk-2 RNAi causes G1 cell cycle arrest and results in nuclear localization of GFP::MCM-3.**

The panels represented in this figure display dissected germline from the *cdk-2* (RNAi) animal represented in Figure 3F (top panel here) with distal end (*) oriented to the left. The bottom panels in this figure represent staining for EdU and Phospho-Histone H3. Upon *cdk-2* RNAi, pH3 (red) and EdU (yellow) are absent, suggesting an arrest on the GSCs. GFP::MCM-3 accumulates in the nuclei of *cdk-2* RNAi mutants. The straight line marks the end of progenitor zone. Scale bar: 40µm.
Figure S7: Cyclin E is expressed continuously throughout wild-type and gsk-3 mutant GSCs.

Dissected germlines from wild-type and gsk-3(nr2047) mutant animals, oriented with distal end (*) to the left. Anti-CYE-1 antibody labels cyclin E in all the GSCs evenly in both wild-type and gsk-3 mutant germlines and is depleted in cye-1 RNAi. End of progenitor zone is labeled with a white line. Scale bar: 40µm.
Figure S8: YFP::CDK-2 rescues EdU incorporation in gsk-3 mutant animals at L4.

Dissected germlines from wild-type and gsk-3(nr2047) mutant animals with ddIs30[YFP::CDK-2/ analyzed for EdU incorporation (green), oriented left to right, with DTC on left. DAPI (white) labels DNA, EdU (green) marks the S phase. Overexpression of YFP::CDK-2 in gsk-3 mutant animals re-initiated S phase in GSCs. Scale bar: 40μm.
Figure S9: DPL-1 binds to “intron 1” of *cdk-2* to inhibit S phase entry and progression

(A) Screen shot of Wormbase Ver WS258 depicting the binding of DPL-1 via Chip analysis to intron 1 of *cdk-2* immediately upstream of Exon 2. (B) qRT-PCR of *dpl-1* mRNA on dissected germlines from each of the genotypes indicated. (C) RNAi mediated depletion of *dpl-1* in *gsk-3* mutants rescues the GSC S phase index to ~35% from <5%.
Figure S10: gsk-3 functions independently of nutritional cues to regulate stem cell divisions.

Progenitor zone region of a germline with the DTC (*) on the left of the photograph, labeled for DNA (DAPI, white), HIM-3 (meiosis, green) and phospho-histone H3 (M phase, red). After 12 hours of starvation, re-feeding of the gsk-3 mutants results in the onset of M-phase cells, similar to the fed control. White bar with blunt ends marks the length of the proliferative region. Scale bar: 40 μm.

REFERENCES