ABSTRACT

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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. PUF family RNA-binding proteins FBF-1 and FBF-2 limit XX spermatogenesis by repressing the male-promoting proteins FEM-3 and GLD-1. For my dissertation research, I 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. I 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 coopted 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, I document non-redundant roles for *Cbr-puf-2* in several aspects of somatic development. I show *Cbr-puf-2* is required for reliable embryonic development, and that it is essential for vulval development and normal progression from early larval stage. I provide evidence suggesting that this latter role is related to pharyngeal muscle physiology. Thus, recently duplicated PUF paralogs, while redundant for some roles, can also rapidly acquire distinct non-redundant functions. This is consistent with theoretical models for the preservation of gene duplicates.

FUNCTIONAL AND MOLECULAR EVOLUTION OF THE PUF FAMILY

By

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Dedication

For Mom and Dad

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For the past five years, I have met and got help from many wonderful people. I would like to thank you all who make this dissertation possible.

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Introduction

1. Evolution of Sex Determination

Sexual reproduction is a universal phenomenon in eukaryotes, with very few exceptions (e.g. Mark and Curtis, 1995; Mark Welch and Meselson, 2000). Sex promotes genetic variability, and facilitates adaptation under stressful conditions (Morran et al., 2009). Therefore, it's reasonable to ask: if it's a widespread phenomenon, does it have a single origin? We cannot yet answer this definitively, but if it does, then we must explain how and why sex determination is among the least conserved of developmental processes (Marin and Baker, 1998).

Generally, there are two categories of primary sex determination mechanisms: environmental sex determination (ESD) systems, in which environmental cues initiate sexual differentiation, and genetic sex determination (GSD) systems, in which genetic components trigger the sexual fate (Hodgkin, 1992). Even among those animals that apply GSD systems, there is a diversity of mechanisms. The two commonest types are chromosomal sex determination systems and single-gene segregating systems. The former ones involve cyto-differentiated sex chromosomes, but the latter ones do not (Wilkins, 2002).

Further downstream of the variety of primary sex determination systems lies even more diversity: the genetic control of sex determination (Marin and Baker, 1998). By a comparison of regulatory pathways in roundworms, fruit flies and vertebrates, the great divergence of sex determination pathways has been revealed. Although both *C. elegans*

and *D. melanogaster* utilize X-chromosome dosage as their primary sex determination signal, the nematode pathway acts through a negative regulatory cascade of transcriptional regulation and cell-cell signaling, but the fly pathway is regulated by alternative mRNA splicing and cell-autonomous control (Cline and Meyer, 1996). In contrast to both of these, vertebrate sex determination is initiated by the testis-determining gene on Y chromosome, *Sry*. This encodes a transcription factor that acts only in the gonad primordium, with non-gonadal sex determination mediated by hormones (Swain and Lovell-Badge, 1999).

Notably few regulatory genes in the sex determination pathway of these three animal groups have homologs, even though within each of their phyla strong homology can be detected. The isolation of *tra-1* from *C. elegans* and the non-rhabditid *Pristionchus pacificus* provides evidence for the conservation of sex determination over the past 100 million years (Pires-daSilva and Sommer, 2004). The equivalence of two genes, tra and dsx, in medfly *Ceratitis capitata*, fruit fly *Drosophila melanogaster* and the silkmoth *Bombyx mori* suggests sex determination has been controlled by the same pathway for at least 280 million years (Ohbayashi et al., 2001; Saccone et al., 1996; Saccone et al., 2002). The same is true for vertebrates, where *Sry* has been involved in sex for at least 130 million years (Marin and Baker, 1998). However, the presence of homologous genes, *dsx*, *mab-3* and *Dmrt1* in *D. melanogaster*, *C. elegans* and vertebrates, respectively, articulates the potential common ancestry of sex determination in metazoans (Raymond et al., 1998; Yi and Zarkower, 1999).

To truly understand the plasticity of sex determination, a detailed comparison of the different sex determination systems of related species is needed. These comparisons

can be used to identify the precise differences and provide the insight of the evolutionary processes (Wilkins, 2002). With their simple laboratory culture and powerful genomic and experimental tools (Haag et al., 2007; Haag and Liu, 2013), closely related *Caenorhabditis* species can serve as such a comparative animal system.

2. Mating system variation among nematode species

Sexual reproduction is the most common reproductive strategy in nematodes and transitions to different systems have occurred many times throughout the phylum (Denver et al., 2011; Kiontke and Fitch, 2005). For example, phylogenetic analysis of eighteen species from genus *Pristionchus* indicated that each of the five androdioecious species most likely evolved independently from distinct dioecious ancestors (Mayer et al., 2007). The other example is from nematode family Cephalobidae. Parthenogenetic and dioecious species were observed for both genera *Acrobeloides* and *Cephalobus*, and they demonstrate a remarkably high degree of flexibility in reproductive mode between dioecy and parthenogenesis (Smythe and Nadler, 2006). The evolutionary advantages associated with sexual reproduction have been extensively studied. One major advantage of sexual reproduction is the avoidance of deleterious mutation accumulation (Barton and Charlesworth, 1998; Otto and Gerstein, 2006). Moreover, sexual reproduction and genetic recombination may be favored by Red Queen coevolution under negative species interactions (Bell and Smith, 1987; Otto and Nuismer, 2004).

However, there are disadvantages related dioecious sexual reproduction, and under certain circumstances the transition from dioecious species to self-fertile

hermaphrodites or parthenogenetic species may be evolutionary selected. It was suggested that hermaphrodites are better early colonists when population sizes are commonly low, such as younger populations colonizing new habitats (Pannell, 2002). Previous work has shown that when the chance of encountering a mate is sufficiently low, selection can favor hermaphroditism and parthenogenesis (Eppley and Jesson, 2008; Tomlinson, 1966). Short-lived, ephemeral rotten fruits are the typical habitat for *Caenorhabditis* nematodes (Felix and Braendle, 2010). Likewise, infection of plant or animal parasitic nematodes often results in very small population sizes in the host environment. The ability to self-fertilize guarantees the production of offspring, which would be extremely beneficial in colonizing transient habitats where population density may be low.

The presence of both male-hermaphrodite (androdioecious) and male-female (gonochoristic) mating strategies in the nematode genus *Caenorhabditis* provides an excellent system to examine how reproductive transitions occur. With the exception of *C. elegans*, *C. briggsae* and *C. sp. 11*, the remaining *Caenorhabditis* species are gonochoristic, and the phylogenetic analysis of nineteen *Caenorhabditis* species in culture suggests the independent origin of self-fertility in these three lineages (Kiontke et al., 2011, Figure 1). The ability to form fertile hybrids between two very closely related sister species, the androdioecious *C. briggsae* and newly discovered gonochoristic *C. sp. 9,* renders this system even more intriguing for dissecting the mating strategy transition (Woodruff et al., 2010).

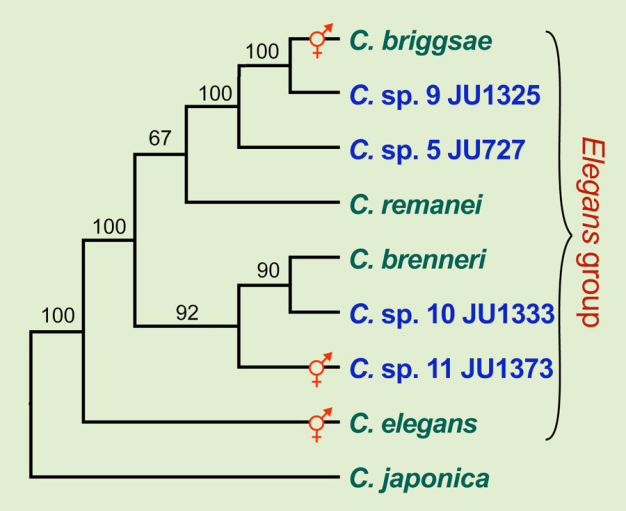


Figure 1: Phylogeny of *Caenorhabditis* species adapted from Kiontke et al., 2011

3. Molecular Evolution of Sex determination in *Caenorhabditis*

Different mating systems allow different developmental strategies for reproduction. Gonochoristic species produce XX females that make oocytes and XO males that make sperm, and are obligated to outcross for reproduction. For androdioecious species, XX hermaphrodites possess a fully female soma, but make sperm transiently before switching to produce oocytes for the rest of their lives, and are capable of either selfing or of crossing with rare XO males. These phenotypic differences are rooted in the developmental programming and genetic interactions in the germ line. By scrutinizing the molecular basis of developmental and genetic processes, we can gain deep insights of how phenotypes are produced. Then we can compare different molecular mechanisms in different lineages and start to understand their evolutionary significance. In the best case scenario, changes at the gene and protein level can be causally linked to phenotypic changes at the organismal level.

Because of the detailed characterization of its sex determination pathway (Ellis, 2008; Zarkower, 2006), *C. elegans* serves as a great reference to identify the precise differences in sex determination among *Caenorhabditis* species (Figure 2). In *C. elegans*,

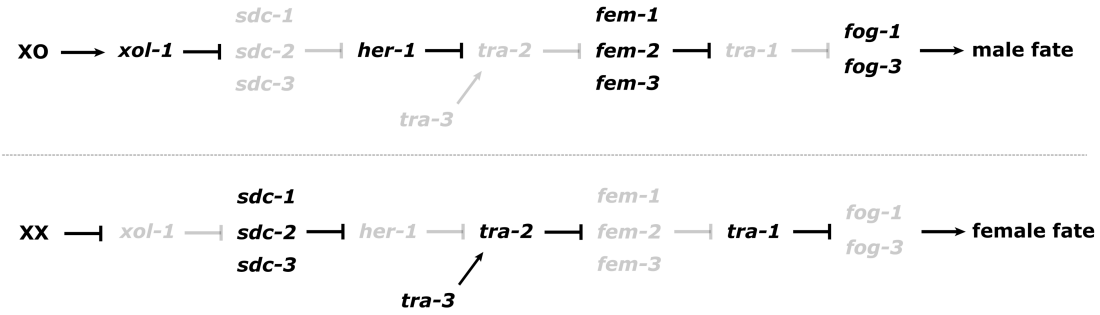


Figure 2. *C. elegans* global sex determination pathway. Genes in their inactivated states are greyed. XO stands for male karyotype, and XX stands for female karyotype.

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 (XX) in hermaphrodite repressing *her-1* transcription and a low ratio (XO) in male activating *her-1* transcription (Dawes et al., 1999; Trent et al., 1991). In the hermaphrodite soma, low *her-1* expression permits TRA-2 membrane protein activity (Perry et al., 1993), and allows it to repress the male-promoting FEM proteins (FEM-1, FEM-2, and FEM-3) (Chin-Sang and Spence, 1996; Mehra et al., 1999). This, in turn, allows the transcription factor TRA-1 to repress genes required for male development (Chen and Ellis, 2000; Conradt and Horvitz, 1999; Mason et al., 2008; Yi et al., 2000).

The global sex determination pathway described above functions in all cells, but is further modified at the post-transcriptional level in the *C. elegans* hermaphrodite germ line to allow transient spermatogenesis (Puoti et al., 2001). The translational repression of *tra-2* by FOG-2 and GLD-1 is required to initiate hermaphrodite spermatogenesis

(Clifford et al., 2000b; Goodwin et al., 1993), and the translational repression of *fem-3* by FBF-1/2 is required for the transition from spermatogenesis to oogenesis (Ahringer and Kimble, 1991; Zhang et al., 1997).

With genetic, molecular, and bioinformatic techniques recently made available in multiple *Caenorhabditis* species, comparative studies have already revealed both of the functional conservation and rapid co-evolution of protein interactions. In *C. remanei*, the functions of *fog-3* and *tra-2* orthologs are conserved (Chen et al., 2001; Haag and Kimble, 2000), although, interestingly, both the *Cr*-FEM-3/*Cr*-TRA-2 and *Cr*-FEM-3/*Cr*-FEM-2 interactions are maintained in somatic sex determination, but with partial or complete species-specific affinity (Haag et al., 2002; Stothard and Pilgrim, 2006). In the two hermaphroditic species that are the best characterized, *C. briggsae* and *C. elegans*, sex determination genes generally have corresponding orthologs (Nayak et al., 2005). However, this doesn't mean these orthologs have similar genetic functions and molecular interactions. For example, though the functions of the *tra* genes are conserved between *C. elegans* and *C. briggsae* (Hodgkin and Brenner, 1977; Kelleher et al., 2008; Kuwabara, 1996), there are exceptions of species-specific genes and discrepancies of orthologous gene functions in the germ line. *fog-2* is a recent, *C. elegans*-specific tandem duplication (Clifford et al., 2000b), and there is no *fog-2* ortholog in the *C. briggsae* genome (Nayak et al., 2005). There are two cases of changed orthologous gene function. *fem* genes promote male soma formation in both species, however, in the germ line, *C. elegans fem* mutations transform germ cells to female mode in both males and hermaphrodites (Hodgkin, 1986), while both XX and XO *C. briggsae Cb-fem-2* and *Cb-fem-3* mutants have hermaphroditic germ lines (Hill et al., 2006). The other case is that of *gld-1*: *gld-1*

loss-of-function hermaphrodites are Fog (feminization of germline; i.e. with oocytes only) in *C. elegans* (Francis et al., 1995a), but are surprisingly Mog (only sperm) in *C. briggsae* as judged by RNAi knock down or loss-of-function mutations (Beadell et al., 2011; Nayak et al., 2005).

Together, these molecular analyses and functional studies reveal that the sex determination pathways of *C. elegans* and *C. briggsae* are different in their genetic regulation in the germ line. This provides the molecular support for the phylogenetic prediction that *C. elegans* and *C. briggsae* independently acquired self-fertility from distinct gonochoristic ancestors (Cho et al., 2004; Kiontke et al., 2004). Since the germ line is where gametogenesis takes place, and the onset of spermatogenesis and the following sperm-to-oocyte switch are key steps of acquiring self-fertility in hermaphrodite, it is reasonable to believe that germ line is where the most changes of sex determination pathways occur.

4. PUF Protein Family and Translational Control

Two key regulators of the hermaphroditic sperm/oocyte switch in *C. elegans* are FBF-1 and FBF-2 (collectively called FBF), which belong to the PUF (Pumilio and FBF) mRNA-binding protein family. The PUF family is conserved across eukaryotes, including fungi, plants and metazoans (Wickens et al., 2002). PUF family members typically share a protein domain formed by eight consecutive PUF repeats, which defines both their mRNA and protein binding specificities (Edwards et al., 2001; Wang et al., 2001). PUF proteins regulate a diverse range of developmental processes, such as anterior/posterior pattern formation in *Drosophila* (Barker et al., 1992; Murata and

Wharton, 1995), germline stem cell proliferation in *C. elegans* (Crittenden et al., 2002), and mating type switch in *S. cerevisiae* (Tadauchi et al., 2001).

PUF family proteins could relate to mating system evolution and its underlying molecular causes. This is likely because of the involvement of FBF proteins in *C. elegans* germline sex determination and their generally prominent role in controlling translational machinery. In the *C. elegans* germ line, FBF allows the sperm/oocyte switch by repressing *fem-3* expression post-transcriptionally. It accomplishes this by binding specifically to *fem-3*-binding elements (FBE) in the 3' untranslated region (UTR) of the *fem-3* mRNA (Haag et al., 2002; Zhang et al., 1997). Depletion of FBF in the germ line eliminates the hermaphrodite switch from spermatogenesis to oogenesis (Zhang et al., 1997). FBF interacts with the *nanos* homolog NOS-3 and the *bicaudal-C* homolog GLD-3 to control the sperm/oocyte switch. NOS-3 acts like a FBF activator, and together they repress *fem-3* expression to promote oocyte fate (Kraemer et al., 1999). In contrast, GLD-3 antagonizes FBF function and this interaction de-represses *fem-3* expression to promote sperm fate (Eckmann et al., 2002).

fbf-1 and *fbf-2* have other important germline functions as well, related to cell cycle control. FBF represses *gld-1* mRNA translation in the distal niche to maintain mitosis of germline stem cells (Crittenden et al., 2002), but is also required for normal meiotic entry (Suh et al., 2009). These essentially opposite functions are achieved by binding of distinct protein partners of FBF: with CCF-1 it activates *gld-1* mRNA translation, and with GLD-2 it represses it (Suh et al., 2009). Moreover, the two *fbf* genes are crucial regulators of the size of the mitotic region, but they have opposite roles in fine-tuning the size (Lamont et al., 2004). Also, *fbf-1* together with another PUF family

gene, *puf-8*, act redundantly to control the hermaphrodite sperm/oocyte switch in *C. elegans* germ line (Bachorik and Kimble, 2005). Overall, it appears that various germline patterning systems that rely upon translational controls are mediated by a combinatorial network of interactions between a limited set of RNA-binding proteins.

These *C. elegans* studies set up a solid base for my comparative study of PUF protein function in other *Caenorhabditis* species. Since the sperm/oocyte switch is a key step of acquiring self-fertility in hermaphrodite, by investigating close relatives of FBFs we can draw a clearer picture about how selfing was achieved in different lineages of *Caenorhabditis*. Also, by studying and comparing PUF translational controls in different species, we can start to understand how translational networks evolve. Translational control and its evolutionary dynamics are presumably important for adaptation in tissues like the germ line, yet it has been little explored (Haag, 2009; Haag and Liu, 2013). An important underlying hypothesis of this project is that by fine-tuning translational efficiency of sex-related mRNA targets through *cis*-acting structural elements, the general translational apparatus, and specific *trans*-acting factors, organisms can produce a number of phenotypic variations. Here, I focus on the germ line of *Caenorhabditis* species, where 3'UTR mediated translational control is the predominant rule of controlling gene expression (Merritt et al., 2008), and specifically on the PUF mRNA binding protein family.

As mentioned above, the general importance of PUF family members in germline biology suggested they may be important in *C. briggsae* germline sex determination. A pilot RNAi screen of all *C. briggsae* PUF family genes found that three related genes, *Cbr-puf-1.1/1.2/2*, showed germline feminization and reduced proliferation phenotypes

when they were knocked down simultaneously (S. Feng, unpublished data). Because *C. briggsae* hermaphrodites lacking *Cbr-fem-3* can still produce sperm and are fertile (Hill et al., 2006), these three PUF genes likely have novel molecular interactions. The different PUF functions in *C. briggsae* and *C. elegans* implied by the above suggested them as a case study to characterize the similarity (or differences) in the translational control networks of these two hermaphrodites. More generally, comparative study of the PUF family allows us to learn how translational controls relate to the evolution of an important novel trait. In my dissertation research, I tried to address these issues through a variety of genetic and molecular methods.

Chapter 1: Context-Dependent Function of A Conserved Translational Regulatory Module

1. Summary

In the Introduction Chapter, I mentioned 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. In the current Chapter, I 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. I 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, whose own role in germline sex is opposite in *C. elegans* and *C. briggsae* (Beadell et al., 2011). Similar to *gld-1*, 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, I show that a *C. briggsae*-specific PUF paralog has already acquired additional essential functions, which may explain why such duplicate genes are so common.

2. Materials and Methods

2.1 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: ftp://ftp.sanger.ac.uk/pub/wormbase/WS213/genomes/). 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 cutoff threshold. Removal of likely alternative alleles in the *C. remanei* and *C. brenneri* predictions (Barriere et al., 2009), reduced family sizes to 10 and 9 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.

54 *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 sub-groups 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 PUF domain with its flanking regions (335 characters) were extracted according to known PUF protein sequence features (Wickens et al., 2002). Maximum likelihood tree search was done 5 times independently using GARLI 2.0 (Zwickl, 2006), and the tree with the best likelihood score was picked. 100 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).

2.2 Nematode culture and genetics

All nematode species were cultured by 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: LG*II: Cbr-puf-2(nm66)*, *Cbr-dpy(nm4)*, *Cbr-tra-2(nm1)*, and *Cbr-tra-2(nm9ts)*; LG*III*: *Cbr-tra-1(nm2)*, *Cbr-let(nm28);* LG*IV*: *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.

2.3 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, Natl. Inst. of Health, Bethesda, MD) was used to amplifying 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 RNA was introduced by maternal microinjection (Haag et al. 2002).

2.4 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 7.5µM 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.

2.5 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 2µl was used as template for quantitative PCR using the 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 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.

2.6 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 one million haploid genomes screened, *Cbr-puf-2* deletion *nm66* and *Cbr-unc-119* deletion *nm67* were isolated. Both alleles were outcrossed 6 times with the unmutagenized AF16 strain.

2.7 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, Natl. Inst. of Health, Bethesda, MD), 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.

2.8 Immunoblots

Triplicate samples for quantitative *Cbr*-GLD-1 immunoblots were 50 L4 worms of *Cbr-puf-2/1.2(RNAi)* or AF16 controls in sodium dodecyl sulfate (SDS) sample buffer (Russell, 2001). Primary antibodies were rabbit anti-GLD-1 polyclonal antibody (gift from T. Schedl, Washington Univ., St. Louis, MO) at 1:2000, and mouse anti-tubulin monoclonal antibody (DM1A, Sigma) at 1:1000. Secondary antibodies were HRP-donkey anti-rabbit IgG (Jackon ImmunoResearch) at 1:1000 and HRP-sheep anti-mouse IgG (GE Healthcare) at 1:1600. ECL signal intensity was quantified using ImageJ

(Abramoff, 2004). Cbr-GLD-1 protein expression was normalized to tubulin.

2.9 Immunohistochemistry

Immunohistochemistry protocol was slightly modified from that of T. Schedl (Washington Univ., St. Louis, MO), using a methanol/formaldehyde fix for 10 min at room temperature. For PH3 staining, 1:200 dilution of rabbit-anti-PH3 was applied (Upstate). Fluorescently conjugated secondary antibody (goat-anti-rabbit IgG, Alexa 488, Invitrogen) was used at 1:2,000 dilution. All gonads were dissected and stained simultaneously and in the same conditions.

2.10 Yeast reporter constructs

DNA encoding the PUF domain and flanking regions of *Cbr-puf-2* (amino acids 92-568) and *Cbr-puf-1.2* (amino acids 108-554) was cloned into the GST fusion protein vector pGEX-4T-1 (GE Healthcare) with *XmaI* and *NotI*. The same fragments were cloned into pACT2-AD (Clontech) using *NcoI* and *XmaI* to allow activation domain fusion protein expression in yeast. Sense and antisense 45bp DNA oligomers (IDT) flanking the putative FBF binding element of *Cbr-gld-1* and *Cbr-fem-3* 3'UTR were annealed and inserted into pIIIA/MS2-2 vector using *XmaI* and *SphI* 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 previously described (Bernstein et al., 2005).

2.11 Gel mobility shift assays

GST fusion proteins were isolated from T7 Express *lysY* Competent E. coli (NEB) and purified using the following elution buffer: 1×PBS, 0.2% Tween-20, 150mM NaCl,

0.1% 2-mercaptoethanol, and 50 mM glutathione (reduced, pH 8.0). 20 femtomoles ^{32}P-end-labeled RNA oligoribonucleotides (Dharmacon) were combined with GST-Cbr-PUF-2 or GST-Cbr-PUF-1.2 at various concentrations as described (Bernstein et al., 2005).

2.12 Yeast three-hybrid assay

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

2.13 Statistics

For yeast three hybrid assay data analysis, standard errors for the ratios of test to vector RNAs (Figure 4 and Figure 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*-testa were applied.

3. Results

3.1 Caenorhabditis PUF family phylogeny reveals ancient sub-family structure

Preliminary experiments with *fbf*-related *C. briggsae* PUF homologs defined by Lamont et al. (2004) suggested they were required for XX sperm production (S. Feng, QL and ESH, 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, I produced an expanded PUF phylogeny using all homologs from the five currently sequenced *Caenorhabditis*. The most likely tree (Figure 1) divides the PUF family into 9

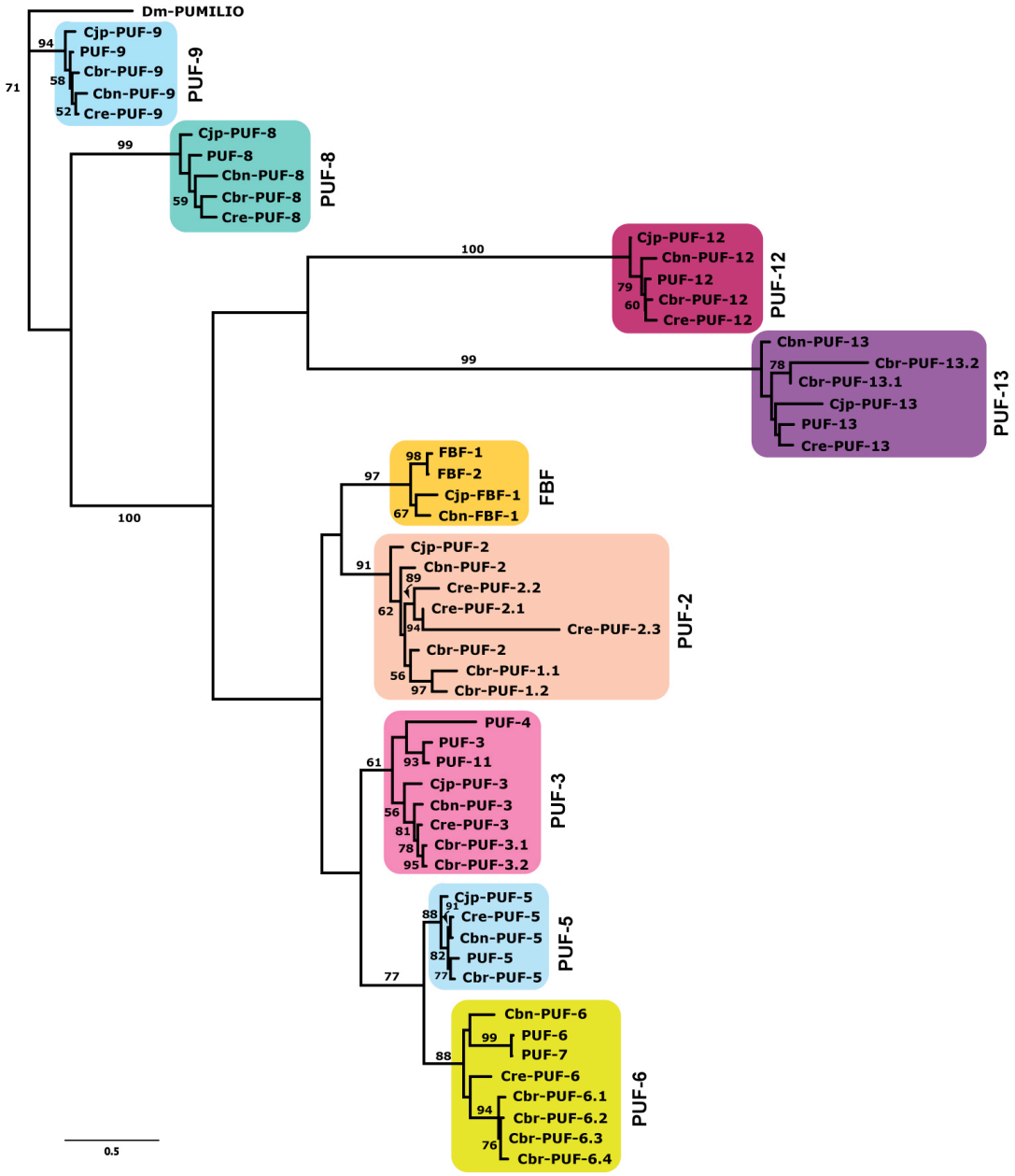


Figure 1. PUF family phylogeny for five *Caenorhabditis* species. Maximum likelihood tree based on the PUF domain and conserved flanking regions. Bootstrap support is given for internal branches. See Appendices for WormBase gene numbers and nomenclature scheme.

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 Figure 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 8 subfamilies were therefore present in the *Caenorhabditis* ancestor, and a more complete genome assembly for *C. japonica* may 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. More certain is that both belong to a well-supported super-clade of seven PUF subfamilies, two of which (PUF-5 and PUF-6/7) are closely related and share a binding preference distinct from FBF (and likely PUF-2) sub-families (Stumpf et al., 2008a). Thus, the *C. elegans* and *C. briggsae* genes whose functions are compared below are not orthologous, but belong to subfamilies that are relatively closely related.

3.2 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 knock-down 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*

Table 1. RNA interference phenotypes of *C. briggsae* PUF-2 sub-family 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%

* proximal or whole-gonadal tumor, malformed germ line and oocytes (all lack sperm)
# Fog animals can produce viable progeny when mated with males
For some of the RNAi experiments, the efficiency of knockdown was measured by qRT-PCR and found to range from 10-90%

and *Cbr-puf-1.2* (but not other combinations) led to a strongly feminized germ line (Figure 2B). *Cbr-puf-2/1.2 (RNAi)* females have normal-sized germ lines, and could mate and produce viable progeny. *Cbr-puf-2/1.2 (RNAi)* males were overtly normal and could

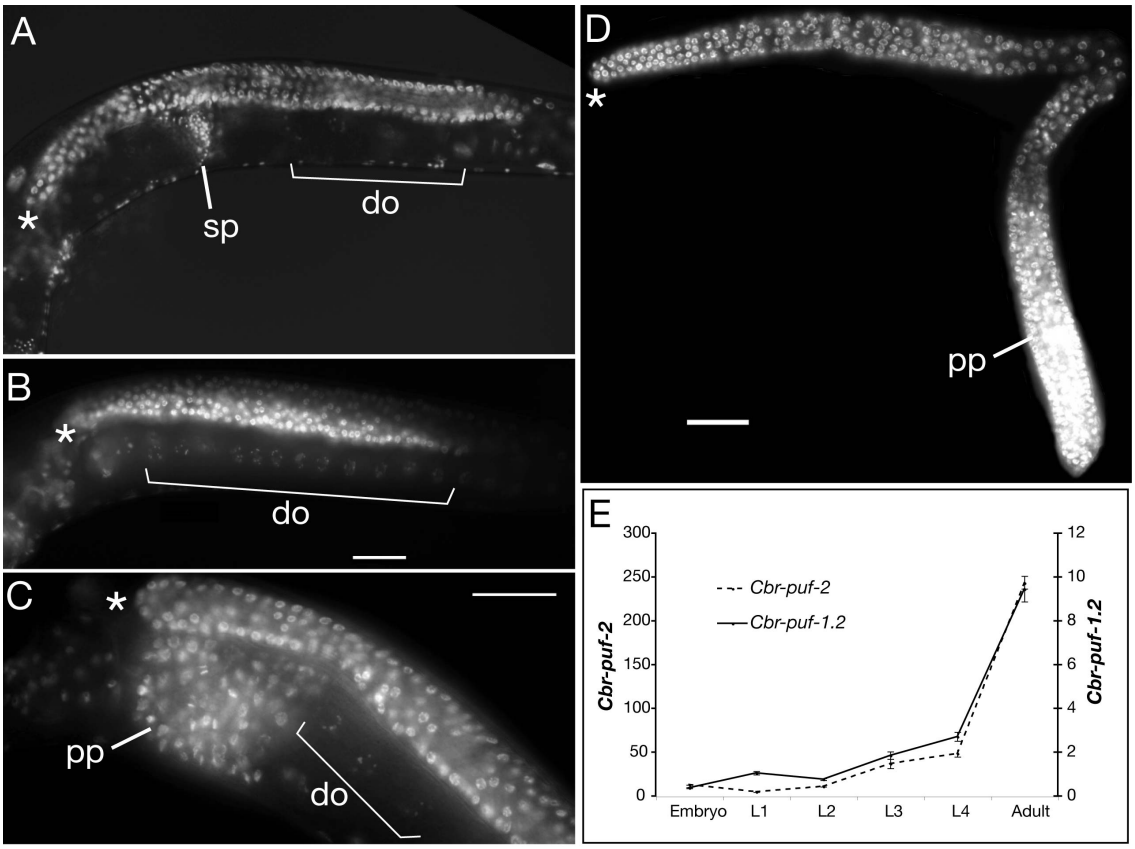


Figure 2. Expression and germline phenotypes of *Cbr-puf-2/1.2(RNAi)*. A. Wild-type *C. briggsae* adult hermaphrodite, stained with Hoechst 33258 to visualize DNA. sp, sperm, do, diakinesis oocytes. Asterisk marks the distal tip of the gonad. B. XX *Cbr-puf-2/1.2(RNAi)* Fog phenotype, commonly seen in low-dose RNAi, revealed by Hoechst staining. C-D. Proximal proliferation (pp) of germ line (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 using 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}).

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 *fbfs* 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 (0.5µg/µl) of dsRNA (Figure 2C,D). When the concentration of dsRNA was increased to 3.0µg/µl, the percentage of Fog animals decreased and more proximal tumors were observed (Table 1). In tumorous gonads, proximal over-proliferated 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 *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 *Cbr-puf-1.2* is involved non-redundantly in oocyte development.

The developmental profile of *Cbr-puf-2* and *Cbr-puf-1.2* mRNA levels (Figure 2E) are qualitatively similar to each other, and are typical of germline-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,* whose transcripts are on the order of 10^{-5} times less abundant (body-wide) than that of total actins.

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

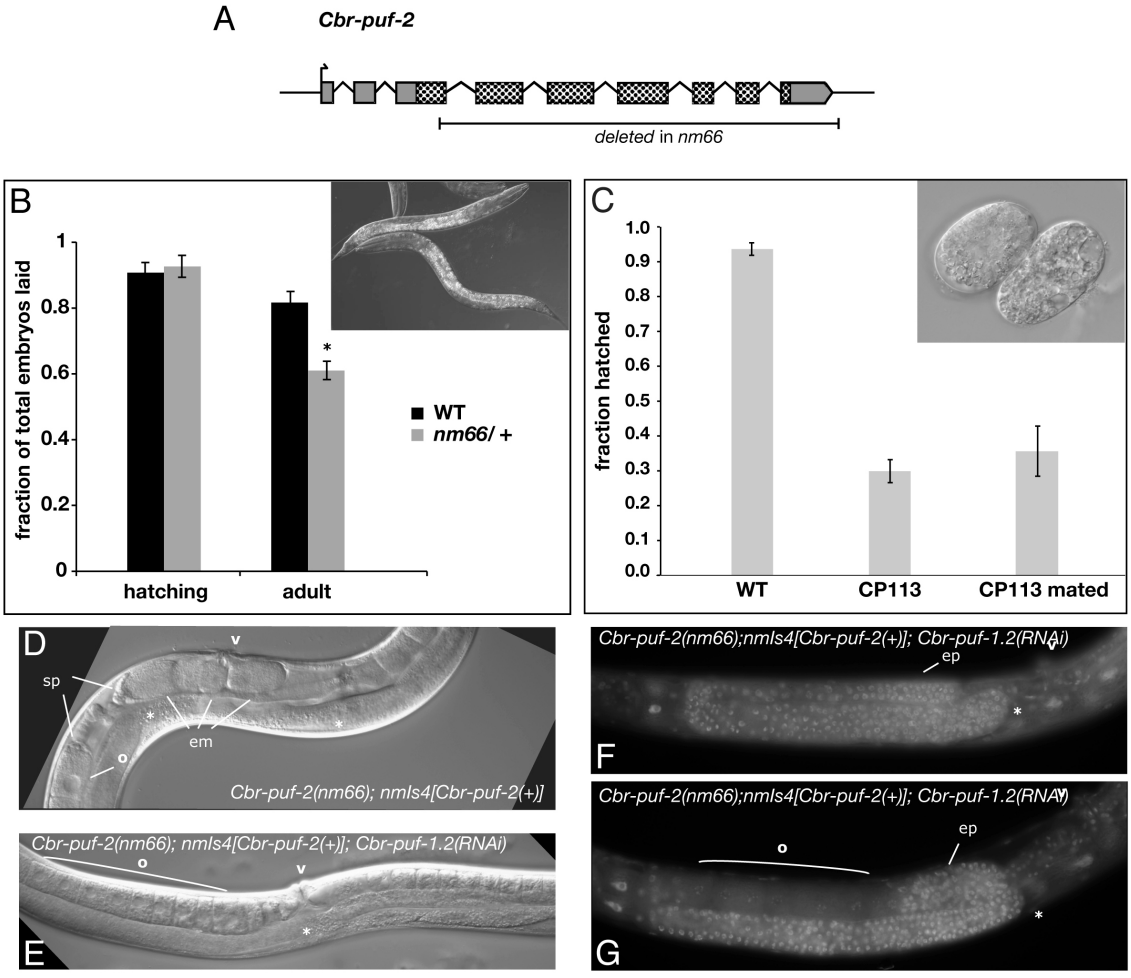


Figure 3. Pleiotropic functions of *Cbr-puf-2* in embryogenesis and larval growth. A. Structure of *Cbr-puf-2* and extent of deletion in allele *nm66*. Rectangles represent exons, with coding sequence for the conserved PUF domain and flanking regions stippled, and other coding sequences in gray. B. *Cbr-puf-2(nm66)* embryos hatch normally, but arrest as larvae. Progeny that reach adulthood were significantly fewer from *nm66/+* mothers than those from WT AF16 mothers (*p*-value: 0.003), *nm66* adults were never observed. Inset: arrested *nm66* larvae. C. Maternally deposited *Cbr-puf-2* promotes embryonic development. CP113 animals hatched at lower rates than AF16 (*p*-value<0.0001), and mating with AF16 males failed to rescue lethality (*p*-value: 0.0002). Inset: representative dead embryos. 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 produces Fog animals (D); while higher doses produce tumors (F and G).

To further study the function of *Cbr-puf-2*, two deletion alleles were isolated. For one 1.9kb genomic deletion, I failed to obtain homozygous adults, and eventually it was lost. The second allele, *nm66*, carries a 1.7kb genomic deletion that removes three quarters of the coding sequence, including the entire PUF domain (Figure 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 at an early larval stage (Figure 3B) five days after hatching at 20°C. Genotyping of arrested larvae confirmed they were *nm66* homozygotes.

To confirm that loss of *Cbr-puf-2* function causes the larval arrest phenotype in *nm66*, I introduced a wild-type *Cbr-puf-2* transgene into *nm66* mutants. This was sufficient to allow *nm66* homozygotes to develop into fertile adults (Figure 3D). The rescued strain, CP113, nevertheless had undetectably low *Cbr-puf-2* mRNA level as measured by RT-PCR. Since germline transgene silencing is a known phenomenon in *C. elegans* (Seydoux and Schedl, 2001), I 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 (Figure 2B). In CP113, however, *Cbr-puf-1.2(RNAi)* alone produces the Fog phenotype (Figure 3E), and at high doses of *Cbr-puf-1.2* dsRNA, germ line tumors became common (Figure 3F, G). These results are consistent with germline silencing of the *Cbr-puf-2* transgene in the CP113 strain. It 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.

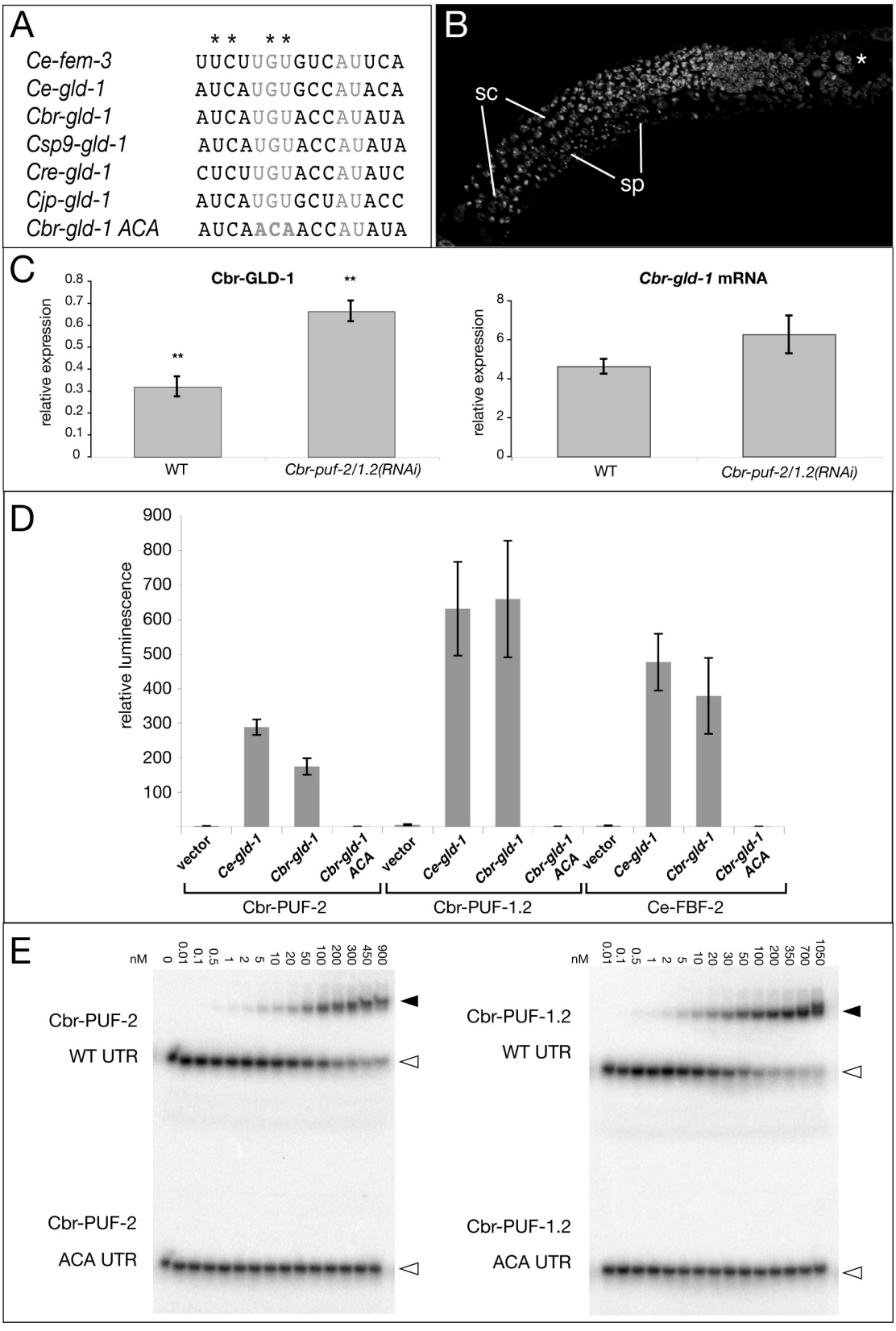
XX CP113 animals also have subtle germline defects. While they are overtly normal and fertile, they have delayed gamete maturation. Newly molted adult CP113 animals have very few yolky oocytes, and spermatocytes are just beginning to differentiate. AF16 animals at this stage generally have fully differentiated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

3.4 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 (FBE) in their 3' UTRs (Crittenden et al., 2002; Suh et al., 2009; Zhang et al., 1997). The binding elements contain a "core" central

Figure 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: invariant core residues mutated in the ACA variant tested in panels D & E. Asterisks: *fem-3(gf)* 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. C. Cbr-GLD-1 protein level is significantly higher in *Cbr-puf-2/1.2(RNAi)* than in wild-type L4 worms, while *Cbr-gld-1* mRNA level is not. Error bars show standard errors; *p*-values (unpaired Student *t*-test) are 0.006 and 0.168 for protein level and mRNA level, respectively. D. Yeast three-hybrid interactions among *C. elegans* and *C. briggsae* PUF proteins and *gld-1* mRNA variants. RNA plasmid pIIIa serves as the plain vector control. 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 arrows indicate mobility-retarded complexes of labeled RNA oligomers and pure PUF proteins, while open arrows indicate free RNA oligomers.

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-nucleotide stretch that is nearly identical to the *C. elegans gld-1* FBE (Figure 4A). Moreover, loss of *Cbr-gld-1* function masculinizes the germ line (Beadell et al., 2011; Nayak et al., 2005), suggesting its normal function is to promote oogenesis. I hypothesized that *Cbr-gld-1* might be hyperactive in *Cbr-puf-2/1.2(RNAi)* animals, and thus completely repress hermaphrodite spermatogenesis.

I 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 (Figure 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) are pproximately twice as high in *Cbr-puf-2/1.2(RNAi)* worms as in wild-type (Figure 4C), a statistically significant result (unpaired student t-test; p=0.006). In contrast, there is no significant difference in *Cbr-gld-1* transcript levels in the two treatments (Figure 3C, 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 (Figure 4D). *C. elegans* FBF-2 also interacts 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, I used synthetic ribo-oligonucleotides encoding the candidate FBE and purified proteins in gel mobility shift assays. Both Cbr-PUF-2 and Cbr-PUF-1.2 bind with high affinity to the *Cbr-gld-1* FBE (Figure 4E), and this interaction required the UGU motif 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.*

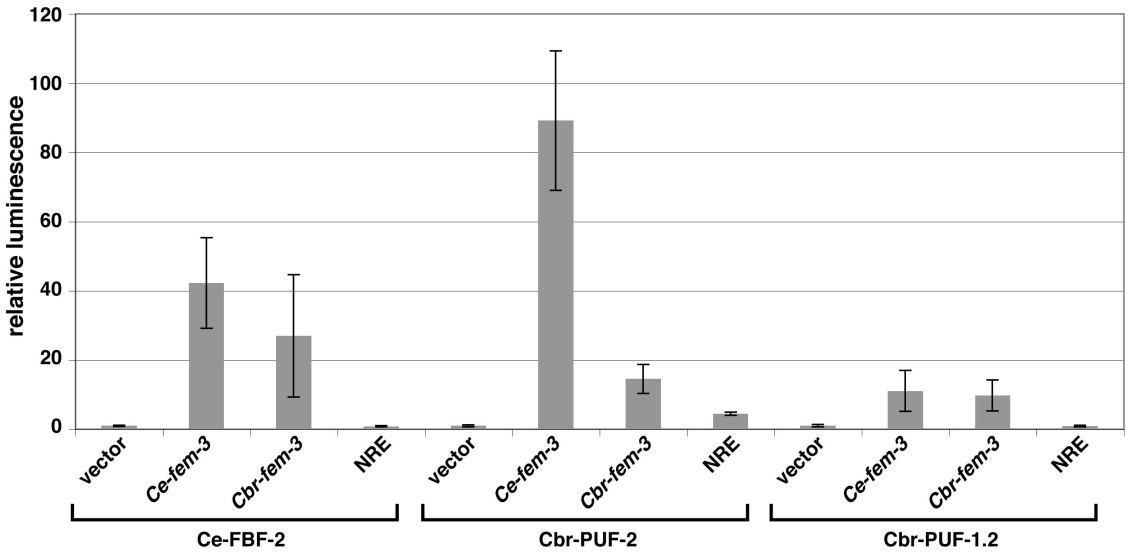


Figure 5. Conservation of *fem-3* PME binding by *C. briggsae* PUF proteins. Yeast three-hybrid assays were performed to assess interaction of the protein and bait RNA fragments indicated. To account for potential variation in expression of a particular test protein, reporter activity for each RNA bait is normalized to that of the vector control.

briggsae fem-3 3' UTR also possesses a well-conserved FBF-like binding site, the point mutation element (PME) (Haag et al., 2002). In yeast three-hybrid assays, Cbr-PUF-1.2 and Cbr-PUF-2 interact specifically with PME-containing fragments from *C. elegans* and *C. briggsae fem*-3, and *C. elegans* FBF interacts with *C. briggsae* and *C. elegans fem-3* PME fragments to a similar extent (Figure 5). 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).

3.5 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, I 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), while 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 (Figure 6C). 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 2) had two gonads full of sperm with no sign of oogenesis (Figure 6D). Genotyping confirmed that these female soma/Mog 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.

Table 2. Interactions between sex determination mutations and *Cbr-puf-1.2/2* knockdown. The first column: the genotypes of the mothers that *Cbr-puf1.2/2* RNAi knockdown was operated. The second and third columns: the somatic (2^{nd}) and germline (3^{rd}) phenotypes of self progeny from mothers listed in the first column. The fourth column: the germline phenotypes of self progeny with RNAi. The fifth column: the number of animals observed showing the phenotype as the fourth column. The sixth column: confirmed *tra* lesion by sequencing.

genotype of injected mother	self progeny class by somatic phenotype[1]	self progeny class by gonad phenotype[1]: non-RNAi	gonad phenotype of progeny: RNAi	Number scored	genotype (T = *nm1* or *nm2*, C = WT)
Cbr-tra-2(nm1)/ Cbr-dpy(nm4)	A class: Tra pseudo-male soma	One armed gonad with sperm only	Sperm+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[2]	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.

Table 2. continued

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 + 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[3]	Two armed gonads with both sperm and oocytes	Fog	57	T/C, C/C
	B class[3]		self-fertile	21	T/T

[1] no RNAi phenotype
[2] poorly formed vulva and tail, undifferentiated germ line
[3] All progeny have female somas due to *Cbr-fem-3(nm63)* - Not genotyped
N. D.: not determined

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 germline 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.

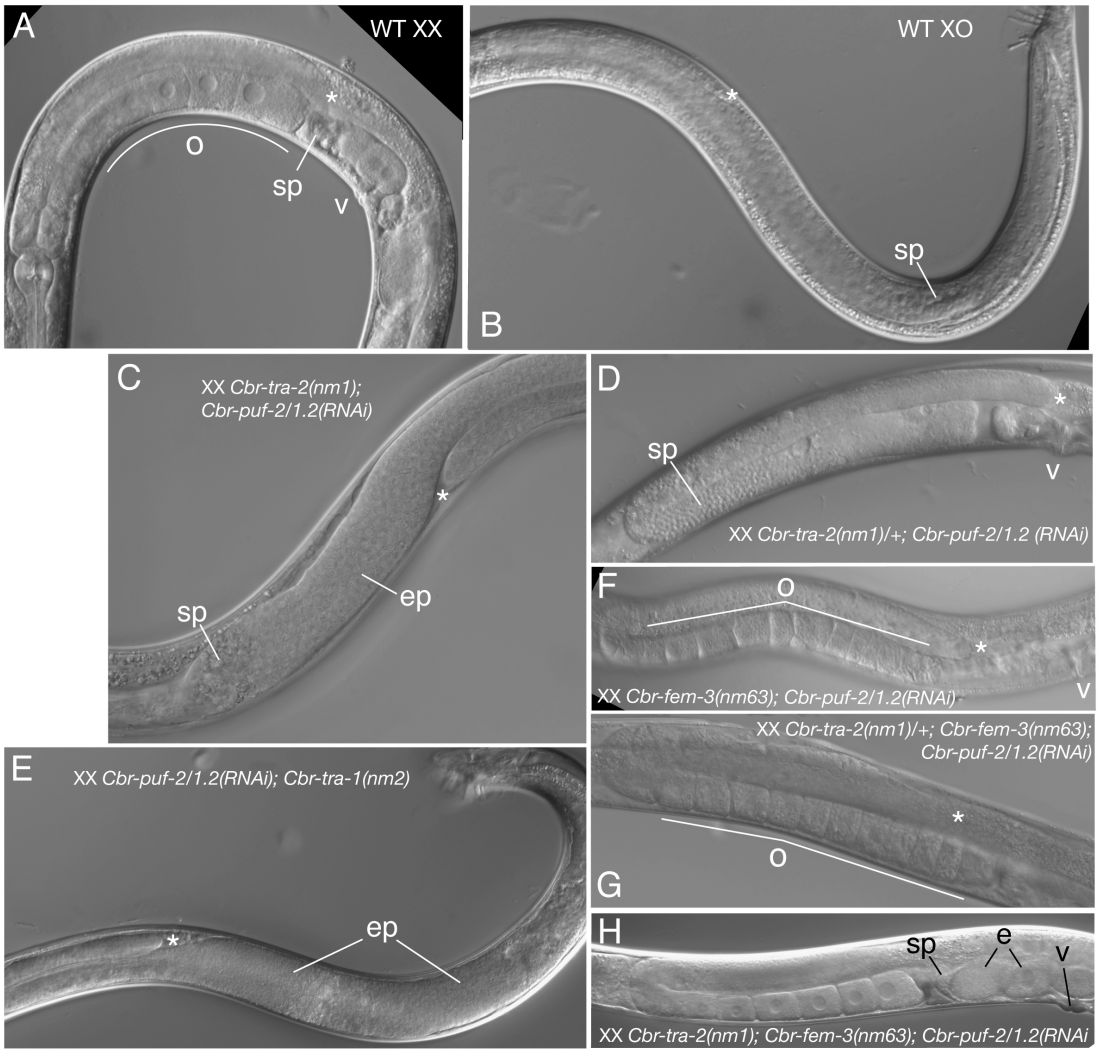


Figure 6. Interaction between *Cbr-puf-2/1.2* knockdown and masculinizing *tra* mutations. A. Wild-type XX *C. briggsae* hermaphrodite gonad arm (anterior), showing mature oocytes (o), sperm (sp) in the spermatheca, and vulva (v). The distal tip of the reflexed arm is marked with an asterisk. B. Wild-type *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 (sp) proximal to ectopically proliferative (ep) germ line 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 tumorous germ line without apparent gametogenesis. F. *Cbr-puf-2/1.2(RNAi);Cbr-fem-3(nm63)* animals are Fog; oocytes (o). 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).

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. 77% developed germline tumors without apparent gametogenesis (Figure 6E), 17% had differentiated oocytes distal to tumorous germ cells, and the rest 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, since wild-type XO males show no such defect.

I 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 (Figure 6F), suggesting that *Cbr-puf-2/1.2* and *Cbr-fem-3* do not have obvious genetic interaction. To further test a simple linear model, I 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, while all *Cbr-tra-2(nm1)/+; Cbr-puf-2/1.2(RNAi);Cbr-fem-3(nm63)* animals were Fog (Figure 6G, 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 (Figure 6H). 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.

3.6 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. I therefore sought to clarify the evolutionary history of FBF and PUF-2 subfamily genes' functions in germline sex determination. RNAi knockdown by direct injection of dsRNA into the germ line is efficient in a range of *Caenorhabditis* species (Winston et al., 2007), so I 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 under-proliferation, ranging from fewer than usual germ cells to complete loss (Figure 7C-J). A notable exception, however, was knockdown of *C. brenneri fbf-1*. In this case, germ cells appear to exit meiosis and reenter the mitotic cell cycle, producing a germ cell tumor (Figure 7E, 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.

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

C. sp. 9	Male		Female	
	Number	Phenotype	Number	Phenotype
Csp9-puf-1.1/2[1]	65	GD	68	GD, OD
Csp9-puf-2/1.2[1]	83	GD	68	GD, OD
Csp9-puf-1.1/1.2[1]	38	GD	58	GD, OD
Csp9-puf-2[1]	50	Normal	50	Normal

Table 3 continued

C. brenneri	Male		Female	
	Number	Phenotype	Number	Phenotype
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	Male		Female	
	Number	Phenotype	Number	Phenotype
puf-2	95	GD[2]	98	GD[2]
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	Male		Female	
	Number	Phenotype	Number	Phenotype
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

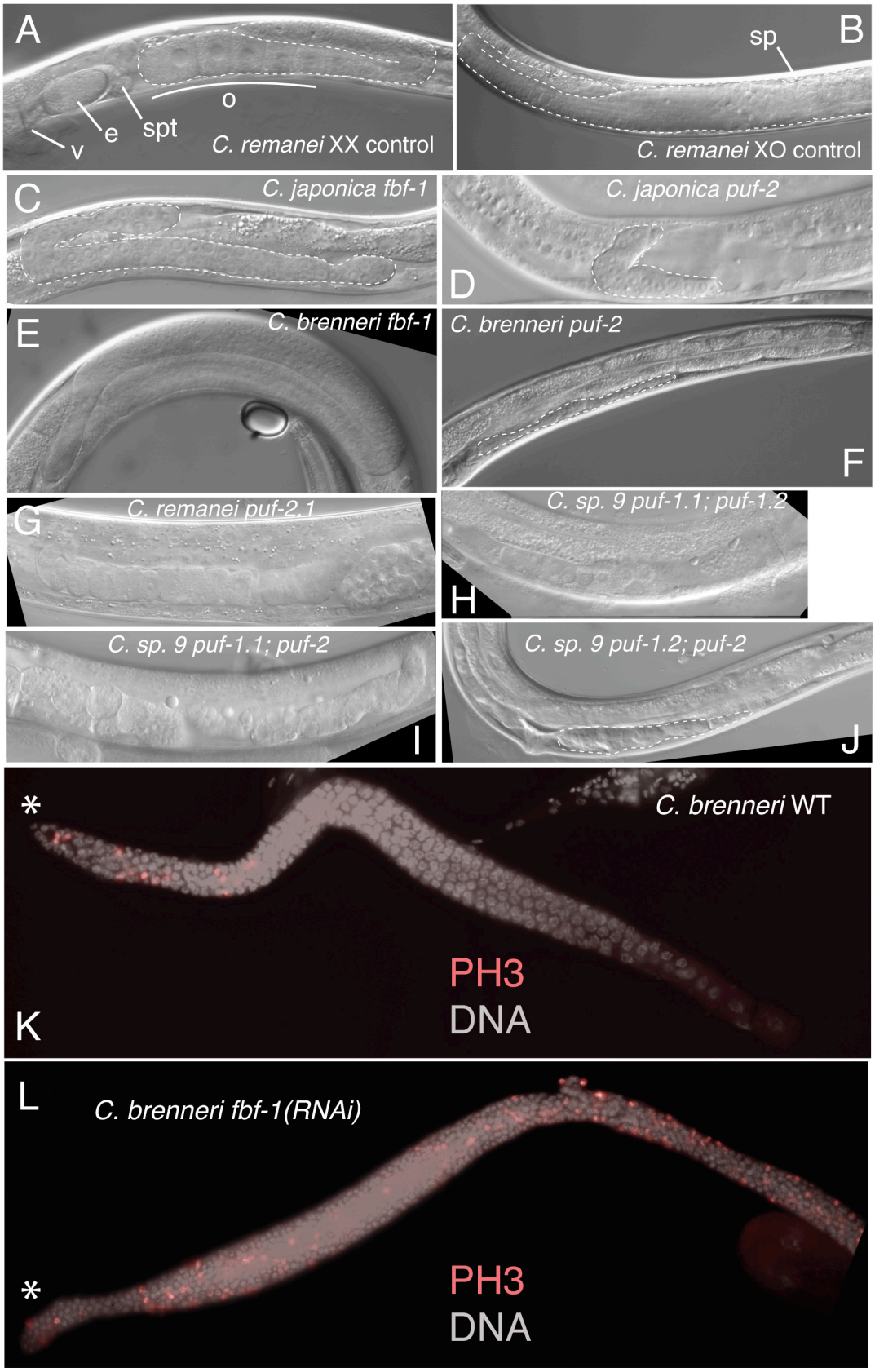
[1] primers designed according to *C. briggsae* orthologs and dsRNA derived from *C.* sp. 9 cDNA
[2] dsRNA derived from *Cre-puf-2.1*, but also has stretches of high similarity to *Cre-puf-2.2*.
F: female, M: male, GD: germline degeneration, OD: oogenesis defect, EL: embryonic lethal, LA: larval arrest, N.A.: not applicable (see Figure 1)
Numbers account for all observations.

4. Discussion

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

Since 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 sub-families are completely unknown. Phylogenetic reconstruction unambiguously groups PUF proteins into nine distinct subfamilies, and shows that Ce-FBF and Cbr-PUF-1.1/1.2/2 are members of different sub-families 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, in hermaphrodite germline sexual patterning, evolved as the *C. elegans* and *C. briggsae* lineages adopted self-fertility (Figure 8A).

Figure 7. PUF family knockdown in gonochoristic *Caenorhabditis*.
A, B. Untreated adult *C. remanei* female and male, with germ lines outlined; e, embryo; o, oocytes, spt, spermatheca; v, vulva. B-J, RNA interference directed against *C. japonica*, *C. brenneri*, *C. remanei* and *C.* sp.*9* (as indicated). PUF homologs generally produced a germline underproliferation (C, D, F, J) or abnormal germline degeneration (G-I). In contrast, *C. brenneri fbf-1(RNAi)* (E) produces a germ cell tumor. K, L. Merged fluorescent images of DNA (gray) and phospho-histone 3 (PH3; red) staining of extruded XX *C. brenneri* gonads from untreated (I) or *Cbn-fbf-1(RNAi)* (J) animals. Mitotic nuclei are localized to the distal stem cell niche (*) in wild-type females (K), but distributed throughout the gonad in *Cbn-fbf-1(RNAi)* (L).


A
v
e
spt
o
C. remanei XX control
B
sp
C. remanei XO control
C
C. japonica fbf-1
D
C. japonica puf-2
E
C. brenneri fbf-1
F
C. brenneri puf-2
G
C. remanei puf-2.1
H
C. sp. 9 puf-1.1; puf-1.2
I
C. sp. 9 puf-1.1; puf-2
J
C. sp. 9 puf-1.2; puf-2
*
C. brenneri WT
PH3
DNA
K
L
C. brenneri fbf-1(RNAi)
*
PH3
DNA

4.2 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., 2000a; 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 I 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 (Figure 1).
3. The sexual transformations of both *gld-1* orthologs and PUF-2/FBF genes (Figure 2) are opposite in *C. elegans* (Clifford et al., 2000b; 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).

I 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.

I 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 both FBF-2 and Cbr-PUF-2 and Cbr-PUF-1.2 (Figure 5). Thus, 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. Though 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 over-expression being the chief mechanism by which *Cbr-puf-2/1.2(RNAi)* feminizes the hermaphrodite germ line.

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

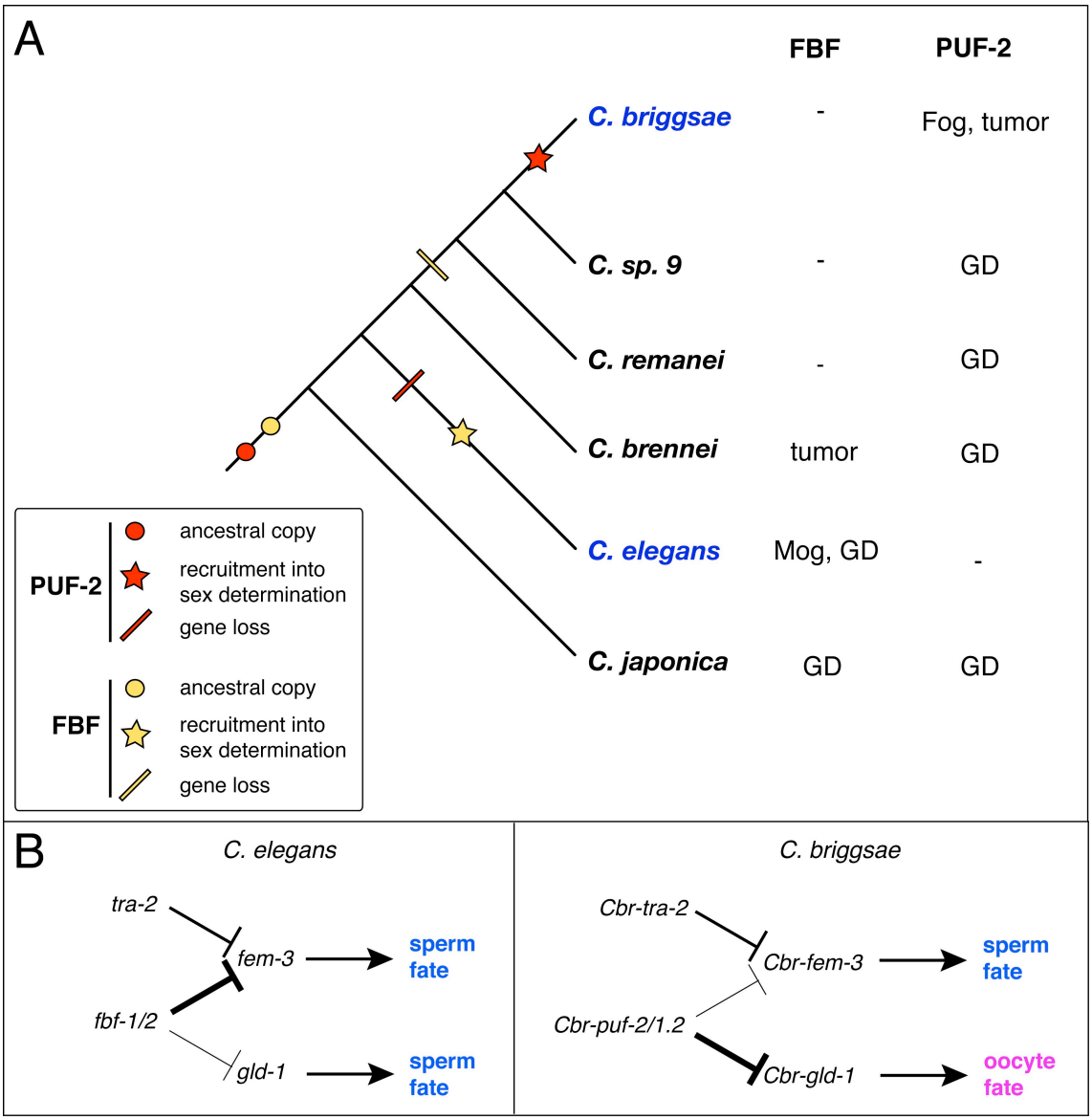


Figure 8. Models of FBF and PUF-2 subfamily evolution. A. Cladogram of *Elegans* group *Caenorhabditis* (Base on Kiontke et al., 2004; Woodruff et al., 2010) and summaries of knockdown phenotypes for FBF and PUF-2/1.2 sub-families from this study (GD, germ line degeneration; Mog, masculinization of germ 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* (left) and *C. briggsae* (right). 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.

PUF-2 and FBF sub-family gene knockdowns (Figure 7) 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. However, I 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. In 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 (Figure 8).

The repeated recruitment of PUF and *gld-1* (Beadell et al., 2011) homologs into hermaphroditic germline sex determination may reflect the general reliance of germline development on posttranscriptional 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 predate the origins of self-fertility. This overlap may 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 not (Figure 4A).

Taken together, it is likely that the last common ancestor of the FBF and PUF-2 sub-families both repressed *gld-1* translation in the service of regulating germline proliferation. Extant *Caenorhabditis* species have then modified this situation by losing one or the other sub-family entirely (but never both) and duplicating genes within a given sub-family. Layered upon this is the cooption of the entire PUF-*gld-1* module into hermaphrodite development. Though this happened in both characterized selfing species (and may 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) may therefore promote their continued regulatory linkage in the face of altered germline phenotypes.

4.4 Evolution of genetic interactions between PUF targets

C. elegans fbf-1/2 hypomorphs or mutants are Mog (Zhang et al., 1997) due to *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 may 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 (Figure 5) suggest simultaneous up-regulation of Cbr-GLD-1 and Cbr-FEM-3 may 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: 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 (Figure 8B). 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 (Figure 6D). I 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 (Figure 8B). 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.

4.5 Pleiotropy and redundancy in the PUF family

The nine PUF subfamilies, while 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 *Cbr-puf-2* is required in the maternal germline 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 represents was ancestral but lost in *C. elegans*, perhaps associated with loss of the PUF-2 sub-family, or 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 may explain their evolutionary persistence (Force et al., 1999).

4.6 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 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 *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 sub-family genes in gonochoristic species led to germline degeneration (Figure 6). This suggests that the ancestral function of both 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 was 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 sub-families 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.

4.7 Evolution of gene regulation at the translational level

Examples of cis-regulatory DNA as the locus of genetic variation underlying novel phenotypes are accumulating, 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 like the germ line, yet it has been little explored (Haag, 2009). 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 may take on altered significance due to changes in the role of target mRNA (as appears to be the case with PUF-*gld-1*) or to variation in RBP protein cofactors that qualitatively or quantitatively modify conserved RBP-mRNA interactions, such as FOG-2, a GLD-1 cofactor in *C. elegans* (Clifford et al., 2000b; Nayak et al., 2005). FBF cofactors have also been reported (Kraemer et al., 1999; Suh et al., 2009). Clarification of the precise biochemical roles(s) of such cofactors is an important subject of future research.

Chapter 2: *C. briggsae puf-2* has multiple essential somatic roles in postembryonic development

1. Summary

In Chapter 1, I found that *Caenorhabditis briggsae* mutant animals with a deletion in the PUF family RNA-binding protein gene *puf-2* fail to reach adulthood and arrest at the second larval stage. This unexpected phenotype has not been reported for any characterized *C. elegans* PUF family member. In this chapter, I describe my detailed characterization of the *Cbr-puf-2(nm66)* mutant phenotype. I find that the larval arrest phenotype of *Cbr-puf-2* mutant animals is caused by inefficient breakdown of bacteria food, which leads to insufficient nutrient intake and developmental arrest. A *Cbr-puf-2* promoter reporter shows transient expression only in pharyngeal muscle cell 7 during a brief window from the late four-fold embryo to the early first larval stage. Other data suggest that *Cbr-puf-2* is involved in sustaining the normal muscular strength of the terminal bulb during larval progression, by acting in cell 7. In addition, I discover that *Cbr-puf-2* functions in the vulval cell lineage to promote vulval development. My study of *Cbr-puf-2* gene unveils a suite of pleiotropic developmental functions, and reveals the amazing functional plasticity of PUF RNA binding proteins.

2. Introduction

Proper physiological control is vital to maintain normal body function and homeostasis. Malfunction of physiological control early in development usually results in severe defects later in life. Some well-known disorders of this kind are muscular dystrophies, genetic diseases that involve progressive muscle weakness and loss of muscle tissue over time (Amato and Griggs, 2011). In *Caenorhabditis elegans*, the pharynx, a bilobed neuromuscular tube that connects stoma to intestine, has been served as a powerful model to study mechanisms of organogenesis and physiological control (Avery and You, 2012; Mango, 2009). Towards the end of embryonic development, *C. elegans* embryos complete their pharyngeal organogenesis (Sulston et al., 1983), and newly hatched larvae have fully functional pharynxes. The pharynx is comprised of seven distinct but functionally integrated cell types (Mango, 2007), and is capable of producing a rapid and coordinated pumping action that is essential for the ingestion and mechanical breakdown of food (Avery and You, 2012). In *C. elegans*, physiological control of pharyngeal function has been understood at the level of firing frequency and timing of pharyngeal muscles and neurons (Avery and You, 2012), and behavioral features about food ingestion and transportation in the pharynx have also been studied in detail (Avery and Shtonda, 2003; Fang-Yen et al., 2009).

The pharynx has three functional parts, which from anterior to posterior are the corpus, the isthmus, and the terminal bulb (Avery and Horvitz, 1989). Structurally, there are eight muscle cell layers extending from anterior to posterior of the pharynx, and each muscle layer is composed of muscle cells arranged as a three-fold symmetric tube encircling the lumen (Albertson and Thomson, 1976). Coordinated contraction and relaxation of these consecutive muscle sectors produces feeding behavior (Avery and

You, 2012). The radially oriented pharyngeal muscle cells (pm), pm1 to pm5, coordinate two sequential pharyngeal motions: pharyngeal pumping and isthmus peristalsis. Each cycle of pharyngeal pumping starts with corpus muscle contraction mediated by pm1 to pm4, which opens the pharyngeal lumen and draws in food particles. Followed by relaxation, the pharynx traps food particles in the anterior isthmus and expels liquid. Then the contraction of isthmus muscle cell pm5 initiates the second pharyngeal motion, isthmus peristalsis. This motion transports food particles from the anterior isthmus to the grinder in the terminal bulb. It is known that the mechanic force provided by pm6 and pm7 in the terminal pharyngeal bulb inverts the plates of the grinder, which breaks up bacteria and passes the debris back to the intestine. However, it is not clear that how muscular strength of the terminal bulb is established and maintained during larval progression and adulthood.

In my dissertation research, I discovered an unexpected pharyngeal function of *C. briggsae puf-2*. *Cbr-puf-2* belongs to a widespread RNA-binding protein family, the PUF family, whose family members control diverse biological processes (Wickens et al., 2002). In Chapter 1, I discovered that *Cbr-puf-2* is required for reliable embryogenesis, and is essential for developmental progression of the newly hatched larva. Since PUF family genes are renowned for their germline functions in *C. elegans*, and none of them are essential in early development, such crucial roles of *Cbr-puf-2* in *C. briggsae* are surprising. In the current research, I find that the larval arrest phenotype of *Cbr-puf-2* mutant animals is caused by inefficient breakdown of bacteria food, which leads to insufficient nutrient intake and developmental arrest. My data suggest that *Cbr-puf-2* is involved in sustaining the normal muscular strength of the terminal bulb during larval

progression, specifically, by acting in pharyngeal muscle cell 7. In addition, I discover that *Cbr-puf-2* functions in vulval cell lineage to promote vulval development. My study of *Cbr-puf-2* gene unveils a full spectrum of its developmental dynamics, and reveals the amazing functional plasticity of PUF RNA binding proteins.

3. Materials and Methods

3.1 Nematode culture and genetics

All nematode species were cultured by using standard *C. elegans* conditions (Wood, 1988), with the use of 2.2% agar plates to discourage burrowing. The *C. briggsae* mutant, *Cbr-puf-2(nm66)*, was derived from the wild isolate AF16, and is on linkage group II (Liu et al., 2012). *nm66* was maintained in a pseudo-balanced strain CP102, whose genotype is *Cbr-cby(nm15)/Cbr-puf-2(nm66)*. Homozygous *Cbr-cby(nm15)* mutant animals are dumpy (Dpy). Strain CP113 (*Cbr-puf-2(nm66)*, *nmIs4*[*Cbr-puf-2(+)*, *Cbr-unc-119 (+)*]) is transgene-rescued *Cbr-puf-2* mutant strain (Liu et al., 2012). The *C. briggsae pha-4* reporter strain, RW20019 (*Cbr-unc-119*, *stIs20019*[*pha-4*∷mCherry, *unc-119 (+)*]), uses the *C. elegans pha-4* promoter and first intron to drive red fluorescence in a conserved pattern (Zhao et al., 2010)) to drive. *C. briggsae* strain CP127, which carried both *nmIs4* and *stIs20019* reporter transgenes, was constructed by crossing RW20019 and *puf-2* reporter strain CP126 (described below).

3.2 Cbr-puf-2 transcription reporter

For constructing a transcription GFP reporter of *Cbr-puf-2*, its 5' and 3' regulatory regions were fused with histone2B-GFP (H2B) chimeric coding sequence via

Gateway cloning technology (Invitrogen) into destination plasmid pCR40 (gift from C. Richie), which also contains the wild-type *Cbr-unc-119* gene. When combined with the complete *Cbr-puf*-2 coding sequence, these regulatory regions successfully rescue the *Cbr-puf-2(nm66)* mutant phenotype (Liu et al., 2012). This plasmid was introduced into *Cbr-unc-119(nm67)* mutants through biolistic bombardment (Praitis et al., 2001). Stable non-Unc lines were examined for transgenic expression, and one strain, CP126 (*Cbr-unc-119(nm67)*, *nm5*[*Cbr-puf-2*::H2B, *Cbr-unc-119 (+)*]), was identified with stable GFP expression.

3.3 Microscopy

Nematode differential interference contrast (DIC) microscopy was followed by standard methods (Wood, 1988). In brief, worms were put on 2% agarose pad, and mounted in M9 buffer or Vectashield (Vector Laboratories) for DIC microscopy or fluorescence microscopy, respectively, using an Axioskop2 *plus* (ZEISS). Images were captured with Axiocam digital camera (ZEISS) and Open Lab software (Improvision) or an LSM710 confocal microscope (ZEISS). In the latter, z-stacks were collapsed for presentation using ZEN lite 2011 (ZEISS).

3.4 Pharyngeal pumping rate assay

Motions of the grinder plate in the terminal bulb were used to count pumping rate. Wild type worms at the second larval stage or *Cbr-puf-2(nm66)* mutant worms 3-day post hatching were placed on a lawn of *E. coli* OP-50 on an agar plate and allowed to acclimate for at least 2 hours. Counts were made at room temperature. Each worm was observed three times for 20 seconds using Axioskop2 *plus* (ZEISS) with a 20× objective. Pumping rate per minute was calculated. Videos were taken with an eye-piece digital

microscope camera (AM-423X, Dino-Eye) at 15 frames per second speed. Videos were slow-played at one fourth of the recorded speed for accurate pumping rate counting.

3.5 Feeding assay

E. coli bacteria strain BL21 expressing recombinant GFP protein (gift from Hamza Lab, UMD) was used to assay pharyngeal function. Wild type AF16 and strain CP113 animals at the second larval stage or *Cbr-puf-2(nm66)* mutant animals 3-day post hatching were placed on a lawn of *E. coli* BL21 strain expressing GFP, and allowed to acclimate for at least 3 hours. Then, worms were put on agarose pad with a drop of 50mM sodium azide, and mounted in Vectashield (Vector Laboratories). Images were taken using Axioskop2 *plus* (ZEISS) with a 63× objective with the same setting.

3.6 Video image analysis

Using video microscopy, I observed animals for differences in terminal bulb grinding behaviors. The assay was adapted from a developed protocol (Chiang et al., 2006). Wild type worms at the second larval stage or *Cbr-puf-2(nm66)* mutant worms 3-day post hatching were transferred to bacterial suspension placed on a thin agarose pad, and a coverslip was placed on top. I typically waited about 30 minutes before making observations to allow animals recovering from the perturbation. Pharyngeal motions were then observed using Axioskop2 *plus* (ZEISS) with a 63× objective. Videos were taken with an eye-piece digital microscope camera (AM-423X, Dino-Eye) at 30 frames per second speed. I viewed image sequences frame by frame and manually extracted consecutive frames corresponding to the cycle of terminal bulb contraction using iMove v7.1.4 (Apple).

3.7 Axenic culture

To grow *Cbr-puf-2(nm66)* mutant animals in axenic culture, I washed embryos laid by *Cbr-cby(nm15)/Cbr-puf-2(nm66)* parents off of NGM plate media with M9 buffer, and bleached them with a mixture of sodium hypochlorite and sodium hydroxide. After bleaching, the clean embryos were washed in M9 for three times to remove traces of bleach solution. The embryos were allowed to hatch and grow in axenic modified *C. elegans* habitation and reproduction (mCeHR-2) medium supplemented with hemin chloride at 24°C with continuous shaking (Nass and Hamza, 2007). 10 ~ 14 days later, worms at the fourth larval and adult stages were collected and singled on fresh agar plates for recovery overnight. On the following day, singled animals were imaged and genotyped for the presence of the *Cbr-puf-2(nm66)* allele using single-worm PCR (Kelleher et al., 2008).

3.8 Phalloidin staining

Wild-type *C. briggsae* worms at the second larval stage or *Cbr-puf-2(nm66)* mutant worms three-day after hatching were collected and rinsed with PBS to remove bacteria. The following protocol was adapted from Shaham (2006). Cleaned worms were moved to an eppendorf tube and frozen in liquid nitrogen, followed by an immediate lyophilization in a speedvac. Then, worms were treated with 3–4 drops ice-cold acetone for 3 minutes. After the removal of acetone and vacuum-dry, 2U fluorescein-conjugated phalloidin (Molecular Probes) diluted in 20ul S mix (0.2M Na-phosphate, pH 7.5; 1mM $MgCl_2$; 0.004% SDS) was added to the dry worms. Worms were stained at room temp in the dark for1 hour. At the end of staining, worms were washed twice in PBBT (PBS + 0.5% BSA+0.5% Tween-20), and mounted for microscopy.

4. Results

4.1 Cbr-puf-2 sustains adequate pharyngeal function in C. briggsae

Shortly after hatching, homozygous *Cbr-puf-2(nm66)* mutant animals moved actively and looked overtly normal, although they had reduced movement when aged. I followed their development for two weeks after hatching, and found that they accumulated refractile vacuoles in their intestine, which increased with time (Figure 1C - 1E). These phenotypes might result from starvation caused by defective pharyngeal function (Schroeder et al., 2007). However, the pharynx of mutant animals did not display morphological abnormalities, and the coordinated pharyngeal pumping movement was also maintained. I hypothesized that *Cbr-puf-2(nm66)* mutants have subtle pharyngeal defects at the cellular or physiological level. To test this, I first examined the frequency of pharyngeal pumping. Since terminal pharyngeal bulb powers the breakdown of food particles, and its movement is coordinated with the whole pharynx (Avery and Horvitz, 1989), I compared the terminal bulb grinding rates of *Cbr-puf-2(nm66)* mutant animals three-day after hatching with wild type animals at the second larval stage. Mutant animals three-day after hatching are developmental equivalents of wild type animals at the second larval stage. While the average grinding rates of wild-type animals were 263±10/min, the average grinding rates of 3-day-old mutants were slightly lower, 215±20/min (Figure 2).

While intriguing, the relatively minor shift of the grinding rate noted above seemed insufficient to cause complete developmental arrest. It also may reflect a side

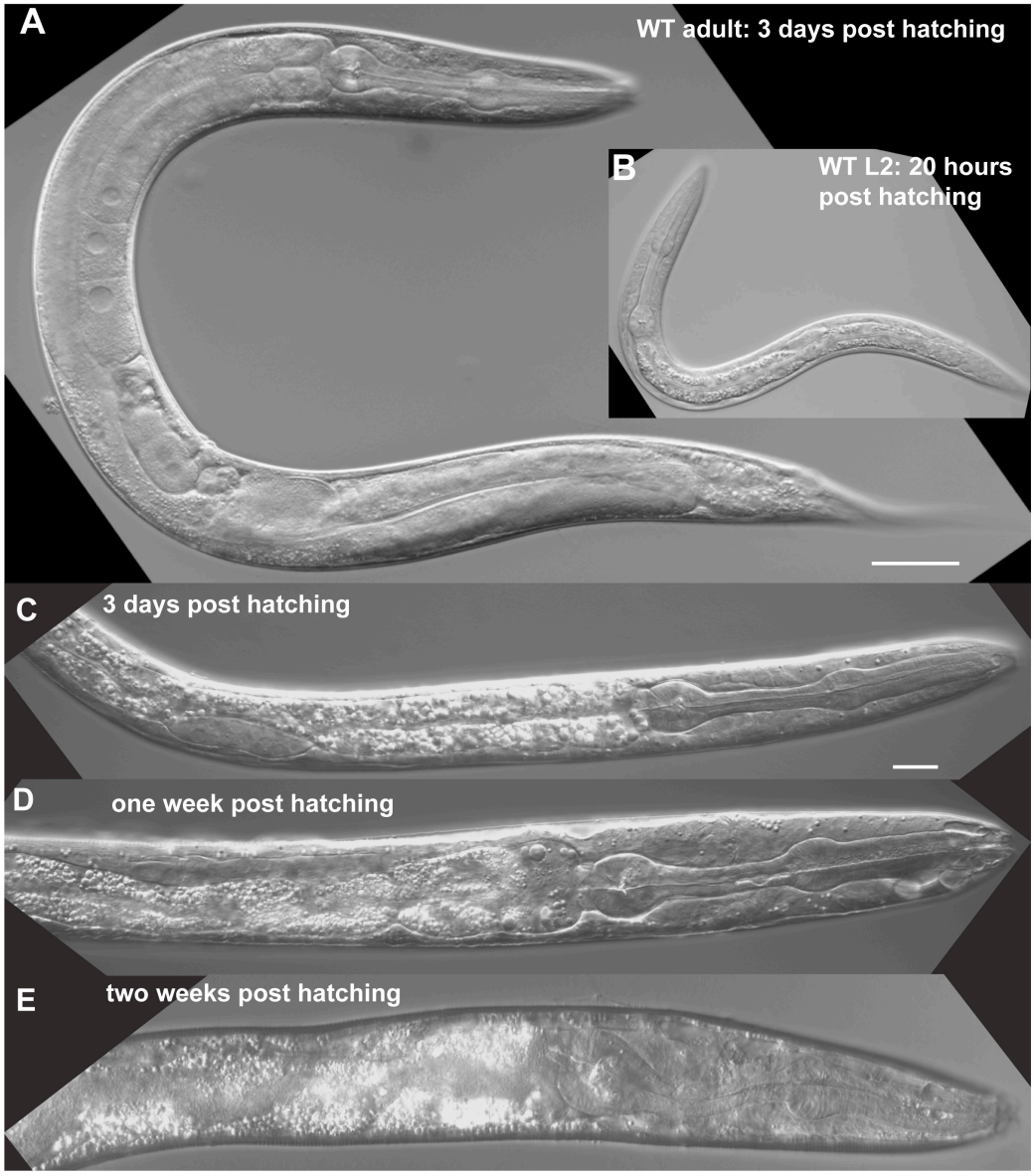


Figure 1. *Cbr-puf-2(nm66)* mutant animals manifest starvation phenotype. (A and B) Wild-type animal at adult stage (A) and the second larval stage (B), scale bar: 50μm. (C-E) *Cbr-puf-2(nm66)* mutant animals of 3 days (C), one week (D) and two weeks (E) after hatching, scale bar: 10μm.

effect of some more fundamental defect, or of starvation, rather than being the cause of

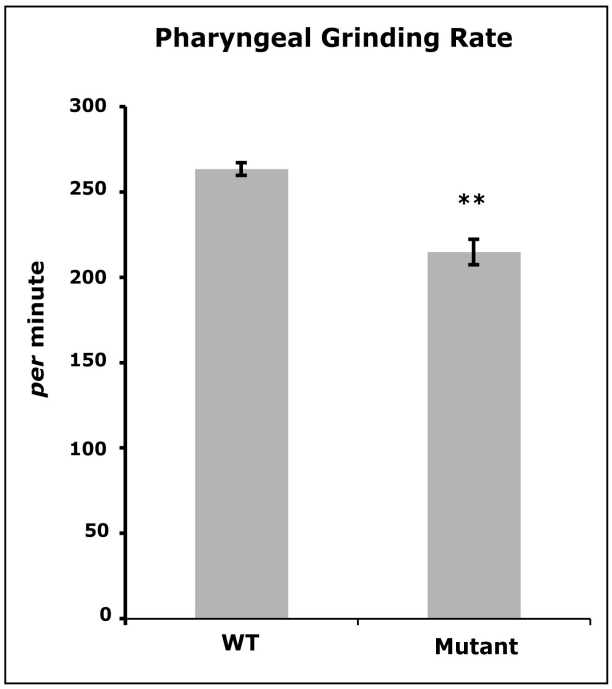


Figure 2. Pharyngeal pumping rates between wild-type animal at the second larval stage (WT column) and their developmental equivalent *Cbr-puf-2(nm66)* mutant animals 3 days after hatching (Mutant column). ** indicates the pumping rates are significantly different (two-tailed P value equals 0.0004, N=5).

the dramatic arrest phenotype. To further test the pharyngeal defect hypothesis, I fed *Cbr-puf-2(nm66)* mutants with *E. coli.* expressing green fluorescent protein (GFP), and analyzed by fluorescence microscopy. For wild-type animals, GFP labeled *E. coli.* cells were drawn in and transported to the terminal bulb, where they were ground open when passing through the grinder. The process was accompanied by the disappearance of intense GFP signal, since constrained GFP molecules became diffused once cell wall was broken up (Figure 3A and 3B). While wild-type *C. briggsae* efficiently swallowed and

broke up ingested bacteria and then expelled the debris and contents back to the intestine, *Cbr-puf-2(nm66)* mutants stuffed their intestine with intact *E. coli*. cells (Figure 3C and

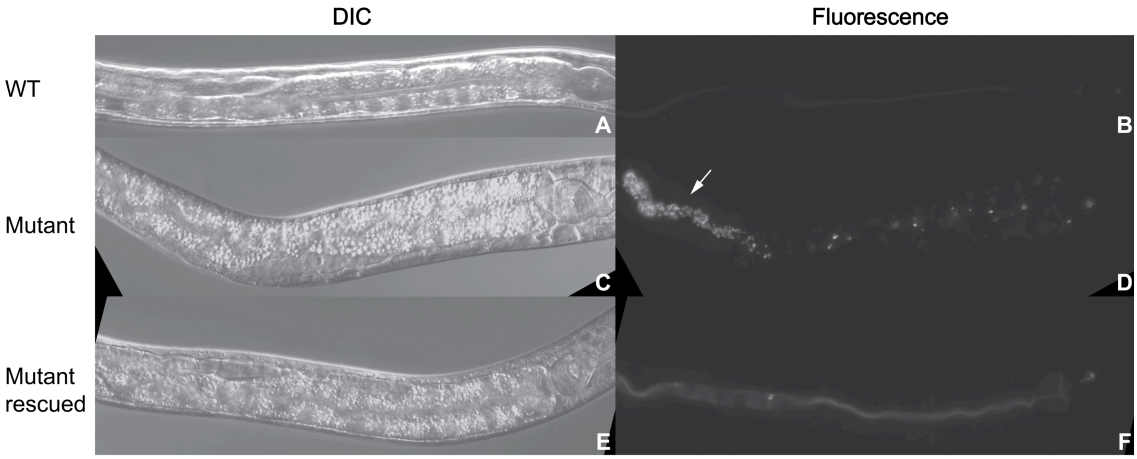


Figure 3. Bacteria grinding efficiency of wild-type and *Cbr-puf-2(nm66)* mutant animals. GFP-expressing BL21 *Escherichia coli* were fed to wild-type animal at the second larval stage (A and B), *Cbr-puf-2(nm66)* mutant animals 3 days after hatching (C and D) and *Cbr-puf-2(nm66;nmIs4)* transgenic rescued animals at the second larval stage (E and F), respectively. The anterior of the worm is facing right. Note that wild-type and *Cbr-puf-2(nm66;nmIs4)* animals are able to mechanically break up bacteria leading to loss of fluorescence in the intestinal tract (B and F). In contrast, *Cbr-puf-2(nm66)* mutant animals are unable to do so leading to accumulation of intense GFP signal in the intestinal tract (D). Arrow indicates intact *E. coli* in the intestinal tract.

3D). This grinding defect was able to be rescued by introducing a wild-type copy of *Cbr-puf*-2 gene into *Cbr-puf-2(nm66)* mutants. *Cbr-puf-2(nm66;nmIs4)* animals, which were transgenic-rescued *Cbr-puf-2(nm66)* mutant animals (Liu et al., 2012), could crack open *E. coli.* efficiently (Figure 3E and 3F). These results suggest *Cbr-puf-2* is involved in the physiological function of terminal pharyngeal bulb in order to support robust food grinding.

4.2 Cbr-puf-2 expresses in the muscle cells of terminal pharyngeal bulb in C. briggsae

To investigate where expression of *Cbr-puf-2* occurs, I introduced a *Cbr-puf-2* reporter plasmid to wild-type *C. briggsae*. This plasmid carries a chimeric gene with *Cbr-puf-2* promoter and 3'UTR regions fused to a fragment coding for histone2B-GFP. I found that the stable transgenic strain CP126 expressed GFP in the terminal bulb of the pharynx (Figure 4B). The GFP signal could only be detected during a brief window from the late four-fold embryo to the early second larval stage. These signals showed 3-fold symmetry, and specifically labeled three nuclei at the posterior part of the terminal bulb

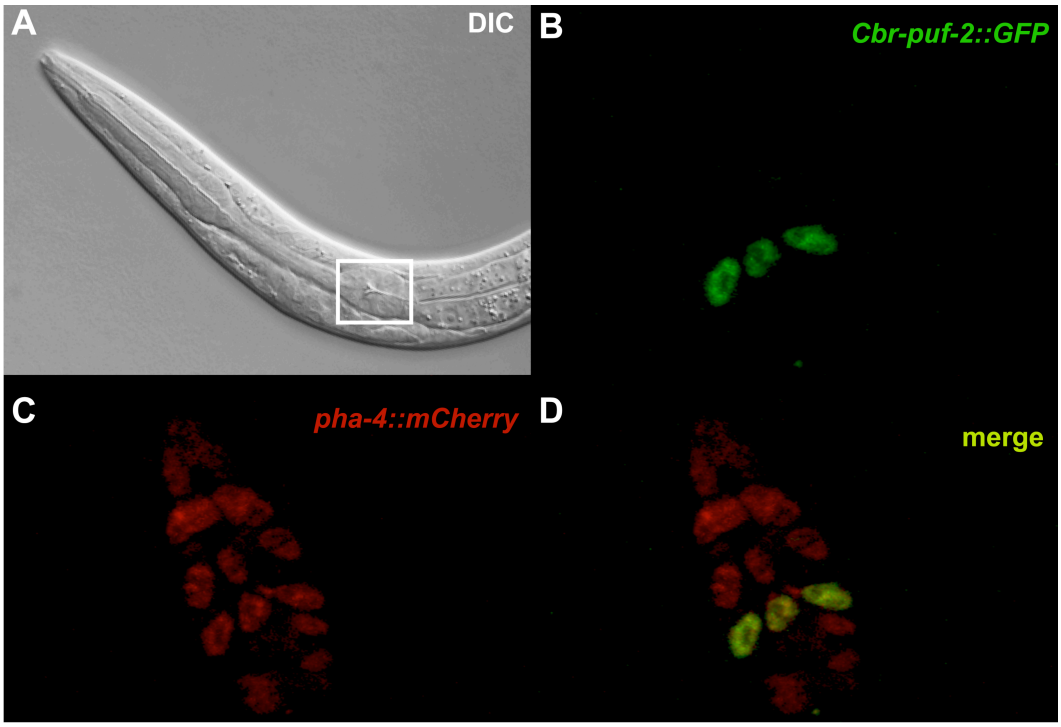


Figure 4. *Cbr-puf-2* expresses in the pharyngeal muscle 7. (A) DIC microscopy of the pharyngeal region of a worm at the second larval stage. The white box highlights the terminal bulb, which is the area shown in figure 4B-4D. (B) *Cbr-puf-2* expresses in three cells at the posterior part of the terminal bulb. (C) Expression pattern of *pha-4* in the terminal bulb. (D) The merge of figure B and C.

(Figure 4B). To further facilitate cell identification, I constructed another strain CP127, which introduced another nuclear localized reporter, *Ce-pha-4*::Histone2B-mCherry (Zhao et al., 2010), into strain CP126 (Figure 4C). *pha-4* is the master cell fate regulator of the pharynx and expresses in all pharyngeal cells, whose expression pattern and function is conserved between *C. elegans* and *C. briggsae* (Mango, 2007; Zhao et al., 2008). I detected that *Cbr-puf-2*::GFP signals overlapped with part of red nuclear signals of *pha-4*::mCherry (Figure 4D). It is known that the pharyngeal cell lineage is conserved between *C. elegans* and *C. briggsae* (Zhao et al., 2008), therefore, as *C. elegans*, *C. briggsae* possesses eight rings of pharyngeal muscles (pm), and its terminal bulb is mainly comprised of pharyngeal muscle pm6, pm7 and pm8 (Avery and Thomas, 1997). Since pm7 muscle cells are located at the posterior of terminal bulb and have three large non-syncytial nuclei, these three *Cbr-puf*-2::Histone2B-GFP cells are pharyngeal muscle 7. My transgenic reporter assay strongly supports the notion that *Cbr-puf-2* is involved in the terminal pharyngeal bulb to promote pharyngeal function.

4.3 Cbr-puf-2 promotes robust muscle contraction of pharyngeal terminal bulb

Because of the grinding defect and the specific expression pattern, I sought to identify the abnormalities associated with the terminal pharyngeal bulb. As I mentioned above, I did not see developmental aberration of the pharynx. Phalloidin staining showed well organized pharyngeal actin filaments in *Cbr-puf-2(nm66)* mutant animals (Figure 5), and the grinding rate of mutants was only slightly less than wild-type animals (Figure 2). These observations indicate the overall integrity of the pharyngeal apparatus, and suggest potential subtle changes that could result in a profound phenotypic consequence. Intuitively, a grinding defect could be a consequence of the malfunction of the “teeth”, a

thick and ridged cuticle layer located at the grinder region of the terminal bulb (Zhang et al., 2005), which is refractile under DIC microscopy. However, I did not find the chitin layer to be missing or obviously different in mutants and wild-type animals by direct DIC imaging (Figure 6).

I next examined the grinding behavior of wild-type and mutant animals in detail by analyzing videos frame-by-frame. These observations first established a normal 3-

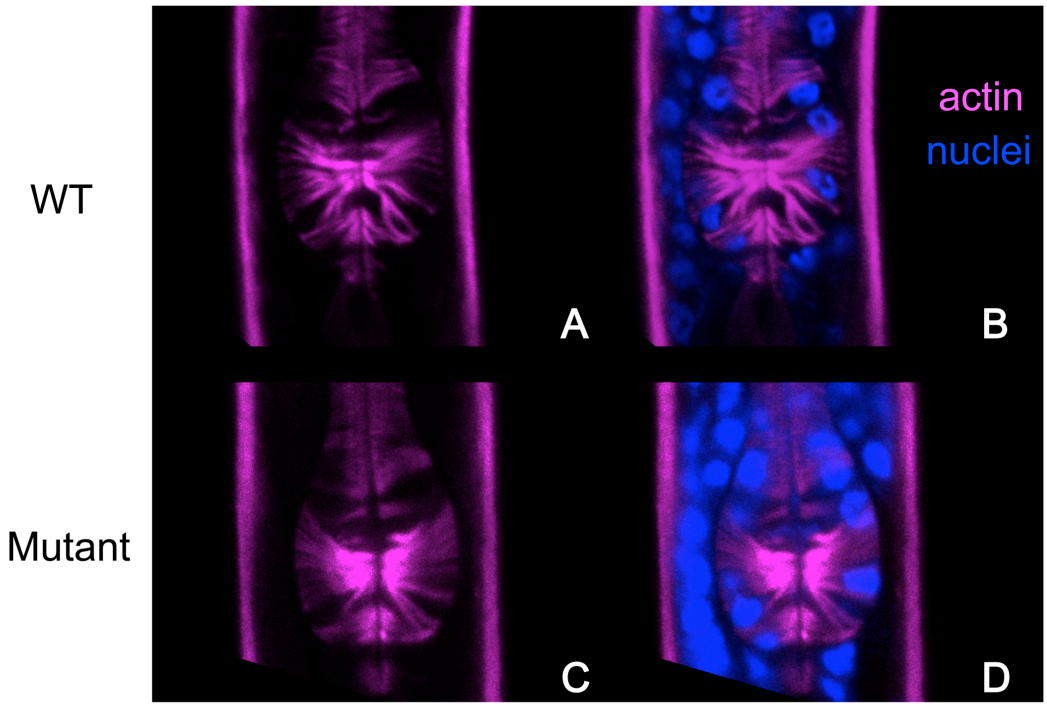


Figure 5. Phalloidin staining of pharyngeal act filament. (A and B) Wild-type *C. briggsae* strain, AF16, at the second larval stage. (C and D) *Cbr-puf-2(nm66)* mutant animals.

step stereotypic movement of the pharyngeal grinding action. At the resting state, the grinder is closed, and each cycle of grinding starts with a coordinated contraction of pharyngeal muscle cells, which opens the lumen of the grinder, and pulls originally separated chitin teeth close together (Figure 6B). Following this initial contraction,

pharyngeal muscle cells contract further, which tightly seals the chitin teeth together, and also opens the valve leading to the intestine lumen (Figure 6C). Afterwards, muscle relaxation returns the grinder back to its rest position (Figure 6D).

For *Cbr-puf-2(nm66)* mutant animals, even though they were able to initiate muscle contraction and open the lumen of the grinder (Figure 6F), they were neither able to fully seal the chitin teeth nor open the pharyngeal-intestinal valve most of time (Figure 6G). This deficiency may be a consequence of the weakening of pharyngeal muscle 7 because they lack a robust pulling force that pushes the posterior of the grinder further backwards, which appears a wider space at the position of pm7 showing in Figure 6G. Nevertheless, occasionally, the grinder of *Cbr-puf-2(nm66)* mutant animals did form small cleft or opening that led to intestinal lumen, which explains how bacteria could still be delivered to the intestine. The incomplete muscle contraction of *Cbr-puf-2(nm66)* mutant animals most likely explains the dramatic deficiency of bacteria grinding I reported above.

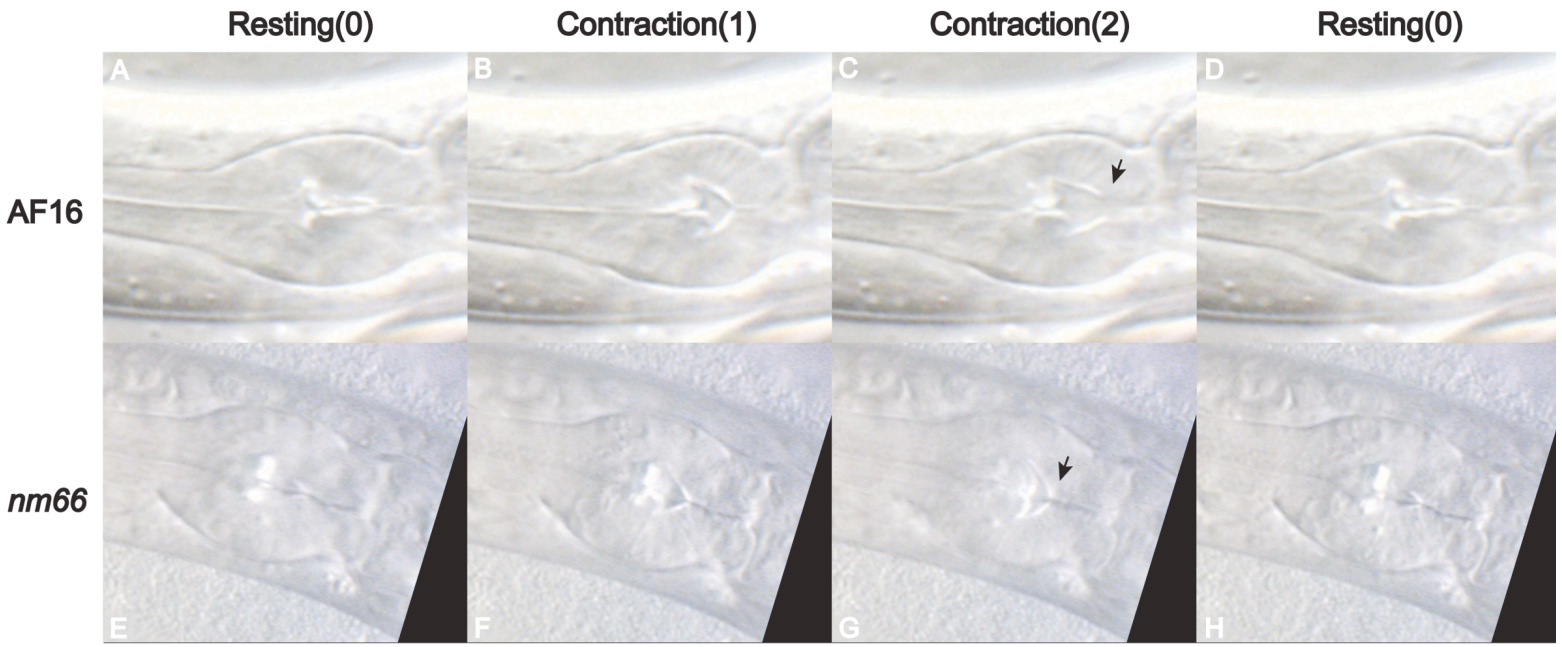


Figure 6. Muscle contraction of the terminal bulb is weaker in *Cbr-puf-2(nm66)* mutant animals. (A-D) One cycle of the grinding motion is shown for wild-type animals at the second larval stage. (E-H) One cycle of the grinding motion is shown for *Cbr-puf-2(nm66)* mutant animals. The motion starts with an initial contraction (B and F), and follows with a pulling, which in wild-type animals opens the valve leading to the intestine lumen (C), but not in mutants (G). Arrow indicates the posterior end of the lumen. Afterwards, muscle relaxation returns the grinder back to its rest position (D and H).

4.4 Cbr-puf-2 promotes faithful vulval development

To confirm that the *Cbr-puf-2(nm66)* larval arrest phenotype was due to starvation, I attempted to rescue development by simply growing mutant animals in

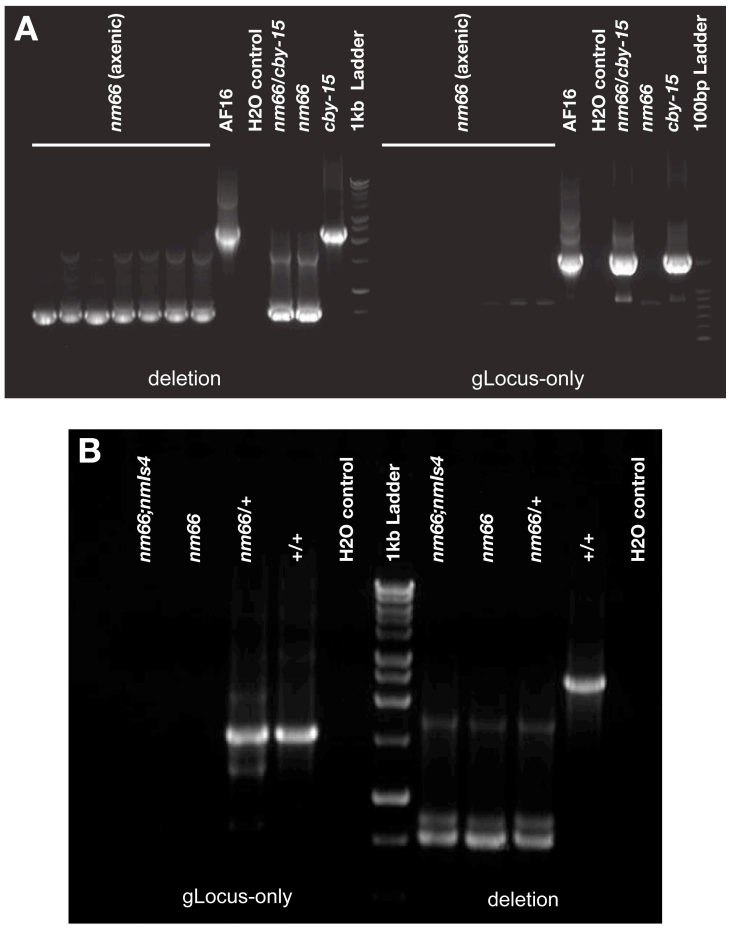


Figure 7. Genotyping results: axenic and transgenic rescues. (A) Genotyping results for axenic rescued *Cbr-puf-2(nm66)* mutant animals. *Cbr-puf-2* PCR primers specific for deletion region (lanes on the left) or intact genomic region (lanes on the right) were used. Lanes are: *nm66*(axenic), adult *nm66* growing in axenic culture; AF16, wild-type *C. briggsae*; H2O control, no-template control; *nm66/cby-15*, parental strain for axenic culture; *nm66*, arrested *Cbr-puf-2* mutant growing on regular bacteria food; *cby-15*, Dyp mutant, pseudo-balancer for *nm66*. (B) Genotyping results for transgenic rescued *Cbr-puf-2(nm66;nmIs4)*. Lanes are: *nm66;nmIs4*, adult *nm66* with integrated wild-type *Cbr-puf-2* genomic region; *nm66/+*, *Cbr-puf-2* mutant allele carrier; +/+, wild-type progeny from *nm66/+*; *nm66*, arrested *Cbr-puf-2* mutant progeny from *nm66/+*; H2O control, no-template control.

axenic liquid medium. This eliminates the link between nutrition and food grinding. Indeed, the mutants could reach adulthood in axenic medium, as confirmed by single-worm PCR genotyping of rescued animals (Figure 7A).

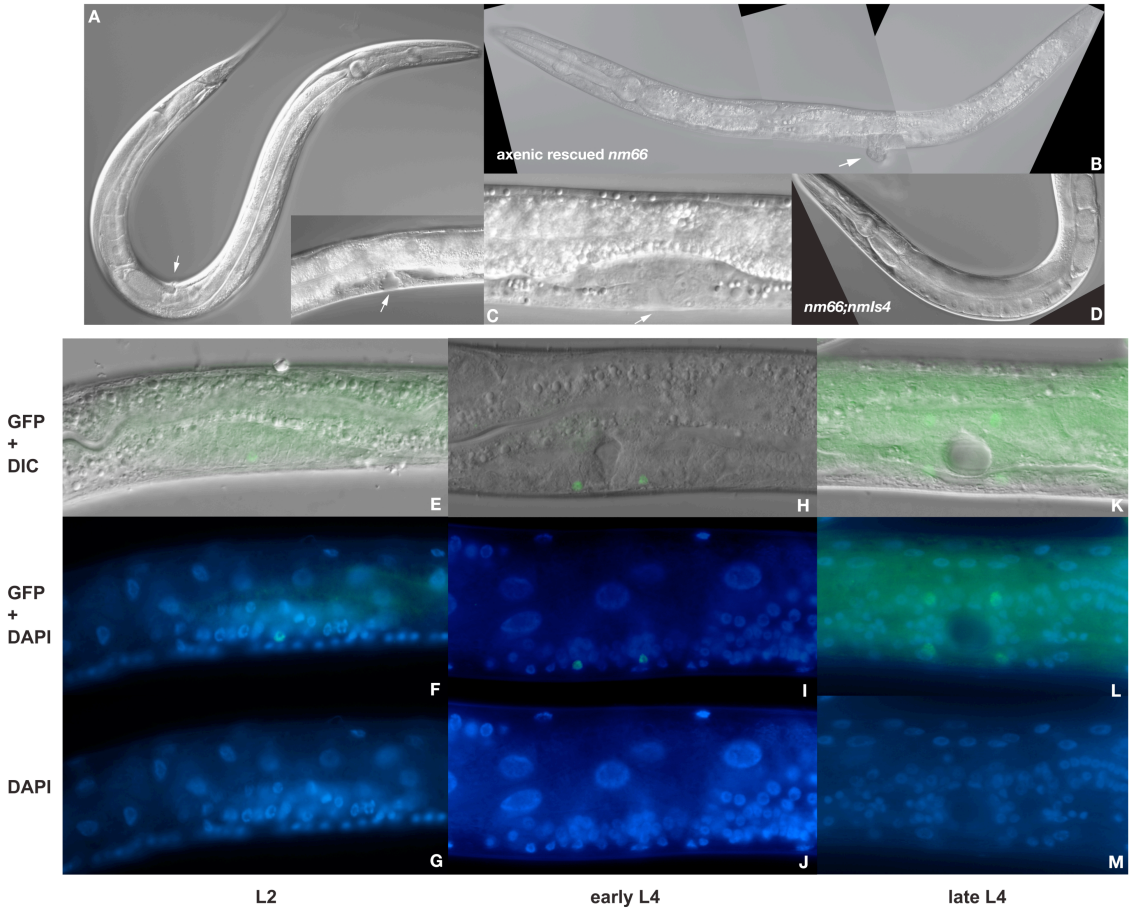


Figure 8. *Cbr-puf-2* is involved in vulval development in *C. briggsae*. (A) Wild-type adult *C. briggsae* hermaphrodites have a vulval opening at their ventral middle body region. Insert, vulval opening of a wild-type animal at L4 stage. Arrow indicates the vulva. (B) *Cbr-puf-2(nm66)* mutant animals are able to complete larval development and reach adulthood in axenic culture. Arrow indicates protruding vulvae. (C) *Cbr-puf-2(nm66)* mutant animal at the same size of wild-type L4 stage animal. Arrow indicates an incomplete vulva. (D) *Cbr-puf-2(nm66)* animal stably transformed with *Cbr-puf-2(+)* transgene (strain CP113) have normal vulval development and are fertile. (E-M) *Cbr-puf-2* transcriptional reporter expresses in the vulval cells. Blue, DAPI nuclear DNA signal. Green, *Cbr-puf-2* reporter signal. (E-G) At the second larval stage, *Cbr-puf-2* expresses in one cell of the vulval lineage. (H-J) At the early fourth larval stages, the green signal expanded to two cells around the vulval opening. (K-M) At the late fourth larval stage, *Cbr-puf-2* expresses in four cells peripheral of vulval opening.

Surprisingly, axenic rescued *Cbr-puf-2(nm66)* animals had severe developmental defects. In wild-type hermaphrodites, each animal possesses a single centrally located, "Christmas tree"-like, vulval opening at the fourth larval stage (Figure 8A, insert), and at the adult stage it develops to a fully functional vulva (Figure 8A). The vulva is the egg-laying and copulatory organ of *Caenorhabditis* species, which is highly conserved developmentally and specified from six vulval precursor cells during larval development (Kiontke et al., 2007; Sternberg and Horvitz, 1986; Sulston and Horvitz, 1977; Sulston and White, 1980). By contrast, axenic rescued *Cbr-puf-2(nm66)* animals had incomplete vulval opening at the fourth larval stage (Figure 8C). At the adult stage, they also had very few germ cells in their gonads, and a protruding vulva in their ventral mid-body region (Figure 8B).

By introducing a wild-type copy of *Cbr-puf-2* gene, vulval and germline phenotypes of *Cbr-puf-2(nm66)* mutant animals could be rescued (Figure 8D and 7B). These data indicate that *Cbr-puf-2* is involved in both vulval and germline development. Supporting this hypothesis, I found that *Cbr-puf-2* reporter gene expressed in the vulval cells. In transgenic *C. briggsae* expressing GFP-H2B driven by *Cbr-puf-2* regulatory regions, animals at the second larval stage showed GFP signal from one cell located at the ventral middle-body region (Figure 8E-8G), and then at the early and late fourth larval stages, the green signal expanded to two and four cells around the vulval opening, respectively (Figure 8H-8M). Judged from the localization of these cells, *Cbr-puf-2* likely functions in 2° vulval cells to promote vulval fate or morphogenesis.

5. Discussion

5.1 C. briggsae puf-2 gene is required to maintain pharyngeal muscle function.

In the first Chapter, I identified an essential role of *C. briggsae puf-2* in larval progression. In this Chapter, I find that the larval arrest phenotype of *Cbr-puf-2* mutant animals is caused by inefficient breakdown of bacteria food, which leads to insufficient nutrient intake and developmental arrest. My data suggest that *Cbr-puf-2* is involved in sustaining the normal muscular strength of the terminal bulb during larval progression. Since I can only detect *Cbr-puf-2* gene expression in three pharyngeal muscle cells during a very narrow range of development, it is surprising to see such a profound consequence of *Cbr-puf-2* function. However, I provide three lines of evidence to support the notion that *Cbr-puf-2* gene expression pattern correlates its *in vivo* function. Firstly, my GFP-labelled *E. coli* feeding assay shows that *Cbr-puf-2* mutant animals accumulate intact bacteria cells in their intestine, which pinpoints the defect is the food grinding. Supporting this conclusion, I did not see any feeding defect by video taping mutant animals. Secondly, axenic growth medium rescues *Cbr-puf-2(nm66)* larval arrest phenotype, which means that by eliminating the requirement for grinding mutant animals can overcome the arrested state and reach adulthood. Thirdly, I identify the obvious grinding defect at muscle cell behavior level. The model for *Cbr-puf-2(nm66)* malfunction is illustrated in Figure 9: mutant animals can't provide the robust muscle contraction that brings pharyngeal teeth tightly together and break up food particles. This sealing force, I suspect, is mainly provided by pharyngeal muscle 7, which are *Cbr-puf-2* expressing cells.

The general morphology of the pharynx in *Cbr-puf-2(nm66)* mutant animals is normal, and no pharynx muscle cell abnormalities are detected by DIC microscopy. It is

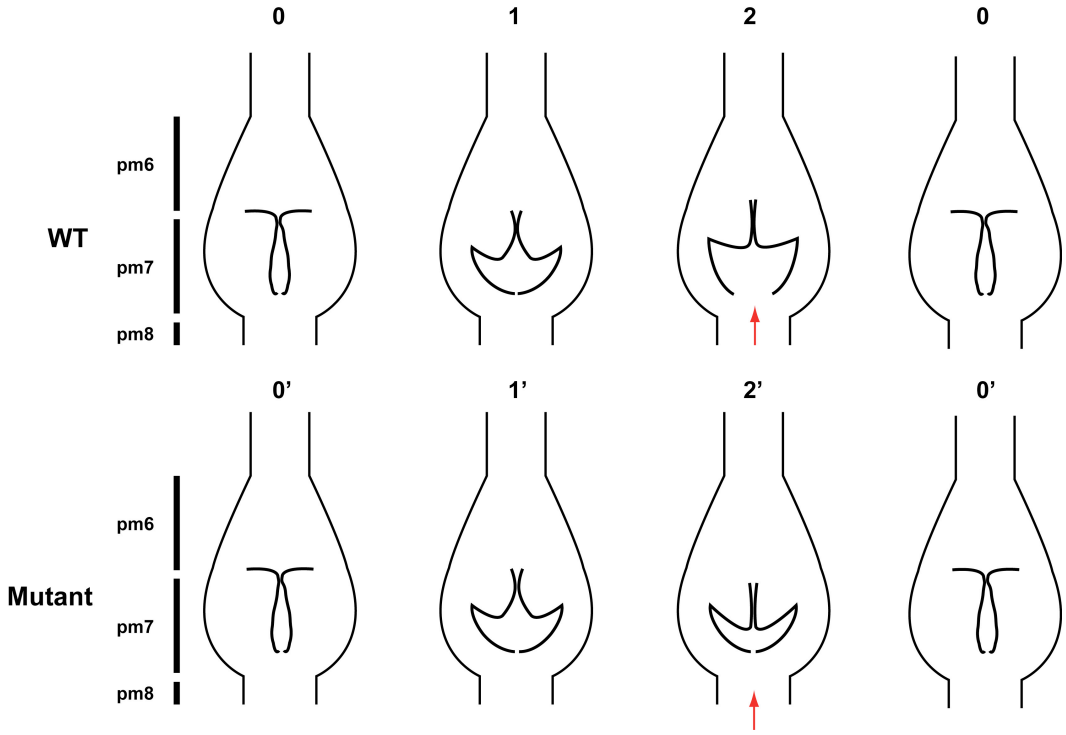


Figure 9. Model of pharyngeal grinding and *Cbr-puf-2(nm66)* mutant defect. The model shows the zoomed view of pharyngeal terminal bulb. The proximal location of pharyngeal muscle cells are marked on the far left side. For wild-type *C. briggsae* strain, AF16, the natural grinding motion starts with a pulling force that seals the teeth together (position 1), follows by a continuing pulling mainly operated by pm7, which not only further seals the teeth but also opens the pharyngeal-intestinal valve (position 2). After that, the grinder returns back to its resting position (position 0). For *Cbr-puf-2(nm66)* mutant animals, the major defect is that the grinder lumen can't be further opened (positon 2'), which may result from the weakening of pm7. As a consequence, the teeth can't be tightly sealed leading to inefficient food process. Red arrows indicate the defective region.

possible that the loss of *Cbr-puf-2* has a subtle effect on muscle filament structure, but the well-formed pharyngeal actin cytoskeleton structure in *Cbr-puf-2(nm66)* mutant animals (Figure 5) is inconsistent with this hypothesis. Alternatively, Cbr-PUF-2 may regulate genes participating in muscle physiological functions, such as titin, myosin-binding

protein C, and myosin light chain kinase, which are structural proteins in establishing the muscle sarcomere or regulators of muscle contraction (Benian et al., 1996). Another likely hypothesis is that *Cbr-puf-2* is involved in some specific aspect of neuromuscular function in pm7. RNA-binding proteins are renowned for their roles in synaptic plasticity (Richter, 2010), and PUF family members have been found to be essential for synapse formation and maintenance in fly, rodents and human (Dubnau et al., 2003; Marrero et al., 2011; Menon et al., 2004; Siemen et al., 2011; Vessey et al., 2010). A unifying thought is that Pumilio modulates synaptic function in neurons or muscle cells via direct repression of mRNA targets including eIF4E and AChE (Marrero et al., 2011; Menon et al., 2004). Interestingly, in *C. elegans*, mutations of either dystrobrevin gene *dyb-1* or dystrophin gene *dys-1* cause muscle degeneration in a sensitized genetic background as well as hyperactivity and increased sensitivity to the neurotransmitter acetylcholine and its inhibitor (Bessou et al., 1998; Gieseler et al., 1999a; Gieseler et al., 1999b; Giugia et al., 1999). In *C. briggsae*, pm7 receive synapses from motor neuron M5, and *Cbr-puf-2* may participate in post-synaptic gene regulation to control cholinergic transmission.

5.2 C. briggsae puf-2 gene controls vulval development

In *C. elegans*, PUF genes function in the soma to control cell fate specification during larval development. In particular, PUF-8, FBF-1 and FBF-2 negatively regulate vulval development in the hermaphrodite (Thompson et al., 2006; Walser et al., 2006). Here, my data suggest that *Cbr-puf-2* functions in 2° vulval cells to promote vulval fate completion. In *C. elegans*, vulval development has been studied extensively, and the process is divided into five steps (Sternberg, 2005): (1) Generation of Vulval Precursor Cells (VPCs) during the L1 and L2 stages; (2) Vulval Precursor Patterning during the L3

stage through a signal from the gonad and signaling among the VPCs; (3) Generation of the adult vulval cells; (4) Anchor cell invasion to form a hole in the epidermis. (5) Morphogenesis of the vulva. *Caenorhabditis* species follow the same conversed vulval fate patterning process (Kiontke et al., 2007). Since axenic rescued *Cbr-puf-2(nm66)* animals are still able to form vulval openings, even though incompletely, it implies that *Cbr-puf-2* most likely functions at the later stage of vulval development to promote faithful morphogenesis.

The vulval fate specification relies on two major evolutionarily conserved signaling pathways, EGF/Ras/MAP Kinase and Notch (Sternberg, 2005), and 2° vulval cell fate adoption requires Notch signaling (Greenwald et al., 1983; Sternberg and Horvitz, 1989). *Caenorhabditis* species use the same conversed signaling network for vulval fate specification (Felix, 2007; Sommer, 2005). In *C. elegans*, *fbf-2* gene has been identified as a GLP-1/Notch target in distal germ line to promote mitotic decision, and as a pleiotropic gene, it also functions in the vulval precursor cells or their descendants to negatively regulate vulval development (Walser et al., 2006). Interestingly, *C. briggsae puf-2* possesses putative LAG-1 binding sites at its 5' regulatory region (Lamont et al., 2004). Therefore, it is reasonable to speculate that, in *C. briggsae* vulva, the activation of Notch signaling pathway in 2° vulval cells leads to nucleus translocation of LAG-1, which turns on *Cbr-puf-2* gene expression. In those cells, *Cbr-puf-2* may regulate genes that promote proper attachment of the vulva to the uterine cells or to the seam cells because of the Protruding vulval phenotype in mutant animals.

5.3 Functional evolution of pleiotropic genes

Together with Chapter 1, my studies unveil the pleiotropic effects of *Cbr-puf-2* function. During early development, maternally deposited *Cbr-puf-2* is important for embryogenesis, and depletion of the gene's transcript in the parental germ line leads to embryonic lethality (Liu et al., 2012). Later in larval development, *Cbr-puf-2* is required for proper physiological control of pharyngeal muscle contraction, and loss of *Cbr-puf-2* results in early larval arrest. In adulthood, *Cbr-puf-2* acts redundantly with *Cbr-puf-1.2*, a closely related paralogous gene, to promote meiotic entry and spermatogenesis in the germ line (Liu et al., 2012). Remarkably, axenic culture of *Cbr-puf-2(nm66)* mutants bypasses the larval arrest phenotype to reveal another novel function of *Cbr-puf-2* in vulval development. Reporter transgene expression suggests that this role of *Cbr-puf-2* is played in vulval precursor cells.

As a member of the regulatory PUF RNA-binding protein family, it is perhaps not surprising that *Cbr-puf-2* can affect multiple traits. Both loss-of-function studies and recent genome-wide systematic surveys show that PUF proteins function in many aspects of tissue development and physiology through translational control of a variety of mRNA targets (Bachorik and Kimble, 2005; Crittenden et al., 2002; Kershner and Kimble, 2010; Lamont et al., 2004; Merritt and Seydoux, 2010; Subramaniam and Seydoux, 2003; Suh et al., 2009; Zhang et al., 1997). Structural and biochemical studies provide further information on how its multi-target association is established at atomic level (Bernstein et al., 2005; Dong et al., 2011; Wang et al., 2009). Therefore, their pleiotropic nature stems from their powerful biochemical ability of physical interactions. Pleiotropy potentially restricts evolution over the long term because of constraints, an effect known as "antagonistic pleiotropy" (Hodgkin, 1998). Nevertheless, in *Caenorhabditis* species, we

see that in the PUF protein family radical differences in gene function have evolved. Understanding how constraint and flexibility are achieved at the molecular level is an interesting topic of future research.

The Pumilio and FBF (PUF) family proteins are known for their regulatory roles in various aspects of germ cell development in *C. elegans* and *C. briggsae* by translational repression of target mRNAs (Ariz et al., 2009; Crittenden et al., 2002; Kalchhauser et al., 2011; Liu et al., 2012; Subramaniam and Seydoux, 2003; Suh et al., 2009; Zhang et al., 1997). However, it is surprising that, as a recent duplicated gene, *Cbr-puf-2* plays multiple essential and non-redundant roles in somatic tissues. According to evolutionary theory, recent duplicated genes usually have overlapping roles (Ohno, 1970; Zhang, 2003). In *C. elegans*, FBF-1 and FBF-2 do have largely redundant functions in the germ line and in somatic tissues (Kalchhauser et al., 2011; Kraemer et al., 1999; Lamont et al., 2004; Merritt and Seydoux, 2010; Thompson et al., 2006; Zhang et al., 1997). This phenomenon is also true for additional PUF subfamilies, PUF-3, PUF-5 and PUF-6 (Hubstenberger et al., 2012; Lublin and Evans, 2007). However, for the *C. briggsae* PUF-2 subfamily, which is comprised of three lineage-specific duplicates, one (Cbr-PUF-2) has already acquired non-redundant functions in somatic development. It is not clear whether these functions are ancestral to the subfamily and now uniquely performed by *Cbr-puf*-2, or if they represent novel roles that evolved recently. In either case, it is very likely that gene duplication relaxes the constraints on Cbr-PUF-2, allowing it diverge functionally from the two other subfamily members (Cbr-PUF-1.1 and Cbr-PUF-1.2). Since Cbr-PUF-2 and Cbr-PUF-1.2 still maintain very similar biochemical binding properties (Chapter 1, Figure 4), their functions seem to be

conserved at the coding sequence level. Therefore, their functional divergence might largely be a process of gaining or losing mRNA targets as well as changing gene expression.

Developmental system drift (DSD) is an evolutionary phenomenon in which divergent genetic variations underlie constant phenotypic traits (True and Haag, 2001). I suggest that, because of the ease with which PUF proteins can gain and lose target mRNAs through changes in *cis*-regulatory RNA and/or changes in PUF protein expression, they may be frequent participants in DSD events. In nematodes, one well-studied case of DSD is the vulval fate determination (Kiontke et al., 2007; Sommer, 2005). The rhabditid nematode vulva is a homologous, highly conserved egg-laying organ. However, significant developmental differences have been uncovered in comparative studies at the resolution of different genera (Kiontke et al., 2007). My current study adds PUF gene function to the variation of nematode vulval development within an even smaller evolutionary divergence. As I discussed before, *C. elegans fbf-2* and *puf-8* genes function in the vulval precursor cells or their descendants to negatively regulate vulval development (Walser et al., 2006). Here, I showed that *C. briggsae puf-2* is involved in positively promoting vulval development. It is intriguing that, although they are all PUF homologs, the detailed genetic networks in which they function are potentially very different. Overall, this work suggests that PUF proteins with conservative biochemical activities can be rapidly incorporated into developmental processes both new (*e.g.* hermaphrodite germ line development) and ancient (*e.g.* vulval morphogenesis).

Conclusions

For my dissertation research, I investigated the functional evolution of PUF gene family in *Caenorhabditis* with respect to their roles in germline development and sex determination. Specifically, I provide genetic, molecular, and biochemical evidence to demonstrate that PUF-2 and FBF sub-family genes were involved in promoting germline proliferation ancestrally, but not in sex determination, in *Caenorhabditis* species. Nevertheless, they are independently co-opted into hermaphroditic germline sexual fate patterning to either promote sperm/oocyte switch or spermatogenesis in *C. elegans* or *C. briggsae*, respectively. Moreover, I found that PUF family genes were coopted 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. Combined with data from our previous work on the evolution of *gld-1* (Beadell et al., 2011), my research indicates that the last common ancestor of the FBF and PUF-2 sub-families repressed *gld-1* translation in the service of regulating germline proliferation, and later, the entire PUF-*gld-1* module was co-opted into hermaphrodite development.

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) may therefore promote their continued regulatory linkage in the face of altered germline phenotypes.

Aside from PUF genes' redundant germline functions, I was surprised to find that, as a recent duplicated gene, *Cbr-puf-2* plays multiple essential and non-redundant roles in somatic tissues. During early development, maternally deposited *Cbr-puf-2* is important for embryogenesis, and depletion of the gene's transcript in the parental germ line leads to embryonic lethality. Later in larval development, *Cbr-puf-2* is required for proper physiological control of pharyngeal muscle contraction, and loss of *Cbr-puf-2* results in early larval arrest. Remarkably, axenic culture of *Cbr-puf-2(nm66)* mutants bypasses the larval arrest phenotype to reveal another novel function of *Cbr-puf-2* in vulval development. According to evolutionary theory, recent duplicated genes usually have overlapping roles (Ohno, 1970; Zhang, 2003). In *C. elegans*, PUF proteins do have largely redundant functions in the germ line and in somatic tissues (Kalchhauser et al., 2011; Kraemer et al., 1999; Lamont et al., 2004; Merritt and Seydoux, 2010; Thompson et al., 2006; Zhang et al., 1997). Although, it is not clear whether these functions are ancestral to the subfamily and now uniquely performed by *Cbr-puf*-2, or if they represent novel roles that evolved recently, it is very likely that gene duplication relaxes the constraints on Cbr-PUF-2, allowing it diverge functionally from the two other subfamily members (Cbr-PUF-1.1 and Cbr-PUF-1.2).

Overall, my work suggests that PUF proteins with conservative biochemical activities can be rapidly incorporated into developmental processes both new (*e.g.* hermaphrodite germ line development) and ancient (*e.g.* vulval morphogenesis). It may be an consequence of evolutionary phenomenon called developmental system drift (DSD)

in which divergent genetic variations underlie constant phenotypic traits (True and Haag, 2001). I propose that, because of the ease with which PUF proteins can gain and lose target mRNAs through changes in *cis*-regulatory RNA and/or changes in PUF protein expression, and because of the relaxed functional constraints through gene duplication, they may be frequent participants in DSD events.

Appendices

Appendix 1: List of primers

Cbr-puf-2 allele *nm66* deletion amplification:
Inner primers:
QL1: CAGCGAGCCACCGTAATACTTTC
QL4: CTACAGAGCTGATGACACTCGAG
Outer primers:
QL5: GCAGCTGGAACAAACGCTTGGA
QL6: GACTGGGTAGGACACATAACAG

Cbr-puf-2 rescue proof (amplify band: w/ WT chromosome, can't amplify: homozygous)
Inner primers:
QL21: GTAACGCCATCATCGAGACCTG
QL53: GAAATCGGCTGAAGATGGCGAC
Outer primers:
QL51: AAGCAATCGCCTCTCGAGTCTTC
QL54: GGACTCCTCATGAATGGGATCG

Cbr-puf-1.2 in vitro mRNA synthesis primers:
QL22: TAATACGACTCACTATAGGGATGCCACCGTACGACGACTCTTC
QL23: TAATACGACTCACTATAGGGATGGCCTATGGGATGGGTGGTAC

Cbr-puf-2 in vitro mRNA synthesis primers:
QL24: TAATACGACTCACTATAGGGCCAGCTTCGATCTTCACTCTCC
QL25: TAATACGACTCACTATAGGGGTAGGCGGGTCAACAGTGTAAG

New *Cbr-puf-1.2* N-terminal *in vitro* mRNA synthesis primers
QL47: TAATACGACTCACTATAGGGAATAGCCAATCTTCGTCGTCCGG
QL48: TAATACGACTCACTATAGGGTCCGCGTCGATTTCCTGAGTGTT

Gateway expression construct primers:

Cbr-puf-2 promoter 840bp
QL 59
*att*B4forward
GGGGACAACTTTGTATAGAAAAGTTG CTCCATCGTTCATCCGTTTCGG
QL 60
*att*B1reverse
GGGGACTGCTTTTTTGTACAAACTTGT
ATCGCGATCCATTTCCTCCTGG

GFP w/ intron 826bp

QL 61
attB1forward
GGGGACAAGTTTGTACAAAAAAGCAGGCTCC
AGTAAAGGAGAAGAACTTTTCAC
QL 62
attB2reverse
GGGGACCACTTTGTACAAGAAAGCTGGGTC
CAAACTCAAGAAGGACCATGTG

Cbr-puf-2 coding + 3'UTR 2880bp
QL 63
*att*B2forward
GGGGACAGCTTTCTTGTACAAAGTGG*GA* GATCGCGATACATTTTCAGACAGC
QL 64
*att*B3reverse
GGGGACAACTTTGTATAATAAAGTTG
TCGTCGACTGTTTGAAAACGAACC

Cbr-puf-1.2 promoter 694bp
QL 65
*att*B4forward
GGGGACAACTTTGTATAGAAAAGTTG GCTGTCGCACTTTCTCTTCTCG
QL 66
*att*B1reverse
GGGGACTGCTTTTTTGTACAAACTTGT
TGAGCCGCGGTCCATGTTTTTCC

mCherry w/ intron 861bp
QL 67
attB1forward
GGGGACAAGTTTGTACAAAAAAGCAGGCTCC
GTCTCAAAGGGTGAAGAAGATAAC
QL 68
attB2reverse
GGGGACCACTTTGTACAAGAAAGCTGGGTC
CTTATACAATTCATCCATGCCACC

Cbr-puf-1.2 coding + 3'UTR 3556bp
QL 69
*att*B2forward
GGGGACAGCTTTCTTGTACAAAGTGGGA
GACCGCGGCTCATTCTCGAACA
QL 70
*att*B3reverse
GGGGACAACTTTGTATAATAAAGTTG
GTGAACTACGCTTTTGCCCGCG

New *Cbr-puf-1.2* coding+ 3'
QL71
attB2forward
GGGGACAGCTTTCTTGTACAAAGTGGGA
AACAGCCGAAGAGCCAGACAC
QL72
attB3reverse
GGGGACAACTTTGTATAATAAAGTTG
CCGCATGGTGTAGTGGTTAGTG

New Cb-puf-2 promoter 1380bp(expanded promoter region)
QL 86-2 (pair with QL60)
*att*B4forward
GGGGACAACTTTGTATAGAAAAGTTG
GAGCTCATTGCTCCAGATGAGTAC

Cb-puf-2 genomic region(expanded promoter) 4237bp
QL 87-2 (pair with QL86-2)
*att*B1reverse
GGGGACTGCTTTTTTGTACAAACTTGT
TCGTCGACTGTTTGAAAACGAACC

In situ mRNA synthesis primers:
Cbr-puf-1.2
QL73: ATGCCACCGTACGACGACTCTTC
QL74: ATGGCCTATGGGATGGGTGGTAC

Cbr-puf-2
QL75: CCAGCTTCGATCTTCACTCTCC
QL76: GTAGGCGGGTCAACAGTGTAAG

Cbr-puf-1.1
QL77: AACTCCTACCACTTTCCATACGGAG
QL78: TGGGACCGATACAAGAAATAAAGGCG

Cbr-puf-8
QL86: TGCGACTTCAGATGACCTGCTAAC
QL87: CGGTTGACTACATGCAGCTCTCAC

SL1: GGTTTAATTACCCAAGTTTGAG
SL2: GGTTTTAACCCAGTTACTCAAG
QL88: TACTCGCTACTACTAGTTTCAC (pair with SL sequences, cross the junction of exon2 and exon3 of *Cbr-puf-2*)
QL89: TCTCGCTTGAATAGATTCTCTTCT (pair with polyT primer, cross the junction of exon8 and 9 of *Cbr-puf-2*)

pCR50 BamHI flanking site sequencing primers:
QL90: TTGCGCAGCCTGAATGG (forward)
QL91: TGTCCTGTCACACTCGCT (reverse)

Multisite Gateway expression vector junction sequencing primers (for GFP-PUF-2 fusion, w/in GFP sequence, both are forward facing)
QL92: GGAAGCGTTCAACTAGC
QL93: ACTCCAATTGGCGATGG

Cbr-puf-8 RT-PCR primers (~320bp):

QL94: GGCTCACCATTAAAGTCCTACGG (exon1-2 junction)
QL95: AGATCGGAACATGCGTGTGACG (exon2-3 junction)

Cbr-puf-1 RT-PCR primers (~475bp):
QL96: ACATCCCTCATCCAGAGACTGG (exon5-6 junction)
QL97: GGTGTCGGTTCATCCATTGACG (exon9-10 junction)
QL 98: ACCGGTCAACGTGATACATTGG (exon6-7 junction, pair with QL97, 372bp)
QL122: GACAATCGATCTCCATTGGTGG (pair with QL98: 215bp, or with QL96: 322bp)

Cbr-glp-1 RNAi fragment (1kb, in exon 7)
QL99: TAATACGACTCACTATAGGGAGAGATTTATCCCAGGACGCTGGC
QL100: TAATACGACTCACTATAGGGCGTGGATCTGGTTGTGGTACTTCAG

Cbr-puf-1 RT-PCR primers (RT-PCR: 193bp; PCR: 1142bp)
QL101: ATACGGAGACTCTCCGTCAGCATC (w/in exon 8)
QL102: CAT TGA CGG AGT ATC CGG GTT CAG (w/in exon 9)

PCR50 Ampcilin resistance sequence (265bp)
QL111: GGTTGAGTACTCACCAGTCACAGA
QL112: GCAACGTTGTTGCCATTGCTACAG

Cbr-puf-2 RT-PCR (transgene expression)
QL114: ACACCAGCTTCAGTCCGATGAACAC
QL115: TTGAAGGAAGTGGCAACCGGACTTG (pairs with QL114; 311bp)
QL116: AGGCTCGAGTTTCTCCAACGCA (pairs with QL49; 514bp)

Primers for RT-PCR *Cbr-puf-2* transgene mutation
QL117: GCTTGGAACTTCTGCTAGTAGTC (FP)
QL118: TTTGAAGGAAGTGGCAACCGGAC (RP)
QL119: CGATCTCGAATGTATGGATCCACG (RP)

Yeast three hybrid pACT2 AD constructs: NcoI-HF+XmaI

Cbr-puf-2 (274-1704bp/92-568a.a.)
QL154: TATCCATGGAGAGAAAAATGCTTGGAACTTCTG
QL155: GCCCCGGGCTAGTCATAGTCGAAATGAGGCTC

Cbr-puf-1.2 (322-1662bp/108-554a.a.)
QL156: TATCCATGGAGAAGAGAACGGCGTCGTCGTC
QL157: GCCCCGGGCTATGAAGAAATCGACGATCGACGTG

PUF domain recombinant protein expression: pGEX-4T-1 constructs: XmaI+NotI-HF

Cbr-puf-2:
QL158: GCCCCGGGCAGAAAAATGCTTGGAACTTCTG
QL159: GCGCGGCCGCCTAGTCATAGTCGAAATGAGGCTC

Cbr-puf-1.2:
QL160: GCCCCGGGCAAGAGAACGGCGTCGTCGTC
QL161: GCGCGGCCGCCTATGAAGAAATCGACGATCGACGTG

C. species 9 cDNA cloning

PUF-2 clade:
QL162: GGTTGCCACTTCCTTCAAAGTAACTAC (outer)
QL163: CTCCCGTCTGGCTCTCACGTAGAGCTT

QL164: CTCCCGGCGTGGGCGCTCGATGAAAAT (inner)
QL165: GTCTAGGATCTTCTTGCCAGAAGAGAA

FBF clade:
QL166: CTGCCAACCTGGTCCTTGGATTCCAAT (fbf-1)
QL167: CTCGATCATCTTTTTGCCGGATGAGAA

QL168: CTCTCTGAAGTTTTGGATAGTGGCGAT (fbf-1)
QL169: AACTCTCGAGTGCAACTTTTTGAGCCA

PUF-8 clade:
QL170: GCTCAACAAATAGTCGATAGCGTCTG
QL171: CTTATCAAGCTTTGCGAGAATGTGCTT

QL172: TTCGGTACACCTCCATCATGCGCGTG
QL173: GACGGTTAGAATCAGTTCACGACGTTG

PUF-9 clade:
QL174: AGTGCGTTCAATGGAATGGTCGATAA
QL175: GTCAGTGTGATCTTCTTGCGATGCTG

QL176: CCACAGGGCACTCCTGACTTCCAGAT

QL177: ATTTGAAGCAATGGAGGGGATGGATC

PUF-5 clade:

QL178: TTCGATGGAGGAGTCTTCTCATCCAAC
QL179: GATCTTCTTTCCAGAGGAGAAGCGCTC

QL180: CTTGACTCCAGCTGACTTCGGTCTCG
QL181: GAACCATTTTGCGTAGAGTCGCATCTC

PUF-3 clade:
QL182: GACAAAAATGGTTGTCGTTTCCTGC
QL183: GACATAGTTTCCGTATTGGTGGAACAG

QL184: ACATGTTCGGAAACTTCTTTGTCCAGC
QL185: GGTGTGGAATGTAACCTTCGAAGATTTC

PUF-6/7 clade:
QL186: CCGATTCGGGAAGTTTCTTTTTCAAC
QL187: CAAATCGTTATCATCTGTTGAACGAC

QL188: ACGGAGTGAAGTTCCTGGAGATCCAC
QL189: AGTGCATCGCGATTCGTCCCATGATC

Cbr-gld-1 pIIIA/MS2-2 cloning (direct annealing)
WT form
QL190:
CCGGGCAGTGCTAGCATAGAATCATGTACCATATATCGTGTATCCATCACGC
ATG
QL191: CGTGATGGATACACGATATATGGTACATGATTCTATGCTAGCACTGC

Mutant form
QL192:
CCGGGCAGTGCTAGCATAGAATCAACAACCATATATCGTGTATCCATCACGC
ATG
QL193: CGTGATGGATACACGATATATGGTTGTTGATTCTATGCTAGCACTGC

Cbr-gld-1 gel shift RNA oligo
WT
QL194: UAGAAUCAUGUACCAUAUAUCGUGUAU

Mutant
QL195: UAGAAUCAACAACCAUAUAUCGUGUAU

Vector sequencing primers:
pGEX 5': GGGCTGGCAAGCCACGTTTGGTG

pGEX 3': CCGGGAGCTGCATGTGTCAGAGG

pACT2 5': TACCACTACAATGGATG
pACT2 3': TGAGATGGTGCACGATG

pIIIa 5': CTGTAATCATTGTCAACAGG
pIIIa 3': AGACATGGGTGATCCTCATG

pCITE-4a-F, CGGGGACGTGGTTTTCCTTTG
pCITE-4a-R, GCTAGTTATTGCTCAGCGGAC

Cbr-gld-1 3'UTR ACA (NheI-PstI fragment)

QL196: CGCTAGCATAGAATCAACAACCATATATCGTGTATCCATCAC
QL197: ATTTGCCAAGAATCTCCTTCTGCAG

dsRNA fragment amplification for PUF-2&FBF clades RNAi

CBN01481 (C-terminus, 730bp)
QL198: TAATACGACTCACTATAGGGCTAATGACATCCGTCACATCCCGT
QL199: TAATACGACTCACTATAGGGGACGAGAAGACTTCAGCTTCATCG

CBN20762 (N-terminus, 610bp)
QL200: TAATACGACTCACTATAGGGACCGCGACACGTTCTCGAATAG
QL201: TAATACGACTCACTATAGGGCTTCTCTCGAACTTGTGGATCCAC

CJA08591 (N-terminus, 614bp)
QL202: TAATACGACTCACTATAGGGATGGACAGTGACTCGTTTTCGAAC
QL203: TAATACGACTCACTATAGGGTTGCATATCGTGAGGAACACTTCG

CJA06848 (N-terminus, 602bp)
QL204: TAATACGACTCACTATAGGGATGGATCAGTCGAAAACGCGAG
QL205: TAATACGACTCACTATAGGGTTGGCCAATTGCATTCTAGCACGC

CRE13610 (C-terminus, 625bp; from the alignment with CRE09826, this primer pair most likely only targets CRE13610)
QL206: TAATACGACTCACTATAGGGATCCAACACATCATCGAGACGCC
QL207: TAATACGACTCACTATAGGGATGGCCAGTCGACGCTTGAAGT
QL242, TAATACGACTCACTATAGGGAAACTCGCGTCCAACGAGTTCG (pair with QL207)

New CBN20762 (Cbn-PUF-2) RNAi fragment C terminus 573bp:
QL210:
TAATACGACTCACTATAGGGCCTATCGAAATGCCAAAAGCTG
QL211:
TAATACGACTCACTATAGGGTCATAATCGACATGTTGTTCGG

Sp.9-PUF-1 (~1078bp)
QL212: AAAGAGTAGTCCATGAGGATCTG
QL213: GAAGAGAATCTCATCAATCGCGC

Sp.9-PUF-12 (~1010bp)
QL214: AGAACACTCAGGAAATCGACGCG
QL215: CGACGAGAATCTTGTCAAACGGC

Sp.9-PUF-2 RNAi: ~900bp
QL216: TAATACGACTCACTATAGGGTTCAACAACTACGGTCGATCGG
QL217: TAATACGACTCACTATAGGGATCTTATGGATCGGCTCCTCAG

Sp.9 PUF-2 clade RNAi:
QL218: TAATACGACTCACTATAGGGCGAGATCGAGTTTCCAGAGAAG
QL219: TAATACGACTCACTATAGGGATGTGTGTATCTCGCTGTCCAG

Sp.9-PUF-1 RNAi (~1078bp)
QL220: TAATACGACTCACTATAGGGAAAGAGTAGTCCATGAGGATCTG
QL221: TAATACGACTCACTATAGGGGAAGAGAATCTCATCAATCGCGC

Sp.9-PUF-12 RNAi (~1010bp)
QL222: TAATACGACTCACTATAGGGAGAACACTCAGGAAATCGACGCG
QL223: TAATACGACTCACTATAGGGCGACGAGAATCTTGTCAAACGGC

Cbr-tra-2 (nm1) lesion sequencing primers:
QL234, ACCTGGCGCAGTAACACTCCATTG (pair with DK12, 273bp)

Cbr-tra-1 (nm2) lesion sequencing primers:
QL235, CGCTGCTCTGATGGATCCGAATGG (pair with DK35, 486bp)

Total actin primers:
EH37, TACCTCATGAAGATCCTCACCG
EH38, CATACCCAAGAAGGATGGCTGG

GFP RNAi:
QL244, TAATACGACTCACTATAGGGAGTAAAGGAGAAGAACTTTTCAC
QL245, TAATACGACTCACTATAGGGCAAACTCAAGAAGGACCATGTG

Ce-fem-3 pIIIA/MS2-2 cloning (direct annealing)
WT form
QL248:
GGGTCTATCTCACTAACGCTTCTTGTGTCATTCACTTTCGAATCCTCTGCATG
QL249:
CAGAGGATTCGAAAGTGAATGACACAAGAAGCGTTAGTGAGATAGACCC

Mutant form
QL250:
GGGTCTATCTCACTAACGCTTTTTGTGTCATTCACTTTCGAATCCTCTGCATG
QL251:
CAGAGGATTCGAAAGTGAATGACACAAAAAGCGTTAGTGAGATAGACCC

C. briggsae-fem-3 pIIIA/MS2-2 cloning (direct annealing) (match to Kimble lab construct)
Mutant form
QL252: GGGCCCACCTCATCCCATCTCCTGTGTCATTGTTCGCATG
QL253: CGAACAATGACACAGGAGATGGGATGAGGTGGGCCC

C. brennei-fem-3 pIIIA/MS2-2 cloning (direct annealing)
WT form
QL254: GGG
CCCCGATCACTCATTCTTCTTGTGTCATTTTTTGATTCGCTTCCTGCATG
QL255: C
AGGAAGCGAATCAAAAAATGACACAAGAAGAATGAGTGATCGGGGCCC

C. remanei-fem-3 pIIIA/MS2-2 cloning (direct annealing)
WT form
QL256: GGG
ACGTCTTATACCCCCACTATTGTGTCATTTCCTCCCTGTGTCTTAGCATG
QL257:
CTAAGACACAGGGAGGAAATGACACAATAGTGGGGGTATAAGACGTCCC

C. sp 9-fem-3 pIIIA/MS2-2 cloning (direct annealing)
WT form
QL258:
GGGCACCTCATCCCATCATCTTCTGTGTCATTTGTTCAATTTTCTACAGCATG
QL259:
CTGTAGAAAATTGAACAAATGACACAGAAGATGATGGGATGAGGTGCCC

C. japonica fem-3 3'RACE
QL260, AGTTATCCGCCTGACCAGCACAAC
QL261, CGGTTGGTATCACGTGTCAATCG

New *C. japonica fem-3* cloning (both pair with QL260), RNA or cDNA as the template
QL262, TGAGTGGGTGTACAGTCTCG
QL263, ATTAGAAGGGGGTGGGTGAG

Verify PUF gene sequences

CRE10385 (RP30447)
QL276, ACAGCGTTGGAGACTCTCGACC
QL277, CGCCAATCATGTCATCCAAAAG

QL278, TTCTGATCACTTACCTGTGC

CJA06848
QL279, GGCTGTCCAGGTTTTCCTCCGG
QL280, GAGATCGAAGAGAGCCGAATG
QL281, GAAGCCACGCCCACGACGGG

CJA08591
QL282, ACGTTCCTCATCCAGACACCGG
QL283, TGGACATTCTGATCTTCCACC

Sequencing CRE10385 (Reverse primer, designed according to 3'RACE: 278.1_RF file)
QL284, AGACACGTCACGATGATGGC

Appendix 2: Revised nomenclature for Caenorhabditis PUF family members

NRec, not recognized; WB, WormBase; subfam, PUF subfamily.

	C. elegans			*C. briggsae*			*C. remanei*		*C. brenneri*		*C. japonica*	
subfam.	Lamont et al.	WB	Liu et al.	Lamont et al.	WormBase	Liu et al.	WB	Liu et al.	WB	Liu et al.	WB	Liu et al.
FBF	*fbf-1*	*fbf-1*	*fbf-1*	N.A.	N.A.	N.A.	N.A.	N.A.	CBN14362	*Cbn-fbf-1*	CJA06848	*Cjp-fbf-1*
FBF	*fbf-2*	*fbf-2*	*fbf-2*	N.A.	N.A.	N.A.	N.A.	N.A.				
PUF-2	N.A.	N.A.	N.A.	CbPUF-2	cbg02702	*Cbr-puf-2*	*Cre-fbf-2* CRE13610	*Cre-puf-2.1*	CBN07343	*Cbn-puf-2*	CJA08591	*Cjp-puf-2*
PUF-2	N.A.	N.A.	N.A.	CbPUF-1	cbg02701	*Cbr-puf-1.1*	CRE09826	*Cre-puf-2.2*				
PUF-2	N.A.	N.A.	N.A.	CbPUF-12	cbg13460	*Cbr-puf-1.2*	CRE30447	*Cre-puf-2.3*				
PUF-3/4	*puf-3*	*puf-3*	*puf-3*	CbPUF-3	*Cbr-puf-11* cbg01774	*Cbr-puf-3*	*Cre-puf-11* CRE01853	*Cre-puf-3*	CBN28060	*Cbn-puf-3*	CJA16871	*Cjp-puf-3*
PUF-3/4	*puf-4*	*puf-4*	*puf-4*	CbPUF-4	cbg09894	*Cbr-puf-4*						
PUF-3/4	*puf-11*	*puf-11*	*puf-11*									
PUF-5	*puf-5*	*puf-5*	*puf-5*	CbPUF-5	*Cbr-puf-5* cbg00661	*Cbr-puf-5*	*Cre-puf-5* CRE05663	*Cre-puf-5*	CBN19189	*Cbn-puf-5*	CJA06598	*Cjp-puf-5*
PUF-6/7	*puf-6*	*puf-6*	*puf-6*	CbPUF-6	cbg13175	*Cbr-puf-6.1*					N.A.	N.A.
PUF-6/7	*puf-7*	*puf-7*	*puf-7*	CbPUF-7	cbg11800	*Cbr-puf-6.2*	*Cre-puf-7* CRE04675	*Cre-puf-6*	CBN14700	*Cbn-puf-6*	N.A.	N.A.
PUF-6/7				CbPUF-10	cbg22348	*Cbr-puf-6.4*					N.A.	N.A.
PUF-6/7				CbPUF-11	cbg06390	*Cbr-puf-6.3*					N.A.	N.A.
PUF-8	*puf-8*	*puf-8*	*PUF-8*	CbPUF-8	*Cbr-puf-8* cbg13130	*Cbr-puf-8*	*Cre-puf-8* CRE26150	*Cre-puf-8*	CBN24464	*Cbn-puf-8*	CJA04875	*Cjp-puf-8*
PUF-9	*puf-9*	*puf-9*	*PUF-9*	CbPUF-9	*Cbr-puf-9* cbg14762	*Cbr-puf-9*	*Cre-puf-9* CRE00692	*Cre-puf-9*	CBN26238	*Cbn-puf-9*	CJA07693	*Cjp-puf-9*
PUF-12	NRec	*puf-12*	*puf-12*	NRec	*Cbr-puf-12*	*Cbr-puf-12*	CRE02049 *Cre-puf-12*	*Cre-puf-12*	CBN00897	*Cbn-puf-12*	CJA14022 *Cjp-puf-12*	*Cjp-puf-12*
PUF-13	NRec	ZK792.5	*puf-13*	NRec	cbg26313	*Cbr-puf-13.1*	CRE09865	*Cre-puf-13*	CBN28884	*Cbn-puf-13*	CJA06649	*Cjp-puf-13*
PUF-13					cbg06093	*Cbr-puf-13.2*						

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