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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 (Ahringer 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., 2009; 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).

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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 *bicaudal 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 germline 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 (Subramaniam 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 translation 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 hermaphrodite 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 hermaphrodite 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 acquired additional essential functions, which might explain why such duplicate genes are so common.

MATERIALS AND METHODS

Phylogenetic analysis

Protein datasets for *C. elegans*, *C. briggsae*, *C. remanei*, *C. brenneri* and *C. japonica* were retrieved from the Nematode Genome Annotation Assessment Project (nGASP, http://www.sanger.ac.uk/projects/C_elegans/Wormbase/current/wormpep_download.shtml). A PUF domain hidden Markov model (HHM; PUF_ls.hmm) from Pfam (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 releases (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(nm66)*, *Cbr-dpy(nm4)*, *Cbr-tra-2(nm1)* and *Cbr-tra-2(nm9ts)*; LGIII: *Cbr-tra-1(nm2)*, *Cbr-let(nm28)*; LGIV: *Cbr-fem-3(nm63)*. *Cbr-tra-2(nm1)/+;Cbr-fem-3(nm63)* animals were the progeny of *Cbr-tra-2(nm1)/+;Cbr-fem-3(nm63)/+* mothers, which came from a cross between *Cbr-tra-2(nm1)/Cbr-dpy(nm4)* and *Cbr-fem-3(nm63)/+* 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 (*C. briggsae*) or cDNA (*C.* sp. 9, *C. remanei*, *C. brenneri* and *C. japonica*) with primers flanked by the T7 promoter and sequenced to verify identity. For *C.* sp. 9, primers designed according to *C. 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 Axiocam digital camera and Open Lab software (Improvision) or an SP5 X confocal microscope (Leica). In the latter, *z*-stacks were collapsed for presentation.

Quantitative RT-PCR

Total RNA from staged 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 I 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.

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′), coding, and 3′ flanking sequences of *Cbr-puf-2* were engineered via Gateway cloning technology (Invitrogen) into destination plasmid pCR40 (gift from C. Richie), which also contains the wild-type *Cbr-unc-119* gene. This plasmid was introduced into *Cbr-unc-119(nm67)* mutants through biolistic bombardment (Praitis et al., 2001). Stable non-Unc lines were crossed with *Cbr-puf-2(nm66)/+* mutants to test for rescue of larval arrest.

Immunoblots

Triplicate 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* (amino acids 108-554) was cloned into the GST fusion protein vector pGEX-4T-1 (GE Healthcare) using *Xma*I and *Not*I. The same fragments were cloned into pACT2-AD (Clontech) using *Nco*I and *Xma*I to allow activation domain fusion protein expression in yeast. Sense and antisense 45 bp DNA oligomers (Integrated DNA Technologies) flanking the putative FBF binding element of the *Cbr-gld-1* and *Cbr-fem-3* 3′UTR were annealed and inserted into the pIIIA/MS2-2 vector using *Xma*I and *Sph*I for hybrid RNA expression in yeast. *Cbr-gld-1* and *Cbr-fem-3* wild-type and ACA mutant forms were made similarly. All constructs were confirmed by direct sequencing. pIIIA/MS2-2-*Ce-fem-3*, pIIIA/MS2-2-*Ce-gld-1*, pIIIA/MS2-2-NRE and pACT2-FBF-2 (amino acids 121-632) are as previously described (Bernstein et al., 2005).

Gel mobility shift assays

GST fusion proteins were isolated from T7 Express *lysY* competent *E. coli* (New England BioLabs) and purified using the following elution buffer: 1×PBS, 0.2% Tween 20, 150 mM NaCl, 0.1% 2-mercaptoethanol, 50 mM glutathione (reduced, pH 8.0). Twenty femtomoles ^{32}P end-labeled oligoribonucleotides (Dharmacon) were combined with GST-Cbr-PUF-2 or GST-Cbr-PUF-1.2 at various concentrations as described (Bernstein et al., 2005).

Yeast three-hybrid assay

In all experiments, RNA plasmids and activation domain fusion plasmids were co-transformed into the YBZ1 yeast strain. The three-hybrid assay was followed as described (Stumpf et al., 2008b). The strength of the interaction was measured using the *beta*-Glo Assay System (Promega) quantified in a luminometer (Turner 20/20n or Spectra Max M5^e).

Statistics

For yeast three-hybrid assay data analysis, standard errors for the ratios of test to vector RNAs (see Fig. 4 and supplementary material Fig. S1) were estimated using the 'delta method', which is based on Taylor series expansions to account for multivariate nonlinear transformations of the data (Powell, 2007). Otherwise, standard two-tail *t*-tests were applied.

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 they 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 *puf-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 puf-10* is a pseudogene with stop codons throughout its former 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.

Importantly for this study, *C. elegans* FBF proteins and *C. briggsae* PUF-2 proteins belong to two distinct clades. Moreover, *C. elegans* lacks a PUF-2 subfamily member, and *C. briggsae* lacks an FBF subfamily ortholog. FBF and PUF-2 subfamilies are marginally supported as sister groups. What is more certain is that both belong to a well-supported superclade of seven PUF subfamilies, two of which (PUF-5 and PUF-6/7) are closely related and share a binding preference distinct from that of FBF (and likely PUF-2) subfamilies (Stumpf et al., 2008a). Thus, the *C. elegans* and *C. briggsae* genes compared below are not orthologous but belong to subfamilies that are relatively closely related.

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, gene-specific 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 germ line (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 *puf-8* in *C. elegans* hermaphrodites, where they promote oogenesis (Bachorik and Kimble, 2005; Zhang et al., 1997).

Cbr-puf-2 and *Cbr-puf-1.2* also function in non-sexual aspects of germline development (Table 1). A minority of *Cbr-puf-2/1.2(RNAi)* worms had proximal germ cell tumors at low concentrations (0.5 μg/μl) of dsRNA (Fig. 2C,D). When the

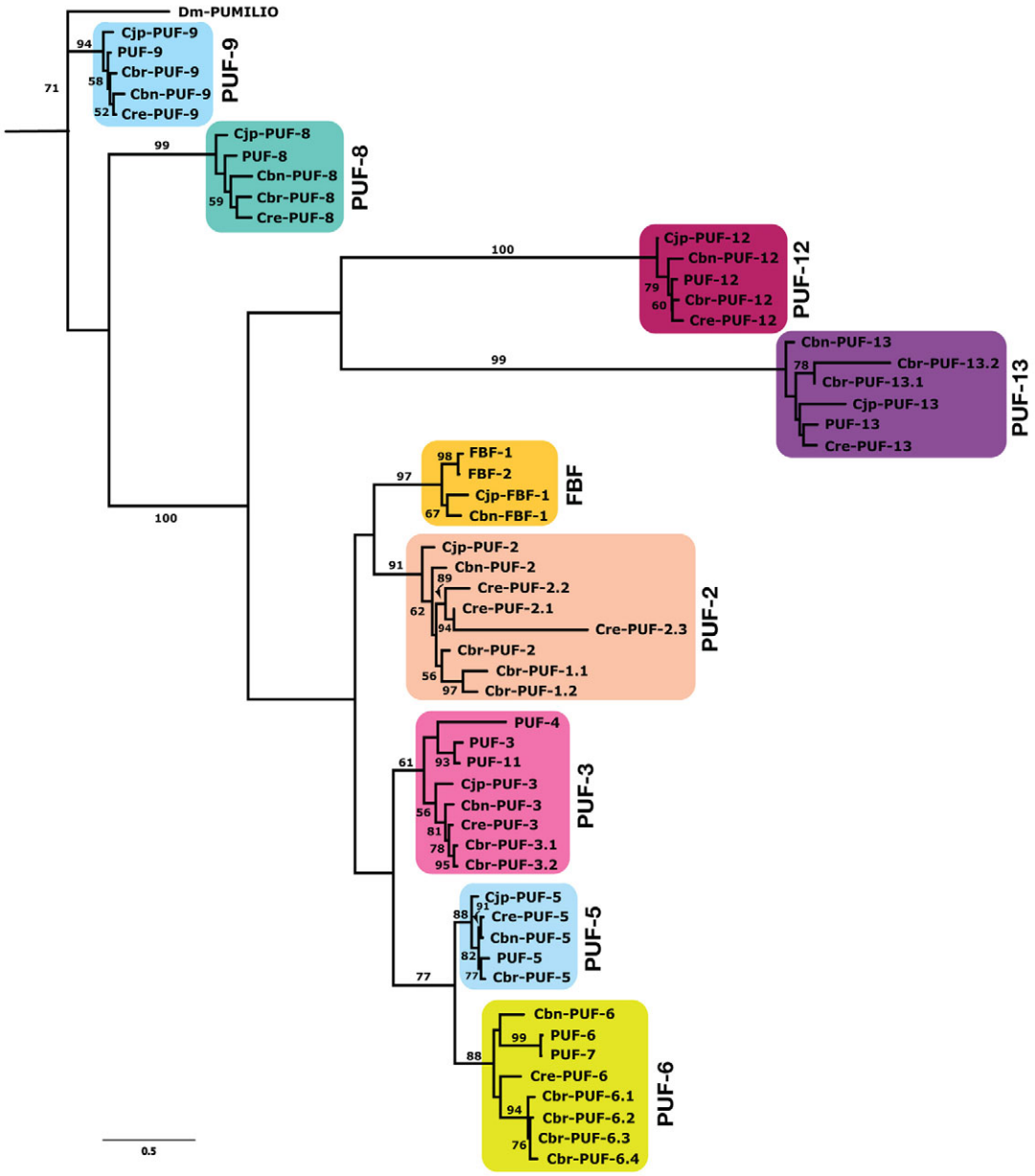

Fig. 1. PUF family phylogeny for five *Caenorhabditis* species. Maximum likelihood tree based on the PUF domain and conserved flanking regions. Bootstrap support is given for internal branches. See supplementary material Table S1 for WormBase gene numbers and nomenclature scheme.

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 *Cbr-puf-2* and *Cbr-puf-1.2* are involved in the control of meiotic progression and/or the prevention of the return to mitosis. In addition, *Cbr-puf-1.2(RNAi)* worms produced fewer and atypically small oocytes, which indicates that *Cbr-puf-1.2* is involved non-redundantly in oocyte development.

The developmental profiles of *Cbr-puf-2* and *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, *Cbr-puf-2* is over 100-fold more abundant than *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.

Cbr-puf-2 mutant reveals pleiotropic roles in embryogenesis and larval somatic development

To study the function of *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, *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 *Cbr-puf-2(nm66)*/+ mothers were arrested

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

Target gene(s)	dsRNA (μg/μl)	Phenotype percentage (*n*>200/treatment)		
		Fog*	Other sterile‡	Self-fertile
Cbr-puf-1.1	~3.0	–	–	100
Cbr-puf-2	~3.0	–	–	100
Cbr-puf-1.2	~3.0	–	–	100 (oocyte defect)
Cbr-puf-1.1 + *Cbr-puf-2*	~4.0/3.0	–	–	100
Cbr-puf-1.1 + *Cbr-puf-1.2*	~4.0/3.0	–	<1	~100
Cbr-puf-2 + *Cbr-puf-1.2*	0.5/0.5	91	9	0
Cbr-puf-2 + *Cbr-puf-1.2*	1.0/1.0	86	14	0
Cbr-puf-2 + *Cbr-puf-1.2*	1.5/1.5	80	20	0
Cbr-puf-2 + *Cbr-puf-1.2*	2.0/2.0	76	24	0
Cbr-puf-2 + *Cbr-puf-1.2*	~3.0/3.0	53	41	6
Cbr-puf-1.1 + *Cbr-puf-1.2* + *Cbr-puf-2*	~4.0/3.0/3.0	25	73	2

*Fog animals can produce viable progeny when mated with males.
‡Proximal or whole-gonadal tumor, malformed germ line and oocytes (all lack sperm).

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 *nm66* mutants. This was sufficient to allow *nm66* homozygotes to develop into fertile adults (Fig. 3D). The rescued strain, CP113, nevertheless had undetectably low *Cbr-puf-2* mRNA levels as measured by RT-PCR. Since germline transgene silencing is a known phenomenon in *C. elegans* (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.

Cbr-PUF-2 and Cbr-PUF-1.2 directly repress *Cbr-gld-1* mRNA to promote spermatogenesis

In *C. elegans*, FBF-1 and FBF-2 directly regulate *gld-1* and *fem-3* mRNA translation via FBF binding elements (FBEs) in their 3′UTRs (Crittenden et al., 2002; Suh et al., 2009; Zhang et al., 1997). The binding elements contain a ‘core’ central region (CGUGUAUUAUA, invariable nucleotides underlined) and flanking sequences, and the core is distinct from that of other PUF proteins (Bernstein et al., 2005). The 3′UTR of *Cbr-gld-1* bears a 15 nt stretch that is nearly identical to the *C. elegans gld-1* FBE (Fig. 4A). Moreover, loss of *Cbr-gld-1* function masculinizes the

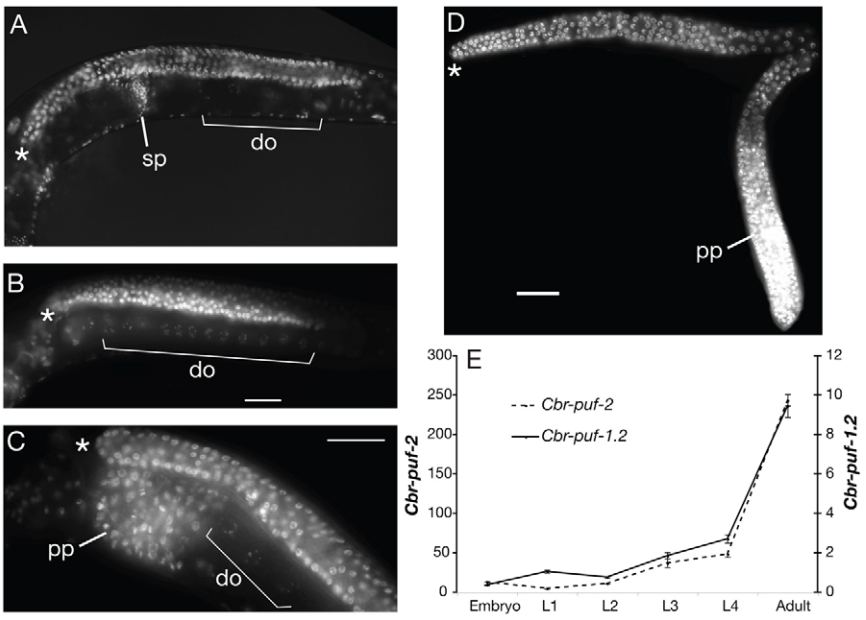

Fig. 2. Expression and germline phenotypes of *Cbr-puf-2/1.2(RNAi)*. (**A**) Wild-type *C. briggsae* adult hermaphrodite, stained with Hoechst 33258 to visualize DNA. Asterisk marks the distal tip of the gonad. (**B**) XX *Cbr-puf-2/1.2(RNAi)* Fog phenotype, commonly seen in low-dose RNAi, revealed by 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^{-4}). Error bars indicate s.e.m. for three biological replicates. sp, sperm, do, diakinesis oocytes; pp, proximal proliferation. Scale bars: 30 μm.

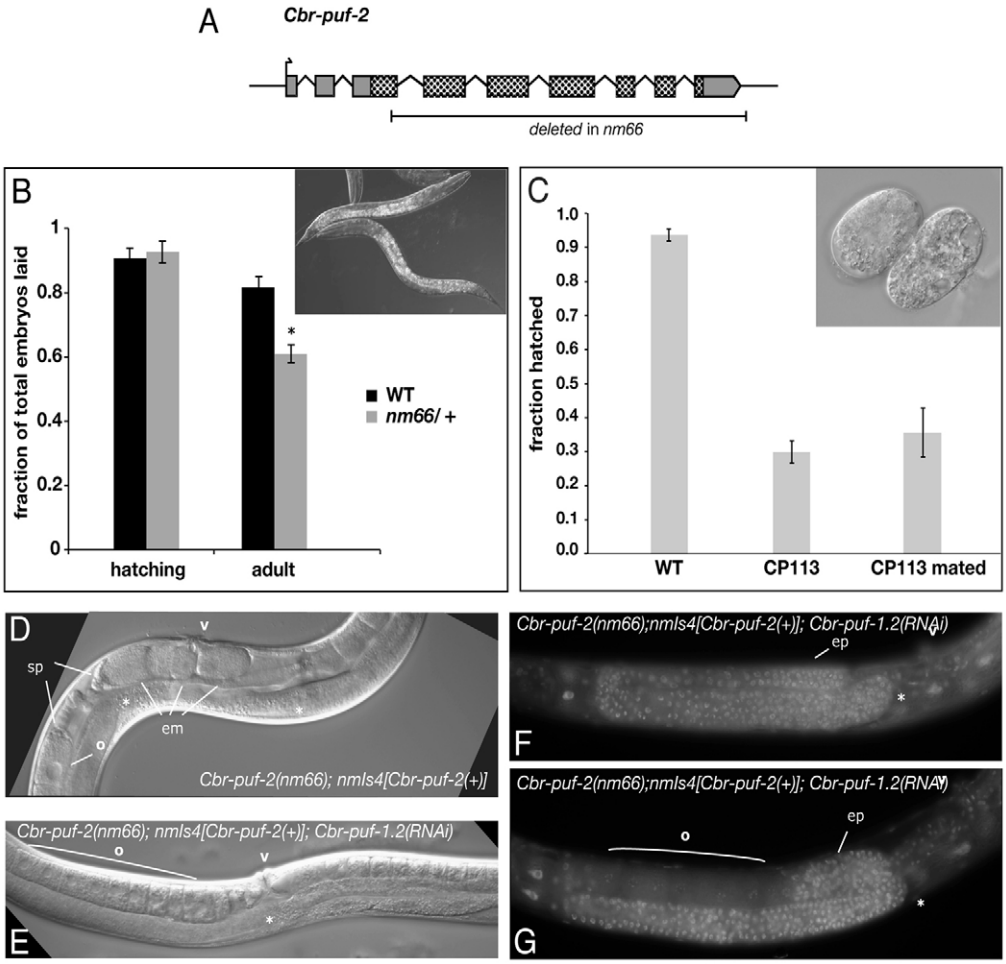

Fig. 3. Pleiotropic functions of *Cbr-puf-2* in embryogenesis and larval growth. (**A**) Structure of *Cbr-puf-2* and extent of deletion in allele *nm66*. Rectangles represent exons, with coding sequence for the conserved PUF domain and flanking regions stippled, and other coding sequences in gray. *, *P*=0.003 (two-tailed *t*-test), for the fraction of embryos from wild-type versus *nm66*/+ mothers that reach adulthood. (**B**) *Cbr-puf-2(nm66)* embryos hatch normally but arrest as larvae. Progeny that reach adulthood were significantly fewer in number from *nm66*/+ mothers than from wild-type (WT) AF16 mothers (*P*=0.003); *nm66* adults were never observed. Inset shows arrested *nm66* larvae. (**C**) Maternally deposited *Cbr-puf-2* promotes embryonic development. CP113 animals hatched at lower rates than AF16 (*P*<0.0001), and mating with AF16 males failed to rescue lethality (*P*=0.0002). Inset shows representative dead embryos. Error bars indicate s.e.m. (**D**) *Cbr-puf-2(nm66)* animals harboring a *Cbr-puf-2(+)* transgene (strain CP113) grow into fertile adults. (**E-G**) Low concentrations of *Cbr-puf-1.2* dsRNA produce Fog animals (E), whereas higher doses produce tumors (F,G). Asterisk indicates the distal tip of the gonad arm. sp, sperm; v, vulva; em, embryos; o, oocytes; ep, ectopically proliferative germline tumor.

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

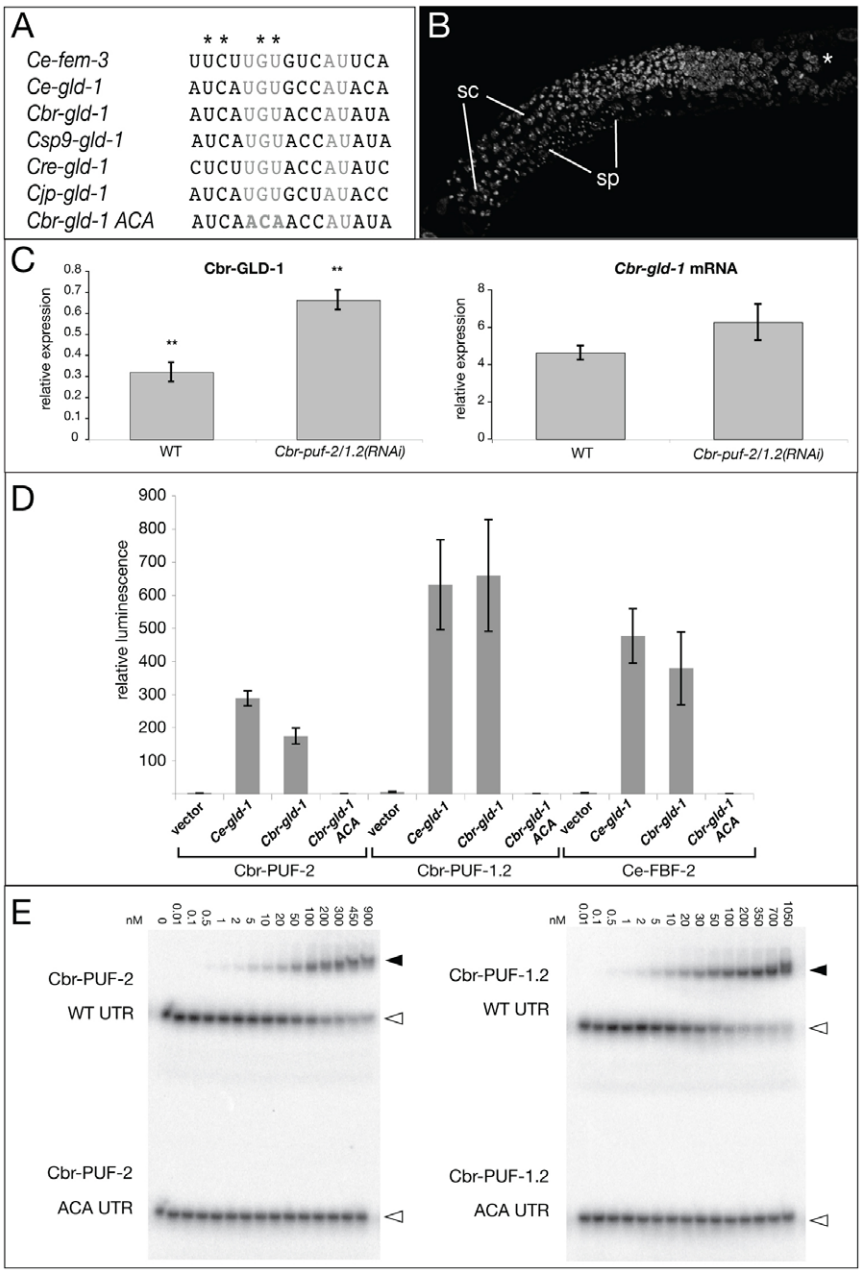

Fig. 4. *Cbr-gld-1* is a direct target of Cbr-PUF-2/1.2. (**A**) Alignment of FBF binding sites of *C. elegans fem-3* and *gld-1* with their orthologs from various *Caenorhabditis* species. Gray indicates the invariant core residues mutated in the ACA variant tested in D,E. Asterisks indicate *fem-3(gf)* (gain-of-function allele) point mutations. (**B**) Masculinization of germ line by *Cbr-gld-1(RNAi);Cbr-puf-2/1.2(RNAi)*. Hoechst staining reveals spermatocytes (sc) at the gonad arm bend and highly condensed sperm (sp) nuclei at the proximal end of the gonad. Asterisk marks the distal tip of the gonad. (**C**) Cbr-GLD-1 level is significantly higher in *Cbr-puf-2/1.2(RNAi)* than in wild-type L4 worms, whereas the *Cbr-gld-1* mRNA level is not. Error bars indicate s.e.; P=0.006 and P=0.168 for protein level and mRNA level, respectively (unpaired Student's t-test). (**D**) Yeast three-hybrid interactions among *C. elegans* and *C. briggsae* PUF proteins and *gld-1* mRNA variants. RNA plasmid pIIIa serves as the vector control. Error bars indicate s.e.m. (**E**) Cbr-PUF-2 and Cbr-PUF-1.2 bind to the putative *Cbr-gld-1* FBE in vitro in a UGU-dependent manner. Black arrowheads indicate retarded complexes between labeled RNA oligomers and pure PUF proteins, and white arrowheads indicate free RNA oligomers.

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 fem-3*, and *C. elegans* 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)/+* and *Cbr-tra-2(+/+)* could also be scored reliably. Surprisingly, most *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

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

Genotype of injected mother	Self progeny class by somatic phenotype*	Self progeny class by gonad phenotype*: non-RNAi	Gonad phenotype of progeny: RNAi	Number scored	Genotype¶
Cbr-tra-2(nm1)/ Cbr-dpy(nm4)	A class: Tra pseudo-male soma	One armed gonad with sperm only	Sperm plus tumor (includes Pro)	38	T/T
			Tumor	45	T/T
			Normal	10	N.D.
	B class: non-Dpy female soma	Two armed gonads with both sperm and oocytes	Single gonad arm, tumor	3	C/T
			Two gonad arms, both Mog	32	C/T
			Abnormal female‡	8	C/T
			Degenerated germ line	3	N.D.
			Self-fertile	1	N.D.
	C class: Dpy female soma	Two armed gonads with both sperm and oocytes	Normal	1	C/C
			Mog	3	C/C
			Degenerated or undifferentiated germ line	10	C/C
Cbr-dpy(nm4)/+	A class: Dpy	Two armed gonads with both sperm and oocytes	Fog	>200	N.D.
	B class: non-Dpy		Fog		N.D.
Cbr-tra-1(nm2)/ Cbr-let(nm28)	A class: Tra pseudo-male soma	One armed gonad with sperm only and late oocyte production	Tumor	89	T/T
			Tumor plus oocytes	20	T/T
			Oocytes	6	N.D.
	B class: female soma	Two armed gonads with both sperm and oocytes	Tumor	3	N.D.
			Abnormal oogenesis	56	T/C
Cbr-fem-3(nm63)	Isogenic	Two armed gonads with both sperm and oocytes	Fog	>100	N.D.
Cbr-tra-2(nm1); Cbr-fem-3(nm63)	Isogenic	Two armed gonads with both sperm and oocytes	Self-fertile	>100	N.D.
Cbr-tra-2(nm1)/+; Cbr-fem-3(nm63)	A class§	Two armed gonads with both sperm and oocytes	Fog	57	T/C, C/C
	B class§		Self-fertile	21	T/T

*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

confirmed that these female soma/Mog (masculinization of germ line) animals were indeed *Cbr-tra-2(nm1)/+*. Since *Cbr-tra-2* germline 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 Mog 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 endomitotic 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 germline 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 germline 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 germline sex determination. RNAi

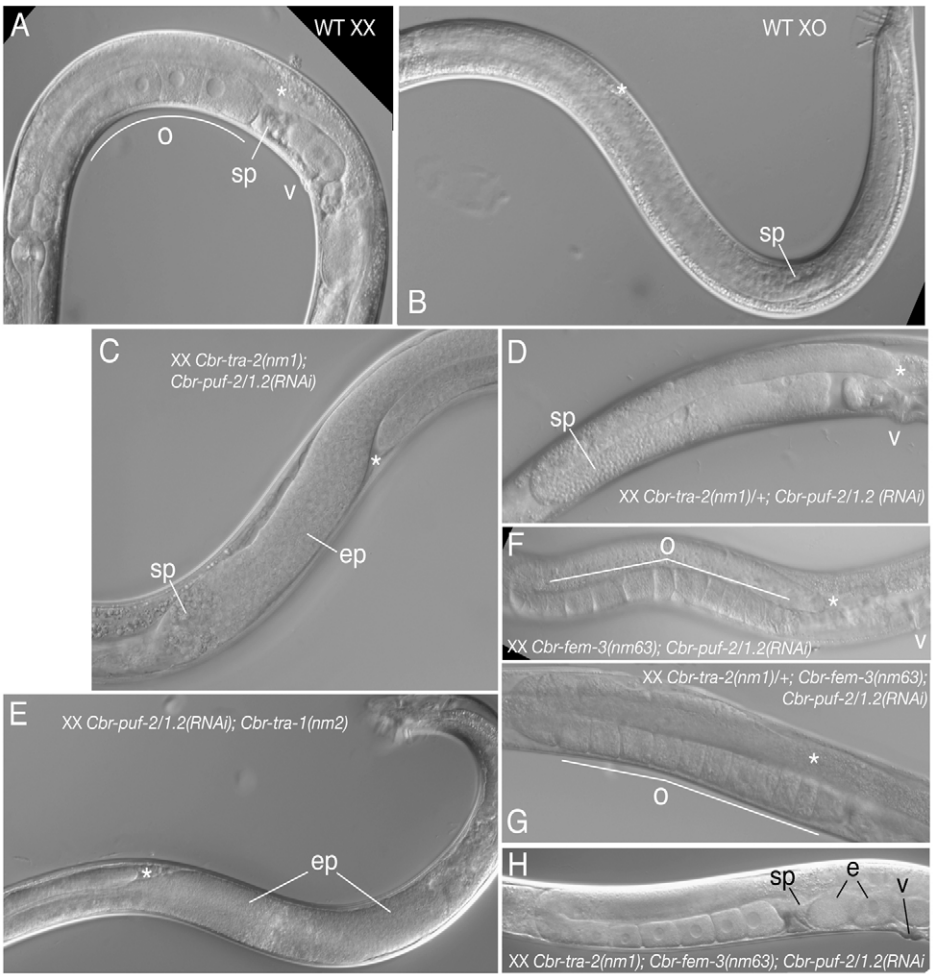

Fig. 5. Interaction between *Cbr-puf-2/1.2* knockdown and masculinizing *tra* mutations. (**A**) Wild-type XX *C. briggsae* hermaphrodite gonad arm (anterior), showing mature oocytes (o), sperm (sp) in the spermatheca, and vulva (v). The distal tip of the reflexed arm is marked with an asterisk. (**B**) Wild-type *C. briggsae* XO male testis (labeled as in A). (**C**) *Cbr-tra-2(nm1);Cbr-puf-2/1.2(RNAi)* XX animals develop male soma, half of which have sperm proximal to an ectopically proliferative (ep) germline tumor. (**D**) *Cbr-tra-2(nm1)/+;Cbr-puf-2/1.2(RNAi)* animals have two gonads full of sperm with no sign of oogenesis. (**E**) *Cbr-puf-2/1.2(RNAi);Cbr-tra-1(nm2)* XX animals have male soma. Among them, 77% developed a tumorous germ line without apparent gametogenesis. (**F**) *Cbr-puf-2/1.2(RNAi);Cbr-fem-3(nm63)* animals are Fog. (**G**) *Cbr-tra-2(nm1)/+;Cbr-puf-2/1.2(RNAi);Cbr-fem-3(nm63)* are Fog. (**H**) *Cbr-tra-2(nm1);Cbr-puf-2/1.2(RNAi);Cbr-fem-3(nm63)* animals are self-fertile hermaphrodites that produce embryos (e).

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-1.1/1.2/2 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*

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

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

Numbers account for all observations.
*Primers 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).

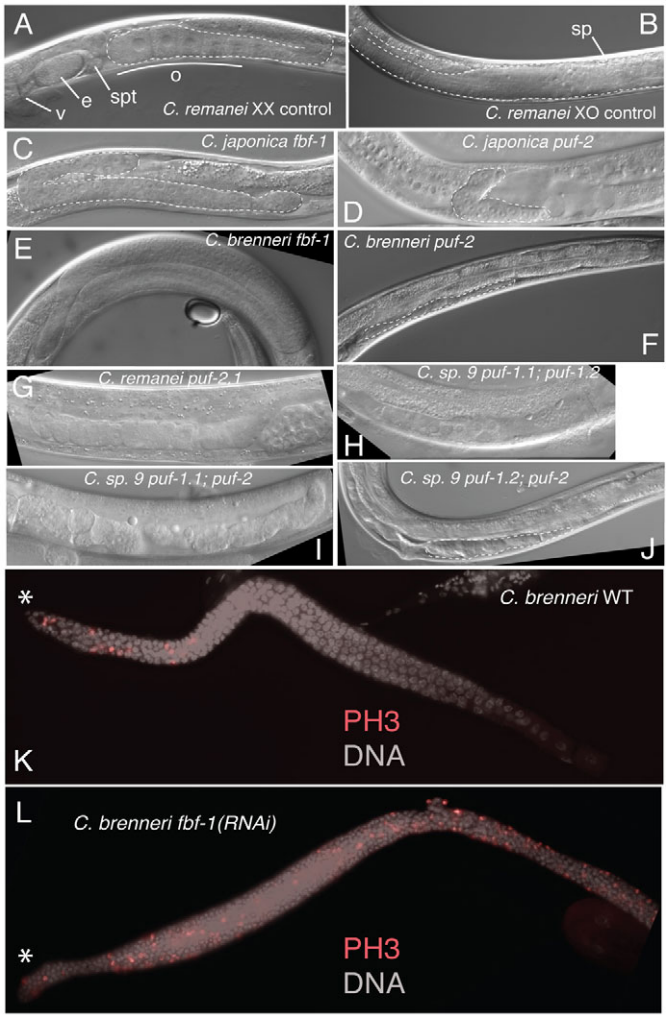

Fig. 6. PUF family knockdown in gonochoristic *Caenorhabditis*. (**A**,**B**) Untreated adult *C. remanei* female and male, with germ lines outlined. (**C-J**) RNAi directed against the *C. japonica*, *C. brenneri*, *C. remanei* and *C.* sp. 9 genes indicated. RNAi of PUF homologs generally produced a germline underproliferation (C,D,F,J) 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 *Cbn-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 *Cbn-fbf-1(RNAi)* (L). e, embryo; o, oocytes, spt, spermatheca; v, vulva.

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. 1); and (3) the sexual transformations of both *gld-1* orthologs and *fbf/puf-2* PUF genes (Fig. 2) are opposite in *C. elegans* (Clifford et al., 2000; Francis et al., 1995a; Francis et al., 1995b; Goodwin et al., 1993; Jan et al., 1999) and *C. briggsae* (Beadell et al., 2011; Nayak et al., 2005).

We hypothesized that loss of FBF and PUF-2 family members in *C. elegans* and *C. briggsae*, respectively, have opposite effects on germline sex primarily because a conserved, negatively regulated target mRNA, *gld-1*, has itself adopted opposite sexual roles.

We have presented several lines of evidence indicating that Cbr-GLD-1 expression is indeed repressed directly by Cbr-PUF-2/1.2. First, the conserved *Cbr-gld-1* FBE can be specifically bound in vitro by Cbr-PUF-2, Cbr-PUF-1.2 and *C. elegans* FBF-2. In yeast, *fem-3* FBEs also interact with FBF-2, Cbr-PUF-2 and Cbr-PUF-1.2 (supplementary material Fig. S1). Thus, the FBF and PUF-2 subfamilies have similar RNA binding properties. Secondly, reduced *Cbr-puf-2/1.2* function elevates Cbr-GLD-1 levels at the stage when spermatogenesis normally occurs. Although it is possible that this effect is indirect, the simplest interpretation is that *Cbr-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.

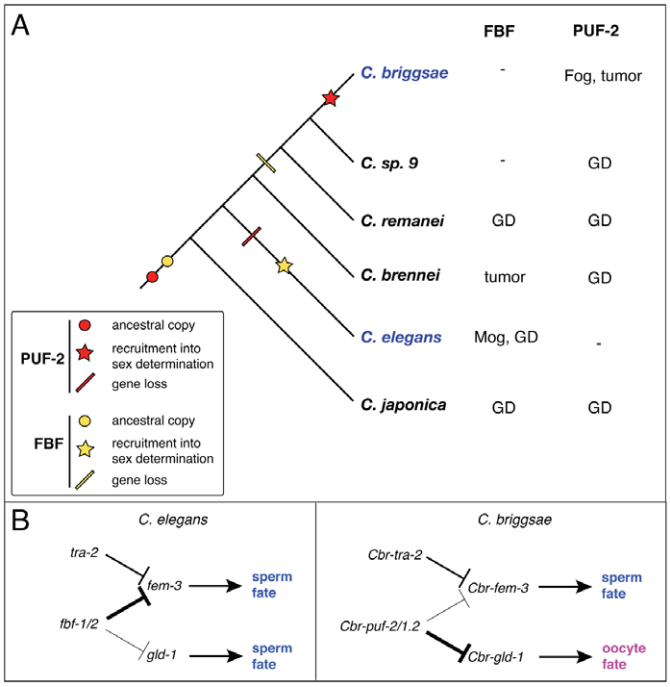

Fig. 7. Models of FBF and PUF-2 subfamily evolution.
(**A**) Cladogram of Elegans group *Caenorhabditis* [based on published data (Kiontke et al., 2004; Woodruff et al., 2010)] and summaries of knockdown phenotypes for FBF and PUF-2/1.2 subfamilies from this study: GD, germline degeneration; Mog, masculinization 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.

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 fbf* and *C. briggsae puf-2* phenotypes (Fig. 7).

The repeated recruitment of PUF and *gld-1* (Beadell et al., 2011) homologs into hermaphroditic 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., 2010; Lublin and Evans, 2007; Subramaniam and Seydoux, 2003; Wickens et al., 2002) might therefore promote their continued regulatory linkage in the face of altered germline phenotypes.

Evolution of genetic interactions between PUF targets

C. elegans fbf-1/2 hypomorphs or mutants are Mog (Zhang et al., 1997) because of *fem-3* hyperactivity (Ahringer and Kimble, 1991; Zhang et al., 1997). *C. elegans* GLD-1 is also hyperactive when *fbf-1/2* activity is reduced (Crittenden et al., 2002; 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

redundant. However, the *nm66* mutation reveals that *Cbr-puf-2* is required in the maternal germ line for reliable embryogenesis, and in the larval soma it is absolutely essential for progression beyond the L2 stage. These roles were not apparent in RNAi knockdown experiments, and similar essential roles have not been reported for any *C. elegans* PUF family member. Whether this somatic function was ancestral but lost in *C. elegans*, perhaps associated with loss of the PUF-2 subfamily, or represents a gain in *C. briggsae*, is unclear. What is clear, however, is that not all functions of recently duplicated PUF proteins are redundant, and this might explain their evolutionary persistence (Force et al., 1999).

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 germline 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 antagonistic 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.

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