The P granule component PGL-1 promotes the localization and silencing activity of the PUF protein FBF-2 in germline stem cells

Ekaterina Voronina, Alexandre Paix and Geraldine Seydoux*

SUMMARY

In the C. elegans germline, maintenance of undifferentiated stem cells depends on the PUF family RNA-binding proteins FBF-1 and FBF-2. FBF-1 and FBF-2 are 89% identical and are required redundantly to silence the expression of mRNAs that promote meiosis. Here we show that, despite their extensive sequence similarity, FBF-1 and FBF-2 have different effects on target mRNAs. FBF-1 promotes the degradation and/or transport of meiotic mRNAs out of the stem cell region, whereas FBF-2 prevents translation. FBF-2 activity depends on the P granule component PGL-1. PGL-1 is required to localize FBF-2 to perinuclear P granules and for efficient binding of FBF-2 to its mRNA targets. We conclude that multiple regulatory mechanisms converge on meiotic RNAs to ensure silencing in germline stem cells. Our findings also support the view that P granules facilitate mRNA silencing by providing an environment in which translational repressors can encounter their mRNA targets immediately upon exit from the nucleus.

KEY WORDS: Germline stem cells, RNA silencing, P granules, PUF domain, PGL-1, Caenorhabditis elegans

INTRODUCTION

Post-transcriptional control of gene expression is common in the germline, where protein expression patterns are often determined by RNA-binding proteins that bind to sequences in the 3' UTR (reviewed by Rangan et al., 2008; Kimble, 2011; Voronina et al., 2011). Germ cells contain a unique type of RNA granule, or 'nuage', that localizes to the cytoplasmic face of the nuclear envelope. In Drosophila, C. elegans and zebrafish, the nuage has been implicated in the biogenesis of small RNAs, as several components of the RNAi machinery, including Argonautes, localize there (reviewed by Voronina et al., 2011). Studies in C. elegans have revealed that the nuage ('P granules') overlay clusters of nuclear pores and are the primary sites of mRNA export from the nucleus, raising the possibility that P granules also regulate mRNAs (Pitt et al., 2000; Sheth et al., 2010). Core components of the P granules include two classes of RNA-binding proteins: the VASA-related RNA helicases GLH-1, GLH-2 and GLH-4 and the RGG domain proteins PGL-1 and PGL-3 (reviewed by Updike and Strome, 2010). In this study, we investigate a connection between PGL-1 and mRNA regulation in germline stem cells.

In adult hermaphrodites, germ cells are arranged in a distal-to-proximal order of differentiation in two U-shaped tubes connected to a common uterus (reviewed by Hubbard and Greenstein, 2005). The distal end of each tube contains ~250 cells that divide by mitosis (mitotic zone). The cells are connected to a common cytoplasmic core (rachis) and are displaced proximally each time a cell divides (Fig. 1A). Cells at the distalmost tip include the stem cells, which continue to divide by mitosis indefinitely into adulthood. Cells in the proximal half of the mitotic zone are considered a transient-amplifying population: they continue to divide by mitosis but begin to accumulate low levels of meiotic proteins (Hansen et al., 2004; Cinquin et al., 2010). Meiotic protein expression peaks in the transition zone, where cells initiate chromosome pairing in preparation for recombination in the pachytene region (Fig. 1A).

Maintenance of mitotically dividing cells in the mitotic zone requires FBF-1 and FBF-2, two 89% identical PUF domain RNA-binding proteins (Crittenden et al., 2002). Immunoprecipitation experiments suggest that FBF-1 and FBF-2 bind thousands of mRNAs, including several meiotic mRNAs that are transcribed but silenced in the mitotic zone (Kershner and Kimble, 2010; Merritt and Seydoux, 2010). In the 3' UTR of meiotic mRNAs, FBF-1 and FBF-2 recognize a motif (UCnUGUnnnAU) required for silencing in the mitotic zone (Bernstein et al., 2005; Merritt and Seydoux, 2010; Qui et al., 2012). In the absence of both FBF-1 and FBF-2, all cells in the mitotic zone express meiotic proteins precociously, enter meiosis and differentiate into sperm (Crittenden et al., 2002). fbf-1 fbf-2 hermaphrodites do not make oocytes and are sterile. fbf-1 and fbf-2 single mutants are fertile but have smaller (fbf-1) or larger (fbf-2) mitotic zones, suggesting that, although redundant for fertility, FBF-1 and FBF-2 also have unique roles (Lamont et al., 2004). Here, we provide further evidence that FBF-1 and FBF-2 have distinct activities and demonstrate a requirement for PGL-1 in FBF-2-dependent mRNA silencing.

MATERIALS AND METHODS

Nematode culture and RNAi

C. elegans strains (supplementary material Table S1) were derived from Bristol N2 and cultured according to standard protocols (Brenner, 1974) at 20°C or 25°C as indicated.

RNAi treatments were performed by feeding L4 hermaphrodites bacteria expressing double-stranded RNA for 24 hours. F1 progeny were transferred to fresh RNAi plates and examined as adults. The following RNAi constructs were used: glh-4, pgl-3 (Kamath and Ahringer, 2003) and glh-1, pgl-1 (genomic fragments covering full coding sequence) cloned in pL4440 (Timmons and Fire, 1998).
Generation of transgenic worms

FBF-1 (Merritt et al., 2008) and FBF-2 (this study) transgenes were constructed by Gateway cloning (Invitrogen) using the pic-1 promoter and pby-1 or pby-2 genomic coding and 3’ UTR sequences, and introduced into worms by microprojectile bombardment (Pratsios et al., 2001). GFP::FBF-1 rescues the sterility of the fbf-1 fbf-2 double mutant (Lee et al., 2007). GFP::FBF-2 rescues the expanded mitotic region of the fbf-2[7238] mutant, but not the shortened region of fbf-1[ok91] (data not shown).

In situ hybridization

In situ hybridizations were performed as described previously (Raj et al., 2008), with the following variations: dissected C. elegans gonads were fixed in −20°C methanol and stored at −20°C. Fixed samples were rehydrated in PBS and washed three times with PBS and twice with 2× SSC. Quasar570 dye-labeled oligonucleotide probes were purchased from Biosearch Technologies and hybridized as described (Raj et al., 2008). Gonads were washed once with PBS and three times with PBS before mounting. In transgenic animals expressing GFP fusion proteins, residual GFP fluorescence was detectable following the in situ hybridization protocol.

Antibody generation and western blots

Polyclonal rabbit antisera PA2388 was generated against the FBF-1 peptide EEGNLRLMRTFSP and affinity purified by Open Biosystems (Huntsville, AL, USA). For immunoblotting, the following primary antibodies were used: rabbit PA2388 anti-FBF-1 at 1:20 (26 μg/ml); mouse monoclonal anti-GFP JLI-8 (Clontech) at 1:200; mouse monoclonal anti-p34 CDC28 (Sigma-Aldrich) at 1:1000; and mouse monoclonal anti-PGL-1/PGL-3 KT3 [Developmental Studies Hybridoma Bank (DSHB), University of Iowa] at 1:10. Secondary reagents were ECL Plex Cy5-conjugated goat anti-rabbit or goat anti-mouse antibodies (GE Healthcare/Amersham) at 1:2500 or HRP-Protein A (BD Biosciences) at 1:2000. Blots reacted with fluorescent secondary antibodies were scanned using HyGlo Quick Spray Reagent (Denville).

To assess the specificity of the anti-FBF-1 antibody, extracts were prepared from synchronized cultures of C. elegans young adults that were either wild type or single mutants for fbf-1[ok91], fbf-2[q738] or pgl-1[ct131] by repeated freeze-thaw in 1× NuPage LDS sample buffer (Invitrogen) containing 200 mM DTT, followed by sonication and heating for 10 minutes at 70°C. Lysates were separated on 7% SDS-PAGE gels (Invitrogen) containing 200 mM DTT, followed by sonication and heating at 1:200; and mouse monoclonal anti-p34 CDC28 (Sigma-Aldrich) at 1:1000, and mouse monoclonal anti-PGL-1/PGL-3 KT3 (Developmental Studies Hybridoma Bank (DSHB), University of Iowa) at 1:10. Secondary reagents were ECL Plex Cy5-conjugated goat anti-rabbit or goat anti-mouse antibodies (GE Healthcare/Amersham) at 1:2500 or HRP-Protein A (BD Biosciences) at 1:2000. Blots reacted with fluorescent secondary antibodies were scanned in a Typhoon 9410 imager (GE Healthcare) and quantified using ImageJ (NIH). Blots reacted with HRP-conjugated secondary reagents were developed using HyGlo Quick Spray Reagent (Denville).

RESULTS

FBF-1 and FBF-2 exhibit different subcellular localizations

We characterized the distribution of FBF-1 and FBF-2 in fixed adult gonads using two complementary sets of reagents: polyclonal antibodies against FBF-1 (see Materials and methods) and FBF-2 (Lamont et al., 2004) to detect endogenous proteins, and anti-GFP antibody to detect GFP::FBF-1 and GFP::FBF-2 fusions in transgenic animals. Both approaches yielded the same results. As reported previously (Lamont et al., 2004), we detected FBF-1 and FBF-2 in the distal arm of the germline (Fig. 1B; supplementary material Fig. S1A). FBF-1 levels were uniformly high throughout the mitotic zone (supplementary material Fig. S1A); by contrast, FBF-2 levels were low in the first four to six rows and increased by 5-fold in subsequent rows (Fig. 1C,E; supplementary material Fig. S1A). FBF-1 and FBF-2 also differed in their subcellular distributions: FBF-1 localized to numerous cytoplasmic and perinuclear foci, whereas FBF-2 localized primarily to perinuclear foci and was more diffusely distributed in the cytoplasm (Fig. 1B).

To determine whether the FBF-1 and/or FBF-2 perinuclear foci correspond to P granules, we co-stained GFP::FBF-1 and GFP::FBF-2 with K76, an antibody against the P granule component PGL-1 (Kawasaki et al., 1998), and used deconvolution microscopy to evaluate colocalization in single confocal slices (Fig. 2; supplementary material Fig. S2E; Materials and methods). The majority (74%) of perinuclear GFP::FBF-1 foci did not overlap with P granules, although many (39%) were immediately adjacent
to a P granule (Fig. 2C). By contrast, 72% of GFP::FBF-2 perinuclear foci overlapped either completely or partially with P granules (Fig. 2B,C). Similar results were obtained comparing the distribution of endogenous FBF-1 with PGL-1 (Fig. 2A) and of the P granule component GLH-2 with endogenous FBF-1 or GFP::FBF-2 (supplementary material Fig. S2C,D). Double staining of FBF-1 and GFP::FBF-2 confirmed that FBF-1 and FBF-2 perinuclear foci are distinct, although they overlap occasionally (supplementary material Fig. S2A). We conclude that FBF-1 localizes to both cytoplasmic and perinuclear foci, the majority of which do not coincide with P granules. By contrast, FBF-2 localizes primarily to perinuclear foci that overlap with P granules and on occasion overlap with FBF-1 foci.

FBF-1 and FBF-2 have been reported to negatively regulate each other’s abundance (Lamont et al., 2004). Consistent with those findings, FBF-1 levels were 2-fold higher in \( fbf-2 \) mutants by western blot analysis and 1.6-fold higher by immunostaining (supplementary material Fig. S1B,D). GFP::FBF-2 levels were also higher in \( fbf-1 \) mutants, but the increase was more modest when measured by western blot (1.5-fold; supplementary material Fig. S1C) and not significant when measured by immunostaining with an antibody to endogenous FBF-2 (supplementary material Fig. S1E). We noticed, however, that the distribution of FBF-2 and GFP::FBF-2 changed in \( fbf-1 \) mutants: FBF-2 and GFP::FBF-2 localized to several large (over 1 \( \mu \)m) aggregates in the central core (rachis) of the mitotic zone of \( fbf-1 \) mutant gonads. These aggregates depend on \( pgl-1 \). (D,F) The percentage of germlines with FBF-2 or GFP::FBF-2 aggregates in the indicated genotypes. N, number of hermaphrodites scored.
aggregates were specific to FBF-2: the P granule components GLH-2 and PGL-1 did not localize to FBF-2 aggregates in /bf/-1 mutants (Fig. 1G), and no FBF-1 aggregates were observed in /bf/-2 mutants (supplementary material Fig. S1A).

**PGL-1 is required for FBF-2 localization to P granules**

To determine whether the subcellular localization of FBF-1 and FBF-2 depends on P granule components, we characterized FBF-1 and FBF-2 distributions in /pgl-1(ct131) mutants raised at the permissive temperature (20°C). At this temperature, /pgl-1(ct131) mutants are fertile and have a small but distinct mitotic zone (Kawasaki et al., 1998) (supplementary material Fig. S3). We found that FBF-1 still localized to numerous foci in /pgl-1 mutants (Fig. 1B). By contrast, FBF-2 became more diffusely distributed in /pgl-1 mutants and no longer colocalized with P granules (detected with an antibody against GLH-2; Fig. 1B). We also observed that FBF-2 formed fewer aggregates in the rachis of /bf/-1;/pgl-1 double mutants (Fig. 1C-F).

GLH-1 is a P granule component required to recruit PGL-1 to P granules (Spike et al., 2008a). We found that GFP::FBF-2 (but not GFP::FBF-1) was delocalized in glh-1(RNAi) gonads (supplementary material Fig. S1F). By contrast, no localization defects were detected in glh-4(RNAi) and /pgl-3(RNAi) gonads, which maintain PGL-1 on P granules (supplementary material Fig. S1F). We conclude that P granule-localized PGL-1 is required to localize FBF-2 to P granules. PGL-1 also contributes to FBF-2 localization to aggregates in /bf/-1 mutants.

**PGL-1 is required for FBF-2 function**

/bf/-1;/bf/-2 double mutants are 100% sterile (Crittenden et al., 2002). We noticed that 41% of /bf/-1;/pgl-1 hermaphrodites were also sterile (Fig. 3A). By contrast, only 5% of /bf/-2;/pgl-1 worms were sterile (see supplementary material Table S1 for full genotypes). Like /bf/-1;/bf/-2 double mutants, sterile /bf/-1;/pgl-1 hermaphrodites produce excess sperm and no oocytes (Fig. 3B), in contrast to wild-type worms, which produce both sperm and oocytes (Fig. 3C). These observations suggested that /pgl-1 might be required for /bf/-2 activity. If so, /bf/-1;/pgl-1 double mutants should show the same range of phenotypes as /bf/-1;/bf/-2 mutants.

To test this prediction, we first examined the expression patterns of meiotic proteins that are silenced by FBF-1 and FBF-2 in the mitotic zone. fog-1 is a direct target of FBF-1 and FBF-2: the fog-1 3’ UTR contains FBF binding sites that silence FOG-1 protein expression in the mitotic region (Thompson et al., 2005). Expression of a GFP::H2B::fog-1 3’ UTR reporter is inhibited in the mitotic zone of wild-type, /bf/-1, /bf/-2 and /pgl-1 single-mutant gonads, but is derepressed in /bf/-1;/bf/-2 double-mutant gonads (Merritt et al., 2008) (supplementary material Fig. S3A). We observed ectopic expression of the fog-1 3’ UTR reporter in the mitotic region of 26% of fertile /bf/-1;/pgl-1 double mutants and 55% of sterile /bf/-1;/pgl-1 double mutants (Fig. 3D,E). We also observed derepression of the fog-1 3’ UTR reporter in 39% of /bf/-1;/glh-1(RNAi) gonads (supplementary material Fig. S3C).

HTP-1 and 2 are two highly homologous synaptonemal complex proteins that are silenced in the mitotic region redundantly by FBF-1 and FBF-2 (Merritt and Seydoux, 2010). We observed ectopic expression of HTP-1/2 in 18% of fertile /bf/-1;/pgl-1 double mutants and in 53% of sterile /bf/-1;/pgl-1 double mutants (Fig. 3F,G). In /bf/-1;/bf/-2 mutants, ectopic HTP-1/2 in the mitotic zone accumulate in aggregates, which persist in pachytene germ cells and interfere with normal HTP-1/2 loading on paired chromosomes (Merritt and Seydoux, 2010). We observed similar aggregates and incomplete chromosome loading in /bf/-1;/pgl-1 mutants (Fig. 3H). /bf/-1;/bf/-2 mutants produce defective gametes with achiasmatic chromosomes (Thompson et al., 2005) leading to embryonic lethality (Luitjens et al., 2000). Similarly, we observed achiasmatic chromosomes in 70% of oocytes produced by fertile /bf/-1;/pgl-1 hermaphrodites.
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FBF-1 and FBF-2 regulate different aspects of meiotic mRNA regulation

The finding that **pgl-1** is required primarily for **fbf-2** but not **fbf-1** activity suggests that, despite their sequence similarity, FBF-1 and FBF-2 use different mechanisms to silence meiotic gene expression. To investigate this possibility further, we visualized meiotic mRNAs in **fbf-1**, **fbf-2** and **pgl-1** single- and double-mutant combinations using fluorescent in situ hybridization. We analyzed the FBF targets **gld-1**, **htp-1/2** and **him-3** and the non-FBF target **tbb-2**, which encodes β-tubulin, as a control. **tbb-2** mRNA was uniformly distributed throughout the distal region in all genotypes (Fig. 4; data not shown). As reported previously for **gld-1** (Jones et al., 1996), in wild-type gonads we detected low levels of **gld-1**, **htp-1/2** and **him-3** mRNAs in the distal half of the mitotic zone, and **pgl-1** is required primarily for **fbf-2** activity.
increasing levels in the proximal half of the mitotic zone, and high levels in the pachytene region (Fig. 4A). The same pattern was observed in fbf-2 and pgl-1 single mutants (Fig. 4A). By contrast, in fbf-1 mutants we detected high levels of gld-1, htp-1/2 and him-3 mRNAs throughout the mitotic zone. In the mitotic zone, these mRNAs accumulated in several large aggregates that exceeded 1 μm in size (Fig. 4A,B). No such aggregates were observed with tbb-2 mRNA (Fig. 4A,B). The RNA aggregates were reminiscent of the FBF-2 aggregates that also form in fbf-1 mutants (Fig. 1E). Colocalization experiments confirmed that gld-1 RNA and GFP::FBF-2 localize to the same aggregates in fbf-1 mutants (Fig. 4C). The aggregates were dependent on both fbf-2 and pgl-1: no, or fewer, RNA aggregates were observed in fbf-1 fbf-2 or fbf-1;pgl-1 double mutants (Fig. 4A,B). In those backgrounds, the mRNAs were uniformly distributed throughout the mitotic zone (Fig. 4A).

We conclude that fbf-1 functions to inhibit the accumulation of meiotic mRNAs in the distal half of the mitotic zone. fbf-2 and pgl-1 are not essential for this process, but are required for aggregation of the ectopic mRNAs that accumulate in fbf-1 mutants. Examination of RNAs derived from transgenes under the control of the pan-germline promoter pie-1 confirmed that these effects are post-transcriptional: GFP mRNA derived from the pie-1 promoter::GFP::H2B::Him-3 3’ UTR reporter was distributed in a low-to-high gradient in the mitotic zone (supplementary material Fig. S4A). By contrast, GFP mRNA derived from the same reporter but with mutations in the FBF-1 binding sites in the him-3 3’ UTR (Merritt and Seydoux, 2010) was distributed uniformly throughout the mitotic zone (supplementary material Fig. S4A).

The above findings suggest that silencing by FBF-1 involves post-transcriptional mechanisms that lower mRNA levels in the distal mitotic zone, whereas silencing by FBF-2 does not affect mRNA levels. If so, mRNA and protein levels should be correlated in fbf-2 mutant gonads (where silencing depends on FBF-1), but not in fbf-1 gonads (where silencing depends on FBF-2). To compare protein and RNA levels in the same gonads, we visualized gld-1 RNA by situ hybridization in gonads expressing a pie-1 promoter::GFP::H2B fusion under the control of the gld-1 3’ UTR (we could not detect GLD-1 protein directly because the in situ protocol is not compatible with immunofluorescence). We found that, as predicted, gld-1 RNA and GFP levels correlated well throughout the mitotic zone in fbf-2 mutant gonads, but not in wild-type or fbf-1 gonads (supplementary material Fig. S4B). In the latter, significant levels of gld-1 mRNA were detected in the distal end of the mitotic zone where GFP fluorescence was undetectable or low (supplementary material Fig. S4B). These observations confirm that silencing by FBF-2 primarily suppresses protein levels (mRNA translation), whereas silencing by FBF-1 also leads to a reduction (or redistribution) of mRNA levels.

Fig. 4. fbf-1 affects the distribution of meiotic mRNAs in the distal gonad. (A) Distal gonads of the indicated genotypes hybridized to fluorescent probes specific for gld-1, htp-1/2, him-3 and tbb-2 mRNAs. Germlines are outlined; vertical dotted lines indicate the transition zone as recognized by DAPI staining (not shown). (B) The percentage of germlines with aggregates of the indicated mRNAs as visualized by in situ hybridization as shown in A. N, the number of gonads scored for each genotype and mRNA. (C) fbf-1 mutant distal gonad double stained for GFP::FBF-2 and gld-1 mRNA. Arrow points to a cytoplasmic aggregate that is positive for both GFP::FBF-2 and gld-1 mRNA.
PGL-1 is required for maximum binding of FBF-2 to target mRNAs

The observation that PGL-1 is required for the formation of FBF-2–mRNA aggregates in fbf-1 mutants raised the possibility that PGL-1 promotes FBF-2 binding to target mRNAs. To test this hypothesis, we immunoprecipitated GFP::FBF-2 with an anti-GFP antibody (or with a control IgG) in lysates prepared from wild-type or pgl-1 mutant worms and quantified the amounts of five known FBF-1/2 target mRNAs in the immunoprecipitates by qRT-PCR. Results were normalized for the amount of GFP::FBF-2 immunoprecipitated in three independent experiments and the amount of non-specific binding to the control IgG antibody (see Materials and methods). We found that target mRNAs were precipitated with higher efficiency from wild-type lysates than pgl-1 lysates (Fig. 5A).

FBF-1 recognizes the same consensus sequence as FBF-2 in vitro (Bernstein et al., 2005) and co-immunoprecipitates at least some of the same RNAs (Merritt and Seydoux, 2010). We immunoprecipitated FBF-1 from the same wild-type and pgl-1 lysates used for the GFP::FBF-2 immunoprecipitations, and observed no decrease in the ability of FBF-1 to interact with the target RNA gld-1 (Fig. 5B). We observed a minor reduction in binding of FBF-1 to htp-1, him-3 and syt-3 mRNAs, but this decrease was observed in only one experimental replicate (data not shown) and was not statistically significant when averaged over all experimental replicates. We also examined the ability of a GLD-1::GFP fusion to interact with gld-1 mRNA in wild-type and pgl-1 lysates and again found no significant differences (Fig. 5C). We conclude that pgl-1 activity is required for maximal association of FBF-2 with target mRNAs, but is not essential for all protein-RNA interactions.

DISCUSSION

In this study we present evidence that FBF-1 and FBF-2 inhibit the expression of meiotic mRNAs in the distal germline by two distinct mechanisms and that PGL-1 is required for FBF-2 activity. We discuss each of these points in turn below.

FBF-1 and FBF-2 silence meiotic mRNAs by distinct mechanisms

In situ hybridization experiments on fixed adult gonads revealed that the gld-1, hip-1/2 and him-3 mRNAs are distributed in a gradient in the mitotic zone, with the lowest signal at the distal end (where the stem cells reside) and the highest signal toward the proximal end (transition zone, where meiosis starts). This pattern does not depend on FBF-2 or PGL-1: fbf-2 and pgl-1 mutants maintain a distal-low/proximal-high gradient of meiotic RNAs. By contrast, in fbf-1 mutants, meiotic mRNAs accumulate in several aggregates throughout the rachis of the mitotic zone, including at the distal end. Because all cells are connected to a central core that runs through the mitotic zone and into the pachytene region (Hubbard and Greenstein, 2005), we do not know whether FBF-1 ‘clears’ meiotic mRNAs from the distal end by promoting their degradation or transport. In the proximal germline, cytoplasmic streaming transports mitochondria and small PGL-1::GFP particles from the pachytene region into oocytes (Wolke et al., 2007). No
such bulk movement, however, has been observed in the mitotic zone. If FBF-1 promotes mRNA transport, it must do so by targeting specific mRNAs, as tubulin mRNA was not in a gradient and the graded distribution of the him-3 3’ UTR reporter mRNA was dependent on FBF-1/2 binding sites.

Expression of GLD-1 and of the meiotic 3’ UTR reporters is partially derepressed in the mitotic zone of fbf-1 mutants and completely derepressed in fbf-1 fbf-2 mutants (Crittenden et al., 2002; Suh et al., 2009; Merritt and Seydoux, 2010). Thus, in the absence of FBF-1, FBF-2 incompletely suppresses the translation of meiotic mRNAs in the distal region. This suppression correlates with the formation of aggregates that contain both FBF-2 and meiotic mRNAs: the aggregates are observed in fbf-1 mutants but not in fbf-1 fbf-2 and fbf-1; pgl-1 double mutants, in which the meiotic mRNAs are no longer silenced. We suggest that the aggregates represent translationally repressed FBF-2–mRNA complexes, which in wild-type gonads are degraded or transported out of the region by FBF-1. Consistent with this hypothesis, as is true for meiotic mRNAs, FBF-2 levels are lowest in the five distalmost rows and this pattern depends on FBF-1 (Fig. 1E).

Our findings indicate that despite their sequence similarity and shared binding sites, FBF-1 and FBF-2 have different effects on the distribution of meiotic mRNAs. One possibility is that binding by either FBF-1 or FBF-2 blocks translation, and binding by FBF-1 additionally leads to RNA degradation or transport. In vitro, both FBF-1 and FBF-2 bind the Pop2 subunit of the CCR4-Pop2-NOT deadenylase complex, and FBF-2 can stimulate deadenylation by the yeast Pop2 complex (Suh et al., 2009). Shortening of the poly(A) tail could in principle lead to both translation inhibition and RNA degradation (Goldstrom et al., 2006; Hook et al., 2007). Loss of Pop2 activity moderately increases GLD-1 levels in the distal gonad, confirming that deadenylation is one of the mechanisms used by the FBFs to silence mRNAs, but not the only one (Suh et al., 2009). Recently, FBF-1 has also been shown to inhibit the GTPase activity of elongation factor eIF1A in a complex with the Argonaute protein CSR-1. In vitro, the FBF-1–CSR-1–eIF1A complex stalls ribosomes during elongation (Friend et al., 2012). Interestingly, in yeast, translational repression by PUF proteins has been linked to localization of the silenced mRNA to distinct subcellular regions. Puf6 promotes the assembly of ‘locasome complexes’ on the ASH1 mRNA by relieving the competition between ribosomes and the localization factor She2 (Gu et al., 2004; Deng et al., 2008). Binding by She2 in turn localizes ASH1 mRNA to the daughter bud. Similarly, Puf5 promotes the localization of the Pex14 mRNA to peroxisomes, and Puf3 localizes its many mRNA targets to mitochondria (reviewed by Quenault et al., 2011).

We suggest that, like other PUFs, FBF-1 and FBF-2 have diverged enough to interact with different factors. FBF-1 and FBF-2 are 95% identical throughout their RNA-binding domain, but only show 87% and 72% identity in their N- and C-termini, respectively. These divergent domains might provide interaction surfaces for distinct co-factors leading to different modes of RNA regulation. Consistent with interacting with different protein partners, FBF-1 and FBF-2 accumulate in different cytoplasmic granules. FBF-1 localizes to many unidentified granules throughout the cytoplasm, whereas FBF-2 localizes primarily to P granules around nuclei and is more diffusely distributed in the cytoplasm. Because FBF-1 and FBF-2 recognize the same sequence, one possibility is that they compete for targets. We suggest that, in the distal end of the mitotic zone, an unknown mechanism, possibly dependent on Notch signaling from the distal tip cell (Kimble, 2011), suppresses FBF-2 activity and/or levels, allowing FBF-1 to cleart meiotic mRNAs from this region. In more proximal rows, FBF-2 competes effectively with FBF-1, causing meiotic mRNAs to accumulate while remaining translationally repressed. Competition between FBF-1 and FBF-2 could also explain why the mutants have opposite effects on the size of the mitotic zone (Lamont et al., 2004). In the absence of FBF-1, meiotic mRNAs accumulate throughout the mitotic zone, leading to premature meiotic entry (shorter mitotic zone). In fbf-2 mutants, FBF-1 keeps meiotic mRNA levels low, delaying meiotic entry (longer mitotic zone). It will be interesting to investigate what keeps FBF-2 activity/levels low in the distalmost mitotic zone: FBF-1 is required, but cannot be the only factor because FBF-1 is also present in the proximal half of the mitotic zone where FBF-2 levels are high (Fig. 1).

### PGL-1 contributes to FBF-2-dependent silencing

Several lines of evidence indicate that PGL-1 functions with FBF-2 to promote the silencing of meiotic mRNAs. Both fbf-2 and pgl-1 mutants are fertile on their own but become sterile when combined with mutations in fbf-1. fbf-1; pgl-1 mutants share several phenotypes with fbf-1 fbf-2 mutants, including sterility, excess sperm, derepression of meiotic mRNAs in the mitotic zone, defective loading of the synaptonemal complex during meiosis, and defects in meiotic recombination. These defects are less penetrant in fbf-1; pgl-1 mutants than in fbf-1 fbf-2 mutants, suggesting that pgl-1 is only partially required for fbf-2 activity, perhaps because of redundancy with other P granule components. Consistent with these findings, FBF-2 binding to target RNAs is reduced, but not completely eliminated, in extracts from pgl-1 mutants. FBF-2 is enriched on P granules and this enrichment is lost in pgl-1 mutants. FBF-2 activity and localization to P granules are also compromised in ghb-1( RN4ji) gonads, where PGL-1 is present but no longer on P granules. These findings suggest that localization to P granules, rather than PGL-1 function per se, is necessary to maximize FBF-2 activity.

P granules are major sites of RNA export from the nucleus (Sheth et al., 2010) and have been proposed to extend the nuclear pore environment into the cytoplasm (Updike et al., 2011). Like nuclear pores, P granules establish a size-exclusion barrier (Updike et al., 2011). Such a barrier could create a privileged environment in which mRNAs are able to interact with regulators such as FBF-2 without interference from ribosomes or other translation-promoting factors. The assembly of multivalent complexes in P granules could also greatly increase the local concentration of RNA-binding proteins (Li et al., 2012). As most RNAs do not appear to accumulate in P granules upon exit from the nucleus (Sheth et al., 2010), we suggest that meiotic mRNAs meet FBF-2 while transiting through the P granules and enter the cytoplasm complexed with FBF-2 in a silenced mRNP (Fig. 5D). Consistent with this hypothesis, in fbf-1 mutants, we observed large FBF-2–meiotic mRNA aggregates in the cytoplasm away from P granules, and formation of these aggregates was dependent on pgl-1.

A role for P granules in mRNA regulation has been suggested ever since their discovery as RNA-rich structures (reviewed by Voronina et al., 2011; Schisa, 2012). Our data demonstrate that PGL-1 facilitates translational silencing by FBF-2, but this is unlikely to be the only role of PGL-1. pgl-1 mutants display several phenotypes not seen in fbf-2 mutants, including resistance to RNAi (Robert et al., 2005; Spike et al., 2008b), a short mitotic zone (supplementary material Fig. S3B) and sterility at 25°C (Kawasaki et al., 1998). PGL-1 is a key structural component of P granules: PGL-1 is...
required (with its homolog PGL-3) for P-granule formation in embryos and can form ectopic granules when expressed in somatic cells (Handazawa et al., 2011; Updike et al., 2011). It will be interesting to determine which RNA-binding proteins besides FBF-2 require PGL-1 to function efficiently in germ cells.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.083980/-/DC1

References


Fig. S1. FBF-1 and FBF-2 have distinct distributions in the distal gonad. (A) Distal young adult (1 day post-L4) gonads of indicated genotypes co-immunostained for FBF-1 or FBF-2 and PGL-1 (OIC1D4 antibody) as indicated. Lack of staining in the mutants confirms the specificity of the FBF-1 and FBF-2 antibodies. Quantification of anti-FBF-2 immunostaining in the first (distalmost) five rows compared with the next five rows revealed a 5.0±1.2-fold increase (N=10 gonads); a similar increase (5.2±0.5-fold; N=9) was observed by quantification of GFP intensity in the GFP::FBF-2 transgenic line. (B,C) Western blots of whole worm lysates of the indicated genotypes. Anti-FBF-1 antibody detects a band on western blot of whole worm lysates, which is absent in fbf-1 mutants. FBF-1 protein amount doubles in fbf-2 lysates, but does not change significantly in pgl-1 lysates. GFP::FBF-2 protein increases 1.5-fold in the fbf-1 mutant background. Tubulin is used as a loading control. (D,E) Quantification of endogenous FBF-1 and FBF-2 protein levels in immunostained germlines. The signal intensity in a 3D stack encompassing the entire mitotic zone (from the distal tip to the transition zone) was summed, and background was subtracted. Three germlines were analysed for each set. Wild-type signal was set to 100%, and the mutant signal was scaled accordingly. For FBF-1, the difference between wild type and mutant is statistically significant (P<0.01). By contrast, for FBF-2, the difference did not reach statistical significance (P=0.299). (F) Live images of distal gonads expressing indicated GFP fusions subjected to the indicated RNAi treatments. Numbers at the bottom right of each panel indicate the percentage of germlines that exhibit the phenotype shown; 10-50 animals were examined per each treatment. glh-1(RNAi) causes dispersal of both PGL-1::GFP and GFP::FBF-2 foci, but not GFP::FBF-1. RNAi of glh-1 and pgl-3 do not affect localization of any GFP fusion.
Figure 1, supplemental

A

wt

fbf-1(ok91)

fbf-2(q738)

B

C

D

E

F

GFP::FBF-2

anti-FBF-2

staining intensity, AU

E

GFP::FBF-1

anti-FBF-1

staining intensity, AU

GFP::FBF-1

GFP::FBF-2

PGL-1::GFP

vector RNAi

glh-1(RNAi)

glh-4(RNAi)

pgl-3(RNAi)
Fig. S2. FBF-1 and FBF-2 localize to distinct perinuclear granules. (A-E) Deconvolved confocal sections of fixed wild-type mitotic zone germ cells double immunostained with the indicated antibodies (nuclei in blue). PGL-1 was detected with K76 antibody; GFP::FBF-1 and GFP::FBF-2 transgenes were detected with anti-GFP antibody.
Figure 2, supplemental
Fig. S3. Meiotic protein expression is derepressed in fbf-1::pgl-1 mutants. Distal gonads are outlined; vertical dotted line indicates transition zone as recognized by DAPI staining (not shown). (A) Distal gonads of indicated genotypes expressing GFP::H2B::fog-1 39 UTR. The reporter is expressed in distalmost cells in the fbf-1::pgl-1 double mutant but not in any of the single mutants. (B) Distal gonads of the indicated genotypes stained with an antibody against HTP-1 and HTP-2. HTP-1/2 are expressed in distalmost cells in the fbf-1::pgl-1 and fbf-1 fbf-2 double mutants but not in any of the single mutants. (C) Distal gonads of the indicated genotypes expressing GFP::H2B::fog-1 39 UTR. The reporter is expressed in distalmost cells in fbf-1::glh-1(RNAi) germlines, but not in fbf-2::glh-1(RNAi) germlines. The graph on the right shows percentage germlines of the indicated genotypes with expression of GFP::H2B::fog-1 39 UTR extending to the distal end of the germline. Numbers at the bottom of each bar indicate number of hermaphrodites scored. (D) The percentage of oocyte nuclei of the indicated genotypes showing excess of six DAPI-stained bodies indicative of failed meiotic recombination. The penetrance of this phenotype is highest in the fbf-1::pgl-1 mutant animals.
Figure 3, supplemental

A  GFP::H2B::fog-1 3'UTR

B  endogenous HTP-1/2

C  GFP::H2B::fog-1 3'UTR

D  % derepressed

% oocytes with >6 DAPI spots

fbf-1; glh-1(RNAi)  53 70 27 25
fbf-2; glh-1(RNAi)  57 59 33 25

% oocytes with >6 DAPI spots

fbf-1; pgl-1  140 70 93
fbf-2; pgl-1  74 74 33 25
pgl-1  74 74
wt  71 71

% derepressed

fbf-1  59
fbf-2  70
fbf-1; pgl-1  70
fbf-2; pgl-1  70
pgl-1  70
wt  70

N  71 74 70 74 140 93
Fig. S4. FBF-1 and FBF-2 have distinct effects on the distribution and repression of meiotic mRNAs. 
(A) Distal gonads of animals expressing indicated transgenes under the control of the pan-germline pie-1 promoter (or no transgene) hybridized to fluorescent probe against GFP. mRNAs containing the wild-type him-3 39 UTR are distributed in a gradient, whereas mRNAs containing the him-3 39 UTR with mutations in the FBF binding sites are distributed uniformly. No signal is detected in the absence of the transgene. (B) Distal gonads of indicated genotypes expressing pie-1::GFP::H2B::gld-1 39 UTR (GFP fluorescence, first row) and hybridized with a fluorescent probe against gld-1 mRNA (second row). Third row shows DAPI-stained nuclei. Signal intensity is represented by a pseudocolor scale ranging from blue (low signal) to yellow (high signal). (C) The relative intensities of GFP (green) and mRNA (red) signals along the length of the gonad. The intensities are scaled from 0 to 1 (maximal). (D) GFP signal versus mRNA signal; each point represents a single position along the length of the gonad. In wild type and fbf-1 mutant, mRNA and GFP intensities are not well correlated (linear regression $R^2=0.46$ and 0.73, respectively, in the examples shown; average of two gonads $R^2=0.51$ and 0.66). In the fbf-2 mutant, mRNA and GFP intensities correlate better ($R^2=0.92$ in the example shown; average of two gonads $R^2=0.88$).
Figure 4, supplemental

A

GFP in situ

GFP::H2B:: him-3 3'UTR

GFP::H2B:: him-3 3'UTR (FBE mut)

no transgene

B

wt

fbf-1

fbf-2

GFP

RNA in situ

DNA

C

Relative signal intensity

distance from distal end, \( \mu m \)

D

wild type

\( R^2 = 0.46 \)

fbf-1

\( R^2 = 0.73 \)

fbf-2

\( R^2 = 0.92 \)
Fig. S5. Controls for immunoprecipitation experiments. (A-C) Western blots of the anti-GFP and anti-FBF-1 immunoprecipitates from wild-type and pg/l-1 lysates showing GFP::FBF-2, FBF-1 and GLD-1::GFP levels. These levels were used to normalize cDNA loading for qPCR reactions. (D) Meiotic mRNA levels do not change significantly in pg/l-1 mutants. Relative levels of the indicated mRNAs in wild-type or pg/l-1 lysates were determined by qPCR. Expression levels were normalized to ama-1 (RNA polymerase II) mRNA. For each wild type versus pg/l-1 pair of lysates, wild-type mRNA levels were set to 1 (horizontal dashed line), and the levels in the pg/l-1 mutant were scaled accordingly. Bars show average relative levels of mRNAs across all experiments; error bars indicate s.e.m.
Figure 5, supplemental

A. wt and pgl-1 expression of GFP::FBF-2

B. wt and pgl-1 expression of FBF-1

C. wt and pgl-1 expression of GLD-1::GFP

D. mRNA abundance in pgl-1 mutant compared to wt (scaled to mRNA level in wt = 1).
### Table S1. Worm strains

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Transgene description</th>
<th>Strain</th>
<th>Reference</th>
</tr>
</thead>
</table>
| **Transgenes: ORF + 3’UTR**
unc-119(ed3) III; axIs1471 [pCM4.06] IV | pie-1 prom::GFP::FBF-1::fbf-1 3’UTR | JH2024 | Merritt et al., 2008 |
unc-119(ed3) III; axIs1702 [pCM4.09] | pie-1 prom::GFP::FBF-2::fbf-2 3’UTR | JH2365 | Merritt et al., 2008 |
unc-119(ed3) III; axIs2000 [pEV1.05] | pie-1 prom::LAP::FBF-2::fbf-2 3’UTR | JH2919 | This study |
fbf-1(ok91) II; axIs2000 [pEV1.05] (unc-119(ed3) III - ???) | pie-1 prom::LAP::FBF-2::fbf-2 3’UTR | JH2929 | This study |
pgl-1(ct131) him-3(e1147) IV; axIs1702 [pCM4.09] (unc-119(ed3) III - ???) | pie-1 prom::GFP::FBF-2::fbf-2 3’UTR | JH2881 | This study |
ozIs5 I; unc-119(ed3) III | GLD-1::GFP | Arur et al., 2009 |
ozIs5 I; pgl-1(ct131) him-3(e1147) IV (unc-119(ed3) III - ???) | GLD-1::GFP | JH2874 | This study |
zuIs242 | nmy-2 prom::PGL-1::GFP::nmy-2 3’UTR | JJ2101 | Wolke et al., 2007 |
| **Transgenes: GFP::H2B::3’UTR**
unc-119(ed3) III; axIs1722 [pCM1.90] | pie-1 prom::GFP::H2B::fog-1 3’UTR | JH2423 | Merritt et al., 2008 |
fbf-1(ok91) II; axIs1722 [pCM1.90] | pie-1 prom::GFP::H2B::fog-1 3’UTR | JH2525 | Merritt et al., 2008 |
fbf-2(q738) II; axIs1722 [pCM1.90] | pie-1 prom::GFP::H2B::fog-1 3’UTR | JH2523 | Merritt et al., 2008 |
fbf-1(ok91) II; pgl-1(ct131) him-3(e1147) IV; axIs1722 [pCM1.90] | pie-1 prom::GFP::H2B::fog-1 3’UTR | JH2883 | This study |
fbf-2(q738) II; pgl-1(ct131) him-3(e1147) IV; axIs1722 [pCM1.90] | pie-1 prom::GFP::H2B::fog-1 3’UTR | JH2877 | This study |
unc-119(ed3) III; axIs1723 [pCM6.36A] | pie-1 prom::GFP::H2B::gld-1 3’UTR | JH2436 | Merritt et al., 2008 |
fbf-1(ok91) II; axIs1723 [pCM6.36A] | pie-1 prom::GFP::H2B::gld-1 3’UTR | JH2513 | Merritt and Seydoux, 2010 |
fbf-2(q738) II; axIs1723 [pCM6.36A] | pie-1 prom::GFP::H2B::gld-1 3’UTR | JH2512 | Merritt and Seydoux, 2010 |
unc-119(ed3) III; axIs1691 [pCM6.52A] | pie-1 prom::GFP::H2B::him-3 3’UTR | JH2336 | Merritt et al., 2008 |
unc-119(ed3) III; axIs1691 [pCM1.101] | pie-1 prom::GFP::H2B::him-3 M1M2 3’UTR | JH2375 | Merritt and Seydoux, 2010 |
| **Mutant strains; no transgene**
fbf-1(ok91) II | – | JK3022 | Crittenden et al., 2002 |
fbf-2(q738) II | – | JK3101 | Lamont et al., 2004 |
fbf-1(ok91) fbf-2(q704) II/mIm1[mls14 dpy-10(e128)] II | – | JK3107 | Crittenden et al., 2002 |
pgl-1(ct131) him-3(e1147) IV | – | SS2 | Kawasaki et al., 1998 |