RESEARCH ARTICLE

Evolutionarily Dynamic Roles of a PUF RNA-Binding Protein in the Somatic Development of *Caenorhabditis briggsae*

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ABSTRACT

Gene duplication and divergence has emerged as an important aspect of developmental evolution. The genomes of *Caenorhabditis* nematodes encode an ancient family of PUF RNA-binding proteins. Most have been implicated in germline development, and are often redundant with paralogs of the same sub-family. An exception is *Cbr-puf-2* (one of three *Caenorhabditis briggsae* PUF-2 sub-family paralogs), which is required for development past the second larval stage. Here, we provide a detailed functional characterization of *Cbr-puf-2*. The larval arrest of *Cbr-puf-2* mutant animals is caused by inefficient breakdown of bacterial food, which leads to starvation. *Cbr-puf-2* is required for the normal grinding cycle of the muscular terminal bulb during early larval stages, and is transiently expressed in this tissue. In addition, rescue of larval arrest reveals that *Cbr-puf-2* also promotes normal vulval development. It is expressed in the anchor cell (which induces vulval fate) and vulval muscles, but not in the vulva precursor cells (VPCs) themselves. This contrasts with the VPC-autonomous repression of vulval development described for the *Caenorhabditis elegans* homologs *fbf-1/2*. These different roles for PUF proteins occur even as the vulva and pharynx maintain highly conserved anatomies across *Caenorhabditis*, indicating pervasive developmental system drift (DSD). Because Cbr-PUF-2 shares RNA-binding specificity with its paralogs and with *C. elegans* FBF, we suggest that functional novelty of RNA-binding proteins evolves through changes in the site of their expression, perhaps in concert with *cis*-regulatory evolution in target mRNAs. *J. Exp. Zool. (Mol. Dev. Evol.) 9999B: 1–13, 2013.* © 2013 Wiley Periodicals, Inc.

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Recently duplicated genes typically have overlapping roles, and their evolutionary fates are determined by a mixture of mutations, functional constraints, and natural selection (Ohno, '70; Zhang, 2003). Under current models of gene evolution, the most common fate of gene duplication is pseudogenization, a mutation accumulation process that erases duplicates from the genome (Lynch et al., 2001). In the nematode *Caenorhabditis elegans*, there is roughly one pseudogene for every eight functional genes (Harrison et al., 2001). Less often, duplicated genes could also gain novel functions, which would promote their retention (Bergthorsson et al., 2007). One well-studied case in humans is in the RNase A superfamily. The eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) genes of humans were generated through gene duplication, and a novel antibacterial activity evolved in the ECP duplicate that is independent of its historical ribonuclease activity (Zhang et al., '98). More often, duplicates can be stably maintained by subfunctionalization, in which duplicates each carry part of their ancestral functions (Orgel, '77; Force et al., '99; Lynch and Force, 2000). For example, RNase1 gene duplication in an Asian

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colobine monkey created RNase1 and RNase1B copies. Although the optimal catalytic pH remains at 7.4 for RNase1, RNase1B specialized to degrade dsRNA at pH 6.3, a secondary capacity of the ancestral RNase1 (Zhang et al., 2002).

The Pumilio and FBF (PUF) family proteins belong to a large protein family that help regulate diverse biological processes (Wickens et al., 2002). In *C. elegans*, PUF proteins are known for their regulatory roles in various aspects of germ cell development by translational repression of target mRNAs (Zhang et al., '97; Crittenden et al., 2002; Subramaniam and Seydoux, 2003; Ariz et al., 2009; Suh et al., 2009; Kalchhauser et al., 2011). *C. elegans* PUF proteins often act together with paralogs to fulfill their various roles. For example, the recent FBF sub-family duplicates FBF-1 and FBF-2 have largely redundant functions (Zhang et al., '97; Kraemer et al., '99; Lamont et al., 2004; Thompson et al., 2006; Walser et al., 2006; Merritt and Seydoux, 2010; Kalchhauser et al., 2011). This phenomenon is also true for additional PUF sub-families, PUF-3, PUF-5, and PUF-6 (Lublin and Evans, 2007; Hubstenberger et al., 2012).

Within Caenorhabditis nematodes, both ancient and lineage-specific duplications and gene losses have occurred within the PUF family (Fig. 1; Lamont et al., 2004; Liu et al., 2012). In our previous and current research, we focus on members of two PUF sub-families, *C. elegans* FBFs and *Caenorhabditis briggsae* PUF-2 genes, which are not strict orthologs, but are nevertheless each other's closest relatives (Fig. 1). We previously reported that the *C. briggsae* PUF-2 sub-family paralog *Cbr-puf-2*, which, like *C. elegans* FBF-1/2 has multiple roles in germline development, is also required for reliable embryogenesis and is absolutely essential for developmental progression of newly hatched larva (Liu et al., 2012). Since *C. elegans* PUF family genes are often redundant with other paralogs, and none are essential for early development, the non-redundant role of *Cbr-puf-2* in larval progression is surprising.

In this study, we demonstrate that larval arrest of *Cbr-puf-2* mutants is caused by pharyngeal malfunction and the starvation resulting from it. In addition to functions in the pharynx, we also show that *Cbr-puf-2* promotes normal vulval development, most likely by acting in the anchor cell. Taken together, our work demonstrates that in the soma, unlike in the germ line, *Cbr-puf-2* has acquired multiple essential roles that are not covered by its two closely related paralogs, *Cbr-puf-1.1* and *Cbr-puf-1.2*. These roles are also not seen in its closest *C. elegans* homologs, indicating that highly conserved organs like the pharynx and vulva experience rapid developmental systems drift (True and Haag, 2001).

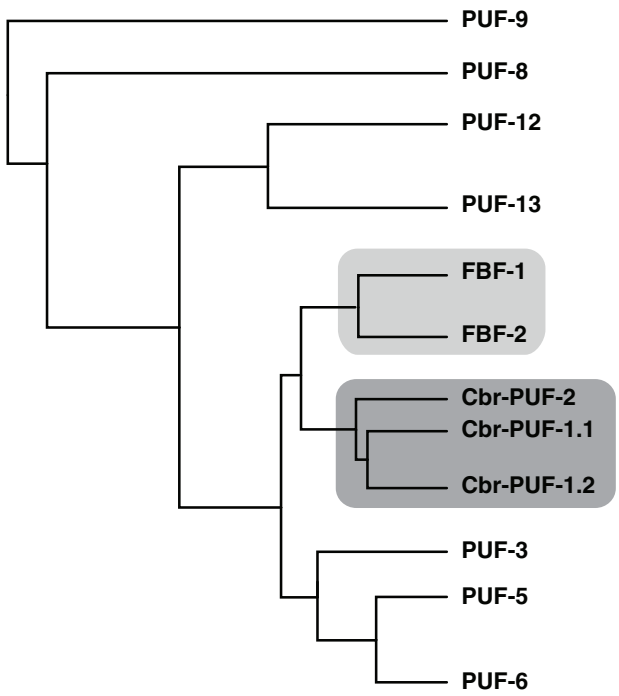

Figure 1. Simplified phylogeny of the Caenorhabditis PUF family. Each of the nine PUF sub-families, which diverged prior to the *C. elegans–C. briggsae* common ancestor, are shown. For the FBF and PUF-2 sub-families (light gray and dark gray, respectively), all *C. elegans* and *C. briggsae* paralogs are indicated, highlighting the complementary losses of PUF-2 in *C. elegans* and of FBF in *C. briggsae*. This figure shows that *C. elegans* FBFs and *C. briggsae* PUF-2 genes are not strict orthologs, but are nevertheless each other's closest relatives. Paralog details are omitted from other sub-families, but *C. elegans* and *C. briggsae* have at least one member of each. Figure modified from Liu et al. (2012).

MATERIALS AND METHODS

Nematode Culture and Genetics

All nematode species were cultured by using standard *C. elegans* conditions (Wood, '88a), 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 *cby-15/Cbr-puf-2(nm66)*. Homozygous *cby-15* mutant animals are dumpy (Dpy). Strain CP113 (*Cbr-puf-2(nm66)*, *nmIs4*[*Cbr-puf-2(+)*, *Cbr-unc-119 (+)*]) is a 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).

Cbr-puf-2 Transcription Reporter

For constructing a transcription GFP reporter of *Cbr-puf-2*, 1,380 nucleotides of 5′ regulatory DNA and the native 3′ untranslated

region were fused with histone2B-GFP (H2B) chimeric coding sequence via Gateway cloning technology. 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.

Microscopy

Nematode differential interference contrast (DIC) microscopy was followed by standard methods (Wood, '88a). In brief, worms were put on 2% agarose pad, and mounted in M9 buffer or Vectashield (Vector Laboratories, Burlingame, CA, USA) for DIC microscopy or fluorescence microscopy, respectively, using an Axioskop2 *plus* (Zeiss, Oberkochen, Germany). Images were captured with Axiocam digital camera (Zeiss) and Open Lab software (Improvision, Lexington, MA, USA) or an LSM710 confocal microscope (Zeiss). In the latter, z-stacks were collapsed for presentation using ZEN lite 2011 (Zeiss).

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 hr. Counts were made at room temperature. Each worm was observed three times for 20 sec using Axioskop2 *plus* (Zeiss) with a 25× oil-immersion objective. Pumping rate per minute was calculated from videos taken with an eye-piece digital microscope camera (AM-423X, Dino-Eye) at 15 frames per second. Videos were slow-played at one fourth of the recorded speed for accurate pumping rate counting.

Feeding Assay

This assay was designed to examine the efficiency of food intake. *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 hr. Then, worms were put on agarose pad with a drop of 50 mM sodium azide, and mounted in Vectashield (Vector Laboratories). Images were taken using Axioskop2 *plus* (Zeiss) with a 63× objective with the same setting.

Video Image Analysis

Video microscopy was used to observe 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 days post-hatching were transferred to bacterial suspension placed on a thin agarose pad, and a coverslip was placed on top. We typically waited about 30 min 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. Image sequences were viewed frame-by-frame, and frames were manually extracted consecutively corresponding to the cycle of terminal bulb contraction using iMovie v7.1.4 (Apple).

Axenic Culture

To grow *Cbr-puf-2(nm66)* mutant animals in axenic culture, embryos laid by *Cbr-cby(nm15)/Cbr-puf-2(nm66)* parents were washed off of NGM plate media with M9 buffer, and bleached 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). Ten to 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).

Phalloidin Staining

Wild-type *C. briggsae* worms at the second larval stage or *Cbr-puf-2(nm66)* mutant worms 3-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 min. After the removal of acetone and vacuum-dry, 2U fluorescein-conjugated phalloidin (Molecular Probes, Eugene, OR, USA) diluted in 20 μL S mix (0.2 M Na-phosphate, pH 7.5; 1 mM $MgCl_2$; 0.004% SDS) was added to the dry worms. Worms were stained at room temp in the dark for 1 hr. At the end of staining, worms were washed twice in PBBT (PBS + 0.5% BSA + 0.5% Tween-20), and mounted for microscopy.

RESULTS

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. We followed their development for 2 weeks after hatching, and found that they accumulated refractile vacuoles in their intestine, which increased with time

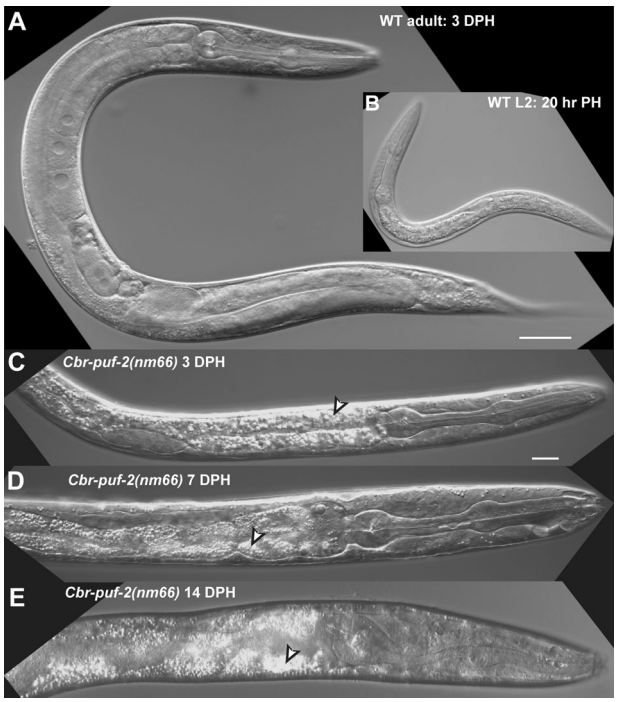

Figure 2. *Cbr-puf-2(nm66)* mutant hatchlings arrest development but are grossly normal. (**A**,**B**) Wild-type animal at adult stage (A) and the second larval stage (B), scale bar: 50 μm. (**C**)–(**E**) *Cbr-puf-2 (nm66)* mutant animals of 3 (C), 7 (D), and 14 (E) days post-hatching (DPH); scale bar: 10 μm. Arrowheads indicate refractory granules in gut.

(Fig. 2). These phenotypes might result from starvation caused by defective pharyngeal function (Schroeder et al., 2007). The Caenorhabditis pharynx is a neuromuscular tube that connects stoma to intestine (Mango, 2009; Avery and You, 2012). Newly hatched *C. elegans* larvae have fully functional pharynxes comprised of seven distinct but functionally integrated cell types (Mango, 2007). The pharynx has eight muscle segments, each forming a three-cell torus encircling the lumen (Albertson and Thomson, '76), which from anterior to posterior are the corpus, the isthmus, and the terminal bulb (Avery and Horvitz, '89). Coordinated contraction and relaxation of consecutive muscle sectors produces feeding behavior (Avery and You, 2012) and mechanical breakdown of food (Avery and You, 2012).

The pharynx of *Cbr-puf-2(nm66)* mutants did not display morphological abnormalities, and coordinated pharyngeal pumping movement was also maintained. We therefore hypothesized that *Cbr-puf-2(nm66)* mutants have subtle pharyngeal defects at the cellular or physiological level. To test this, we 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, '89), we compared the terminal bulb grinding rates of *Cbr-puf-2(nm66)* mutant animals 3-day after hatching with wild-type animals at the second larval stage. Mutant animals 3-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 (Fig. 3A).

While intriguing, the relatively minor shift of the grinding rate noted above seemed insufficient to cause a complete developmental arrest. It also may reflect a side effect of some more fundamental defect, or of starvation, rather than being the cause of the dramatic arrest phenotype. To further test the pharyngeal defect hypothesis, we examined *Cbr-puf-2(nm66)* mutants fed with *E. coli* expressing green fluorescent protein (GFP) with fluorescence microscopy. For wild-type animals, GFP labeled *E. coli* cells are drawn in and transported to the terminal bulb, where they are crushed upon passage through the grinder. The process is accompanied by the disappearance of intense GFP signal, presumably because GFP molecules are dispersed and inactivated after the cell wall is broken up (Fig. 3B,C). In contrast to wild-type *C. briggsae*, *Cbr-puf-2(nm66)* mutants accumulate intact *E. coli* cells in their intestine (Fig. 3D,E). This defect was rescued by introduction of a wild-type copy of *Cbr-puf-2* into *Cbr-puf-2 (nm66)* mutants. *Cbr-puf-2(nm66;nmIs4)* animals (Liu et al., 2012) can break open *E. coli* efficiently (Fig. 3F,G). These results suggest *Cbr-puf-2* is involved in the physiological function of terminal pharyngeal bulb, and is required to support robust food grinding.

Cbr-puf-2 Is Expressed in the Muscle Cells of Terminal Pharyngeal Bulb

To investigate where expression of *Cbr-puf-2* occurs, we introduced a *Cbr-puf-2* reporter plasmid into wild-type *C. briggsae*. This chimeric gene fuses the *Cbr-puf-2* promoter and 3′-UTR regions to sequence coding for histone 2B-GFP. We found that the stable transgenic strain CP126 expressed GFP in the terminal bulb of the pharynx (Fig. 4B). The GFP signal could only be detected during a brief window from the late fourfold embryo to the early second larval stage, and labeled three nuclei at the posterior part of the terminal bulb (Fig. 4B). To further facilitate cell identification, we constructed another strain CP127, which introduced another nuclear localized reporter, *Ce-pha-4*::Histone2B-mCherry (Zhao et al., 2010), into strain CP126 (Fig. 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). *Cbr-puf-2*::GFP-positive nuclei overlap with a subset of the *pha-4*::mCherry nuclei (Fig. 4D). The pharyngeal cell lineage is conserved between *C. elegans* and *C. briggsae* (Zhao et al., 2008). The resulting organ is composed of eight rings of pharyngeal muscles (pm), and its terminal bulb is mainly

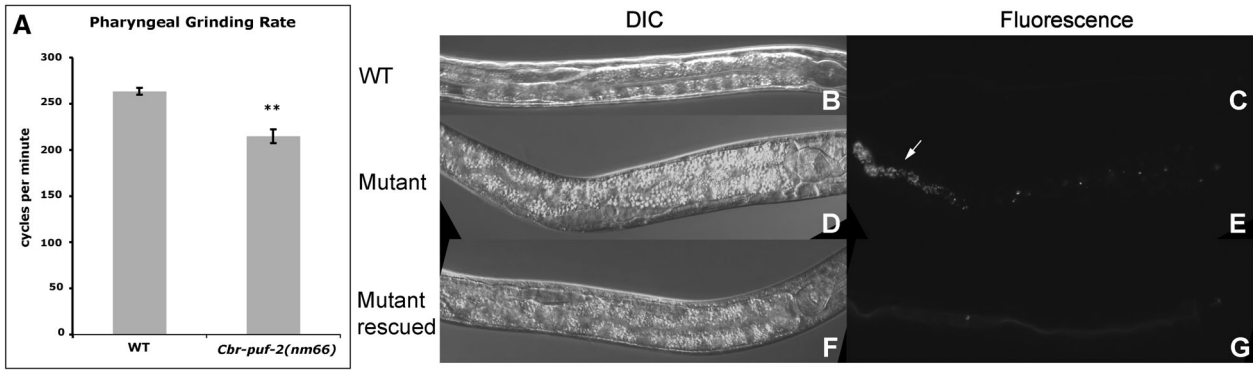

Figure 3. Pharyngeal defects of *Cbr-puf-2(nm66)* mutants. **(A)** Pumping rates between wild-type animal at the second larval stage (WT column) and *Cbr-puf-2(nm66)* mutant animals 3 days after hatching (Mutant column). **Indicates the pumping rates are significantly different (two-tailed *t*-test, $P = 0.0004$, $N = 5$). **(B)–(G)** 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 (B,C), *Cbr-puf-2(nm66)* mutant animals 3 days after hatching (D,E) and *Cbr-puf-2(nm66;nmls4)* transgenic rescued animals at the second larval stage (F,G), respectively. The anterior of the worm is facing right. Note that wild-type and *Cbr-puf-2(nm66;nmls4)* animals are able to mechanically break up bacteria leading to loss of fluorescence in the intestinal tract (C,G). In contrast, *Cbr-puf-2(nm66)* mutant animals are unable to do so leading to accumulation of intense GFP signal in the intestinal tract (E). Arrow indicates intact *E. coli* in the intestinal tract.

comprised of pharyngeal muscle pm6, pm7, and pm8 (Avery and Thomas, '97). 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. These results support the notion that *Cbr-puf-2* acts in the terminal bulb to promote normal pharyngeal function.

Cbr-puf-2 Promotes Robust Muscle Contraction of Pharyngeal Terminal Bulb

Because of the grinding defect and the terminal bulb-specific expression pattern, we sought to characterize the grinding abnormalities of *Cbr-puf-2* mutants. As mentioned above, we did not see developmental aberration of the pharynx, the grinding rate of mutants was only slightly less than wild-type animals (Fig. 3A), and pharyngeal actin filaments in *Cbr-puf-2(nm66)* mutant animals appear grossly normal (Fig. S1). These observations suggest the defect is subtle, yet of profound phenotypic consequence. Intuitively, a grinding defect could be a consequence of the malfunction of the "teeth," thick and ridged cuticle deposits lining the lumen of the grinder (Zhang et al., 2005), which are refractile under DIC microscopy. However, the chitin layer is not obviously different in mutant and wild-type animals by DIC microscopy (Fig. 5) or staining with a fluorescent chitin-binding protein (data not shown).

The pharyngeal muscle (pm) cells, pm1–pm5, coordinate each cycle of pharyngeal pumping (Avery and You, 2012). The pharyngeal lumen first opens and draws in food particles, and traps them in the anterior isthmus and expels liquid. Food particles are then transported from the anterior isthmus to the grinder in the terminal bulb by isthmus peristalsis. Force provided by pm6 and pm7 inverts the plates of the grinder to break up bacteria and pass debris to the intestine. We examined the grinding behavior of wild-type and mutant animals in detail by analyzing videos frame-by-frame. These observations first established a normal three-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 (Fig. 5B). 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 (Fig. 5C). Afterwards, muscle relaxation returns the grinder back to its rest position (Fig. 5D).

Though *Cbr-puf-2(nm66)* mutant animals are able to initiate muscle contraction and open the lumen of the grinder (Fig. 5F), they were neither able to fully seal the chitin teeth nor open the pharyngeal–intestinal valve most of time (Fig. 5G). This deficiency may be caused by an inability of pharyngeal muscle 7 to push the posterior of the grinder further backwards during the second phase of the cycle, which appears a wider space at the position of pm7 (Fig. 5G). Occasionally the grinder of *Cbr-puf-2(nm66)* mutant animals does form a small cleft or opening that led to intestinal lumen, explaining how bacteria are still delivered to the intestine. The incomplete muscle contraction of *Cbr-puf-2(nm66)* mutant animals explains the dramatic deficiency of bacteria grinding reported above.

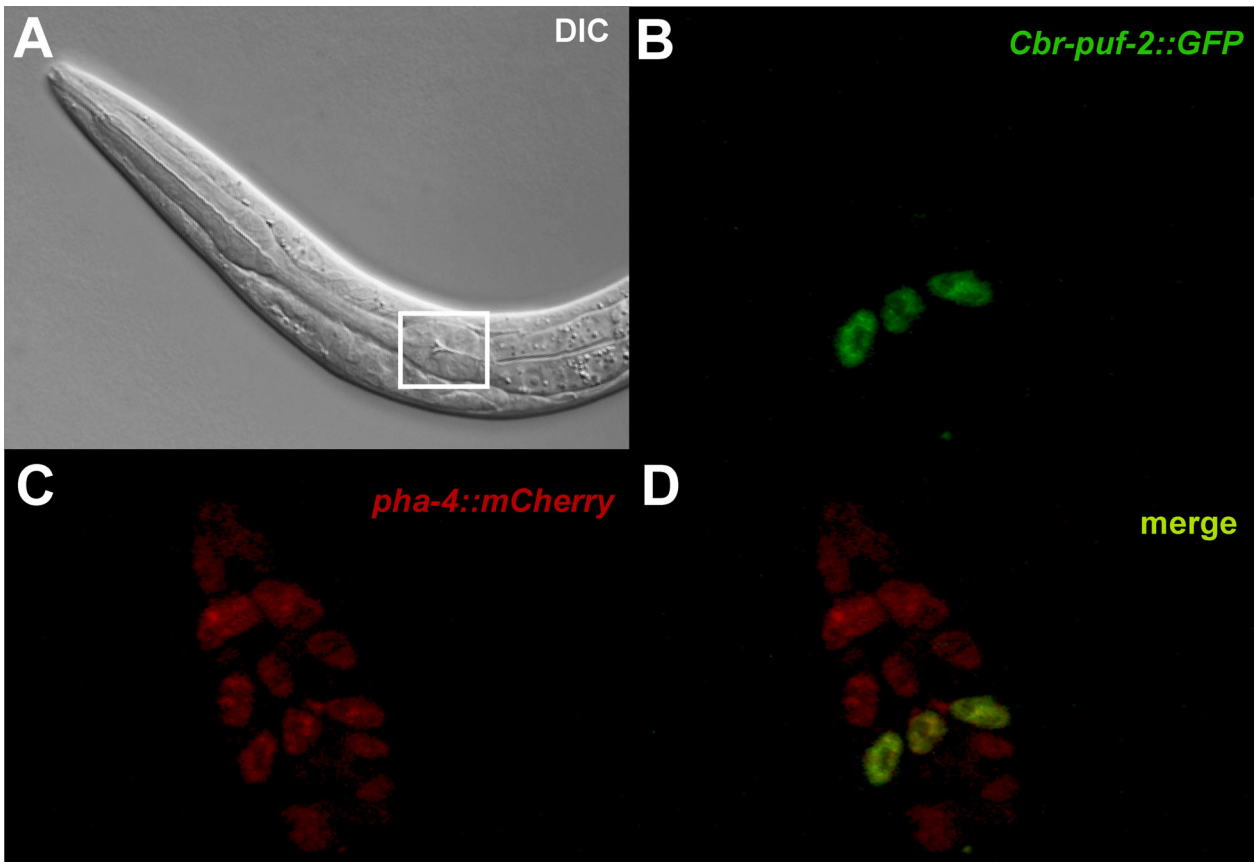

Figure 4. *Cbr-puf-2* expresses in the pharyngeal muscle 7. (**A**) DIC microscopy of the pharyngeal region of a worm at the second larval stage. The white box highlights the terminal bulb, which is the area shown in Figure 5B–D. (**B**) *Cbr-puf-2* expresses in three cells at the posterior part of the terminal bulb. (**C**) Expression pattern of *pha-4* in the terminal bulb. (**D**) The merge of (B,C).

To confirm that the *Cbr-puf-2(nm66)* larval arrest phenotype was due to starvation, we grew mutant animals in axenic liquid medium (Nass and Hamza, 2007) to eliminate the link between nutrition and food grinding. Fifty-nine progeny of *Cbr-puf-2 (nm66)/cby-15* mothers that reached the L4 stage were scored by DIC 1 day later. Seven were Pvl and sterile, all of which were *nm66* homozygotes as judged by single-worm PCR assay (Fig. S2A). Seventy-seven non-Pvl siblings returned to plate culture all produced chubby (Cby) offspring, indicating none were *nm66* homozygotes. These numbers suggest that roughly half of *nm66* homozygotes reach adulthood in axenic medium with typical developmental timing, but with abnormal vulval development. We also examined whether liquid culture per se was sufficient to rescue development by rearing *nm66* homozygotes in S medium with suspended *E. coli* for food (Wood, '88b). No improvement over plate culture was seen, regardless of whether mutants were hatched in S medium or placed there 2 days post-hatching (data not shown).

Cbr-puf-2 Promotes Faithful Vulval Development

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 (Sulston and Horvitz, '77; Sulston and White, '80; Sternberg and Horvitz, '86; Kiontke et al., 2007). In wild-type hermaphrodites, each animal possesses a single centrally located, "Christmas tree"-like, vulval opening at the fourth larval stage, and at the adult stage it develops to a fully functional vulva (Fig. 6A). Though axenic rescued *Cbr-puf-2(nm66)* animals grew to adulthood, they had an asymmetrical developing vulva at the fourth larval stage (Fig. 6C). At the adult stage, the vulva was protruding (Fig. 6B). These protruding vulva (Pvl) animals also had very few germ cells in their gonads.

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 (Figs. 6D and S2B). These data indicate that *Cbr-puf-2* plays important, non-redundant roles in both vulval and germline

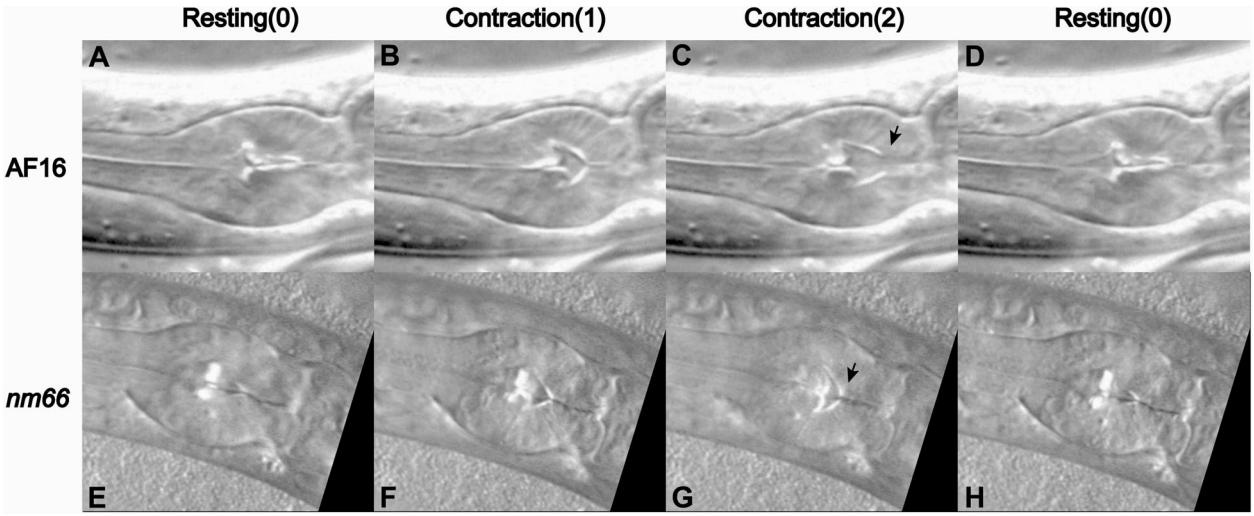

Figure 5. Muscle contraction of the terminal bulb is weaker in *Cbr-puf-2(nm66)* mutant animals. (**A**)–(**D**) One cycle of the grinding motion is shown for wild-type animals at the second larval stage. (**E**)–(**H**) One cycle of the grinding motion is shown for *Cbr-puf-2(nm66)* mutant animals. The motion starts with an initial contraction (B,F), and follows with a pulling, which opens the valve leading to the intestine lumen (arrow) in wild-type animals (C), but not in mutants (G). Afterwards, muscle relaxation returns the grinder back to its rest position (D,H).

development, in addition to redundant roles in the germ line that were previously described (Liu et al., 2012). Supporting this hypothesis, the *Cbr-puf-2* reporter is expressed in the anchor cell (AC) of the somatic gonad in L2 larvae (Fig. 6E–G). The AC induces vulval fate in the vulval precursor cells (VPCs) during late L2 and L3 stages (Sternberg, 2005). The *Cbr-puf-2* reporter also marks four cells around the vulval opening at the early and late L4 stage (Fig. 6H–M), which are likely to be the vm1 vulval muscle quartet. Judging from the mutant phenotype and reporter gene expression, we conclude that *Cbr-puf-2* likely functions to promote normal vulval development by acting in the anchor cell and/or vulval muscles, rather than in the VPCs themselves.

DISCUSSION

A Novel Role for *C. briggsae puf-2* in Pharyngeal Muscle Function

We previously identified an essential role of *C. briggsae puf-2* in larval progression (Liu et al., 2012). Three lines of evidence presented here suggest that the larval arrest phenotype of *Cbr-puf-2* mutants is due to starvation caused by inefficient breakdown of bacteria food. First, our GFP-labeled *E. coli* feeding assay shows that *Cbr-puf-2* mutant animals accumulate intact bacteria cells in their intestine, demonstrating that the defect is in food grinding. Further supporting this conclusion, video analysis of mutant animals did not reveal any feeding defects. Second, axenic medium rescues *Cbr-puf-2(nm66)* larval arrest phenotype, indicating that when the requirement for grinding is eliminated, mutant animals can overcome arrest and reach adulthood. Third, we identified an obvious grinding defect involving the same cells that express the *Cbr-puf-2* GFP reporter. We propose that *Cbr-puf-2(nm66)* pharynx malfunction is caused by an inability to provide the robust muscle contraction that brings pharyngeal teeth tightly together and break up food particles (Fig. 7). This sealing force, we suspect, is mainly provided by pharyngeal muscle 7, which express *Cbr-puf-2*.

The general morphology of the pharynx in *Cbr-puf-2(nm66)* mutant animals is normal. It is possible that the loss of *Cbr-puf-2* has a subtle anatomical effect, but the well-formed pharyngeal actin cytoskeleton structure in *Cbr-puf-2(nm66)* mutant animals (Fig. S1) is inconsistent with this hypothesis. Alternatively, Cbr-PUF-2 may regulate genes participating in muscle physiological functions, such as structural proteins in establishing the muscle sarcomere or regulators of muscle contraction (Benian et al., '96). Another hypothesis is that *Cbr-puf-2* is involved in neuromuscular function in pm7. RNA-binding proteins play important roles in synaptic plasticity (Richter, 2010), and PUF family members are essential for synapse formation and maintenance in *Drosophila*, rodents, and humans (Dubnau et al., 2003; Menon et al., 2004; Vessey et al., 2010; Marrero et al., 2011; Siemen et al., 2011). For example, a founding PUF family member, Pumilio, modulates synaptic function in neurons or muscle cells via direct repression of mRNA targets including eIF4E and AChE (Menon et al., 2004; Marrero et al., 2011). In Caenorhabditis, pm7 receive synapses from motor neuron M5. One possibility is that *Cbr-puf-2* may participate in post-synaptic gene regulation, for example to repress mRNA involved in neurotransmission.

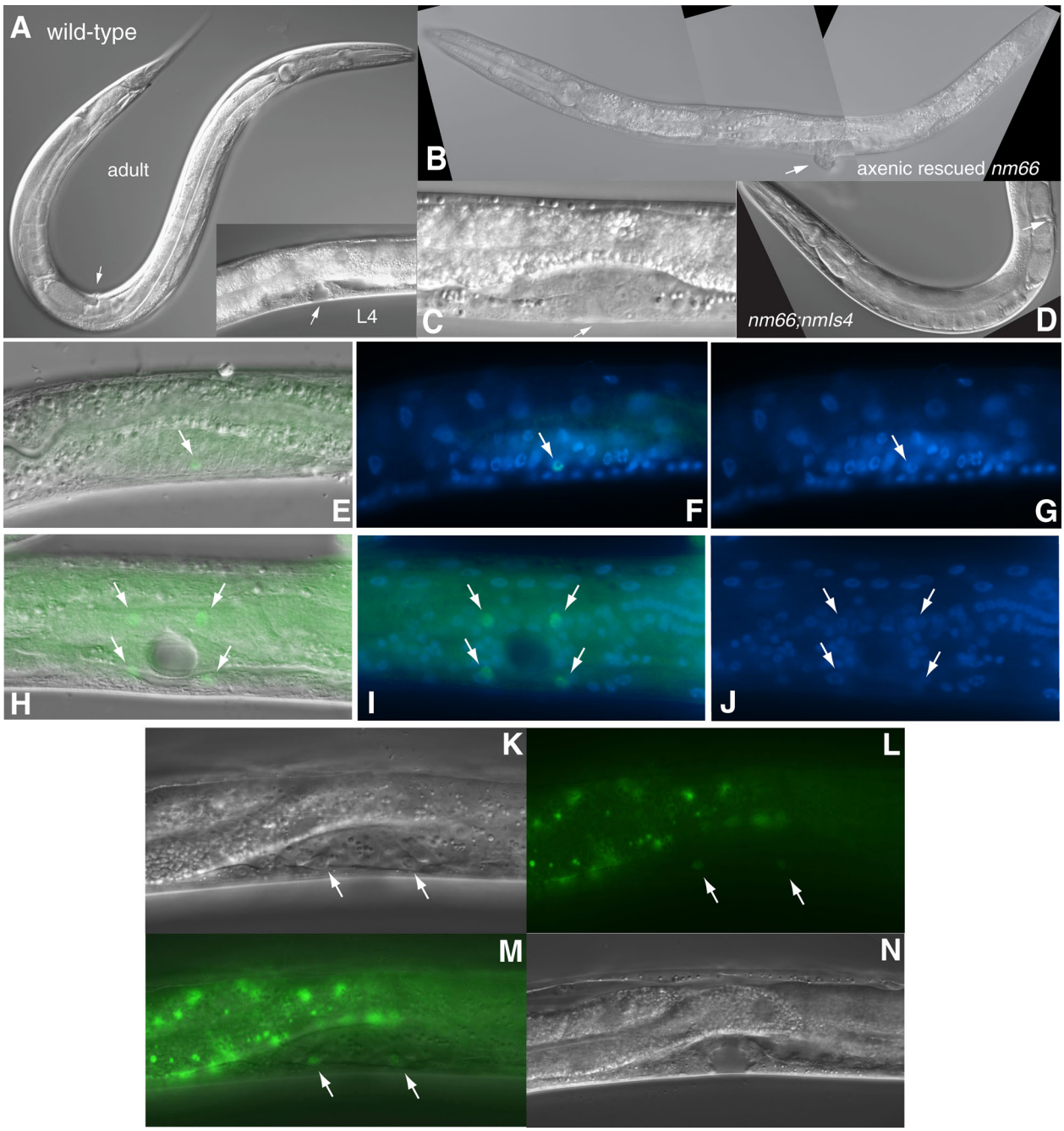

Figure 6. *Cbr-puf-2* is expressed during vulval development in *C. briggsae*. (**A**) Wild-type adult *C. briggsae* hermaphrodite, with a ventral vulva roughly half way between head and tail (arrow). Inset shows the developing vulva (arrow) of a wild-type L4 larva. (**B**) *Cbr-puf-2(nm66)* mutant animals grow to adulthood in axenic culture, but with protruding vulvae (arrow). (**C**) The developing vulva (arrow) of L4 *Cbr-puf-2(nm66)* mutants reared in axenic culture has an abnormal shape. (**D**) *Cbr-puf-2(nm66)* transformed with a *Cbr-puf-2(+)* transgene (strain CP113) have normal vulval development (arrow) and are fertile. (**E**)–(**N**) *Cbr-puf-2* transcriptional GFP reporter expression in ventral cells. (E)–(G) Fixed, Hoechst-stained L2 worm visualized with merged DIC and GFP (E), GFP merged with Hoechst DNA stain (F), and Hoechst stain alone (G), with the single GFP-positive anchor cell indicated with an arrow. (H)–(J) In early L4, reporter expression is seen in four vulval muscle cells (arrows, panels as in E–G). (K)–(N) A living late L4 hermaphrodite expressing *Cbr-puf-2* in four cells lateral to the vulval opening, as seen in DIC (K), GFP (L), and DIC and GFP merged (M). A medial focal plane of the same animal (N) shows that the cells in (K)–(M) are lateral to the vulva proper.

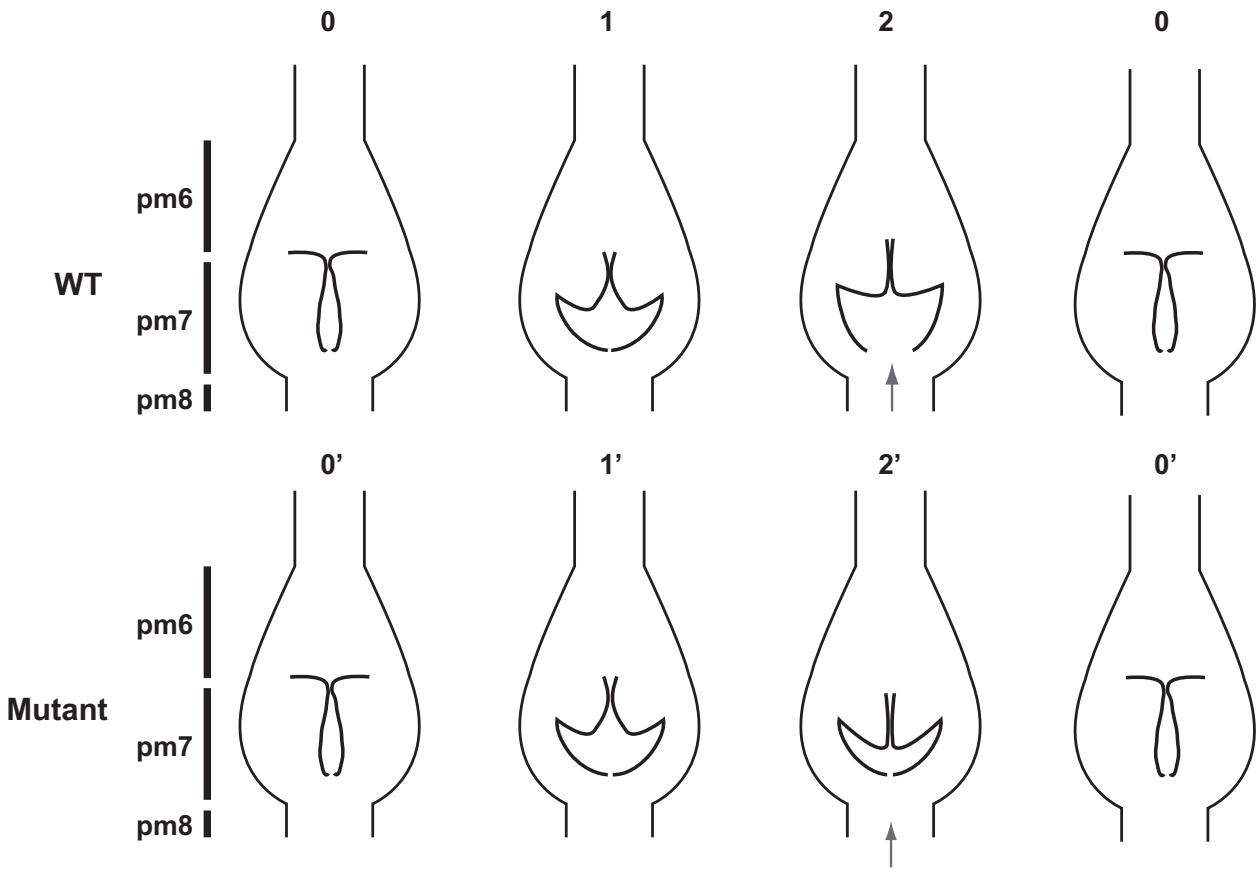

Figure 7. Model of pharyngeal grinding in normal and *Cbr-puf-2(nm66)* mutant *C. briggsae*. The model shows the zoomed view of pharyngeal terminal bulb. The proximal location of pharyngeal muscle cells are marked on the far left side. For wild-type *C. briggsae* strain, AF16, the natural grinding motion 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 cannot be further opened (positon 2′), which may result from the weakening of pm7. As a consequence, the teeth cannot be tightly sealed leading to inefficient food process. Grey arrows indicate the defective region.

While a role in pharyngeal physiology has not been reported for any *C. elegans* PUF proteins, the control of pharyngeal gene expression is well studied. PHA-4 is the master transcriptional regulator of pharyngeal fate (Mango, 2009). PHA-4 and its targets function coordinately to modulate gene expression in different pharyngeal cell types and at different developmental stages (Gaudet et al., 2004). We identified four putative PHA-4-binding motifs in the vicinity of *Cbr-puf*-2 matching the two consensus motifs for embryonic promoters (Zhong et al., 2010), and somewhat looser consensus (Gaudet and Mango, 2002) found in *C. elegans*. These lie at 250, 735, 1,098, and 1,309 nucleotides upstream of the start codon, all of which are present in our GFP reporter construct. *Cbr-puf-1.1*, *Cbr-puf-1.2* and *C. elegans fbf-1* and *fbf-2* also harbor 1–3 putative embryonic PHA-4 binding sites, but only of the TGTBTSY (where B = [TGC], S = [GC], Y = [TC]) motif family. *Cbr-puf-2* is thus unique in possessing the second (GAGAGAS) embryonic motif. Because *fbf-1* and *fbf-2* are not direct targets of PHA-4 (Zhong et al., 2010), it is possible that the GAGAGAS motif evolved recently in *C. briggsae puf-2*, imparting embryonic PHA-4 regulation as a result. Alternatively, a role in pharyngeal function may be ancestral to the primordial PUF gene that gave rise to the FBF and PUF-2 sub-families of which *C. elegans fbf-1/2* and *Cbr-puf-2* are part, respectively (Liu et al., 2012). This scenario is less parsimonious, because it implies that *C. elegans fbf-1/2* and *Cbr-puf-1.1* and *Cbr-puf-1.2* independently lost an ancestral function in pharyngeal physiology, but cannot yet be rejected. Because RNA interference is incapable of revealing the *Cbr-puf-2* pharynx defect,

distinguishing between these two alternatives will require generating *puf* gene knockout mutations in gonochoristic Caenorhabditis, which is so far not straightforward.

C. briggsae puf-2 Is Also a Novel Regulator of Vulval Development

C. elegans vulva development occupies most of larval development, starting with specification of the VPCs during L1 and L2 stages. Subsequently, induction of vulval fates by AC signaling occurs during L2, specific, induction-dependent cell lineages are executed in L3, and morphogenesis occurs during the L4 stage (Sternberg, 2005). *Caenorhabditis* species follow the same patterning process, though the underlying signaling pathways are subject to quantitative variations among species (Kiontke et al., 2007; Hoyos et al., 2011) and mutagenesis of *C. briggsae* produces a distinct spectrum of vulval defects (Sharanya et al., 2012). Though the *C. elegans* PUF proteins PUF-8, FBF-1, and FBF-2 were initially characterized as germ line-acting genes, they also negatively regulate vulval induction (Thompson et al., 2006; Walser et al., 2006). In *C. briggsae*, we found the PUF gene *Cbr-puf-2* also functions in vulval development, likely by promoting faithful cell fate specification. This conclusion is supported by the observation that axenic rescued *Cbr-puf-2(nm66)* mutants consistently have protruding vulvae and by the expression of a *Cbr-puf-2* GFP reporter in the AC at the time of vulval induction.

Because abnormal patterning of VPC lineages is sufficient to explain the Pvl phenotype (Eisenmann and Kim, 2000) and no reporter expression occurs in the VPCs themselves, the Pvl phenotype is likely due to lack of AC expression. In Caenorhabditis, the chief role of the anchor cell in early larva is to produce an EGF-type LIN-3 signal to the VPCs (Hill and Sternberg, '92). Cbr-PUF-2 may therefore regulate the timing or level of LIN-3 expression, either directly or indirectly. However, *Cbr-puf-2* reporter expression is also seen in four vulval muscle (vm) cells starting in L4, and in certain sensitized backgrounds muscle cells can influence gonad patterning in *C. elegans* (Moghal et al., 2003). It thus remains possible that vm function contributes to the Pvl phenotype of *Cbr-puf-2(nm66)* mutants.

Pleiotropy, Redundancy, and the Evolution of mRNA-binding Proteins

Cbr-puf-2 plays multiple important roles in development, acting both alone and in concert with closely related paralogs of the PUF-2 sub-family at different times and in different tissues. Maternally deposited *Cbr-puf-2* gene products are important for reliable embryogenesis, and L1 larvae require *Cbr-puf-2* function to grow (Liu et al., 2012). In this study, we show that this latter function lies in the physiological control of pharyngeal muscle contraction. In later larval stages, this study also reveals an unexpected function of *Cbr-puf-2* in vulval development. Finally, in adulthood *Cbr-puf-2* acts redundantly with *Cbr-puf-1.2*, a closely related paralog, and with the more distantly related *Cbr-puf*-8, to promote meiotic entry and spermatogenesis in the hermaphrodite germ line (Beadell et al., 2011; Liu et al., 2012). Structural and biochemical studies suggest that this pleiotropy of PUF proteins stems from the minimal sequence requirements for target recognition (Bernstein et al., 2005; Opperman et al., 2005; Wang et al., 2009; Dong et al., 2011), which allow them to affect a variety of traits through translational control of a large number of mRNA targets (Zhang et al., '97; Crittenden et al., 2002; Subramaniam and Seydoux, 2003; Lamont et al., 2004; Bachorik and Kimble, 2005; Ariz et al., 2009; Suh et al., 2009; Kershner and Kimble, 2010; Merritt and Seydoux, 2010; Kalchhauser et al., 2011).

Recently duplicated genes are thought to initially possess overlapping roles (Ohno, '70; Zhang, 2003). Consistent with this, in both *C. elegans* and *C. briggsae* the major germline functions of the related FBF and PUF-2 sub-families (respectively) are shared among recently duplicated paralogs (Zhang et al., '97; Kraemer et al., '99; Lamont et al., 2004; Thompson et al., 2006; Merritt and Seydoux, 2010; Kalchhauser et al., 2011; Liu et al., 2012). Similar germline redundancy is also seen for the PUF-3, PUF-5, and PUF-6 sub-families (Lublin and Evans, 2007; Hubstenberger et al., 2012). However, at least some somatic functions of these same genes are not redundant. For example, *C. elegans fbf-1* is alone required to promote expression of the cGMP-dependent kinase EGL-4, which in turn is necessary for olfactory adaptation (Kaye et al., 2009). The present study infers similar paralog-specific somatic functions for *Cbr-puf-2*, specifically in the pharynx and vulva. Cbr-PUF-2 has RNA-binding properties similar to those of the two other *C. briggsae* PUF-2 sub-family members (Cbr-PUF-1.1 and Cbr-PUF-1.2), and are similar to the FBF sub-family as well (Liu et al., 2012). Therefore, the variable roles of PUF proteins are likely due to the gain and/or loss of mRNA targets. This could occur by *cis*-regulatory changes in the mRNA population of a conserved cell type (such as the germ line). Alternatively, changing the site of the PUF protein's expression through *cis*-regulatory changes in its promoter region could expose a population of mRNAs to PUF control for the first time.

The paralog-specific functions of *Cbr-puf-2* and other PUF genes may be ancestral to the sub-family and now uniquely performed by a single paralog, or represent novel roles that evolved recently. However, because somatic expression of *Cbr-puf-2* and *C. elegans fbf-1* is important for the function of specific cell types not described for any other homologs, we favor the latter. If so, a model emerges in which duplicated PUF genes retain common expression in the germ line, where they remain functionally redundant, while acquiring paralog-specific roles in the soma. Common germline expression may derive in part from the relatively minor role that transcriptional regulation plays in germline gene regulation (Merritt et al., 2008). However, paralogs may acquire novel somatic sites of expression through changes to their *cis*-regulatory DNA. New somatic expression domains may eventually be co-opted into the regulation of gene expression in new cell types. Once such novel roles are essential, the formerly

redundant paralogs can no longer be easily lost from the genome. This sort of "neofunctionalization" may explain why most older gene duplicates persist (Lynch et al., 2001). More generally, this becomes a mechanism for developmental system drift (DSD; also called "phenogenetic drift"), in which divergent genetic variations underlie constant phenotypic traits (Weiss and Fullerton, 2000; True and Haag, 2001). The vulva is a highly conserved organ with some significant developmental differences within and between nematode genera (Sommer, 2005; Kiontke et al., 2007; Hoyos et al., 2011). This study adds PUF gene function to the variations of nematode vulval development. Further, we can now add the pharynx as another highly conservative organ built (at least in part) through species-specific developmental mechanisms. Similar unexpected divergence between *C. elegans* and *C. briggsae* is also seen in the signaling mechanisms producing the highly congruent early embryonic cell lineage (Lin et al., 2009). Taken together, the pervasiveness of DSD in the face of even the most extreme morphological conservation is impressive.

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