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A Protein•Protein Interaction Platform Involved in Recruitment of GLD-3 to the FBF•*fem-3* mRNA Complex

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Abstract

The *Pumilio* and *FBF* (PUF) family of RNA-binding proteins interacts with protein partners to post-transcriptionally regulate mRNAs in eukaryotes. The interaction between PUF family member *fem-3*-binding factor (FBF) and germline development defective-3 protein (GLD-3) promotes spermatogenesis in *Caenorhabditis elegans* by increasing expression of the *fem-3* mRNA. Defined here in these studies is the molecular basis for this critical interaction. A 10 amino acid region within GLD-3 is required for FBF binding, while a 7 amino acid loop in FBF between PUF repeats 7 and 8 is necessary for GLD-3 binding. These short sequences are conserved, as other FBF-binding proteins bear sequences similar to those in GLD-3 and other *C. elegans* PUF proteins contain sequences similar to those in FBF. The FBF-binding region of GLD-3 forms a ternary complex with FBF on the point mutation element (PME) in the *fem-3* 3' untranslated region (UTR) and formation of this GLD-3•FBF complex does not impact the RNA-binding activity of FBF. These data raise the possibility of alternative models involving the formation of a GLD-3•FBF•RNA complex in the regulation of germline mRNAs.

Keywords

RNA-binding proteins; PUF protein; *fem-3*; RNA•protein interactions; germline development

Introduction

RNA-binding proteins regulate the location, stability, and translation of developmental mRNAs to control cell division and differentiation in eukaryotes^{1–4}. Many of these regulatory events are directed by multi-protein complexes formed on the 3' untranslated region (UTR) of the mRNA through the recruitment of protein factors by the RNA-binding proteins^{5–7}. Relatively little is known about how these complexes are assembled or change during eukaryotic development. The *Caenorhabditis elegans* germline provides an ideal

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system for understanding these regulatory complexes, since mRNA regulation plays an integral role in germline development and many of the regulatory proteins and their mRNA targets in this system have been genetically identified and characterized. *C. elegans* is sexually dimorphic: males continuously produce sperm throughout their lifetime, whereas hermaphrodites produce sperm for a short period during larval development and switch to oogenesis in adulthood^{10–12}. Germline development in both sexes involves the coordination of mitosis, meiosis, and germ cell differentiation by the RNA-binding *Pumilio* and *FBF* (PUF) family member *fem-3*-binding factor (FBF)^{13–16}. FBF interacts with many different protein partners; among the identified proteins are: GLD-2, GLD-3, CPB-1, NOS-3, CCF-1, RACK-1, and CSR-1•EFT-3^{16–20}. These interactions are critical *in vivo* to the role of FBF in regulating the stability and translation of specific germline mRNAs.

Many of the *C. elegans* PUF protein binding partners and their interactions with PUF proteins are also biologically important in other organisms. For instance, GLD-3 is a member of the Bicaudal-C protein family, which has key roles in vertebrate embryogenesis, left-right asymmetry, and renal development^{21–24}; GLD-2 is a non-canonical poly(A) polymerase important in mammalian gametogenesis, cellular senescence, and memory^{25–28}; and CPB-1 is a member of the CPEB protein family, which has been implicated in human diseases such as insulin resistance and cancer. Despite the importance of PUF protein binding partners and their interactions with PUF proteins, little is known about the molecular or structural basis of these interactions.

The PUF protein family is characterized by a crescent shaped *Pumilio*-homology domain (PUM-HD), composed of eight α -helical PUF repeats flanked at the N- and C-termini by Csp1 and Csp2 caps (known as R1' and R8' in FBF), that binds to RNA on the inner surface (Figure 1(a) and 1(b))^{31–40}. FBF comprises two exemplary PUF proteins, FBF-1 and FBF-2, which are nearly identical in sequence, exhibit the same RNA-binding activities, and are largely redundant in biological function. In adult hermaphrodites, FBF promotes the switch to oogenesis by binding to the *fem-3* mRNA and repressing FEM-3 protein expression¹³. FBF specifically recognizes a UGUGUCAU sequence within the point mutation element (PME) in the *fem-3* 3' UTR (Figure 6(a))^{40–42}.

In males and larval hermaphrodites, the formation of a complex between the germline development defective-3 protein (GLD-3) and FBF is required for spermatogenesis⁴³. Previous yeast three-hybrid studies showing a modest reduction in FBF binding to the PME regulatory element in the presence of GLD-3 led to the hypothesis that GLD-3 association with FBF promotes spermatogenesis by disrupting the FBF•*fem-3* mRNA interaction¹⁹. Additional yeast two-hybrid studies showed that FBF interacts with the 860 to 949 amino acid region of GLD-3 (Figure 1(b))¹⁹; however, the sequence for this region does not resemble that of any known protein•protein interaction domain structures. These same studies showed that GLD-3 interacts with the RNA-binding PUM-HD domain of FBF (Figure 1(a) and 1(b))¹⁹. The biological importance of PUF protein•binding partner and FBF•GLD-3 complexes as well as the intriguing model proposed for the activation of the *fem-3* mRNA by the FBF•GLD-3 complex served as the motivation for examining these interactions.

In this report, both biochemical and cell-based assays were used to identify the molecular interface between the PUF protein, FBF, and its binding partner, GLD-3. Their interaction is mediated by short, specific sequences in each protein. These sequences are conserved, as other FBF-binding proteins bear sequences similar to those in GLD-3 and other *C. elegans* PUF proteins contain sequences similar to those in FBF. The 90 amino acid region of GLD-3 (residues 860 to 949) that binds FBF tightly and specifically does not impact FBF-binding to the PME RNA. Rather, this small region forms a ternary complex with FBF on

the PME RNA. The formation of this FBF, GLD-3 FBF-binding region, RNA complex casts doubt on the hypothesis that GLD-3 activates *fem-3* mRNA expression by disrupting the FBF•PME RNA complex and raises the possibility of alternative mechanisms by which the GLD-3•FBF complex regulates *fem-3* mRNA expression, as discussed herein.

Results

The N-terminus of the GLD-3 FBF-Binding Region is Necessary for FBF-2 Interaction

Previous yeast two-hybrid studies mapped the FBF-binding region of GLD-3 to residues 860 to 949 (Figure 1(b)), and suggested that a smaller portion of this region may be sufficient for FBF binding¹⁹. To examine this possibility, a complex of FBF-2 PUM-HD and the GLD-3 FBF-binding region (GLD-3 (860–949)) was subjected to limited trypsin proteolysis. The GLD-3 FBF-binding region demonstrated a high susceptibility to trypsin digestion that was reduced in the presence of FBF-2 PUM-HD. To determine the potential stretches within the GLD-3 FBF-binding region being protected by FBF-2 PUM-HD, a complex of GLD-3 (860–949) and polyhistidine-tagged FBF-2 PUM-HD was formed, lightly digested with trypsin, and afterwards subjected to nickel affinity chromatography to capture FBF-2 and associated protected GLD-3 FBF-binding region fragments. These GLD-3 fragments were identified using mass spectrometry. A region of GLD-3 consisting of residues 860 to 909 was observed to be protected and pulled down by the polyhistidine-tagged FBF-2 PUM-HD (data not shown).

To evaluate whether the smaller 860 to 909 amino acid region of GLD-3 is sufficient for interaction with FBF, the binding of FBF-2 PUM-HD to the full GLD-3 FBF-binding region (GLD-3 (860–949)) and the two halves of the GLD-3 FBF-binding region (GLD-3 (860–909) and GLD-3 (910–949)) were examined using a nickel affinity pulldown assay (Figure 1(c)). Mixtures of FBF-2 PUM-HD with polyhistidine-tagged GLD-3 (860–949), GLD-3 (860–909), and GLD-3 (910–949) were subjected to nickel affinity chromatography. Interaction between the GLD-3 and FBF-2 proteins is indicated by the co-elution of the two proteins from the nickel column at high imidazole concentrations. The comparable elution profiles of FBF-2 PUM-HD with polyhistidine-tagged GLD-3 (860–949) and GLD-3 (860–909) shows that GLD-3 residues 860 to 909 are sufficient for FBF-2 binding (Figure 1(c)), consistent with the results from the pulldown of the partially proteolyzed GLD-3•FBF-2 complex. Since certain GLD-3 trypsin sites in the GLD-3•FBF-2 complex may not have been cleaved due to preferential cleavage of other sites by trypsin or steric hindrance caused by FBF-2 binding, it is possible that a smaller region between residues 860 and 909 is sufficient for interaction with FBF-2. To evaluate this possibility, a series of polyhistidine-tagged GLD-3 (860–909) C-terminal truncations were evaluated for binding to FBF-2 PUM-HD using the above nickel affinity pulldown assay. N-terminal truncations of GLD-3 (860–909) were not examined, since previous yeast two-hybrid studies indicated that residues 860 to 872 are essential for FBF binding¹⁹. This additional experiment pared down the region of GLD-3 sufficient for FBF-2 interaction to residues 860 to 899 (data not shown).

The ideal size of the newly identified FBF-binding region of GLD-3 for fluorescence polarization spectroscopy enabled the examination of the GLD-3•FBF interaction quantitatively. The 40 amino acid GLD-3 protein (consisting of residues 860 to 899) was labeled at its N-terminus with the fluorophore Alexa Fluor 488 and used in fluorescence polarization (FP) binding and competition assays (Figure 2(a)). Using the FP binding assay, FBF-1 PUM-HD and FBF-2 PUM-HD were observed to bind to GLD-3 with similar low affinities (FBF-1 PUM-HD $K_{d,app} = 2.5 \pm 0.7 \mu M$ and FBF-2 PUM-HD $K_{d,app} = 0.9 \pm 0.1 \mu M$) (Figure 2(b)). The FP competition assay was used to measure and compare the abilities of the full-length and N-terminal fragments of the GLD-3 FBF-binding region to compete with fluorescent GLD-3 (860–899) for FBF-2 PUM-HD binding (Figure 2(c)). The full-

length and N-terminal residues 860 to 899 of the FBF-binding region competed comparably (GLD-3 (860–949) and GLD-3 (860–899) in Figure 2(c)), confirming that residues 900 to 949 are dispensable for the GLD-3•FBF-2 interaction. Similar competition was also observed for N-terminal residues 860 to 894 of the FBF-binding region, further narrowing down the minimal region sufficient for FBF-2 binding (GLD-3 (860–894) in Figure 2(c)). An additional FP competition experiment was performed with the N- and C-terminal halves of the GLD-3 860 to 894 amino acid region to further localize the FBF-binding site (GLD-3 (860–879) and GLD-3 (879–894) in Figure 2(c)). Although both segments showed decreased competition, GLD-3 (879–894) exhibited a dramatic 13-fold decrease in competition when compared with GLD-3 (860–949). Collectively, these results identify within the GLD-3 FBF-binding region a 35 amino acid region (residues 860 to 894) that is sufficient for interaction with FBF-2 and a smaller 20 amino acid stretch (residues 860 to 879) that is required for FBF-2 binding.

A Specific Sequence at the N-terminus of the FBF-Binding Region in GLD-3 is Required for FBF-2 Binding

To identify residues important for FBF binding within the newly determined minimal GLD-3 FBF-binding region, an unbiased double alanine scan of GLD-3 residues 860 to 894 was conducted. The FP competition assay was used to measure and compare the abilities of the sequential double alanine mutants to compete with fluorescent GLD-3 (860–899) for binding to FBF-2 PUM-HD (Figure 3, Supplemental Figure 1). Double alanine mutants located within a ten amino acid region between residues 864 and 874 (KTILEPRARVE) of GLD-3 significantly affected FBF-2 binding (Figure 3). The KT864,865AA, IL866,867AA, and RR870,872AA mutants exhibited significant 5- to 7-fold decreases in competition compared to wild-type (Figure 3). These results are consistent with those from the previous FP competition experiment, which positioned the GLD-3 FBF-binding site between residues 860 and 879 at the N-terminus of the FBF-binding region (Figure 2(C)).

To identify key residues in the newly identified GLD-3 FBF-binding site necessary for the GLD-3•FBF interaction, a single alanine scan of the KTILEPRARVE sequence was performed. The FP competition assay was used to measure and compare the abilities of single alanine mutants to compete with fluorescent GLD-3 (860–899) for binding to FBF-2 PUM-HD (Figure 4(a)). The yeast two-hybrid assay was also used to evaluate the binding of these GLD-3 mutants to FBF-2 (Figure 4(b)). The results from the *in vitro* and cell-based assays generally correlated well and showed that single alanine mutations in the KTIL and RAR sequences significantly affected GLD-3 binding to FBF-2 (boxed in the GLD-3 wild-type amino acid sequence in Figure 4(a)).

The K864A, T865A, I866A, and L867A mutations exhibited the greatest effect in both assays. In the FP competition assay, the K864A, T865A, and I866A mutants showed 5- to 9-fold lower competition in comparison to wild-type, while the L867A mutant did not compete at all (Figure 4(a)). These alanine mutations showed LacZ activities similar to that of the negative control (No FBF-2), in which no FBF-2 was expressed, in the yeast two-hybrid experiment (Figure 4(b)). Significant but less dramatic decreases in FBF-2 binding were observed with alanine substitutions in the RAR sequence. The R870 and R872 mutants showed 4-fold decreases in competition in comparison to wild-type in the FP competition assay (Figure 4(a)). These mutations showed similar effects in the yeast two-hybrid assay, reducing LacZ activities by 10- to 50-fold in comparison to wild-type (Figure 4(b)). Minor effects on FBF-2 binding were observed with alanine mutations of P869 and E874. The P869A mutation showed 2-fold lower competition in comparison to wild-type in the FP competition assay and 3-fold lower LacZ activity in comparison to wild-type in the yeast two-hybrid assay (Figure 4). The same trend was observed with the EP868,869AA double mutant, which exhibited a 2-fold decrease in competition in comparison to wild-type in the

FP competition assay (Figure 3). Surprisingly, the E874A mutant demonstrated a 2-fold increase in competition in comparison to wild-type in both the double alanine and single alanine scan FP competition experiments (Figure 3 and Figure 4(a)). In the yeast two-hybrid assay, the E874A mutation showed a slight increase in LacZ activity in comparison to wild-type (Figure 4(b)). These results suggest that while glutamic acid is tolerated at residue 874, hydrophobic residues such as alanine may be preferred for this interaction. The single alanine scan of the FBF-binding site (residues 864 to 874) shows the contributions of individual residues within this sequence to FBF-2 binding, with: K864, T865, I866, and L867 being critical; both R870 and R872 being important; and P869 and E874 playing a minor role.

Although the single alanine scan FP competition experiment show L867 as being indispensable for GLD-3 binding to FBF-2, the double alanine scan FP competition experiment indicated I866 and L867 as being critical but not vital (Figure 4(a) and Figure 3). To resolve these contradictory results, the L867A mutation was introduced into the same background as the double alanine mutants (GLD-3 (860–899)) and examined using the above FP competition assay (Supplemental Figure 2). The GLD-3 (860–899) L867A mutant was able to compete with fluorescent GLD-3 (860–899) for FBF-2 PUM-HD binding and exhibited a 5-fold decrease in competition in comparison to wild-type GLD-3 (860–899) (Supplemental Figure 2). The different effects of the L867A mutation in the two GLD-3 backgrounds (GLD-3 (860–879) and GLD-3 (860–899)) suggests that residues 879 to 894 may also be involved in FBF-2 binding and can compensate partially for the L867A mutation. An alignment of GLD-3 regions 864 to 874 (KTILEPRARVE) and 879 to 889 (KISLEHKTKYS) suggests that there may be a second FBF-binding site in the GLD-3 879 to 894 amino acid region. However, the FP competition experiment with the various fragments of the GLD-3 FBF-binding region and sequential double alanine mutants indicate that this is a very weak FBF-binding site. Removal of the second FBF-binding site between residues 879 and 894 resulted in a small 3-fold decrease in competition in comparison to both the full and minimal GLD-3 FBF-binding region (GLD-3 (860–879), GLD-3 (860–894), and GLD-3 (860–949) in Figure 2(c)). Also, the double alanine mutants in the second FBF-binding site (residues 879 to 889) exhibited only minor 2-fold decreases in competition in comparison to wild-type (KI879,880AA, KT885,886AA, and KY887,888AA in Supplemental Figure 1). Collectively, these results show that the primary FBF-binding site in GLD-3 is between residues 864 and 874 and that a second very weak FBF-binding site may exist between residues 879 and 889.

Specific Residues in the FBF-2 PUF Repeat 7 and 8 Loop are Required for GLD-3 Binding

The loop between PUF repeats 7 and 8 in the PUM-HD is required for FBF-2 interaction with protein partner CPB-1 (Figure 5(a)). To determine whether this loop is also required for FBF-2 interaction with GLD-3, an FBF-2 protein in which the loop (residues 479 to 485) had been removed was tested for GLD-3 binding using the yeast two-hybrid assay. Removal of the loop abolished GLD-3 binding to FBF-2 (data not shown). Previous RNA-binding studies showed that this deletion does not affect the FBF-2•RNA interaction, indicating that removal of the loop affects FBF-2 association with protein partners without disrupting the FBF-2 protein structure.

To identify specific residues in the FBF-2 PUF repeat 7 and 8 loop important for the GLD-3•FBF-2 interaction, an alanine scan of the loop was performed. Single alanine mutations introduced into the loop of FBF-2 PUM-HD were analyzed for effects on GLD-3 binding using the previously described FP binding and yeast two-hybrid assays (Figure 5(b) and 5(c)). In both the *in vitro* and cell-based assays, alanine substitutions of Y479, I480, and T485 at the N- and C-terminal ends of the loop dramatically affected FBF-2 binding to GLD-3. The GLD-3 binding affinities of the Y479A and T485A mutants were more than 20-

fold lower than that of wild-type in the FP binding assay (Figure 5(b)). In the yeast two-hybrid assay, these alanine mutants exhibited LacZ activities comparable to that of the negative control (No FBF-2), in which no FBF-2 was expressed (Figure 5(c)). The I480A mutant exhibited a 7-fold lower GLD-3 binding affinity in comparison to wild-type in the FP binding assay and a 120-fold decrease in LacZ activity in comparison to wild-type in the yeast two-hybrid experiment (Figure 5(b) and 5(c)). Minor effects on GLD-3 binding were observed for single alanine mutations of P481, H482, and P483 residues in the center of the loop sequence. P481A, H482A, and P483A mutations resulted in 2- to 3-fold lower GLD-3 binding affinities in comparison to wild-type in the FP binding assay and 2- to 13-fold lower LacZ activities in the yeast two-hybrid experiment (Figure 5(b) and 5(c)). The two different assays show that FBF-2 PUF repeat 7 and 8 loop residues Y479, I480, and T485 are critical for GLD-3 binding (boxed in the sequence of the FBF-2 wild-type PUF repeat 7 and 8 loop in Figure 5(b)) and that P481, H482, and P483 play a minor role in GLD-3 binding.

GLD-3 Interaction with FBF-2 Does Not Impact FBF-2 RNA-binding

Previous yeast three-hybrid studies showed a modest 2-fold decrease in FBF binding to PME RNA (the *fem-3* 3' UTR regulatory element) in the presence of GLD-3, leading to the hypothesis that GLD-3 promotes spermatogenesis by associating with FBF and disrupting the interaction between FBF and the PME RNA¹⁹. This hypothesis was tested *in vitro* by equilibrating pre-formed complexes of either FBF-1 PUM-HD or FBF-2 PUM-HD and ³²P-labeled PME RNA with increasing concentrations of GLD-3 (860–949). The FBF-binding region (residues 860 to 949) of GLD-3 was used in this experiment since full-length GLD-3 could not be purified. FBF binding to PME RNA was monitored using an electrophoretic mobility shift assay (EMSA). Dissociation of either FBF PUM-HD•PME RNA complex was not observed even at the highest concentration of GLD-3 used (10 μM) (Supplemental Figure 3).

It is plausible that full-length GLD-3 or additional factors from yeast not present in this *in vitro* assay are necessary to reproduce the previously reported results. However, it is also possible that GLD-3 is capable of binding to the FBF in the FBF•PME RNA complex and forming a GLD-3•FBF•PME RNA complex. To assess this possibility, a large maltose binding protein (MBP) tag was attached to the N-terminus of the GLD-3 (860–949) construct. Using the MBP-tagged GLD-3, a distinct GLD-3•FBF•PME RNA complex band was observed by EMSA. The different EMSA mobilities of the two complexes are indicated in Lanes 1 (MBP-GLD-3•FBF-2 PUM-HD•PME RNA complex) and 2 (FBF-2 PUM-HD•PME RNA complex) in Figure 6(b). This ternary complex was not observed previously, likely due to the small size difference between the ternary GLD-3•FBF•PME RNA complex and the binary FBF•PME RNA complex (Supplemental Figure 3).

To evaluate the effect of GLD-3 on FBF-2 binding to the PME RNA, the binding of both the FBF-2 PUM-HD and pre-formed MBP-GLD-3•FBF-2 PUM-HD complex to ³²P-labeled PME RNA was compared using EMSA (Figure 6(b)). FBF-2 PUM-HD and the MBP-GLD-3•FBF-2 PUM-HD complex showed similar affinities for the PME RNA (FBF-2 PUM-HD $K_{d,app} = 14.0 \pm 5.4$ nM and MBP-GLD-3•FBF-2 PUM-HD $K_{d,app} = 17.0 \pm 4.9$ nM) (Figure 6(c)). The observation of a stable ternary complex formed between the GLD-3 FBF-binding region, FBF-2, and PME RNA and the comparable affinities of FBF-2 and the FBF-2•GLD-3 FBF-binding region complex for the PME RNA raises doubts that binding of the GLD-3 FBF-binding region to FBF-2 disrupts the FBF-2•PME RNA interaction and about the mechanism by which the GLD-3•FBF complex promotes spermatogenesis.

Discussion

PUF proteins and FBF interact with many different protein partners⁴⁶ (see Introduction). However, to date there is no structural and very little biochemical information describing the interaction between PUF proteins, including FBF, and their binding partners. Here in these studies is a description of the molecular basis of the biologically important interaction between *C. elegans* PUF protein FBF and its binding partner GLD-3. The first measured affinities of the interaction between FBF proteins (FBF-1 and FBF-2) and GLD-3 are reported here (Figure 2(b)). Also identified here are the specific regions on the GLD-3 and FBF proteins required for formation of the GLD-3•FBF complex. GLD-3 association with FBF-2 requires a short amino acid sequence between residues 864 and 874 (KTILEPRARVE), within the minimal GLD-3 FBF-binding region that spans residues 860 to 894 (Figure 2(c), Figure 3, and Figure 4). Within this newly identified GLD-3 FBF-binding site, three of the residues critical for the GLD-3•FBF-2 interaction (K864, T865, and L867) are also present in the FBF-binding proteins CPB-1 and RACK-1 (boxed in Figure 7(a)). The KTXL sequence motif has been shown in previous and subsequent studies to also be required for CPB-1 binding to FBF-2. It is possible that additional FBF-binding proteins may share this sequence motif and a mode of FBF-binding similar to GLD-3 and CPB-1.

FBF-2 association with GLD-3 requires the loop connecting PUF repeats 7 and 8 in the RNA-binding PUM-HD, and specifically residues Y479, I480, and T485 located in this loop (Figure 5). Notably, while this loop is necessary for the GLD-3•FBF-2 interaction, it is dispensable for the RNA•FBF-2 interaction. While Y479 and T485 are conserved between the two FBF proteins, I480 in FBF-2 is a methionine at the corresponding position in FBF-1 (figure 7(b)). The minor difference in FBF-1 and FBF-2 binding affinities for GLD-3 may be due to the slight difference in the residues at this position (Figure 2(b)). In PUF-8, a *C. elegans* PUF protein shown not to interact with GLD-3 in yeast two-hybrid studies, the amino acid sequence of the loop is dramatically different from that of FBF-1 and FBF-2 (Figure 7(b))¹⁹. The sequence differences in the PUF repeat 7 and 8 loop between PUF-8 and FBF may explain why GLD-3 interacts with FBF and not PUF-8, suggesting that the amino acid sequence of the loop specifies the PUF proteins with which GLD-3 interacts (Figure 7(b)). The same loop is also required for FBF binding to the CPB-1 protein and the association of *Drosophila melanogaster* PUF protein Pumilio with both Nanos and Brat proteins. Additionally, individual residues in the PUF repeat 7 and 8 loop critical for the formation of these three PUF protein•binding partner complexes have been identified. Collectively, these and previous studies demonstrated that the PUF repeat 7 and 8 loop is an important platform for the interaction of PUF proteins with their binding partners.

GLD-3 activates expression of the *fem-3* mRNA and spermatogenesis in the *C. elegans* germline. Previous studies using a yeast three-hybrid system suggested that full-length GLD-3 disrupted binding of FBF to the PME regulatory element in the *fem-3* 3' UTR, which led to the proposal of the Inhibition Model in Figure 7(c). These studies here show that the FBF-binding region of GLD-3 (residues 860 to 949) neither disrupts the formation of an FBF-2•PME RNA complex (Supplemental Figure 3), nor affect the affinity of FBF-2 for PME RNA (Figure 6). Several differences between the two studies may cause these discrepancies. The previous studies used full-length GLD-3 and an RNA with tandemly repeated FBF-binding PME sites¹⁹; while these studies use the FBF-binding region of GLD-3 and a single FBF-binding PME site, as naturally occurs in the *fem-3* 3' UTR. In addition, factors present in yeast and not in this *in vitro* system with purified proteins may be an important variable. Regardless, the findings presented here suggest the formation of a ternary complex consisting of the GLD-3, FBF, and the PME RNA, and raises the possibility of alternative mechanisms by which the GLD-3•FBF complex regulates *fem-3* mRNA expression.

Two other possible models for the activated expression of the *fem-3* mRNA involving the formation of a GLD-3•FBF•PME RNA complex are shown in Figure 7(c). In the first alternative model, GLD-3 binds to the FBF•PME RNA complex and recruits activator proteins, such as the poly(A) polymerase GLD-2, to the *fem-3* mRNA (Recruitment Model in Figure 7(c)). Activation of the germline developmental mRNA *gld-1* has been hypothesized to occur through this mechanism¹⁶. In a second alternative model, GLD-3 binds to the FBF•PME RNA complex and blocks the binding of repressor proteins to FBF (Occlusion Model in Figure 7(c)). Both GLD-3 and CPB-1 proteins have been shown to bind to the PUF repeat 7 and 8 loop of FBF (Figure 5); similarly in *D. melanogaster*, both Brat and Nanos proteins also bind to this loop in Pumilio. These three models in Figure 7(c) are not mutually exclusive and a combination of all three may be involved in the regulation of *fem-3* and other germline mRNAs.

Materials and Methods

Protein Constructs and Purification

The FBF-1 Pumilio-homology domain (residues 164–566) and FBF-2 Pumilio-homology domain (residues 164–575) was PCR amplified and inserted into a modified pET-22b(+) vector (Novagen) in which a polyhistidine tag and TEV protease cleavage site was introduced before the multiple cloning site. The FBF-2 loop single alanine substitutions were introduced using Quickchange (Stratagene). The point mutations were introduced into FBF-2 since FBF-1 and FBF-2 exhibit minor differences in GLD-3 binding affinities and the quantities of FBF-2 needed for biochemical studies could be readily produced. All FBF-1 and FBF-2 proteins were expressed and purified in the same manner from plasmids transformed into the BL21-Gold(DE3) *E. coli* strain. Cells were grown in LB medium containing 100 µg/mL ampicillin at 37°C until reaching an OD₆₀₀ of 0.4 to 0.6. Protein was expressed by adding 0.5 mM IPTG and reducing the temperature to 18°C for 18–24 h. Cells were harvested by centrifugation and stored at –80°C. Cell pellets were resuspended in lysis buffer (20 mM Tris (pH8.6), 1 M NaCl, 10 % glycerol, 10 mM imidazole, 10 mM β-me, cOmplete EDTA-free Protease Inhibitor Cocktail Tablets (Roche)) and sonicated. The lysate was centrifuged to remove cell debris and the soluble fraction was mixed with Ni-NTA Superflow resin (Qiagen) in a column. The column was washed with lysis buffer and 20 mM imidazole buffer (20 mM Tris (pH7.5), 150 mM NaCl, 20 mM imidazole, 10 mM β-me) before being eluted into 300mM imidazole buffer (20 mM Tris (pH7.5), 150 mM NaCl, 300 mM imidazole, 10 mM β-me). The eluted protein was dialyzed overnight at 4°C in: 20 mM Tris (pH7.5), 150 mM NaCl, 0.5 mM EDTA, 10 mM β-me to remove the imidazole and subjected to TEV protease cleavage to remove the polyhistidine tag. The polyhistidine tag and TEV protease were removed using the Ni-NTA Superflow resin (Qiagen) in a column. The protein was further purified using a HiTrap Heparin HP column (GE Healthcare) and the previously described procedure⁴⁰.

Constructs for GLD-3 (860–949), GLD-3 (860–899) and GLD-3 (860–894) were amplified using PCR and inserted into a modified pET-22b(+) vector (Novagen) in which a thrombin cleavage site was introduced after the multiple cloning site and before the polyhistidine tag. The double alanine substitutions and L867A single mutant were introduced into the GLD-3 (860–899) construct using Quickchange (Stratagene), since GLD-3 (860–899) could be easily expressed and purified from *E. coli*. These GLD-3 proteins were all expressed and purified in the same manner from plasmids transformed into the BL21-Gold (DE3) *E. coli* strain. The cells were grown in LB medium supplemented with 100 µg/mL ampicillin at 37°C until reaching an OD₆₀₀ of 0.4 to 0.6. Protein expression was induced for 3 h at 37°C with 0.5 mM IPTG. The cells were harvested using centrifugation and stored at –80°C. The cell pellets were resuspended in lysis buffer (20 mM Tris (pH8.8), 250 mM NaCl, 5% glycerol, 10 mM imidazole, cOmplete EDTA free Protease Inhibitor Cocktail Tablets

(Roche)) and sonicated. Cell debris was removed from the lysate by centrifugation and the soluble fraction was mixed with Ni-NTA Superflow resin (Qiagen) in a column. The column was washed with lysis buffer, high NaCl buffer (20 mM Tris (pH8.8), 1 M NaCl, 5% glycerol, 10 mM imidazole) and low imidazole buffer (20 mM Tris (pH8.8), 5% glycerol, 20 mM imidazole). Protein was eluted from the column using buffer containing 300 mM imidazole (20 mM Tris (pH8.8), 5% glycerol, 300 mM imidazole). The eluted protein was dialyzed overnight at 4°C in: 20 mM Tris (pH8.8), 5% glycerol to remove the imidazole. The protein was further purified using a HiTrap Q HP column (GE Healthcare) with a 0–100 mM NaCl gradient. GLD-3 (860–879), GLD-3 (879–894) and GLD-3 (860–879) single alanine point mutants were too short for *E. coli* expression and were instead purchased from Genescript.

GLD-3 (860–949) was also inserted into a modified pMAL-c2x vector (New England Biolabs) in which a TEV cleavage site was introduced between the MBP tag and the multiple cloning site and a polyhistidine tag was placed after the multiple cloning site. The MBP-tagged GLD-3 was expressed identically to the other GLD-3 proteins in the previous paragraph. The cell pellet was resuspended in lysis buffer (20 mM Tris (pH8.0), 200 mM NaCl, 5% glycerol, 20 mM imidazole, cComplete EDTA-free Protease Inhibitor Cocktail Tablets (Roche)) and sonicated. The lysate was centrifuged and the soluble fraction was mixed with Ni-NTA Superflow resin (Qiagen) in a column. The column was washed with lysis buffer, high NaCl buffer (20 mM Tris (pH8.0), 1 M NaCl, 5% glycerol, 20 mM imidazole) and low imidazole buffer (20 mM Tris (pH8.0), 100mM NaCl, 5% glycerol, 40 mM imidazole). Protein was eluted from the Ni-NTA Superflow resin column using 300 mM imidazole buffer (20 mM Tris (pH8.0), 100 mM NaCl, 5% glycerol, 300 mM imidazole) directly onto an amylose resin column (New England Biolabs). The amylose resin column was washed with low NaCl buffer (20 mM Tris (pH8.0), 20 mM NaCl, 5% glycerol) before being eluted into the same buffer supplemented with 10 mM maltose.

Nickel Pulldown Assays

16 μ M FBF-2 (164–575) was combined individually with 24 μ M polyhistidine-tagged GLD-3 (860–949), GLD-3 (860–909), and GLD-3 (909–949) in 400 μ L of binding buffer (20 mM Tris (pH8.6), 100 mM NaCl, 5% glycerol, 10 mM β -me). The reactions were equilibrated at room temperature for 1 h before being loaded onto prepacked His SpinTrap columns (GE Healthcare). As a negative control, 400 μ L of 16 μ M FBF-2 (164–575) was also loaded onto a prepacked His SpinTrap column (GE Healthcare). The columns were washed with binding buffer before eluting the protein using binding buffer supplemented with: 20 mM, 40mM, 80 mM, 150 mM, 200 mM, 250 mM, and 300 mM imidazole. Visualized the protein using SDS-PAGE.

Fluorophore Labeling of GLD-3

Three serines and a cysteine were introduced at the N-terminus of the GLD-3 (860–899) construct using Quickchange (Stratagene). GLD-3 (860–899) was selected for fluorophore labeling since it was the smallest FBF-2 binding GLD-3 peptide that could easily be expressed and purified from *E. coli*. The protein was expressed and purified similar to the wild-type GLD-3 (860–899); however, the buffers were modified to include 2 mM DTT to prevent disulfide bridges from forming between GLD-3 molecules. To ensure that the cysteines were fully reduced, the protein was incubated overnight at room temperature in degassed reducing buffer (50 mM HEPES (pH7.0), 100 mM NaCl, 5% glycerol, 0.5 mM TCEP). The reduced GLD-3 was loaded onto a column of Ni-NTA Superflow resin (Qiagen). The resin was washed with 10 column volumes of degassed reducing buffer and 10 column volumes of degassed labeling buffer (50 mM HEPES (pH7.0), 100 mM NaCl, 5% glycerol). Nitrogen gas was used to flush the buffers through the column to expel

oxygen. A 5-fold molar excess of Alexa Fluor 488 C₅ maleimide (Invitrogen) was added to the GLD-3 on the column and incubated overnight at room temperature. Unreacted dye was washed from the column using 10 column volumes of labeling buffer supplemented with 0.5 mM DTT and 10 column volumes of low imidazole buffer (50 mM HEPES (pH7.0), 100 mM NaCl, 5% glycerol, 20 mM imidazole, 0.5 mM DTT). The protein was eluted from the column using 300 mM imidazole buffer (50 mM HEPES (pH7.0), 100 mM NaCl, 5% glycerol, 300 mM imidazole, 0.5 mM DTT). The protein was dialyzed overnight at 4°C in: 20 mM Tris (pH8.8), 5% glycerol, 2 mM DTT to remove the imidazole and exchange the protein into buffer suitable for purification by anion exchange chromatography. The labeled and unlabeled proteins were separated using a HiTrap Q HP column (GE Healthcare) with a 0–500 mM NaCl gradient. Fractions containing labeled GLD-3 were pooled and dialyzed into 2% acetic acid for 72 h at 4°C. The labeled protein was dehydrated using a SpeedVac and stored at –80°C. The labeled protein was resuspended in buffer containing: 20 mM Tris (pH8.8), 100 mM NaCl, 5% glycerol, 2 mM TCEP, cOmplete EDTA-free Protease Inhibitor Cocktail Tablets (Roche).

Fluorescence polarization assays

For the FP binding experiments, 5 nM Alexa Fluor 488 conjugated GLD-3 was equilibrated with 0.01–39.00 μ M FBF. The reactions were set up in a total volume of 100 μ L in the following conditions: 20 mM Tris (pH8.6), 100 mM NaCl, 0.1 mM EDTA, 5% glycerol, 2 mM DTT, 0.1 mg/mL tRNA, 50 μ g/mL BSA. The samples were set up in 96-well opaque FLUOTRAC 200 microtiter plates (Greiner Bio-One) and incubated for 1 h at room temperature. Polarization values for the reactions were determined using the Envision plate reader (Perkin Elmer). The $K_{d,app}$ for the binding of FBF to GLD-3 was calculated by fitting the data to a modified version of the Hill equation using IGOR (Wavemetric) as previously described⁵⁰. Each reported $K_{d,app}$ and Hill coefficient value is the average of at least three independent experiments. The P value showing the significance of differences in $K_{d,app}$ values was calculated using the student's t-test function in Excel (Microsoft). The confidence intervals were calculated using the confidence function in Excel (Microsoft).

For the FP competition experiments, 5 nM Alexa Fluor 488 labeled GLD-3 and 2 μ M FBF-2 (164–575) was equilibrated with a range of non-fluorescent GLD-3 competitor concentrations using the same set up as the FP binding experiments. The IC₅₀ values for the various GLD-3 competitors were determined by fitting the data to a sigmoidal dose response function in IGOR (Wavemetrics) as previously described⁵⁰. Each reported IC₅₀ value is the average of at least three independent experiments. The P value showing the significance of differences between IC₅₀ values was calculated using the student's t-test function in Excel (Microsoft). The confidence intervals were calculated using the confidence function in Excel (Microsoft).

Yeast two-hybrid assay

Minor modifications were made to the previously described assay¹⁹. GLD-3 (850–900) was cloned into pBTM116 and FBF-2 (121–632) into pACT2. Luminescence data was collected using the β -Glo reagent (Promega). Measurements for wild-type and mutant proteins were taken for three individual yeast transformants.

Electrophoretic mobility shift assays

The PME RNA was ordered from Dharmacon and handled according to the manufacturer's protocol. The RNA was labeled and purified using a previously described procedure⁵¹. For the experiments assessing the effect of GLD-3 on FBF binding to PME RNA, 110 nM FBF-1 (164–566) or FBF-2 (164–575) was incubated with 0.1 nM ³²P-labeled PME RNA for 2 h at room temperature before titrating in 0.01–10000.0 nM GLD-3 (860–949). The

RNA was heated to 65°C for 1 minute and cooled on ice for 10 minutes before being added to FBF. The reactions were set up in a total volume of 20 μ L using the same conditions as for the fluorescence polarization experiments and adjusted to have a constant concentration of 110 nM FBF-1 or FBF-2. The reactions were incubated for 1 h at room temperature prior to adding 4 μ L of loading buffer (15% Ficoll 400,000, 30% glycerol, 0.08% bromophenol blue and 0.08% xylene cyanol). In parallel, reactions for the titration of 0.2–3000.0 nM FBF into 0.1 nM 32 P-labeled PME RNA were also set up using the procedure described below. An 8 μ L portion of each reaction was loaded onto a pre-run native polyacrylamide gel (8% 29:1 acrylamide:bisacrylamide, 5% glycerol, 0.5x TBE) with the voltage set at 100 V. The gel was run at 600 V for 1 h at 4°C and dried before being exposed to a phosphorimager screen (Molecular Dynamics) overnight. The screen was scanned on a Storm 820 Phosphorimager (Molecular Dynamics).

In the experiments measuring the affinities of FBF-2 and MBP-GLD-3•FBF-2 for the PME RNA, a 0.1–2000.0 nM range of FBF-2 (164–575) and FBF-2 (164–575)•MBP-GLD-3 (860–949) was equilibrated with 0.1 nM 32 P-labeled PME RNA. The FBF-2 (164–575) was incubated with 15 μ M MBP-GLD-3 (860–949) for 1 h at room temperature to form the complex before adding RNA. The reactions were set up similarly to the above EMSA experiments, except that the reaction conditions for the GLD-3•FBF-2 complex were adjusted such that a constant concentration of 15 μ M MBP-GLD-3 (860–949) was present in all of the reactions. The reactions were incubated for 2 h at room temperature prior to adding 4 μ L of loading dye. The samples were loaded and run on the native polyacrylamide gel as described above. The fraction of bound and free RNA was determined using ImageQuant software (Molecular Dynamics) and the $K_{d,app}$ calculated by fitting the data to a modified version of the Hill equation using IGOR (Wavemetrics) as previously described. The reported $K_{d,app}$ and Hill coefficient are the average of at least three independent experiments. The P value showing the significance of differences between $K_{d,app}$ values was calculated using the student's t-test function in Excel (Microsoft). The confidence intervals were calculated using the confidence function in Excel (Microsoft).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

UTR	untranslated region
FBF	<i>fem-3</i> binding factor
PUF	Pumilio and FBF
PUM-HD	Pumilio-homology domain
GLD-3	germline development defective-3
GLD-2	germline development defective-2

PME	point mutation element
FP	fluorescence polarization
EMSA	electrophoretic mobility shift assay
MBP	maltose binding protein

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Highlights

- It is unknown how the RNA-binding PUF protein FBF interacts with binding partners.
- Short sequences on FBF and binding partner GLD-3 mediate the FBF•GLD-3 interaction.
- GLD-3 and FBF form a complex on the PME in the 3' UTR of the *fem-3* mRNA.
- Sequences mediating the GLD-3•FBF interaction are present in other proteins.
- *fem-3* mRNA regulation may be mediated by a GLD-3•FBF•PME RNA complex.

Figure 1.

The N-terminal half of the GLD-3 FBF-binding region is sufficient for interaction with the FBF Pumilio homology domain. (a) Structure of the FBF-2 PUM-HD bound to PME RNA on the inner surface (PDB ID: 3K64). The PUM-HD is composed of 8 PUF repeats (R1-R8) flanked by caps (R1' and R8'). (b) The FBF PUM-HD is contained within residues 166 to 565 in FBF-1 and residues 168 to 567 in FBF-2. GLD-3 is composed of five KH domains, a serine-rich region, and an FBF-binding region that spans residues 860 to 949. (c) Residues 860 to 909 are sufficient for GLD-3 binding to FBF-2 PUM-HD. SDS-PAGE gels for the nickel affinity pulldown experiments with FBF-2 PUM-HD and mixtures of FBF-2 PUM-HD with polyhistidine-tagged GLD-3 (860–949), GLD-3 (860–909), and GLD-3 (910–949).

The Load lane shows that equal amounts of GLD-3 and FBF-2 protein are loaded onto the nickel column for each experiment. The subsequent lanes show the stepwise elution of the protein from the nickel resin using 20 mM to 300 mM imidazole. Bands corresponding to FBF-2 and GLD-3 proteins are labeled. The portion of the GLD-3 FBF-binding region used in each experiment is shown to the right of the gels. Each GLD-3 protein has a thrombin cleavage site (THR) and a polyhistidine tag (6H) at the C-terminus.

Figure 2.

GLD-3 residues 860 to 879 are required for FBF-2 interaction. (a) Amino acid sequence of the Alexa Fluor 488 N-terminal conjugated GLD-3 (860–899) used in the FP binding and competition assays. The green star at the N-terminus of the amino acid sequence represents the Alexa Fluor 488 dye. (b) FBF-1 and FBF-2 PUM-HD bind to GLD-3 with similar low affinities. To the left are the characteristic traces for the titration of FBF-1 and FBF-2 PUM-HD into fluorescent GLD-3 (860–899) obtained using the FP binding assay. To the right is the figure legend for the graph, mean apparent equilibrium dissociation constants ($K_{d,app}$), and mean Hill coefficients for the FBF-1•GLD-3 and FBF-2•GLD-3 interactions. The mean $K_{d,app}$ and Hill coefficient values and standard deviations are calculated from at least three

independent experiments. The mean $K_{d,app}$ values are within the 95% confidence interval and the difference in the mean $K_{d,app}$ values of FBF-1 PUM-HD and FBF-2 PUM-HD are not statistically significant ($P > 0.05$). (c) Identification of a minimal region of GLD-3 necessary for FBF-2 interaction. The ability of the GLD-3 FBF-binding region and fragments of this region to compete with fluorescent GLD-3 (860–899) for FBF-2 PUM-HD binding was measured using the FP competition assay. Fluorescent GLD-3 was equilibrated with a constant concentration of FBF-2 PUM-HD to achieve 70% binding of the fluorescent GLD-3 and variable concentrations of nonfluorescent GLD-3 competitor. At the top is the plot of normalized polarization as a function of competitor concentration for the full and partial GLD-3 FBF-binding region proteins. Beneath is the figure legend for the graph, diagrams showing the portion of the FBF-binding region used in each experiment and their mean IC_{50} values, and IC_{50} ratios comparing the mean IC_{50} values for the GLD-3 FBF-binding region fragments to the full GLD-3 FBF-binding region (GLD-3 (860–949)). The mean IC_{50} values and standard deviations are calculated from at least three independent experiments. The mean IC_{50} values are within the 95% confidence interval. The difference in mean IC_{50} s between the full GLD-3 FBF-binding region (GLD-3 (860–949)) and the GLD-3 FBF-binding region fragments is not statistically significant for GLD-3 (860–899) ($P \gg 0.1$), but are significant for GLD-3 (860–894) ($P = 0.02$), GLD-3 (860–879) ($P \ll 0.01$), and GLD-3 (879–894) ($P \ll 0.01$).

GLD-3	Sequence	IC ₅₀ (μM)	IC ₅₀ ratio
○ Wild-type	NGVT <u>KTILEPRARVE</u> KDYGKISLEHKTKYSNEYGD	6.4 ± 0.6	-
▲ KT864,865AA	NGVT <u>AA</u> ILEPRARVEKDYGKISLEHKTKYSNEYGD	31.4 ± 1.4	4.9
■ IL866,867AA	NGVTKT <u>AAE</u> PRARVEKDYGKISLEHKTKYSNEYGD	41.7 ± 2.8	6.5
◆ EP868,869AA	NGVTKT <u>ILA</u> ARARVEKDYGKISLEHKTKYSNEYGD	9.4 ± 0.4	1.5
× RR870,872AA	NGVTKTILEP <u>AA</u> AVEKDYGKISLEHKTKYSNEYGD	38.8 ± 3.8	6.1
● VE873,874AA	NGVTKTILEPRAR <u>AA</u> KDYGKISLEHKTKYSNEYGD	3.2 ± 0.3	0.5

Figure 3.

The KTILEPRARVE sequence at the N-terminus of the FBF-binding region is necessary for GLD-3 interaction with FBF-2. Sequential double alanine mutations between residues 860 and 894 were introduced into GLD-3 (860–899). The ability of the GLD-3 double alanine mutants to compete with fluorescent GLD-3 (860–899) for FBF-2 PUM-HD binding was measured using the FP competition assay. Double alanine mutations between residues 864 and 874 dramatically decreased GLD-3 binding to FBF-2. At the top is the plot of normalized polarization as a function of competitor concentration for GLD-3 wild-type and double alanine mutants between residues 864 and 874. Beneath is the figure legend for the graph, amino acid sequences (of residues 860 to 894) and mean IC₅₀ values for each GLD-3 competitor, and IC₅₀ ratios comparing the mean IC₅₀ values of each mutant to wild-type GLD-3. The mean IC₅₀ values and standard deviations are calculated from at least three independent experiments. The mean IC₅₀ values are within the 95% confidence interval and the difference in mean IC₅₀s between GLD-3 wild-type and the double alanine mutants is statistically significant ($P < 0.01$).

Figure 4.

A KTILXXRARXX sequence is required for GLD-3 binding to FBF-2. (a) GLD-3 single alanine mutations between residues 864 and 874 were introduced into GLD-3 (860–879). The FP competition assay was used to measure the ability of GLD-3 single alanine mutants to compete with fluorescent GLD-3 (860–899) for FBF-2 PUM-HD binding. At the top is the plot of normalized polarization as a function of competitor concentration for GLD-3 wild-type and representative alanine mutants. The table beneath shows the amino acid sequence (of residues 864 to 874) and mean IC₅₀ value for each GLD-3 competitor as well as the mean IC₅₀ ratios comparing the IC₅₀ values of each mutant to wild-type GLD-3. The IC₅₀ for GLD-3 L867A could not be fitted because no displacement of the fluorescent

GLD-3 from FBF-2 was observed in these studies even at competitor concentration as high as 1200 μ M ($\gg$ 1200.0). As such, the IC_{50} ratio is likely considerably greater than 60-fold ($\gg$ 60.0). The mean IC_{50} values and standard deviations are calculated from at least three independent experiments. The mean IC_{50} values are within the 95% confidence interval. The difference in mean IC_{50} s between GLD-3 wild-type and the single alanine mutants are significant ($P \ll 0.01$), except for the V873 mutant ($P = 0.05$). The alanine point mutations are underlined. Boxed in the GLD-3 wild-type amino acid sequence are the residues that when mutated to alanines show the greatest decreases in competition. (b) The same alanine point mutations were also introduced into GLD-3 (850–900) and evaluated for effects on the GLD-3•FBF-2 interaction using yeast two-hybrid assay. Yeast were transformed with FBF-2 and either GLD-3 wild-type or a single alanine mutant. As a negative control, yeast were also transformed with wild-type GLD-3 and an empty vector (No FBF-2). The graph shows the mean relative LacZ activities and errors for the various FBF-2 and GLD-3 pairs. The mean relative LacZ activity values and standard deviations are calculated from measurements collected from three individual yeast transformants. To the right of the graph are the LacZ ratios comparing the mean relative LacZ activities of wild-type to each alanine mutant. The boxed GLD-3 alanine mutants are those that demonstrate the greatest decreases in relative LacZ activities.

Figure 5.

A specific sequence in the PUF repeat 7 and 8 loop of FBF-2 is required for GLD-3 binding.

(a) The loop connecting PUF repeats 7 and 8 in the FBF-2 PUM-HD is non-essential for RNA-binding but required for GLD-3 interaction. Shown in the diagram are the FBF-2 PUM-HD and the amino acid sequence for the PUF repeat 7 and 8 loop (residues 479 to 485). The PUF repeats and flanking caps are labeled (R1-R8, R1', and R8'). To assess the contributions of individual residues in the PUF repeat 7 and 8 loop to GLD-3 binding, single alanine substitutions were introduced into FBF-2 PUM-HD and evaluated for effects on GLD-3 binding using the FP binding assay (b) and yeast two-hybrid assay (c). (b) For the FP binding assay, the single alanine mutations were introduced into the FBF-2 (164–575)

construct. At the top are the characteristic traces for the titration of FBF-2 PUM-HD wild-type and alanine mutants into fluorescent GLD-3 (860–899) obtained using the FP binding assay. Beneath is the figure legend for the graph, loop amino acid sequences (residues 479 to 485) and mean $K_{d,app}$ values for FBF-2 PUM-HD wild type and single alanine mutants, and $K_{d,app}$ ratios comparing the mean $K_{d,app}$ for each single alanine mutant to wild-type. The $K_{d,app}$ ratio for Y479A is listed as > 44.0 , since the apparent $K_{d,app}$ is $> 40.0 \mu\text{M}$. The mean $K_{d,app}$ values and standard deviations are calculated from at least three independent experiments. The mean $K_{d,app}$ values are within the 95% confidence interval and the difference in mean $K_{d,app}$ values between FBF-2 PUM-HD wild-type and single alanine mutants is statistically significant ($P < 0.01$). The alanine mutations are underlined. The boxed residues in the wild-type loop amino acid sequence greatly affect the GLD-3 binding affinity when mutated to alanines. (c) In the yeast two-hybrid experiments, the single alanine mutations were introduced into FBF-2 (121–632). Yeast were transformed with GLD-3 and either FBF-2 wild-type or single alanine mutants. As a negative control, yeast were also transformed with wild-type GLD-3 and an empty vector (No FBF-2). The graph shows the mean relative LacZ activities and errors for the various GLD-3 and FBF-2 pairs. The mean relative LacZ activity values and standard deviations are calculated from measurements collected from three individual yeast transformants. To the right of the graph are the LacZ ratios comparing the mean relative LacZ activities of wild-type to each alanine mutant. The boxed GLD-3 alanine mutants are those that demonstrate the greatest decreases in relative LacZ activities.

Figure 6.

GLD-3•FBF-2 and FBF-2 have similar affinities for the PME RNA. (a) Sequence of the PME RNA used in these studies with the nucleotides recognized by FBF underlined. (b) Binding of FBF-2 PUM-HD (left) and MBP-GLD-3•FBF-2 PUM-HD (right) to 32 P-labeled PME RNA monitored by EMSA. The MBP-GLD-3•FBF-2 PUM-HD complex was formed by incubating FBF-2 PUM-HD with 15 μ M MBP-GLD-3. The protein complex was then equilibrated with the 32 P-labeled PME RNA and the reactions were adjusted to contain a total constant concentration of 15 μ M MBP-GLD-3. Holding the GLD-3 at a constant concentration 15 times higher than the $K_{d,app}$ for the GLD-3•FBF-2 complex ensures that FBF-2 will be associated with GLD-3 even at the lowest FBF-2 concentrations used in these experiments. The order of the addition for each component is shown beneath the gels. Bands on the gels corresponding to PME, FBF-2•PME and GLD-3•FBF-2•PME are labeled to the right of the gels. Lanes 1 and 2 in the right gel show the difference in migration of the

GLD-3•FBF-2•PME and FBF-2•PME complexes, respectively, in the gel. (c) To the left are the typical traces for the binding of FBF-2 PUM-HD and MBP-GLD-3•FBF-2 PUM-HD to the PME RNA obtained from the EMSA experiments. To the right is the figure legend for the graph and mean $K_{d,app}$ values as well as mean Hill coefficients for the FBF-2 PUM-HD•PME and MBP-GLD-3•FBF-2 PUMHD•PME complexes. The mean $K_{d,app}$ and Hill coefficient values and standard deviations are calculated from at least three independent experiments. The mean $K_{d,app}$ values are within the 95% confidence interval and the difference in mean $K_{d,app}$ values for FBF-2 PUM-HD and MBPGLD-3•FBF-2 PUM-HD are not statistically significant ($P \gg 0.05$).

(a)

FBF Binding Proteins

GLD-3	864	K	T	I	L	E	P	R	A	R	V	E	874
CPB-1	44	K	T	N	L	D	I	S	V	S	F	L	54
RACK-1	40	K	T	I	L	V	W	D	V	D	S	V	50

(b)

C. elegans PUF proteins

FBF-1	477	Y	M	P	H	P	G	T	483
FBF-2	479	Y	I	P	H	P	D	T	485
PUF-3	419	Y	I	P	H	P	D	T	425
PUF-8	454	H	Q	E	G	S	V	P	460
PUF-11	422	Y	I	P	H	P	D	T	428

(c)

Spermatogenesis Models

Figure 7.

The short sequences mediating the GLD-3•FBF interaction are present in other proteins and the possible mechanisms for the activation of spermatogenesis by GLD-3 association with FBF. (a) FBF-binding proteins CPB-1 and RACK-1 may share a common FBF interaction similar to GLD-3. Sequence alignment of the GLD-3 FBF-binding site with the potential FBF-binding sites of CPB-1 and RACK-1. The KTXL sequence in GLD-3 critical for FBF-2 binding is also present in the hypothetical FBF-binding sites of CPB-1 and RACK-1 (boxed). (b) GLD-3 may interact with a subset of other *C. elegans* PUF family members in addition to FBF-1 and FBF-2. Sequence alignment of the PUF repeat 7 and 8 loop of: FBF-1, FBF-2, PUF-3, PUF-8, and PUF-11. The residues in the PUF repeat 7 and 8 loop critical for FBF-2 binding to GLD-3 (Y479, I480, and T485)

are also present at corresponding positions in the PUF repeat 7 and 8 loop of PUF-3 and PUF-11 (boxed), but absent from the PUF-8 PUF repeat 7 and 8 loop. (c) Three possible models for how GLD-3 association with FBF promotes *fem-3* mRNA expression and spermatogenesis: the Inhibition Model, the Recruitment Model, and the Occlusion Model. In the Inhibition Model, FBF binding to the *fem-3* 3' UTR regulatory element (PME) represses FEM-3 protein expression and GLD-3 interacts with FBF in order to disrupt the FBF•PME RNA interaction and release *fem-3* from FBF-mediated repression. In the Recruitment Model, GLD-3 associates with the FBF•PME RNA complex and recruits activator proteins, such as the poly(A) polymerase GLD-2 to lengthen the *fem-3* poly(A) tail. In the Occlusion Model, GLD-3 binds to the FBF•PME RNA complex and impedes the interaction of FBF with repressor proteins.