Context-dependent function of a conserved translational regulatory module

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SUMMARY

The modification of transcriptional regulation is a well-documented evolutionary mechanism in both plants and animals, but post-transcriptional controls have received less attention. The derived hermaphrodite of *C. elegans* has regulated spermatogenesis in an otherwise female body. The PUF family RNA-binding proteins FBF-1 and FBF-2 limit XX spermatogenesis by repressing the male-promoting proteins FEM-3 and GLD-1. Here, we examine the function of PUF homologs from other Caenorhabditis species, with emphasis on *C. briggsae*, which evolved selfing convergently. *C. briggsae* lacks a bona fide fbf-1/2 ortholog, but two members of the related PUF-2 subfamily, Cbr-puf-2 and Cbr-puf-1.2, do have a redundant germline sex determination role. Surprisingly, this is to promote, rather than limit, hermaphrodite spermatogenesis. We provide genetic, molecular and biochemical evidence that Cbr-puf-2 and Cbr-puf-1.2 repress Cbr-gld-1 by a conserved mechanism. However, Cbr-gld-1 acts to limit, rather than promote, XX spermatogenesis. As with gld-1, no sex determination function for fbf or puf-2 orthologs is observed in gonochoristic *Caenorhabditis*. These results indicate that PUF family genes were co-opted for sex determination in each hermaphrodite via their long-standing association with gld-1, and that their precise sex-determining roles depend on the species-specific context in which they act. Finally, we document non-redundant roles for *Cbr-puf-2* in embryonic and early larval development, the latter role being essential. Thus, recently duplicated PUF paralogs have already acquired distinct functions.

KEY WORDS: PUF proteins, *Caenorhabditis*, Evolution, Translation, Germ cells, Hermaphroditism

INTRODUCTION

In convergent evolution, different lineages acquire similar phenotypes independently. However, the extent to which key modifications to development and physiology are reproduced in convergent lineages is only beginning to be addressed. In the nematode family Rhabditidae, self-fertile hermaphrodites have evolved from female ancestors at least ten times (Kiontke and Fitch, 2005). Even for the closely related *Caenorhabditis elegans* and *Caenorhabditis briggsae*, for which XX spermatogenesis is similar in extent and timing, self-fertility has evolved convergently (Cho et al., 2004; Kiontke et al., 2004). Because germline sex determination is well studied in *C. elegans*, comparisons between *C. elegans* and *C. briggsae* offer an experimentally tractable way to explore the molecular and genetic details of convergent evolution.

Genetic comparisons between *C. elegans* and *C. briggsae* reveal the conservation of the global sex determination pathway (Hill et al., 2006; Kelleher et al., 2008). The sex determination cascade is initiated in the early embryo by the ratio between the number of X chromosomes and sets of autosomes (X:A ratio) (Nigon, 1951), with a high ratio (2X:2A) in the hermaphrodite repressing *her-1* transcription and a low ratio (1X:2A) in the male activating *her-1* transcription (Dawes et al., 1999; Trent et al., 1991). In hermaphrodites, low *her-1* expression permits activity of the membrane protein TRA-2, which represses the male-promoting FEM proteins (Chin-Sang and Spence, 1996; Mehra et al., 1999). The resulting lower *fem* activity allows accumulation of the transcription factor TRA-1, which represses genes required for male development (Chen and Ellis, 2000; Conradt and Horvitz, 1999; Mason et al., 2008; Yi et al., 2000). This global sex determination pathway is modified at the post-transcriptional level in the *C. elegans* XX hermaphrodite germ line to allow transient spermatogenesis. Translational repression of *tra-2* by the STAR family RNA-binding protein (RBP) GLD-1 and its co-factor, the F-box protein FOG-2, is required to initiate hermaphrodite spermatogenesis (Clifford et al., 2000; Goodwin et al., 1993; Jan et al., 1999; Schedl and Kimble, 1988), and the translational repression of *fem-3* by the PUF (Pumilio and FBF) (Wickens et al., 2002) family RBPs FBF-1/2 is required for the transition from spermatogenesis to oogenesis (Ahhringer and Kimble, 1991; Zhang et al., 1997).

Despite overall conservation of the global pathway, species-specific germline sex determination genes and gene regulation have been described in convergent hermaphrodites. Both *C. elegans* and *C. briggsae* utilize F-box genes (fog-2 and she-1, respectively) to promote spermatogenesis, but they are both species-specific gene duplicates (Guo et al., 2009b; Nayak et al., 2005). Another example is the role of the FEM genes. Although they promote male somatic fate in both species, their germline sex determination function differs. In *C. elegans*, *fem* mutations transform spermatocytes into oocytes in both males and hermaphrodites (Hodgkin, 1986), whereas XX *C. briggsae* Cbr-­fem-2 and Cbr-fem-3 mutants are normal hermaphrodites and XO counterparts are transformed to hermaphrodites, not to females as in *C. elegans* (Hill et al., 2006). The different genetic architecture at the level of *fem-3* regulation suggests that the *C. briggsae* homologs of *fbf-1/2* might have different roles in germline sex determination, or none at all (Haag, 2009b).
In *C. elegans*, the PUF genes *fbf-1*, *fbf-2* and *puf-8* are key regulators of the sperm/oocyte switch. The nearly identical FBF paralogs bind specifically to the conserved FBF binding elements (FBEs) at the 3’ untranslated region (UTR) of *fem-3*, and enable the sperm/oocyte switch by repressing *fem-3* translation (Zhang et al., 1997). FBF interaction with the nanos homolog NOS-3 and the *bicucullin C* homolog GLD-3 is also required for the sperm/oocyte switch. NOS-3 acts like an FBF activator, and together they repress *fem-3* expression to promote oocyte fate (Kraemer et al., 1999). By contrast, GLD-3 antagonizes FBF function and this interaction derepresses *fem-3* expression to promote sperm fate (Eckmann et al., 2002). The sex determination function of *puf-8* is less clear, but it acts redundantly with *fbf-1* to allow oogenesis (Bachorik and Kimble, 2005). Aside from their roles in germline sex determination, *fbf-1*, *fbf-2* and *puf-8* are also important regulators of the transition from mitosis to meiosis. FBF-1 and FBF-2 act together to promote germ line cell proliferation by directly repressing translation of *gld-1* mRNA (Crittenden et al., 2002). *Puf-8* also promotes faithful meiotic entry in spermatocytes at elevated temperatures (Subramanian and Seydoux, 2003).

The above observations indicate that PUF proteins pattern germline development by working with a limited set of other RBPs to form a combinatorial network of translational controls. This important role for transition is consistent with its general prominence in regulating gene expression in the *C. elegans* germ line (Merritt et al., 2008). The comparison of PUF functions in different species thus provides an opportunity to study regulatory evolution at the translational level. Here we present genetic and molecular analyses of PUF family genes in *C. briggsae* and other, gonochoristic *Caenorhabditis* species, focusing on their roles in germline sex determination. We find that two homologs of *fbf*, *Cbr-puf-2* and *Cbr-puf-1.2*, act redundantly to promote hermaphroditic spermatogenesis, much as *fbf-1/2* act to promote oogenesis in *C. elegans*. Cbr-PUF-2/1.2 directly repress the expression of *GLD-1*, which itself has opposite roles in germline sex determination in *C. elegans* and *C. briggsae* (Beadell et al., 2011). Similar to *gld-1* (Beadell et al., 2011), PUF protein involvement in germline sex determination coincides phylogenetically with the origin of hermaphroditic development. Thus, *C. briggsae* and *C. elegans* PUF genes have opposite effects on germline sex determination because the role of a conserved target mRNA has diverged. Finally, we show that a *C. briggsae*-specific PUF paralog has already released (e.g. WS190 and many prior releases) had the correct prediction, and Cja-*fbf-1* was corrected in WormBase release WS227. The corrected coding sequence for *Cbr-puf-1.2*, however, has not been reported elsewhere, and has been submitted to GenBank as accession JQ655294.

Fifty-four *Caenorhabditis* PUF proteins were aligned with PUMILIO, the unique PUF protein in *Drosophila melanogaster*. Multiple sequence alignment quality was improved by first aligning sequences in three separate subgroups using MUSCLE v3.6 (Edgar, 2004) with default settings, after which the three alignments were combined using the Profile-profile alignment in MUSCLE v3.6. The combined alignment was manually curated using Se-Al v2.0 (http://tree.bio.ed.ac.uk/software/seal/), and the PUF domain with its flanking regions (335 characters) was extracted according to known PUF protein sequence features (Wickens et al., 2002). Maximum likelihood tree search was performed five times independently using GARLI 2.0 (D. J. Zwickl, PhD thesis, The University of Texas at Austin, 2006), and the tree with the best likelihood score was picked. One hundred non-parametric bootstrap runs were generated using GARLI 2.0. Trees were read in PAUP* (Swofford, 2002) for majority-rule consensus branch values, which were manually mapped onto the best tree and visualized in Dendroscope v2.6.1 (Huson et al., 2007).

### Nematode culture and genetics

All nematode species were cultured using standard *C. elegans* conditions (Wood, 1988), with the use of 2.2% agar plates to discourage burrowing. All *C. briggsae* mutants were derived from the wild isolate AF16, and included: LGII: *Cbr-puf-2*(nn66), *Cbr-dpy*(nn4), *Cbr-tra-2*(nn1) and *Cbr-tra-2*(nn9); LGIII: *Cbr-tra-1*(nn2), *Cbr-let*(nn28); LGIV: *Cbr-fem-3*(nn63). *Cbr-tra-2*(nn1)/+, *Cbr-fem-3*(nn63) animals were the progeny of *Cbr-tra-2*(nn1)/+, *Cbr-fem-3*(nn63)/+ mothers, which came from a cross between *Cbr-tra-2*(nn1)/+, *Cbr-dpy*(nn4)/+, and *Cbr-fem-3*(nn63)/+ males. The final genotype was confirmed by sequencing of diagnostic PCR amplicons.

### RNA interference

Gene-specific templates for in vitro transcription were PCR amplified from genomic DNA (*Briggsae* or *C. remanei*) and *C. brenneri* and *C. japonica*) with primers flanked by the T7 promoter and sequenced to verify identity. For *C. sp.*, primers designed according to *Briggsae* sequences were used. Plasmid pCR50 (gift from C. Richie, National Institutes of Health, Bethesda, MD, USA) was used to amplify green fluorescent protein (GFP) coding sequence, and pharyngeal GFP strain CP105 was used for the triple RNA interference (RNAi) efficacy test. For all experiments, double-stranded (ds) RNA was introduced by maternal microinjection (Haag et al., 2002).

### Microscopy

Worms were mounted for differential interference contrast (DIC) microscopy by standard methods (Wood, 1988). For nuclear staining, worms were fixed in cold methanol, washed with M9, stained with 7.5 μM Hoechst 33258 in M9, rinsed with several changes of M9, and mounted in Vectashield (Vector Laboratories) for fluorescence microscopy. Images were captured with a Zeiss Axioscam digital camera and Open Lab software (Improvement) or an SP5 X confocal microscope (Leica). In the latter, z-stacks were collapsed for presentation.

### Quantitative RT-PCR

Total RNA from stage worms was extracted in Trizol Ambion and purified according to the manufacturer’s instructions. For *Cbr-gld-1* expression, RNA from 50 L4 *Cbr-puf-2/1.2 (RNAi)* worms was extracted. cDNA was reverse transcribed from total mRNA using Superscript III (Invitrogen), and 2 μl was used as template for quantitative PCR using a LightCycler 480 and SYBR Green 1 Master (Roche) as described (Hill and Haag, 2009). Exon-exon junction primers were used for *Cbr-gld-1*, *Cbr-puf-1.2* and *Cbr-puf-2*, and pan-actin was used as an internal standard. Raw data were analyzed using LinRegPCR (11.0) (Ruijter et al., 2009), which calculates the starting concentration of the sample from the mean PCR efficiency per amplicon and the Ct value per sample (Ramakers et al., 2003). For each sample, expression was normalized to actin expression.

### MATERIALS AND METHODS

#### Phylogenetic analysis

Protein datasets for *C. elegans*, *Briggsae*, *C. remanei*, *C. brenneri* and *C. japonica* were retrieved from the Nematode Genome Annotation Project (nGASP, http://www.sanger.ac.uk/projects/C_elegans/Wormbase/current/wormpep_download.shtml). A PUF domain hidden Markov model (HHMM; PUF ls.hmm) from P fam (Sonnhammer et al., 1998) was used to search for PUF domain proteins using HMMER v2.3.2 (Eddy, 1998). Based on test searches for known *C. elegans* PUF homologs, an E-value of 1.0 was used as the cut-off threshold. Removal of likely alternative alleles in the *C. remanei* and *C. brenneri* predictions (Barriere et al., 2009) reduced family sizes to ten and nine sequences, respectively. To validate predictions with unexpected features, some sequences were reverse transcribed using FirstChoice RLM-RACE kit (Ambion) from total RNA, PCR amplified and sequenced. This revealed errors in the WS213 splicing predictions for *Cbr-puf-2*, *Cbr-puf-1.2* and Cja-*fbf-1* and confirmed the structure for *Cre-puf-1.2*. For *Cbr-puf-2*, earlier WormBase
Deletion mutant screen and transgenic rescue

A *C. briggsae* AF16 deletion library was produced and screened following standard *C. elegans* methods (Edgley et al., 2002) without the ‘poison primer’ modification. From 10^6 haploid genomes screened, *Cbr-puf-2* deletion *nm66* and *Cbr-unc-119* deletion *nm67* were isolated. Both alleles were outcrossed six times with the unmutagenized AF16 strain.

Production of *Cbr-puf-2* transgene

Regulatory (5' bound), coding, and 3' flanking sequences of *Cbr-puf-2* were engineered via Gateway cloning technology (Invitrogen) into destination vector using *I* for hybrid RNA expression in yeast.

Immunoblots

Triple samples for quantitative Cbr-GLD-1 immunoblots comprised 50 L4 worms of *Cbr-puf-2/1.2 (RNAi)* or AF16 controls in SDS sample buffer (Sambrook and Russell, 2001). Primary antibodies were rabbit anti-GLD-1 polyclonal (gift from T. Schedl, Washington University, St Louis, MO, USA) at 1:2000 and mouse anti-tubulin monoclonal (DM1A, Sigma) at 1:1000. Secondary antibodies were HRP-linked donkey anti-rabbit IgG (Jackson ImmunoResearch) at 1:1000 and HRP-linked sheep anti-mouse IgG (GE Healthcare) at 1:1600. ECL signal intensity was quantified using ImageJ (Abramoff et al., 2004). Cbr-GLD-1 protein expression was normalized to tubulin.

Immunohistochemistry

The immunohistochemistry protocol was slightly modified from that of T. Schedl, using a methanol/formaldehyde fix for 10 minutes at room temperature. For PH3 staining, rabbit anti-PH3 (Upstate) was used at 1:200. Fluorescently conjugated secondary antibody (goat anti-rabbit IgG, Alexa 488, Invitrogen) was used at 1:2000. All gonads were dissected and stained simultaneously and under the same conditions.

Yeast reporter constructs

DNA encoding the PUF domain and flanking regions of *Cbr-puf-2* (amino acids 92-568) or *Cbr-puf-1.2* at various concentrations as described (Bernstein et al., 2005). One or GST-Cbr-PUF-1.2 at various concentrations as described (Bernstein et al., 2005).

**RESULTS**

*Caenorhabditis* PUF family phylogeny reveals an ancient subfamily structure

Preliminary experiments with *fbf*-related *C. briggsae* PUF homologs defined by Lamont et al. (Lamont et al., 2004) suggested the were required for XX sperm production (S. Feng, Q.L. and E.S.H., unpublished), the opposite role of *C. elegans* *fbf-1*, *fbf-2* and *pfu-8* (Bachorik and Kimble, 2005; Zhang et al., 1997). To guide more precise experiments, we produced an expanded PUF phylogeny using all homologs from five sequenced *Caenorhabditis*. The most likely tree (Fig. 1) divides the PUF family into nine monophyletic subfamilies, two of which, PUF-12 and PUF-13, are newly defined here. The previously described *C. elegans* *pfu-10* is a pseudogene with stop codons throughout its coding region and highly divergent sequence, and thus does not appear in Fig. 1. Relative to the two-species analysis of Lamont et al. (Lamont et al., 2004), one *C. elegans* gene and three *C. briggsae* genes are added. The PUF-9 subfamily is basal, with highly conserved orthologs in all sequenced species. The remaining eight subfamilies represent a more recent radiation, yet all but one has an ortholog in *C. japonica*, the outgroup to the other species (Cho et al., 2004; Kiontke et al., 2004). At least eight subfamilies were therefore present in the *Caenorhabditis* ancestor, and a more complete genome assembly for *C. japonica* might reveal additional PUF family genes.

Opposite functions of PUF homologs in convergent hermaphrodites

Because PUF-2 orthologs are absent from *C. elegans* their specific functions in *C. briggsae* are not readily predicted. Therefore, genespecific knockdown of *Cbr-puf-1.1*, *Cbr-puf-1.2* and *Cbr-puf-2* was performed separately and in various combinations (Table 1). *Cbr-puf-2 (RNAi)* alone had little effect, but simultaneous knockdown of *Cbr-puf-2* and *Cbr-puf-1.2* (but not other combinations) led to a strongly feminized germline (Fig. 2B). *Cbr-puf-2/1.2 (RNAi)* females had normal size germ lines and could mate and produce viable progeny. *Cbr-puf-2/1.2 (RNAi)* males were overtly normal and could sire viable progeny (not shown). Thus, *Cbr-puf-2* and *Cbr-puf-1.2* act synthetically and specifically to promote spermatogenesis in *C. briggsae* hermaphrodites, but not in males. This contrasts with the role of FBF genes and *pfu-8* in *C. elegans* hermaphrodites, where they promote oogenesis (Bachorik and Kimble, 2005; Zhang et al., 1997).
concentration of dsRNA was increased to 3.0 μg/μl, the percentage of Fog (feminization of germ line) animals decreased and more proximal tumors were observed (Table 1). In tumorous gonads, proximal overproliferated cells were followed distally by oogenic cells at various meiotic stages or abnormal pachytene cells. This oogenic region is often small and located at the bend of the gonad arm, which can be easily missed in whole-mounts. This tumor phenotype indicates that \textit{Cbr-puf-2} and \textit{Cbr-puf-1.2} are involved in the control of meiotic progression and/or the prevention of the return to mitosis. In addition, \textit{Cbr-puf-1.2} (RNAi) worms produced fewer and atypically small oocytes, which indicates that \textit{Cbr-puf-1.2} is involved non-redundantly in oocyte development.

The developmental profiles of \textit{Cbr-puf-2} and \textit{Cbr-puf-1.2} mRNA levels (Fig. 2E) are qualitatively similar and are typical of germ line-expressed genes: low expression from embryo to L2 stages, slightly increasing expression at L3 and L4, and peak levels in adults. However, \textit{Cbr-puf-2} is over 100-fold more abundant than \textit{Cbr-puf-1.2}, the transcripts of which are in the order of $10^{-5}$ times less abundant (body-wide) than those of total actins.

\textbf{\textit{Cbr-puf-2} mutant reveals pleiotropic roles in embryogenesis and larval somatic development}

To study the function of \textit{Cbr-puf-2} further, two deletion alleles were isolated. For one, a 1.9 kb genomic deletion, we failed to obtain homozygous adults and it was eventually lost. The second allele, \textit{nm66}, carries a 1.7 kb genomic deletion that removes three-quarters of the coding sequence, including the entire PUF domain (Fig. 3A), and is thus a likely null allele. Again, homozygous adults could not be identified, and close inspection revealed that one-quarter of progeny from \textit{Cbr-puf-2(nm66)/+} mothers were arrested.
at an early larval stage (Fig. 3B), 5 days after hatching at 20°C. Genotyping of arrested larvae confirmed that they were nm66 homozygotes.

To confirm that loss of Cbr-puf-2 function causes the larval arrest phenotype in nm66, we introduced a wild-type Cbr-puf-2 transgene into homozygotes to develop into fertile adults (Fig. 3D). The rescued CP113 strain, nevertheless had undetectably low Cbr-puf-2 mRNA levels as measured by RT-PCR. Since germline transgene silencing is a known phenomenon in Caenorhabditis (Seydoux and Schedl, 2001), we hypothesized that CP113 was a somatic-rescued but germline-null Cbr-puf-2 mutant.

In a wild-type genetic background, both Cbr-puf-2 and Cbr-puf-1.2 must be knocked down to feminize the germ line and produce tumors (Fig. 2B). In CP113, however, Cbr-puf-1.2(RNAi) alone produced the Fog phenotype (Fig. 3E), and at high doses of Cbr-puf-1.2 dsRNA germline tumors became common (Fig. 3F,G). These results are consistent with germline silencing of the Cbr-puf-2 transgene in the CP113 strain. This also suggests that very low levels of Cbr-puf-2 expression are sufficient in somatic tissues to allow progression from larval stages to adulthood. This could also explain the observation that Cbr-puf-2(RNAi) animals did not undergo larval arrest.

XX CP113 animals also had subtle germline defects. Although they were overtly normal and fertile, they had delayed gamete maturation. Newly molted adult CP113 animals had very few yolky oocytes, with spermatocytes just beginning to differentiate. AF16 animals at this stage generally have fully differentiated sperm, and oocytes fill the proximal gonad arms. Also, ~70% of CP113 eggs died at various embryonic stages (Fig. 3G), and this embryonic lethal phenotype could not be rescued by a paternal copy of Cbr-puf-2(+) . We interpret this to be a maternal effect of nm66 caused by lack of Cbr-puf-2 activity in the maternal germ line.

### Table 1. RNAi phenotypes of C. briggsae PUF-2 subfamily paralogs

<table>
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<th>Target gene(s)</th>
<th>dsRNA (μg/μl)</th>
<th>Phenotype percentage (n&gt;200/treatment)</th>
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<tr>
<td>Cbr-puf-1.1</td>
<td>~3.0</td>
<td>Fog* 100 Other sterile‡ Self-fertile</td>
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<tr>
<td>Cbr-puf-2</td>
<td>~3.0</td>
<td>– 100</td>
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<tr>
<td>Cbr-puf-1.2</td>
<td>~3.0</td>
<td>– 100</td>
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<tr>
<td>Cbr-puf-1.1 + Cbr-puf-2</td>
<td>~4.0/3.0</td>
<td>– 100 (oocyte defect)</td>
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<tr>
<td>Cbr-puf-1.1 + Cbr-puf-1.2</td>
<td>~4.0/3.0</td>
<td>– 100 (oocyte defect)</td>
</tr>
<tr>
<td>Cbr-puf-2 + Cbr-puf-1.2</td>
<td>0.5/0.5</td>
<td>– 100</td>
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<td>– 100</td>
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<tr>
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<td>~4.0/3.0/3.0</td>
<td>25 73 2</td>
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*Fog animals can produce viable progeny when mated with males.
‡Proximal or whole-gonadal tumor, malformed germ line and oocytes (all lack sperm).

Figure 2. Expression and germline phenotypes of Cbr-puf-2/1.2(RNAi). (A) Wild-type C. briggsae adult hermaphrodite, stained with Hoechst 33258 to visualize DNA. Asterisk marks the distal tip of the gonad. (B) XX Cbr-puf-2/1.2(RNAi) Fog phenotype, commonly seen in low-dose RNAi, revealed by Hoechst staining. (C-D) Proximal proliferation of germline (Pro) phenotype in XX Cbr-puf-2/1.2(RNAi) animals. Tumors were observed proximal to either small populations of well-differentiated diakinesis oocytes (C) or undifferentiated germ cells (D). (E) Developmental profile of Cbr-puf-1.2 and Cbr-puf-2 mRNA levels assessed by quantitative RT-PCR. Expression levels were normalized to total actin expression and scaled (unit for Cbr-puf-2, 10^-3; for Cbr-puf-1.2, 10^-3). Error bars indicate s.e.m. for three biological replicates. sp, sperm, do, diakinesis oocytes; pp, proximal proliferation. Scale bars: 30 μm.
germ line (Beadell et al., 2011; Nayak et al., 2005), suggesting that its normal function is to promote oogenesis. We hypothesized that Cbr-gld-1 might be hyperactive in Cbr-puf-2/1.2(RNAi) animals and would thus completely repress hermaphrodite spermatogenesis. We investigated the epistatic relationship of Cbr-gld-1 and Cbr-puf-2/1.2 through triple RNAi knockdown. A preliminary experiment was conducted to demonstrate the efficacy of Cbr-puf-2/1.2(RNAi) in a triple knockdown. A myo-2::gfp transgenic strain injected with a mixture of Cbr-puf-2/1.2 and gfp dsRNA had a feminized germ line with compromised pharyngeal GFP expression (data not shown). XX Cbr-gld-1(RNAi);Cbr-puf-2/1.2(RNAi) adults had masculinized germ lines (Fig. 4B), indicating that sperm production (to excess) in Cbr-puf-2/1.2 is restored when Cbr-gld-1 function is reduced. Also consistent with repression of Cbr-gld-1 by Cbr-PUF-2/1.2, Cbr-GLD-1 protein levels at the late L4 stage (when wild-type worms are at their peak of sperm production) in Cbr-puf-2/1.2(RNAi) worms were approximately double those in wild type (Fig. 4C), a statistically significant result (P=0.006, unpaired Student’s t-test). By contrast, there was no significant difference in Cbr-gld-1 transcript levels in the two treatments (Fig. 4C; P=0.168) at this stage. These results are consistent with Cbr-PUF-2/1.2 acting at the level of translation to promote spermatogenesis via direct repression of Cbr-GLD-1 expression.

Binding of Cbr-PUF-2/1.2 to the candidate FBE in the Cbr-gld-1 3’UTR was first measured using the yeast three-hybrid assay, in which interaction of an RBP activation domain fusion protein with a ‘bait’ RNA leads to activation of a reporter (Bernstein et al., 2002). Reporter activity was much higher with wild-type than with mutated versions of Cbr-gld-1 FBE bait RNA (Fig. 4D). C. elegans FBF-2 also interacted strongly and in an FBE-dependent manner with the Cbr-gld-1 bait RNA. To verify that the interactions between the Cbr-gld-1 FBE and the Cbr-PUF-2 and Cbr-PUF-1.2 proteins were direct, we used synthetic oligoribonucleotides encoding the candidate FBE and purified proteins in gel mobility
shift assays. Both Cbr-PUF-2 and Cbr-PUF-1.2 bound with high affinity to the Cbr-gld-1 FBE (Fig. 4E), and this interaction required the UGU motif that is essential for FBE binding by FBF in C. elegans (Bernstein et al., 2005).

The above assays indicate that Cbr-PUF-2 and Cbr-PUF-1.2 interact with the Cbr-gld-1 FBE directly and with properties similar to those of the FBF subfamily. The C. briggsae fem-3/3’UTR also possesses a well-conserved FBF-like binding site termed the point mutation element (PME) (Haag et al., 2002). In yeast three-hybrid assays, Cbr-PUF-1.2 and Cbr-PUF-2 interacted specifically with PME-containing fragments from C. elegans and C. briggsae FBF interacted with C. briggsae and C. elegans fem-3 PME fragments to a similar extent (supplementary material Fig. S1). This suggests that both PUF-2 and FBF PUF subfamilies can recognize a similar RNA motif conserved in fem-3 orthologs, but the biological significance of a Cbr-PUF–Cbr-fem-3 mRNA interaction was initially unclear (see below).

Nonlinear interactions between Cbr-PUF-2/1.2 and the core sex determination pathway

In an effort to place Cbr-PUF-2/1.2 activity in the sex determination pathway, we performed approximations of epistasis tests by combining Cbr-PUF-2/1.2(RNAi) with tra (masculinizing) mutants (Table 2). XX Cbr-tra-2(nm1) homozygotes develop imperfect male bodies and produce only sperm (Kelleher et al., 2008), whereas heterozygotes are normal hermaphrodites. All XX Cbr-tra-2(nm1);Cbr-PUF-2/1.2(RNAi) animals developed male somas, but roughly half of these had tumorous germ lines lacking differentiated gametes and half produced sperm proximal to a tumor (Fig. 5C). None had obvious oocytes. Using the Cbr-dpy(nm4) marker closely linked to Cbr-tra-2 in trans, Cbr-tra-2(nm1)/+;Cbr-PUF-2/1.2(RNAi) animals (Table 3) had two gonads full of sperm with no sign of oogenesis (Fig. 5D). Genotyping
confirmed that these female soma/Mog (masculinization of germ line) animals were indeed Cbr-tra-2(nm1)/+. Since Cbr-tra-2 germ line masculinization is normally recessive and Cbr-puf-2/1.2(RNAi) has a feminizing effect, the masculinization of this combination is unexpected.

Also unexpected was the lack of differentiated gametes seen in the Dpy progeny with two wild-type zygotic copies of Cbr-tra-2. To control for possible effects of the Cbr-dpy(nm4) marker, Cbr-dpy(nm4)+/+ mothers lacking any Cbr-tra-2 mutation were injected with Cbr-puf-2/1.2 dsRNA. Here, all selfed progeny, including Dpy homozygotes, were Fog. Therefore, the Fog phenotype of Cbr-tra-2(nm1)/+; Cbr-puf-2/1.2(RNAi) animals requires a maternal nm1 allele, and the poorly differentiated germ line of their Cbr-dpy(nm4); Cbr-puf-2/1.2(RNAi) siblings is a dominant maternal effect of the Cbr-tra-2(nm1) mutation. Another Cbr-tra-2 allele, nm9ts (Kelleher et al., 2008), produced the same result, suggesting that the interaction between Cbr-tra-2 and Cbr-puf-2/1.2 is general.

The strong loss-of-function mutation Cbr-tra-1(nm2) causes XX animals to develop a male body and a mixture of sperm and endodermic oocytes (Hill and Haag, 2009; Kelleher et al., 2008). Similar to Cbr-tra-2(nm1); Cbr-puf-2/1.2(RNAi), all Cbr-tra-1(nm2); Cbr-puf-2/1.2(RNAi) XX animals had a fully male soma, consistent with Cbr-puf-2/1.2 acting to determine sex exclusively in germ cells. Seventy-seven percent developed germ line tumors without apparent gametogenesis (Fig. 5E), 17% had differentiated oocytes distal to tumorous germ cells, and the remainder had only oocytes with an otherwise normal germ line (Table 2). Suppression of the abundant sperm development characteristic of Cbr-tra-1(nm2) by Cbr-puf-2/1.2(RNAi) is surprising because wild-type XO males show no such defect.

We also examined interactions between Cbr-puf-2/1.2(RNAi) and the likely null Cbr-fem-3 mutant nm63, which on its own has no effect on XX hermaphrodites but sex-reverses XO animals (Hill et al., 2006). XX Cbr-puf-2/1.2(RNAi); Cbr-fem-3(nm63) animals are Fog (Fig. 5F), suggesting that Cbr-puf-2/1.2 and Cbr-fem-3 do not have obvious genetic interaction. To further test a simple linear model, we reduced Cbr-tra-2 levels via the nm1 mutation with the expectation that, in the absence of Cbr-fem-3, loss of all or part of Cbr-tra-2 activity would have no effect. However, whereas all Cbr-tra-2(nm1)/+; Cbr-puf-2/1.2(RNAi); Cbr-fem-3(nm63) animals were Fog (Fig. 5G, Table 2), homozygosity for Cbr-tra-2(nm1) restored self-fertility to the otherwise Fog Cbr-puf-2/1.2(RNAi); Cbr-fem-3(nm63) animals (Fig. 5H). Thus, the germ line sex determination activity of Cbr-puf-2/1.2 is sensitive to Cbr-tra-2 dose even in the absence of Cbr-fem-3, which is inconsistent with a linear epistasis model for gene activity.

### Functions of puf-2 and fbf orthologs in gonochoristic Caenorhabditis

But for the production of sperm, females of gonochoristic Caenorhabditis are very similar to C. elegans and C. briggsae hermaphrodites, and males are anatomically identical. We therefore sought to clarify the evolutionary history of FBF and PUF-2 subfamily gene function in germ line sex determination. RNAi

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**Table 2. Interactions between sex determination mutations and Cbr-puf-1.2/2 knockdown**

<table>
<thead>
<tr>
<th>Genotype of injected mother</th>
<th>Self progeny class by somatic phenotype*</th>
<th>Self progeny class by gonad phenotype*: non-RNAi</th>
<th>Gonad phenotype of progeny: RNAi</th>
<th>Number scored</th>
<th>Genotype§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cbr-tra-2(nm1)/Cbr-dpy(nm4)</td>
<td>A class: Tra pseudo-male soma</td>
<td>One armed gonad with sperm only</td>
<td>Sperm plus tumor (includes Pro)</td>
<td>38</td>
<td>T/T</td>
</tr>
<tr>
<td></td>
<td>B class: non-Dpy female soma</td>
<td>Two armed gonads with both sperm and oocytes</td>
<td>Normal</td>
<td>45</td>
<td>T/T</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Single gonad arm, tumor</td>
<td>10</td>
<td>T/T</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Two gonads, both Mog</td>
<td>32</td>
<td>T/T</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Abnormal female‡</td>
<td>8</td>
<td>C/T</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Degenerated germ line</td>
<td>3</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Self-fertile</td>
<td>1</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mog</td>
<td>1</td>
<td>C/C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Degenerated or undifferentiated germ line</td>
<td>10</td>
<td>C/C</td>
</tr>
<tr>
<td>Cbr-dpy(nm4)+</td>
<td>A class: Dpy</td>
<td>Two armed gonads with both sperm and oocytes</td>
<td>Fog</td>
<td>&gt;200</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>B class: non-Dpy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cbr-tra-1(nm2)/Cbr-let(nm28)</td>
<td>A class: Tra pseudo-male soma</td>
<td>One armed gonad with sperm only and late oocyte production</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B class: female soma</td>
<td>Two armed gonads with both sperm and oocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cbr-fem-3(nm63)</td>
<td>Isogenic</td>
<td>Two armed gonads with both sperm and oocytes</td>
<td></td>
<td>&gt;100</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cbr-tra-2(nm1); Cbr-fem-3(nm63)</td>
<td>Isogenic</td>
<td>Two armed gonads with both sperm and oocytes</td>
<td></td>
<td>&gt;100</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cbr-tra-2(nm1)+; Cbr-fem-3(nm63)</td>
<td>A class§</td>
<td>Two armed gonads with both sperm and oocytes</td>
<td></td>
<td>57</td>
<td>T/C, C/C</td>
</tr>
<tr>
<td></td>
<td>B class§</td>
<td></td>
<td></td>
<td>21</td>
<td>T/T</td>
</tr>
</tbody>
</table>

*No RNAi phenotype.
‡Poorly formed vulva and tail, undifferentiated germ line.
§All progeny have female somas due to Cbr-fem-3(nm63).
¶T, nm1 or nm2; C, WT.
N.D., not determined
knockdown by direct injection of dsRNA into the germ line is efficient in a range of Caenorhabditis species (Winston et al., 2007), so we applied this to the gonochoristic C. brenneri, C. remanei, C. japonica and C. sp. 9 (Table 3). In nearly every case, puf RNAi caused pronounced germline underproliferation, ranging from fewer germ cells than usual to complete loss (Fig. 6C-J). A notable exception, however, was knockdown of C. brenneri fbf-1. In this case, germ cells appeared to exit meiosis and re-enter the mitotic cell cycle, producing a germ cell tumor (Fig. 6E,L). The phenotype is reminiscent of loss of gld-1 function in both C. elegans (Francis et al., 1995a) and C. briggsae (Beadell et al., 2011; Nayak et al., 2005). However, straightforward germline sex determination phenotypes were not observed in either XX or XO animals.

**DISCUSSION**

**Additional taxa clarify the size and evolutionary history of the Caenorhabditis PUF family**

Since Lamont et al. (Lamont et al., 2004) produced the first phylogeny for C. elegans and C. briggsae PUF gene family members, the genomes of three gonochoristic Caenorhabditis (C. remanei, C. brenneri and C. japonica) have been sequenced and annotated (and others now have preliminary assemblies). Searches of all five genomes revealed two PUF protein families not present in this earlier analysis: PUF-12 and PUF-13. The functions of these two newly added PUF subfamilies are unknown. Phylogenetic reconstruction unambiguously groups PUF proteins into nine distinct subfamilies and shows that C. elegans FBF and Cbr-PUF-1.1/1.2 are members of different subfamilies that existed prior to the divergence of C. japonica from the Elegans group species. Nevertheless, the FBF and PUF-2 subfamilies retain common RNA-binding site preferences and roles in regulating germline proliferation. Their most striking difference, which is in hermaphrodite germline sexual patterning, evolved as the C. elegans and C. briggsae lineages adopted self-fertility (Fig. 7A).

**The sex determination function of Cbr-puf-2/1.2 is mediated by a conserved PUF-gld-1 interaction**

The PUF and GLD-1 RBPs are pleiotropic regulators with complex interactions with other factors. In C. elegans, FBF-1/2 regulate germ cell sexual fate (Zhang et al., 1997) and the entry into meiosis (Crittenden et al., 2002; Lamont et al., 2004) through repression of hundreds of target mRNAs (Kershner and Kimble, 2010). In addition, in the soma FBF-1 can act as a positive regulator of target gene expression (Kaye et al., 2009). GLD-1 is also a translational repressor (Jan et al., 1999) with many target mRNAs (Wright et al., 2010) and roles in both sex determination and meiotic progression (Francis et al., 1995b). gld-1 is itself both positively and negatively regulated at the mRNA (Crittenden et al., 2002; Suh et al., 2009; Suh et al., 2006) and protein (Clifford et al., 2000; Jeong et al., 2010) levels. Further, in a sensitized background C. elegans gld-1...
mutations can have an unexpected strong masculinizing effect (Kim et al., 2009), and FBF associates with molecular complexes that have both repressive and stimulatory effects on gld-1 expression (Suh et al., 2009). These complexities suggest a number of ways that a PUF–gld-1 regulatory linkage could be modified such that homologous PUF mutants have opposite sexual phenotypes. However, in this study we tested a simple hypothesis based on three initial observations: (1) gld-1 is repressed by FBF in C. elegans (Crittenden et al., 2002; Suh et al., 2009); (2) the FBF and PUF-2 subfamilies are related (Fig. 2); and (3) the sexual transformations of both subfamilies have similar RNA binding properties. Secondly, it is possible that this effect is indirect, the simplest interpretation is that

Table 3. Summary of puf RNAi knockdown experiments in gonochoristic Caenorhabditis

<table>
<thead>
<tr>
<th>Species</th>
<th>Male Number</th>
<th>Male Phenotype</th>
<th>Female Number</th>
<th>Female Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. sp. 9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Csp9-puf-1.1/2*</td>
<td>65</td>
<td>GD</td>
<td>68</td>
<td>GD, OD</td>
</tr>
<tr>
<td>Csp9-puf-2/1.2*</td>
<td>83</td>
<td>GD</td>
<td>68</td>
<td>GD, OD</td>
</tr>
<tr>
<td>Csp9-puf-1.1/1.2*</td>
<td>38</td>
<td>GD</td>
<td>58</td>
<td>GD, OD</td>
</tr>
<tr>
<td>Csp9-puf-2*</td>
<td>50</td>
<td>Normal</td>
<td>50</td>
<td>Normal</td>
</tr>
<tr>
<td>C. brenneri</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>puf-2</td>
<td>50</td>
<td>GD, EL, LA</td>
<td>50</td>
<td>GD, EL, LA</td>
</tr>
<tr>
<td>All puf-2 paralogs</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>fbf</td>
<td>50</td>
<td>Tumor</td>
<td>50</td>
<td>Tumor</td>
</tr>
<tr>
<td>puf-2 + fbf</td>
<td>50</td>
<td>GD, EL, LA</td>
<td>50</td>
<td>GD, EL, LA</td>
</tr>
<tr>
<td>C. remanei</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>puf-2</td>
<td>95</td>
<td>GD</td>
<td>98</td>
<td>GD</td>
</tr>
<tr>
<td>All puf-2 paralogs</td>
<td>72</td>
<td>GD</td>
<td>70</td>
<td>GD</td>
</tr>
<tr>
<td>fbf</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>puf-2 + fbf</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>C. japonica</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>puf-2</td>
<td>26</td>
<td>GD, mild</td>
<td>30</td>
<td>GD, mild</td>
</tr>
<tr>
<td>All puf-2 paralogs</td>
<td>12</td>
<td>GD, mild</td>
<td>9</td>
<td>GD, mild</td>
</tr>
<tr>
<td>fbf</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>puf-2 + fbf</td>
<td>82</td>
<td>GD, severe</td>
<td>85</td>
<td>GD, severe</td>
</tr>
</tbody>
</table>

Numbers account for all observations.

*R1mers designed according to C. briggsae orthologs and dsRNA derived from C. sp. 9 cDNA.

*dsRNA derived from Cre-puf-2.1, but also has stretches of high similarity to Cre-puf-2.2.

F, female; M, male; GD, germline degeneration; OD, oogenesis defect; EL, embryonic lethal; LA, larval arrest; N.A., not applicable (see Fig. 1).

Independent recruitment of a PUF–gld-1 regulatory module during evolution of hermaphroditism

Fig. 6. PUF family knockdown in gonochoristic Caenorhabditis. (A,B) Untreated adult C. remanei female and male, with germ lines outlined. (C-J) RNAi directed against the C. japonica, C. brenneri, C. remanei and C. sp. 9 genes indicated. RNAi of PUF homologs generally produced a germline underproliferation (C,D,F,I), or abnormal germline degeneration (G-I) phenotype. By contrast, C. brenneri fbf-1(RNAi) produced a germ cell tumor (E). (K,L) Merged fluorescent images of DNA (gray) and phospho-histone 3 (PH3, red) staining of extruded XX C. brenneri gonads from untreated (K) or Cbr-fbf-1(RNAi) (L) animals. Mitotic nuclei are localized to the distal stem cell niche (asterisk) in wild-type females (K), but distributed throughout the gonad in Cbr-fbf-1(RNAi) (L). e, embryo; o, oocytes, spt, spermatheca; v, vulva.

Chr–gld-1 translation is increased. Finally, Cbr-gld-1(RNAi) suppression of Cbr-puf-2/1.2(RNAi) feminization is consistent with GLD-1 overexpression being the chief mechanism by which Cbr-puf-2/1.2(RNAi) feminizes the hermaphrodite germ line.

Independent recruitment of a PUF–gld-1 regulatory module during evolution of hermaphroditism

PUF-2 and FBF subfamily gene knockdowns (Fig. 6) revealed defects in proliferation control, but not in sex determination, in gonochoristic Caenorhabditis, whereas both C. elegans and C. briggsae show strong masculinization or feminization, respectively. This could suggest the independent co-option of PUF proteins into C. elegans and C. briggsae hermaphroditic germline patterning.
PUF evolution in Caenorhabditis

However, we have recently described complementary changes in gld-1 function in the same species (Beadell et al., 2011). Specifically, the *C. elegans* *tra-2* 3′UTR evolved to support an unusually strong in vivo association with GLD-1 that is required for XX spermatogenesis. By contrast, *C. briggsae* *gld-1* evolved to limit XX sperm production through regulation of *Cbr-puf-8*. These changes in the targets of gld-1, when combined with the existence of a conserved PUF–gld-1 module described here, are largely sufficient to explain the differences in *C. elegans* *fbl* and *C. briggsae* *puf-2* phenotypes (Fig. 7).

The repeated recruitment of PUF and *gld-1* (Beadell et al., 2011) homologs into hermaphrodite germline sex determination might reflect the general reliance of germline development on post-transcriptional gene regulation (Leatherman and Jongens, 2003), especially via mRNA 3′ UTRs (Merritt et al., 2008). PUF proteins are pleiotropic germline mRNA-binding proteins (Ariz et al., 2009; Lublin and Evans, 2007; Subramaniam and Seydoux, 2003; Wickens et al., 2002), and are thus a priori on a short list of candidates for mediating germline sex determination. Also, germline sex determination has spatial and temporal overlap with events regulating germline meiotic entry and gamete differentiation, which pre-date the origins of self-fertility. This overlap might increase the probability of recruiting genes regulating these events into hermaphrodite patterning. Consistent with this, the 3′ UTR motif that allows *C. elegans* FBF repression of *gld-1* mRNA to promote germ cell proliferation (Crittenden et al., 2002) is conserved among all sequenced *Caenorhabditis* species, hermaphroditic or otherwise (Fig. 4A).

Taken together, it is likely that the last common ancestor of the FBF and PUF-2 subfamilies repressed *gld-1* translation in the service of regulating germline proliferation. Extant *Caenorhabditis* species have then modified this situation by losing one or other subfamily entirely (but never both) and duplicating genes within a given subfamily. Layered upon this is the co-option of the entire PUF–*gld-1* module into hermaphrodite development. Although this occurred in both characterized selfing species (and might be true of others), the exact role of the module is variable and dependent upon the overall context in which it occurs.

Computer simulations of evolving, unconstrained genetic networks show that participation of genes in multiple traits leads to modular regulation, and that pre-existing modules have a tendency to be utilized as raw materials for subsequent evolutionary innovation (Espinosa-Soto and Wagner, 2011). The multiple developmental functions of PUF family genes and *gld-1* (Ariz et al., 2009; Crittenden et al., 2002; Francis et al., 1995a; Jeong et al., 1995a; Jones et al., 1996), which might synergize with excess FEM-3 to reinforce male fate. In *C. briggsae*, conservation of the fem-3 PME (Haag and Kimble, 2000) and its interaction with *Cbr-PUF-2/1.2* (supplementary material Fig. S1) suggest simultaneous upregulation of *Cbr-GLD-1* and *Cbr-FEM-3* might also occur when *Cbr-puf-2/1.2* activity is reduced. If so, why would the GLD-1 side dominate phenotypically in *C. briggsae*? fem-3 plays a different germline role in the two species (Hill et al., 2006), so regulation of *Cbr-fem-3* by *Cbr-puf-2/1.2* could be inconsequential with respect to hermaphrodite sex determination. However, the genetic interactions between *Cbr-puf-2/1.2* and *Cbr-tra-2* suggest an alternative explanation: excess Cbr-FEM-3 is masculinizing on its own, but the simultaneous hyperactivity of Cbr-GLD-1 that occurs in the *Cbr-puf-2/1.2* knockdown suppresses it via a parallel pathway (Fig. 7B). Consistent with this, loss of a single copy of *Cbr-tra-2*, which has no effect on its own (Kelleher et al., 2008), completely masculinizes the germ line of *Cbr-puf-2/1.2* (RNAi) animals (Fig. 5D). We propose that reduced function of both *Cbr-tra-2* and *Cbr-puf-2/1.2* synergize to activate *Cbr-fem-3* to the point where this dominates over the *Cbr-gld-1*-mediated feminizing effect of *Cbr-puf-2/1.2* alone (Fig. 7B). This is an interesting example of the inherently bi-stable nature of germline sex determination, in which subtle differences in dosage cause complete sex reversal.

Pleiotropy and redundancy in the PUF family

The nine PUF subfamilies, although generally stable, show some recent duplications and loss in particular lineages. That germline feminization requires simultaneous loss of both *Cbr-puf-2* and *Cbr-puf-1.2* function initially suggested that these genes would be wholly

---

**Fig. 7. Models of FBF and PUF-2 subfamily evolution.**

(A) Cladogram of Elegans group *Caenorhabditis* (based on published data (Kiontke et al., 2004; Woodruff et al., 2010) and summaries of knockdown phenotypes for FBF and PUF-2/1.2 subfamilies from this study. GD, germline degeneration; Mog, masculinization of germ line; Fog, feminization of germ line. Because of lineage-specific subfamily loss, some species-subfamily combinations have no data. (B) Genetic model for regulatory interactions between *fbf, puf-2* and other sex determination factors in the hermaphrodite germ line of *C. elegans* and *C. briggsae*. The weight of the repression bars downstream of *fbf* and *Cbr-puf-2/1.2* is indicative of the relative significance of the interaction for sex determination. Note that *C. elegans* *gld-1* promotes spermatogenesis by directly regulating *tra-2* (Jan et al., 1999), but this is not shown here.
Dynamic functions of puf-2 and fbf orthologs in regulation of germ cell proliferation

Cbr-puf-1.2/2 also promote germ cell meiotic progression. This effect is independent of sexual fate, as it is not fully suppressed in the XO male germ line and is never suppressed in Cbr-tra-1 and Cbr-tra-2 pseudo-males. In this respect, the role of Cbr-puf-2/1.2 is distinct from that of C. elegans fbf-1/2, which promote proliferation and repress meiotic entry (Crittenden et al., 2002). With the exception of C. brenneri fbf-1, RNAi knockdown of PUF-2 and FBF subfamily genes in gonochoristic species led to germine degeneration (Fig. 6). This suggests that the ancestral function of both the PUF-2 and FBF subfamilies is the maintenance of germline proliferation and/or integrity. If so, then Cbr-puf-2/1.2 acquired a distinct tumor-suppressing role in the C. briggsae lineage, perhaps as it acquired a role in hermaphrodite sex determination. Whether these two changes were functionally linked is unclear. In addition, in C. brenneri FBF and PUF-2 subfamilies have taken on opposite roles in regulating proliferation, with the former limiting it and the latter promoting it. If they also have similar RNA binding properties, then understanding what mediates their apparently antagonist functions will help clarify the overall logic of PUF regulation.

Evolution of gene regulation at the translational level

Cis-regulatory DNA has emerged as a common locus of genetic variation underlying novel phenotypes, presumably because this avoids deleterious pleiotropic effects (Carroll, 2008; Stern, 2000; Stern and Orgogozo, 2008). Translational control and its evolutionary dynamics are presumably important for adaptation in tissues such as the germ line, yet it has been little explored (Haag, 2009a). The in vitro PUF–gld-1 cross-species interaction described here suggests that, at the protein sequence level, Cbr-PUF-2/1.2 and FBF are interchangeable. We recently reported similar results for GLD-1 (Beadell et al., 2011). These studies provide evidence that conserved RBP-mRNA interactions might take on altered significance due to changes in the role of the target mRNA (as appears to be the case with PUF–gld-1) or to variation in RBP protein co-factors that qualitatively or quantitatively modify conserved RBP-mRNA interactions, such as FOG-2, a GLD-1 co-factor in C. elegans (Clifford et al., 2000; Nayak et al., 2005). FBF co-factors have also been reported (Kraemer et al., 1999; Suh et al., 2009). Clarification of the precise biochemical roles(s) of such co-factors is an important subject of future research.

Acknowledgements

We thank A. Beadell, G. Woodruff and M. A. Félix for sharing results and reagents prior to publication; J. Ross for strain CP105; L. Pick and A. Bely for reagents; N. Andrews for use of a luminescent meter; M. Cummings and C. Delwiche for phylogenetics advice; and S. Feng for assistance with preliminary experiments that motivated this work.

Funding

This work was supported by a research fellowship from the University of Maryland Graduate School (to Q.L.) and National Institutes of Health (NIH) grants [GM79414 to E.S.H. and GM50942 to M.W.] Deposited in PMC for release after 12 months.

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.070128/-/DC1

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