Germline Stem Cells and Their Regulation in the Nematode *Caenorhabditis elegans*

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Abstract

C. elegans germline stem cells exist within a stem cell pool that is maintained by a single-celled mesenchymal niche and Notch signaling. Downstream of Notch signaling, a regulatory network governs stem cells and differentiation. Central to that network is the FBF RNA-binding protein, a member of the widely conserved PUF family that functions by either of two broadly conserved mechanisms to repress its target mRNAs. Without FBF, germline stem cells do not proliferate and they do not maintain their naïve, undifferentiated state. Therefore, FBF is a pivotal regulator of germline self-renewal. Validated FBF targets include several key differentiation regulators as well as a major cell cycle regulator. A genomic analysis identifies many other developmental and cell cycle regulators as likely FBF targets and suggests that FBF is a broad-spectrum regulator of the genome with >1,000 targets. A comparison of the FBF target list with similar lists for human PUF proteins, PUM1 and PUM2, reveals ~200 shared targets. The FBF hub works within a network controlling self-renewal vs. differentiation. This network consists of classical developmental cell fate regulators and classical cell cycle regulators. Recent results have begun to integrate developmental and cell cycle regulation within the network. The molecular dynamics of the network remain a challenge for the future, but models are proposed. We suggest that molecular controls of *C. elegans* germline stem cells provide an important model for controls of stem cells more broadly.

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The *C. elegans* germline provides an exceptionally simple and tractable system for studying stem cells and their regulation. Asymmetric stem cell lineages are pervasive in somatic tissues of this small nematode [1–8], but are not the rule in the germline. Instead, a pool of stochastically dividing stem cells drives generation, maintenance and regeneration of the germline tissue [9–12]. *C. elegans* germline stem cells (GSCs) are therefore of particular value for understanding how a stem cell pool accomplishes both self-renewal and generation of differentiated progeny.

In this review we focus on the regulation of GSC self-renewal and differentiation in adult hermaphrodites and emphasize progress over the past 5 or so years plus directions for the future. We refer readers to other reviews for background information and for GSC controls in larvae and males [4, 13–22].

3.1 Brief Overview of *C. elegans* Germline Stem Cells and Their Niche

Understanding the regulation of *C. elegans* GSCs requires a brief background to germline anatomy and development. We focus on hermaphrodites raised under 'standard' conditions (Petri plates with ample food at 20 °C). Adult hermaphrodites possess ~1,000 germ cells in each of two U-shaped gonadal arms (Fig. 3.1a). This actively reproducing germline has achieved a stable state—its cell number remains constant despite continuous loss to fertilization and cell death. Sperm made in larvae are stored in adults and used to fertilize oocytes within the same animal to generate the next generation. In each gonadal arm, germ cells are organized with self-renewing stem cells at one end and differentiating oocytes at the other (Fig. 3.1a). Each arm is capped by a mesenchymal 'distal tip cell' (DTC) that provides the niche for GSCs (Fig. 3.1a, red). GSCs reside within the 'mitotic zone' and, as their daughter cells move away from the mitotic zone, they enter meiotic prophase and progress into oogenesis (Fig. 3.1a). This essentially linear spatial organization—from stem cell to terminal differentiation—is similar to that seen in the intestinal crypt, which also relies on a pool of stochastically-dividing stem cells [24–27].

The mitotic zone germ cells continually replenish the germline and therefore are responsible for self-renewal. The zone consists of ~225 actively dividing germ cells with no quiescent cells [10, 28–30]. Divisions are not oriented; however, germ cells residing in the distal one-third of the mitotic zone, next to the DTC, remain undifferentiated, whereas germ cells in the proximal two-thirds of the mitotic zone increasingly express markers of early differentiation [e.g. 31]. Therefore, the mitotically dividing germ cells are not equivalent with respect to their state of differentiation. Indeed, two pools of germ cells exist within the mitotic zone: a distal pool of ~45–70 essentially uniform cells in an undifferentiated state, and a proximal pool of ~150 cells that have been triggered to differentiate and are maturing in a gradient, from undifferentiated to differentiated, as they progress through the pool (Fig. 3.1b) [12]. Starved adult hermaphrodites retain a pool of ~35 GSCs capable of regenerating a fully functional germline upon refeeding [11]. The emerging model is that a pool of ~30–70 undifferentiated germ cells with stem cell potential resides in the distal-most germline (Fig. 3.1b). It seems likely that germ cells become capable of differentiation once niche signaling drops below a certain threshold and that their transition from an undifferentiated state to overt differentiation occurs as they progress through the proximal pool of the mitotic zone.

C. elegans embryos produce two primordial germ cells or GSCs, and those GSCs proliferate during four larval stages (L1–L4) to generate ~2,000 germ cells in adults. During the first two larval stages, all GSCs divide uniformly, but in L3, a pattern emerges that persists through adulthood: distal germ cells divide mitotically while more

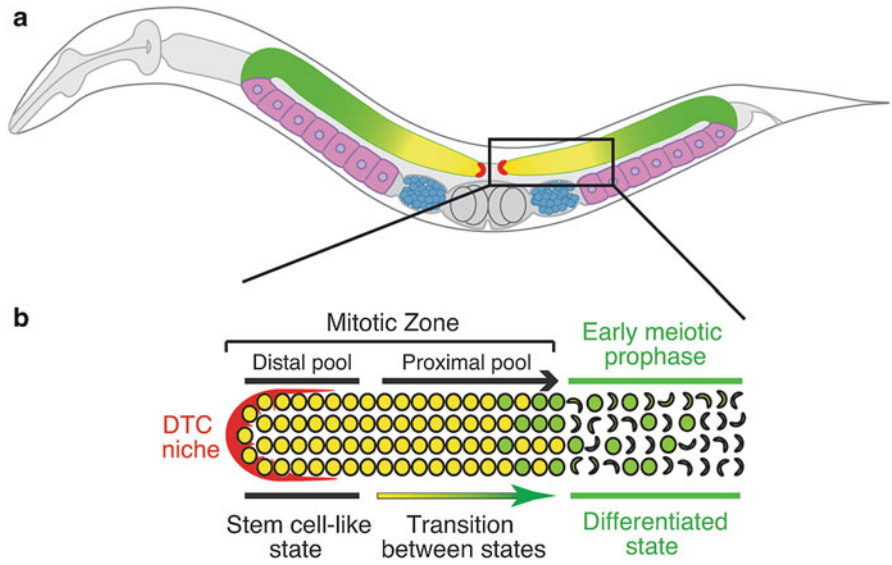

Fig. 3.1 Overview of *C. elegans* GSC biology. (**a**) The adult hermaphrodite gonad contains two U-shaped arms, each capped by a mesenchymal cell, the distal tip cell (DTC, *red*). The Mitotic Zone is adjacent to the DTC (*yellow*). As germ cells move out of the Mitotic Zone, they enter the meiotic cell cycle (*green*). Further proximally, germ cells start overt gametogenesis, oogenesis (*rose*) in the adult hermaphrodite. Sperm (*blue*) are made earlier in development and are stored for use in the adult. (**b**) The adult hermaphrodite distal gonad. The DTC niche (*red*) maintains the Mitotic Zone, which is composed largely of germ cells in the mitotic cell cycle (*yellow*), including GSCs. Some cells in the most proximal mitotic zone have entered meiotic S-phase (*green circles*). As germ cells move out of the proximal pool, they enter meiotic prophase. In early meiotic prophase, the chromosomes take on a distinctive crescent-shaped morphology (*green crescents*). Note that within the mitotic zone a germ cell's state of differentiation cannot be deduced from its cell cycle: the distal pool of mitotically-dividing germ cells are in an undifferentiated stem cell-like state [12], whereas the proximal pool of mitotically-dividing germ cells increase expression of differentiation markers [e.g. 23]

proximal germ cells enter the meiotic cell cycle and differentiate. The differentiating germ cells make sperm in L4s and oocytes in adults. *C. elegans* germ cells can arrest their divisions at several points during development in response to environmental cues; their arrest upon starvation of late stage larvae is accompanied in adults by a dramatic germline shrinkage, which can be reversed upon feeding to restore the germline to its normal adult size and reproductive state [11, 31].

3.2 GSC Regulation by the Niche and Notch Signaling

3.2.1 The Mesenchymal DTC Niche and Its Specification

The mesenchymal DTC provides the niche for both larval and adult GSCs [reviewed in 32]. Briefly, DTC removal causes all GSCs to differentiate and hence results in loss of GSC self-renewal. Moreover DTC repositioning or duplication forms a new or ectopic axis harboring stem to differentiated cells [33–36]. Therefore the DTC is essential for both GSC maintenance and initiation of the germline maturation gradient.

Understanding how the niche itself is specified is critical for understanding stem cell control. Each DTC arises from the asymmetric division of a somatic gonadal precursor cell during early larval development [9]. A divergent Wnt signaling pathway [37] activates transcription of the CEH-22/Nkx2.5 homeodomain transcription factor to specify the DTC niche fate [35, 36]. Loss of the Wnt pathway or the CEH-22/Nkx2.5 transcription factor eliminates DTCs and GSCs, while overexpression drives production of extra DTCs and ectopic GSCs. It is not known if this mode of niche specification is conserved—few niches are

well defined and fewer still have been subjected to analyses of specification controls.

3.2.2 Notch Signaling Controls GSC Maintenance

The DTC uses Notch signaling to maintain GSCs. Of the two Notch receptors encoded in the *C. elegans* genome, GLP-1/Notch is both necessary and sufficient for GSC maintenance [13]. Briefly, when GLP-1 is removed completely in null mutants, the GSCs in newly hatched L1s divide only once or twice before differentiating, mimicking the effect of DTC ablation [38]. When GLP-1 is depleted in larvae or adults using temperature-sensitive *glp-1* mutants, GSCs again are lost to differentiation. By contrast, when GLP-1 is unregulated in gain-of-function *glp-1* mutants, the number of undifferentiated germ cells expands dramatically [39, 40]. Thus, GLP-1/Notch signaling is both necessary and sufficient to maintain GSCs. This system is an unusually tractable one to analyze how Notch signaling controls stem cells and differentiation, because the signaling is triggered from a well-defined source, the DTC, and because it is continuous with a simple cellular readout, maintenance of the mitotic zone. Yet our understanding of how Notch signaling controls stem cells remains in its infancy.

Canonical mammalian and *Drosophila* Notch ligands contain both DSL (Delta, Serrate, LAG-2) and DOS (Delta and OSM-11) motifs, and both domains are critical for Notch activation [41]. In *C. elegans*, the DSL and DOS domains reside in distinct proteins—ten DSL-containing proteins [42] and five DOS-containing proteins [43]. Indeed, DSL- and DOS-containing proteins work together to activate Notch signaling in neurons [43, 44]. Therefore, *C. elegans* may have developed a bipartite ligand system utilizing separate DSL and DOS ligands to influence the strength or fidelity of Notch signaling. This model provides an attractive explanation for the diversity of *C. elegans* Notch ligands, most of which have not yet been investigated for DTC expression or control of GSCs. Only two DSL ligands are known to be expressed in the DTC and to activate GLP-1/Notch signaling for GSC maintenance. These include LAG-2 [45, 46] and APX-1 [47], both DSL motif-containing ligands.

In addition to the Notch control of GSCs, insulin signaling drives robust germline proliferation in larvae [48], and TGF-beta signaling maintains germ cell number in the mitotic zone [49]. Germ cell number is reduced by about one-half in L4 larvae defective for insulin signaling, and germ cell number is reduced by about one-half in adult mitotic zones defective for TGF-beta signaling. Thus, insulin or TGF-beta signaling are important for modulating the number of undifferentiated germ cells.

3.2.3 GLP-1/Notch Target Genes Control Stem Cells and Differentiation

Once the Notch ligand triggers signaling, the GLP-1/Notch receptor is cleaved to generate a 'Notch intracellular domain', or NICD, that is transported to the nucleus. In the nucleus, the NICD assembles into a ternary complex with the LAG-1 DNA-binding protein and the LAG-3/SEL-8 transcriptional co-activator. This ternary complex activates transcription, presumably by a mechanism similar to that seen in other organisms [50]. Notch signaling in other organisms employs not only this canonical transcriptional mechanism but also non-canonical mechanisms that are poorly understood [e.g. 51]. The use of non-canonical mechanisms of Notch signaling has not yet been investigated in the *C. elegans* germline.

To date, two GLP-1/Notch target genes have been identified, *fbf-2* and *lip-1* [52, 53]. The *fbf-2* gene encodes a key regulator of GSC maintenance (see below), and *lip-1* encodes a dual-specificity phosphatase of the MAP kinase phosphatase (MKP) family, which directly inhibits activated MAP kinases [54]. LIP-1 activity is critical for size of the mitotic zone but not for self-renewal *per se* [53]. Therefore LIP-1 normally controls the extent of proliferation, a role shared by vertebrate MKP homologs [55].

It is likely that many GLP-1/Notch target genes have not yet been discovered. Chromatin immunoprecipitation (ChIP) analyses of Notch signaling target genes in human and *Drosophila*

cell culture identified 134 and 262 potential target genes, respectively [56, 57]. Similar experiments have not been done in *C. elegans* for either of its two Notch receptors, LIN-12 or GLP-1. However, bioinformatic analysis has identified 163 potential Notch targets in the *C. elegans* genome [58]. In addition, one recent study found 202 genes upregulated in *glp-1* gain-of-function mutants compared to wild-type animals [59], but this approach does not distinguish between genes activated directly and those activated indirectly. Therefore, identification of the GLP-1/Notch target genes responsible for GSC maintenance remains a critical line of investigation for the future.

3.3 Controls of GSCs and Differentiation: FBF-1 and FBF-2

3.3.1 FBF Represents a Conserved Post-transcriptional Mechanism for Stem Cell Maintenance

FBF-1 and FBF-2 (collectively termed FBF) are nearly identical PUF (for Pumilio and FBF) family mRNA-binding proteins that control GSC maintenance [60, 61]. Single mutants lacking either *fbf-1* or *fbf-2* have only subtle germline defects, maintain GSCs and are fertile, but *fbf-1 fbf-2* double mutants fail to maintain GSCs and are sterile [52, 61]. Double mutant germlines proliferate normally during most of larval development, but in L4s, germ cells that normally would continue in the mitotic cell cycle instead enter meiosis and differentiate as sperm. Stem cell loss also occurs when *fbf-1* and *fbf-2* are depleted from adult hermaphrodites using RNA interference. *fbf-1 fbf-2* males also fail to maintain GSCs. Thus, FBF is essential for GSC maintenance, regardless of gender, but its effect is limited to late larvae and adults.

A role for FBF in larval GSCs was observed when an additional germline regulator, FOG-1, was removed from an *fbf-1 fbf-2* double mutant [62]. FOG-1 belongs to the CPEB family of RNA-binding proteins and its primary biological role is sperm specification [13]. However, in *fog-1; fbf-1 fbf-2* triple mutants, GSCs are lost in L2s when germ cells enter meiotic prophase. This effect reveals a role for FBF in larval stem cell divisions in addition to its role in late larval and adult GSC divisions.

PUF proteins control stem cell maintenance in several organisms. For example, *Drosophila* Pumilio is essential for GSC maintenance in adult ovaries [63, 64], and DjPum maintains totipotent stem cells called neoblasts in planaria [65]. The role of PUF proteins in vertebrate stem cells is not yet understood. Microarray analyses reveal that mRNAs encoding both mammalian PUF proteins, Pum1 and Pum2, are present in virtually all mammalian stem cells investigated, including embryonic stem cells, hematopoietic stem cells, neuroblasts and multipotent mesenchymal cells among others [66], a finding consistent with a conserved role of PUF proteins in stem cells. Moreover, loss of Pum2 causes a reduction of murine testis size with at least some agametic seminiferous tubules [67]. Because Pum1 and Pum2 may substitute for each other in murine GSCs, an effect well established for *fbf-1* and *fbf-2* in *C. elegans*, it seems likely that both Pum1 and Pum2 must be removed to learn their function in vertebrate GSCs. Regardless, the FBF mechanism of GSC control is likely to represent a broadly conserved mechanism with implications for vertebrates.

3.3.2 PUF Proteins Are Largely Post-transcriptional Repressors

PUF proteins, including FBF, repress mRNA activity, either by controlling mRNA stability or translation (Fig. 3.2) [reviewed in 68]. PUF proteins in virtually all eukaryotes bind regulatory elements in the 3′ untranslated region (3′UTR) of their target mRNAs and repress their targets by conserved mechanisms. Best understood is recruitment of the Ccr4/Not deadenylase complex, potentially via interaction with CCF-1, a Ccr4/Not component [69, 70]. In addition, PUF proteins can repress mRNAs via a newly discovered deadenylation-independent mechanism to inhibit translation [71]. This mechanism relies on formation of a ternary complex composed of a

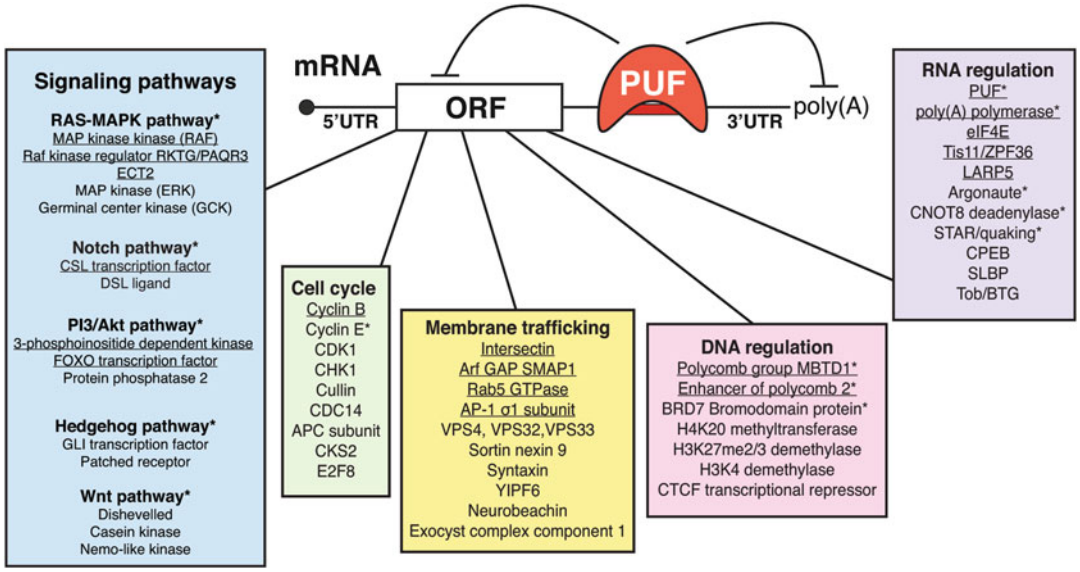

Fig. 3.2 Worm and human PUF proteins share key target mRNAs. PUF proteins (*red*) bind regulatory elements in the 3′ untranslated region (3′UTR) of their target mRNAs. PUF proteins repress mRNAs, either by shortening the poly(A) tail or blocking translational elongation (see text). Shared PUF target mRNAs have been identified by comparison of putative targets identified in genome-wide studies for *C. elegans* FBF-1, *Drosophila* Pumilio and human PUM1 and PUM2 (see text). Shown here are selected targets shared by *C. elegans* and human PUFs, grouped by function. Each is represented using the human gene name; an *asterisk* marks those with important roles in stem cell self-renewal and/or differentiation of stem cell progeny; targets shared by *C. elegans*, human and *Drosophila* PUFs are *underlined*

PUF protein (FBF in *C. elegans* or PUM2 in humans), an Argonaute protein and the core translation factor EFT-3/eEF1A. In reticulocyte lysates, the PUF–Ago–eEF1A complex does not dramatically affect ribosome loading but instead arrests ribosomes during elongation. The roles of these two conserved repressive mechanisms in stem cell control are not yet understood.

At least two PUF proteins can also act as mRNA activators—*C. elegans* FBF and trypanosome PUF9 [70, 72, 73]. Although the major mode of FBF control appears to be repression, it is also capable of activation via recruitment of the cytoplasmic poly(A) polymerase GLD-2 [70]. One attractive idea is that PUF repression and activation are part of a dynamic sequence underlying first GSC maintenance and self-renewal and then differentiation of GSC daughters. One can imagine that PUF recruitment of a deadenylase might destabilize its target mRNAs in stem cells, that PUF recruitment of a core translation elongation factor might permit translational initiation but leave its target mRNAs in an arrested state of translational elongation in transit-amplifying cells, and that PUF recruitment of a poly(A) polymerase might activate those translationally arrested mRNA when triggered for overt differentiation. Although these ideas remain speculative, they provide an important model for the dynamics of PUF-centered macromolecular complexes during development—a model that will guide future investigations.

3.3.3 FBF Target mRNAs: Lessons from a Candidate mRNA Approach

Several FBF target mRNAs have been identified using a candidate mRNA approach. Evidence supporting their identification has relied on a number of criteria, including the following: (1) FBF targets possess one or more consensus FBF binding elements (FBEs) in their 3′UTRs; (2) at least one FBE binds FBF *in vitro*; (3) FBF targets co-purify with FBF from worm extracts; (4) their proteins increase *in vivo* when FBF is removed,

suggesting a repressive mode of FBF control; and (5) their 3′UTRs repress expression in an FBE-dependent and/or FBF-dependent manner when assayed using transgenic reporters [13, 74–77]. Although not all FBF target mRNAs have been subject to all five tests, most are supported by at least four assays.

The FBF target mRNAs identified on a candidate mRNA basis demonstrate that FBF promotes GSC maintenance in two major ways. First, FBF regulates the mitotic and meiotic cell cycles themselves. FBF promotes GSC mitotic divisions by repressing a Cip/Kip family cyclin-dependent kinase inhibitor *cki-2*, a negative regulator of the mitotic cell cycle [77], and FBF represses structural components of the meiotic machinery for chromosomal synapsis and recombination (e.g., *him-3*, *syp-2*, and *syp-3*) [75]. Second, FBF regulates differentiation. FBF prevents germline differentiation by repressing *gld-1* and *gld-3* [61, 74, 78], which regulate meiotic entry (see below) and by repressing *fem-3* and *fog-1* [60, 62, 74, 79–81], key regulators of sperm differentiation [82, 83]. FBF prevents differentiation more broadly by repressing *mpk-1* [53], the *C. elegans* ERK/MAP kinase that promotes differentiation in both somatic and germline tissues [84, 85]. And one FBF target, the *lin-3* TGF-alpha ortholog [86, 87], regulates somatic differentiation. Therefore, from this limited set of ~15 mRNA targets, FBF emerges as a broad-spectrum repressor of mRNAs critical for continued mitotic divisions and maintenance of an undifferentiated stem cell state.

3.3.4 FBF Target mRNAs: Lessons from a Genomic Approach

Many more FBF target mRNAs have been found using a genome-wide approach [76]. In this study, FBF was immunoprecipitated together with its associated mRNAs, which were identified on microarrays. The resulting list of putative targets was whittled from >4,000 mRNAs with a 2.25 % false discovery rate to a smaller list of the 1,350 most enriched mRNAs cut off at the *gld-3S* mRNA, which had been previously validated as an FBF target [78]. The 1,350 target mRNAs were enriched for mRNAs containing FBF binding elements (FBE) in their 3′UTRs. This list included all validated FBF germline targets known at the time of its publication and all FBF germline targets validated since (i.e., *cki-2*, *him-3*, *syp-2*, and *syp-3*) [75, 77]; however, the list did not include a neuronal FBF target, *egl-4*/*pkg-1* [72]. Therefore, most are likely *bona fide* FBF target mRNAs and we refer to them as FBF targets for simplicity. Although their identification via FBF association provides no clue about whether the targets are repressed or activated, it seems likely that most are repressed based on our knowledge of validated targets.

The identities of the 1,350 FBF target mRNAs complement and extend lessons learned from the candidate mRNA approach. In addition to *cki-2*, the 1,350 include other key cell cycle genes, including *cye-1*. In addition to *him-3* and *syp* mRNAs, a battery of other components of the meiotic machinery were on the FBF target list: among 247 genes annotated for involvement in meiosis, 84 (34 %) are FBF targets, suggesting a broad control of the meiotic program. In addition to *gld-1* and *gld-3*, the *gld-2* regulator of meiotic entry is on the target list. In addition to *fog-1*, the *fog-3* and *rnp-8* regulators of gamete specification are on the target list. And in addition to *mpk-1*, several other core components of the MAP kinase signaling cascade as well as components of other key developmental signaling pathways appear to be FBF targets. Other prominent targets include central components of intracellular trafficking and cell death mRNAs. Therefore, FBF appears to be a broad-spectrum regulator of the genome, targeting 7 % of its protein coding capacity, with a distinct enrichment for mRNAs encoding diverse regulators of differentiation.

3.3.5 Conserved PUF Targets

Genome-wide studies of PUF protein mRNA targets have been conducted in yeast, *Drosophila* ovaries and embryos, mouse testis, and human

HeLa cells [88–92]. These studies together with the aforementioned FBF study [76] demonstrate unequivocally that PUF proteins are broad-spectrum regulators of the genome. For example, mammalian Pum1 and Pum2 individually associate with ~700–1,500 unique mRNAs [90–92], and these mRNAs represent a range of biological activities, including cell signaling, cell death, cell cycle, and transcription factors. One surprising upshot from these studies is that, despite being carried out in diverse cell and tissue types, PUF proteins from different organisms regulate many of the same developmental pathways and, indeed, many orthologous mRNAs. Remarkably, 197 *C. elegans* FBF targets are orthologous to a human PUM target, and this overlap is significant [76]. The common targets encompass a range of biological activities, including major developmental signaling pathways and key cell cycle regulators (Fig. 3.2). In addition to sharing specific targets, *C. elegans* FBF and human PUM also regulate additional components of the same pathways, albeit not the same individual proteins. Interestingly, several shared targets and pathways regulate stem cells. Given the conserved link between PUF proteins and stem cell control, one intriguing idea is that PUF repression of developmental signaling pathways is an ancient regulatory module for stem cell control.

3.4 Molecular Regulation of Germline Differentiation

3.4.1 Key Regulators of Meiotic Entry: GLD-1 and GLD-2

When GSC progeny differentiate, they enter the meiotic cell cycle and specialize as either sperm or oocyte. In this review, we focus on regulators of meiotic entry, which are the best understood regulators of germline differentiation. The two primary regulators of meiotic entry are GLD-1 and GLD-2, distinct proteins that function in parallel to drive germ cells into the meiotic cell cycle [93].

GLD-1 is an RNA-binding protein of the STAR/Quaking family [94] and a translational repressor of two key mitosis-promoting factors, the GLP-1/Notch receptor and the cyclin E/CYE-1 cell cycle regulator [95–101]. Genomic analyses have identified >400 GLD-1 target mRNAs, which are enriched for cell division-promoting factors [100, 101]. Therefore, GLD-1 emerges as a broad-spectrum repressor of the mitotic cell cycle and crucial counterweight to the FBF broad-spectrum repressor of the meiotic cell cycle. Although the mechanism of GLD-1 repression remains unclear, genome-wide mapping of *in vivo* sites of GLD-1 occupancy reveal binding either in 3′UTRs or at start codons, suggesting the existence of multiple mechanisms [101–103].

GLD-2 is the catalytic subunit of cytoplasmic poly(A) polymerase (PAP) and a translational activator of meiotic entry [78, 104–106]. GLD-2 functions with either of two RNA-binding proteins, GLD-3 or RNP-8; the GLD-2/GLD-3 heterodimer promotes meiotic entry and spermatogenesis, while GLD-2/RNP-8 promotes oogenesis [78, 107]. Most relevant here, GLD-2/GLD-3 polyadenylates and activates *gld-1* mRNA [106, 107]. GLD-4, another cytoplasmic PAP, forms a complex with GLS-1 and possibly GLD-3, and also activates *gld-1* mRNA [108, 109]. Thus, the dual activation of *gld-1* mRNA by GLD-2 and GLD-4 PAPs provides a robust positive feed-forward loop to drive meiotic entry. In addition, the *gld-2* mRNA itself associates with GLD-2 protein, suggesting positive autoregulation [107]. GLD-2 must also control other mRNAs to drive the meiotic program, because GLD-2 is sufficient to promote meiotic entry in the absence of *gld-1* [93]. Although GLD-2 reproducibly associates with >500 mRNAs from worm extracts [107], additional GLD-2 targets critical for entry into the meiotic cell cycle have not yet been identified. NOS-3, a member of the Nanos family of mRNA-binding proteins, also promotes abundant GLD-1 and promotes meiotic entry, but its mechanism is not yet known [105].

3.4.2 Other Regulators of Meiotic Entry

Regulation of pre-mRNA splicing has recently emerged as another critical node in the control of meiotic entry. Over 50 splicing factors have been implicated in meiotic entry, most notably PRP-17, TEG-1, TEG-4, and six MOG proteins [110–116]. These splicing factors have been proposed to promote activity of the GLD-1 branch of the meiotic entry pathway [114, 116]. The meiotic defects in splicing mutants are not likely due to a general decrease in gene expression because germline depletions of RNA Pol II or ribosomal genes did not have the same effect as removal of the splicing factors [114]. One idea is that splicing (possibly alternative splicing) of specific key mRNAs is essential for meiotic entry. However, no such targets have been identified.

Numerous regulators influence the balance between mitosis and meiosis, with subtle effects on the position at which meiotic entry occurs. Any such regulators not implicated in control of self-renewal are beyond the scope of this review. For example, the LIP-1 dual specificity phosphatase and inhibitor of MAP kinase activity controls the number of germ cells in the mitotic zone [53], but no genetic background has yet been found in which LIP-1 is essential for germline self-renewal. A similar situation exists for many other regulators, including ATX-2/Ataxin [117, 118], EGO-1/RdRP [119, 120], the Piwis PRG-1 and PRG-2 [121, 122]; PAB-1/Pab, EFT-3/eEF1A, and the L11 ribosomal subunit RPL-11.1 [123].

3.5 Integration of Cell Cycle and Developmental Regulators

The regulation of cell divisions must be integrated with regulation of developmental programs to maintain stem cells and produce functional tissues. Although germ cells in the mitotic zone differ in their differentiation state (see above), they divide at approximately the same rate throughout the zone with no observed quiescence [10, 28–30, reviewed in 124]. The standard cell cycle machinery controls germ cell divisions as might be expected [reviewed in 21], and mechanisms integrating that machinery with developmental regulators are now emerging.

The developmental regulator FBF represses the cell cycle regulator, *cki-2*, to drive continued mitotic divisions [77]. CKI-2, in turn, is likely to control CDK-2/CYE-1, the *C. elegans* counterpart of Cdk2/cyclin E, which has recently emerged as a pivotal bridge between cell cycle and developmental controls [30, 125]. In the absence of CDK-2 or CYE-1, all germ cells stop mitotic divisions and some enter the meiotic cell cycle earlier than normal. More importantly, *cye-1* and *cdk-2* mutants enhance weak *glp-1* and null *fbf-1* mutants so that the double mutants possess no GSCs and germline self-renewal is lost. Other cell cycle mutants do not similarly affect germline self-renewal, suggesting that CDK-2/CYE-1 has a specific role in that process. At a molecular level, CDK-2/CYE-1 keeps GLD-1 levels low in the distal germline, likely by direct GLD-1 phosphorylation [125]. Thus, the CYE-1/CDK-2 cell cycle regulator acts in the mitotic zone to negatively regulate the GLD-1 developmental regulator and to promote GSC self-renewal.

Once germ cells have acquired GLD-1 and entered the meiotic cell cycle, the reciprocal regulatory relationship is observed between GLD-1 and CDK-2/CYE-1. During meiotic prophase, GLD-1 represses translation of *cye-1* mRNA [99]. An additional brake on CYE-1/CDK-2 in meiotic cells is provided by *cki-2*, which is freed from FBF repression and available to repress CDK-2 activity [77]. Therefore, the combination of post-transcriptional and post-translational controls ensures that CKI-2 and GLD-1 are repressed in the mitotic zone and that CYE-1 is repressed in meiotic germ cells.

The mutual repression between GLD-1 and CYE-1 constitutes a double-negative feedback loop, a classical network motif and toggle switch for decisions between two states [126]. In this case, the two states are germline proliferation and differentiation. Importantly, CYE-1 or CDK-2 removal does not flip all germ cells from one state

to the other but rather shifts the balance between the two states. Elimination of all mitotically dividing germ cells, which is essential for abolishing germline self-renewal, requires decreased GLP-1/Notch signaling or FBF-1 removal in addition to loss of CYE-1 or CDK-2. Therefore, multiple layers of regulation must be peeled away to reveal effects on stem cell self-renewal.

3.6 A Self-Renewal vs. Differentiation Regulatory Network: Motifs and Properties

Figure 3.3a diagrams the major components of the network regulating germ cells to remain in an undifferentiated stem cell-like state or to

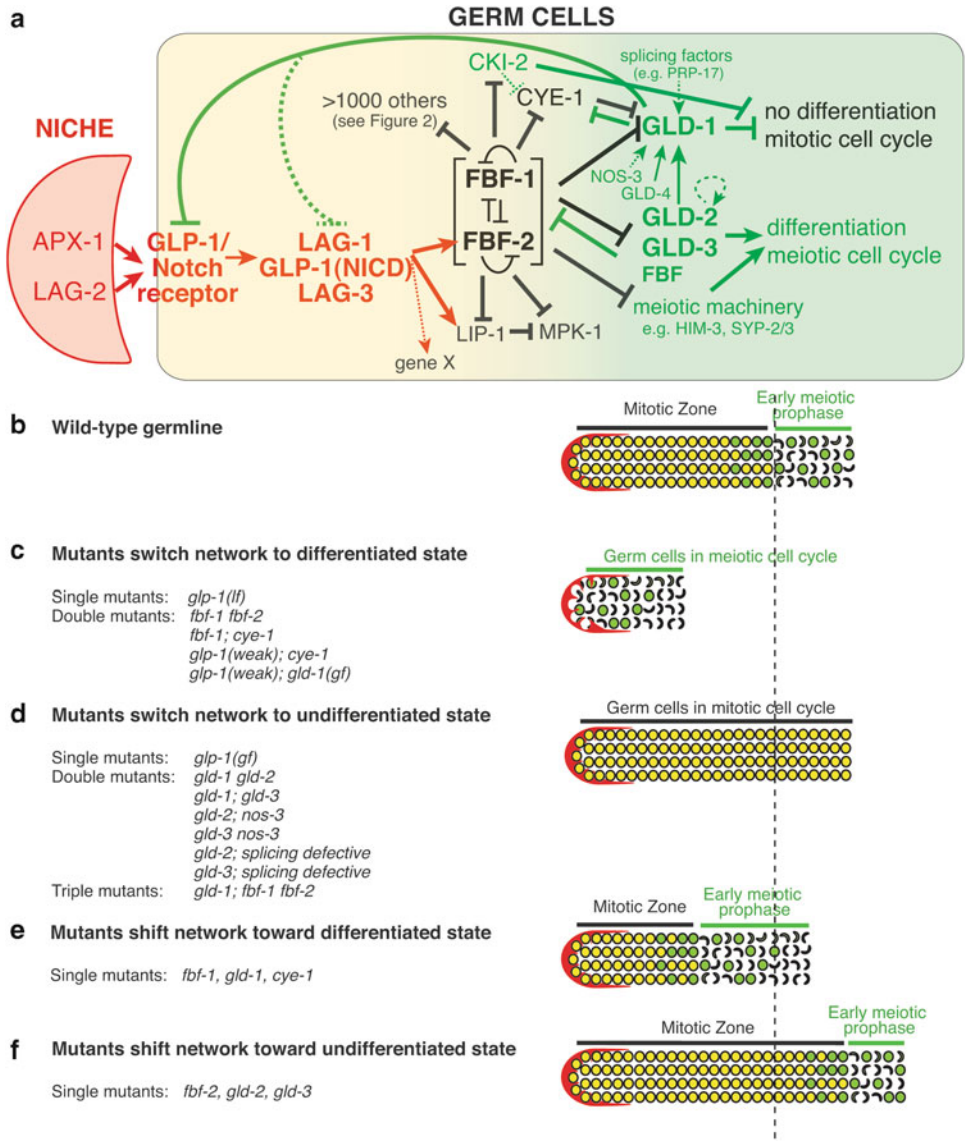

Fig. 3.3 Regulatory network controlling self-renewal versus differentiation. (**a**) Regulatory network for decision between self-renewal (undifferentiated, stem cell state) and differentiation (entry into the meiotic cell cycle). Specifics of the depicted network are simplified and a work in progress. Briefly, GLP-1/Notch signaling (*red text*) from the niche (*red shading*) activates the GLP-1/Notch receptor in germ cells to activate transcription of

differentiate (enter the meiotic cell cycle). In this diagram, nodes are regulatory proteins and edges are regulatory relationships, which can be either positive (arrow) or negative (bar). Most individual elements of the network are described above. Here we bring together those individual elements to discuss emergent regulatory motifs and properties.

A variety of network motifs work together to regulate the decision between the undifferentiated stem cell-like state and differentiation. These motifs combine transcriptional regulation (Notch signaling), post-transcriptional controls (FBF, GLD-1, GLD-2) and post-translational controls (CYE-1, LIP-1). The existence of these network motifs provides the backbone for switching between two states plus refinements that likely regulate the time and rate of switching. Mathematical modeling of the network remains a critical direction for the future. Briefly the major motifs include the following:

- Negative cross-regulation and likely autoregulation influence FBF-1 and FBF-2 levels [52]. *fbf-1* and *fbf-2* mRNAs possess FBEs in their 3′UTRs, and removal of either FBF protein results in an increase of the other. It seems likely that the two FBF proteins also autoregulate because their binding properties appear the same, but this idea has not been explicitly tested. One rationale for FBF negative cross- and auto-regulation is maintenance of a level sufficiently low to be vulnerable to signals initiating the switch to differentiation.
- Positive auto-regulation likely promotes robust GLD-2 protein accumulation. *gld-2* mRNA associates with GLD-2 protein from worm extracts [107]. Notably, GLD-2 autoregulation occurs in vertebrates [129]. This autoregulation likely reinforces the switch into the meiotic cell cycle.
- One double-negative feedback loop provides a toggle between GLP-1/Notch and the GLD-1 translational repressor [60, 97, 105]. GLP-1/Notch downregulates GLD-1, at least partially via FBF repression of *gld-1* mRNA activity, and conversely, GLD-1 represses *glp-1* mRNA [97] and also likely *lag-1* mRNA [100, 101]. This toggle integrates Notch signaling with the differentiation response.
- A second double-negative feedback loop provides a toggle between the CYE-1/cyclin E cell cycle regulator and the GLD-1 translational repressor [99, 125]. CYE-1 inhibits GLD-1 post-translationally, and GLD-1

Fig. 3.3 (continued) regulators that promote the undifferentiated state (*black text*); those regulators in turn repress regulators that promote differentiation (*green text*). *Solid lines* mark a direct biochemically validated regulatory relationship; *dashed lines* mark postulated or indirect regulation. Gene X represents predicted GLP-1/Notch target genes. See text for details. (**b–d**) Robustness and plasticity in the network controlling self-renewal versus differentiation can be observed by a shift in the balance between germ cells in the mitotic cell cycle and meiotic cell cycle (see text for more discussion). Conventions as in Fig. 3.1b. (**b**) Wild-type germline. (**c**) Genes critical for GSC self-renewal, revealed by a mutant phenotype of GSC loss: *glp-1* [38]; *fbf-1 fbf-2* double mutant [61]; *fbf-1; cye-1* double mutant [125]; *glp-1(weak); cye-1* double mutant [30]; *glp-1(weak); gld-1(gf)* double mutant [105]. (**d**) Genes critical for differentiation of GSC progeny, revealed by a mutant phenotype of differentiation loss: *gld-1 gld-2* double mutant [93]; *gld-1; gld-3* double mutant [78]; *gld-3 nos-3* double mutant [78]; *gld-2; gld-3* double mutant [78]; *gld-2; nos-3* double mutant [105]; double mutants lacking either *gld-3* or *gld-2* and one of several splicing factors (e.g., *prp-17*) [111–116]; *gld-1; fbf-1 fbf-2* triple mutants [61]. Note that this diagram is simplified and the degree of differentiation loss can vary, suggesting the existence of additional regulators not yet known [127]. Additional mutants that are not depicted here cause a failure in meiotic progression and result in a reentry into the mitotic cell cycle. Such mutants include *gld-1* single mutants [128] and *gld-2 gld-4* double mutants [108]. (**e**) Genes identified as critical for GSC renewal or differentiation in double mutants (**c**, **d**) but that as single mutants shift the balance toward differentiation, revealed by the phenotype of a shortened mitotic zone: *fbf-1* [52]; *gld-1* [78]; *cye-1* [125]. Importantly, GSC loss does not occur in these single mutants; therefore this phenotype is interpreted as a shift in the balance of the network controlling self-renewal and differentiation. (**f**) Genes identified as critical for GSC renewal or differentiation in double mutants (**c**, **d**) but that as single mutants shift the balance of the network away from differentiation, revealed by the phenotype of a lengthened mitotic zone: *fbf-2* [52]; *gld-2* [78]; *gld-3* [78]. Importantly, differentiation loss does not occur in these single mutants; therefore this phenotype is interpreted as a shift in the balance of the network controlling self-renewal and differentiation

represses *cye-1* translation. This second toggle integrates cell cycle and developmental regulators.

- A Coherent type 2 positive feed forward loop [130] from FBF and GLD-2 onto GLD-1 drives forward the decision to differentiate. In this motif, FBF inhibits both GLD-1 and GLD-2, but GLD-2 activates GLD-1 to help overcome FBF repression and ensure the switch to differentiation.
- An Incoherent type 1 positive feed forward loop [131] likely exists from GLP-1/Notch to LIP-1. Via this motif, GLP-1/Notch activates *lip-1* transcription and also activates transcription of the *lip-1* repressor, *fbf-2*. Interestingly, Notch signaling also employs similar regulatory logic in *Drosophila* [58].

The primary network property emerging from the *C. elegans* self-renewal/differentiation regulatory circuitry is robustness, the resilience to stochastic failure of individual elements. Indeed, the *C. elegans* network is rife with 'redundant' regulators that provide buffering capacity. Examples of GSC-promoting redundant regulators include FBF-1 and FBF-2 [61], and FBF-1 and CYE-1 [125]. Examples of differentiation-promoting redundant regulators include GLD-1 and GLD-2 [93], GLD-3 and NOS-3 [78, 105], and GLD-2 and GLD-4 [108]. This pervasive robustness insulates the network from perturbation, allowing GSCs to be maintained and the switch to differentiation to proceed despite genetic deficiencies or stochastic defects. Robustness also provides the network with multiple points of regulation that can be turned up or down without abolishing either GSC self-renewal or differentiation.

A second emergent network property is plasticity. Evidence for plasticity derives from measurable shifts in network readout observed upon removal of individual elements (Fig. 3.3b–f). For example, *fbf-1* single mutants possess fewer undifferentiated germ cells than normal (Fig. 3.3e), and *gld-3* single mutants possess more undifferentiated germ cells than normal (Fig. 3.3f). A critical next step is to understand how plasticity is structured within the network. For example, does it result from a change in the differentiation trigger or from a change in the rate of network transition from the undifferentiated to differentiated state? Understanding the network at this level will provide new ways of thinking about how stem cell networks are structured and can be manipulated.

3.7 Transition from an Undifferentiated Stem-Cell-Like State to Overt Differentiation

A regulatory network must be dynamic to both maintain GSCs in an undifferentiated state and transition GSC daughters towards an overtly differentiated state. One mode of the network governs stem cells and a different mode drives overt differentiation. A key question is how the network is regulated to shift from one mode to the other and the mechanistic basis of that transition. *C. elegans* provides an optimal entrée into this important question because of its exceptional *in vivo* accessibility and the growing knowledge of critical network components and their regulatory functions.

Figure 3.4 shows a speculative model for stem cell network dynamics. This model has grown out of earlier models [12, 13] and will surely change as more is learned. Central to the model is the idea that the network must switch from FBF-mediated mRNA repression for GSC maintenance (Fig. 3.4b) to GLD-mediated regulation driving differentiation (Fig. 3.4). The proposed dynamics include increases and decreases in major regulators as follows.

- GLP-1/Notch signaling is proposed to decrease as germ cells leave the niche. In support of that idea, germ cells more than 6–8 rows from the distal end of the germline can differentiate in the presence of GLP-1/Notch signaling [12], transcripts of key GLP-1/Notch target genes are found in the distal-most region of the mitotic zone (A. Kershner, H. Shin and J. Kimble, unpublished) and GLP-1/Notch ligands in the

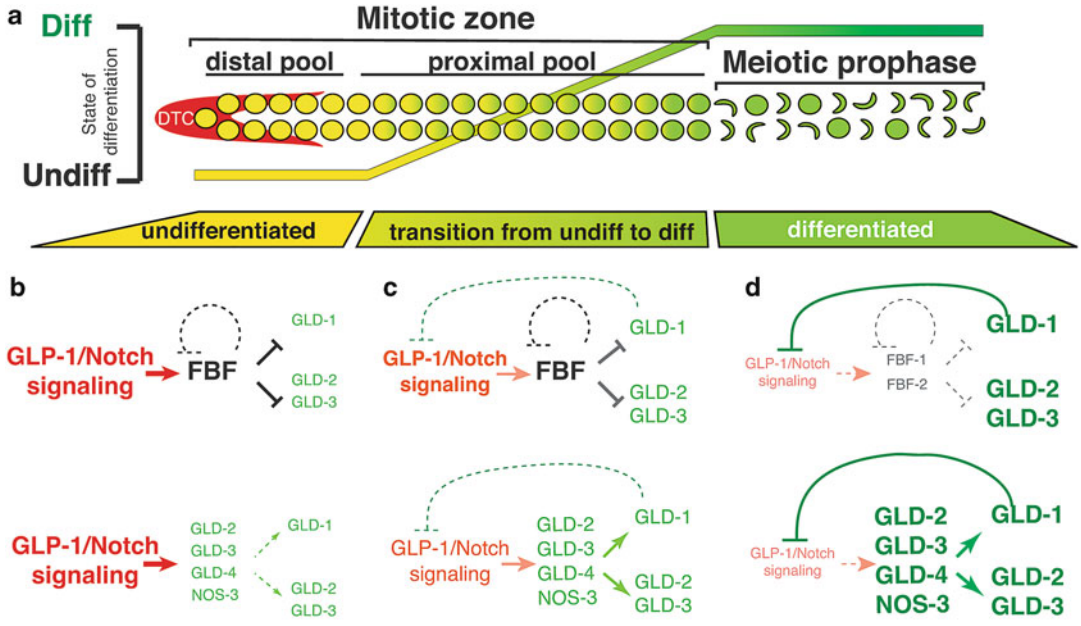

Fig. 3.4 Model for network transition from stem cell to differentiation. (**a**) Cartoon of progression from stem cell to overt differentiation in the distal germ line. *Left*, axis of differentiation with undifferentiated, stem cell-like state (Undiff) at *bottom* and differentiated (Diff) at *top*. As cells move proximally, they leave the undifferentiated pool and begin the transition toward differentiation. Cells in stem cell pool (*yellow*); cells in meiotic cell cycle (*green*); cells transitioning from stem to differentiated state (gradient from *yellow* to *green*); DTC, distal tip cell (*red*). (**b–d**) Model for network dynamics. (**b**) Stem cells are maintained in an undifferentiated state by strong GLP-1/Notch signaling, which activates FBF to repress generation of GLD proteins. (**c**) As cells progress from the niche, GLP-1/Notch signaling attenuates, tipping the network such that GLD proteins start to reinforce their own expression and repress GLP-1/Notch signaling. (**d**) Abundant GLD proteins continue to reinforce their own expression and repress GLP-1/Notch signaling, promoting differentiation (entry into the meiotic cell cycle)

DTC have transmembrane domains, suggesting that they are signaling locally [45, 47].

- FBF activity is proposed to decrease once germ cells have left the mitotic zone. FBF-1 and FBF-2 are both abundant in the mitotic zone and taper off as germ cells enter the meiotic cell cycle [52, 61]. In addition, FBF represses its target mRNAs in the mitotic zone and therefore is active in that region (see above). The mechanism limiting FBF to the mitotic zone is not yet understood.
- GLD activities are proposed to increase as germ cells progress through the mitotic zone [12, 23, 105]. Low GLD abundance at the distal end is accomplished by FBF repression of *gld* mRNAs together with CYE-1 repression of GLD-1 protein accumulation [61, 78, 125]. In addition to controls on protein abundance, post-translational regulation of GLD activity could be an important mode of regulation that remains to be explored. The massive increase in GLD-1 abundance as germ cells progress through the proximal pool is likely due to the cumulative effect of the GLD-2 and GLD-4 poly(A) polymerases, which act directly on *gld-1* mRNA [106, 108] together with the effects of the NOS-3 Nanos-like RNA-binding protein [105] and splicing factors [116].

The idea that FBF transitions into an activating macromolecular complex as germ cells transit from the niche towards differentiation is not included in Fig. 3.4 for simplicity. This idea is based on several findings: FBF acts genetically in the GLD-2/GLD-3 branch of the pathway, FBF binds GLD-2 *in vitro*, FBF promotes GLD-2 poly(A) polymerase activity *in vitro* and FBF co-immunoprecipitates with GLD-2 from worm extracts [61, 70]. The primary prediction of this model is that, upon FBF removal, the abundance

of FBF targets should decrease in the region where FBF functions within an activating complex [70]. This prediction holds true in male but not female germlines. The simplest interpretation is that FBF activates differentiation in male but not hermaphrodite germlines. Alternatively, FBF activation of GLD-2 could be redundant to other means of GLD-2 activation in oogenic germlines. Thus the role of FBF in activation is not clear at this point.

3.8 Conclusions and Future Directions

The analysis of *C. elegans* GSC regulation has been instrumental for understanding basic mechanisms of stem cell regulation. The mesenchymal DTC is an exceptionally well-defined and simple stem cell niche; the use of GLP-1/Notch signaling for stem cell maintenance provides a powerful model for unraveling Notch-dependent stem cell controls; and the regulatory network acting downstream of Notch signaling demonstrates the importance of post-transcriptional regulation for both stem cell maintenance and differentiation. These broad conclusions set the stage for the continued mining of principles of stem cell regulation.

Several major questions with broad implications are now poised for attack in this tractable system. What controls the extent of niche influence for control of a stem cell pool? How does Notch signaling govern stem cell maintenance? How are stem cell daughters triggered to embark on the path to differentiation? What are the biological roles of the various molecular mechanisms of mRNA control used by PUF proteins? How prevalent is post-transcriptional regulation in stem cell control? And how does the environment impact this stem cell molecular network? Answers to these fundamental questions in nematodes will likely lead to discovery of mechanisms of stem cell control that are widely conserved, including in humans.

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