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Soma-Germline Interactions That Influence Germline Proliferation in *Caenorhabditis elegans*

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Abstract

Caenorhabditis elegans boasts a short lifecycle and high fecundity, two features that make it an attractive and powerful genetic model organism. Several recent studies indicate that germline proliferation, a prerequisite to optimal fecundity, is tightly controlled over the course of development. Cell proliferation control includes regulation of competence to proliferate, a poorly understood aspect of cell fate specification, as well as cell-cycle control. Furthermore, dynamic regulation of cell proliferation occurs in response to multiple external signals. The *C. elegans* germ line is proving a valuable model for linking genetic, developmental, systemic, and environmental control of cell proliferation. Here, we consider recent studies that contribute to our understanding of germ cell proliferation in *C. elegans*. We focus primarily on somatic control of germline proliferation, how it differs at different life stages, and how it can be altered in the context of the life cycle and changes in environmental status.

Keywords

L1 diapause; renewal; dauer; Notch; insulin

OVERVIEW OF GERMLINE PROLIFERATION IN *CAENORHABDITIS ELEGANS*

Germ cell fate is exclusively relegated to one cell in the embryo (P4) that ultimately gives rise to thousands of adult germ cells (for recent general reviews see Kimble and Crittenden, 2005, 2007; Hansen and Schedl, 2006). The P4 blastomere undergoes one further symmetric division early in embryogenesis ~2.5 hr after the first cleavage to produce Z2 and Z3 (Fig. 1). At hatching, Z2 and Z3 are flanked by the somatic gonad precursors Z1 and Z4. Together with their surrounding basement membrane, these four cells constitute the gonad primordium. These four cells remain quiescent until the mid-L1 stage, when they begin to proliferate. Therefore, despite the demand on the germ line for gamete production later in life, germ cells do not begin to proliferate until more than one-third of the egg-to-egg life cycle has elapsed.

During the next third of the worm's lifecycle, a subset of germ cells continues to proliferate while others differentiate. Prior to the mid-L3 stage, no overt differentiation can be detected. In the mid-L3, the proximal-most germ cells begin to enter meiosis. This initial onset of

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meiosis defines the germline pattern, establishing a distal proliferative (or mitotic) zone and a more proximal region where meiotic germ cells differentiate by entering prophase of meiosis I and ultimately undergoing gametogenesis. In the L3 and L4 stages, germline proliferation in the distal zone is robust, ultimately producing a distal population of ~200 cells in the mitotic cell cycle in each of the two arms of the adult hermaphrodite gonad (Fig. 2).

Gametogenesis in hermaphrodites occurs in two stages. The first ~35 germ cells that differentiate in each gonad arm adopt the male germ cell fate and differentiate as sperm, setting the total self-progeny brood size at ~300 (4 sperm differentiate from each male-fated germ cell). All subsequent germ cells are female and either undergo programmed cell death or differentiate as oocytes. When hermaphrodites deplete their reserves of self-sperm, they continue to produce mature oocytes in response to insemination by males. Distal germ cell proliferation slows in late-larval and adult stages and maintains gametogenesis. The germ line is the only tissue in *C. elegans* in which cell divisions occur into adulthood.

In summary, distinct aspects of germline proliferation occur in the embryonic, larval, and adult stages. In the embryo, one symmetric cell division of the exclusive germ cell precursor P4 generates primordial germ cells (PGCs) Z2 and Z3. (Since germ cell fate is separate from somatic fate in P4, this cell is *the* “primordial germ cell.” By analogy with other organisms, however, Z2 and Z3 are also referred to as “primordial germ cells” since they are the cells that ultimately interact with the somatic gonad precursors.) In larval stages, the proliferation-competent germ cell population is expanded, initially in the context of all undifferentiated germ cells and later in the context of both undifferentiated and differentiated germ cells. Larval germline proliferation establishes the proper number of cells in the adult mitotic region. Finally, the adult germ line is maintained by a lower average rate of cell division.

CELL FATE DECISIONS AND GERMLINE PROLIFERATION: TO DIFFERENTIATE OR NOT TO DIFFERENTIATE?

In the *C. elegans* germline, a conserved Notch signaling pathway controls a critical cell fate decision, to retain proliferative capacity or to differentiate (mitosis vs. meiosis, in this context). The molecular details of GLP-1/Notch signaling and germline-autonomous events downstream of Notch signaling are the subject of intense investigation. Several excellent recent reviews address this topic (Hansen and Schedl, 2006; Kimble and Crittenden, 2007; Byrd and Kimble, 2009), and it will only be considered briefly here.

Signaling from the distal tip cell (DTC) activates the Notch family receptor GLP-1 in the germ line, thereby maintaining a population of cells close to the DTC in the mitotic cell cycle and/or preventing them from entering meiosis. As a result, post-embryonic germline proliferation (with the curious exception of the first few cell divisions), requires the activity of the Notch signaling pathway. Withdrawal of signaling causes all germ cells to enter meiosis (precociously, if signaling is blocked early) and differentiate as gametes (Austin and Kimble, 1987). Although GLP-1/Notch signaling specifies a zone where differentiation is inhibited, Notch is not required for proliferation, per se, since highly proliferative GLP-1-independent tumors can form, for example, in the absence of downstream factors GLD-1 and GLD-2 (Kadyk and Kimble, 1998).

The emerging picture is that GLP-1 activity feeds into a web of functionally interacting RNA-binding proteins. Like other Notch family receptors, in response to ligand-binding, GLP-1 likely undergoes a series of cleavage events that release the intracellular domain of the receptor (Crittenden et al., 1994). The intracellular domain then acts with LAG-1 and

SEL-8, to activate transcription of target genes in the germ line. Important positive factors genetically downstream of GLP-1 include FOG-1, a cytoplasmic polyadenylation element binding protein (CEBP) and two pumilio-related proteins FBF-1 and FBF-2. Genes encoding these three proteins are required for continued maintenance of the mitotic region: loss of all three results in early larval differentiation of all germ cells while loss of *fbf-1* and *fbf-2* results in loss of the mitotic region in late-larval and adult stages (Crittenden et al., 2002; Lamont et al., 2004; Thompson et al., 2005). Additionally, the *fbf-2* gene appears to be a direct GLP-1 target (Lamont et al., 2004). Ultimately, GLP-1 activation interferes with at least three partially redundant differentiation-promoting pathways, including the GLD-1 and GLD-2 pathways (Kadyk and Kimble, 1998; Hansen et al., 2004). In the absence of both *gld-1* and *gld-2*, most germ cells are in the mitotic cell cycle and over-proliferate in a *glp-1*-independent manner (Kadyk and Kimble, 1998). GLD-1 is a maxi-KH/STAR domain-containing RNA-binding protein (Jones and Schedl, 1995) and GLD-2 is a cytoplasmic poly(A) polymerase catalytic subunit (Wang et al., 2002) that is stimulated by interaction with the BicC-related protein GLD-3 (Eckmann et al., 2002, 2004; Wang et al., 2002).

Two recent studies add significantly to this picture. First, a possible role for GLD-2 in micro-RNA (miRNA) stabilization comes from studies of the mammalian homolog (Katoh et al., 2009). This result opens the exciting possibility that miRNA control may be an important key to this differentiation decision. Second, GLD-4 was identified as another partially redundant poly(A) polymerase. GLD-4 catalytic activity is enhanced by interaction with GLS-1, a GLD-3-interacting protein. Like *gld-1* mutants, *gld-2 gld-4* double mutants appear defective in meiotic maintenance, and *gld-2* and *gld-4* are required for proper accumulation of GLD-1 protein levels (Schmid et al., 2009).

Consistent with the multi-faceted role GLD-1 plays in germline development, this protein binds many RNA targets (Lee and Schedl, 2001). Of relevance to germline cell cycle control, GLD-1 is critical for meiotic entry and maintenance of the meiotic program. In the absence of GLD-1, germ cells embark on meiotic entry but do not complete meiotic prophase and, instead, re-initiate mitosis (Francis et al., 1995). Cyclin E (*cye-1*) mRNA was recently identified as an important GLD-1 target. GLD-1 normally represses translation of *cye-1*, preventing cells from inappropriately reverting to the mitotic cell cycle (Biedermann et al., 2009). The re-initiation of mitosis accompanies another phenotype in germ lines with reduced *gld-1* activity: the inappropriate expression of somatic fates. This effect is enhanced by loss of another germline KH-domain-containing RNA-binding protein, MEX-3 (Ciosk et al., 2006). Interestingly, the aberrant return to mitosis in GLD-1 mutants appears to trigger precocious expression of early embryonic genes (Biedermann et al., 2009). This work suggests the intriguing possibility that loss of meiotic maintenance, a return to the mitotic cell cycle, and precocious expression of somatic fates in the germ line may be features of teratomas in mammals as well as worms (Biedermann et al., 2009).

Another pumilio family member, PUF-8, was recently characterized for its partially redundant role with MEX-3 in germline-autonomous control of proliferation (Ariz et al., 2009). Although each mutant alone causes only a modest decrease in germ cell number, the double mutant causes a severe reduction in germ cell number in both larval and adult stages. Consistent with a role for these genes in mitotic competence, as opposed to a role in establishing or maintaining germ cell fate or in preventing meiotic entry, germ cells in the double mutant express germ cell markers, but express neither mitosis nor meiosis epitopes (recognized under certain fixation conditions by anti-REC-8 and anti-HIM-3, respectively). Moreover, in the triple mutant with a loss-of-function allele of *glp-1*, the germ cells all enter meiosis and differentiate as sperm (as they do in the *glp-1* single mutant). Therefore, germ cells mutant for both *puf-8* and *mex-3* are capable of meiosis and differentiation, but do not proceed through meiosis in the presence of *glp-1* (Ariz et al., 2009). This phenotype

contrasts with that of mutations in *glp-4* (Beanan and Strome, 1992) and in *glp-3/eft-3* (Kadyk et al., 1997), two other genes required for germline proliferation. Mutations in these genes block mitosis and meiosis since germ cells in these mutants are not capable of meiotic entry even in the absence of *glp-1*. Further, loss of both *puf-8* and *mex-3* is epistatic to tumors that normally form in response to constitutive activation of *glp-1* or simultaneous depletion of *gld-1* and *gld-2*. In summary, *puf-8 mex-3* double mutants exhibit a phenotype consistent with the hypothesis that germline proliferation is not simply a consequence of preventing meiotic entry, but requires *puf-8*, *mex-3*, and their targets to promote the mitotic cell cycle.

Another example of the complexity of the interaction between proliferation and differentiation is exemplified by studies of *mett-10*, a gene that encodes a putative methyltransferase. METT-10 promotes differentiation in several cell types including the germ line, but also permits the progression of the mitotic cell cycle (Dorsett et al., 2009).

The germline cell cycle itself has only come under close scrutiny relatively recently (Ashcroft and Golden, 2002; Lamitina and L'Hernault, 2002; Brodigan et al., 2003; Crittenden et al., 2006; Maciejowski et al., 2006; Jaramillo-Lambert et al., 2007; Michaelson et al., 2010). Studies on the germline cell cycle in general have been challenging since, compared with somatic cells, obtaining stable expression of transgenes in the germ line is laborious, and the cell division pattern is variable from individual to individual. Moreover, techniques to genetically mark cell lineages have only recently become available (Voutev and Hubbard, 2008). Nevertheless, technical improvements continue and these bode well for many interesting discoveries in the near future.

GERMLINE PROLIFERATION IN THE EMBRYO: P4 GIVES RISE TO Z2 AND Z3

Similar to germ cell development in other organisms, several molecular mechanisms ensure that the early germ line maintains transcriptional quiescence and does not adopt somatic fates. These mechanisms include transcriptional control and chromatin-based repression (for recent reviews, see Blackwell, 2004; Schaner and Kelly, 2006; Seydoux and Braun, 2006; Strome and Lehmann, 2007). Briefly, in early *C. elegans* germline blastomeres P0 and P1, two TIS11 zinc finger-containing proteins OMA-1 and OMA-2 repress transcription by sequestering the TAF-4 protein (TATA binding associated factor 4) in the cytoplasm (Guen-Ozkan et al., 2008). Though present from P0-P4, the activity of the CCCH zinc finger protein PIE-1 becomes paramount in P2 to maintain transcriptional quiescence up to the division of P4. In the presence of maternal PIE-1, germ cells do not express the RNAP C-terminal repeat domain (CTD) phospho-Ser2 epitope associated with active transcriptional elongation. Consistent with the hypothesis that PIE-1 globally represses transcription by interfering with transcriptional elongation, PIE-1 interacts with and inhibits p-TEFb (the kinase complex that phosphorylates Ser2) in cell culture, where a competitive substrate mimic mechanism acts to inhibit RNAP CTD phosphorylation (Zhang et al., 2003). Surprisingly, more recent structure-function studies aided by improved germline transgene expression technology suggest that the block in Ser2 phosphorylation by PIE-1 is not absolutely essential for transcriptional silencing nor for germline specification. Rather, an additional mechanism localized outside the competitive substrate sequence is important to limit phosphorylation of Ser5 on the RNAP CTD. Moreover, PIE-1-dependent inhibition of Ser5 phosphorylation appears to correlate better with PIE-1's functions in transcription inhibition and promoting germ cell fate (Ghosh and Seydoux, 2008). Though PIE-1 is not conserved in other organisms, other organisms employ similar global repression of RNAP activity in early germ cells (Seydoux and Braun, 2006).

Upon division of P4 to Z2 and Z3, PIE-1 is degraded. However, transcriptional repression is maintained by a chromatin-based mechanism that likely prevents further germ cell division (Schaner et al., 2003). In brief, histone modifications differ in newly born Z2 and Z3 compared to surrounding somatic cells. In particular, the H3K4methylation mark of active chromatin is globally reduced. This mark does not accumulate in the PGCs until just prior to the onset of their proliferation in the L1. Similar to loss of *nanos* in *Drosophila*, in the absence of the *nanos*-like RNA-binding proteins NOS-1 and NOS-2, H3meK4 accumulates prematurely. Interestingly, interference with NOS activity leads to premature germ cell death and to aberrant proliferation control (see below), suggesting that the control of early germ cell proliferation is intimately connected to other aspects of germ cell fate specification and development.

CONTROL OF GERMLINE PROLIFERATION IN THE L1

Despite their close apposition with cells that express the LAG-2 ligand for GLP-1/Notch, PGCs remain quiescent until the mid-L1 stage. Consistent with the idea that GLP-1/Notch is not critical for restarting the cell cycle at this stage, Z2 and Z3 undergo several rounds of cell division in null *glp-1* mutants prior to premature differentiation (Austin and Kimble, 1987). What triggers the re-entry of Z2 and Z3 into the cell cycle? While the picture is still incomplete, at least two independent criteria must be met: communication with Z1 and Z4 and the availability of food.

First, the somatic gonad precursors must be present. If Z1 and Z4 are ablated in the early L1, Z2 and Z3 do not proliferate (Kimble and White, 1981). The identity of the signal from the somatic gonad precursors and how this signal promotes cell division competence in the PGCs is unknown. In theory, a genetic screen for genes that regulate this process would be straightforward: mutations that permit proper germ cell fate specification and localization of Z2 and Z3 to the gonad primordium but that interfere with subsequent proliferation of Z2 and Z3 should identify relevant components, provided the same genes were not required for specification of the somatic gonad precursors or the PGCs. It is also possible that interfering with the growth and/or division of Z1 and Z4 alone can interfere with division of Z2 and Z3. For example, mutations in the TRP cation channel-encoding gene *gon-2* can prevent Z1 and Z4 division even though Z1 and Z4 are properly specified. In these mutants, a range of phenotypes is observed, but failure of Z2 and Z3 proliferation correlates with failure of Z1 and Z4 to divide (Sun and Lambie, 1997). The current model is that Mg⁺⁺ uptake is impaired in Z1 and Z4 in *gon-2* mutants, preventing their proper growth and cell-cycle progression (Kemp et al., 2008).

Genetic screens for mutations that specifically interfere with the communication from Z1 and Z4 to Z2 and Z3 were stymied by an unexpected and interesting twist of germline transcriptional silencing and genome organization (Maciejowski et al., 2005). In short, while Z2 and Z3 are liberated from chromatin-based transcriptional silencing just prior to their reentry into the cell cycle in the presence of food, most of the X chromosome remains silenced throughout subsequent germline development. This silencing is largely dependent upon the MES proteins: *mes-2*, *-3*, and *-6* encode components of a histone methyltransferase complex that is excluded from autosomes by MES-4 (Fong et al., 2002). As a result, for “cell-essential” genes that are duplicated in the genome such that one copy is on an autosome and one copy is on the X chromosome (e.g., genes encoding the poly-A binding proteins PAB-1 and PAB-2, elongation factors GLP-3/EFT-3 and EFT-4, or ribosome components RPL-11.1 and RPL-11.2), the autosome copy becomes vital for germ cell development since the X-linked copy is likely silent (Maciejowski et al., 2005). Hence, many autosome-linked housekeeping genes that contain functionally redundant X-linked copies appear to be germlinespecific proliferation factors. A similar phenomenon occurs in

male mammals as the unpaired X in males is silenced during germline development (Handel, 2004), and some autosomal paralogs of X-linked genes are required for male fertility (see Maciejowski et al., 2005, and references therein).

Importantly, subfunctionalization of X/autosome-duplicated genes may not be limited to housekeeping genes. Signaling genes also display some phenotypic differences in the soma and germ line, with the autosome copy displaying a stronger germline role. Examples include the presenilin genes *sel-12* and *hop-1* (Pepper et al., 2003a), and the Akt/PKB kinase genes *akt-1* and *akt-2* (Fukuyama et al., 2006).

A second criterion for PGCs to initiate proliferation in the L1 gonad primordium is that larvae must feed. The release of the PGCs from chromatin-based transcriptional silencing to permit cell-cycle progression depends on food availability, suggesting that a food-related signal triggers germline chromatin changes that permit the requisite transcriptional activation (Schaner et al., 2003). Additionally, in embryos lacking both *nos-1* and *nos-2*, PGCs often proliferate inappropriately in the absence of food (Subramaniam and Seydoux, 1999). Communication of Z2 and Z3 with somatic gonad precursors and food availability are likely independent triggers of early germline proliferation. This possibility is suggested by the observation that in animals with reduced *nos-2* activity, one of the PGCs sometimes does not become incorporated into the gonad primordium, and the unincorporated PGC will inappropriately proliferate in the absence of food with the same frequency as the incorporated PGC in the *nos-1* and *nos-2* double (Subramaniam and Seydoux, 1999).

Both somatic and germ cell proliferation is repressed in L1 larvae that hatch in the absence of food. This “L1 diapause” allows worms to suspend development until conditions improve when they resume development (Johnson et al., 1984). Remarkably, ~70% of wild-type worms can survive L1 starvation for as long as 3 weeks in S basal media, a bacteria-free solution consisting of sodium chloride, potassium phosphate, and cholesterol (Baugh and Sternberg, 2006). L1 arrest can be induced by starvation, high temperature, or interference with the DAF-2/Insulin-IGF-like receptor (IIR) signaling pathway (Baugh and Sternberg, 2006). During L1 diapause, somatic cells are arrested in G1, and this arrest requires the cyclin-dependent kinase inhibitor *cki-1* (Hong et al., 1998; Fukuyama et al., 2003). A link from IIR signaling to *cki-1* transcription was recently made in *C. elegans*. Similar to other organisms, active IIR signaling interferes with the activity of a FOXO transcription factor (DAF-16 in worms) by way of a conserved PI3K signaling cascade that activates Akt/PKB kinase and is opposed by PTEN. When L1 animals hatch in the absence of food, *cki-1* reporter expression in a subset of cells (e.g., the seam cells) is elevated relative to well-fed animals. This “physiologically regulated” *cki-1* expression is largely *daf-16*-dependent, suggesting that DAF-16 normally interferes with the cell cycle in response to L1 starvation by activating *cki-1* (Baugh and Sternberg, 2006).

Although nutrient-regulated L1 cell-cycle arrest in the soma and the germ line are both dependent on IIR signaling, the control of PGC division in response to food availability differs from the control of somatic cells (Fukuyama et al., 2006). First, DAF-16 is dispensable for germline cell cycle control during L1 diapause, suggesting that the IIR/PI3K pathway interferes with a different factor to prevent germline proliferation in the L1 diapause (Fukuyama et al., 2006). Thus, while PGCs inappropriately initiate proliferation under L1 diapause conditions in the absence of the PTEN ortholog DAF-18, unlike somatic cells, they do not inappropriately initiate proliferation in the absence of DAF-16. This observation was somewhat surprising given that IIR signaling in *C. elegans* converges on *daf-16* regulation in many different contexts, including later larval germline proliferation control (see below). Second, consistent with a lack of dependence on *daf-16*, unlike somatic cells that arrest prior to replication in a *cki-1*-dependent manner, germ cell arrest occurs in

the post-replication (G2) phase in a *cki-1*-independent manner (Fukuyama et al., 2003., 2006). Third, *akt-1* (but not *akt-2*) mediates the germline arrest signal (Fukuyama et al., 2006). As mentioned above, since *akt-1* is autosomal and *akt-2* is X-linked, this difference might result from expression differences that culminate in a more germline-specific role for *akt-1* in L1 cell-cycle arrest.

The spindle assembly checkpoint (SAC) gene *mdf-1* has also been implicated in the response of PGCs to nutritional cues in the L1 (Watanabe et al., 2008). MDF-1 is the worm homolog of MAD1 and binding partner of the worm MAD2 ortholog, MDF-2 (Kitagawa et al., 2002). In the presence of MAD1, a MAD2-CDC20 complex forms on unattached kinetochores, triggering SAC and halting the cell cycle. In *C. elegans*, reduction of *mdf-1* activity (in *mdf-1*/+ heterozygotes or homozygous progeny of heterozygous mothers) prevents PGC cellcycle arrest in the absence of food. Counter-intuitively, a more severe reduction of *mdf-1* (in homozygotes) prevents proper reactivation of the cell cycle when starved L1s are reintroduced to food. These results suggest that the precise levels of *mdf-1* are important for proper PGC cellcycle response to L1 nutritional status. MDF-1 can be phosphorylated by AKT (Watanabe et al., 2008). However, the full functional significance of this phosphorylation for L1 arrest remains to be determined. Furthermore, the role of MDF-1 in L1 proliferation control may not be specific to the germ line.

CONTROL OF GERMLINE PROLIFERATION IN THE DAUER

Near the end of the L1 stage, a critical life history decision is made: to continue in the “reproductive” developmental mode or enter the dauer, a non-feeding, stress-resistant, non-aging, and developmentally arrested stage (for recent reviews, see Hu, 2007; Fielenbach and Antebi, 2008). Under harsh environmental conditions, including high population density (sensed by pheromone), high temperature, and lack of food, rather than undergoing the normal L2 molt, animals undergo an alternate “L2d” molt from which they can progress to the L3 if conditions improve or to dauer if conditions remain unfavorable. Genetic analysis of dauer-constitutive (Daf-c) and dauer-defective (Daf-d) mutants has defined four distinct pathways that regulate dauer arrest, including guanylyl cyclase, transforming growth factor- β (TGF β)-like, insulin-like, and a steroid hormone pathway. When environmental conditions improve, dauer exit is initiated, and the recovering dauer eventually molts into an L4 larva and continues development normally. Entry into and exit from the dauer state coincide with reversible arrest or slowing of cell division in the whole organism including the germ line (Ren et al., 1996).

What prevents germline proliferation during dauer? Germline proliferation is repressed in dauer even when GLP-1/Notch is constitutively active, suggesting alternate mechanisms control proliferation (Narbonne and Roy, 2006). Narbonne and Roy (2006) used a clever screening strategy to find mutants that inappropriately proliferate the germ line during dauer based on the observation that the distance between the DTC in the two arms of the gonad is reproducibly short in dauer larvae in which proliferation has slowed, but increases when the germ line inappropriately proliferates. The screen yielded a reduction-of-function mutation in *aak-2*, one of the two AMPK-encoding genes, suggesting that AMPK normally prevents germline proliferation during dauer. AMPK activity in all eukaryotes is sensitive to AMP:ATP ratios, allowing it to act as a cellular energy monitor. Subsequent analysis indicated that both *aak-1* and *aak-2* limit germline proliferation in response to dauer entry, that *aak-2(RNAi)* is largely independent of *rrf-1* (suggesting germline-autonomous function), and that *aak-1* acts in response to the AMPKK component LKB/*par-4*, a conserved activator of AMPK. The data are consistent with a model in which active TGF β and IIR signaling pathways prevent PAR-4, DAF-18, and the AAKs from inhibiting germline proliferation, as they do in dauer-promoting conditions.

CONTROL OF GERMLINE PROLIFERATION UNDER REPLETE CONDITIONS IN THE L2, L3 AND L4

When environmental conditions are favorable, worms bypass dauer entry and proceed into a “reproductive” developmental pathway during which germline proliferation is still controlled by somatic signals, both local and global.

Several distinct cell–cell interactions between the somatic gonad and the germ line regulate germline proliferation in larval stages. At the end of the L1 stage, the somatic gonad consists of the DTCs positioned at the distal tips of each gonad arm expressing the DSL ligand LAG-2, and 10 more central somatic gonad cells, two of which also express low levels of LAG-2, Z1.ppp and Z4.aaa. Prior to the formation of the hermaphrodite somatic gonadal primordium (SPh) in the center of the gonad (Kimble and Hirsh, 1979), Z1.ppp and Z4.aaa contact germ cells and, as demonstrated by cell ablation studies, they contribute to the maintenance of the mitotic fate of early germ cells (Pepper et al., 2003b). By the early L3, the SPh has displaced germ cells to anterior and posterior gonad “arms” and robust germline proliferation builds the numbers of proliferative germ cells.

Recently, expression of a second DSL ligand, APX-1, was found to contribute, together with LAG-2, to activation of the GLP-1 receptor in the germ line and to germline proliferation (Nadarajan et al., 2009). The DTC begins expressing *apx-1* in the early L3, whereas *lag-2* expression starts in the L1. Partial redundancy of the two is suggested by the result that *apx-1(RNAi)* increases the penetrance of the Glp-1-like sterile phenotype observed in *lag-2(q420ts)* mutants and vice versa [e.g., *lag-2(RNAi)* in an *apx-1(or3)* background]. Consistent with a contribution of APX-1 to GLP-1 activation, reduction of *apx-1* reduces the number of nuclei in the adult germline proliferative zone by ~25% in the mutant and 40% by RNAi. These two ligands are also redundant for a role in GLP-1-mediated oocyte size control (Nadarajan et al., 2009).

From the early L3 to the end of the L4 stage, the number of germ cells in the mitotic cell cycle expands from ~20 to ~200 cells per gonad arm (Fig. 2; Killian and Hubbard, 2005). During the L3 and L4, concurrent with this period of robust germline proliferation, the SPh gives rise to the somatic gonadal sheath, the spermatheca, and the uterus. The expansion of the proliferative zone in the L3 and L4 sets up the adult pool of mitotic germ cells and influences fecundity. Indeed, the distance that the DTC travels toward the anterior and posterior in the L3 (away from the center of the worm) is largely dependent on the extent to which the germ line proliferates as well as its own intrinsic early migration program (Tamai and Nishiwaki, 2007; McGovern et al., 2009). As the gonad elongates during the L3 and L4 larval stages, the number of mitotic germ cells increases sharply before reaching a plateau during adulthood (Fig. 2).

Recent studies indicate that the larval amplification of the pool of proliferation-competent germ cells depends on at least two additional signals that are distinct from signals exerted by the DTC via GLP-1/Notch signaling: a local signal from the distal sheath cells (McCarter et al., 1997; Killian and Hubbard, 2005) and an IIR-mediated signal (Michaelson et al., 2010).

Both cell ablation studies and genetic analysis implicate the somatic gonadal sheath in proliferation control, distinct from the mitosis/meiosis decision. Sheath cells arise from the sheath/spermatheca precursor (SS) cells, and ultimately form five pairs of thin elongated cells. Each pair of sheath cells occupies a stereotyped position along the gonad proximaldistal axis. The distal-most pair, Sh1, is born in the mid-L3 while the remaining pairs are born in the early L4 (Kimble and Hirsh, 1979). The position of Sh1 changes with respect to proliferating germ cells. In the L3 and L4, Sh1 cells are in contact with the distal

proliferative zone, while in the adult, the end result of growth and morphological changes in both the sheath cells and the germ line positions the Sh1 cells proximal to the mitosis/meiosis border (Killian and Hubbard, 2005; Fig. 3A). Ablation of either both SS cells in a gonad arm or the pair of Sh1 cells alone results in a 50–60% reduction in the number of cells in the adult proliferative zone, without alteration of the overall pattern of proliferation and differentiation (Figs. 2 and 3B; McCarter et al., 1997; Killian and Hubbard, 2005). Ablation of precursors to the remaining sheath/spermatheca cells does not cause a similar reduction in proliferation, thereby implicating Sh1 as the source of a germline proliferation-promoting signal during larval germline amplification (Fig. 2; Killian and Hubbard, 2005).

Genetic studies of mutations that caused proximal germline tumors unexpectedly implicated the sheath in larval germline proliferation. Mutations in several genes were identified that cause both a reduction in larval germline proliferation and a proximal tumor. Through a series of events, inadequate larval germline proliferation can set up inappropriate soma-germline contacts that, counter-intuitively, cause the formation of a proximal germline tumor (Killian and Hubbard, 2004, 2005; Voutev et al., 2006). Briefly, inadequate larval proliferation delays differentiation since the gonad does not elongate properly and germ cells remain in proximity to the DTC. If differentiation is delayed beyond the mid-L4 while somatic gonad development proceeds normally, GLP-1-responsive undifferentiated germ cells come into contact with proximal sheath cells that express two DSL ligands, APX-1 and ARG-1. These ligands then interact with GLP-1, and act as a niche for germcell renewal, creating a mirror-image pattern defect in germline development (McGovern et al., 2009). Therefore, defects in larval germline proliferation, provided they do not interfere with the ability of the germ line to proliferate per se, can cause the formation of a localized germline tumor of unregulated proliferating cells.

The upshot of extensive genetic analysis was that genes involved in several different aspects of ribosome biogenesis are required largely in the soma to promote germline proliferation. For example, PRO-1, PRO-2, and PRO-3 are related to yeast IPI3, NOC2, and SDA1, proteins involved in rRNA processing, a nucleolus-nucleus complex, and later stages of ribosome biogenesis, respectively. Molecular analysis confirmed a role for *pro-1* in worm rRNA processing (Killian and Hubbard, 2004; Voutev et al., 2006). Genetic mosaic studies narrowed the focus of activity of the *pro-1* gene to the SS lineage (Killian and Hubbard, 2004), and sheath cell defects were observed after reducing the activity of several ribosome biogenesis genes (Voutev et al., 2006). Taken together with the cell ablation studies that pinpoint Sh1 as the source of a critical germline proliferation-promoting cell–cell interaction, these data suggest that optimal ribosome biogenesis in the distal pair sheath cells, Sh1, is important for proper larval germline proliferation. The precise molecular link between the distal sheath and the germ line, however, remains unidentified.

Based on an assay designed to identify genes that promote robust larval proliferation, a candidate screen for signaling pathways that promote larval germline proliferation identified the IIR signaling pathway as an important molecular player (Michaelson et al., 2010). Although previous studies indicated that reducing IIR signaling reduced fertility (Gems et al., 1998; Tissenbaum and Ruvkun, 1998; Dillin et al., 2002), it was unclear whether these influences were separable from the restriction on germline proliferation imposed by dauer. A combination of experimental approaches (anatomically restricted expression studies, temperature-shift, RNAi manipulations, and mosaic analysis) suggests that IIR signaling acts in the germ line through the DAF-2 receptor and the canonical PI3K pathway to promote the germline cell cycle by inhibition of the transcription factor DAF-16/FOXO. This role for IIR signaling is separable from the well-characterized role of this pathway in preventing dauer entry. The observed defects in germline cell cycle caused by reduced IIR signaling are consistent with a reduced rate of larval germline cellcycle progression (reduced

mitotic and S-phase indices) as well as a shift in the number of cells in late S and G2 at the expense of cells in G1 (Michaelson et al., 2010).

The role of IIR signaling in larval germline proliferation under replete conditions is largely in response to the activity of *ins-3* and *ins-33*, two somatically acting putative IIR ligands. The data support a model in which IIR acts in parallel with Notch signaling, the latter affecting the number of cells in the proliferative zone, but not their mitotic index. Finally, sheath cell ablation studies indicate that the IIR signaling pathway can not be the sole essential sheath-cell influence on germline proliferation (Fig. 4; Michaelson et al., 2010). In summary, results are consistent with a model where under replete reproductive conditions the larval germ line responds to insulin signaling to ensure robust germline proliferation that amplifies the germline stem cell population. Taken together with recent studies showing that reducing *daf-2* activity lowered the mitotic index in adult germline tumors caused by a reduction of *gld-1* activity (Pinkston et al., 2006), these results suggest that germline tumor growth control in this mutant may resemble that of the larval germ line.

CONTROL OF ADULT GERMLINE MITOSIS AND MAINTENANCE

In contrast to the larval stages, the sheath cells do not contact much of the proliferative zone in adult hermaphrodites (Killian and Hubbard, 2005), nor does the IIR pathway appear to elevate the cell cycle (Michaelson et al., 2010). Consequently, germline proliferation in the adult maintenance phase lacks sheath cell and IIR regulation, and may rely more exclusively on GLP-1 activity and its downstream consequences (Fig. 4). Based on mutant analysis, it has been suggested that mitosis itself may feed back on *glp-1* activity (Berry et al., 1997; Fig. 4).

ADULT REPRODUCTIVE DIAPAUSE

That the germ line might be capable of renewal was not unexpected given the fecundity of *C. elegans* under replete conditions. However, a recent study suggests that this tissue is capable of an unexpectedly high degree of plasticity for reconstitution after starvation late in larval development (Angelo and Van Gilst, 2009). When early L4 larvae are placed under conditions of extreme starvation and high density, they either arrest as L4's, produce a few embryos that hatch inside of the mother, or undergo "adult reproductive diapause" (ARD). In ARD, the germ line degenerates over the course of 10 days with the exception of ~35 distal cells per gonad arm. Even after 30 days of starvation, upon reintroduction of food the germ line undergoes a very impressive regrowth in as little as 72 hr and viable progeny can be produced. This ability of worms to undergo ARD is largely dependent on the activity of the nuclear hormone receptor *nhr-49* (Angelo and Van Gilst, 2009). These observations are very exciting and suggest the existence of hormonal control of germline plasticity.

PERSPECTIVES

There are number of interesting open questions that, when addressed, will offer considerable additional insight into the control of germline proliferation in *C. elegans*, and likely cell proliferation control in general.

Further characterization of the germline cell cycle at all developmental stages and under different life history conditions is necessary. These investigations will benefit from the characterization of additional cell-cycle markers.

The exact cellular and molecular mechanisms underlying the spatial control of GLP-1 activity and how it impacts individual germ cells in the distal mitotic region remains to be determined. Processes extend from the DTC, but they do not always correlate with the

differentiation border and their precise role, if any, in mediating the GLP-1 signal is not known (Fitzgerald and Greenwald, 1995; Hall et al., 1999; Crittenden et al., 2006). The situation is complicated by the fact that the germ “cells” are actually incompletely cellularized and possess an opening to a core of shared cytoplasm (Hirsh et al., 1976). The role of this core, if any, in transducing the GLP-1 signal is unclear. Although GLP-1 protein accumulates on the surface of germ cells in the distal mitotic region, a direct correlation between the frequency of cell divisions and the accumulation of GLP-1 on the surface of germ cells is not observed (Crittenden et al., 1994; Maciejowski et al., 2006; Ariz et al., 2009). On the contrary, the cells closely opposed to the DTC body appear to divide less frequently than those a few cell-diameters away.

To understand better how GLP-1 signaling impacts fate specification and proliferation, direct targets of GLP-1 and/or reagents to monitor GLP-1 activity (rather than its accumulation alone) need to be identified. Two targets have been found thus far, *fbf-2* and *lip-1* (Lamont et al., 2004; Lee et al., 2006), but they do not account for all the effects of *glp-1* activity. Therefore, it is likely that other targets exist. The network of RNA-binding proteins acting downstream of GLP-1 will undoubtedly continue to provide important mechanistic insights into the control of proliferation and differentiation in the germ line.

Additional questions remain open regarding germline proliferation in response to external factors. The precise mechanisms that prevent cell-cycle progression of Z2 and Z3 in the absence of Z1 and Z4 (or their proliferation) or in the absence of food await identification, as do the signals that promote larval proliferation in response to the gonadal sheath. The recent discoveries of germline-specific responses to nutritional signals provide interesting avenues of investigation into the molecular signals that relay food availability to germ cells at all stages.

While beyond the scope of this review, the proliferating germ line also signals to the soma, most notably in the context of aging (Mukhopadhyay and Tissenbaum, 2007; Berman and Kenyon, 2006).

In general, now that many of the core components of the cell cycle have been identified, investigation of the intersection of cell-cycle control and cell-fate specification *in vivo* is an exciting area (see reviews by Kohlmaier and Edgar, 2008; Budirahardja and Gonczy, 2009). There is both precedent for interplay between cell-cycle and -fate specification, and cases in which the two are genetically separable yet interact to promote proper developmental responses. There is broad precedent for changes in cell-cycle control during development and aging, and for important roles for systemic and environmental factors in this control (Drummond-Barbosa, 2008).

If the past is any indication, studies of proliferation and differentiation in the *C. elegans* germ line will continue to offer valuable molecular and cellular discoveries and paradigms with influence well beyond this fast-growing highly fecund worm.

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Fig. 1.

The *C. elegans* life cycle, germline proliferation, and major diapause points. Adapted from Wood et al. (1980) with permission from the publisher. Developmental time in hours at 25°C. Hatching and molts are indicated in lines intersecting the life-cycle circle. Diapause opportunities are indicated as exit from the life cycle in black arrows, with return to the life cycle in dotted black arrows. Hermaphrodite germ-cell divisions and approximate hermaphrodite germcell counts (total counts including both gonad arms) are indicated in blue. prolif, germ cells in the proliferative zone or distal mitotic region; diff, differentiated germ cells in meiosis or gametogenesis. Circular arrow indicates continuous renewal of adult germ cells.

Fig. 2. Changes in the number of germ-cell nuclei in the proliferative zone over time. Modified from Killian and Hubbard (2005) with permission of the publisher. Dotted line indicates the number of nuclei in the wild type. The effects of sheath cell ablation on the number of proliferative cells in the adult is indicated (either paired SS cell ablation [SS ablation], or distal pair ablation [Sh1 ablation], or ablation of the proximal daughters of the SS cells [Sh2-5 ablation]). X marks the time of ablation while ovals mark the time of germ-cell nuclei quantitation.

Fig. 3.

The distal sheath cells and the proliferative zone. **A:** One of the pair of distal sheath cells (L3-adult) and distal tip cell (L4 and adult) as visualized with *lim-7::GFP (tnIs6)* and *lag-2::GFP(qIs19)*, respectively. Dashed lines outline the L3 germ cells and dotted-line boxes show the area enlarged in the inset. The green bar indicates the approximate reach of the Sh1 cell and arrowheads indicate the position of the border of cells in the proliferative zone and those that have entered meiosis. Distal is to the right and proximal to the left. **B:** Adult gonads after ablation of SS cells or Sh1 cells compared to an unablated control. Reproduced from Killian and Hubbard (2005) with permission of the publisher. Scale bar = ~25 μ m in both panels.

Fig. 4.
Model for influences on larval versus adult germline proliferation. See text for details.