Diverse Roles of PUF Proteins in Germline Stem and Progenitor Cell Development in C. elegans

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Stem cell development depends on post-transcriptional regulation mediated by RNA-binding proteins (RBPs) (Zhang et al., 1997; Forbes and Lehmann, 1998; Okano et al., 2005; Ratti et al., 2006; Kwon et al., 2013). Pumilio and FBF (PUF) family RBPs are highly conserved post-transcriptional regulators that are critical for stem cell maintenance (Wickens et al., 2002; Quenault et al., 2011). The RNA-binding domains of PUF proteins recognize a family of related sequence motifs in the target mRNAs, yet individual PUF proteins have clearly distinct biological functions (Lu et al., 2009; Wang et al., 2018). The C. elegans germ line is a simple and powerful model system for analyzing regulation of stem cell development. Studies in C. elegans uncovered specific physiological roles for PUFs expressed in the germline stem cells ranging from control of proliferation and differentiation to regulation of the sperm/oocyte decision. Importantly, recent studies started to illuminate the mechanisms behind PUF functional divergence. This review summarizes the many roles of PUF-8, FBF-1, and FBF-2 in germline stem and progenitor cells (SPCs) and discusses the factors accounting for their distinct biological functions. PUF proteins are conserved in evolution, and insights into PUF-mediated regulation provided by the C. elegans model system are likely relevant for other organisms.

Keywords: germline, C. elegans, pumilio and fem-3-binding factor, RNA regulation, stem cells

INTRODUCTION

Post-transcriptional regulation of gene expression governs the rate of protein production through the control of key steps in mRNA life cycle. In eukaryotes, RNA-binding proteins (RBPs) play critical roles in mRNA biogenesis, stability, function, transport, and cellular localization essential for post-transcriptional regulation (Glisovic et al., 2008). RBPs expressed in stem cells contribute to the regulation of stem cell self-renewal and differentiation (Zhang et al., 1997; Forbes and Lehmann, 1998; Okano et al., 2005; Ratti et al., 2006; Kwon et al., 2013), while misregulation of RBP activity can lead to tumors (Rezza et al., 2010; Degrawwe et al., 2016). Post-transcriptional regulation in stem cells relies on the combined activities of many RBPs (Eckmann et al., 2004; Arvola et al., 2017). Investigating the basic mechanisms of RBP function in stem cells will advance our understanding of abnormal post-transcriptional regulation relevant to human diseases, such as cancer.

Pumilio and FBF family RBPs are highly conserved eukaryotic posttranscriptional regulators (Wickens et al., 2002; Quenault et al., 2011). The name of this family comes from the first identified PUF proteins, *Pumilio* in *D. melanogaster* and fem-3-binding factor (FBF) in *C. elegans*. 
PUF proteins control diverse biological processes including oogenesis (Parisi and Lin, 1999), organelle biogenesis (García-Rodriguez et al., 2007), neuronal function (Mee et al., 2004), and memory formation (Dubnau et al., 2003; Zhang et al., 2017). In addition to these roles, PUF proteins share an evolutionarily conserved role in stem cell maintenance. Mutation of Pumilio induces loss of female germline stem cells in Drosophila due to differentiation to cystoblasts and then egg chambers (Lin and Spradling, 1997; Forbes and Lehmann, 1998). Similarly, loss of PUF proteins in C. elegans results in germline stem cells entering meiosis and undergoing spermatogenesis (Zhang et al., 1997; Crittenden et al., 2002; Haupt et al., 2019b) and knockdown of planarian homolog DjPum by RNA interference induces loss of totipotent stem cells called neoblasts (Salvetti et al., 2005). In mammals, PUM proteins contribute to stem cell maintenance across multiple tissues (Shigunov et al., 2012; Naudin et al., 2017; Zhang et al., 2017).

Canonical PUF proteins are characterized by a conserved RNA-binding domain (Pumilio homology domain, PUM-HD) with eight consecutive α-helical PUM repeats (Zamore et al., 1997; Zhang et al., 1997; Wang et al., 2001; Hall, 2016). Crystal structures of the classical PUM-HD uncover a crescent arrangement of PUM repeats. Single-stranded RNA binds to the inner concave surface of PUM-HD. Typically, one PUM repeat contacts one RNA base. A five-amino-acid motif in the second alpha helix of a PUM repeat determines the sequence specificity of RNA base recognition (Wang et al., 2002; Cheong and Hall, 2006; Campbell et al., 2014). Three key residues in the motif directly interact with RNA, thus comprising the tripartite recognition motifs (TRMs) (Wang et al., 2002; Campbell et al., 2014; Hall, 2016). Although individual PUF proteins preferentially associate with RNA motifs of distinct lengths and sequences, the canonical target motifs share the core UGU triplet (Lu et al., 2009; Wang et al., 2018).

Pumilio and FBF proteins control stability and translation of their target mRNAs by binding to their 3′UTRs (Zamore et al., 1997; Zhang et al., 1997). The best-documented mechanism of PUF-mediated regulation is through deadenylation of the target mRNAs that results in translational repression or mRNA decay (Wreden et al., 1997; Goldstrohm et al., 2006; Kadyrova et al., 2007; Van Etten et al., 2012; Weidmann et al., 2014). Alternatively, PUFs can interfere with recognition of cap structure by translation initiation factors through directly binding to the cap (Cao et al., 2010) or through recruiting cap-binding cofactors (Cho et al., 2005, 2006). Additionally, PUFs might attenuate translational elongation through an interaction with Argonaute family proteins (Friend et al., 2012). For all PUFs investigated to date, high-throughput approaches have suggested a large number of putative regulatory targets. Putative PUF-regulated transcripts have been identified in yeast, Drosophila, C. elegans, and humans by using RIP (RNA Immunoprecipitation)-Chip, RIP-seq, and CLIP (Cross-linking Immunoprecipitation)-seq (Gerber et al., 2004, 2006; Morris et al., 2008; Hafner et al., 2010; Prasad et al., 2016; Porter et al., 2019). The conservation of a number of PUF targets between nematodes and other species including humans was first reported in a microarray study (Kershner and Kimble, 2010) and then confirmed and expanded by CLIP-seq analysis (Prasad et al., 2016; Porter et al., 2019). The shared PUF target mRNAs are enriched for biological process GO terms such as cell cycle, cell division, and nuclear division. Cell cycle regulation is central to stem cell maintenance (Boward et al., 2016), and mRNA target conservation reflects PUF proteins’ ancient role in stem cell maintenance.

The C. elegans germline is a powerful model that revealed many aspects of PUF protein function in germline stem cells. Ten PUF proteins identified in C. elegans are clustered into 4 subfamilies: PUF-8/9, FBF-1/2, PUF-3/11/4, and PUF-5/6/7 (Wickens et al., 2002; Stumpf et al., 2008; Hubstenberger et al., 2012; Liu et al., 2012). Five of these PUF proteins, FBF-1 and FBF-2, as well as PUF-8, PUF-3, and PUF-11 are enriched in germline stem cells and support stem cell maintenance (Crittenden et al., 2002; Lamont et al., 2004; Ariz et al., 2009; Racher and Hansen, 2012; Voronina et al., 2012; Haupt et al., 2019b), yet each is functionally distinct. In-depth studies of C. elegans germline PUF proteins provided novel insights into the mechanisms mediating this functional specialization. This review provides an overview of C. elegans germline stem cells and focuses on the contribution of PUF-8, FBF-1, and FBF-2 to germline stem and progenitor cell function, since PUF-3 and PUF-11 are less well-studied. We then discuss recent advances in uncovering the determinants that mediate the divergence of PUF biological functions.

C. elegans GERMLINE, A POWERFUL MODEL FOR STEM CELL STUDIES

Overall Structure of C. elegans Germline

The C. elegans germline is a simple but very powerful model system for studying stem cell biology (Figure 1A). C. elegans can exist as hermaphrodites or males, and in this review, we are focusing on hermaphrodites, although mechanisms regulating germline stem cells are similar in the two sexes. A C. elegans adult contains two symmetric U-shaped germlines. Most of the C. elegans germline, except for late oocytes, is a syncytium, where individual germ cells have an opening to a central shared cytoplasmic core (Hirsh et al., 1976). Although germ cells have access to continuous cytoplasm, the communication between cells is limited and neighboring germ cells can be seen at distinct stages of cell cycle or differentiation. Similar to the germlines of other organisms, the C. elegans germline is maintained by a population of proliferative stem cells in the stem cell niche at its distal end (Figure 1A; Pazdernik and Schedl, 2013). When progenitor cells leave the niche, they enter meiosis followed by differentiation into sperm during larval development and into oocytes in adulthood. Maintenance of stem and progenitor cells (SCPs) in the mitotic zone is critical for C. elegans germline development and worm fertility.

Germline Stem and Progenitor Cells

The proliferative zone of the C. elegans germline extends about 20 cell diameters from the distal tip, and contains cells in a mitotic cell cycle and cells that have entered meiotic S-phase (Crittenden et al., 2006; Jaramillo-Lambert et al., 2007; Fox et al., 2011).
SPCs. The collectively refer to the cells that have not entered meiosis as equivalent cells (Fox and Schedl, 2015). In this review, we amplifying cells, the proliferative zone contains developmentally Wang and Voronina PUFs in Stem Cells

GLP-1, signaling components such as the receptor and Kimble, 1987). Loss-of-function mutations of GLP-1/Notch is highly conserved in most multi-cellular organisms (Austin and Kimble, 1987; Henderson et al., 1997; Nadarajan et al., 2009; Kershner et al., 2014). By contrast, germ cells of the germ line are found in RNA granules (Noble et al., 2008). PUF proteins expressed in the C. elegans germ line are found in RNA granules (Noble et al., 2008; Ariz et al., 2009; Voronina et al., 2012; Haupt et al., 2019b). PUF-5 colocalizes with processing body components (Noble et al., 2008), PUF-8 and FBF-2 localize to P granules (Ariz et al., 2009; Voronina et al., 2012; Wang et al., 2016), and the identities of RNA granules containing FBF-1 or PUF-3 and PUF-11 are currently unknown.

**Cytoplasmic Organization of RNA Regulation**

Many RBPs that mediate post-transcriptional regulation of germ cell development are found enriched at cytoplasmic foci called RNA granules. Germ cells have a number of RNA granule subtypes (Figure 1A), including germ granules or P granules in C. elegans, processing bodies, and stress granules (Voronina et al., 2011). The processing bodies and stress granules are distributed throughout the cytoplasm in somatic cells as well as in germ cells (Boag et al., 2005; Noble et al., 2008; Hubstenberger et al., 2017; Lechler et al., 2017). By contrast, P granules are perinuclear cytoplasmic RNA granules specific to germ cells and present throughout germ line development, excluding mature sperm (Strome and Wood, 1982). All PUF proteins expressed in the C. elegans germ line are found in RNA granules (Noble et al., 2008; Ariz et al., 2009; Voronina et al., 2012; Haupt et al., 2019b). PUF-5 colocalizes with processing body components (Noble et al., 2008), PUF-8 and FBF-2 localize to P granules (Ariz et al., 2009; Voronina et al., 2012; Wang et al., 2016), and the identities of RNA granules containing FBF-1 or PUF-3 and PUF-11 are currently unknown.

**REGULATORY ROLES OF PUF PROTEINS IN C. ELEGANS GERMLINE STEM AND PROGENITOR CELLS**

**PUF Function in Maintaining Germline SPCs**

Germ line stem cells are maintained by promoting proliferation and/or inhibiting cell death and differentiation. FBF-1 and FBF-2 are redundantly required for maintaining germline SPCs in adult hermaphrodites since a C. elegans double mutant for both FBFs lose their germ line stem cells by 24 h after the last larval stage (Crittenden et al., 2002). Several FBF targets have been proposed to contribute to FBFs’ role in SPC maintenance (Figure 2A). First, FBFs are suggested to repress expression of MPK-1, a homolog of Mitogen-activated protein kinase (MAPK)/ERK, and mpk-1 mRNA contains two FBF binding elements in its 3’UTR (Lee et al., 2007a). This repression was

**RNA-Binding Protein Network Downstream of GLP-1/Notch**

Post-transcriptional control is a widespread mechanism for regulating gene expression in the C. elegans oogenic germline (Merritt et al., 2008). Downstream of GLP-1/Notch, germline stem cell development is regulated by a network of posttranscriptional regulators that includes a large number of RBPs, a subset of which is shown in Figure 1B. FBF-1 and FBF-2, PUF family RBPs expressed in the distal germ line, control stem cell maintenance and sex fate (Zhang et al., 1997; Crittenden et al., 2002). Additionally, four RNA regulators, including three GLD proteins and NOS-3, act in two parallel pathways that inhibit mitosis and promote meiosis (Hansen et al., 2004), while the cytoplasmic poly(A) polymerase formed by the complex of GLD-2 [the poly(A) polymerase enzyme] and GLD-3 (a homolog of Bicaudal-C RBP) constitutes a translational activation pathway (Eckmann et al., 2004).

Unlike other stem cell systems with distinct stem cells and transit amplifying cells, the proliferative zone contains developmentally equivalent cells (Fox and Schedl, 2015). In this review, we collectively refer to the cells that have not entered meiosis as SPCs. The C. elegans germ line SPC zone is maintained within a niche formed by a single mesenchymal cell, called the distal tip cell (DTC), which caps the distal end of the germ line and extends its cytoplasmic processes proximally (Kimble and White, 1981; Crittenden et al., 2006; Byrd et al., 2014). The DTC preserves the mitotic identity and promotes mitotic division of SPCs through the canonical GLP-1/Notch signaling that is highly conserved in most multi-cellular organisms (Austin and Kimble, 1987). Loss-of-function mutations of GLP-1/Notch signaling components such as the receptor glp-1, ligands lag-2 and apx-1, and downstream transcriptional targets lst-1 and sygl-1 cause germ line stem cells to enter meiosis prematurely, which is similar to the DTC removal (Kimble and White, 1981; Austin and Kimble, 1987; Henderson et al., 1997; Nadarajan et al., 2009; Kershner et al., 2014). By contrast, germ cells of the glp-1(oz112gf) gain-of-function mutant with constitutive GLP-1 signaling fail to exit from the mitotic cell cycle leading to tumorous germ lines (Berry et al., 1997).
hypothesized to be important for stem cell maintenance since RNAi-mediated knockdown of mpk-1 increased the number of mitotic germ cells, while promoting MPK-1 activity by a Ras gain-of-function mutation let-60(n1046) decreased the number of mitotic germ cells (Lee et al., 2006). Similarly, MAPK repression is observed to promote self-renewal of embryonic stem cells and skeletal muscle stem cells (Burdon et al., 1999; Bernet et al., 2014). However, in addition to repressing MPK-1, FBFs repress the expression of its negative regulator, MAPK inactivating phosphatase LIP-1 (Lee et al., 2006). Therefore, an fbf mutation would derepress both MPK-1 and LIP-1 that inhibits MAPK activity and thus might not result in abnormal activation of MPK-1 in SPCs. Instead, such mutation would result in a sensitized background that might deregulate MPK-1 following additional genetic lesions. Regulation of MAPK by PUF homologs appears conserved in evolution, and was also documented in human embryonic stem cells as well as in mouse spermatocytes (Lee et al., 2007a; Chen et al., 2012). Second, FBFs promote self-renewal of germline stem cells by repressing expression of CKI-2 (Kalchhauser et al., 2011), a Cyclin-dependent kinase inhibitor that regulates cell cycle entry/exit decisions (Buck et al., 2009). Removing cki-2 partially rescues the germline stem cell depletion phenotype in fbf-1 fbf-2 double mutant adult hermaphrodites (Kalchhauser et al., 2011), suggesting that repression of cki-2 is not the only mechanism by which FBFs promote stem cell proliferation. CIP/KIP family cyclin-dependent kinase inhibitors are conserved targets of PUF proteins as they were found to be regulated by PUFs in mouse embryos and human cells (Kedde et al., 2010; Lin et al., 2019). Interestingly, genes encoding diverse cell cycle regulators, beyond cki-2 and its homologs, are enriched among target mRNAs pulled down with FBFs as well as PUF proteins from other organisms (Hafner et al., 2010; Kershner and Kimble, 2010; Chen et al., 2012; Prasad et al., 2016; Porter et al., 2019), suggesting a conserved mechanism of PUF-mediated control of cell proliferation. Third, FBFs prevent premature meiotic entry of SPCs by inhibiting expression of target mRNAs that encode differentiation-promoting regulators, such as GLD-1 (Crittenden et al., 2002), GLD-2 (Millonigg et al., 2014), and GLD-3 (Eckmann et al., 2004), as well as structural components of meiotic chromosomes, such as HTP-1, -2 orthologs of human HOR1 and 2 (Merritt and Seydoux, 2010).

PUF-8 promotes germline SPC proliferation by acting redundantly with a KH domain-containing RBP MEX-3 (Figure 2A; Ariz et al., 2009). This function might be explained by PUF-8-dependent translational control of cell cycle regulators, but the analysis of GLP-1/Notch receptor in the mutant germlines uncovered mislocalization of GLP-1 protein (Ariz et al., 2009). It appears that PUF-8 facilitates translation of the endoplasmic reticulum protein FARL-11 that is required for GLP-1 membrane localization (Maheshwari et al., 2016), suggesting another potential mechanism of PUF-8 promoting SPC proliferation.

**PUF Function in Inhibiting Mitotic Cell Fate of SPCs and Promoting Differentiation**

In addition to facilitating stem cell maintenance, both FBFs and PUF-8 were unexpectedly found to limit stem cell numbers by promoting stem cell exit from mitosis and differentiation (Figure 2B). The GLP-1/Notch signaling within the distal niche maintains the mitotic cell fate of germline SPCs (Hansen and Schedl, 2006; Kimble and Crittenden, 2007). Temperature-sensitive glp-1(gf) mutant animals with excessive GLP-1 activity have slightly enhanced proliferation of germline SPCs at the permissive temperature, and produce tumorous germlines at the restrictive temperature (Berry et al., 1997; Pepper et al., 2003; Wang et al., 2012). Interestingly, puf-8 knockout strongly enhances germ cell over-proliferation of several glp-1(gf) mutants at the permissive temperature. This suggests that puf-8 might inhibit mitotic cell fate of SPCs through negatively regulating the GLP-1/Notch signaling or by functioning parallel to it (Racher and Hansen, 2012). One relevant target mRNA for PUF-8-mediated inhibition of the mitotic cell fate is *C. elegans* RAS. 

![Figure 2](https://www.frontiersin.org)
homolog LET-60. Loss of puf-8 promotes accumulation of both endogenous LET-60 and a GFP:H2B reporter under the control of the let-60 3′UTR in mitotic germ cells as well as in early meiotic cells, suggesting direct regulation of let-60 by PUF-8 (Vaid et al., 2013). Increased levels of LET-60 in puf-8 mutant are not sufficient to ectopically activate MPK-1 in SPCs (Vaid et al., 2013). However, additional loss of LET-60 negative regulator gap-3 in the puf-8; gap-3 double mutant leads to activation of MPK-1 throughout the germline, abnormal mitotic proliferation, and tumorous germlines (Vaid et al., 2013). Interestingly, tumor formation in this genetic background was dependent on MAPK signaling and was repressed by RNAi-mediated depletion of MAPK pathway components (Vaid et al., 2013). It appears that MAPK activation doesn’t always cause the proliferative response of SPCs, since the presence of activated MPK-1 in a different double mutant background (fbf-1; lip-1) fails to elicit abnormal proliferation (Lee et al., 2007a). This brings up a question whether MAPK signaling promotes SPC proliferation (Lee et al., 2006) or differentiation (Vaid et al., 2013). Analysis of null mutants in lin-45/Raf; mek-2/MEK, and mpk-1/ERK suggested that MAPK components are not essential for SPC maintenance, but each leads to a decrease in SPC numbers especially as animals age (Lee et al., 2007b). Additionally, null mutants in lin-45/Raf, mek-2/MEK, and mpk-1/ERK enhance premature meiotic entry defect of a temperature-sensitive gdp-1 loss-of-function allele at the permissive temperature, suggesting that MAPK signaling promotes SPC proliferation (Lee et al., 2007b). On the other hand, RNAi depletion of mpk-1 increased SPC numbers (Lee et al., 2006). The null mutations and RNAi treatment might not affect gene function with the same efficiency, and disparate results obtained by the two approaches might point to the critical differences in specific levels and developmental dynamics of MAPK activity underlying each phenotype. Considering this, regulation of multiple genes affecting the levels of MPK-1-mediated signaling by FBFs and PUF-8 (Lee et al., 2006, 2007a; Vaid et al., 2013) might allow SPCs to maintain precise control of MPK-1 activity during development.

Genetic evidence suggests that FBFs act to promote meiotic entry of SPCs through the GLD-2, GLD-3 genetic pathway (Crittenden et al., 2002). GLD-1, NOS-3 and GLD-2, GLD-3 are the two main pathways that redundantly promote SPC meiotic entry (Figure 1B; Kimble and Crittenden, 2007). In the absence of gdp-1, FBFs are no longer required to sustain germline proliferation and the gdp-1; fbf-1 fbf-2 mutant worms have tumorous germline with all mitotic cells (Crittenden et al., 2002). This tumorous germline phenotype is similar to the tumors observed in gdp-1; gld-2 and gdp-1; gld-3 mutants (Kadyk and Kimble, 1998; Eckmann et al., 2004), suggesting a possibility that FBFs proteins function through the GLD-2, GLD-3 genetic pathway to promote meiotic entry. Direct interaction of FBF with GLD-3 that might underlie this function is discussed further in section “Protein Cofactors That Change PUF Regulatory Outcome.”

The fact that PUF proteins appear to regulate both proliferation and differentiation is enigmatic and has promoted several interpretations. For example, PUF-8 represses some mRNAs associated with proliferation while facilitating expression of other targets promoting proliferation in the same cells. As a result, it is possible that the overall effect of PUF-8 on germline proliferation is minor, and it acts to fine-tune SPC proliferation rather than as an all-or-none switch specifying stem cell fate. In a similar vein, functional annotation of mRNAs co-isolated with FBFs suggests that they associate with and repress mRNAs required for both differentiation as well as cell cycle progression of germ cells (Prasad et al., 2016; Porter et al., 2019). One intriguing interpretation is that this allows FBFs to simultaneously control the rate of both SPC proliferation and differentiation, thus maintaining the balance between these two cell fates. In order to maintain stem cell numbers over time, their self-renewal needs to be matched with differentiation (Morrison and Kimble, 2006). In C. elegans, SPC homeostasis is controlled at a population level, where some progenitor cells are lost through differentiation, while other cells proliferate, with both outcomes observed at the same frequency (Kimble and Crittenden, 2007). Although C. elegans SPCs proliferate continuously, the rate of SPC proliferation changes during development and is responsive to environmental conditions and nutrition (Hubbard et al., 2013). Simultaneous control of SPC proliferation and differentiation by FBFs might work to match the output of the stem cell compartment to the proliferative demands of the germline, while keeping the two fates in a balance.

**PUF Function in Controlling the Sperm/Oocyte Decision in Germline Mitotic Zone**

The mechanism underlying sperm/oocyte decision has been a long-standing question in all animals (Casper and Van Doren, 2006; Kimble and Page, 2007). In C. elegans hermaphrodites, germlines first produce sperm and then oocytes, but it is still not clear when, where, and how the sperm/oocyte switch is executed. As recently reviewed (Zanetti and Puoti, 2013), the germline sex determination is executed through an elaborate pathway involving more than 30 regulators for sperm or oocyte specification, part of which is shown in Figure 2C. These regulators, including GLD-1, TRA-1 (GLI transcription factor homolog; Hodgkin, 1987), and FOG-1 (feminization of the germline, a member of cytoplasmic polyadenylation element binding protein family; Jin et al., 2001) are expressed in the proximal mitotic region and transition zone, suggesting that the commitment of germ cells to the sperm or oocyte fate might occur in distal germline. Studies of sex determination in a temperature-sensitive fog-1 mutant suggested that germ cells might become committed to the sperm or oocyte fate when they enter meiosis (Barton and Kimble, 1990). Further analysis of sex determination in puf-8; lip-1 worms that permit chemical manipulation of the sperm/oocyte decision supported these earlier conclusions by mapping the sex fate determination to the progenitor cells moving proximally to transition zone (Morgan et al., 2013). PUF-8, FBF-1, and FBF-2 contribute to the control of the sperm/oocyte decision by regulating expression of sex-determination regulators (Figure 2C).

FBF-1 and FBF-2 are redundantly required for controlling the sperm/oocyte switch. Nematodes mutant for individual fbf
genes produce both sperm and oocytes, but the \( \text{fhl} \) double mutants fail to switch from spermatogenesis to oogenesis and only produce sperm (Zhang et al., 1997). The two FBF paralogs promote oogenesis by repressing several target mRNAs including \( \text{fem-3, fog-1} \), and possibly \( \text{fog-3} \) that are positive regulators for sperm fate decision (Zhang et al., 1997; Thompson et al., 2005). Additionally, Nanos homolog NOS-3 physically interacts with FBF proteins and participates in the FBF-mediated sperm/oocyte switch through forming a regulatory complex that represses the translation of \( \text{fem-3} \) mRNA (Kraemer et al., 1999; Arur et al., 2011). The binding between NOS-3 and FBF-1 is disrupted by MPK-1/ERK-dependent phosphorylation of NOS-3 to limit formation of the functional complex to the distal germine (Arur et al., 2011). Lastly, functional splicing machinery promotes efficient sperm/oocyte switch (Kerins et al., 2010), and a combination of \( \text{fhl} \) single mutants and splicing factor knockdown results in enhanced germline masculinization, suggesting that the splicing machinery facilitates FBF function in sex determination (Novak et al., 2015).

PUF-8 and FBF-1 also redundantly promote the germline sperm/oocyte switch (Bachorik and Kimble, 2005). A mutation in \( \text{pfu-8} \) results in a low percentage of germlines that develop excess sperm and fail to switch to oogenesis, whereas most of the \( \text{fhl-1 pfu-8} \) double mutants result in germlines with a failed sperm/oocyte switch. GLD-1 and FOG-2 proteins can physically interact (Clifford et al., 2000), and both are required for the sperm fate determination (Jan et al., 1999; Clifford et al., 2000; Hu et al., 2019). The dramatic increase in FOG-2 protein abundance in \( \text{fhl-1 pfu-8} \) double mutants and rescue of oogenesis in \( \text{fhl-1 pfu-8} \) suggests that FBF-1 and PUF-8 function upstream of FOG-2 in the sex determination pathway (Bachorik and Kimble, 2005). In addition, PUF-8 acts redundantly with MEX-3 to promote the sperm/oocyte switch (Ariz et al., 2009). Although \( \text{pfu-8, mex-3} \) mutant germlines have severe proliferation defects and never produce any gametes, 34% of \( \text{pfu-8, mex-3(+/-)} \) mutant worms produce only sperm (Ariz et al., 2009). This suggests that MEX-3 contributes to the sperm/oocyte switch in the absence of PUF-8, although the relevant regulatory targets have not yet been identified (Ariz et al., 2009).

One of the many functions of MAPK/ERK signaling pathway in \( C. \) elegans is to promote the sperm fate (Lee et al., 2007b). Therefore, regulation of MPK-1 activity by PUF-8 and FBF-1 reviewed above contributes to germline sex determination. Hyperactivation of MPK-1 and excessive spermatogenesis were observed in \( \text{pfu-8, lip-1} \) as well as in \( \text{fhl-1, lip-1} \) genetic backgrounds (Morgan et al., 2010; Sorokin et al., 2014). In these genetic backgrounds, spermatogenesis was dependent on MPK-1 activity and repressed by a small molecule MEK inhibitor U0126 (Morgan et al., 2010; Sorokin et al., 2014). Activation of MPK-1 in \( \text{fhl-1, lip-1} \) genetic background likely results from the loss of FBF-mediated repression of \( \text{mpk-1} \) translation and the loss of LIP-1-mediated post-translational inhibition of MPK-1 (Lee et al., 2007a). On the other hand, PUF-8 limits ERK activity by repressing LET-60/RAS (Vaid et al., 2013), and the \( \text{pfu-8, lip-1} \) double mutant results in hyperactivation of MPK-1/ERK at meiotic entry in the transition zone (Morgan et al., 2013).

**PUF-8 Function in Protecting Germ Cell Fate**

In multicellular organisms, diverse factors and mechanisms, including posttranscriptional regulation, contribute to the maintenance of germ cell fate and protect germ cells from reprogramming toward somatic cells (Strome and Updike, 2015). To protect germ cell identity, PUF-8 suppresses the expression of \( \text{pal-1} \) in germline stem cells of \( C. \) elegans (Figure 2D; Mainpal et al., 2011). PAL-1 is a somatic homeodomain transcription factor that activates transcription of its downstream targets such as \( \text{hhl-1} \) (Hunter and Kenyon, 1996; Lei et al., 2009). In turn, \( \text{hhl-1} \) encodes the myogenic regulatory factor HLH-1/MyoD homolog that is normally expressed in the embryonic muscle lineage (Krause et al., 1990). Deletion of \( \text{pfu-8} \) results in derepression of \( \text{PAL-1} \) in germline SPCs and PAL-1-dependent misexpression of HLH-1 in germ cells (Mainpal et al., 2011). These findings suggest that PUF-8 protects germline SPCs from the impact of somatic differentiation factors such as PAL-1.

**MECHANISMS BEHIND FUNCTIONAL DIVERGENCE OF PUF PROTEINS**

The highly conserved RNA-binding domain of canonical PUF family proteins recognizes stereotypical consensus binding sites in target mRNAs (Wang et al., 2018). Yet, as reviewed in the previous section, individual PUF proteins have clearly distinct regulatory functions. In \( C. \) elegans germline stem cells, activities of multiple PUF proteins combine to promote many aspects of healthy stem cell function. This made \( C. \) elegans germine an excellent model to probe the mechanisms mediating functional specialization of PUFs. Here we will survey the recent insights into the mechanisms specifying unique non-redundant aspects of RNA regulation mediated by FBF-1, FBF-2, and PUF-8.

**Structural Differences in RNA-Binding Domains Determine the Specificity of Binding RNA**

All canonical PUF proteins contain a highly conserved RNAS-binding domain, PUF domain (also known as PUM-HD), with eight consecutive \( \alpha \)-helical repeats. Crystal structures of the PUM-HDs from different organisms bound to short target RNA motifs revealed that the PUM-HD forms a crescent shape molecule with eight \( \alpha \)-helical repeats ( Edwards et al., 2001; Wang et al., 2001, 2002, 2009; Zhu et al., 2009). Mutational analysis of \( Drosophila \) Pumilio revealed the amino acids mediating contacts with the mRNA and protein partners (Zamore et al., 1997; Wharton et al., 1998; Sonoda and Wharton, 1999, 2001). Subsequent structural studies of \( Drosophila, C. \) elegans, and mammalian PUFs extended the genetic and biochemical data and have identified the TRMs that contact RNA on the concave surface as well as the sites on the convex surface that interact
with protein partners (Edwards et al., 2001; Wang et al., 2002, 2009; Campbell et al., 2014; Bhat et al., 2019; Qiu et al., 2019). Differences in the PUF RNA-binding domains result in distinct RNA motifs bound by PUF homologs.

In vitro biochemical studies using isolated PUF domains found that C. elegans FBFs bind to the same RNA motif, a 9-nt motif (5′-UGURHHAUA-3′; Bernstein et al., 2005; Opperman et al., 2005; Campbell et al., 2012), while PUF-8 recognizes an 8-nt motif (5′-UGUANAUA-3′; Opperman et al., 2005; Bhat et al., 2019). Crystal structures of FBF and PUF-8 PUM-HDs in complex with their preferred RNA oligonucleotides uncovered RNA-binding modes for each protein. FBF's PUM repeats R8–R6 bind to the 5′-UGU sequence and PUM repeats R1–R3 bind to the AUA-3′ element. The purine in the fourth position is recognized by PUM repeat R4, while bases in positions five and six turn away from the RNA-binding surface. Interactions between base five and the protein depend on the identities of the fourth and fifth bases, and base six does not interact with PUM-HD at all (Wang et al., 2009). By comparison, PUF-8's PUM repeats R8–R5 bind to the 5′-UGA sequence, while PUM repeats R3–R1 bind to the AUA-3′ sequence with central fifth base stacked with the fourth base and not recognized by the protein (Bhat et al., 2019). Distinct binding site preferences between FBFs and PUF-8 are expected to result in specific mRNA populations associated with these proteins. FBF-1 and FBF-2 share most of their target mRNAs, which has been demonstrated by immunoprecipitation followed by CLIP-seq analyses (Prasad et al., 2016; Porter et al., 2019).

Initial characterization of PUF-8 target mRNAs was carried out through a pull-down with recombinant protein followed by micro-array analysis (Mainpal et al., 2011). Although PUF-8 target data is less extensive than those available for FBFs, several notable observations emerge. A number of PUF-8 targets are also present in FBF target lists and some, such as ubc-6, are regulated by both PUF-8 and FBFs (Mainpal et al., 2011; Prasad et al., 2016). However, overall the mRNAs bound to PUF-8 and FBFs are largely distinct. While further studies will determine the extent of PUF-8’s targets overlapping with those of FBFs, these initial results provide an attractive model of specifying distinct but redundant functions of FBFs and PUF-8 in the germline.

If distinct binding preferences of PUF proteins underlie the differences in their function, one might expect to elicit functional changes in PUFs through changing the RNA-binding interface. Recent structural study revealed that the RNA-binding preference of FBF-2 can be changed to become similar to that of PUF-8 through mutations in TRM of PUM repeat R5 (Bhat et al., 2019). The FBF-2 R5 variant was tested for its ability to mediate a PUF-8-specific function, namely promoting SPC differentiation in a genetic background of a temperature-sensitive glp-1(gf) mutation. While 98% of glp-1(gf) germlines with the wild-type FBF-2 developed tumors upon puf-8 knockdown, over-proliferation was only observed in 36% germlines expressing FBF-2 R5 variant (Bhat et al., 2019). This partial rescue supports the importance of PUF domain RNA-binding preference in specifying function, but it still remains to be determined whether FBF-2 R5 variant truly elicits its new effect through associating with and regulating PUF-8 targets in vivo.

**Protein Cofactors That Change RNA Target Preference**

While determination of in vivo FBF targets confirmed FBF preferential association with mRNAs containing canonical FBF-binding element identified in vitro, many of the identified targets did not contain the canonical motif, suggesting that FBF binding specificity may be altered in vivo (Prasad et al., 2016; Porter et al., 2019). Biochemical and structural studies of PUFs in complex with their partner proteins revealed that several PUF interacting partners can affect the RNA-binding affinity and specificity of PUF proteins (Figures 3A,B). Crystal structure of Nanos-Pumilio-RNA complex from Drosophila suggested that Nanos embraces Pumilio and RNA, contributes to sequence-specific contacts, and increases Pumilio affinity for hunchback mRNA (Weidmann et al., 2016; Malik et al., 2019). By contrast, association of Pumilio with mothers against dpp (mad) mRNA requires Bm and Bgcn proteins, but not Nanos (Malik et al., 2019). In C. elegans, both FBF proteins physically interact with CPB-1, a cytoplasmic polyadenylation element binding protein (Luitjens et al., 2000; Menichelli et al., 2013). The assay investigating binding of FBF-2 PUF domain to target mRNA in the presence of a 40-amino-acid fragment of CPB-1 outside of the RNA-binding domain demonstrated that association with CPB-1 fragment alters FBF’s preference for specific RNA sequences (Campbell et al., 2012; Menichelli et al., 2013). Additional FBF interaction partners include novel proteins SYGL-1 and LST-1 that are required for FBF-dependent target mRNA repression in germline SPCs (Kershner and Kimble, 2010; Brenner and Schedl, 2016; Shin et al., 2017; Haupt et al., 2019a). Using SEQRs (in vitro selection, high-throughput sequencing of RNA, and sequence specificity landscapes), analysis of RNA-binding preference of FBF-2 PUF domain bound to a 150-amino-acid LST-1 fragment...
containing one of FBF-binding sites revealed a distinct RNA-binding specificity of the FBF-2/LST-1 complex (Qiu et al., 2019). Crystal structure of FBF-2 in complex with a 24- amino-acid fragment of LST-1 and an 8-nucleotide RNA oligo isolated by in vitro selection showed that FBF-2 PUF domain changes its RNA-binding mode to 1:1 association of PUM repeats R4-R5 with GA in positions four and five (Qiu et al., 2019). However, the structural basis for the changes in the RNA-binding specificity is not entirely clear since association with LST-1 peptide appeared to weaken FBF-2 affinity for all tested target sequences (Qiu et al., 2019). Further studies are necessary to understand whether association with full-length LST-1 has similar effects on FBF-2 binding to its targets.

Protein Cofactors That Change PUF Regulatory Outcome

Pumilio and FBF proteins lack enzymatic activity and often mediate their regulatory influence by recruiting specific cofactors to their target mRNAs (Sonoda and Wharton, 1999; Eckmann et al., 2002; Cho et al., 2005, 2006; Goldstrohm et al., 2006; Kadyrova et al., 2007; Suh et al., 2009; Friend et al., 2012). PUF proteins typically reduce expression of their target mRNAs by repressing translation or promoting RNA decay (Wreden et al., 1997; Olivas and Parker, 2000; Crittenden et al., 2002; Goldstrohm et al., 2006; Cao et al., 2010; Weidmann and Goldstrohm, 2012). This repressive function of PUF proteins in C. elegans and other species can be mediated by CCR4-NOT deadenylase that promotes RNA deadenylation and decay (Figure 3C), and FBF-1, FBF-2, and PUF-8 all bind deadenylase enzyme CCF-1 (Goldstrohm et al., 2006; Suh et al., 2009). One alternative repressive mechanism suggested for FBFs relies on PUF domain interaction with Argonautes resulting in attenuated translational elongation (Friend et al., 2012).

In several cases, PUF proteins appear to activate translation: FBFs are suggested to promote GLD-1 expression in spermatogenic germline as well as translation of EGL-4 in neurons, while PUF-8 facilitates translation of FARL-11 in germline SPCs (Kaye et al., 2009; Suh et al., 2009; Maheshwari et al., 2016). A search for cofactors of FBFs uncovered an interaction with poly(A) polymerase complex identifying one potential mechanism for translational activation (Figure 3D; Eckmann et al., 2002; Kimble and Crittenden, 2007). FBFs interact with the GLD-3 subunit of GLD-3/GLD-2 cytoplasmic poly(A) polymerase complex (Eckmann et al., 2002). FBFs also interact with the GLD-2 subunit in the RNA-independent manner, and this interaction is facilitated by formation of a larger complex including GLD-3 (Suh et al., 2009). Interaction with GLD-3 does not affect FBFs binding to their target mRNA, and is instead hypothesized to switch the regulatory outcome from repression to activation (Wu et al., 2013).

It is still unknown what cofactors are required for PUF-8-mediated translational activation. Since FBF interacts with GLD-3 via its conserved RNA-binding domain (Wu et al., 2013), it is possible that PUF-8 RNA-binding domain might interact with GLD-3 as well. Additionally, a recent study found that PUF-8 promotes accumulation of several of its target mRNAs through interaction with mRNA processing/export machinery components, such as the nuclear cap-binding protein NCBP-2 (Pushpa et al., 2013).

Distinct PUF Localization

FBF-1 and FBF-2 are nearly identical in primary sequence, share most of the target mRNAs (Prasad et al., 2016; Porter et al., 2019), and function redundantly in maintaining germline SPCs. Nevertheless, they differentially affect germline SPC gene expression. Therefore, the mechanisms behind diverse biological activities of PUFs are required for many aspects of germline SPCs, development, and each facilitates specific aspects of SPC function. Studies in C. elegans resulted in considerable advances in understanding the mechanisms behind diverse biological activities of PUFs as shown in Figure 3. The next challenge to the field is to uncover the mechanisms directing PUF protein’s choice of specific cofactors and influencing PUFs function as negative or positive translational regulators in stem cells.

CONCLUSION

Pumilio and FBF family RBPs have evolved as essential post-transcriptional regulators of stem cell development in eukaryotes. PUF-mediated RNA regulation is achieved through recognizing target mRNAs and subsequently changing their rates of degradation or translation. Three PUF proteins, PUF-8, FBF-1 and FBF-2, expressed in C. elegans germline mitotic region are required for many aspects of germline SPCs development, and each facilitates specific aspects of SPC function. Studies in C. elegans resulted in considerable advances in understanding the mechanisms behind diverse biological activities of PUFs as shown in Figure 3. The next challenge to the field is to uncover the mechanisms directing PUF protein’s choice of specific cofactors and influencing PUFs function as negative or positive translational regulators in stem cells.
PUF protein Puf3p at the N-terminal low complexity region

their cofactors. In yeast, nutrient-responsive phosphorylation of activation. Since GLD-3 becomes expressed as SPCs transition doesn’t affect FBF target selection, but rather might change FBF this binding site is also shared by GLD-3, a protein that across development. Both CPB-1 and LST-1 appear to bind to the same site on FBF RNA-binding domain. Interestingly, might result in a shifting repertoire of FBF regulatory targets (Shin et al., 2017). Their stage-specific association with FBFs to the same site on FBF RNA-binding domain. As a result, FBF might become recruited to different sites on RNA transcripts, leading to the activation or repression of different target genes. This flexibility in RNA-binding specificity could provide an additional layer of regulation that affects FBF protein activity.

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XW and EV contributed equally to writing of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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