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The *C. elegans pumilio* homolog, *puf-9*, is required for the 3'UTR mediated repression of the *let-7* microRNA target gene, *hbl-1.*

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Abstract

The Puf family of RNA-binding proteins directs cell fates by regulating gene expression at the level of translation and RNA stability. Here, we report that the *C. elegans pumilio* homolog, *puf-9*, controls the differentiation of epidermal stem cells at the larval-to-adult transition. Genetic analysis reveals that loss-of-function mutations in *puf-9* enhance the lethality and heterochronic phenotypes caused by mutations in the *let-7* microRNA (miRNA), while suppressing the heterochronic phenotypes of *lin-41,* a *let-7* target and homolog of *Drosophila* Brat. *puf-9* interacts with another known temporal regulator *hbl-1,* the *C. elegans* ortholog of *hunchback*. We present evidence demonstrating that *puf-9* is required for the 3'UTR-mediated regulation of *hbl-1,* in both the hypodermis and the ventral nerve cord. Finally, we show that this regulation is dependent on a region of the *hbl-1* 3'UTR that contains putative Puf family binding sites as well as binding sites for the *let-7* miRNA family, suggesting that *puf-9* and *let-7* may mediate hypodermal seam cell differentiation by regulating common targets.

Keywords

puf-9; hbl-1; let-7; C. elegans; Pumilio; hunchback; development; microRNA

Introduction

As early as the first embryonic cell divisions, spatial and temporal control mechanisms establish asymmetric gene expression patterns necessary for proper cell fate decisions during development. In multi-cellular organisms cell fates are often determined by the precise control of gene expression at a post-transcriptional level. In many cases cis-regulatory elements in the 3' untranslated region (3'UTR) of an mRNA direct these control mechanisms by the binding of specific regulatory factors.

The Puf family of of RNA-binding proteins is named for its founding members, *Drosophila* Pumilio and *C. elegans fem-3* Binding Factor (FBF) (Zhang et al., 1997), and is comprised of a complex group of proteins that are found in diverse organisms, including yeast, flies, nematodes, frogs, and humans (Edwards et al., 2001; Wang et al., 2001; Zamore et al., 1997). Despite their widespread abundance, the molecular mechanism by which Puf proteins regulate their target RNAs is still largely unknown. Previously characterized Puf proteins have been

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found to act by binding to specific regulatory elements in the 3'UTR's of their target RNAs, leading to either translational repression or degradation of the target mRNA (Wickens et al., 2002). Puf proteins share an evolutionarily conserved C-terminal motif, termed the Pumilio-homology domain (Pum-HD) (Zamore et al., 1997). This domain consists of eight imperfect repeats and two flanking conserved regions that form an arc of supercoiled α-helices and retains the RNA binding activity of the protein (Zamore et al., 1997). *Drosophila* Pumilio (Pum) is one of the most well characterized members of the Puf family and has been assigned multiple developmental roles, including the establishment of axis formation and germline stem cell maintenance (Wickens et al., 2002). For example, Pum negatively regulates the maternal gap gene *hunchback* in the embryonic syncytium (Murata and Wharton, 1995; Tautz, 1988) to establish a gradient of Hunchback protein that is essential for the formation of anterior structures of the fly (Wu et al., 2001). Pum participates in the post-transcriptional repression of *hunchback* mRNA by binding to a defined 36nt motif in the *hunchback* 3' UTR called the Nanos Response Elements (NRE) (Murata and Wharton, 1995; Wharton and Struhl, 1991) and acts in a multi-protein complex that includes Nanos (Nos) and Brain Tumor (Brat) (Sonoda and Wharton, 2001).

The *C. elegans* genome encodes nine *pumilio* and three *nanos* homologs (Zhang et al., 1997). Like *Drosophila,* the characterized *C. elegans pumilio* genes play definitive roles in regulating stem cell maintenance in the germline. For instance, *puf-8* is required to maintain germline cells in a meiotically arrested state during spermatogenesis (Subramaniam and Seydoux, 2003). In PUF-8 depleted animals, primary spermatocytes dedifferentiate and proliferate, leading to germline tumors (Subramaniam and Seydoux, 2003). FBF-1 and FBF-2, two other *C. elegans* Puf proteins collectively referred to as FBF, target the *fem-3* 3'UTR and play a largely redundant role to promote mitotic divisions and define the contributing region of stem cells in the adult germline (Crittenden et al., 2002; Lamont et al., 2004). Deletion of the *fbf-1* and *fbf-2* gene products leads to the failure of germline stem cell maintenance, due to premature entry into meiosis (Crittenden et al., 2002).

The *C. elegans* developmental timing, or heterochronic, pathway also displays many examples of negative gene regulation at the post-transcriptional level. Heterochronic genes coordinately establish cell fate profiles unique to a particular stage and act as timing switches to control cellular identities in many different tissues (Ambros and Horvitz, 1984; Banerjee and Slack, 2002). In heterochronic mutants, the hypodermal seam cells, which are a set of specified cells that run down the length of the animal on each side, will inappropriately adopt cellular fates in either a retarded or precocious manner relative to wild type developmental stages. During wild type development the seam cells divide in a stem-cell-like manner, such that they are continuously renewed during each larval stage. Like stem cells, seam cells are characterized by asymmetric cell divisions where one daughter cell differentiates and one retains its stem cell identity. For example, following each division, the anterior seam cell daughter differentiates and fuses with the large hypodermal syncytium (hyp7), while the posterior seam cell daughter retains its identity and continues to divide at the subsequent larval stages. This self-renewing division cycle continues until the fourth larval stage when the seam cells terminally differentiate and permanently exit the cell cycle, thus defining the larval-to-adult (L/A) switch.

The L/A switch is controlled by a cascade of heterochronic gene interactions and heterochronic mutants often display defects in the cuticle of the animal at the L/A switch. The first larval (L1) and adult stages of *C. elegans* post-embryonic development are characterized by an epidermal structure called lateral alae, which are cuticular ridges that run down each side of the animal. Alae are secreted by the underlying seam cells in response to a variety of differentiation factors, including the *lin-29* transcription factor (Ambros, 1989; Papp et al.,

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1991) and the *let-7* miRNA (Reinhart et al., 2000), which act to promote adult fates in the hypodermis (Ambros, 1989; Pasquinelli et al., 2000).

Loss-of-function (lf) mutations in *let-7* and *lin-29* result in retarded developmental phenotypes where the seam cells continue to divide at the adult stage, thereby failing to differentiate and secrete alae (Ambros, 1989; Reinhart et al., 2000). Strong *let-7(lf)* mutants also display an adult lethality due to vulval bursting (Reinhart et al., 2000). RNA interference (RNAi) of two known *let-7* target genes, *hbl-1* and *lin-41*, can partially suppress the lethality and alae defects associated with *let-7* mutant animals (Abrahante et al., 2003; Lin et al., 2003; Pasquinelli et al., 2000; Slack et al., 2000), thus supporting the idea that *let-7* acts in an opposing manner to *hbl-1* and *lin-41* to regulate the L/A switch. *let-7* inhibits its targets, *hbl-1* and *lin-41* in a post-transcriptional manner by binding to sequences in their 3'UTRs (Vella et al., 2004a) and has been shown *in vivo* to bind directly to the *lin-41* 3'UTR at specific regulatory sequences called *let-7* complementary sites (LCS) (Vella et al., 2004a; Vella et al., 2004b).

In *hbl-1* or *lin-41* loss-of-function mutants the seam cells precociously exit the self-renewing cell cycle, differentiate and secrete alae during the fourth larval stage (Abrahante et al., 2003; Lin et al., 2003; Pasquinelli et al., 2000; Slack et al., 2000). Thus, *hbl-1* and *lin-41* normally prevent cells from attaining adult fates prematurely. HBL-1 is the ortholog of the *Drosophila* transcription factor, Hunchback (Hb) (Fay et al., 1999), while LIN-41 is closely related to *Drosophila* Brain Tumor (Brat) (Slack and Ruvkun, 1998)

In a wild type animal, *hbl-1::gfp* is expressed in the main body hypodermis (hyp7) until the L3 stage and in the ventral nerve cord (VNC) until the early-L4 stage of development (Abrahante et al., 2003; Lin et al., 2003). Previous work has demonstrated that *hbl-1* down-regulation in epidermal and neural tissues is dependent on the *hbl-1* 3'UTR (Abrahante et al., 2003; Lin et al., 2003), similar to *Drosophila hunchback*. When the heterologous *unc-54* 3'UTR was substituted for the native *hbl-1* 3'UTR, proper down-regulation was abrogated and GFP expression was maintained in both the hyp7 and the VNC in the adult stage (Lin et al., 2003). Potential cis-regulatory elements in the *hbl-1* 3'UTR have been identified, including complementary sites for the miRNAs *let-7* (LCS) and *lin-4* (LCE), and their family members (Lin et al., 2003). The 3'UTR mediated regulation of *hbl-1* in the hypodermis at the L2/L3 stage requires the *let-7* family microRNAs, *mir-48, mir-84* and *mir-241* (Abbott et al., 2005), while *let-7* and *lin-4* are needed for *hbl-1* 3'UTR reporter down-regulation in the ventral nerve cord (VNC) at the L4 stage (Lin et al., 2003). Therefore, *hbl-1* plays a role in defining both the early (L2/L3) and late (L4/Adult) transitions (Abbott et al., 2005; Abrahante et al., 2003; Lin et al., 2003). When the *let-7* family members are mutated, *hbl-1* continues to be partially down-regulated in both the hypodermis and the VNC (Lin et al., 2003), suggesting that additional factors are required for the regulation of *hbl-1*. However, protein co-factors that function with the *let-7* family to mediate *hbl-1* regulation remain unidentified.

There are many intriguing molecular parallels between the *Drosophila* spatial patterning genes and *C. elegans* temporal patterning genes. In *Drosophila*, pattern formation in the early embryo is established through the spatially restricted expression of a complicated network of transcription factors, including *hunchback (hb)* (Irish et al., 1989; Wu et al., 2001). In *C. elegans*, *hbl-1* is highly expressed during early larval stages and is down-regulated over time in a variety of tissues (Abbott et al., 2005; Abrahante et al., 2003; Fay et al., 1999; Lin et al., 2003) and is thus regulated along a temporal rather than a spatial gradient. In *Drosophila,* the transcription factor *kruppel* is a direct target of *hunchback* and acts in a cascade of multiple transcription factors to ultimately establish segmentation patterning of the fly (Wu et al., 2001). The *C. elegans kruppel* homologue, *lin-29*, is analogously regulated along a temporal gradient with its highest expression peaking in the later larval stages (Bettinger et al., 1996). Interestingly, in addition to their functions in body plan formation, *hunchback* and *kruppel*

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have been shown to play a temporal role in establishing neuroblast identity during cortical layer formation in *Drosophila* (Isshiki et al., 2001). Finally, LIN-41 and *Drosophila* Brat share high structural similarities and belong to the same RBCC-NHL family of translational regulatory proteins (Slack and Ruvkun, 1998). The possibility exists that additional *Drosophila* spatial patterning homologs may function to regulate developmental timing in *C. elegans*. We hypothesized that one or more of the *C. elegans pumilio* homologs may interact with the late-stage timing factors, *lin-41* and *hbl-1*, to regulate the stem cell-like proliferation and differentiation of seam cells at L/A transition.

Here, we report that the *C. elegans pumilio* homolog, *puf-9*, acts to control the 3'UTR mediated expression of *hbl-1* in the hypodermis and the ventral nerve cord (VNC). We demonstrate that this repression requires a region of the *hbl-1* 3'UTR that contains potential Puf protein binding sites (NRE) and *let-7* family complementary sites (LCS), suggesting that Pumilios and miRNAs may cooperate at the 3'UTRs of target genes to control their translation. We show that *puf-9* genetically interacts with known regulators of the L/A switch, including the *let-7* miRNA and two *let-7* target genes, *lin-41* and *hbl-1*. Our data reveals that *puf-9* is required for seam cell morphology and adult cuticle formation, expanding a somatic role for the Puf family proteins in *C. elegans* development.

Materials and methods

Strain maintenance and RNAi

C. elegans strains were maintained on NGM plates containing *E. coli* OP50 bacterial lawns. Experiments were performed at 20°C, unless otherwise indicated. Gene knock-down was achieved through RNAi by feeding using previously published reagents and protocols (Kamath et al., 2003; Timmons et al., 2001). All RNAi experiments were done by plating the indicated worm strain onto plates containing *E. coli* HT115(DE3) bacteria lawns expressing the appropriate dsRNA. RNAi constructs for *fbf-2, puf-3, puf-5, puf-7, puf-8, puf-9, nos-1, nos-2,* and *nos-3* (Subramaniam and Seydoux, 1999) contained genomic fragments of coding sequence cloned into the MCS of pL4440 RNAi vector (Timmons et al., 2001). RNAi constructs for *fbf-2* and *puf-7* also knock-down *fbf-1* and *puf-6* levels, respectively (K. Subramaniam, personal communication). For double and triple-RNAi experiments, bacteria containing each single RNAi construct were diluted with an equal number of cells (determined by optical density at 600nm) containing the indicated RNAi plasmid or an control empty vector (pL4440) RNAi plasmid. In the *puf-9(RNAi)* analyses, late L4 stage animals were exposed to RNAi to allow for depletion of maternal *puf-9* during oogenesis. The *puf-9* depleted progeny were then scored at the adult stage for the presence of alae and additional defects. For *let-7 (n2853ts);puf-9(RNAi)* alae and survival analysis, a population of *let-7(n2853ts)* animals were synchronized as starved L1s, exposed to *puf-9(RNAi)* and scored for alae or survival at the young adult stage. The *puf-9(ok1136)* deletion strain was obtained from the *C. elegans* Gene Knockout Consortium and was back-crossed to wild type (N2) animals four times prior to mutant characterization.

puf-9::gfp Construct Design and Expression Analysis

PCR was used to generate a 1.9 kb genomic fragment from the WO6B11 cosmid (Genebank accession number U39854) including the predicted *puf-9* promoter, using primers PUF9A and PUF9GFP. Primers GFPAK9 and GFP2C were used to amplify a 1.8 kb fragment containing *gfp* followed by the *unc-54* 3'UTR from pPD95.75 vector. The *puf-9* promoter PCR product was then fused in frame to *gfp::unc-54* 3'UTR using overlap extension PCR using primers PUF9B and GFP2C to create MJC20. The resulting PCR products for MJC20 were co-injected directly into N2 animals at a concentration of 50ng/ul along with the pRF4 (*rol-6*) marker at

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100ng/ul. Individually isolated lines were established and used for *puf-9::gfp* expression analysis.

X-gal assay and transgenic lines

For all plasmids with designation "pSJA#" and "pMJC#": PCR fragments were amplified from genomic DNA, digested with *Sac*II and *Nco*I and ligated into the *unc-54* 3'UTR of the B29 vector (Reinhart et al, 2000) cut with the same restriction enzymes. Transgenic animals were created by co-injecting the *hbl-1* 3'UTR constructs (pSJA2, pSJA3, pSJA4, pSJA5, pMJC2, pMJC12 and pFS1038) at 5 ng/ul and the pKP13 (*goa-1::gfp)* marker at 50 ng/ul into wild type (N2) animals. Refer to published methods for pFS1038 (Lin et al., 2003). *puf-9(ok1136)* mutant animals were crossed into the existing ZaEx6 strain, containing the pFS1038 *hbl-1* 3'UTR construct in a wild type background. Independent lines, carrying the extrachromosomal arrays, were tested for β-galactosidase expression. Wild type lines expressing the control reporter construct (pFS1038) were assayed in parallel with either the *puf-9(ok1136)* mutant background or wild type animals expressing the truncated reporter constructs for each separate experimental trial. The average of the individual trials is shown.

The inserted *hbl-1* 3'UTR sequence for pSJA2 was amplified using primers F13D11(16) and SJA2; pSJA3 was amplified using primers F13D11(16) and SJA3; pSJA4 was amplified using primers F13D11(16) and SJA4; and pSJA5 was amplified using primers SJA2 and SJA5. pMJC2 was created by overlap extension PCR using primers F13D11(16) and ONS2, and F13D11(17) and ONS1. X-gal staining of animals expressing the *lacZ* transgene was performed as described (Vella et al., 2004a). All primers were synthesized by the Keck Facility, Yale University Medical School (see Supplemental Fig. 9 for oligonucleotide sequences).

Quantification of hbl-1::gfp expression

GFP levels were quantified in BW1932 and *puf-9(ok1136);*BW1932 lines using the Carl Zeiss AxioVision Release 4.4 program. All fluorescence images used for GFP quantitation were taken at 40x objective and at 750 ms exposure. From these images, multiple representative VNC neuron cell bodies were selected within a single animal and an average Mean GFP value (MeanG) was recorded for each individual animal. The single animal average MeanG values were then used to calculate an average MeanG for the L1 or adult stages for each background.

Results and Discussion

Mutations in puf-9 enhance let-7 lethal and heterochronic alae phenotypes

Based on the parallels between the *Drosophila* spatial patterning genes and the genes known to be involved in the *C. elegans* heterochronic pathway, we investigated a possible post-embryonic role for the *C. elegans* homologs of *nanos (nos)* and *pumilio (fbf/puf)* in regulating developmental timing and epidermal stem cell development. In *C. elegans*, *hbl-1* regulation through its 3'UTR is, in part, dependent upon *let-7* miRNA family members (Abbott et al., 2005; Abrahante et al., 2003; Lin et al., 2003). Therefore, we reasoned that if the *puf* or *nos* homologs regulated *hbl-1,* similar to *Drosophila hunchback,* then they might also genetically interact with the miRNA *let-7*. We utilized the *let-7(n2853ts)* strain that had been shown previously to display 100% lethality due to vulval bursting at the non-permissive temperatures of 20°C and 25°C, while a significant number of animals are able to survive through adulthood at 15°C (Reinhart et al., 2000). A candidate RNAi screen knocking down each of the *C. elegans pumilio, fbf/puf,* and *nanos, nos,* homologs, identified *puf-9* as being able to enhance the vulva bursting phenotype seen in *let-7(n2853ts)* mutant animals (Fig. 1A and Supplemental Fig. 1A). For instance, at the permissive temperature, only 12% of *let-7(n2853ts)* animals survived when exposed to *puf-9(RNAi)* compared to 87% survival in *let-7(n2853)* animals on mock RNAi (Fig. 1A). None of the other *puf* homologs affected the survival rate of *let-7(n2853ts)* mutants

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(Supplemental Fig. 1A). While this analysis does not rule out a role for other Puf proteins in the heterochronic pathway, we decided to concentrate our efforts on the characterization of *puf-9*.

In addition to bursting, *let-7(n2853ts)* animals also display a retarded developmental phenotype where the lateral hypodermal seam cells fail to secrete alae at the young adult stage (Reinhart et al., 2000). Because the bursting phenotype of *let-7(n2853)* animals may not be directly related to their heterochronic phenotypes, we tested for enhancement of the retarded alae phenotype of *let-7* mutant animals on *puf-9(RNAi)*. In a *let-7(lf)* background, parental depletion of *puf-9* during oocyte formation resulted in an almost complete adult lethality in their progeny (Fig. 1A). Therefore, we exposed *let-7(n2853ts)* animals to *puf-9(RNAi)* post-embryonically, and scored these animals for alae at the young adult stage. Consistent with the increased bursting data, *puf-9(RNAi)* also enhanced the delayed adult alae defect of *let-7(n2853ts)* mutants (Fig. 1F), while *puf-9(RNAi)* of wild type animals and *let-7(n2853ts)* mutants on mock RNAi displayed a similar number of adult stage animals lacking alae (Fig. 1F). Due to the dramatic enhancement of *let-7(lf)* phenotypes by *puf-9(RNAi),* we conclude that *puf-9* plays an important role in tissues affected by *let-7* regulation.

The findings from our RNAi screen suggested that *puf-9* acted independently of the *nanos* homologs, *nos-1, nos-2* and *nos-3,* to affect *let-7* mutant bursting induced lethality (Supplemental Figs. 1A and 1B). This was confirmed by the observation that *let-7(n2853ts)* animals exposed to RNAi simultaneously targeting the three *nos* genes, phenocopied *let-7* mutants alone for adult survival under all conditions tested (Supplemental Fig. 1B). Furthermore, *let-7* mutants on RNAi targeting *puf-9* in combination with the three *nos* genes, phenocopied *let-7(n2853ts);puf-9(RNAi)* animals (Supplemental Fig. 1B). These data suggest that the *nos* genes are not involved in the *puf-9* directed enhancement of the *let-7* mutant bursting phenotype. However our analysis does not rule out a subtle role for the *nos* genes. There are many examples of Puf proteins acting independently of a Nanos protein. For instance, *Drosophila* Pumilio regulates a second mRNA target, *bicoid,* in the syncitial embryo in a Nanos independent manner (Gamberi et al., 2002).

Previous work has shown that expression of at least three of the *C. elegans* Pufs, *puf-8, fbf-1* and *fbf-2,* are primarily detected in the germline (Subramaniam and Seydoux, 2003; Zhang et al., 1997). Of the characterized Puf genes in *C. elegans, puf-8* is the most closely related to *puf-9* (Wickens et al., 2002). In contrast to *puf-8*, *puf-9* levels were not affected in mutants that lack a germline (Subramaniam and Seydoux, 2003). *puf-9* is thus unique among the *C. elegans* Puf genes in that it appears to be predominantly expressed in somatic tissues. Therefore, it is not surprising that *puf-9* would be the main, if not the only, *C. elegans* Puf gene to show an interaction with *let-7* in the hypodermis. The enhancement of the *let-7* phenotypes by *puf-9* and the phenotypic similarities between the two individual mutant backgrounds (see below) suggests that *puf-9* could be acting in the same pathway as *let-7* and that these two regulatory factors may function in a similar manner to specify hypodermal fates.

puf-9 null mutants display let-7-like vulval bursting phenotypes

To better understand the function of *puf-9* and to confirm the results seen with *puf-9(RNAi)*, we obtained the *puf-9(ok1136)* deletion allele from the *C. elegans* Gene Knockout Consortium. The *puf-9(ok1136)* deletion removes the entire conserved Pum-HD (Fig. 1B). Of the nine *C. elegans* Pumilio proteins, PUF-9 is the most similar to *Drosophila* Pumilio (Spassov and Jurecic, 2003;Wickens et al., 2002) and based on the importance of the Pum-HD for the function of *Drosophila* Pumilio, we predict that the *ok1136* deletion mutant is a molecular null (see Supplemental Figure 2 for the sequence conservation of the PUF-9 Pum-HD). Interestingly, when *puf-9* was reduced through the *puf-9(ok1136)* deletion or through *puf-9 (RNAi)* exposure, a similar level of lethality was seen as with *let-7(n2853ts)* mutants on mock

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RNAi (Fig. 1A). Of the animals that died, the phenotypes observed for both *let-7* (Reinhart et al., 2000) and *puf-9* mutants were essentially identical at the gross level, with severe hemorrhaging (bursting) through the vulva (Fig. 1D and 1E). *puf-9(ok1136)* homozygotes showed abnormalities similar to those seen in *puf-9(RNAi)* animals in all assays performed, indicating that *puf-9* gene levels were effectively reduced upon exposure to *puf-9(RNAi)*.

puf-9 is required for proper adult cuticle formation and seam cell fusion

We next examined the effects of *puf-9* depletion on hypodermal development, independent of the *let-7* mutant background. We observed that *puf-9* mutants exhibited an almost completely penetrant alae defect where both *puf-9(ok1136)* and *puf-9(RNAi)* animals showed alae breaks at the adult stage (Fig.2A). In the majority of *puf-9* mutant animals, alae structures were generally rough in appearance and cover only a few cell lengths at the head and tail of an individual animal (Fig. 2D and Supplemental Fig. 3B). Occasionally degenerative alae covered one half of the length of the animal and were only rarely observed at the cells over the mid-section of the animal. The lack of completely formed adult alae in *puf-9(ok1136)* mutant animals is reminiscent of *let-7* mutants (Reinhart et al., 2000), however, their individual mutant alae phenotypes differ slightly. In order to determine the underlying cause for the alae defects observed, we utilized a *wIs79* reporter strain that has *gfp* marking the seam cell borders (adherens junctions) and seam cell nuclei (Lin et al., 2003). This marker allows us to visualize seam cell exit from the cell cycle as well as the extent and timing of seam cell fusion. When the *wIs79* strain was crossed into *puf-9(ok1136)* mutants or was exposed to *puf-9(RNAi)* we found that the number of seam cells in *puf-9* mutants was comparable to wild type at all larval stages (data not shown). Thus, this analysis did not reveal a distinct reiteration of L4 seam cell divisions as is often noted in late-acting retarded mutants such as *let-7* and *lin-29* (Ambros, 1989;Reinhart et al., 2000). Additionally, at the L/A transition in a wild type animal, the seam cells fuse to form a linear syncytium (Fig. 2E). We observed that the timing of the initiation of seam cell fusion appeared altered in a subset of animals deficient for *puf-9*. For example, in *puf-9(ok1136)* and *puf-9(RNAi)* animals, approximately half of the animals scored displayed incomplete seam cell fusion at the L/A transition (Figs. 2B and 2F).

We also observed a large number of *puf-9(RNAi)* adults with abnormally spaced seam cell nuclei (Fig. 2F) and instances where the seam nuclei were positioned next to each other in a dorsal-to-ventral orientation (Fig. 2H) rather than the typical anterior-to-posterior orientation (Fig. 2G), indicating that perhaps seam cell divisions in *puf-9(RNAi)* animals occur in the wrong axial plane. The seam cell defects are consistent with our previous observation that the seam cells fail to correctly secrete alae at the adult stage. In fact, in most cases observed, sections of an animal that lacked alae corresponded to regions of abnormal fusion and seam cell spacing (data not shown).

To determine if the phenotypes observed in *puf-9* mutants were due to altered *let-7* expression, we compared *let-7* miRNA levels in wild type and *puf-9(ok1136)* young adult animals. By northern analysis, *let-7* levels were not reduced in a *puf-9(ok1136)* mutant background (Supplemental Fig. 4), suggesting that *puf-9* does not function through *let-7* miRNA processing and most likely acts synergistically with *let-7* for example, to control downstream targets.

The alae and fusion defects seen in *puf-9* mutants suggest that *puf-9* activity is required for the proper timing of seam cell fusion and for the seam cells to differentiate and attain adult fates, but not for exit from the cell cycle. These results support the previously expressed idea that seam cell fusion, cell cycle exit, and alae formation are independently controlled developmental events (Banerjee et al., 2005; Lin et al., 2003). This is also consistent with our observations that removal of *puf-9* activity resulted in a failure of some of the seam cells to fuse at the correct time, but did not show an observable reiteration of larval cell divisions in the hypodermal seam cells, as is seen in *let-7* and *lin-29* retarded loss-of-function mutants (Ambros, 1989; Papp et

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al., 1991; Reinhart et al., 2000). While a *puf-9* mutation does not cause classic retarded heterochronic phenotypes on its own, it is possible that *puf-9* still modulates the activity of genes in the heterochronic pathway.

puf-9 interacts genetically with the let-7 targets, lin-41 and hbl-1

To further establish a role for *puf-9* in adult cuticle formation, we tested the ability of *puf-9* to interact with additional heterochronic genes involved in the in the L/A switch. We hypothesized that if *puf-9* was acting in a similar manner to *let-7*, then *puf-9* might also interact genetically with the known *let-7* downstream targets, *lin-41* and *hbl-1*, in the hypodermis.

lin-41(RNAi) and the strong loss-of-function *(ma104)* allele display a partially penetrant alae defect in which alae are precociously observed at the L4 stage (Pasquinelli et al., 2000). Upon exposure to *puf-9(RNAi)*, which was indistinguishable from *puf-9(ok1136)* in our previous work, the *lin-41(ma104)* precocious alae defect was completely suppressed (Fig. 3A). Therefore, the *lin-41* precocious alae phenotype in the hypodermis requires a wild type copy of *puf-9*, suggesting that *puf-9* acts downstream of, or in parallel to, *lin-41* in the heterochronic pathway. Additionally, *lin-41(RNAi)* was unable to suppress *puf-9(ok1136)* adult alae defects (data not shown). This further supports our hypothesis that *puf-9* is downstream of *lin-41*.

Furthermore, animals carrying a full-length *puf-9::gfp* translational fusion construct often displayed a potential over-expression phenotype where the animals have a dumpy (Dpy) appearance (Fig. 3B). This Dpy phenotype is a characteristic of precocious heterochronic mutants, such as *lin-41* and *hbl-1* loss-of-function mutants, which could again signify that *puf-9* is functioning in an opposing manner to both *lin-41* and *hbl-1*, and acting similarly to *let-7*.

Previous reports showed that *lin-41* and *hbl-1* gene products can partially substitute for one another to regulate the L/A transition (Abrahante et al., 2003; Lin et al., 2003). Therefore, we tested our hypothesis that *puf-9* could also act opposite to *hbl-1* genetically, by looking at the effect of *hbl-1* depletion on the adult alae phenotype of *puf-9* mutants. We observed that *hbl-1 (RNAi)* was able to suppress the adult alae phenotype of *puf-9(ok1136)* mutants (Fig. 4A). Thus, the *puf-9* adult alae defects require a wild type copy of *hbl-1*. The reciprocal RNAi experiment showed that the majority of *hbl-1(ve18)* mutant animals (Abrahante et al., 2003) exposed to *puf-9(RNAi)* continued to display adult type alae in the early L4 stage characteristic of strong *hbl-1(lf)* mutants (left data set, Fig. 4B). Thus, *puf-9(lf)* is unable to suppress *hbl-1 (ve18)* for the precocious alae defect, suggesting that *hbl-1* acts downstream of, or in parallel to, *puf-9* in the heterochronic pathway. This is consistent with our hypothesis that *puf-9* acts to negatively regulate *hbl-1* in a manner similar to *let-7*.

When the *hbl-1(mg285)* allele, or *hbl-1(RNAi)* by feeding was used to reduce *hbl-1* levels, we found that a subsequent loss of *puf-9* was able to partially suppress the formation of precocious alae (Fig. 4B). One explanation for the observed mutual suppression is that in a weak *hbl-1* allele, loss of PUF-9 activity leads to overexpression of residual *hbl-1* and thus restores partially active *hbl-1* levels. It is possible that in the absence of PUF-9, overexpression of residual *hbl-1* was able to prevent alae formation in a portion of the animals. When wild type animals are exposed to *hbl-1(RNAi)* during embryogenesis, a high degree of embryonic and larval lethality is observed (Fay et al., 1999). Therefore, it was necessary in our analysis to expose animals to *hbl-1(RNAi)* at the first larval stage, which could also allow for residual *hbl-1* during development. Another possible explanation for the observed partial suppression of *hbl-1(lf)* preocious alae is that *puf-9* likely suppresses additional targets, other than *hbl-1*, involved in promoting larval cell fates at the L/A switch. Therefore, in the *hbl-1(lf);puf-9* double mutant, de-repression of additional *puf-9* targets may be able to substitute for the loss of *hbl-1* and, along with any residual *hbl-1,* could result in the failure to secrete alae and the observed

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suppression of the *hbl-1* precocious alae phenotype. It is also possible that *hbl-1* negatively feeds back on *puf-9* activity or expression.

In order to determine epistatic relationships it is ideal to use null alleles. However, the *puf-9* genetic interaction analysis described here was performed using a combination of genetic mutants and RNAi for several reasons. First, *lin-41* null animals display a completely sterile phenotype, thus making this strain difficult to generate and maintain. Second, true *hbl-1 (null)* alleles confer an embryonic lethal phenotype (Fay et al., 1999), which would make larval analysis of *hbl-1(null)* mutants impossible. Therefore strong loss-of-function alleles were used in combination with RNAi to carry out semi-epistatic genetic analysis with *puf-9*. Thus, due to the variability of RNAi and non-null alleles, interpretation of the genetic interactions presented here must take into consideration that residual gene activity could remain and that the proposed genetic pathway order may have alternative interpretations.

Taken together, our genetic data suggests a relationship among *let-7, lin-41*, *hbl-1* and *puf-9,* where *puf-9* negatively regulates *hbl-1* and is itself negatively regulated by *lin-41*. Our observation that the bursting and alae phenotypes of *let-7;puf-9* double mutants is more severe than either *let-7* or *puf-9* single mutants suggests that *puf-9* acts in a parallel pathway to reinforce *let-7* activity and to perhaps regulate downstream genes in the hypodermal seam cells. The placement of *puf-9* upstream of *hbl-1* brings up the interesting possibility that *puf-9* may directly regulate *hbl-1* in the heterochronic pathway.

A wild type copy of puf-9 is required for regulation of a hbl-1 3'UTR reporter in the seam cells

To test our hypothesis that *hbl-1* is down-stream of and negatively regulated by *puf-9* in the heterochronic pathway, we investigated whether the 3'UTR-mediated regulation of *hbl-1* is dependent on a wild type copy of *puf-9.* A heterologous *lacZ* reporter gene, driven by the hypodermally expressed *col-10* promoter and containing the full-length *hbl-1* 3'UTR (pFS1038, Fig. 5A) (Lin et al., 2003), was crossed into *puf-9(ok1136).* In a wild type background the *hbl-1* 3'UTR directs down-regulation of the reporter gene beginning in the L3 stage and continues down-regulation at later stages of development, such that β-galactosidase staining is almost completely undetectable by the adult stage (left data set, Fig. 5B and 5C) (Lin et al., 2003). In the *puf-9(ok1136)* mutant background, *hbl-1* down-regulation was incomplete, such that *puf-9(ok1136)* mutant animals retained significant β-galactosidase activity at the adult stage (right data set, Fig. 5B and 5C). These data show that a wild type copy of *puf-9* is necessary for the *hbl-1* 3'UTR directed regulation of a *lacZ* reporter gene in the hypodermis and suggests that PUF-9 likely regulates *hbl-1 in vivo*. The observed remaining partial regulation indicates that additional trans-factors are required to work with PUF-9 to fully regulate *hbl-1* at the adult stage. We can conclude that trans-factors required for *hbl-1* 3'UTR regulation, including PUF-9, are present and active in the seam cells during the later larval and adult stages.

puf-9 is required for hbl-1 down-regulation in the VNC

To test the hypothesis that PUF-9 is also required in tissues where *hbl-1* expression is known to be regulated at the post-transcriptional level (Abrahante et al., 2003; Fay et al., 1999; Lin et al., 2003), we utilized the BW1932 strain, which carries an integrated array consisting of the *hbl-1* promoter driving expression of the first 133 amino acids of HBL-1 and a GFP reporter, followed by the *hbl-1* 3'UTR (Fay et al., 1999). The wild type GFP expression pattern in this strain showed a distinct temporal expression in the VNC. Beginning in the early L1 stage, the GFP signal progressively decreased until it was essentially undetectable by the late L4 stage (Abrahante et al., 2003; Lin et al., 2003). A failure to properly down-regulate *hbl-1* expression in the VNC at the late L4 and adult stages was observed in a *puf-9(ok1136)* background where the majority of late L4 and adult stage animals expressed detectable GFP levels (Figs. 5D and

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Supplemental Fig. 5). Further quantitation of GFP levels in individual neurons from wild type and *puf-9(ok1136)* animals confirmed de-repression of HBL-1/GFP in *puf-9(ok1136)* adults (Fig. 5E). While VNC GFP expression was essentially the same in the *puf-9(ok1136)* and wild type backgrounds at the L1 stage, *puf-9* mutant animals showed a significant increase in the relative amount of GFP signal in the VNC compared to wild type at the adult stage (Fig. 5E). The failure to appropriately down-regulate *hbl-1::gfp* demonstrates that a wild type copy of *puf-9* is required in a subset of the neurons, as well as the hypodermis, for the correct 3'UTR mediated regulation of *hbl-1*.

Interestingly, *Drosophila hunchback (hb)* is temporally expressed during cortical laminar formation and is required to promote first born neuron fates (Cleary and Doe, 2006; Isshiki et al., 2001). Mis-expression of Hb in later born neuroblasts can drive the inappropriate transformation of neurons to the first born fate (Isshiki et al., 2001). Therefore, the appropriate expression of Hb is essential for proper neuron identity and cortical layering in *Drosophila*. However, the factors required for *hb* regulation during neurogenesis have not been identified. Our work could provide evidence for a conserved mechanism for *hunchback* regulation by Pumilio in neural tissues.

hbl-1 requires multiple cis-sequences in its 3'UTR for proper regulation

The dependence on PUF-9 for the proper 3'UTR-mediated regulation of *hbl-1* brings up the interesting possibility that PUF-9 could bind directly to sequences within the *hbl-1* 3'UTR. Previous work identified many putative cis-regulatory elements in the *hbl-1* 3'UTR, including potential binding sites for the miRNAs *let-7* (LCS) and *lin-4* (LCE), and their family members (Abrahante et al., 2003; Lin et al., 2003). Additionally, the *hbl-1* 3'UTR contains sequences that resemble Nanos response elements (NREs) (Supplemental Fig. 6), which are known binding sites for Pumilio in *Drosophila* (Murata and Wharton, 1995; Wharton and Struhl, 1991), and represent potential Puf family binding sites.

We observed that a highly conserved 169 nt region of the *hbl-1* 3'UTR, containing two putative NRE sites (termed NRE1 and NRE2) flanking a predicted LCS and closely preceded by a potential LCE (Supplemental Fig. 6), which we called the "NRE region" was sufficient to form PUF-9 dependent complexes *in vitro* (Supplemental Fig. 7). This molecular data suggests that the NRE-region of the *hbl-1* 3'UTR binds, either directly or in a complex, to PUF-9 *in vivo*.

In order to determine the importance of the NRE region on *hbl-1* 3'UTR down-regulation in the hypodermis, we generated various deletions of the *hbl-1* 3'UTR (Fig. 6A) from the pFS1038 construct described above (Fig. 5A), and injected the constructs into wild type animals. Multiple independent lines were generated for each deletion construct and were scored for β-galactosidase activity in the hypodermis at all post-embryonic stages (Fig. 6B). We observed that inclusion of the intact NRE region (pSJA2 and pSJA5), containing the two NRE-like sequences and the LCS2 and LCE1 sites, lead to substantial reporter down-regulation, suggesting that a combination of NRE1, NRE2, and/or LCS 2 are able to mediate partial regulation of the *hbl-1* 3'UTR reporter gene at the adult stage. Removal of the majority of the *hbl-1* 3'UTR, such that only LCS1 remained (pSJA4), resulted in almost complete loss of reporter gene down-regulation, indicating that the LCS1 site and upstream sequences are not sufficient to direct regulation of the *lacZ* reporter in the hypodermis. The partial de-repression observed in pSJA2 and pSJA5 constructs indicates that sequences downstream of NRE2 are also necessary, in addition to the NRE region, for full *hbl-1* 3'UTR repression. This idea is supported by the staining pattern seen when just the NRE region by itself is deleted (pMJC2) where we observed that keeping the 3'UTR intact, outside of the NRE region, resulted in some adult stage down-regulation of the reporter.

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We have subsequently identified multiple miRNA complementary sites (LCS and LCE), as well as additional conserved NRE-like sequences downstream of the defined NRE region (Supplemental Fig 6A). Any number of these sites could be required in addition to the NRE region for complete down-regulation of *hbl-1* at the adult stage. It is possible that PUF-9 and a *let-7* family member are able to bind at multiple regulatory elements along the length of the *hbl-1* 3'UTR to mediate wild type down-regulation *in vivo*. It is also possible that other factors can partially substitute for *puf-9* to regulate targets, such as *hbl-1*, in the seam cells.

Our hypothesis that sequences outside of the NRE region are important for *hbl-1* regulation during development was further supported by the observation that mutating the individual NRE1 and NRE2 sequences resulted in levels of staining that were not significantly changed from wild type (Supplemental Fig. 8B). While there appeared to be a trend toward mis-regulation of the reporter at the adult stage in pMJC12 lines, the variability of staining between trials does not allow us to definitively conclude that there is significant adult up-regulation in the mutated transgenes.

Taken together, our data suggests that PUF-9 may regulate *hbl-1* by binding to a region of the *hbl-1* 3'UTR that contains modified Nanos Response Elements (NRE1 and NRE2) and a *let-7* family complementary site (LCS2). However, it is unclear whether PUF-9 binds directly to sequences in the NRE region, *in vivo*. It is possible that PUF-9 binds a combination of sites throughout the *hbl-1* 3'UTR to regulate *hbl-1* expression in different tissue types. Given that *let-7* and *puf-9* individually repress *hbl-1* expression via its 3'UTR (Abrahante et al., 2003; Lin et al., 2003), the genetic enhancement of *let-7* by *puf-9(RNAi)*, and the close association of LCS and NRE-like sequences in the *hbl-1* 3'UTR, it is intriguing to speculate that post-transcriptional *hbl-1* regulation is achieved through cooperative binding of PUF-9 and a *let-7* family member.

puf-9 is expressed in the hypodermis and the neurons

To see if *puf-9* is expressed in tissues where *hbl-1* is also expressed, we analyzed the post-embryonic expression pattern of a *puf-9::gfp* transcriptional fusion reporter. Promoter activity with distinct GFP expression in lateral hypodermal seam cells (Fig. 7A, arrows) and in the non-seam cell hypodermis (Fig. 7A, diffuse gfp) was observed throughout development. The observed hypodermal GFP expression pattern is consistent with the noted seam cell and alae defects in *puf-9* mutants, supporting a function for *puf-9* in these tissues. Additionally, expression was noted in many neurons, including the ventral nerve cord (VNC) (Figure 7B, arrows) and the hermaphrodite specific neuron (HSN) (Figure 7C, arrow head). GFP was also observed in various somatic gonad tissues including the anchor cell in larval stages and adult vulval muscle cells, the distal tip cells, a subset of the vulval precursor cells, uterine cells, and spermatheca (not shown). PUF-9/GFP translational fusion lines, containing the entire PUF-9 coding region, recapitulated the expression pattern seen in the transcriptional fusion lines described above, including in the hypodermis and neurons (not shown).

The promoter driven expression pattern of *puf-9* supports our hypothesis that PUF-9 regulates *hbl-1* in both the hypodermis and the VNC. The *puf-9::gfp* constructs above do not appear to be temporally regulated. However, we cannot rule out possible post-transcriptional temporal regulation of PUF-9 expression.

Conclusions and implications

Puf mediated RNA regulation has been co-opted by many different organisms during evolution, illustrating the importance of this highly adaptable family of gene regulators. However, despite their widespread abundance, the molecular mechanisms by which Puf proteins regulate their target RNAs are still largely unknown. The work presented in this study could elucidate a

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general mechanism of translation regulation by Puf proteins, relevant to both the temporal and spatial control of gene expression.

Puf proteins have an evolutionarily conserved function in promoting stem cell proliferation and self-renewal (Crittenden et al., 2002; Wickens et al., 2002). For example, in the adult fly, a wild type copy of *pumilio* (*pum*) is required to maintain a population of germline stem cells (GSCs) and in Pum deficient animals, the GSCs will differentiate after a few rounds of division, leading to small or empty ovary phenotypes (Forbes and Lehmann, 1998). In *pumilio* mutant fly larvae, germline progenitor cells, called pole cells, fail to migrate to the presumptive gonad, exit their proliferative state, and prematurely reinitiate mitosis (Asaoka-Taguchi et al., 1999; Gilboa and Lehmann, 2004). Our work shows that in *C. elegans, puf-9* may promote adult hypodermal fates by acting to repress genes, such as *hbl-1*, which prevent cell differentiation and promote continued cell divisions. Therefore, this work may give further insight into the role of Puf proteins during the regulation of stem cell fate determination.

The transcription factor *hbl-1* is a candidate target of PUF-9 for a variety of reasons. First, *hbl-1* is the homolog of *Drosophila hunchback* (Fay et al., 1999), which is a known target of repression by Pumilio in *Drosophila*. Second, *hbl-1* and *puf-9* expression coincides in many tissues, including the hypodermis (specifically the seam cells), the VNC and the HSN (Abrahante et al., 2003; Fay et al., 1999; Lin et al., 2003). Third, our genetic evidence and reporter gene assays indicate that PUF-9 negatively regulates *hbl-1* in the hypodermis and the VNC. Finally, PUF-9 is able to interact *in vitro* with a region of the *hbl-1* 3'UTR containing potential Puf protein family binding sites.

In *C. elegans* there are four *let-7* family members, including *let-7, mir-48, mir-84,* and *mir-241* (Lim et al., 2003). However, since *hbl-1::gfp* is down-regulated by the L2 stage in the hypodermis (Abrahante et al., 2003; Fay et al., 1999; Lin et al., 2003) and *let-7* RNA is not expressed until the L3 stage (Abbott et al., 2005; Esquela-Kerscher et al., 2005; Johnson et al., 2005; Johnson et al., 2003; Reinhart et al., 2000), other factors most likely contribute to the early regulation of *hbl-1*. In support of this, data from Lin et al. (2003) indicate that loss of *let-7* had no detectable effect on early *hbl-1* expression in the hypodermis. Therefore, it is possible that *puf-9* may act with *mir-48, mir-84,* or *mir-241* at early larval stages to repress a subset of RNA targets, including *hbl-1*, to control adult seam fates. At later stages, *puf-9* may act with *let-7* to regulate *hbl-1* and additional targets required for the larval-to-adult transition.

Our genetic enhancement data supports the idea that *let-7* and *puf-9* work together to promote adult fates during *C. elegans* development. *let-7* is highly conserved in both invertebrate and vertebrate organisms (Pasquinelli et al., 2000) and multiple *let-7* homologs have been found in humans (Lim et al., 2003), underscoring the evolutionary importance of this regulatory molecule. Due to their high abundance and evolutionary conservation, the importance of miRNAs as translational regulators is apparent. However, the binding partners and exact mechanism by which miRNAs act to regulate their mRNA targets is still unclear. Therefore, finding binding partners that work with miRNAs may give insight into the mechanism by which these regulatory RNAs act on their gene targets. Our data suggests that the important translational regulatory families, Pumilios and microRNAs, may cooperate at the 3'UTRs of target genes to control their translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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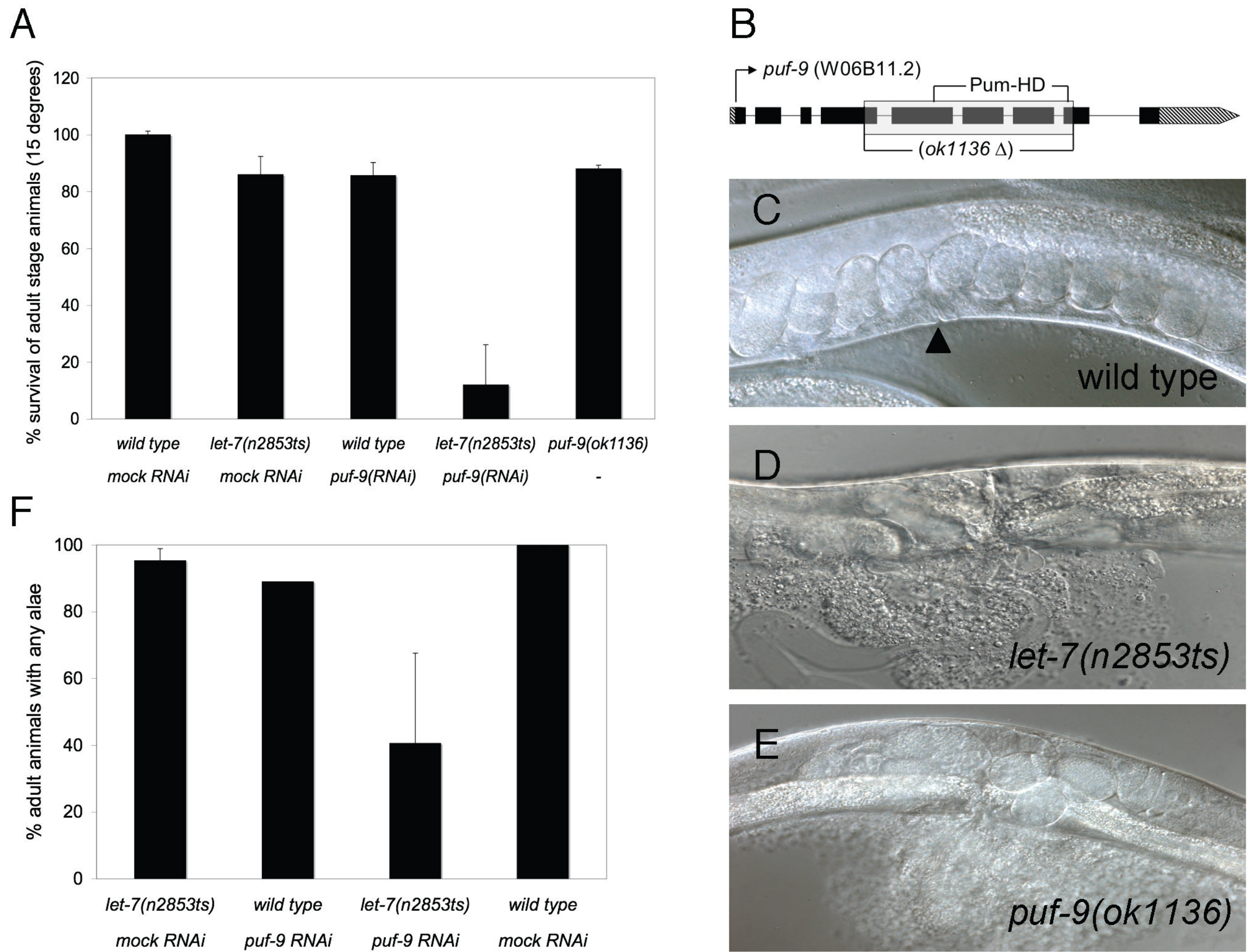

Figure 1.

puf-9 depletion enhances the lethal bursting and heterochronic phenotypes of *let-7(ts)* mutants. (A) *let-7(n2853ts)* animals exposed to *puf-9(RNAi)* show an increase in the number of animals that die due to bursting at the adult stage (n=533) compared to *let-7(n2853ts)* animals grown on a control mock RNAi (n=1114). *puf-9(ok1136)* mutants (n=746) and wild type animals on *puf-9(RNAi)* (n=483) showed similar survival rates as *let-7(n2853ts)* animals on mock RNAi. 100% survival rate was seen in wild type animals grown on mock RNAi (n=652). (B) A cartoon of the intron/exon structure of the *puf-9* gene (contained on the W06B11.2 cosmid). The relative position of the region deleted in the *puf-9(ok1136)* allele is boxed and shaded. The Pum-HD region is indicated within the *ok1136* deletion region. The *puf-9(ok1136)* deletion removes 1581 nt beginning 154 nt into exon 4 and ending 77 nt into exon 8 (nt 5727 to 7309 in the W06B11.2 cosmid sequence). This deletion ends 632 nt upstream of the predicted stop codon and causes a frame-shift to create a premature stop codon 29 nt downstream of the deletion break. (C-E) Depletion of *let-7* and *puf-9* gene products results in a similar vulval bursting phenotype. Nomarski DIC images of a wild type adult (C, vulva is marked with an arrow), an adult stage *let-7(n2853ts)* animal with a burst vulva (D), and a *puf-9(ok1136)* adult animal showing a bursting phenotype similar to *let-7(lf)* mutants (E). (F) *puf-9(RNAi)* enhances the lack of adult alae phenotype of *let-7(lf)* mutants. When grown on *puf-9(RNAi),* less than half of the *let-7(n2853ts)* animals displayed any alae at the young adult stage (n=40) compared to wild type animals exposed to *puf-9(RNAi)* (n=27) or *let-7(n2853ts)* mutants on mock RNAi (n=61).

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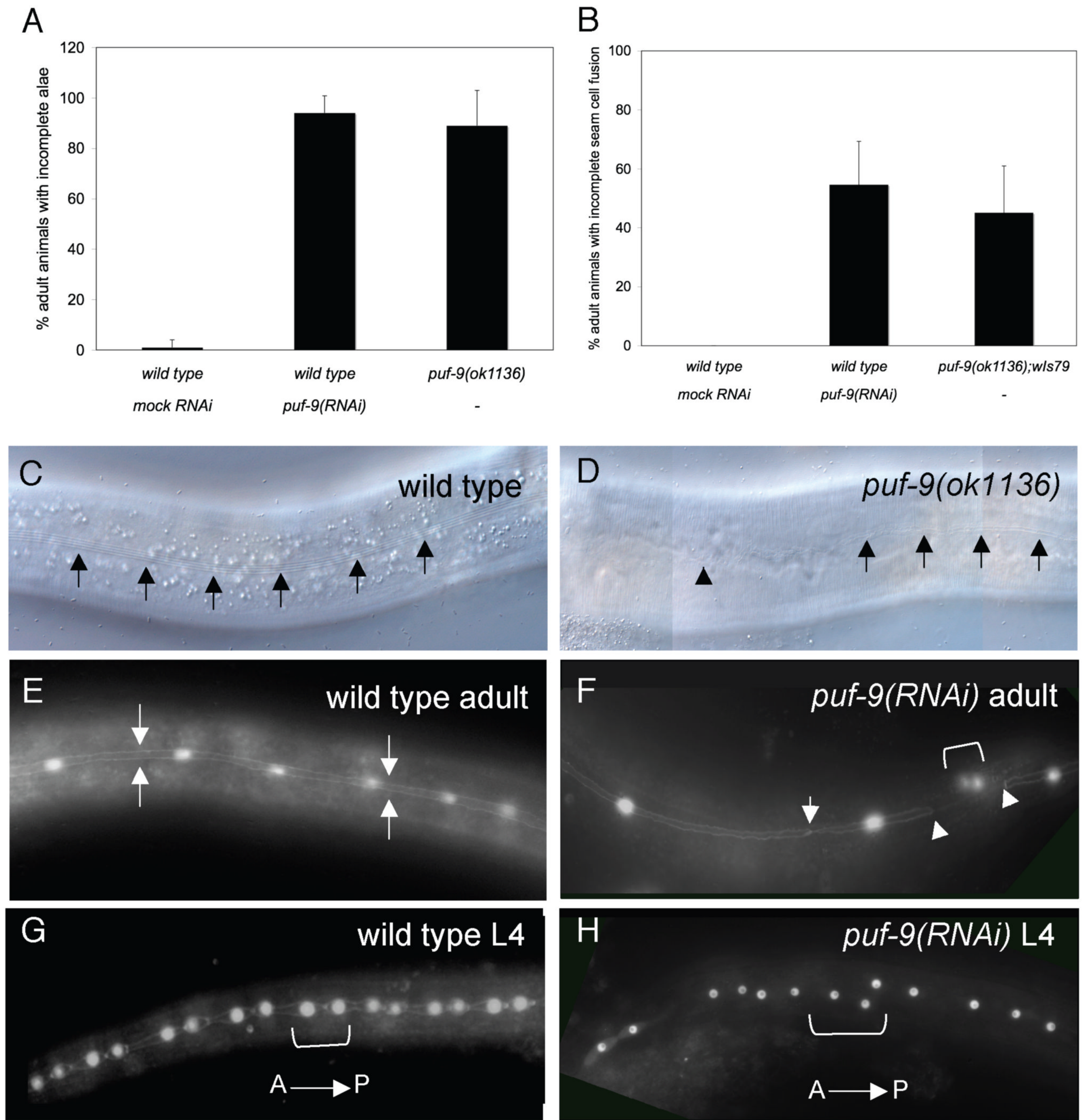

Figure 2.

puf-9 deletion mutants display abnormal alae and seam cell fusion phenotypes. (A) *puf-9 (ok1136)* mutants (n=88) and *puf-9(RNAi)* animals (n=157) show incomplete alae at the adult stage (89% and 94%, respectively) compared to 1% in wild type animals (n=129) (C,D) Nomarski DIC images of adult stage alae in a wild type animal (C, alae marked with arrows) and an adult stage *puf-9(ok1136)* animal showing incomplete alae (D, alae marked with arrows and the alae break marked with an arrow head). (B, E-H) Seam cell analysis of *wIs79* animals carrying a *gfp* reporter marking the seam cell nuclei and adherens junctions. (B) Approximately half of the *wIs79* animals exposed to *puf-9(RNAi)* (n=96) or in a *puf-9(ok1136)* mutant background (n=21) showed incomplete seam cell fusion at the young adult stage compared to

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wIs79 on mock RNAi (n=63). (E-H) GFP images of *wIs79;puf-9(RNAi)* animals. Fusion defects included gaps along the length of the seam cell boundary syncytium (F, gap edges marked by arrow heads) and single seam cell nuclei separated from the rest of the syncytium (F, the anterior boundary of a single unfused seam cell is marked by the arrow), indicating that a number of seam cells failed to properly fuse to the seam cell syncytium. *puf-9(RNAi)* animals also show seam cell nuclei that are abnormally spaced (F, set of nuclei between arrow heads and H, nuclei within bracket) and are often spatially displaced relative to one another (H, bracketed nuclei). (E) Wild type adult seam cell fusion (seam cell syncytium between arrows) and (G) anterior-posterior seam cell nuclei orientation (a seam cell pair is in the bracket). Anterior is to the left and ventral down in all images.

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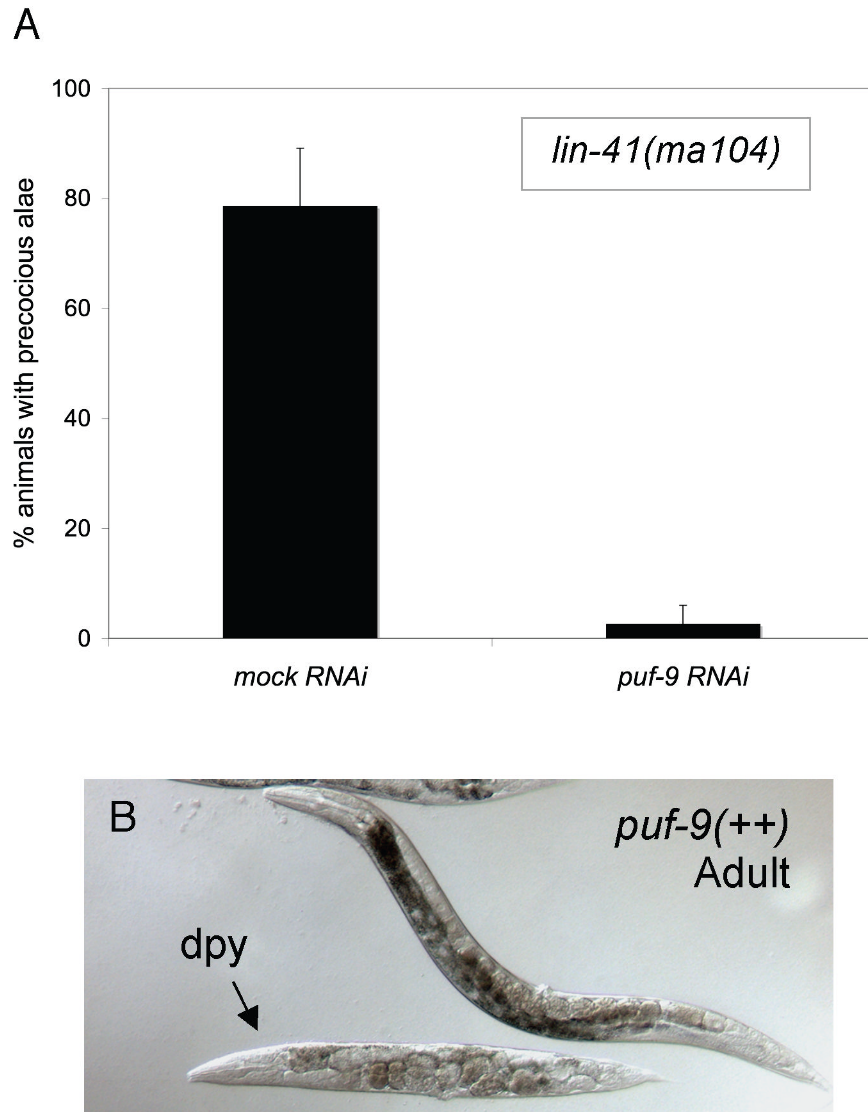

Figure 3.

puf-9(RNAi) by feeding suppresses *lin-41(lf)* precocious alae. (A) *lin-41(ma104)* animals exposed to *puf-9(RNAi)* (n=35) showed an almost complete reduction in the number of animals that displayed alae in the early L4 stage compared to animals on mock RNAi (n=21). (B) Full length *puf-9::gfp* over-expressing animals showed dumpy (Dpy) phenotypes reminiscent of *lin-41* and *hbl-1* loss-of-function mutants. The transgenic animal in (B) is indicated by an arrow.

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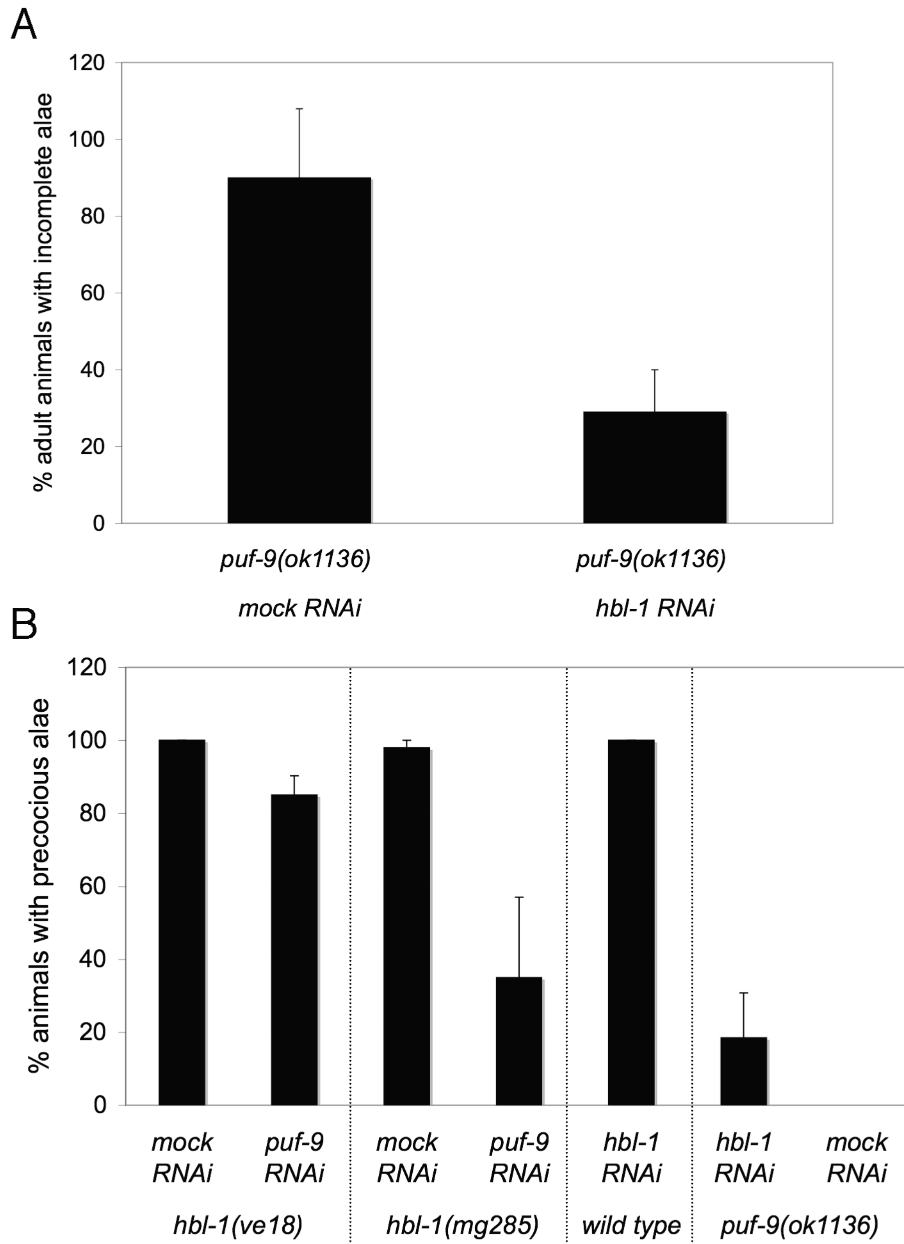

Figure 4.

hbl-1(RNAi) suppresses *puf-9(ok1136)* adult alae phenotypes while *puf-9(RNAi)* is unable to suppress a strong loss-of-function *hbl-1* allele. (A) *hbl-1(RNAi)* suppresses the *puf-9 (ok1136)* adult alae phenotypes. The number of *puf-9(ok1136)* animals displaying incomplete alae was decreased on *hbl-1(RNAi)* (n=55) compared to *puf-9(ok1136)* mutants alone (n=59). (B, left data sets) *puf-9* is unable to suppress the *hbl-1(ve18)* precocious alae phenotype. *hbl-1 (ve18)* mutants on mock RNAi (n=76) show a complete penetrance of precocious alae in the early L4 stage and nearly all *hbl-1(ve18); puf-9(RNAi)* animals (n=96) continued to display this precocious alae defect. (B, middle and right data sets) The precocious alae phenotypes of a weak *hbl-1* allele and *hbl-1(RNAi)* are partially suppressed by removal of *puf-9*. *hbl-1 (mg285)* animals exposed to *puf-9(RNAi)* (n=133) show a reduction in the number of animals displaying precocious alae at the early L4 stage, compared with mock RNAi (n=77). Similarly,

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puf-9(ok1136) mutants on *hbl-1(RNAi)* (n=75) suppressed the precocious alae seen with *hbl-1 (RNAi)* exposure in a wild type background (n=69). *puf-9(ok1136)* mutant animals did not show any precocious alae on their own when fed mock RNAi (n=43). Error bars indicate standard deviations.

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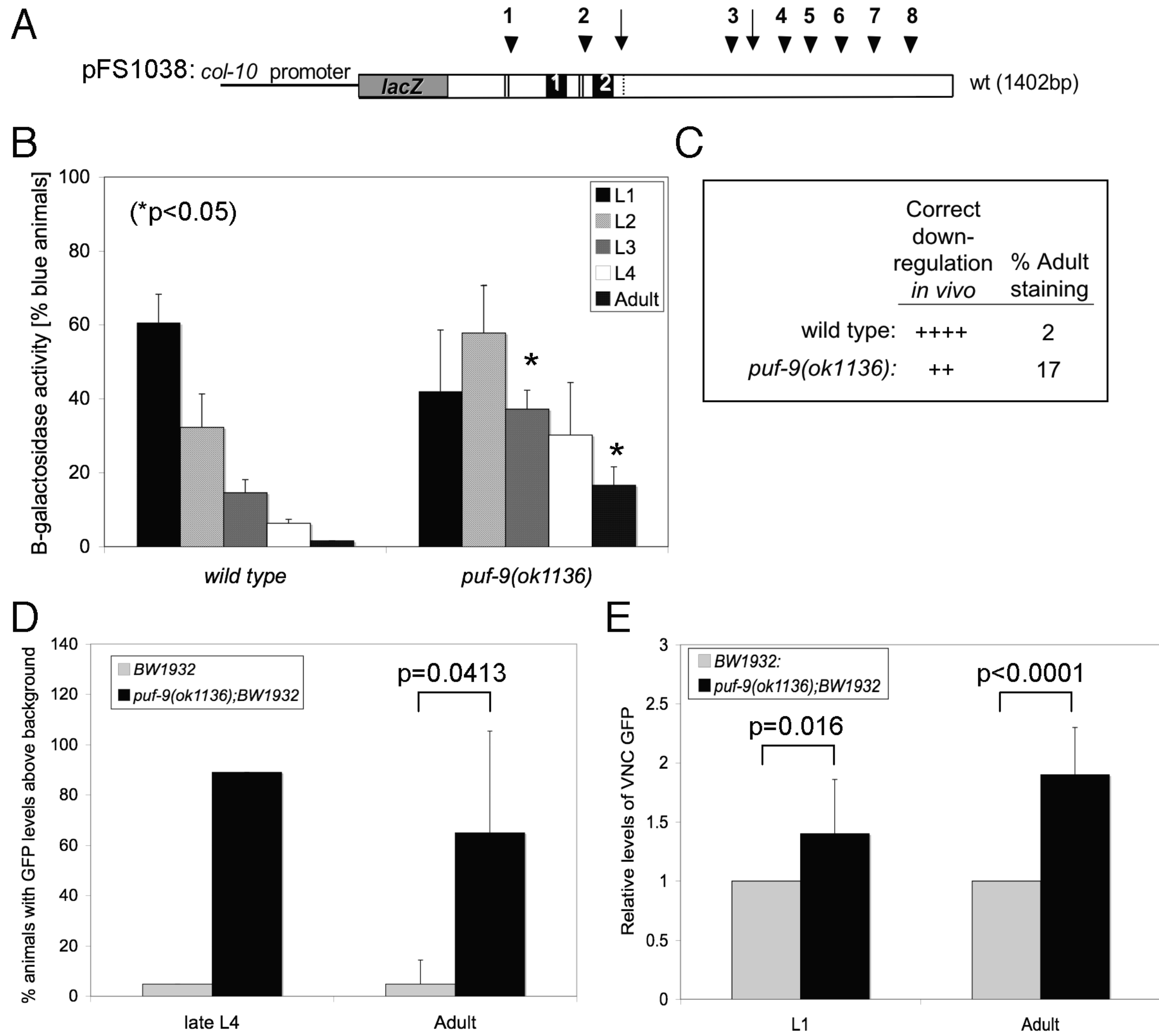

Figure 5.

A wild type copy of *puf-9* is required for *hbl-1* 3'UTR reporter down-regulation in the hypodermis and the neurons. (A) A diagram of the heterologous reporter gene (pFS1038) used to assay *hbl-1* 3'UTR-directed regulation in the hypodermis, consisting of the wild type *hbl-1* 3'UTR fused downstream of the *Escherichia coli lacZ* gene and driven by the hypodermally expressed *col-10* promoter (not to scale). (B, C) *puf-9(ok1136)* mutant animals carrying the pFS1038 reporter showed maintenance of β-galactosidase activity in the adult stage (17%), while the same construct in wild type animals showed expected levels of reporter gene down-regulation (2%) at the adult stage. Three independent staining trials were performed. The number of lines scored for each strain are, wild type: 1 line and *puf-9 (ok1136)*: 5 lines, n > 270 for all stages scored. For statistical analysis, an unpaired Student's t test was performed between corresponding stages for the wild type and *puf-9(ok1136)* backgrounds. Error bars indicate standard deviations. (C) The correct down-regulation for each construct was compared to wild type and assigned values as follows: (++++) 0%−5% of animals with hypodermal expression at the adult stage; (+++) 5%−10%; (++) 10%−20%; (+) 20% −60%; (−) >60%. (D, E) A wild type copy of *puf-9* is required for adult stage *gfp::hbl-1* 3'UTR reporter down-regulation in the VNC. (D) Late L4 (n=18) and adult stage (n=67) *puf-9 (ok1136)* mutants showed an increase in the number of animals with bright HBL-1/GFP in the VNC compared to the same stages in wild type animals (L4, n=21 and adult, n=63). Wild type

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and *puf-9(ok1136)* animals were scored in three independent trials for adult GFP expression, and one trial for L4 GFP expression. (E) Quantification of wild type and *puf-9(ok1136)* GFP levels in the VNC was performed for a sample of L1 and adult stage animals. *puf-9(ok1136)* mutants showed a significant up-regulation of GFP at the L1 (n=8) and adult stages (n=12) compared to wild type animals (L1, n=4 and adult, n=10). The levels of GFP expression were calculated by normalizing the quantity of average GFP signal in *puf-9(ok1136)* mutants against the average GFP signal in wild type animals (for L1 and adult stages). Statistics were performed using an unpaired Student's t test.

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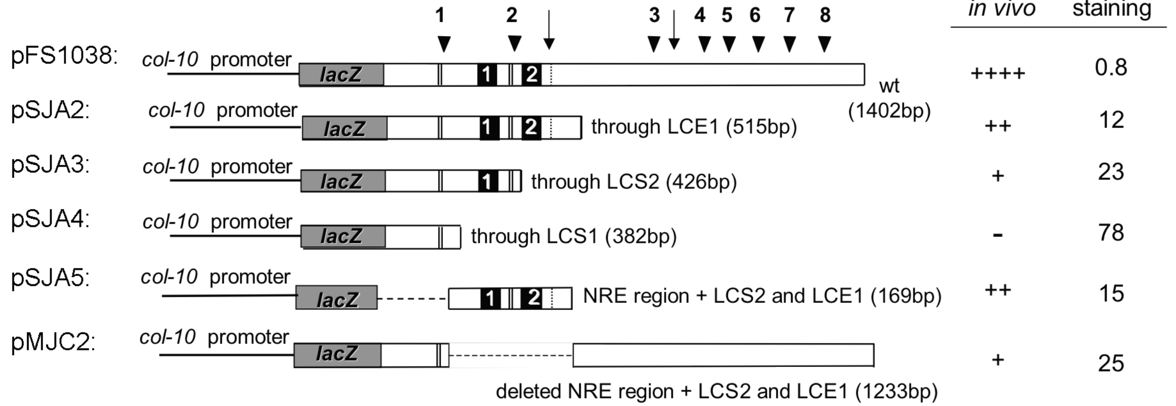

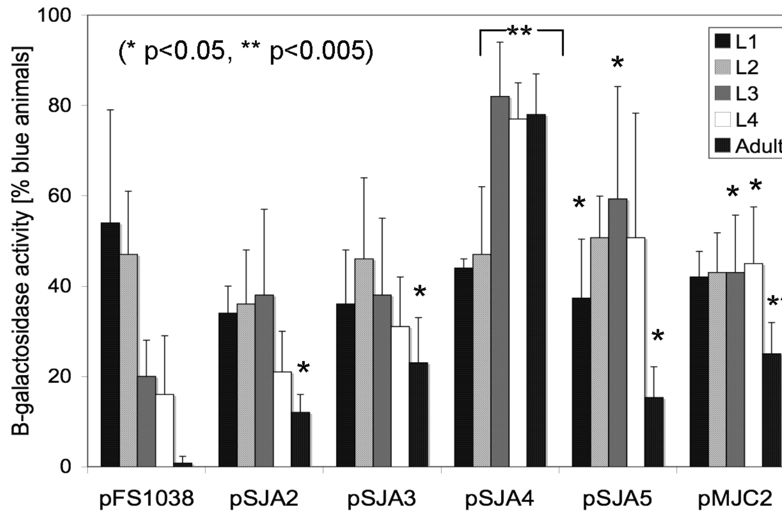

Figure 6.

hbl-1 is regulated by multiple sequences, including the NRE region, in its 3'UTR. (A) Schematic of the *hbl-1* 3'UTR deletion constructs used to determine the necessary regulatory region of the *hbl-1* 3'UTR. Each construct shares a common *col-10* promoter and contains the indicated portions of the *Caenorhabditis elegans hbl-1* 3'UTR fused to the *Escherichia coli lacZ* reporter gene. LCS are designated by arrowheads, with LCS 1 and LCS 2 specified by the double lines. LCE are shown as arrows, with LCE 1 designated as a dashed line. NRE1 and NRE2 are shown as black, numbered boxes within the construct. The values for correct down-regulation were assigned as in Figure 5. pFS1038 is the full-length reporter construct and contains the wild type 1402 bp *hbl-1* 3'UTR. (A, B) The deletion constructs all showed statistically significant up-regulation at the adult stage compared to wild type (students t test). The number of lines scored for each construct in (B) are pFS1038: 1 line (4 trials), pSJA2: 3 lines, pSJA3: 4 lines, pSJA4: 2 lines, pSJA5: 3 lines, and pMJC2: 4 lines, n > 50 for all stages scored. Error bars indicate standard deviations.

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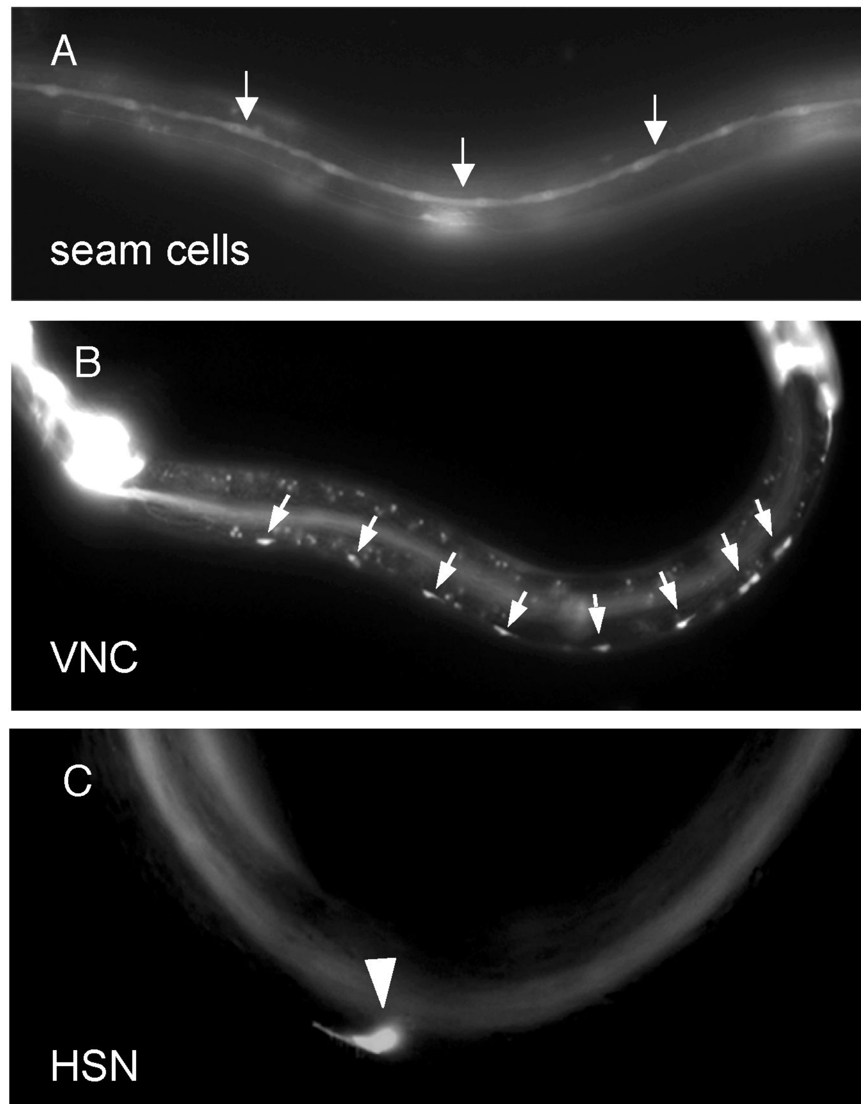

Fig. 7.
puf-9::gfp is expressed in the hypodermis and a subset of the neurons. Animals carrying a *puf-9::gfp* transcriptional fusion (MJC20) showed larval GFP expression in the lateral hypodermal seam cells (A, arrows) and non-seam cell hypodermis (A, diffuse staining). Additionally, GFP expression was noted in various neural cells including the ventral neural cord (VNC) (B, cell bodies marked by arrows), and the hermaphrodite specific neurons (HSN) (C, marked by an arrowhead).