Toward Identifying Subnetworks from FBF Binding Landscapes in Caenorhabditis Spermatogenic or Oogenic Germlines

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ABSTRACT Metazoan PUF (Pumilio and FBF) RNA-binding proteins regulate various biological processes, but a common theme across phylogeny is stem cell regulation. In Caenorhabditis elegans, FBF (fem-3 Binding Factor) maintains germline stem cells regardless of which gamete is made, but FBF also functions in the process of spermatogenesis. We have begun to “disentangle” these biological roles by asking which FBF targets are gamete-independent, as expected for stem cells, and which are gamete-specific. Specifically, we compared FBF iCLIP binding profiles in adults making sperm to those making oocytes. Normally, XX adults make oocytes. To generate XX adults making sperm, we used a fem-3(gf) mutant requiring growth at 25°; for comparison, wild-type oogenic hermaphrodites were also raised at 25°. Our FBF iCLIP data revealed FBF binding sites in 1522 RNAs from oogenic adults and 1704 RNAs from spermatogenic adults. More than half of these FBF targets were independent of germline gender. We next clustered RNAs by FBF-RNA complex frequencies and found four distinct blocks. Block I RNAs were enriched in spermatogenic germlines, and included validated target fog-3, while Block II and III RNAs were common to both genders, and Block IV RNAs were enriched in oogenic germlines. Block II (510 RNAs) included almost all validated FBF targets and was enriched for cell cycle regulators. Block III (21 RNAs) was enriched for RNA-binding proteins, including previously validated FBF targets gld-1 and htp-1. We suggest that Block I RNAs belong to the FBF network for spermatogenesis, and that Blocks II and III are associated with stem cell functions.

KEYWORDS FBF germline stem cell iCLIP sperm oocyte

RNA regulatory networks — defined by genome-wide interactions between RNA-binding proteins and their RNA targets — are central to biological control (Keene 2007; Ascano et al. 2013; Ule and Darnell 2006; Ivshina et al. 2014). Among RNA-binding proteins analyzed at a genomic level for target RNAs, the PUF RNA-binding proteins (for Pumilio and FBF) have served as paradigms because of exquisite sequence-specificity and high affinity for their binding elements (Wang et al. 2001; Wang et al. 2002; Wang et al. 2009; Qiu et al. 2012; Zhu et al. 2009). For example, each of five PUF proteins in Saccharomyces cerevisiae binds a battery of mRNAs, with some redundancy for targets in those networks but with key biological functions associated with each particular PUF (Gerber et al. 2004; Porter et al. 2015; Wilinski et al. 2015). Metazoans also have one or more PUF proteins with multiple biological roles. An ancient and apparently common function of metazoan PUFs is stem cell maintenance (Wickens et al. 2002), but PUFs can also regulate sex determination, embryonic polarity, neurogenesis and learning, among their varied biological roles (Lin and Spradling 1997; Spradling et al. 2001; Crittenden et al. 2002; Wickens et al. 2002; Spassov 2004; Salvetti et al. 2005; Kaye et al. 2009; Vessey et al. 2010; Campbell et al. 2012; Lander et al. 2012; Zhang et al. 1997; Zhang et al. 2017; Darnell 2013; Follwaczny et al. 2017). Moreover, mutations in
the human PUM1 gene can lead to both developmental delay and seizures (Gennarino et al. 2018). The challenge now is to identify metazoan PUF subnetworks with distinct biological roles and to define those mRNAs whose regulation is critical for stem cells.

The C. elegans PUF paralogs, FBF-1 and FBF-2 (collectively known as FBF), are exemplars of metazoan PUF regulation. FBF-1 and FBF-2 are major regulators of germ cell stem maintenance (Crittenden et al. 2002), the hermaphrodite sperm-to-oocyte switch (Zhang et al. 1997), and the process of spermatogenesis (Luitjens et al. 2000). FBF preferentially binds its targets in the 3′ UTR in a sequence-specific fashion (Prasad et al. 2016). The FBF binding element (FBE) is UGUNNNAU with the optimal FBE being UGUDHHAU, where D is A, U, or G and H is A, U, or C (Bernstein et al. 2016); however, FBF can also activate mRNAs (Kaye et al. 2009; Suh et al. 2009) and has been proposed to mediate the transition from self-renewal to differentiation via a switch from its repressive to its activating mode (Kimble and Crittenden 2007). Consistent with this idea, a regulated transition from PUF-mediated repression to activation was recently found for a yeast PUF (Lee and Tu 2015).

Previous genomic analyses of the network of RNAs associated with FBF-1 and FBF-2 focused on adult oogenic germines (Kershner and Kimble 2010; Prasad et al. 2016). Most relevant to this work were the iCLIP studies showing that FBF-1 and FBF-2 associate with largely the same mRNAs via the same binding sites (Prasad et al. 2016). Therefore, FBF-1 and FBF-2 are not only biologically redundant for regulation of stem cells (Crittenden et al. 2002), but these nearly identical proteins also control a common "FBF network".

Here we use iCLIP to compare FBF-bound RNAs in spermatogenic and oogenic germines with the goal of identifying subnetworks responsible for individual FBF biological functions. Because FBF is essential for spermatogenic and oogenic germines, we used genetic crosses to introduce the temperature gain-of-function allele, fem-3(q22 ts gf), and thereby generated JKS140 and JKS545. This fem-3 mutant is spermatogenic when grown at 25° from the first larval stage (L1) (Barton et al. 1987), and strains generated with tagged FBF were similarly spermatogenic at 25°. This fem-3 allele is a T-to-C mutation near one of two 3′ UTR FBEs (CGCTTCTGTGTCTAT to CGCTCCTGTGTCTAC; FBF underlined, mutation underlined and italicised). To compare oogenic and spermatogenic iCLIP datasets, both oogenic and spermatogenic animals were maintained at 15° for propagation and shifted to 25° from the L1 stage for iCLIP.

**MATERIALS AND METHODS**

**Nematode strains used in this study**

| Strain         | Description                                      |
|----------------|--------------------------------------------------|
| JKS4561        | fem-3(q22 ts gf)                                 |
| JKS181        | flb-1(ok91) qsi232[3xFLAG::fbf-1] II             |
| JKS182        | flb-2(q738) qsi75[3xFLAG::fbf-2] II             |
| JKS404        | flb-1(ok91) qsi232[3xFLAG::fbf-1] II; fem-3(q22 ts gf) IV/ nT1[qkB51][IV;V] |
| JKS545        | flb-2(q738) qsi75[3xFLAG::fbf-2] II; fem-3(q22 ts gf) IV/ nT1[qkB51][IV;V] |

**Generation and maintenance of strains carrying epitope-tagged FBF-1 and FBF-2 transgenes**

Strains JKS181 and JKS182 were generated previously (Prasad et al. 2016). Briefly, the qsi232 (3xFLAG::FBF-1) and qsi75 (3xFLAG::FBF-2) transgenes were created by the method of Mos1-mediated single copy insertion (MosSCI) (Frokjaer-Jensen et al. 2008) and placed into strains lacking fbf-1 or fbf-2 respectively. Like wild-type, these strains are oogenic as adults; the primary difference is that they carry a FLAG-tagged FBF so that we can do iCLIP. To generate spermatogenic adults, we used genetic crosses to introduce the temperature gain-of-function allele, fem-3(q22 ts gf), and thereby generated JKS140 and JKS545. This fem-3 mutant is spermatogenic when grown at 25° from the first larval stage (L1) (Barton et al. 1987), and strains generated with tagged FBF were similarly spermatogenic at 25°. This fem-3 allele is a T-to-C mutation near one of two 3′ UTR FBEs (CGCTTCTGTGTCTAT to CGCTCCTGTGTCTAC; FBF underlined, mutation underlined and italicised). To compare oogenic and spermatogenic iCLIP datasets, both oogenic and spermatogenic animals were maintained at 15° for propagation and shifted to 25° from the L1 stage for iCLIP.

**iCLIP**

iCLIP was carried out with modifications for C. elegans, as previously described (Huppertz et al. 2014; Prasad et al. 2016). Single-end sequencing was performed on an Illumina HiSeq 2000. Data are available in the NCBI GEO database, accession GSE83695.

**Data processing**

Fastq files were split by barcode and 3′ linker and reverse transcription primer sequences were clipped. The barcode was then removed from the read sequence and moved to the read name. Reads were mapped to the genome using STAR and CSEQ parameters (Dobin et al. 2013; Kassuhn et al. 2016), except alignment was local, rather than end-to-end. Reads mapping to multiple places by STAR or mapping with a STAR-reported score below 20 were removed. Duplicates were removed using scripts from Weyn-Vanhentenryck et al. (2014) applied to the barcode sequence found in read names. Reads were assigned to RNAs by HTSeq (Anders et al. 2015) and initial differential expression analysis was performed by DESeq2 (v. 1.18.1). Peaks were called as described previously (Prasad et al. 2016), except that two reads-in-peak cutoffs were applied: one cutoff by per-million normalized read number (twofold or higher), and one by un-normalized read number (tenfold or higher). The exact cutoffs varied between datasets and are included in File S2. We used the same criteria as described previously to determine cutoffs (Prasad et al. 2016). Specifically, cutoffs were chosen to retain all validated targets, maximize enrichment of the binding site, and identify as many potential targets as possible. HOMER (Heinz et al. 2010), v. 4.9.1, was performed using the highest 500 peaks, with the single parameter “-rana”.

**Generation of “FBF” replicates for 25° datasets**

One FBF-1 biological replicate from 25° oogenic worms had fewer reads than the other five for this strain (two for FBF-1 and three for FBF-2), although the other FBF-1 replicates are large enough that the FBF-1 dataset is still larger than the FBF-2 dataset (Figure S1A, File S2). To generate replicates of more comparable size, we combined iCLIP reads for FBF-1 and FBF-2 to generate three more equally sized FBF replicates. We did not face a similar problem with replicates from spermatogenic animals (Figure S1B), but similarly combined these as well.

**Clustering method**

We first normalized each of our iCLIP datasets to reads-per-million so that reads-per-RNA represented the frequency of binding at a given RNA. We then subtracted the average of the negative controls from each FBF iCLIP dataset, and finally converted all counts to a log₂ scale.
We calculated distances between binding frequencies for each RNA by Euclidean distance and clustered those distances by simple hierarchical clustering (Eisen et al. 1998). Euclidean distance is a generalization of the notion of distance as the length of a straight line between two points. In our case, the distance between two RNAs A and B is the distance between the vectors of FBF binding (each FBF iCLIP replicate being one dimension of the vector) at A and B. We used Euclidean distance between reads-per-million counts, rather than normalizing each RNA to have the same average number of reads, so that we could cluster according to both frequency of binding and the dependency of binding on germline gender. Distance metrics were used to generate clusters using pairwise average-linkage cluster analysis (Sokal and Michener 1958), in which distances between clusters are simply defined as the average of all distances between elements in a cluster A with all elements in a cluster B.

DESeq2
A read was assigned to an RNA if and only if it overlapped with an exon of the corresponding gene. DESeq2 (v. 1.18.1) results were generated as described in the DESeq2 documentation, using default parameters of the DESeq function, which set minimum read depths based on maximizing the genes passing a given FDR. We used an FDR of 0.01. DESeq-reported p-values are Benjamini–Hochberg-adjusted. We applied DESeq2 analysis to compare the effect of both gender and of temperature. In either case, we restricted our analysis to those RNAs identified as part of the spermatogenic or oogenic program (Noble et al. 2016).

Worm-human PUF target comparison
We used a compendium of C. elegans genes with human orthologs (Shaye and Greenwald 2011) to identify which FBF target RNAs encode proteins with human counterparts. We compared these FBF targets to PUM2 targets identified by PAR-CLIP in human embryonic kidney cells (Hafner et al. 2010) and to PUM1 and PUM2 targets identified by iCLIP during mouse neurogenesis (Zhang et al. 2017). An FBF target was defined as shared with PUM if (1) any mammalian ortholog was targeted by PUM, (2) there were no more than ten mammalian orthologs (such limits have been used previously for cross-phyla comparison (Hogan et al. 2015), and (3) there were no more than ten C. elegans genes orthologous to the same mammalian ortholog. We treated orthology as a transitive property: if nematode genes “A” and “B” are listed as orthologs in Shaye and Greenwald (2011) to mammalian genes that overlap by at least one gene, then “A” and “B” were treated as if they were a single gene for calculating overlap. The same method of combining orthologs was applied to the mammalian gene set (if two mammalian genes overlap in worm orthologs, they were combined).

Statistical analysis
All statistical methods for determining FBF-RNA interactions were as described in Prasad et al. (2016). Briefly, reads in the 500-bp region around a peak were placed in 50-bp bins for both FBF iCLIP and no-antibody iCLIP control data. The negative control was modeled as a Gaussian to calculate a p-value as the chance of observing a peak at the given height from the negative control data. All p-values were then Benjamini-Hochberg corrected and an FDR cutoff of 1% applied, before applying the two ratio cutoffs described above. Statistics used for DESeq2 fold-change estimates and target comparison are described above.

Data Availability
Strains are available upon request. Scripts used to analyze the data were uploaded to github.com/dfporter/FBF_gendered_gl. Sequencing data are available in the NCBI GEO database, accession GSE83695. To replicate the combined 25°C FBF datasets from individual FBF-1 and FBF-2 replicates, first obtain the individual replicates from GSE83695, and concatenate 25°C oogenic FBF-1/FBF-2 replicates in the order 1/3, 2/2, and 3/1; then concatenate 25°C spermatogenic FBF-1/FBF-2 replicates in the order 2/1, 1/2, and 3/3. The 20°C FBF iCLIP data from Prasad et al. (2016) is available at GSE76136. File S1 contains FBF iCLIP peaks. File S2 contains metrics such as complexity for FBF iCLIP peaks. File S3 contains GO terms for FBF targets. File S4 describes RNAs significantly differing between spermatogenic and oogenic in FBF iCLIP. File S5 contains the dataset displayed in Figure 3A, namely FBF binding per gene for 2,111 FBF target RNAs. File S6 contains the blocks defined in Figure 3. Finally, File S7 contains FBF targets overlapping with the human PUF protein PUM2. Supplemental material available at Figshare: https://doi.org/10.25387/g3.7146899.

RESULTS AND DISCUSSION

Generation of FBF iCLIP datasets from spermatogenic and oogenic germlines
We generated FBF-1 and FBF-2 iCLIP datasets from animals with somatic tissues of the same gender but germline tissue of opposite gender (Figure 1, A and B). All animals were chromosomally XX and had hermaphroditic somatic tissues, including the somatic gonad; they also had comparable numbers of germline stem cells but those stem cells generated either only oocytes or only sperm, depending on the strain. For each FBF, we used an N-terminal 3XFLAG-tagged single copy transgene in a strain lacking the endogenous gene (e.g., FLAG::FBF-1 in an fbf-1 null mutant). As reported before (Prasad et al. 2016), fbf(0) FLAG::FBF XX animals are essentially wild-type. Moreover, each tagged FBF rescues fbf-1 fbf-2 double mutants from 100% sterility due to lack of GSCs to 100% fertility due to rescue of the GSC defect (Prasad et al. 2016). These tagged FBFs should therefore interact in an essentially normal fashion with their target RNAs.

XX adults with a spermatogenic germline were obtained using a temperature sensitive gain-of-function (gf) fem-3 mutant (Barton et al. 1987). We crossed transgenes encoding 3XFLAG-tagged FBF-1 or FBF-2 into the fem-3(gf) mutant strain, and again removed the corresponding endogenous fbf gene in each strain. As expected, the final strains, fbf-1(0) FLAG::FBF-1; fem-3 (gf) and fbf-2(0) FLAG::FBF-2; fem-3 (gf), were self-fertile at permissive temperature (15°), but fully spermatogenic at restrictive temperature (25°). Because the previously reported oogenic FBF iCLIP was done with animals raised at 20° (Prasad et al. 2016), we repeated it here with animals grown at 25°. Thus, we performed FBF iCLIP from adults that were either oogenic or spermatogenic, both raised at 25°. For each strain (each FBF, each germline gender), we processed three biological replicates. In parallel, we produced three negative control replicates for each germline gender by omitting the FLAG antibody from the beads during immunopurification.

Targets, networks and subnetworks: definitions
Throughout this work, we define the term “target RNAs” empirically as RNAs that interact with FBF after cross-linking in living animals, followed by immunoprecipitation from lysate and deep sequencing (CLIP). We define “network” to encompass all RNA targets observed by CLIP, and “sub-network” as a subset of that broader network. We refer to RNAs whose expression is regulated by FBF as “validated targets”. Such validation relies on genetic, biochemical and cellular analyses that have been done by ourselves and others in previous
studies. We note that virtually all validated FBF targets are among the targets identified in this work by FBF CLIP (see below).

**Peak calling and generation of quality datasets for comparison**

This work takes advantage of three sets of FBF iCLIP data (Figure 1B). To analyze these datasets, we modified our earlier peak calling pipeline (Prasad et al. 2016) to include a step that collapses duplicate reads while accounting for sequencing errors (Weyn-Vanhentenryck et al. 2014) (see Materials and Methods). This modified pipeline generated lists of FBF-1 and FBF-2 target RNAs in oogenic animals raised at 25° and spermatogenic animals raised at 25°, as well as revised lists of FBF-1 and FBF-2 targets in oogenic animals raised at 20°. File S1 lists iCLIP peaks obtained for each condition, and File S2 presents metrics of dataset size and quality.

The primary motivation for this work was comparison of FBF targets in spermatogenic and oogenic germlines, with the goal of identifying gamete-independent and gamete-specific targets that might inform about FBF subnetworks. Such comparisons are best done with datasets of comparable size. For iCLIP data of animals raised at 25°, we initially called peaks for FBF-1 and FBF-2 separately (File S1, Figure S1A-C), but one 25° FBF-1 replicate from oogenic germlines had a low number of unique and uniquely mapping reads (13,486, File S2). Because of the similarity of FBF-1 and FBF-2 binding (Prasad et al. 2016; this work) and the increased sensitivity of using larger datasets, we combined the FBF-1 and FBF-2 25° iCLIP datasets to generate “FBF” datasets for each gender (see Materials and Methods). Although the differences between FBF-1 and FBF-2 merit future investigation, combining datasets allowed us to more easily compare FBF binding at 25° between genders. These FBF target lists comprised 1,522 RNAs for oogenic animals, and 1,704 RNAs for spermatogenic animals (Figure 2A, File S1).

The quality of the datasets analyzed in this work was high by two key criteria. First, the majority of peaks in each dataset contained the canonical FBE (UGUNNNAU), a percentage that rose to roughly 90% for the top 500 peaks (Figure 1C). For each dataset, we scored all peaks (bars marked “total”) as well as the top 500 peaks (bars marked “top 500”). (D) For each dataset, the canonical FBE was the most significant motif in the top 500 FBF peaks, according to HOMER. (E) Number of distinct FBF target RNAs identified for indicated datasets. (F) Few RNAs are differentially bound by FBF between oogenic worms raised at 25° and 20°, as judged by DESeq2 analysis of reads-per-gene for 5,768 genes with an average of least 20 reads in oogenic FBF iCLIP and which are expressed in the germline (Noble et al. 2016). The x-axis denotes the fold change of FBF binding (reads-per-gene) in 25° worms over 20° worms, while the y-axis denotes the statistical significance of differential binding. The dashed line indicates a p-value of 0.01. Red dots are the 1% (54) of genes with >2 fold change and p-value < 0.01.
Target lists include all expected experimentally validated FBF targets. FBF targets in oogenic germlines included 13/15 validated FBF targets (fbf-1, fbf-2, fem-3, fog-1, gla-1, gla-3, him-3, htp-1, htp-2, syp-2, syp-3, lip-1, and mpk-1), but were missing the two not expected: fog-3 is sperm-specific and therefore not expressed in oogenic germlines (Chen and Ellis 2000), and egl-4 has only been established as an
FBF target in neurons (Kaye et al. 2009) and was not detected in previous genomic analyses of FBF targets (Kershner and Kimble 2010; Prasad et al. 2016). Similarly, FBF targets in spermatogenic germlines included 14/15 validated targets: all those in oogenic germlines plus 503. Finally, both size and complexity of the datasets (File S2) were similar to those for CLIP studies of other PUFs (Hafner et al. 2010; Freeberg et al. 2013; Porter et al. 2015; Wilinski et al. 2015) and consistent with our previous report on FBF targets in oogenic germlines (Prasad et al. 2016). Thus, all target lists include well over a thousand RNAs (Figure 1E).

Our modified peak calling method revises FBF-1 and FBF-2 target lists in oogenic germlines at 20°, but all major conclusions of our previous study (Prasad et al. 2016) were confirmed and revised lists were similar in content. An additional, spermatogenic germline-specific lincRNA linc-36 was identified for the first time in this analysis along with three previously reported lincRNAs (linc-7, linc-4, and linc-29). As in our initial report, the cell cycle is one of the most significantly enriched GO term associated with FBF targets in all of our datasets (File S3). The revised 20° lists contain, respectively, 69% and 84% of FBF-1 and FBF-2 targets reported previously, and the overlap between the FBF-1 and FBF-2 lists remained similar (68–83% of each paralog’s target list overlapped, File S2, Figure S1C). Peak heights for FBF-1 and FBF-2 were highly correlated (Pearson R 0.86), similar to that found previously (Pearson R 0.82) (Prasad et al. 2016), confirming the considerable molecular redundancy of these two nearly identical paralogs. Thus, FBF-1 and FBF-2 bind to largely the same target RNAs and largely to the same sites within those RNAs, as concluded previously.

As might be expected, temperature affected the FBF binding landscape but many metrics were comparable: (1) reads-per-gene counts correlated well between temperatures (Figure 1F, Figure S2, average Spearman rho 0.94 between 25° and 20° replicates), (2) a variety of additional metrics were similar (File S2), and (3) targets overlapped heavily (Figure S1D). Figure 1F shows the similarity of reads-per-gene counts for FBF binding at 25° vs. 20° by DESeq2 analysis: the 1% of RNAs that are significant at a p < 0.01 and fold change of ≥2 are indicated in red. Our peak caller detected peaks in more RNAs in the 20° datasets than in the 25° datasets (Figure 1E), because the 20° datasets have more reads (File S2) and our peak caller has greater sensitivity to detect peaks at higher read depths, despite the distribution of reads-per-gene being similar (Figure 1F).

Germline gender has a strong influence on the FBF binding landscape

We first compared target RNA identities between iCLIP of spermatogenic and oogenic animals, both grown at 25°. Over half of the FBF target RNAs were shared, but significant fractions were also found only in one germline gender or the other (Figure 2A). Differences due to germline gender were thus greater than differences due to temperature (Figure S2, and Figure 2D compared with Figure 1F). Among the 2114 total FBF targets, 2069 were mRNAs and 45 were non-coding RNAs. For mRNA targets, 1092 were common to both genders (53%), 582 were spermatogenic-specific (28%), and 395 were oogenic-specific (19%); for non-coding RNA targets, 20 were common (44%), 10 were spermatogenic-specific (22%), and 15 were oogenic-specific (33%).

We next gauged differences between spermatogenic and oogenic FBF RNA-binding profiles quantitatively. If each iCLIP sequencing read were derived from a single FBF-RNA interaction in vivo, then the number of iCLIP reads mapping to a given RNA, as a fraction of all reads, would serve as an estimate of the frequency of FBF-RNA binding at that RNA (Porter et al. 2015). Based on this reasoning, we assessed FBF-RNA binding frequency at each target as the number of FBF iCLIP reads (per million) at a given RNA. We then used Spearman’s rank-order correlation coefficients to compare FBF-RNA binding frequencies across all targets (Figure 2B, Figure S2). Comparisons of the 25° datasets revealed that FBF-RNA binding frequencies correlated well among spermatogenic replicates (Figure 2B, mean correlation 0.99) and among oogenic replicates (Figure 2B, mean 0.96), but more poorly between spermatogenic and oogenic replicates (Figure 2B, mean 0.89, two-tailed p-value 10−7 indicating significant difference in correlation between genders compared to within genders by t-test). We broadened this analysis to include FBF binding in oogenic germlines at 20° with similar results (Figure S2). We conclude that FBF binding frequencies correlate well for replicates of the same germline gender but are distinct in spermatogenic and oogenic germlines.

One possibility is that gender-specific differences in FBF binding were simply a reflection of underlying RNA abundances. To investigate this possibility, we assessed the correlations between spermatogenic/oogenic ratios in FBF binding and spermatogenic/oogenic ratios in RNA abundance (Figure 2C). RNA abundances were obtained using RNA-seq data from dissected oogenic and spermatogenic gonads (Ortiz et al. 2014). The dashed diagonal line in Figure 2C (slope = 1) represents the case in which a given fold-difference in a transcript’s abundance between germlines resulted in the same fold-difference in FBF binding frequency with that RNA. The RNA abundances were obtained using RNA-seq data from dissected oogenic and spermatogenic gonads (Ortiz et al. 2014). The dashed diagonal line in Figure 2C (slope = 0) represents the case in which FBF binding frequency had no dependence on germline gender or changes in transcript abundance. The data lie between these extremes. FBF binding frequencies changed between germline genders, and mostly in the same direction as RNA abundance changes. However, changes in FBF binding frequencies were not well correlated with changes in RNA abundance (Pearson R 0.64, Spearman 0.69). In other words, FBF-RNA binding frequencies differ markedly between genders, and do not simply reflect differences in RNA abundance. We conclude that this comparative analysis identifies gamete-specific and gender-neutral FBF targets that begin to outline potential subnetworks.

Gamete-specific FBF-RNA complex frequencies reflect gamete-specific programs

We asked how the potential FBF subnetworks relate to gamete RNA programs, defined by RNA-seq (Noble et al. 2016). Briefly, each program includes gamete-specific RNAs plus gamete-independent RNAs; for example, the full spermatogenic program includes RNAs expressed only in spermatogenic germlines plus those expressed in germlines making either gamete. We note the spermatogenic program was obtained from worms with a fem-3 gain-of-function allele (to produce adult spermatogenic animals), as in this work. We asked how FBF-RNA complex frequencies (reads-per-RNA) compare with these spermatogenic and oogenic RNA programs. Out of the total of 12,839 RNAs expressed in the germline (Noble et al. 2016), 6,873 possessed an average of at least 20 reads per RNA in FBF iCLIP, and 768 (12%) were bound differentially between the two genders by at least twofold (p < 0.01 by DESeq2 (Love et al. 2014); Figure 2D; File S4). Figure 2D depicts the agreement between differentially bound FBF-RNA complex frequencies and gamete programs: RNAs in the spermatogenic program (blue) separate from RNAs in the oogenic program (red) when plotted by the ratio of their differential FBF-RNA complex frequencies. Our results are therefore consistent with previous assignment of RNAs to gamete programs. Interestingly, 557 out of 768 differentially bound RNAs are enriched in spermatogenic germlines, indicating that FBF has...
a more complex interaction network in spermatogenic germlines than oogenic germlines, as it gains more new RNA targets.

Search for distinct biological roles associated with FBF subnetworks

We next asked if gamete-neutral, spermatogenic-specific and oogenic-specific targets were enriched for either distinct GO terms or germline phenotypes. No striking difference was found (File S3; Figure 2E). Regardless of germline gender, each group of FBF targets was enriched for genes with similar GO terms and phenotypes. Thus, these groups could not be linked to distinct biological roles.

Conservation of PUF targets

PUF proteins from diverse branches of Eukarya can perform similar biological functions, including stem cell maintenance (Wickens et al. 2002). Moreover, previous studies revealed that they share some of the same target mRNAs, including those regulating the cell cycle and programmed cell death (Kershen and Kimble 2010; Prasad et al. 2016; Lee et al. 2007). To complement those studies with the expanded and refined FBF target datasets reported in this work, we compared them to the PUM2 PAR-CLIP dataset, obtained from human embryonic kidney cells (HEK293) (Hafner et al. 2010) and the PUM1 and PUM2 iCLIP datasets, obtained from neonatal mouse brains (Zhang et al. 2017). We collapsed orthologous genes to ortholog groups, and discarded ortholog groups that did not exist in both humans and worms (see Methods). Comparison with the PUM2 dataset from HEK293 cells showed that 28% of all ortholog groups were shared with PUM2, while 33–35% of ortholog groups targeted by FBF were shared with PUM2 (Figure 2F, File S7). More striking, among the FBF targets in the top 500 peaks, 40–44% were shared (Figure 2F, File S7). FBF targets in spermatogenic and oogenic germlines had similar overlap. By contrast, comparison of FBF targets with PUM1 and PUM2 targets in mouse neonatal brain were less striking, with an overlap of 15–17% with FBF targets, vs. 14% of all ortholog groups (Figure S3, File S7). We also compared targets of the C. elegans RNA-binding protein GLD-1, which controls the differentiation of germline stem cells, with PUM targets (Jungkamp et al. 2011). The GLD-1 target dataset was smaller than the FBF target dataset, but had a similar overlap with human targets (Figure 2F), consistent with the overall number of shared targets reflecting similar molecular and biological functions. For both FBF and GLD-1, target RNAs are more abundant than the average RNA (Figure S4), and this likely also contributes to a higher target overlap with PUM2 than with randomly selected worm genes. This delineation of shared targets provides a resource for further studies.

Clustering FBF-RNA complex frequencies reveals cores of FBF subnetworks

A common method in systems biology is to cluster the expression of genes across conditions to reveal functionally related groups (Eisen et al. 1998). Using that logic, we hypothesized that clustering of the FBF-RNA complex frequencies might also reveal functionally related groups. We began with our list of 2,114 FBF target RNAs and clustered their FBF binding frequencies (Figure 3A; File S5) (see Methods). The actual number of RNAs in Figure 3A and File S5 is 2,111, because our peak caller assigns peaks to the ncRNA if a peak overlaps both mRNA and ncRNA, while such reads were discarded as ambiguous our peak caller assigns peaks to the ncRNA if a peak overlaps both mRNA and ncRNA, while such reads were discarded as ambiguous. Regardless of germline gender, each group of FBF targets was enriched for genes with similar GO terms and phenotypes. Thus, these groups could not be linked to distinct biological roles.

Figure 3D illustrates these clustered blocks of RNAs together with our earlier DESeq2 analysis of FBF-binding. As expected, Block I and Block IV RNAs were differentially bound in spermatogenic and oogenic animals, respectively (blue and pink dots, Figure 3B), while Block II (green dots, Figure 3D) and Block III (purple dots, Figure 3D) RNAs were either gamete-nonspecific or enriched in oogenic germlines. A major difference between Block II and Block III RNAs was number of reads mapping to the average RNA, which was much greater for Block III than for Block II (Figure 4A). We conclude that distinct groups of RNAs emerge by this clustering method. Below we examine each block in turn.

Block I RNAs (File S6)

Block I contains 75 RNAs that are enriched in FBF iCLIP from spermatogenic but not oogenic germlines (Figure 3A). Block I RNAs therefore likely belong to a spermatogenesis FBF subnetwork. Consistent with that idea, Block I RNAs include the key sperm fate regulator fog-3 (Ellis and Kimble 1995), and 70/75 Block I RNAs belong to the spermatogenic program identified by RNA-seq (Noble et al. 2016). However, most Block I RNAs encode proteins whose functions have not yet been characterized and no GO terms were enriched (p-value < 0.01). To pare down Block I RNAs, we applied two criteria: the highest peak is at least modestly high (25 reads/million) and contains an FBE. This allowed identification of 29 RNAs (Table 1) that encode a diverse array of proteins, some with conserved domains, including a phosphatase and five kinases. This is consistent with FBF serving as a “regulator of regulators” (Kershen and Kimble 2010). We note Block I also contains a novel IncRNA FBF target, line-36. We conclude that Block I RNAs belong to the FBF spermatogenesis subnetwork and that 29 RNAs within Block I are likely to be major FBF targets in that subnetwork.

Block II RNAs (File S6)

Block II contains 510 RNAs, most of which were found in FBF iCLIP RNAs common to spermatogenic and oogenic germlines (Figure 3A). Importantly, among target RNAs, Block II RNAs account for half of all FBF iCLIP reads and hence for half of all FBF interactions (Figure 4B). The Block II cluster includes 10 validated FBF target RNAs (fog-1, syp-2, fem-3, gld-3, htp-2, mpk-1, him-3, lpl-1, fbf-1, and fem-2). Stem cell maintenance is the major FBF function and this function is not gamete-specific (Crittenden et al. 2002). Consistent with the idea that Block II RNAs might be central to stem cell maintenance, they include key
self-renewal regulators \textit{fbf-1} and \textit{fbf-2} (Crittenden et al. 2002), and are most enriched for the biological process GO terms of cell cycle (\textit{p}-value $10^{-22}$), cell division ($10^{-18}$) and mitotic nuclear division ($10^{-17}$), embryo development ending in birth or egg hatching ($10^{-53}$) and reproduction ($10^{-27}$; all GO terms in File S3). Cell cycle regulation is central to stem cell maintenance (e.g., Orford and Scadden 2008).
and we suggest that Block II is enriched in RNAs belonging to the FBF subnetwork responsible for stem cell maintenance.

**Block III RNAs (Table 2, File S6)**

Block III contains 21 RNAs that are common to FBF iCLIP from spermatogenic and oogenic germlines, similar to Block II RNAs (Figure 3A). Block III RNAs stand out from Block II RNAs by their much higher frequency of FBF binding (Figure 4A). Because the frequency of RNA-protein complexes is a function of both affinity and abundance, we expected Block III RNAs to be abundant and to possess canonical FBF binding sites. Indeed, all Block III RNAs were abundant (Figure S4) and all had at least one canonical FBE under its highest peak: 15/21 had

![Figure 4](image)

**Figure 4 Block RNAs analyzed by germline gender.** (A) Percentage of reads mapping to block RNAs. Blue boxplots, reads in spermatogenic animals; pink boxplots, reads in oogenic animals. (B) Percentage of reads in target RNAs from iCLIP in either gender mapping to the indicated block. Thus, roughly 50% of reads in all target RNAs belong to Block II RNAs. The 21 Block III RNAs account for roughly 10% of FBF interactions with target RNAs. Blue bars indicate reads from FBF iCLIP in spermatogenic animals, and pink bars indicate reads from FBF iCLIP in oogenic animals.

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**Table 1 Major spermatogenesis-specific FBF targets, from Block I**

| RNA   | Biochemical function | Biological function | References                                      |
|-------|----------------------|---------------------|-------------------------------------------------|
| 1 fog-3 | Tob/BTG RNA regulator | Sperm fate specification | Ellis and Kimble (1995) |
| 2 F58F12.2 | Predicted transmembrane protein | Unknown | Krogh et al. (2001); Petersen et al. (2011) |
| 3 gsk-3 | MOK protein kinase | Development | Mulder et al. (2003) |
| 4 W02B12.12 | Testis-specific serine/threonine-protein kinase | Unknown | WormBase |
| 5 C56G2.5 | Kinase | Unknown | WormBase |
| 6 ZK822.1 | Ortholog of human FER tyrosine kinase | Unknown | WormBase |
| 7 C35E7.10 | FER tyrosine kinase (non-receptor) | Viability | WormBase |
| 8 moa-1 | Tyrosine phosphatase | Unknown | Ewald et al. (2012) |
| 9 osta-1 | Solute carrier family 51, alpha subunit | Cilia morphology | Olivier-Mason et al. (2013) |
| 10 glo-4 | Guanine nucleotide exchange factor | Viability | Hermann et al. (2005) |
| 11 YS7G11A.2 | Vitelline membrane outer layer protein | Unknown | WormBase |
| 12 snpc-1.3 | Ortholog of human SNAPC1 | Unknown | WormBase |
| 13 ZK973.8 | BTB/POZ domain protein | Neural development | WormBase |
| 14 F27C8.5 | BTB/POZ domain protein | Unknown | Mulder et al. (2003) |
| 15 linc-36 | lincRNA | Unknown | WormBase |
| 16 glct-6 | Glucuronyltransferase | Life span | Kim and Sun (2007) |
| 17 pit-5 | Phosphate transporter | Unknown | WormBase |
| 18 ZK686.5 | C2H2-like zinc finger | Unknown | WormBase |
| 19 C42C1.3 | Novel | Fertility | Sun et al. (2011) |
| 20 D1081.12 | Novel | Unknown | WormBase |
| 21 C06C3.10 | Novel | Unknown | WormBase |
| 22 ttr-9 | Transthyretin-like protein | Unknown | WormBase |
| 23 ZK637.12 | Novel | Unknown | WormBase |
| 24 C35A11.2 | Novel, with signal peptide | Unknown | WormBase |
| 25 C44B9.2 | Novel | Unknown | WormBase |
| 26 C09H10.9 | Novel | Unknown | WormBase |
| 27 F42G4.2 | Novel | Unknown | WormBase |
| 28 F52F12.5 | Novel | Unknown | WormBase |
| 29 T05F1.5 | Novel | Unknown | WormBase |

*Biochemical functions are predicted from protein domains or homology with characterized proteins. Biological functions are deduced from mutant phenotypes. Protein contains an N-terminal transmembrane helix.*
two or more canonical FBEs under that peak and 17/21 had an FBE with -1 or -2 C (known to enhance affinity) under that peak. Therefore, Block III RNAs emerge as exceptionally frequent FBF interactors due to both RNA abundance and high affinity FBF binding.

Block III RNAs also stand out by molecular function. Most striking is that 10/21 encode RNA regulatory proteins and 6/21 localize to P-granules (Table 2). GO terms (File S3) included P-granule (10/21) and negative regulation of translation (<0.01). Block III includes two previously validated targets, htp-1 and gld-1, the latter of which encodes a STAR RNA binding protein that localizes to P-granules, functions as a translational repressor and promotes differentiation (Francis et al. 1995; Jan et al. 1999). In addition, 3/21 were protein kinases (Table 2). The association of these molecular functions with Block III RNAs, and hence with exceptionally frequent FBF targets, emphasizes the role of FBF as a regulator of other regulators and in particular, a high-level regulator of other post-transcriptional regulators.

Based on various functional studies, 16/21 Block III RNAs are required for gametogenesis or embryogenesis (Table 2). Roles in oogenesis and embryogenesis are best documented, perhaps because they have been analyzed more intensively than spermatogenesis. GO terms for oogenesis and embryo development ending in birth or egg hatching were both significant (p-values 10^-6 and <0.01, respectively) Remarkably, 6/21 Block III mRNAs affect germ cell apoptosis (Table 2), a homeostatic mechanism common to worms and mammals, and the GO term apoptotic process was enriched (p-value <0.01). This finding underscores an earlier finding that FBF regulates MAPK-driven apoptosis in the germ line (Lee et al. 2007), a function conserved with murine PUM1 (Chen et al. 2012). Thus, many Block III RNAs are key regulators of gametogenesis, strengthening the notion that FBF maintains stem cells by repressing differentiation-promoting mRNAs. In addition, three Block III mRNAs are likely to regulate niche signaling in addition to promoting differentiation. The stem cell niche in this system relies on Notch signaling to maintain stem cells (Kubagawa et al. 2005). An attractive idea is that FBF represses expression of CAR-1 and CGH-1 in germline stem cells to enhance niche signaling.

We suggest that Block III RNAs also belong to the FBF subnetwork responsible for stem cell maintenance. Among these mRNAs, FBF appears to promote stem cell self-renewal in part by enhancing niche signaling and in part by repressing differentiation.
Block IV RNAs (File S6)
Block IV contains 24 RNAs and represents RNAs enriched in oogenic germlines over spermatogenic germlines (Figure 2A). Of the 20/24 Block IV RNAs categorized by Noble et al. (2016), all were in the oogenic program and 18/20 were only in the oogenic program. Interestingly, Block IV includes the snoRNA ZK858.10, which, being a snorNA, is not found in Noble et al. (2016), but which might still be an authentic part of the oogenic program. Consistent with oogenesis-related functions, Block IV also includes RNAs for the yolk receptor RME-2 and the ABC transporter MRP-4, the latter of which is expressed in oocytes to attract sperm (Kubagawa et al. 2006). However, Block IV, like Block I, includes many uncharacterized genes and no GO terms were enriched. Block IV likely represents an oogenesis-specific subnetwork.

Conservation of Block I-IV PUF targets
We compared the RNAs in Blocks I-IV with the PUM2 iCLIP dataset from human embryonic kidney cells, as done for oogenic and spermatogenic FBF datasets described above (Figure 2F). Most striking was the 60% overlap of Block III RNA ortholog groups with PUM2 targets. Block I had the lowest overlap among the three blocks with only ~10%. Of the 21 high-frequency gender neutral Block III targets, 15/21 had human orthologs and 9 were also targets of human PUM2: cgh-1, ncl-1, car-1, ima-3, ncl-1, and cgh-1, ima-3, ifet-1, respectively. Our analyses delineate clusters of FBF-bound RNAs that likely represent regulatory RNAs. In support of this, ~24% of the FBF-bound RNAs are also targets of human PUM2: cgh-1, ncl-1, and cgh-1, ima-3, ifet-1, respectively. These results together with the conservation of these targets provide additional evidence for a conserved role of PUF proteins in regulating other RNA targets.

Conclusions
Our analyses delineate clusters of FBF-bound RNAs that likely represent FBF subnetworks for spermatogenic (Block I), oogenic (Block IV), and stem cell (Blocks II and III) functions. Clearly this is only a first step in understanding the diverse roles of FBF regulation. Because stem cell regulation is a conserved function of metazoan PUFs and many RNAs in the FBF stem cell subnetwork are also targets of human PUM2, a next focus should be to learn whether phylogenetically conserved targets are subject to PUF regulation across phyla, and if so, how and where they are regulated.

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Volume 9 January 2019 | FBF Subnetworks | 163
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Volume 9 January 2019 | FBF Subnetworks | 165

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