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Chapter 4. Stem cell proliferation versus meiotic fate decision in *C. elegans*

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Abstract

The *C. elegans* germ line has emerged as an important model for understanding how a stem cell population is maintained throughout the life of the animal while still producing the gametes necessary for propagation of the species. The stem cell population in the adult hermaphrodite is relatively large, with stem cells giving rise to daughters that appear intrinsically equivalent; however, some of the daughters retain the proliferative fate while others enter meiotic prophase. While machinery exists for cells to progress through the mitotic cell cycle and machinery exists for cells to progress through meiotic prophase, central to understanding germline development is identifying the genes and regulatory processes that determine whether the mitotic cell cycle or meiotic prophase machinery will be utilized; in other words, the genes that regulate the switch of germ cells from the proliferative stem cell fate to the meiotic development fate. Whether a germ cell self-renews or enters meiotic prophase is largely determined by its proximity to the distal tip cell (DTC), which is the somatic niche cell that caps the distal end of the gonad. Germ cells close to the DTC have high levels of GLP-1 Notch signaling, which promotes the proliferative fate, while cells further from the DTC have high activity levels of the GLD-1 and GLD-2 redundant RNA regulatory pathways, as well as a third uncharacterized pathway, each of which direct cells to enter meiotic prophase. Other factors and pathways modulate this core genetic pathway, or work in parallel to it, presumably to ensure that a tight balance is maintained between proliferation and meiotic entry.

Keywords

C. elegans, germ line; stem cell; proliferative fate; meiotic fate; GLP-1 Notch; GLD-1 pathway; GLD-2 pathway

1. Introduction

Studies focused on understanding the behavior and control of stem cells have garnered much interest over the past few decades due to the fundamental roles that stem cells play in development and tissue homeostasis, as well as for their therapeutic potential. Certain stem cell systems have arisen as important models in pursuing fundamental questions relating to stem cell function, such as the *Drosophila* germ line (Losick et al. 2011), the mouse hematopoietic stem cell system (Bianco 2011), and as discussed here, the *C. elegans*

hermaphrodite germ line. Studying these models has, for example, helped in understanding the molecular factors and physiological attributes that regulate whether a given daughter of a dividing stem cell will remain a stem cell (self-renew), or enter a path to differentiation. While differences have emerged between stem cell systems, such as the utilization of different signaling pathways, or the number of stem cells maintained, similarities between these systems have also become apparent. Perhaps one of the most unifying themes of these stem cell systems is their reliance on a stem cell niche to regulate stem cell behavior.

The *C. elegans* germ line stem cell niche was first identified ~30 years ago when ablation of the distal tip cell (DTC), a somatic cell that caps the distal end of the gonad, resulted in all proliferative cells switching to the meiotic fate (Kimble and White 1981). Much of the work since has involved identifying the molecular factors that emanate from the niche, the factors in the germ cell that perceive and execute this signal, the factors that promote meiotic entry, and determining how these factors work together to control the balance between the proliferative stem cell fate and meiotic differentiated fate (for prior reviews see (Seydoux and Schedl 2001; Hansen and Schedl 2006; Hubbard 2007; Crittenden et al. 2003; Kimble and Crittenden 2007). The factors and mechanisms that regulate the balance between the proliferative and the meiotic fates can be separated from those that are necessary for mitotic cell cycle progression or meiotic prophase progression. In other words, there is a difference between executing the switch that determines the cell fate (which is the primary focus of this review), and fulfilling the normal functions of cells adopting a given fate, although some genes may function separately in the two. The factors involved in regulating the balance between the proliferative and meiotic fates have primarily been identified through the use of powerful genetic screens, generating mutations that contribute either to a loss of the stem cell population, due to premature meiotic entry, or to over-proliferation of the stem cell population, often resulting in tumor formation, due to a failure of meiotic entry. A significant theme that has emerged is the existence of substantial redundancy within the genetic network controlling this balance. This redundancy has made it difficult to identify factors and tease apart their regulatory relationships. However, it has also revealed a system that can be finely tuned. This redundancy may be common to other stem cell systems, providing a barrier to identify and characterize all involved factors, especially in systems that do not have the genetic tools that are available in the *C. elegans* model.

While genetic analysis has revealed significant insight into mechanisms regulating the proliferation vs. meiotic entry decision in *C. elegans*, cell biological analysis of the stem cell population has proved more difficult. This difficulty is due, in part, to the syncytial nature of the germ line; each nucleus and surrounding cytoplasm is not fully enclosed by membranes (although collectively these are commonly referred to as a cell). Therefore, cells within the gonad are interconnected, making transplantation and repopulation experiments virtually impossible, as well as complicating lineage tracing approaches. However, recent work using S-phase labeling approaches to examine cell kinetics has helped in understanding cell behaviors within the proliferative region. These studies have reinforced the idea that, unlike stem cell systems that contain a very small number of asymmetrically dividing stem cells, the *C. elegans* germ line contains a relatively large population of stem cells that give rise to equivalent daughters, but which may eventually assume different fates depending on their position relative to the niche signal. Here we discuss our current understanding of the molecular mechanisms that control the balance between the proliferative fate and meiotic entry in the adult *C. elegans* germ line. Although mechanisms that control this decision appear very similar between the male and hermaphrodite, in this review we will primarily focus on the adult hermaphrodite germ line. Additionally, we focus on how this balance is maintained under optimal laboratory growth conditions (“feasting”), that provides a baseline upon which environmental conditions modify this balance for optimal reproduction, which is discussed in Chapter 5, (Hubbard et al. 2012).

2. Anatomy/cytology of the gonad

As the *C. elegans* hermaphrodite larvae hatches, the gonad primordium consists of the Z2 and Z3 germ cells, the Z1 and Z4 somatic cells, and a surrounding basement membrane. Midway through the first larval stage the germ cells begin to proliferate, with all cells remaining proliferative until midway through the third larval stage, when the first cells to enter meiosis can be detected. By this time two gonad arms have begun to develop, each capped by the somatic DTC (descendants of Z1 and Z4), with the meiotic cells found at the proximal end of each gonad arm, thereby establishing a polarity that will be maintained throughout the remaining reproductive life of the animal. The timing of the onset of germ cell differentiation is tightly controlled relative to somatic gonad development in order for this polarity to be properly established and maintained (McGovern et al. 2009). By the time the worm is nearing the end of larval development, the first differentiated cells have developed as sperm. Approximately 150 sperm are made in each gonad arm, with all subsequent germ cells differentiating as oocytes.

The adult gonad is an assembly line, with germ cells progressing from an undifferentiated stem cell fate in the distal end, to a fully differentiated gamete at the proximal end. This linear progression of gamete formation simplifies the analysis of the progression of germ cells through the various stages of gamete formation. For convenience, the hermaphrodite gonad has been divided into different regions, or zones, in which are cells at specific stages of germ cell development (Figure 1). Cells in the distal end of the gonad arm are in the proliferative, or mitotic, zone, which extends ~20 germ cell diameters from the distal tip (Crittenden et al. 1994; Hansen et al. 2004a). As cells move proximally, out of the proliferative zone, they enter the transition zone, where cells first show evidence of entering into meiotic prophase. Transition zone nuclei have a distinct crescent moon-shaped DNA morphology as the chromosomes pair in leptotene/zygotene of meiotic prophase (Dernburg et al. 1998; Francis et al. 1995a; MacQueen and Villeneuve 2001). Not all cells enter meiotic prophase at precisely the same location in the gonad arm. For example, transition zone nuclei can be found at the same distance from the distal end as cells that are undergoing progression through the mitotic cell cycle, as judged by M-phase figures (Figure 1)(Hansen et al. 2004a; Crittenden et al. 2006; Fox et al. 2011). The transition zone has been defined as the region containing the most distal transition zone nucleus to the most proximal transition zone nucleus (Crittenden et al. 1994; Hansen et al. 2004a). Cells that move proximally from the transition zone enter into the pachytene region, followed by gamete formation.

Some markers have aided in the analysis of cells in these zones. For example, antibodies against the REC-8 protein, when used with mild fixation conditions, stain the nucleoplasm of proliferative cells (Figure 1) (Pasierbek et al. 2001; Hansen et al. 2004a). Likewise, antibodies specific to HIM-3 highlight the meiotic chromosome axis, marking cells that have entered into meiotic prophase (Zetka et al. 1999). The use of these and other markers has emphasized the uneven border between the proliferative and transition zones in wild type animals (Figure 1), as well as allowed assessment of germ cell fate in gonads with various phenotypic defects (Figure 2) or whose DNA morphology is abnormal, such as occurs in cell cycle arrest.

3. The adult proliferative/mitotic zone

The adult proliferative zone is a steady state system of ~230 cells (200–250 range), with M-phase nuclei found throughout the proliferative zone, although at lower frequency more proximally (Crittenden et al. 1994; Hansen et al. 2004a; Francis et al. 1995b; Maciejowski et al. 2006; Jaramillo-Lambert et al. 2007). The cell-cycle kinetics of cells within the

proliferative zone has not been closely examined in real-time in living animals due to the lack of the necessary reagents and techniques; however, experiments using various labeling schemes with BrdU, EdU and fluorescent-nucleotides, each of which are incorporated into DNA during S-phase, have provided insight into the cycling behavior of this cell population (Crittenden et al. 2006; Jaramillo-Lambert et al. 2007; Fox et al. 2011). Currently there are no markers to distinguish between cells in mitotic S-phase from those in meiotic S-phase; however, kinetic analysis of the ~230 cells within the proliferative zone suggests that ~130–160 of these cells are actively cycling, with the remaining ~70–90 cells, residing in the proximal part of the proliferative zone, are in meiotic S-phase (Fox et al. 2011). Overall, the population of ~230 cells produces an output of ~20 cells that enter meiosis each hour (Fox et al. 2011). Two groups have analyzed the total cell cycle length, using similar methods; however, different measures of cell cycle length were obtained, 6.5–8 hrs or 16–24 hrs (Crittenden et al. 2006; Fox et al. 2011). It is unclear as to the cause of this difference in estimates. Cell cycle kinetics analysis indicates that G1-phase is either very short, or absent in cycling adult proliferative zone cells. G2 may thus be a major phase for cell cycle regulation of *C. elegans* germ cells. Consistent with this idea, germ cells in L1 diapause and dauer worms, following starvation, are arrested in G2 (Narbonne and Roy 2006; Fukuyama et al. 2006).

In the *Drosophila* ovary and testis, the stem cell daughter displaced from the niche and committed to differentiation undergoes four stereotypic cystoblast/gonialblast transit-amplifying divisions prior to their entry into meiosis (Losick et al. 2011). Of the ~130–160 mitotically cycling cells within the *C. elegans* adult hermaphrodite proliferative zone it is not known if there are two populations of proliferating cells (stem cells and transit amplifying cells), or a single developmentally equivalent population of cells (see (Seydoux and Schedl 2001)). There are no obvious cytological features, such as asymmetric cell divisions or stereotypic division patterns, as well as companion somatic cells, which might distinguish stem cells from possible transit-amplifying cells (Crittenden et al. 2006). In some stem cell systems (e.g. mammalian hair follicle), the stem cells are slow cycling relative to their transit-amplifying counterparts (Fuchs 2009); however, there are no significant differences in cell cycle length among proliferative zone cells (Hansen et al. 2004a; Maciejowski et al. 2006; Jaramillo-Lambert et al. 2007; Crittenden et al. 2006; Fox et al. 2011). There are no markers that distinguish between specific cells in the proliferative zone, although GLD-1 protein (see below) is very low/absent in germ cells in the distal part of the proliferative zone and then rises to a high level as the cells enter the transition zone (Jones et al. 1996). Recently, suggestive evidence that there are two pools of cells with distinct behaviors has been reported from analysis of the proliferative zone following blocking germ cell movement through cell cycle arrest (Cinquin et al. 2010). However, it is unclear to what extent the phenomena observed following cell cycle arrest can be extended to the behavior of proliferative zone cells in a reproductively active germ line.

The lack of markers that differentiate cells within the proliferative zone has also made it difficult to determine if putative transit-amplifying cells or meiotic S-phase cells are capable of returning to a stem cell fate if they come back in contact with the niche cell (DTC), as has been observed in the *Drosophila* germ line (Kai and Spradling 2004; Brawley and Matunis 2004). Through processes that extend between or around germ cells, the DTC is only in close contact with the most distal three or four rows of germ cells, which together comprise approximately 30 germ cells (Fitzgerald and Greenwald 1995; Crittenden et al. 2006; Hall et al. 1999), although the influence of the DTC appears to extend farther than the region of germ cells it immediately surrounds. The close contact between the germ cells and the DTC processes is likely important for cells to self-renew; however, the factors that mediate this interaction are yet to be identified. The interaction between the DTC and germ cells is likely quite dynamic, which could explain why junctional contacts have not been detected between

the DTC and germ cells (Hall et al. 1999). Given the large amount of bulk flow from distal to proximal, with 20 new cells generated per hour, any return of cells to a more distal position is probably a low frequency event.

Where in the distal germline does the decision to enter meiotic prophase occur? Cells in meiotic S-phase are largely REC-8 positive, HIM-3 negative (Jaramillo-Lambert et al. 2007; Fox et al. 2011), indicating that the bulk of HIM-3 loading onto meiotic chromosome axes occurs after meiotic S-phase. In budding yeast and mouse, the decision to enter meiotic prophase can be viewed as a switch from a mitotic S-phase to a meiotic S-phase (Baltus et al. 2006; Honigberg and Purnapatre 2003). The decision point is likely the same for the *C. elegans*. Thus, cytological markers such as HIM-3 or crescent shaped nuclear DNA morphology assess fate based on when cells enter leptotene, which is temporally displaced from when the switch was initiated at entry into meiotic S-phase. Therefore, a major challenge for the field is to generate molecular markers that subdivide proliferative zone cells into those that are mitotically cycling, in meiotic S-phase, stem cells and transit amplifying cells (if they exist).

The adult hermaphrodite proliferative zone containing 130–160 mitotically cycling cells represents a large stem cell system, relative to those found in other organisms, even if some of these cells are transit-amplifying cells. This large number of stem cells is likely a consequence of the requirement to produce many meiotic cells, most of which function as nurse cells for the forming oocytes prior to their undergoing physiological germ cell apoptosis in late pachytene (Chapter 9, Bailly and Gartner 2012; Chapter 10, Kim et al. 2012). Analysis of the gametogenesis output during oogenesis under optimal growth conditions leads to estimates that 90% or more of pachytene germ cells undergo physiological apoptosis (McCarter et al. 1999; Jaramillo-Lambert et al. 2007; Fox et al. 2011). Thus the, large rapidly dividing, proliferative zone may be necessary to generate the ~20 cells entering meiotic prophase per hour, 90% of which will undergo apoptosis after providing cytoplasmic constituents to the forming oocytes.

4. Phenotypic consequence of a disruption in the balance

The primary method used to study the mechanism by which the balance is maintained between specification of the proliferative fate and meiotic entry is to disrupt this balance. Physical manipulation of the somatic gonad, or disruption of normal gene function, can cause the balance to be shifted to either excessive proliferation or premature meiotic entry. In the most extreme cases of excess proliferation, a germline tumor is formed that only consists of proliferative cells, with no cells entering meiotic prophase (Berry et al. 1997; Hansen et al. 2004a). Conversely, animals with premature meiotic entry, occurring very early in larval development, have a complete depletion of proliferative cells and only ~16 sperm being formed per gonad arm (Kimble and White 1981; Austin and Kimble 1987). Study of the physical manipulations and genetic mutations that result in these extreme phenotypes, as well as more moderate phenotypes, has been essential in teasing apart the molecular mechanisms that control the tight balance between proliferation and meiotic entry.

4.1. Premature meiotic entry results in a Glp phenotype

Significant insight into the proliferative fate versus meiotic entry decision was obtained through ablating the DTC with a laser microbeam (Kimble and White 1981). Ablating the DTC in a given gonad arm resulted in all germ cells prematurely entering meiotic prophase and concomitant loss of the proliferative cell population. Whether ablation occurred in early larval development or later in adulthood, all the proliferative cells entered into meiotic prophase resulting in meiotic/differentiated cells extending to the distal end of the gonad.

This phenotype is referred to as ‘Germ line proliferation abnormal’, or Glp. That ablation of the DTC resulted in Glp animals suggests that the DTC is necessary to promote the proliferative fate and/or inhibit the meiotic fate. Additionally, mis-positioning of the DTC to a more proximal position in the gonad arm resulted in the establishment of a proliferative population of cells adjacent to the new location of the DTC (Kimble and White 1981), suggesting that the DTC is also sufficient to promote the proliferative fate.

Various genetic mutations have been isolated that cause a Glp phenotype, as discussed below. Another class of mutations, as well as environmental treatments, results in an adult proliferative zone that contains fewer cells than wild type. In some cases this is due to a disruption in the proliferation versus meiotic entry cell fate decision (e.g. a *glp-1* temperature sensitive (ts) allele at the permissive temperature). In other cases it is not due to a disruption in this decision and additional experiments are necessary to tease apart the process that is disrupted. For example, RNAi knockdown of numerous cell cycle genes results in a smaller proliferative zone, but this appears to be due directly to a disruption in the mitotic cell cycle (Fox et al. 2011). From the L3 stage to adulthood, the proliferative zone expands from ~20 cells to more than 200 (Killian and Hubbard 2005). The larval proliferative zone expansion is regulated by food status, controlled in part through DAF-2 insulin-like signaling and TGFbeta signaling (Chapter 5, Hubbard et al. 2012). Reduced food or disruption of either pathway leads to an adult containing fewer proliferative zone cells. Mutations that directly or indirectly affect pathways involved in environmental signals controlling larval expansion could thus produce adults with a smaller proliferative zone.

4.2 Over-proliferation results in a germline tumor

As mentioned, a germline tumor is formed when the balance between the proliferative fate and the meiotic fate is shifted toward excess specification of the proliferative fate. However, the severity of the tumor can differ depending on a number of factors, including the degree to which a shift in the balance occurs, or when during development the shift is most pronounced (Figure 2). The most severe tumor, or a complete tumor, contains only proliferative cells with no evidence of any cells entering into meiotic prophase (Berry et al. 1997; Hansen et al. 2004a). Fully tumorous germ lines are thought to be due to a complete failure of cells to switch from the proliferative fate to meiotic prophase. Classification of a tumor as complete generally requires monitoring the germline cells during the development of the animal to ensure that some cells do not enter meiosis early during development. Incomplete tumors can be sufficiently robust that even though some cells enter meiosis early during development, proliferative cells quickly fill the entire gonad, making it difficult to see interspersed meiotic or differentiated cells in the adult.

Animals that have excess germline proliferation, but also contain some cells that enter meiotic prophase, often have what is referred to as a late-onset tumor. Although the severity of a late-onset tumor can vary, in most cases germ cells in these animals initially enter meiosis normally during larval and young adult development, but then a disruption occurs such that proliferative self-renewal outpaces entry into meiosis, resulting in a progressively increasing distal proliferative zone (Berry et al. 1997). The size of the distal proliferative zone is often measured as the number of cell diameters from the distal end of the gonad until the first (most distal) meiotic cell; however, counting the total number of proliferative cells within the proliferative zone is more accurate.

Mutant animals can also have tumors in the proximal end of the gonad, referred to as Pro tumours (Figure 2). Pro tumors are usually due to a disruption of the proliferation versus meiotic entry decision in larval development. Some mutant animals can have both late-onset and Pro tumors, resulting in a region of over-proliferation in the distal end of the gonad, followed by cells in meiotic prophase or possibly differentiated gametes (usually sperm),

and finally another region of over-proliferation in the proximal end of the gonad, next to the spermatheca. Pro tumors are often very robust, quickly filling the entire proximal end of the gonad with proliferative cells. While there appear to be diverse molecular and physiological origins for proximal tumor formation, most, if not all, seem to result in inappropriate contact between undifferentiated proximal germ cells and gonadal sheath cells (Killian and Hubbard 2004; Pepper et al. 2003; Seydoux et al. 1990; Voutev et al. 2006). During normal larval development, there is a period when all germ cells are proliferative, prior to when some proximal cells enter meiotic prophase. The timing of when differentiation first occurs, relative to the timing of somatic gonad formation, is crucial for distal to proximal polarity to be established. If there is a delay in when proximal cells first begin to differentiate, the somatic sheath cells develop to a point in which they produce a signal (GLP-1 Notch ligands) that promotes the proliferative fate (McGovern et al. 2009). These sheath cells function as a 'latent niche', in that normally these sheath cells would not come in contact with proliferating cells, and therefore would not promote the proliferative fate (McGovern et al. 2009). However, since there is delayed differentiation in these mutants, proliferative cells do contact the sheath cells and receive a signal to continue to proliferate, resulting in the formation of a Pro tumor.

The germline tumors discussed thus far are due to a disruption in the balance between specification of the proliferative fate and meiotic entry; therefore, studying the causative mutations has provided tremendous insight into the molecular mechanisms involved in controlling this balance (see below). However, it should be emphasized that germline tumors can be formed that are unrelated to the proliferative fate vs. meiotic entry decision. For example, animals that are mutant for *gld-1* alone, or *puf-8* alone, can form de-differentiation tumors (Francis et al. 1995a; Subramaniam and Seydoux 2003). In these mutant animals, a proliferative population of cells exists in the distal end of the gonad arm that is very similar in size to wild-type animals, and cells appear to enter into meiotic prophase normally. However, after entry into meiotic prophase the cells fail to properly progress through meiosis (oogenesis for *gld-1* and spermatogenesis for *puf-8*), and return to a mitotic cell cycle. This results in a proliferative population of cells in the proximal end of the gonad. These proximal mitotic cells receive a signal from the 'latent niche', described above, that contributes to the robustness of this proximal tumor, but is not required for its formation (Francis et al. 1995b). As the animal ages, the tumor continues to grow such that it is no longer restricted to the proximal end. Therefore, if the gonads are analyzed when the animals are quite old, it is possible to misinterpret the cause of the tumor as being due to a defect in the proliferative fate vs. meiotic entry decision.

5. The core genetic pathway

5.1. GLP-1 Notch signaling promotes the proliferative fate

The DTC ablation experiments, which resulted in a Glp phenotype, identified the DTC as the major regulator of the proliferative fate in the germ line (Kimble and White 1981). Genetic screens were performed to determine the molecular signal that promotes the proliferative fate. From these screens, components of the conserved Notch signaling pathway were identified as being the molecular mechanism through which the DTC exerts its influence (Figure 3). Disruption in the activities of the DSL (Delta/Serrate/LAG-2) LAG-2 and APX-1 ligands (Henderson et al. 1994; Tax et al. 1994; Nadarajan et al. 2009), GLP-1 Notch receptor (Austin and Kimble 1989; Yochem and Greenwald 1989), or CSL (CBF1/Suppressor of Hairless/LAG-1) LAG-1 transcription factor (Christensen et al. 1996), all conserved components of the Notch signaling pathway, result in Glp animals (Lambie and Kimble 1991; Austin and Kimble 1987; Yochem and Greenwald 1989). The basic model of how GLP-1 Notch signaling promotes the proliferative fate is as follows. When LAG-2 and APX-1, which are expressed in the DTC (Henderson et al. 1994; Nadarajan et

al. 2009), come in contact with the GLP-1 receptor, which is expressed on the surface of the germ cells (Crittenden et al. 1994), the intracellular domain of GLP-1, referred to as GLP-1(ICD) or GLP-1(INTRA), is thought to move to the nucleus and complex with the LAG-1 transcription factor and the SEL-8 (a.k.a. LAG-3) transcriptional co-activator (Mumm and Kopan 2000; Petcherski and Kimble 2000; Doyle et al. 2000). This complex is then thought to activate transcription of genes necessary for the proliferative fate. As germ cells migrate proximally, away from the DTC, they no longer contact the DTC and bound ligand; therefore, GLP-1 Notch signaling levels are thought to decrease, allowing for cells to enter into meiotic prophase.

However, it should be noted that certain aspects of GLP-1 Notch signaling described above have not fully been demonstrated in the *C. elegans* germ line, but rather are based on a description of Notch signaling in other systems. For example, although GLP-1 has been detected on the surface of distal germ cells (Crittenden et al. 1994), GLP-1(ICD) has not been visualized in the germ line; therefore, its temporal distribution is currently unknown. Furthermore, a direct read-out of GLP-1 Notch signaling in the germ line has not yet been established. Therefore, the spatial pattern of GLP-1 Notch signaling is not precisely known.

5.2. Redundant genetic pathways function downstream of GLP-1 Notch signaling

The major mechanism by which GLP-1 Notch signaling maintains cells in the proliferative fate is through inhibiting the activities of RNA regulatory pathways in the distal end of the gonad that serve to promote meiotic entry and/or inhibit the proliferative fate (Figure 3). The two main pathways are the GLD-1 and GLD-2 pathways, named for the first genes identified in each pathway (Kadyk and Kimble 1998; Francis et al. 1995a; Francis et al. 1995b). The GLD-1 and GLD-2 pathways function redundantly, such that if the activity of only one of the two pathways is reduced or eliminated, the balance between proliferation and meiotic entry is similar to wild-type; however, if the activities of both pathways are reduced or eliminated, over-proliferation occurs resulting in a germline tumor (Kadyk and Kimble 1998; Hansen et al. 2004b; Eckmann et al. 2004). Therefore, either pathway alone is sufficient to cause germ cells to enter meiosis. *gld-1* encodes a translational inhibitor that is homologous to mouse Quaking (Jones and Schedl 1995; Lee and Schedl 2010), and has been shown to bind the 3 UTRs of mRNAs to inhibit their translation (Jan et al. 1999; Lee and Schedl 2001; Marin and Evans 2003; Lee and Schedl 2004; Wright et al. 2011; Jungkamp et al. 2011). Also within the *gld-1* pathway is the *nos-3* gene (Hansen et al. 2004b), which encodes a protein related to the *Drosophila* translational regulator Nanos (Kraemer et al. 1999). Within the *gld-2* pathway are *gld-2* and *gld-3* (Kadyk and Kimble 1998; Eckmann et al. 2004); *gld-2* encodes the catalytic portion of a poly(A) polymerase (Kadyk and Kimble 1998), while *gld-3* encodes a BicC homologue (Eckmann et al. 2002). GLD-2 and GLD-3 bind to one another and GLD-3 enhances GLD-2 activity (Eckmann et al. 2004).

The overall model of how the GLD-1 and GLD-2 pathways regulate the proliferation vs. meiotic entry decision is that their activities are thought to be low or absent in the distal end of the gonad where GLP-1 Notch signaling levels are high, but as cells move more proximally, away from the influence of the DTC, GLP-1 Notch signaling levels decrease, causing GLD-1 and GLD-2 pathway activity to increase, which in turn allows cells to enter into meiotic prophase (Figure 3). Perhaps the regulation of this reciprocal level of activity between GLP-1 Notch signaling and the GLD-1 and GLD-2 pathways is best described for GLD-1. GLD-1 protein accumulation levels are low or absent in the distal end of the gonad, but rise gradually in the proliferative zone until reaching maximum levels in the distal portion of the transition zone, where cells show the first signs of having entered into meiotic prophase (Jones et al. 1996). If GLP-1 Notch signaling levels are eliminated, GLD-1 levels increase more than 10 fold in the distal portion of the gonad (Hansen et al. 2004b), suggesting that GLP-1 Notch signaling suppresses GLD-1 accumulation in the distal end.

Furthermore, increasing GLD-1 levels in the distal end causes cells to enter meiosis prematurely (Hansen et al. 2004b), suggesting that the low level of GLD-1 in the distal end allows cells to remain proliferative. This also suggests that the rise in GLD-1 levels in wild-type animals promotes the transit of cells from the proliferative fate to meiotic prophase. While GLD-2 and GLD-3 also show non-uniform accumulation patterns in the gonad (Wang et al. 2002; Eckmann et al. 2002; Eckmann et al. 2004), the variations in their spatial distribution patterns is significantly more subtle than the GLD-1 accumulation pattern, and the importance of their spatial distributions in regulating the proliferative fate vs. meiotic entry decision has not been established.

GLD-1 and NOS-3 are RNA binding translational inhibitors, while GLD-2 is a poly(A) polymerase catalytic subunit that appears to be targeted to certain mRNAs by GLD-3 (Chapter 8, Nousch and Eckmann 2012). Therefore, genes downstream of these pathways are likely to be regulated at the level of translational control and/or mRNA stability. However, the identification of genes that function downstream of these pathways is still in its early stages. Also, there is cross-talk between the two pathways with GLD-2/GLD-3 promoting the activity of *gld-1* (Hansen et al. 2004b; Suh et al. 2006). However, GLD-2/GLD-3 must have targets in addition to *gld-1*, otherwise a *gld-2* or *gld-3* single mutant would have the same phenotype as a *gld-2 gld-1* or *gld-1; gld-3* double mutant. Immunoprecipitation of GLD-1 has identified many mRNAs that are bound by GLD-1 (Lee and Schedl 2001; Wright et al. 2011; Jungkamp et al. 2011); however, only a limited number of these are likely to be involved in the proliferative fate vs. meiotic entry decision. GLD-1 has many functions in germline development, including sex-determination and meiotic prophase progression of female germ cells (Francis et al. 1995a; Francis et al. 1995b). Therefore, any given mRNA may have a role specific to only one of GLD-1's functions in germline development. Given our current understanding of the molecular functions of GLD-1 and GLD-2/GLD-3, with GLD-1 being an RNA binding translational inhibitor and GLD-2/GLD-3 functioning as a poly(A) polymerase that likely stabilizes mRNAs, GLD-1 and GLD-2/GLD-3 likely have RNA targets with opposite functions: GLD-1 likely represses the translation of mRNAs that promote the proliferative fate, while GLD-2 likely promotes the translation of mRNAs that promote the meiotic fate.

5.3. A third genetic pathway functions redundantly with the *gld-1* and *gld-2* pathways

Although the GLD-1 and GLD-2 pathways are the major known players functioning downstream of GLP-1 Notch signaling to regulate the balance between proliferation and meiotic entry, GLP-1 Notch signaling inhibits the activity of an additional unknown factor(s) that inhibits the proliferative fate and/or promotes meiotic entry. We suggest that there is a third redundant pathway that functions in parallel to the GLD-1 and GLD-2 pathways (Hansen et al. 2004a; Fox et al. 2011). Perhaps the strongest evidence for this third pathway comes from analysis with the CYE-1/CDK-2 cell cycle regulator, which will be discussed in more detail below. In brief, CYE-1/CDK-2 promotes the proliferative fate in the *C. elegans* germ line (Fox et al. 2011; Jeong et al. 2011). In animals that have mutations in both the GLD-1 and GLD-2 pathways, and also have reduced *cye-1/cdk-2* activity, significantly more meiotic entry is observed than in just the GLD-1 and GLD-2 pathway double mutant (Fox et al. 2011). However, meiotic cells are not observed in the most distal end of the gonad in these triple mutants, where GLP-1 Notch signaling is thought to be at its highest level. When GLP-1 Notch signaling is also removed in these triple mutants, meiotic cells become apparent in the distal end of the gonad. Therefore, even when the activities of the GLD-1 and GLD-2 pathways are eliminated, GLP-1 Notch signaling still promotes the proliferative fate in the distal end of the gonad by inhibiting the activity of this third pathway. However, the relative strength of this third pathway may be lower than the other two pathways because its activity alone is not sufficient to cause cells to enter meiotic

prophase; *gld-3 nos-3* double mutants have completely tumorous germ lines, with no evidence of cells entering meiotic prophase, even though the third pathway should still be active (Fox et al. 2011). No components of this third pathway have yet been identified.

5.4. GLP-1 Notch signaling functions, in part, through FBF

Since Notch signaling is thought to *activate* the transcription of downstream genes, but GLP-1 Notch signaling *inhibits* the activities of the GLD-1 and GLD-2 pathways, this inhibition must be indirect. Inhibition of the GLD pathways is accomplished, in part, through the activities of the *fbf-1* and *fbf-2* genes. FBF-1 and FBF-2, collectively referred to as FBF, are homologous to the *Drosophila* Pumilio translation regulator (Zhang et al. 1997) and have largely redundant functions relative to the proliferation vs. meiotic entry decision. *fbf-2* is directly regulated by GLP-1 Notch signaling; it contains LAG-1 binding sites in its promoter region that can be bound by LAG-1 *in vitro*, and FBF-2 protein accumulates in the distal region of the gonad arm, including where GLP-1 Notch signaling levels are thought to be the highest (Crittenden et al. 2002). Interestingly, even though the functions of *fbf-1* and *fbf-2* are largely redundant, only *fbf-2* appears to be directly regulated by LAG-1. When the activities of both *fbf-1* and *fbf-2* are eliminated, germ cells enter into meiosis prematurely (Crittenden et al. 2002). However, the premature meiotic entry, or Glp, phenotype is not as severe as when GLP-1 Notch signaling is eliminated; the proliferative population of cells is not fully depleted until adulthood, resulting in many more differentiated gametes in *fbf-1 fbf-2* double null mutants than in *glp-1* null mutants, and depletion does not occur at all at higher temperatures (Crittenden et al. 2002). This weaker Glp phenotype suggests that other factors function redundantly with FBF, or at different points in development, to promote the proliferative fate. One of these redundant factors is FOG-1 (Thompson et al. 2005), which is an RNA binding protein of the CPEB family (Jin et al. 2001; Luitjens et al. 2000). While the Glp phenotype of *fbf-1 fbf-2* double mutants is less severe than when GLP-1 Notch signaling is eliminated, *fog-1; fbf-1 fbf-2* triple mutants display the more severe Glp phenotype (Thompson et al. 2005). This strong Glp phenotype reveals a redundant function for FBF in promoting the proliferative fate during early larval development, when germ cells are undergoing male development. However, FOG-1 is absent in late larvae and adults when germ cells are undergoing female development, so there must be additional genes that are redundant with *fbf-1 fbf-2*. While FBF functions redundantly with FOG-1, it also appears to promote FOG-1 activity by maintaining FOG-1 levels at low level: if FOG-1 levels increase above a threshold, FOG-1 no longer promotes the proliferative fate (Thompson et al. 2005).

The *fbf-1 fbf-2* double mutant Glp phenotype is consistent with these genes functioning redundantly to promote the proliferative fate; however, the single mutant phenotype of *fbf-2* is not consistent with the predicted phenotype of a direct LAG-1 target. As mentioned, GLP-1 Notch signaling is thought to transcriptionally activate target genes that promote the proliferative fate. Therefore, the predicted loss of function phenotype of LAG-1 target genes would be a decrease in proliferation. However, *fbf-2* single mutants have a larger proliferative zone as compared to wild-type animals, implying that *fbf-2* may function to inhibit the proliferative fate (Lamont et al. 2004). This inconsistent phenotype may have to do with inhibitory feedback between FBF-1 and FBF-2, as they bind to each other's 3 UTRs (Lamont et al. 2004). Therefore, in an *fbf-2* mutant, FBF-1 levels may increase above normal levels, thereby causing an increase in the proliferative fate. Alternatively, FBF-2 may function to both promote the proliferative fate and inhibit the proliferative fate. Indeed, *gld-1; fbf-1 fbf-2* triple null mutants have a tumorous germline (Crittenden et al. 2002), suggesting that *fbf* may function in the GLD-2 pathway to inhibit the proliferative fate (Hansen and Schedl 2006). Additionally, data suggests that FBF also *promotes* GLD-1 accumulation (Suh et al. 2009). Recently, >1000 mRNA targets of FBF were identified, although most still need to be verified (Kershner and Kimble 2010). Interestingly,

identification of certain targets has revealed that FBF not only has targets involved in regulating the switch between the proliferative and meiotic fates (see below), but also has targets directly involved in the execution of meiotic development. For example, FBF binds to and regulates the translation of *him-3*, *htp-1*, *htp-2*, *syp-2* and *syp-3* mRNAs, each of which encode structural components of the synaptonemal complex that forms between paired meiotic chromosomes (Merritt and Seydoux 2010).

5.5 FBF inhibits *gld-1* mRNA translation

FBF appears to promote the proliferative fate, at least in part, by inhibiting the activities of the GLD-1 and GLD-2 pathways, thereby providing the link between GLP-1 Notch signaling and the downstream GLD-1 and GLD-2 redundant pathways. FBF binds to the 3' UTR of *gld-1* mRNA (Suh et al. 2009), and reducing GLD-1 dose partially suppresses the FBF Glp phenotype (Crittenden et al. 2002). Furthermore, GLD-1 levels increase in the distal end in *fbf-1* single mutants. Together, these data suggest that FBF binds to *gld-1* mRNA in the distal end of the gonad, preventing the accumulation of GLD-1 protein. Likewise, FBF appears to inhibit the activity of the GLD-2 pathway by binding to the 3' UTR of *gld-3* and preventing the accumulation of GLD-3 protein in at the distal end of the gonad in larvae (Eckmann et al. 2004), although it is unclear if this regulation occurs in the adult. Given that both the GLD-1 and GLD-2 pathways are each sufficient for cells to enter meiotic prophase, complete activation of either of these pathways should result in a strong Glp phenotype. Therefore, since the *fbf-1 fbf-2* double mutant Glp phenotype only occurs in adults, FBF cannot be the sole inhibitor of these pathways. Furthermore, in certain genetic backgrounds that include a lack of FBF activity (e.g. *fog-3; fbf-1 fbf-2 nos-3* animals), germ cells proliferate, enter meiosis and control GLD-1 levels similar to what is observed in wild-type animals (Hansen et al. 2004b), providing additional support for the existence of players, in addition to FBF, in inhibiting the GLD-1 and GLD-2 pathways.

6. Connection between the mitotic cell cycle and the proliferation vs. meiotic entry decision

Two studies have implicated the cell cycle regulators cyclin E (CYE-1) and Cdk2 (CDK-2) in regulating the proliferative fate vs. meiotic entry decision (Fox et al. 2011; Jeong et al. 2011). RNAi against *cye-1* or *cdk-2* enhances the premature meiotic entry defect of a weak *glp-1* loss-of-function allele (Fox et al. 2011). RNAi against other cell cycle regulators leads to cell cycle arrest and a smaller proliferative zone size but does not enhance *glp-1*, suggesting that it is not simply a disruption of the cell cycle that causes premature meiotic entry, but rather that *cye-1/cdk-2* are specifically involved in regulating this process (Fox et al. 2011). GLD-1 is a likely phosphorylation target of CYE-1/CDK-2, and reduction of *cye-1* activity results in an increase in GLD-1 accumulation in the distal end of the gonad (Jeong et al. 2011), suggesting that *cye-1/cdk-2* may function through *gld-1* to regulate the proliferative fate vs. meiotic entry decision. However, reduction of *cye-1/cdk-2* causes premature meiotic entry even in the absence of *gld-1* and *gld-2* activity (Fox et al. 2011). Therefore, *cye-1/cdk-2* cannot solely function through *gld-1* in regulating the proliferation vs. meiotic entry decision; *cye-1/cdk-2* must have an additional target downstream, or parallel to, the GLD-1 and GLD-2 pathways. One proposed model is that CYE-1/CDK-2 may limit the amount of time in certain parts of the cycle when cells may be receptive to differentiation factors. Alternatively, CYE-1/CDK-2 may have phosphorylation targets, like GLD-1, that are involved in regulating the proliferative fate vs. meiotic entry decision, but which do not have a direct role in controlling the cell cycle.

CYE-1 protein levels and activity (as judged by pCDC-6 staining) are high throughout the proliferative zone and fall abruptly as cells enter the transition zone (Brodigan et al. 2003;

Fox et al. 2011). The loss of CYE-1 appears to be through redundant mechanisms of GLD-1 mediated translational repression (Biedermann et al. 2009) and SCF mediated proteosomal degradation (Fox et al. 2011). However, ectopic CYE-1 in transition zone cells due to removal of both inhibitory processes did not result in continued proliferation, suggesting that yet an additional inhibitory mechanism is in play. The additional mechanism may involve CKI-2, a Cip/Kip protein that has been shown to inhibit Cyclin E/Cdk2 in other systems (Kalchhauser et al. 2011). CKI-2 levels are low or absent in the proliferative zone, due to repression by FBF, but increase as cells enter meiotic prophase, suggesting a model in which CKI-2 inhibits CYE-1/CDK-2 activity to allow cells to enter into meiotic prophase, although inhibition of CYE-1/CDK-2 by CKI-2 has not yet been shown to occur in the germ line. Supporting this model, loss of *cki-2* activity suppresses the premature meiotic entry phenotype of *fbf-2 fbf-1* double mutants (Kalchhauser et al. 2011). However, *cki-2* single mutants do not have a proliferative fate vs. meiotic entry defect (Buck et al. 2009; Kalchhauser et al. 2011), consistent with the presence of multiple redundant mechanisms. A key remaining puzzle is that the fall in CYE-1 level and activity, as well as rise in CKI-2 level, occurs when cells enter meiotic prophase at the transition zone, after completion of meiotic S-phase, and presumably, after implementation of the cell fate switch. Perhaps alterations in yet another factor within the proliferative zone, which controls CYE-1/CDK-2 substrate specificity, for example, triggers the switch to meiotic S-phase in the proximal region of the proliferative zone.

7. Splicing cascade functions in the proliferative fate vs. meiotic entry decision

A number of splicing factors have been implicated in regulating the proliferative fate vs. meiotic entry decision (Belfiore et al. 2004; Kerins et al. 2010; Mantina et al. 2009; Zanetti et al. 2011; Puoti and Kimble 1999, 2000; Wang et al. 2012). Mutants in genes encoding these factors all show similar interactions with the genetic pathway regulating this decision. A complete reduction of the activity of many splicing factors is lethal; however, a partial reduction of splicing factor activity, or more complete reduction of the activities of non-essential splicing factors, enhances the over-proliferation phenotype of *glp-1(gf)* mutants. Additionally, splicing factor mutants show a synthetic over-proliferation phenotype with mutants of the GLD-2 pathway genes, *gld-2* and *gld-3*, suggesting that they likely function, at least in part, in the GLD-1 pathway. Consistent with them functioning in the GLD-1 pathway, downstream of GLP-1 Notch signaling, the synthetic over-proliferation phenotype with *gld-3* null is epistatic to a *glp-1* null mutant.

An RNAi screen of 114 splicing factors identified 31 that enhance the over-proliferation phenotype of *glp-1(gf)*, in addition to some that cause a synthetic tumor in *gld-3(0)* animals (Kerins et al. 2010). These splicing factors are found throughout the splicing cascade, suggesting that there is not a single aspect of splicing that has been co-opted for a function in the proliferative fate vs. meiotic entry decision. Indeed, it is likely that many more splicing factors are involved in this decision but were not identified in the RNAi screen because there is likely a 'sweet spot' of gene reduction in which over-proliferation can be observed. Splicing factor activity would need to be reduced enough to cause a phenotype; however, if it is reduced too much, many cell functions may be disrupted and mask a proliferative fate vs. meiotic entry phenotype. Therefore, it is likely that many more splicing factors than those identified are involved in regulating the proliferative fate vs. meiotic entry balance.

Certainly, the primary question regarding the involvement of splicing factors in regulating this decision pertains to the precise mechanism by which they exert their influence. Perhaps the most straight-forward model is that the reduction in splicing factor activity results in

reduced splicing efficiency, and that certain genes functioning in the proliferative fate vs. meiotic entry decision are particularly susceptible to lower levels of splicing efficiency. Lowering of splicing efficiency could result in mis-splicing, or inappropriate alternative splicing. However, attempts to identify mis-splicing in these mutants have thus far been unsuccessful. These attempts have primarily involved a candidate gene approach; therefore, it is possible that the right candidate(s) has not yet been tested. It is also possible that the level of mis-splicing of a target needed to cause the over-proliferation phenotype may be quite low, and that the techniques used are not sensitive enough to detect this low level of mis-spliced product.

An alternative model for the involvement of splicing factors in regulating the proliferative fate vs. meiotic entry decision is that lowering of splicing factor activity could affect aspects of mRNA function other than splicing, such as the ability of the mRNA to be translated or translationally regulated in the cytoplasm. It is known that splicing factors are involved in formation of ribonucleo-protein complexes on the mRNA, such as the Exon Junction Complex (EJC). If splicing factor activity is reduced, this may affect the ability of such protein complexes to form and the mRNA to be properly transported, translated or translationally regulated.

8. Additional factors and/or pathways involved

The basic model for how the proliferation vs. meiotic entry decision is regulated consists of high GLP-1 Notch signaling in the distal end promoting the proliferative fate, and high levels of RNA regulatory genes more proximally allowing for meiotic entry (Figure 3). However, many other factors and pathways have been identified that interact with this basic genetic network. For many of these factors, it is only in highly sensitized genetic backgrounds that a role in the proliferative fate vs. meiotic entry decision is revealed.

For example, from a mutant screen that utilized a *gld-2* null sensitized background a partial loss-of-function allele of a proteasome subunit was isolated (MacDonald et al. 2008). Genetic analysis revealed that the proteasome functions in at least two places in the proliferative fate vs. meiotic entry genetic pathway to inhibit the proliferative fate. First, it inhibits GLP-1 Notch signaling, presumably to degrade GLP-1(ICD). Proteasomal degradation of the intracellular portion of Notch has been identified as an important means of decreasing Notch signaling levels in many systems (Lai 2002; Andersson et al. 2011). Second, it functions in the GLD-1 pathway. As mentioned above, GLD-1 protein levels increase as cells enter meiotic prophase, and it functions as a translational inhibitor. Therefore, GLD-1 likely acts to repress the translation of the genes that are necessary for the proliferative fate. However, since cells are moving along the gonad arm, proliferative fate promoting proteins, which were made while the cell was in the proliferation zone, may persist in the cell as it moves proximally to the transition zone, even though GLD-1 prevents new translation of these proliferative proteins. Therefore, the current model is that the proteasome degrades these proliferative proteins that persist as the cell moves proximally, allowing the cell to enter meiotic prophase. Indeed, this model holds true for at least one GLD-1 target, *cye-1*, as described above.

Many of the factors that have been implicated in regulating the proliferation vs. meiotic entry decision also have other roles in germ line development and function. For example, HIM-17 was first identified due to its role in the execution of double strand breaks necessary for meiotic recombination (Reddy and Villeneuve 2004). While the single mutant appears to have a normal balance between proliferation and meiotic entry, *him-17* mutants enhance the tumorous phenotype of a *glp-1* gain-of-function allele (Bessler et al. 2007). The position of HIM-17 in the cell fate decision is uncertain, but appears not to function in the GLD-1 or

GDL-2 pathways as a synthetic tumorous phenotype was not observed in double mutants with canonical null alleles of *gld-1* or *gld-2* pathway genes (A. Mohammad and TS, unpublished).

METT-10, the ortholog of vertebrate METT10D methyltransferase, also interacts with the core genetic pathway to regulate the proliferative fate vs. meiotic entry decision. A function for *mett-10* in this decision was first identified through the novel *mett-10(oz36)* allele, whose poison protein product results in a partially penetrant late-onset germline tumor (Dorsett et al. 2009). This tumor is dependent on GLP-1 Notch signaling, suggesting that METT-10 normally inhibits the proliferative fate by negatively regulating GLP-1 Notch signaling. Loss-of-function alleles of *mett-10* do not cause a germ line tumor to form; however, they do enhance over-proliferation in animals carrying weak *glp-1* gain-of-function alleles (Dorsett et al. 2009). Therefore, it is only through a poison protein product, or in a sensitized genetic background, that a role for *mett-10* in the proliferative fate vs. meiotic entry decision is apparent, further emphasizing the redundancy that exists in the genetic pathway regulating this decision.

puf-8 was recently identified as being an inhibitor of the proliferative fate, as loss of PUF-8 activity strongly enhanced the tumorous phenotype of weak *glp-1* gain-of-function alleles (H. Rancher and DH, unpublished). PUF-8 belongs to the same family of Pumilio homologues as FBF-1 and FBF-2 (Crittenden et al. 2002), which function to promote the proliferative fate and/or promote meiotic entry; therefore, it is intriguing that Pumilio homologs appear to function in both directions in the balance between the proliferative fate and meiotic entry. As mentioned earlier, in a proportion of *puf-8* single mutants male germ cells fail to properly progress through meiotic prophase, dedifferentiate and enter the mitotic cell cycle, forming a proximal tumor (Subramaniam and Seydoux 2003). This role of *puf-8* is distinct from its role in regulating the balance between the proliferative fate and meiotic entry. Therefore, *puf-8* inhibits the proliferative fate in at least two points in germline development.

While the factors discussed thus far in this section all enhance over-proliferation when their activity is reduced, suggesting that they normally function to inhibit the proliferative fate and/or promote meiotic entry, other factors have been identified that normally function in the opposite direction, to promote the proliferative fate and/or inhibit meiotic entry. For example, five *ego* genes were identified in a genetic screen for mutations that enhance the Glp phenotype of a *glp-1* partial loss-of-function allele (Qiao et al. 1995). EGO-1 is an RNA-dependent RNA polymerase and functions in parallel to GLP-1 Notch signaling (Vought et al. 2005; Smardon et al. 2000). EGO-1 also has roles in other aspects of germline development, including proper P-granule and nuclear pore complex assembly (Vought et al. 2005). Additionally, *ego-1* mutants disrupt the effectiveness of RNA interference and proper heterochromatin assembly (Maine et al. 2005; Smardon et al. 2000). It is currently unclear if each of these functions for EGO-1 is independent, or if EGO-1 has one (or few) function that influences the other processes.

Like the *ego* genes, *atx-2* also promotes the proliferative fate. Dominant mutations in the human homologue of *atx-2* cause the neurodegenerative disorder spinocerebellar ataxia type 2 (SCA2) (Sanpei et al. 1996); however, its molecular function is not well understood. A reduction in *atx-2* function enhances the Glp phenotype of a weak *glp-1* loss-of-function allele (Maine et al. 2004); however, ATX-2 does not appear to function as a positive regulator of GLP-1 Notch signaling (Ciosk et al. 2004; Maine et al. 2004). Rather, it may function parallel to GLP-1 Notch signaling, or it could be involved in the translational regulation of mRNA targets of GLD-1 (Ciosk et al. 2004).

These are just some of the factors that influence the activity of, or work in parallel to, the core genetic pathway that regulates the proliferation vs. differentiation decision. While the activities of some of these factors may be spatially regulated and directly involved in regulating the transition from the proliferative fate to meiotic entry, others may ensure that the field of cells is competent to respond to the regulatory signals provided by the core genetic pathway.

8. Teratoma, totipotency and reprogramming

Germ cells are totipotent; from germ cells, all cells and tissues of offspring are formed. In order for totipotency to be maintained in the germ line, the activities of differentiation genes must be suppressed. However, after fertilization, the activities of many of these differentiation genes must increase in order to drive embryogenesis. Therefore, many genes necessary for embryogenesis are transcribed in the gonad, but are masked by RNA binding translational repressors until the activities of these genes are needed for embryogenesis. GLD-1, in addition to its role in regulating the proliferative fate vs. meiotic entry decision, is also involved in masking maternal mRNAs of a number of differentiation genes. Loss of *gld-1* activity results in female germ cells failing to properly progress through meiotic prophase, and subsequently re-entering the mitotic cell cycle (Francis et al. 1995a). These mitotic cells then proliferate, forming a proximal tumor. Using somatic cell markers, it was found that a number of the mitotic cells within a *gld-1* tumor were not undifferentiated totipotent cells, but rather were differentiating as somatic cells, analogous to those in a human teratoma (Biedermann et al. 2009; Ciosk et al. 2006). The extent of teratoma formation is increased in *gld-1* mutants if the activity of *mex-3*, another translational regulating RNA binding protein, is also reduced (Ciosk et al. 2006). It is thought that MEX-3 and GLD-1 normally bind to and repress target mRNAs in order to repress differentiation genes, thereby preventing teratoma formation. At least one of these mRNA targets is *pal-1*, which encodes a homeobox transcription factor that promotes muscle development (Ciosk et al. 2006). Both MEX-3 and GLD-1 inhibit *pal-1* translation, and PAL-1 ectopic expression in *mex-3 gld-1* double mutants contributes to teratoma formation (Mootz et al. 2004; Ciosk et al. 2006).

The cells that become the teratoma in *mex-3 gld-1* animals successfully entered into meiotic prophase, but then failed to progress through meiosis and re-entered the mitotic cell cycle. However, cells within the proliferative zone also must maintain their totipotency. Recently, it was found that over-expression of the transcription factors CHE-1, UNC-3 or UNC-30 resulted in the reprogramming of mitotic germ cells to glutamatergic, cholinergic or GABAergic neurons, respectively (Tursun et al. 2011). However, this conversion only occurred if the activity of LIN-53, a histone chaperone, was also removed. Therefore, it is thought that one mechanism to maintain totipotency in germ cells is through chromatin factors that render the DNA inaccessible to differentiation promoting transcription factors.

9. Compare and contrast with the *Drosophila* germ line

In order for dividing stem cells to be maintained in any system, a balance must be maintained between the number of cells that self-renew, and the number of cells that enter a differentiation pathway. As we have described, in *C. elegans* this balance is achieved on a population basis, with the proximity to the DTC niche being the primary determinant of the proliferative or meiotic fates (Figure 4). While this system may appear quite different than many other systems that rely on asymmetric division to specify fate, there are common themes between these systems. The *Drosophila* female germ line is a prime example of a stem cell system that relies on asymmetric cell division (Figure 4). In this system, the niche consists of a Cap cell, working with the terminal filament and escort cell. The GSC directly

contacts the Cap cell, and is held in place via adherens junctions. The Cap cell utilizes the Jak/Stat pathway to signal the GSC, preventing the transcription of the *bag-of-marbles* differentiation gene. When the GSC divides, one daughter (usually the proximal), remains attached to the Cap cell, while the other daughter is positioned on the opposite side of its sister from the Cap cell. This difference in contact to the Cap cell results in an asymmetric outcome; with the sister contacting the Cap cell remaining a GSC, while the other cell enters a differentiation pathway. However, it should be emphasized, and as has been recently discussed (Losick et al. 2011), the asymmetry between sister cells does not appear to be due to intrinsic differences between the cells, but rather due to differences, based on position, in extrinsic signals. Space constraints around the Cap cell usually limit only one of the sister cells to remain attached to the Cap cell (Figure 4). The *Drosophila* male germline stem cells also utilize asymmetric division to maintain the balance between the proliferative fate and differentiation. A regulated spindle orientation ensures that one daughter cell of the dividing stem cell remains attached to the hub cells, which make up the niche, while the other daughter cell is one cell diameter removed from the hub cells. However, as with the female, the proliferating vs. differentiating fate of the cells appears to rely on differences in external signals rather than on intrinsic differences.

Comparing these *Drosophila* stem cell systems to the *C. elegans* system reveals some differences. For example, in the *C. elegans* proliferation zone the total number of stem cells appear to be larger, the stem cells do not appear to require direct contact with the niche cell to self-renew, and dividing stem cells do not result in asymmetric outcomes (Figure 4). However, these differences should not distract from the overarching themes that are common to these, and perhaps most, stem cell systems. For example, that the proximity to the niche is the primary determinant of whether daughters of a dividing stem cell self-renew or differentiate. In *Drosophila*, the niche zone of influence is only one cell diameter (Figure 4), with many factors, including the extracellular matrix, ensuring that its influence does not extend beyond this boundary. In *C. elegans*, the DTC does not appear to require direct contact with cells to specify their fate, although the presence of extended DTC membrane and processes is a distinguishing feature. Additionally, factors have been identified, such as the proteasome, which appear to limit the range of signaling. As a second example of similarity between these systems, both stem cell daughters are capable of self-renewal, and that it is differences in external signals that determines fate. In *Drosophila*, that both daughters are intrinsically capable of self-renewal is best exemplified by cells that are in the early stages of differentiation regaining their stem cell properties if they come in contact with the niche cells (Brawley and Matunis 2004; Sheng et al. 2009). In *C. elegans*, any given stem cell may give rise to two self-renewing cells, or two differentiating cells, depending on their proximity to the niche (Figure 4).

Therefore, although differences exist between the asymmetric and symmetric stem cell systems exemplified by the *Drosophila* and *C. elegans* germ lines, respectively, both utilize the proximity of equally competent daughter cells to the niche to determine cell fate. While these systems differ in the number of stem cells maintained, the signaling pathways utilized, and the zone of influence of the niche, each are able to maintain a balance between the proliferative fate and differentiation.

10. Conclusions

The *C. elegans* germ line has emerged as an important model in understanding how a balance is achieved between the proliferative fate and differentiation. The transparency of the animal has allowed the system to be visualized in living animals, which has brought significant power to genetic screens. These screens have aided in providing a molecular understanding of how the DTC signals to the proliferative zone cells and how these cells

interpret this signal, as well as identified many of the factors involved in differentiation once the cell is out of the range of the signal. Characterization of the factors involved has revealed a significant level of genetic redundancy in the system. Perhaps this level of redundancy is needed to fine-tune the signal emanating from the niche in a relatively large stem cell system, where the signal is likely received over a range of cells, rather than just received by cells in direct contact with the niche cell. Additionally, as conditions encountered by *C. elegans* in the environment can drastically alter reproductive strategies (Chapter 5, Hubbard et al. 2012), the redundant mechanisms provide a diverse set of nodes by which environmental signaling can modify the proliferation vs. meiotic cell fate decision and mitotic cell cycle progression. Certainly, major advances in our understanding of this system will come once we are able to specifically identify true stem cells, putative transit amplifying cells, and cells in meiotic S-phase. Distinguishing between these cell types will aid in determining what cells are truly committed to differentiation, and help to refine our understanding of the molecular mechanisms involved in this commitment.

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Figure 1. Dissected adult hermaphrodite gonad

A dissected hermaphrodite adult gonad stained with DAPI (blue) to visualize nuclear morphology, anti-REC-8 antibodies (green) to mark mitotic cells, and anti-HIM-3 antibodies to mark meiotic cells. The distal end is to the left (asterisk). Germ cells in the distal end of the gonad are proliferative, but enter meiotic prophase as they move out of the proliferative zone and into the transition zone, where they display pairing and synapsis indicative of leptotene/zygotene (Chapter 6, Lui and Colaiácovo 2012). The somatic distal tip cell (DTC) is at the very distal end of the gonad arm (arrow). Cells at similar distances from the distal end of the gonad do not necessarily enter meiosis at precisely the same location. Rather, some cells show evidence of having entered into meiotic prophase while some neighboring cells do not (inset).

Figure 2. Different classes of germline tumors

Diagrams (left) and pictures (right) of hermaphrodite gonad arms illustrating different classes of germline tumors. The distal tip cell (DTC) caps the distal end of the gonad. In wild-type animals (A) proliferative cells (green) are restricted to an ~ 20 cell diameter region in length from the distal end of the gonad, then enter into meiosis (red) as they move proximally, progress through meiotic prophase, and eventually differentiate into sperm (blue) or oocytes (orange). In gonads with a late-onset tumor (B & C), the region containing proliferative cells is much longer than in wild type. A Pro (C) tumor consists of proliferating cells in the proximal end of the gonad, while a complete tumor (D) contains proliferative cells throughout the entire gonad arm. Dissected gonad arms are stained with anti-REC-8 antibodies (green) to mark proliferative cells. Genotypes used (A) Wild-type (N2) (B) *glp-1(gf)/glp-1(0)* [Actual genotype *unc-32(e189) glp-1(oz112)/unc-36(e251) glp-1(q175)*] (C) *glp-1(gf) teg-1(0)* [Actual genotype *unc-32(e189) glp-1(ar202) teg-1(oz230)*] (D) *glp-1(gf)/glp-1(gf)/glp-1(+)* [Actual genotype *dpy-19(e1259) unc-32(e189) glp-1(oz112)/dpy-19(e1259) unc-32(e189) glp-1(oz112); qDp3[dpy-19(+)] glp-1(+)*]]. Scale bar = 20 Microns

Figure 3. Genetic control of the proliferation vs. differentiation decision

Core genetic pathway controlling the proliferation vs. differentiation decision (top) with factors that promote the proliferative fate and/or inhibit meiotic entry shown in green, while factors that promote meiotic entry and/or inhibit the proliferative fate shown in red. In this model, the LAG-2 and APX-1 ligands, which are expressed on the distal tip cell (DTC), interact with the GLP-1 Notch receptor, which is expressed on the germ cells. This causes the intracellular portion of GLP-1 to translocate to the nucleus and interact with the LAG-1 CSL transcription factor and SEL-8 Mastermind co-activator. These are all conserved components of the Notch signaling pathway. The GLP-1 pathway inhibits the activities of the redundant GLD-1 and GLD-2 genetic pathways, whose components all appear to regulate mRNA stability and/or translatability. An additional redundant pathway that is inhibited by GLP-1 signaling has been uncovered genetically (not shown), although genes that reside in this pathway have not been identified. The basic model of how signaling from the DTC regulates the proliferation vs. differentiation decision is shown (bottom). While germ cells are in the distal-most end of the gonad arm, close to the DTC (yellow), GLP-1 Notch signaling levels are high (green), keeping the activities of the GLD-1 and GLD-2 pathways low (red), resulting in cells remaining in the proliferative fate. As cells move proximally, GLP-1 Notch signaling levels decrease, causing the activities of the GLD-1 and GLD-2 pathways to increase, resulting in cells entering into meiotic prophase.

Figure 4. Comparison of *Drosophila* ovarian and *C. elegans* hermaphrodite gonad stem cell systems

In the *C. elegans* germ line, the distal tip cell (DTC) functions as the niche cell, and its zone of influence (yellow gradient) extends beyond its physical location. Within this system, stem cells (green) are thought to divide symmetrically, giving rise to intrinsically similar daughter cells. It is not until cells reach a certain distance from the DTC that they begin to differentiate by entering meiotic prophase (red). In *Drosophila*, the zone of influence (yellow gradient) of the niche Cap cell extends only to the cells that it directly contacts. The dividing stem cell (green), gives rise to intrinsically equivalent daughter cells; however, the daughter cell (red) that does not contact the Cap cell is outside of the zone of influence; therefore, it adopts the differentiating fate. With the two daughter cells receiving different extrinsic signals results in an asymmetric cell division.