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Distinct roles of the Pumilio and FBF translational repressors during *C. elegans* vulval development

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There were errors published in *Development* **133**, 3461-3471.

We have discovered that the expression pattern of the PUF-8::GFP reporter *zhEx61* shown in Fig. 2B-L on p. 3466 of this article, is based on an incorrect reporter construct that carries the insert in the reverse orientation. The corrected Fig. 2 below shows the PUF-8::GFP expression pattern that is observed with the correct PUF-8::GFP reporter *zhEx274.1*, which carries the insert in the correct orientation. Also shown is a corrected supplementary Fig. S1, in which a quantification of the expression pattern of *zhEx274.1* is shown (A-C). Although the overall expression pattern observed with the PUF-8::GFP reporter *zhEx274.1* is similar to that obtained with the reverse reporter *zhEx61* shown in Fig. 2B-L of Walser et al. (2006), there are four differences in its expression, which are accounted for in the text changes detailed below. Also provided are new methods for the generation of *zhEx274.1*. These corrections do not change the overall conclusions of this paper. The page, paragraph and line numbers below refer to the PDF version of the article.

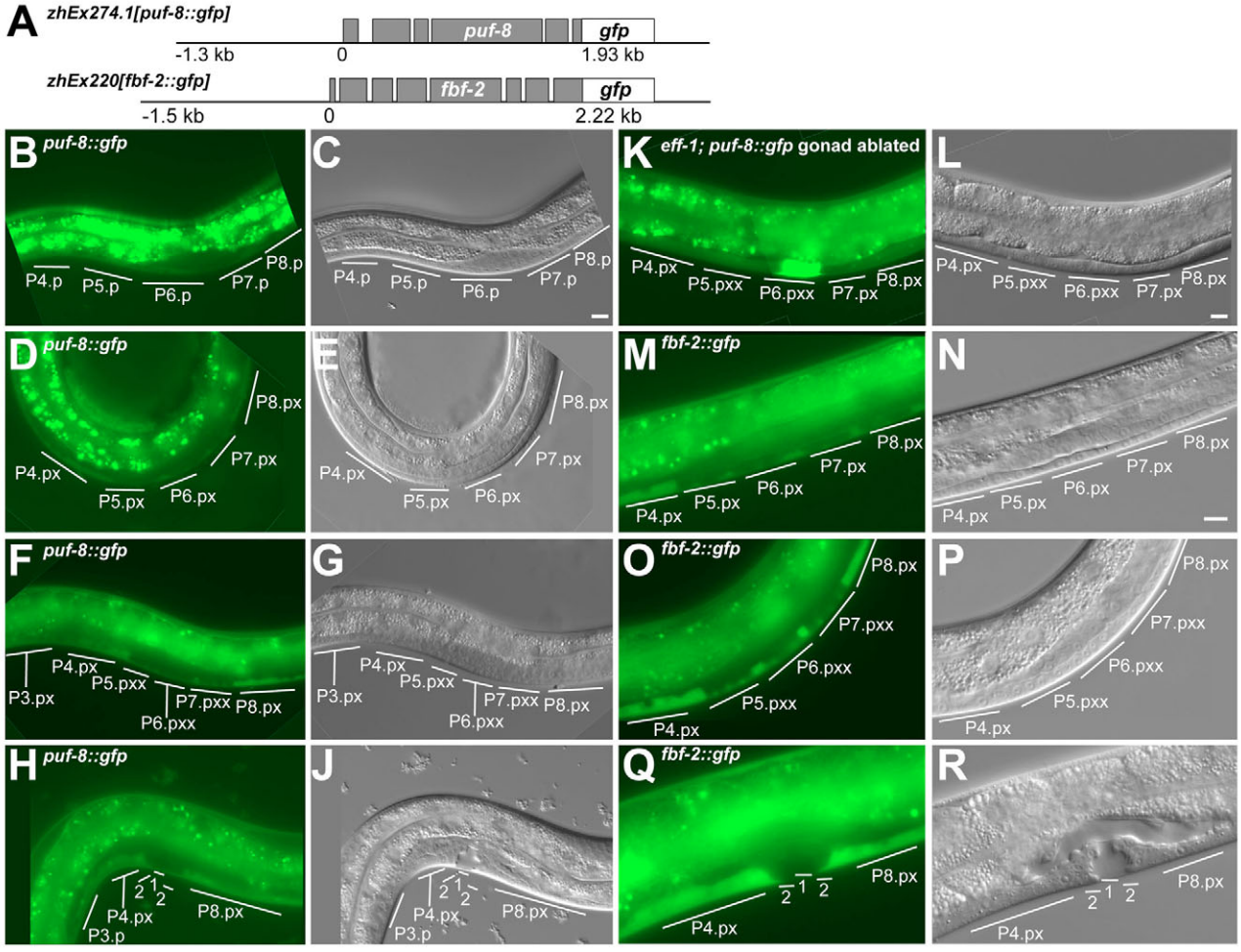

Fig. 2. PUF-8::GFP and FBF-2::GFP expression during vulval development. (**A**) Structure of the translational *puf-8::gfp* and *fbf-2::gfp* reporters. (**B**,**D**,**F**,**H**) Time-course analysis of PUF-8::GFP expression in the vulval cells from the L2 until the L4 stage with (**C**,**E**,**G**,**J**) the corresponding Nomarski images. For a semi-quantitative analysis of the expression patterns, see Fig. S1 in the supplementary material. (**K**,**L**) PUF-8::GFP expression in gonad-ablated *eff-1(hy21)* animals, and the corresponding Nomarski image. All VPC descendants showed PUF-8::GFP expression with a strong increase in the descendants of P6.p. Note that despite the extra round of cell divisions in P5.p and P6.p descendants of gonad-ablated *eff-1* mutants, no vulval differentiation was observed. (**M-R**) FBF-2::GFP expression, and the corresponding Nomarski images, from the early L3 until the L4 stage. In all panels, anterior is to the left and ventral is to the bottom. Scale bars: 10 μm.

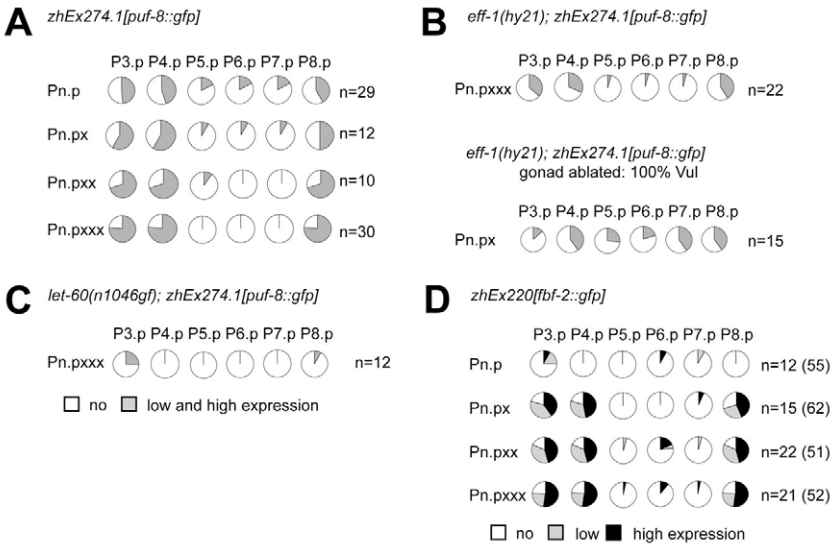

Fig. S1. PUF-8::GFP and FBF-2::GFP expression analysis. (**A**) Semi-quantitative time-course analysis of PUF-8::GFP expression in wild-type animals. The two daughter cells after the first cell division are termed Pn.px for all VPCs. The descendants of the second cell divisions of induced VPCs are termed Pn.pxx, and after the third round of cell divisions Pn.pxxx cells. Gray areas indicate the proportion of PUF-8::GFP-positive vulval cells, white areas the proportion of PUF-8::GFP-negative cells. (**B**) Analysis of PUF-8::GFP expression pattern in (top row) *eff-1(hy21)* mutants at the Pn.pxxx stage without gonad ablation and (bottom row) gonad-ablated *eff-1(hy21)* mutants at the Pn.px stage. Both conditions were analyzed in L4 larvae, but since VPCs in gonad-ablated animals are not induced to adopt vulval cell fates, they divide once and arrest at the Pn.px stage or occasionally divide a second time, as shown in Fig. 2K,L. (**C**) Analysis of the PUF-8::GFP expression pattern in *let-60(n1046gf)* L4 larvae at the Pn.pxxx stage. Since *let-60(n1046gf); zhEx274.1[puf-8::gfp]* animals developed into sterile adults for unknown reasons, the *let-60(n1046); zhEx274.1[puf-8::gfp]* animals were maintained as heterozygotes, and their multivulva progeny homo- or heterozygous for *let-60(n1046gf)* were scored at the Pn.pxxx stage. (**D**) Semi-quantitative time-course analysis of FBF-2::GFP expression in wild-type animals. Only animals showing bright FBF-2::GFP expression in somatic tissues were used for the analysis. White indicates no FBF-2::GFP expression, grey low expression and black high expression.

Correction to the text on p. 3462, paragraph 6

Extrachromosomal transgenic arrays [transgenes; co-transformation marker; pBS: Bluescript (concentration in ng/μl)] were generated by microinjection of DNA into young adult worms (Mello et al., 1991):

Correction to the text on p. 3462, paragraph 7, line 6

…zhEx220[fbf-2::gfp; lin-48::gfp (100;50)], zhEx274.1[puf-8::gfp; lin-48::gfp (80;50)].

Correction to the text on p. 3464, paragraph 2, from line 4

PUF-8::GFP was expressed in various tissues including the hypodermis, the ventral cord motor neurons (not shown) and the vulval cells (Fig. 2B-J and see Fig. S1A in the supplementary material). Before vulval induction in L2 larvae, PUF-8::GFP was expressed in all six vulval precursor cells, although expression was more frequently observed in the distal VPCs (P3.p, P4.p and P8.p) than in the proximal VPCs (P5.p, P6.p and P7.p, Fig. 2B,C, and row with Pn.p cells in Fig. S1A in the supplementary material). After vulval induction in early L3 larvae, PUF-8::GFP expression persisted in the descendants of the 3° distal VPCs (P3.p, P4.p and P8.p), while expression faded in the 1° and 2° descendants of the proximal VPCs (P5.p, P6.p and P7.p, Fig. 2D-J, Fig. S1A in the supplementary material, rows Pn.px to Pn.pxxx).

Correction to the text on p. 3464, paragraph 3, from line 1

We hypothesized that PUF-8::GFP expression in the descendants of the distal 3° VPCs might persist because these cells fuse with the hyp7 hypodermis that also expresses PUF-8::GFP. To test if the expression of PUF-8::GFP in the descendants of the 3° VPCs is a consequence of their fusion with hyp7, we examined PUF-8::GFP expression in an *eff-1(hy21)* background, in which no cell fusions occur (Mohler et al., 2002).

Correction to the text on p. 3464, paragraph 3, from line 10
In most gonad-ablated *eff-1(hy21)* animals, PUF-8::GFP expression was observed in the VPCs and their descendants (Fig. 2K,L and see Fig. S1B in the supplementary material). Moreover, in *let-60 ras(gf)* animals, in which the distal VPCs frequently adopt the 1° or 2° induced cell fates, PUF-8::GFP expression was often absent in the distal VPCs and their descendants (see Fig. S1C in the supplementary material) (Beitel et al., 1990; Greenwald et al., 1983). We conclude that PUF-8::GFP is expressed in the descendants of VPCs that have adopted the uninduced 3° cell fate independently of their fusion with hyp7.

Correction to the text on p. 3466, paragraph 2, line 1
The expression of PUF-8::GFP in the distal 3° vulval cells raises the possibility that PUF-8 might regulate the competence of the distal vulval cells to respond to the inductive signal.

Correction to the text on p. 3469, paragraph 2, line 7
A PUF-8::GFP reporter transgene is expressed predominantly in the distal VPCs (P3.p, P4.p and P8.p) and their descendants that have adopted the 3° fate.

The authors apologise to readers for these mistakes and are grateful to Dave Hansen for discovering the error in the plasmid used to generate *zhEx61*.

Publisher's note: Although the mistake reported in this corrigendum has resulted in several corrections being made to Walser et al. (2006) and in an unusually lengthy corrigendum, we would like to reassure readers that expert opinion has confirmed that the minor changes in expression that are seen between the incorrect reporter *zhEx61* and the correct reporter *zhEx274.1* do not alter or affect the conclusions drawn by this paper.

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Distinct roles of the Pumilio and FBF translational repressors during *C. elegans* vulval development

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The *C. elegans* PUF and FBF proteins regulate various aspects of germline development by selectively binding to the 3′ untranslated region of their target mRNAs and repressing translation. Here, we show that *puf-8, fbf-1* and *fbf-2* also act in the soma where they negatively regulate vulval development. Loss-of-function mutations in *puf-8* cause ectopic vulval differentiation when combined with mutations in negative regulators of the EGFR/RAS/MAPK pathway and suppress the vulvaless phenotype caused by mutations that reduce EGFR/RAS/MAPK signalling. PUF-8 acts cell-autonomously in the vulval cells to limit their temporal competence to respond to the extrinsic patterning signals. *fbf-1* and *fbf-2*, however, redundantly inhibit primary vulval cell fate specification in two distinct pathways acting in the soma and in the germline. The FBFs thereby ensure that the inductive signal selects only one vulval precursor cell for the primary cell fate. Thus, translational repressors regulate various aspects of vulval cell fate specification, and they may play a conserved role in modulating signal transduction during animal development.

KEY WORDS: *Caenorhabditis elegans*, Vulva, Pumilio, Translational control, Signal transduction

INTRODUCTION

The spatial and temporal regulation of gene expression can occur either at the level of gene transcription or at the level of mRNA export, stability or translation through RNA-binding proteins or micro RNAs (de Moor et al., 2005; Kuersten and Goodwin, 2003). Work on model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* has contributed much to our current understanding of post-transcriptional gene regulation during development. Translational control by RNA binding proteins is frequently used in the *C. elegans* germline and early embryo, but translational regulation has also been observed during larval development (Kuersten and Goodwin, 2003; Rougvie, 2001). Many mRNAs contain sequence motifs in their 5′ or 3′ untranslated regions (5′UTRs or 3′UTRs) that serve as binding sites for regulatory proteins controlling different aspects of mRNA localization, translation or stability.

The PUF gene family is conserved from yeast to humans. PUF proteins function as translational repressors that bind to specific elements in the 3′UTRs of their target mRNAs (reviewed by Wickens et al., 2002). The first characterized members of this family were *Drosophila* Pumilio and the two *C. elegans* FBF proteins. Hence, this family is referred to as PUF for Pumilio and FBF repeat proteins (Zhang et al., 1997). Typical PUF proteins contain eight PUF repeats of approximately 40 amino acids with a core consensus sequence containing aromatic and basic residues. The PUF repeats directly bind to the target mRNAs and recruit additional proteins such as Nanos, Brain tumor and CPEB (Kraemer et al., 1999; Luitjens et al., 2000; Sonoda and Wharton, 1999; Sonoda and Wharton, 2001). The cis-regulatory elements in the 3′ UTRs of their target mRNAs contain a UGUR tetra nucleotide sequence motif termed a *Nanos* response element (NRE). The binding specificity of the individual PUF proteins is thought to be determined by additional flanking nucleotides (Murata and Wharton, 1995; Tadauchi et al., 2001; Wharton et al., 1998; Zamore et al., 1997; Zhang et al., 1997).

Pumilio, the only PUF protein in *Drosophila melanogaster*, controls, together with Nanos, the establishment of the anterior-posterior axis of the embryo by repressing the translation of maternal *hunchback* mRNA (Barker et al., 1992; Murata and Wharton, 1995). Pumilio and Nanos also inhibit *cyclin B* translation in migrating pole cells allowing them to arrest in G2 until they reach the gonads (Asaoka-Taguchi et al., 1999). In addition to its roles during development, *Drosophila* Pumilio was recently shown to be necessary for the activity-dependent expression of the voltage-gated sodium channel Paralytic in the central nervous system (Mee et al., 2004). The human and mouse genomes each encode two PUF proteins with unknown functions (Spassov and Jurecic, 2002; Spassov and Jurecic, 2003).

The *C. elegans* genome contains the surprisingly high number of eleven PUF genes (*fbf-1* and *fbf-2*, *puf-3* to *puf-11*). PUF-8 forms, together with PUF-9, a distinct subgroup among the *C. elegans* PUF proteins, as PUF-8 and PUF-9 are more similar to the *Drosophila* and to the two vertebrate pumilio proteins than to the other *C. elegans* PUF proteins (Wickens et al., 2002). FBF-1 and FBF-2 (*fem-3*-binding factor-1 and -2) are two closely related proteins that regulate the sperm/oocyte switch in the hermaphrodite germline by binding to the PME (point mutation element) in the 3′ UTR of *fem-3* mRNA (Ahringer and Kimble, 1991; Kraemer et al., 1999; Zhang et al., 1997). In addition, FBF-1 and FBF-2 both regulate the mitosis versus meiosis decision in the distal region of the germline by repressing *gld-1* translation in the mitotic region to prevent the stem cells from entering meiosis (Crittenden et al., 2002; Kadyk and Kimble, 1998). Furthermore, FBF and PUF proteins are required for germ cell survival, germ cell migration and the mitotic arrest of germ cells during embryogenesis (Kraemer et al., 1999; Subramaniam and Seydoux, 1999). PUF-8 is necessary for the meiotic division of the primary spermatocytes in hermaphrodites and males (Subramaniam and Seydoux, 2003).

Here, we show that the same PUF proteins that control germline development also act in the soma during vulval induction. During larval development, the hermaphrodite vulva is formed out of 22

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cells that are generated by three out of six equivalent vulval precursor cells (VPCs; P3.p through P8.p) (Greenwald, 1997). To induce vulval differentiation, the anchor cell (AC) in the somatic gonad sends an epidermal growth factor signal (LIN-3) to the adjacent VPCs (Hill and Sternberg, 1992). This inductive AC signal activates the LET-23 EGFR signalling pathway in the nearest VPC (P6.p) to specify the primary (1°) cell fate. P6.p then sends a lateral signal to the neighbouring VPCs, P5.p and P7.p, via the LIN-12 NOTCH pathway (Greenwald et al., 1983; Sternberg, 1988). LIN-12 signalling inhibits the 1° fate specification in P5.p and P7.p and instead instructs the secondary (2°) fate in these cells (Ambros, 1999; Sternberg, 1988). Multiple inhibitory signalling pathways antagonize the EGFR/RAS/MAPK pathway to control the cell fate choice in the VPCs (reviewed by Fay and Han, 2000). These inhibitors ensure that the distal VPCs (P3.p, P4.p and P8.p), which receive little or no inductive and lateral signals, adopt the tertiary (3°) non-vulval cell fate. After the vulval cell fates have been specified, the VPCs undergo stereotypic patterns of cell divisions before they differentiate and form the mature organ. Three rounds of symmetric cell divisions generate eight 1° descendants, of which four adopt the VulE and four the VulF subfate. The last of the three cell divisions in the 2° lineage generates only seven descendants that further differentiate into the VulA, VulB, VulC and VulD subfates (Inoue et al., 2002; Sternberg and Horvitz, 1986). The 3° cells divide only once and then fuse with the surrounding hypodermal syncytium (hyp7).

Our analysis indicates that *puf-8*, *fbf-1* and *fbf-2* negatively regulate vulval induction in parallel with the known inhibitors of the EGFR/RAS/MAPK pathway. *puf-8* restricts the temporal competence of the vulval cells by promoting the fusion of the uninduced 3° cells with hyp7, while *fbf-1* and *fbf-2* control the 1° versus 2°/3° cell fate decision.

MATERIALS AND METHODS

Nematode strains and general methods

All strains were derivatives of Bristol strain N2 of *Caenorhabditis elegans* and grown under standard conditions at 20°C (Brenner, 1974) or at the temperature indicated in the table footnotes. Unless noted otherwise, the mutations used have been described previously (Riddle et al., 1997) and are listed below by their linkage group.

LGI: *lin-10(e1438), unc-13(e1091)* to *cis*-link *lin-10(e1438).* LGII: *fbf-1(ok91)* (Crittenden et al., 2002), *fbf-2(q738)* (Lamont et al., 2004), *fbf-2(q704)* (Crittenden et al., 2002), *puf-8(zh17)* (this work), *puf-8(ga145)* (this work), *puf-8(ok302)* (Subramaniam and Seydoux, 2003), *rrf-3(pk1426)* (Sijen et al., 2001), *eff-1(hy21)* (Mohler et al., 2002), *lin-7(e1413), unc-4(e120)* to *cis*-link *puf-8* alleles, *puf-8(ok302)* and the *fbf* mutations were balanced with *mIn1(mIs14 dpy-10(e128))* (Edgley and Riddle, 2001). LGIII: *unc-119(e2498), unc-119(ed4)* for *syIs90.* LGIV: *ark-1(sy247)* (Hopper et al., 2000), *dpy-20(e1282)* to cis-link *ark-1, let-60(n1046gf), let-60(n2021).* LGX: *gap-1(ga133)* (Hajnal et al., 1997), *lin-2(n105ts), lin-15(n765ts), sli-1(sy143).*

Integrated transgenic arrays (transgenes; co-transformation marker): *syIs90[egl-17::yfp + unc-119(+)]* III (Inoue et al., 2002), *swIs79[ajm-1::gfp, unc-119(+)]* IV (Mohler et al., 1998).

Extrachromosomal transgenic arrays [transgenes; co-transformation marker; pBS: Bluescript (concentration in ng/μl)] were generated by microinjection of DNA into young adult worms (Mello et al., 1991), except for the *zhEx61[puf-8::gfp; unc-119(+)]* extrachromosomal line, which was generated by microparticle bombardment using 0.1 mg of 1 μm gold beads coated with 16 μg *puf-8::gfp* and 8 μg *unc-119(+)* plasmids as described previously (Praitis et al., 2001):

zhEx173.1-3[P_{bar-1}::puf-8; sur-5::gfp; pBS (50;50;50)], zhEx175.1-3[P_{bar-1}::fbf-1; sur-5::gfp; pBS (50;50;50)], zhEx174.1-3[P_{bar-1}::fbf-2; sur-5::gfp; pBS (50;50;50)], zhEx170.1[P_{dpy-7}::puf-8; sur-5::gfp; pBS (10;20;120)], zhEx172.1-2[P_{dpy-7}::puf-8; sur-5::gfp; pBS (50;50;50)], zhEx176.1-3[P_{dpy-7}::fbf-2; sur-5::gfp; pBS (50;50;50)], zhEx220[fbf-2::gfp; lin-48::gfp (100;50)].

GFP and YFP expression was observed under fluorescent light illumination with a Leica DMRA microscope equipped with a cooled CCD camera (Hamamatsu ORCA-ER) controlled by the Openlab 3.0 software (Improvision). Animals were mounted on 3% agarose pads in M9 solution containing 15 mM NaN_3. Larvae were first inspected using Nomarski optics to identify the position of the Pn.p cells or their descendants, and GFP or YFP expression was then scored under fluorescent light illumination using the same exposure settings for a particular transgene in all different genetic backgrounds. For the PUF-8::GFP FBF-2::GFP and the EGL-17::YFP experiments, three semi-quantitative classes were made: no expression if the fluorescence was not distinguishable from the background staining, low expression if there was a weak but clearly visible signal, and high expression if the fluorescence signal was strong. The images of PUF-8::GFP and FBF-2::GFP at the L4 stages needed a correction to prevent overexposure.

The induction index of the VPCs was scored under Nomarski optics and the average number of 1° or 2° induced VPCs per animal was calculated as described previously (Dutt et al., 2004).

Laser ablation of the somatic gonad precursors Z1 and Z4 and germline precursors Z2 and Z3 were done as described by Kimble (Kimble, 1981), and induction was scored in L4 larvae.

Genetic screens and positional molecular cloning of *puf-8*

gap-1 enhancer screen to isolate *puf-8(ga145)*: young adult *gap-1(ga133)* hermaphrodites were mutagenized with 50 mM ethyl-methanesulfonate (EMS) for 4 hours at room temperature, and the F2 generation was screened for mutants displaying a multivulva (Muv) phenotype. Approximately 30,000 haploid genomes were screened (Canevascini et al., 2005).

Non-complementation screen to isolate *puf-8*(*zh17*): *gap-1(ga133)* males were mutagenized with EMS as described above, mated with *unc-4(e120) puf-8(ga145); gap-1(ga133)* hermaphrodites and the nonUnc F1 progeny was screened for Muv animals. After screening 2,000 haploid genomes one Muv non-Unc animal was identified and propagated. *ga145* was mapped with three-factor mapping between *dpy-10* and *unc-4* on LGII and further narrowed down by transformation rescue experiments using YACs and cosmids to the cosmid clone C30G12. RNAi analysis of the genes encoded by C30G12 in a *gap-1(ga133)* background identified the *puf-8* gene as candidate, and DNA sequencing of the *puf-8* coding region in the *ga145* and *zh17* alleles identified the molecular lesions.

RNA interference analysis

RNA interference analysis (RNAi) was performed by feeding animals dsRNA-producing bacteria as described previously (Kamath and Ahringer, 2003) with the following modifications. During the cloning of *puf-8*, dsRNA-producing bacteria were grown on plates containing 1 mM IPTG and 5-10 adult P0 *gap-1(ga133)* animals were put on each plate. For the *syIs90; gap-1(ga133)* strain, bacteria were induced with 6 mM IPTG, and for all other RNAi experiments, 5-15 P0 animals were put, as L1 larvae or as adults, on plates containing bacteria grown on 3 mM IPTG. Vulval induction was scored in the F1 progeny at the L4 larval stage to count the number of induced VPCs or in adults to count the percentage of Muv animals (indicated in the table footnotes). All dsRNA-producing bacteria were from the Ahringer library (Kamath and Ahringer, 2003), except for the *fem-3* RNAi bacteria, which were a gift from C. Eckmann.

Plasmids and PCR fusion constructs

For the *puf-8::gfp* translational reporter, a 3.3 kb *Sal*I genomic fragment containing a 1.3 kb upstream promoter fragment and the entire C30G12.7 open reading frame was cloned into the *Sal*I site of plasmid pPD95.75 (a gift from A. Fire). For the *fbf-2::gfp* translational reporter, a 3.73 kb *Bam*HI genomic fragment containing a 1.5 kb upstream promoter fragment and the entire *fbf-2* open reading frame was cloned into the *Bam*HI site of plasmid pPD95.75. All the *dpy-7* and *bar-1* promoter fusions were generated by the PCR fusion method (Hobert, 2002). Details on the primers used and constructions of the *gfp* reporters and promoter fusions are available on request.

RESULTS

Identification of *puf-8* as a negative regulator of vulval development

Single mutants in negative regulators of vulval induction often exhibit a wild-type vulval phenotype because these genes are mostly genetically redundant. We therefore performed a forward genetic screen in a *gap-1(ga133)* loss-of-function background to identify synthetic mutations in additional inhibitors of vulval induction (Canevascini et al., 2005). *gap-1* encodes a GTPase-activating protein that stimulates the intrinsic GTPase activity of LET-60 RAS and thus inhibits the transduction of the inductive signal (Hajnal et al., 1997). *gap-1(ga133)* single mutants exhibit an elevated activity of the EGFR/RAS/MAPK signalling pathway, yet they develop a wild-type vulva (Fig. 1B and Table 1, row 2). After screening approximately 30,000 haploid genomes, we isolated 27 mutants that displayed a synthetic multivulva (Muv) phenotype in a *gap-1(ga133)* background and defined at least four complementation groups. The *ga145* mutation found in this screen caused a 60% penetrant Muv phenotype in the *gap-1(ga133)* background, but no obvious vulval phenotype as a single mutant (Table 1, rows 3 and 5). To identify additional alleles of this complementation group, we performed a non-complementation screen (for details see Materials and methods) that yielded a new allele (*zh17*) displaying an equally penetrant synthetic Muv phenotype (Table 1, rows 4 and 6 and Fig. 1C). The corresponding gene was mapped to LGII between *dpy-10* and *unc-4* and further narrowed down by transformation rescue experiments to the cosmid C30G12. The six genes on this cosmid were tested by RNAi analysis. Feeding *gap-1(ga133)* animals with bacteria producing dsRNA derived from the C30G12.7 open reading frame caused a Muv phenotype of 80% penetrance (Table 1, row 8 and Table 2, row 7). This gene has previously been named *puf-8*, as it encodes one of the two *C. elegans* Pumilio homologues (Wickens et al., 2002). Sequencing the *puf-8* coding region revealed a stop mutation at position 485 of the ORF (CAA to TAA) before the PUF repeats in *zh17,* and a G to A (GGA to AGA) transition at position 1174, replacing glycine 317 with arginine in the fourth PUF repeat in *ga145* animals (Fig. 1A). The glycine mutated in *ga145* is conserved in PUF-9, *Drosophila* Pumilio and the vertebrate PUF proteins. This glycine is adjacent to an asparagine residue that is directly involved in binding to the target mRNA (Opperman et al., 2005). In addition to the vulval phenotype, both *puf-8* alleles we isolated showed the same partially penetrant sterile phenotype at 20°C as the *puf-8(ok302)* deletion strain (Fig. 1A) (Subramaniam and Seydoux, 2003), and the *puf-8(ok302)* deletion caused a Muv phenotype in a *gap-1(ga133)* background of similar penetrance to *zh17* or *ga145* (Table 1, row 7). Thus, *zh17* and *ga145* are strong reduction-of-function or null alleles of *puf-8*.

Genetic interaction of *puf-8* with the EGFR/RAS/MAPK pathway

We examined the genetic interaction of *puf-8(zh17)* with mutations that either reduce or increase the activity of the EGFR/RAS/MAPK signalling pathway. *puf-8(zh17)* partially suppressed the vulvaless (Vul) phenotype caused by mutations in *lin-2*, *lin-7*, *lin-10* and *let-60*, which reduce but do not inactivate the inductive signal (Table 1, rows 9-16) (Kaech et al., 1998). We also combined *puf-8(zh17)* with mutations in inhibitors of the EGFR/RAS/MAPK pathway such as *ark-1*, *sli-1* or *lin-15* that exhibit a wild-type or only a very weak Muv phenotype as single mutants (Herman and Hedgecock, 1990; Hopper et al., 2000; Jongeward et al., 1995; Yoon et al., 1995). With

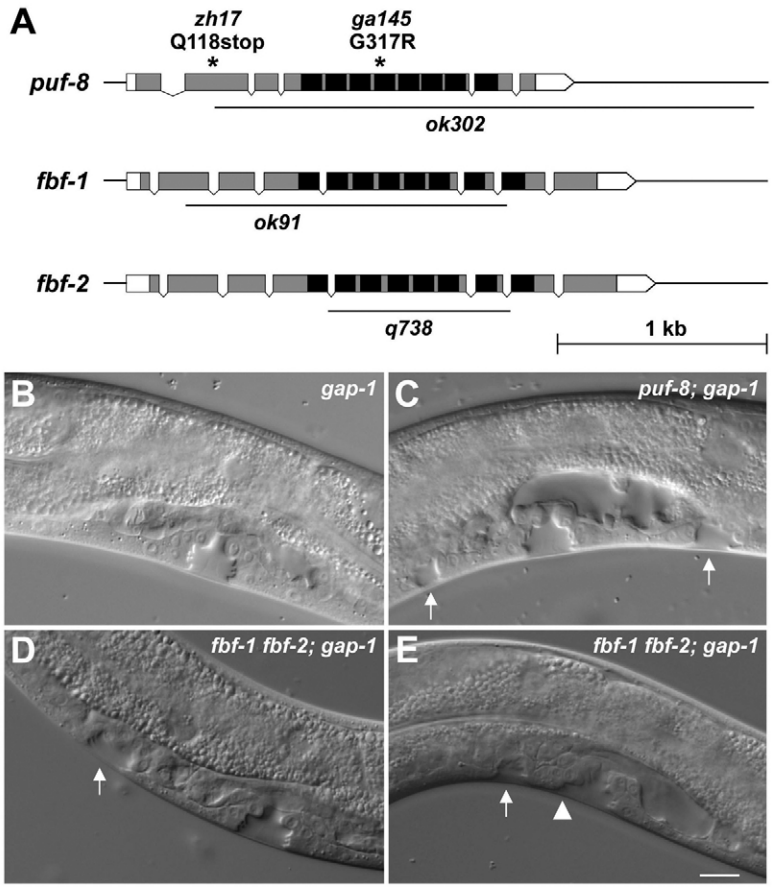

Fig. 1. PUF proteins that negatively regulate vulval development. (**A**) Intron-exon structure and alleles of *puf-8*, *fbf-1* and *fbf-2*. White boxes indicate the 5′UTRs, white boxes with arrowheads the 3′UTRs, grey boxes the coding regions and black boxes the PUF repeats. (**B-E**) Nomarski images of the vulval cells in L4 larvae of (B) *gap-1(ga133),* (C) *puf-8(zh17); gap-1(ga133),* and of (D,E) *fbf-1(ok91) fbf-2(q704); gap-1(ga133)* animals. In all panels, anterior is to the left and ventral is to the bottom. Note the ectopic induction of P4.p and P8.p (arrows in C,D,E). Arrowhead in E indicates an example of defects in the 2° cell lineage generated by P5.p resulting in the detachment of the P5.p descendants from the cuticle in a *fbf-1(ok91) fbf-2(q704); gap-1(ga133)* larva. Scale bar: 10 μm.

Table 1. *puf-8* negatively regulates vulval development

Row	Genotype*	% Muv†	% Vul‡	Induction§	*n*
1	Wild-type	0	0	3.0	many
2	*gap-1(ga133)*	0	0	3.0	30
3	*puf-8(ga145)*	0	0	–	176
4	*puf-8(zh17)*	0	0	3.0	36
5	*puf-8(ga145); gap-1(ga133)*	60	0	4.0	28
6	*puf-8(zh17); gap-1(ga133)*	59	0	3.9	46
7	*puf-8(ok302); gap-1(ga133)*	80	0	4.2	24
8	*gap-1(ga133); puf-8 RNAi*	83	0	4.2	30
9	*lin-2(n105)*¶	0	56	1.6	18
10	*puf-8(zh17); lin-2(n105)*¶	0	18	2.5	22
11	*lin-7(e1413)*	0	95	0.6	38
12	*puf-8(zh17); lin-7(e1413)*	0	58	1.7	36
13	*lin-10(e1438)***	2	83	1.3	42
14	*lin-10(e1438); puf-8(zh17)***	0	41	2.1	29
15	*let-60(n2021)*	0	44	2.6	133
16	*puf-8(zh17); let-60(n2021)*	0	16	2.8	224
17	*ark-1(sy247)*††	0	0	3.0	33
18	*puf-8(zh17); ark-1(sy247)*††	55	0	3.6	42
19	*sli-1(sy143)*	0	0	3.0	37
20	*puf-8(zh17); sli-1(sy143)*	11	0	3.1	38
21	*lin-15(n765ts)*‡‡	0	0	3.0	32
22	*puf-8(zh17); lin-15(n765ts)*‡‡	23	0	3.4	30
23	*eff-1(hy21)*	29	0	3.3	21
24	*eff-1(hy21); gap-1(ga133)*	40	0	3.4	20

*All the strains carrying the *puf-8* mutations *ga145, zh17* or *ok302* carried the cis-linked marker *unc-4(e120)*.
†% Muv indicates the fraction of animals with more than three induced VPCs.
‡% Vul indicates the fraction of animals with less than three induced VPCs.
§Induction indicates the average number of induced VPCs per animal, *puf-8(ga145)* was scored under a dissection microscope.
¶These strains were grown at 25°C.
**lin-10(e1438)* was cis-linked with *unc-13(e1091)*.
††These strains had *ark-1(sy247)* cis-linked with *dpy-20(e1282)*.
‡‡These strains were kept at 14°C before scoring.

each of these mutations, *puf-8(zh17)* caused a synthetic Muv phenotype as described above for *gap-1(ga133)* (Table 1, row 6 and rows 17-22). Thus, *puf-8* either encodes a negative regulator of the EGFR/RAS/MAPK pathway, or alternatively, *puf-8* regulates the competence of the VPCs to respond to the inductive signal.

PUF-8::GFP is expressed in vulval cells and the surrounding epidermis

To analyze the expression pattern of PUF-8, we constructed a translational *puf-8::gfp* reporter by fusing a genomic DNA fragment covering 1.3 kb of 5′ regulatory sequences up to the next gene and the entire *puf-8* coding sequence to a GFP cassette (Fig. 2A). PUF-8::GFP was expressed in various tissues including the pharyngeal muscles, the hypodermis, the ventral cord motor neurons (not shown) and the vulval cells (Fig. 2B-J and Fig. S1A in the supplementary material). Before vulval induction in L2 larvae, PUF-8::GFP was expressed in all six vulval precursor cells at equal levels (Fig. 2B,C and row with Pn.p cells in Fig. S1A in the supplementary material). After vulval induction in early L3 larvae, PUF-8::GFP was upregulated in the descendants of the 3° distal VPCs (P3.p, P4.p and P8.p), while expression faded in the 1° and 2° descendants of the proximal VPCs (P5.p, P6.p and P7.p, Fig. 2D-J, Fig. S1A in the supplementary material, rows Pn.px to Pn.pxxx). In addition, PUF-8::GFP expression was detected in the VulC sublineage of the 2° cells at the Pn.pxxx stage (inset in Fig. 2H,J and Fig. S1A in the supplementary material).

We hypothesized that the increase in PUF-8::GFP expression in the descendants of the distal 3° VPCs might occur because these cells fuse with the hyp7 hypodermis that also expresses PUF-8::GFP. To test if the upregulation of PUF-8::GFP in the descendants of the 3° VPCs is a consequence of their fusion with hyp7, we examined PUF-8::GFP expression in an *eff-1(hy21)* background, in which no cell fusions occur (Mohler et al., 2002). Since *eff-1(hy21)* animals exhibit excess vulval induction (Table 1, row 23), we additionally ablated the somatic gonad precursors Z1 and Z4 to prevent induction by the anchor cell. In most gonad-ablated *eff-1(hy21)* animals, PUF-8::GFP expression was upregulated in all VPCs and their descendants, except for the P8.p descendants (Fig. 2K,L and Fig. S1B in the supplementary material). Moreover, in *let-60 ras(gf)* animals, in which the distal VPCs frequently adopt the 1° or 2° induced cell fates, PUF-8::GFP expression often remained low in the distal VPCs and their descendants (Fig. S1C in the supplementary material) (Beitel et al., 1990). We conclude that PUF-8::GFP is upregulated in the descendants of VPCs that have adopted the uninduced 3° cell fate independently of their fusion with hyp7.

fbf-1 and *fbf-2* negatively regulate vulval development

To examine whether additional *C. elegans* PUF proteins besides PUF-8 play a role in regulating vulval development, we performed an RNA interference (RNAi) analysis by feeding *rrf-3(pk1426); gap-1(ga133)* animals with dsRNA-producing bacteria derived from the other *puf* genes (Kamath and Ahringer, 2003). The *rrf-3(pk1426)* mutation was used to increase the sensitivity for RNAi (Simmer et al., 2002). Of the six other PUF proteins that were tested, RNAi against *fbf-1* and *fbf-2* induced a penetrant Muv phenotype, whereas RNAi against *puf-9*, which is most similar to *puf-8*, did not cause a Muv phenotype (Table 2, rows 1-8). Because of the high degree of sequence similarity between the two *fbf* genes (over 90% identity at the nucleotide level), RNAi against either *fbf* gene most likely reduces both *fbf-1* and *fbf-2* expression. We therefore tested whether *fbf-1* or *fbf-2* single mutants or only the *fbf-1 fbf-2* double mutant show a Muv

Table 2. *fbf-1* and *fbf-2* redundantly regulate vulval induction

Row	Genotype	RNAi	% Muv*	*n*	Induction†	*n*
1	*gap-1(ga133)*‡	*gfp*	0	50	3.0	40
2	*gap-1(ga133)*‡	*fbf-1*	85	110	4.1	21
3	*gap-1(ga133)*‡	*fbf-2*	56	100	3.6	21
4	*gap-1(ga133)*‡	*puf-3*	0	31	–	–
5	*gap-1(ga133)*‡	*puf-5*	3	32	–	–
6	*gap-1(ga133)*‡	*puf-7*	0	41	–	–
7	*gap-1(ga133)*‡	*puf-8*	79	104	3.9	24
8	*gap-1(ga133)*‡	*puf-9*	0	40	–	–
9	*fbf-1(ok91)*	–	0	200	3.0	27
10	*fbf-1(ok91); gap-1(ga133)*	–	1	157	3.0	55
11	*fbf-2(q738)*	–	0	80	3.0	25
12	*fbf-2(q738); gap-1(ga133)*	–	0	63	3.0	24
13	*fbf-1(ok91) fbf-2(q704)*	–	28	74	3.6	26
14	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*	–	94	282	4.9	282
15	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*	*gfp*	94	154	4.8	36
16	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*	*gld-1*¶	19	168	3.3	29
17	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*	*fem-3*¶	91	80	–	–
18	*puf-8(zh17); gap-1(ga133)*§	*gfp*	76	162	3.6	27
19	*puf-8(zh17); gap-1(ga133)*§	*gld-1*¶	82	232	4.0	22
20	*puf-8(zh17); gap-1(ga133)*§	*fem-3*¶	69	108	–	–

*% Muv indicates the fraction of animals showing ectopic vulval induction under a dissection microscope.
†Induction indicates the average number of induced VPCs per animal.
‡These strains carried the *rrf-3(pk1426)* mutation, which made them more sensitive to RNAi (Simmer et al., 2002).
§These strains were cis-linked with *unc-4(e120)*.
¶RNAi against *fem-3* and *gld-1* was additionally checked for presence of the germline phenotype.

phenotype when combined with *gap-1(ga133)*. *fbf-1(ok91); gap-1(ga133)* and *fbf-2(q738); gap-1(ga133)* animals both developed a wild-type vulva, but *fbf-1(ok91) fbf-2(q704); gap-1(ga133)* triple mutants showed a strong Muv phenotype (Fig. 1D,E and Table 2 rows 9-14). Interestingly, even in a *gap-1(+)* background *fbf-1(ok91) fbf-2(q704)* double mutants were weakly Muv (Table 2, row 13). Finally, we tested for a possible redundancy among the *puf* genes by performing *puf-3*, *puf-5*, *puf-7*, *puf-8* and *puf-9* RNAi in the *puf-8(zh17)* and *fbf-1(ok91) fbf-2(q704)* backgrounds, but observed no synthetic Muv phenotypes among the other PUF genes (data not shown). Thus, besides *puf-8* the two *fbf* genes encode functionally redundant negative regulators of vulval development.

fbf-1 and *fbf-2* inhibit specification of the 1° vulval cell fate

We next determined whether PUF-8 or the FBF proteins regulate the specification of the 1° vulval cell fate using the *egl-17::yfp* reporter as a marker for the 1° cell fate (Inoue et al., 2002). *egl-17* encodes a fibroblast growth factor (FGF) homolog that is normally expressed in P6.p and its descendants from the time of induction until the Pn.pxx stage (Fig. 3A,B) (Burdine et al., 1998; Inoue et al., 2002). In L4 larvae at the Pn.pxxx stage, EGL-17::YFP expression disappears in the 1° cells and appears in the VulC and VulD cells of the 2° lineage (Fig. 3C,D) (Burdine et al., 1998; Inoue et al., 2002). Both the early (1° fate-specific) and late (2° subfate-specific) EGL-17::YFP expression depend on inductive signalling (Burdine et al., 1998).

We observed a slight expansion of the early, 1°-specific EGL-17::YFP expression in *gap-1(ga133)* animals causing the descendants of P5.p and P7.p and occasionally also of P8.p to express EGL-17::YFP (Fig. 3E,F), although, *gap-1(ga133)* mutants exhibit normal vulval induction and correct 2° cell fate specification in P5.p and P7.p (Fig. 3G,H).

Surprisingly, in *puf-8(zh17); gap-1(ga133)* double mutants or *puf-8* RNAi-treated *gap-1(ga133)* animals we observed no increase – and sometimes even a reduction – in the 1°-specific EGL-17::YFP expression in the proximal VPC descendants compared to *gap-1(ga133)* single mutants (Fig. 3J,K). Moreover, the descendants of P5.p and P7.p adopted a proper 2° cell fate, as they generated seven descendants that exhibited a normal morphology and a normal EGL-17::YFP expression pattern in the VulC and VulD subfates (compare Fig. 3G with L). In the distal cells (the P3.p, P4.p and P8.p descendants) we observed only a very mild increase in the early, 1°-specific or the late, 2°-specific EGL-17::YFP expression that did not match the frequency of ectopic vulval induction observed in this background (Fig. 3J-M). However, it should be noted that also in other mutant backgrounds such as *let-60(n1046gf)* the frequency and strength of ectopic EGL-17::YFP expression does not mirror the level of ectopic vulval induction (Burdine et al., 1998).

In contrast to *puf-8* mutants, *fbf-1(ok91) fbf-2(q704); gap-1(ga133)* triple mutants displayed a clear upregulation of the early, 1°-specific EGL-17::YFP expression in all VPCs and their descendants (Fig. 3N,O). Especially in the descendants of P5.p and P7.p, the 1°-specific EGL-17::YFP expression was much stronger than in *gap-1(ga133)* single mutants. In addition to the late EGL-17::YFP expression in the ectopically induced pseudovulvae, *fbf-1(ok91) fbf-2(q704); gap-1(ga133)* mutants also exhibited an expansion of the 2°-specific EGL-17::YFP expression to 2° subfates that normally do not express the marker (e.g. VulA and VulB in Fig. 3P,Q). This aberrant EGL-17::YFP expression pattern within the 2° lineage was accompanied by morphological changes of the P5.p and P7.p descendants that are characteristic of a partial transformation towards the 1° fate (note the detachment of the P5.p descendants in Fig. 1E and Fig. 3P) (Berset et al., 2005). Such defects in the 2° cell lineage were only rarely observed in *puf-8(zh17); gap-1(ga133)* animals (Fig. 3M).

Thus, PUF-8 and the FBF proteins perform clearly distinct roles during vulval cell fate specification. FBF-1 and FBF-2 inhibit 1° fate-specific gene expression and are required for proper 2° fate execution in P5.p and P7.p, whereas PUF-8 does not regulate 1°-specific gene expression and appears to regulate vulval induction through a different mechanism.

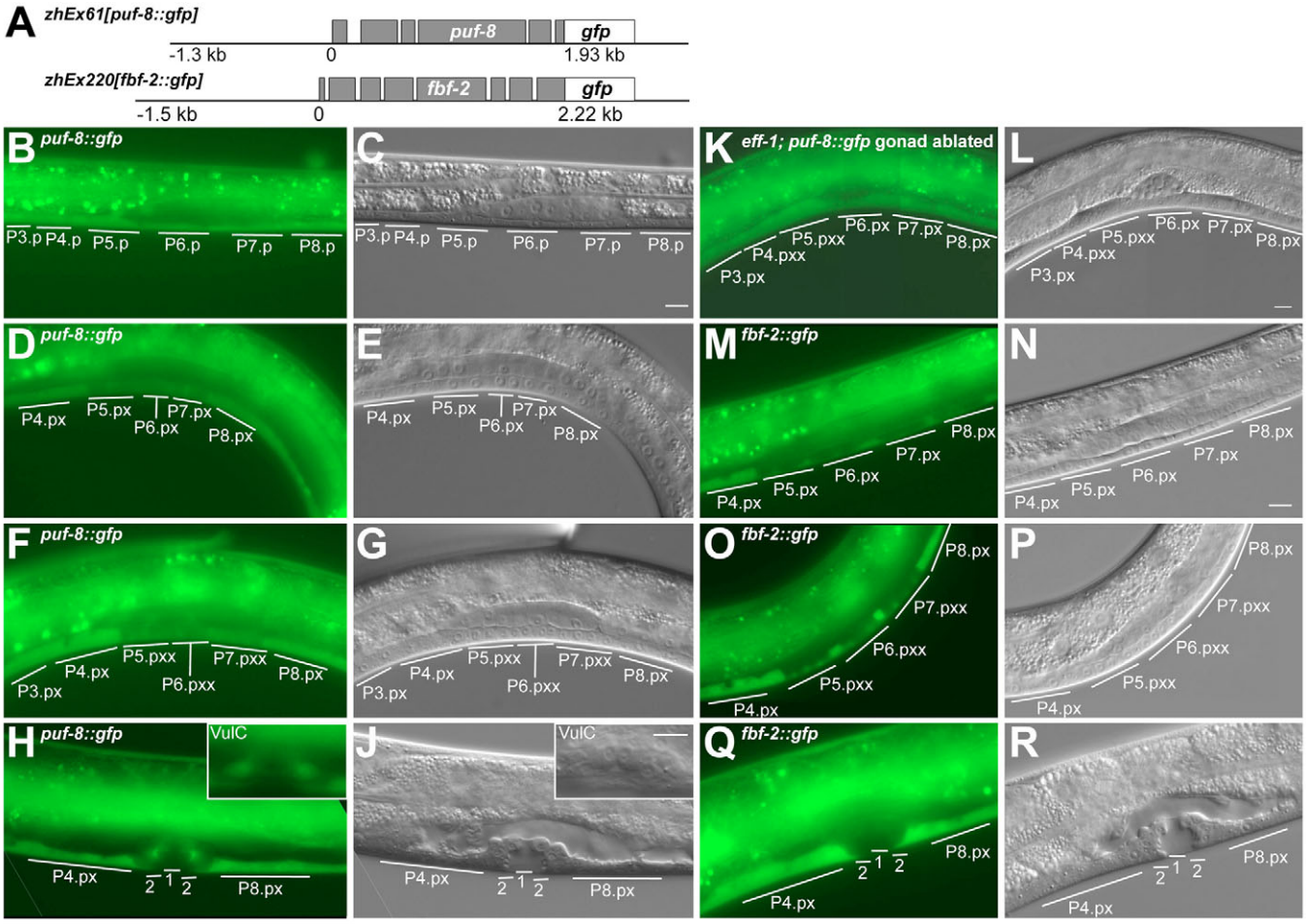

Fig. 2. PUF-8::GFP and FBF-2::GFP expression during vulval development. (**A**) Structure of the translational *puf-8::gfp* and *fbf-2::gfp* reporters. (**B**,**D**,**F**,**H**) Time-course analysis of PUF-8::GFP expression in the vulval cells from the L2 until the L4 stage with (**C**,**E**,**G**,**J**) the corresponding Nomarski images. For a semi-quantitative analysis of the expression patterns, see Fig. S1 in the supplementary material. (**K**,**L**) PUF-8::GFP expression in gonad-ablated *eff-1(hy21)* animals, and the corresponding Nomarski image. Note that despite the extra round of cell divisions in P4.p and P5.p descendants of gonad-ablated *eff-1* mutants no vulval differentiation was observed. (**M-R**) FBF-2::GFP expression, and the corresponding Nomarski images, from the early L3 until the L4 stage. In all panels, anterior is to the left and ventral is to the bottom. Scale bars: in C,L,N and in the inset of J, 10 μm.

gld-1 is an FBF target during vulval development

Since PUF proteins function as translational repressors, the Muv phenotype caused by *puf-8* and *fbf-1* and *fbf-2* mutations is probably caused by enhanced translation of their target mRNAs. Thus, RNAi against a target mRNA that encodes a positive regulator of vulval development should suppress the Muv phenotype of *puf-8(zh17); gap-1(ga133)* and/or *fbf-1(ok91) fbf-2(q704); gap-1(ga133)* mutants. In the germline, *gld-1* and *fem-3* are direct FBF targets that function in mitosis/meiosis and sperm/oocyte decision, respectively (Crittenden et al., 2002; Zhang et al., 1997). No targets of PUF-8 have so far been found. RNAi against *gld-1* suppressed the *fbf-1(ok91) fbf-2(q704); gap-1(ga133)* but not the *puf-8(zh17); gap-1(ga133)* Muv phenotype, whereas RNAi against *fem-3* had no effect on the Muv phenotype of either strain (Table 2, rows 15-20). Thus, the FBF proteins negatively regulate vulval induction by repressing, among others, *gld-1* expression. PUF-8, however, appears to act through a distinct set of yet unknown target genes.

puf-8 controls the timing of 3° cell fusions

The upregulation of PUF-8::GFP in the distal 3° vulval cells raises the possibility that PUF-8 might regulate the competence of the distal vulval cells to respond to the inductive signal. Since the 3° cell fate is only sealed after the Pn.px cells have fused with hyp7 (Wang and Sternberg, 1999), the *puf-8(lf)* mutations might allow distal vulval cells to stay unfused and hence receive the inductive signal over a longer time period, which in combination with a second mutation in a negative regulator of the EGFR/RAS/MAPK pathway would result in excess vulval induction.

To observe the timing of vulval cell fusions, we used the *ajm-1::gfp* reporter, which labels the adherens junctions of the VPCs and their descendants as long as they have not fused with hyp7 (Mohler et al., 1998). In wild-type animals, the uninduced distal VPCs divide once and then rapidly fuse with hyp7. Therefore, in the majority of wild-type larvae we analyzed at the Pn.px stage, the descendants of P3.p, P4.p and P8.p had already fused with hyp7 as demonstrated by the loss of AJM-1::GFP staining (Fig. 4A-C). In *puf-8(zh17)* mutants, however, the fusion of P4.p and P8.p descendants was significantly delayed, as in approximately 50% of the animals AJM-1::GFP staining was still present in P4.px and P8.px (Fig. 4D-F). Note that despite the delay in cell fusion *puf-8(zh17)* single mutants never showed ectopic induction of the distal VPCs (Table 1, row 4). In *fbf-1(ok91) fbf-2(q704)* mutants, P4.p and P8.p descendants were unfused in approximately 20% of the cases (Fig. 4G-J). Since 28% of *fbf-1(ok91) fbf-2(q704)* double mutants exhibit a Muv phenotype in a

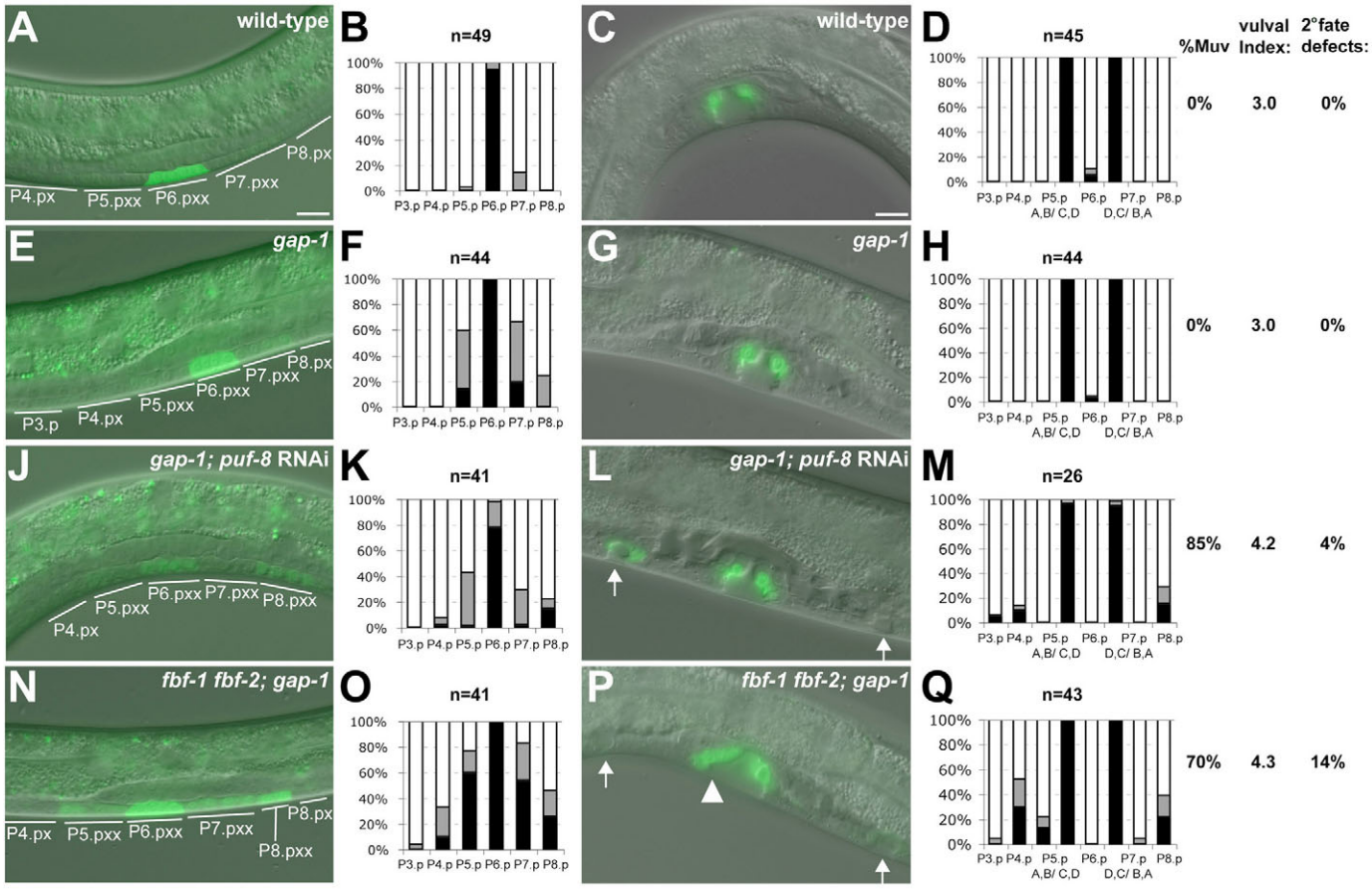

Fig. 3. *fbf-1 and fbf-2* inhibit 1° cell fate specification. Analysis of EGL-17::YFP expression in mid-L3 larvae at the Pn.px or Pn.pxx stage (left side) and in L4 larvae at the Pn.pxxx stage (right side). (**A-D**) Wild-type, (**E-H**) *gap-1(ga133)*, (**J-M**) *gap-1(ga133); puf-8* RNAi and (**N-Q**) *fbf-1(ok91) fbf-2(q704); gap-1(ga133)* larvae. In all panels, anterior is to the left and ventral is to the bottom. In the graphs, white indicates no EGL-17::YFP expression, grey low expression and black high expression. The arrows in L and P indicate ectopic induction of distal vulval cells; the arrowhead in P indicates an example with expanded EGL-17::YFP expression in VulA and VulB, and the resulting defect in the 2° fate execution. Scale bars: in A,C, 10 μm.

gap-1(+) background (Table 2, row 13), the distal cells were probably unfused because they had adopted a 1° or 2° vulval cell fate in these animals. PUF-8 therefore inhibits vulval development by promoting the fusion of the 3° cells with the surrounding hyp7 hypodermis.

Similar to *puf-8(lf)*, a mutation in the effector of cell fusion *eff-1* that blocks all cell fusions causes a weak Muv phenotype that was further enhanced by the *gap-1(ga133)* background (Table 1, rows 23 and 24) (Mohler et al., 2002). However, it should be noted that *eff-1(hy21); gap-1(ga133)* double mutants display a weaker Muv phenotype than *puf-8(zh17); gap-1(ga133)* animals (Table 1, compare rows 6 and 24), indicating that *puf-8* is likely to have additional functions besides controlling the timing of 3° cell fusions.

fbf-1 and *fbf-2* act in the germline and in the soma

Thompson et al. (Thompson et al., 2006) recently reported that feminized *fbf-1 fbf-2* mutants (i.e. *fbf-1 fbf-2; fog-1* or *fbf-1 fbf-2; fog-3* triple mutants) display a strong Muv phenotype that is completely suppressed by ablation of the germ cell precursors Z2 and Z3. This observation indicated that *fbf-1* and *fbf-2* inhibit vulval induction in a non cell-autonomous manner, probably by repressing the translation of a positive regulator of vulval development in the germ cells. We performed similar gonad precursor cell ablations, but used the *fbf-1(ok91) fbf-2(q704); gap-1(ga133)* background. Ablation of Z2 and Z3 resulted in a partial suppression of the Muv phenotype (Table 3, row 3 and Fig. S2B in the supplementary material), and ablation of the somatic gonad precursors Z1 and Z4, which give raise to the AC, resulted in a suppression of the Muv phenotype to nearly wild-type levels of vulval induction (Table 3, row 4 and Fig. S2C in the supplementary material). Even after ablation of all four gonad precursor cells (Z1 to Z4), we observed gonad-independent vulval induction in 19% of the animals (Table 3, row 5 and Fig. S2D in the supplementary material). Since the *gap-1(ga133)* mutation alone does not cause any gonad-independent vulval induction (Hajnal et al., 1997), *fbf-1* and *fbf-2* inhibit vulval differentiation not only by repressing specific target genes in the germ cells but also in somatic cells outside of the gonad. Supporting this hypothesis, a translational FBF-2::GFP reporter showed an expression pattern similar to the PUF-8::GFP pattern described above. Expression of FBF-2::GFP was first observed at the Pn.px stage in the 3° descendants of the distal VPCs, and it persisted throughout the L4 stage (Fig. 2A,M-R and Fig. S1D in the supplementary material).

puf-8, fbf-1 and *fbf-2* act in the vulval cells

We next sought to identify the somatic tissue in which *puf-8* and *fbf-1* and *fbf-2* act. Since *puf-8::gfp* and *fbf-2::gfp* are both expressed in the vulval cells as well as in the hyp7 hypodermis, we tested whether *puf-8*, *fbf-1* and *fbf-2* act cell-autonomously in the VPCs and their descendants or non cell-autonomously in hyp7. To distinguish between these two possibilities, we expressed *puf-8* and

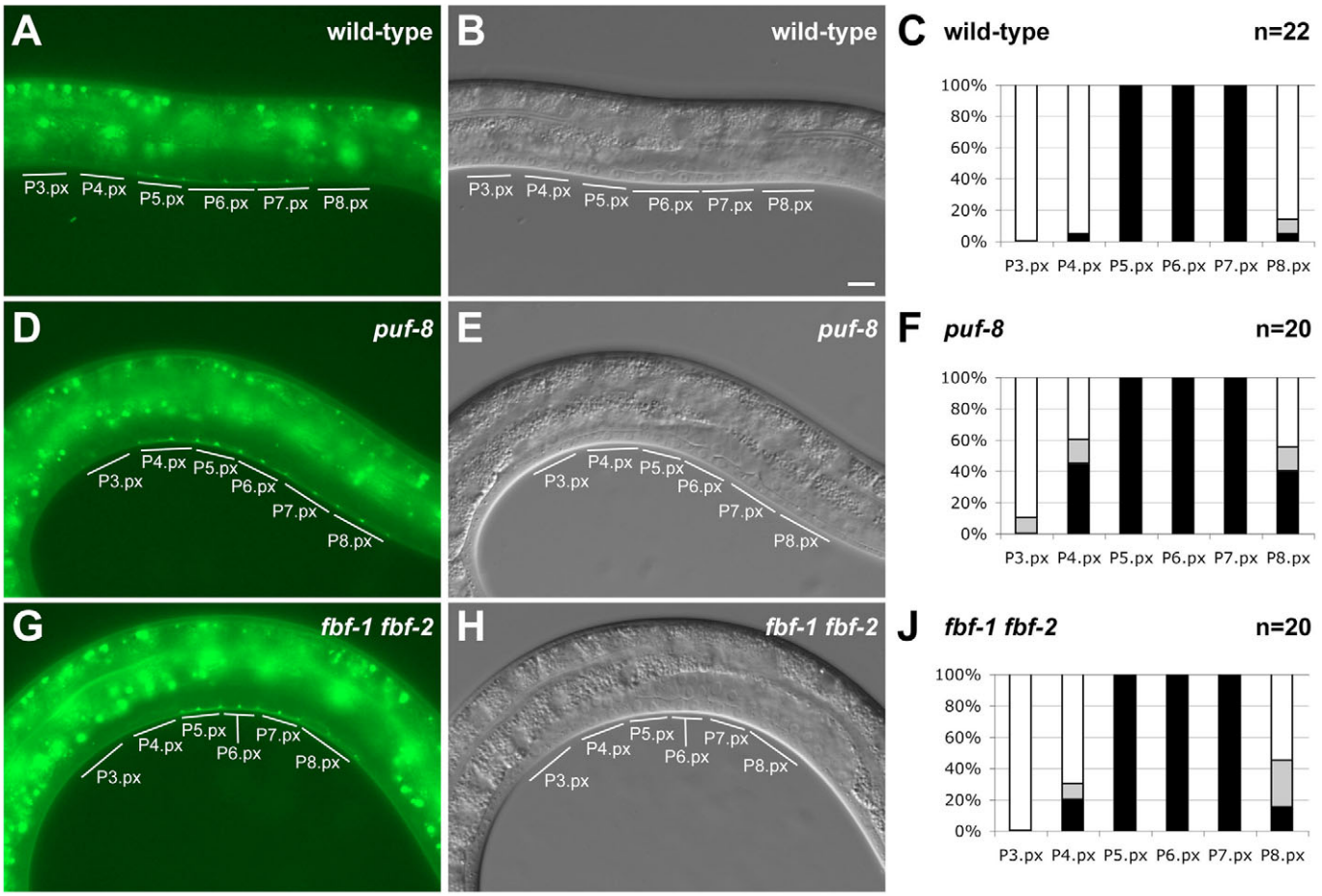

Fig. 4. *puf-8* regulates the fusion of the distal vulval cells. Vulval cell fusion was analyzed at the Pn.px stage using AJM-1::GFP as a cell junction marker for unfused cells. (**A-C**) Wild-type, (**D-F**) *puf-8(zh17)* single mutants and (**G-J**) *fbf-1(ok91) fbf-2(q704)* double mutants. In all panels, anterior is to the left and ventral is to the bottom. In the graphs, white represents fused Pn.px cells, grey indicates fusing Pn.px cells that have started to dissolve their junctions as can be seen for the P8.px cells in G, and black indicates unfused cells with intact AJM-1::GFP-positive junctions. Note that the fraction of unfused cells in *fbf-1(ok91) fbf-2(q704)* double mutants matches the frequency of ectopically induced distal cells that give rise to the 28% penetrant Muv phenotype (see Table 2, row 13). Scale bar in B: 10 μm.

fbf-2 under the control of the hypodermal *dpy-7* promoter (e.g. $P_{dpy\text{-}7}$*::puf-8*) (Gilleard et al., 1997), and each of the three genes under control of a 3.1 kb *bar-1* promoter fragment that drives expression in the vulval cells, the gonadal sheath cells and in the adult seam cells (e.g. $P_{bar\text{-}1}$*::puf-8*) (Natarajan et al., 2004). Neither the sheath cells nor the seam cells are in contact with the vulval cells, making it very unlikely that expression of a gene in these tissues could affect vulval induction. None of the three $P_{dpy\text{-}7}$*::puf-8* transgenes tested caused a significant rescue of *puf-8(ok302); gap-1(ga133)* Muv phenotype, but two out of three $P_{bar\text{-}1}$*::puf-8* lines exhibited partial rescue, and the third line showed a weak reduction of the Muv phenotype (Table 4, rows 5-11). It should be noted that even injection of a cosmid spanning the entire *puf-8* locus never gave complete rescue of the Muv phenotype (Table 4, rows 1-4). Moreover, co-injection of $P_{bar\text{-}1}$*::puf-8* with $P_{dpy\text{-}7}$*::puf-8* did not cause a stronger rescue than injection of $P_{bar\text{-}1}$*::puf-8* alone (data not shown).

Similarly, all but one of the $P_{bar\text{-}1}$*::fbf-1* and $P_{bar\text{-}1}$*::fbf-2* transgenes reduced the penetrance of the *fbf-1(ok91) fbf-2(q704); gap-1(ga133)* Muv phenotype from 90% down to 55-60%, and only one of the three $P_{dpy\text{-}7}$*::fbf-2* transgenes had a slightly significant effect (Table 4, rows 12-21). The incomplete rescue with the different constructs is consistent with the model that *fbf-1, fbf-2* as well as *puf-8* have an additional focus in the germline, since the multicopy extrachromosomal arrays we used for these experiments are normally silenced in the germ cells. Thus, *puf-8*, *fbf-1* and *fbf-2* negatively regulate vulval development at least partly in the VPCs or their descendants.

DISCUSSION

PUF proteins control somatic development

Translational repressors of the Pumilio/FBF (PUF) family regulate various aspects of germ cell development in *C. elegans* by controlling the translation of maternally provided mRNAs (Crittenden et al., 2002; Zhang et al., 1997). Here, we show that three of the eleven *C. elegans* PUF genes also function in the soma to control cell fate specifications during larval development. In particular, we have found that PUF-8, FBF-1 and FBF-2 negatively regulate vulval development in the hermaphrodite. Like most previously identified inhibitors of vulval development, single mutants in one of these three *puf* genes do not change the normal pattern of vulval cell fates. However, when combined with another mutation in an inhibitor of the inductive EGFR/RAS/MAPK pathway, *puf-8* or *fbf* mutants exhibit a hyperinduced multivulva phenotype. Genetic epistasis analysis indicates that *fbf-1* and *fbf-2* perform a redundant function to inhibit 1° vulval fate specification,

Table 3. *fbf-1* and *fbf-2* act in the soma and the germline

Row	Genotype	Ablation	% Muv*	% Vul†	Induction‡	*n*
1	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*	Unablated	84	0	4.1	48
2	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*	Mock ablated	74	0	4.0	31
3	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*	Z2/Z3 (germ line)	27	0	3.3	22
4	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*	Z1/Z4 (somatic gonad)	8	8	3.0	12
5	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*	Z1-Z4 (somatic gonad + germline)	0	81	0.6	21

*% Muv indicates the fraction of animals with more than three induced VPCs.
†% Vul indicates the fraction of animals with less than three induced VPCs.
‡Induction indicates the average number of induced VPCs per animal.

whereas *puf-8* plays a distinct role in regulating the temporal competence of the vulval cells to respond to the inductive and lateral signals.

PUF-8 regulates the temporal competence of the vulval cells

Loss-of-function mutations in *puf-8* partially suppress the Vul phenotype caused by mutations that reduce but do not inactivate the EGFR/RAS/MAPK signalling pathway. Although this observation does not prove a direct involvement of PUF-8 in regulating the inductive EGFR/RAS/MAPK signalling pathway, it indicates that in the absence of PUF-8 lower levels of inductive signal are sufficient to induce vulval differentiation. A PUF-8::GFP reporter transgene is initially expressed in all VPCs at equal levels, but after vulval induction PUF-8::GFP expression increases in the descendants of the distal VPCs (P3.p, P4.p and P8.p) that have adopted the 3° fate. This expression pattern correlates well with the observed delay in the fusion of the distal 3° cells with the hyp7 hypodermis in *puf-8* mutants. All vulval cells are competent to respond to the inductive AC and lateral Notch signals until they fuse with hyp7 (Wang and Sternberg, 1999). Even after the first round of vulval cell divisions, a single pulse of MAPK activity can reprogram a 2° or 3° cell to adopt the 1° cell fate (Berset et al., 2005). It thus appears that by promoting the fusion of the 3° cells with hyp7, PUF-8 limits the time period during which the vulval cells can receive and integrate the vulval patterning signals. In the absence of PUF-8, the vulval cells can receive the inductive signal over a longer time period, which may result in the accumulation of higher levels of activated MAPK in the distal vulval cells. When combined with a mutation in a direct inhibitor of the EGFR/RAS/MAPK pathway such as *gap-1*, this results in the ectopic vulval differentiation and a Muv phenotype. Supporting this idea, a mutation in the effector of cell fusion *eff-1*, which blocks all cell fusions, caused a weak Muv phenotype (Mohler et al., 2002). However, *puf-8* mutants exhibit more ectopic vulval induction in the *gap-1* background than *eff-1* mutants, which points to additional functions of PUF-8 besides controlling the timing of cell fusions.

The distal VPC descendants fuse with hyp7 shortly after they have been born, suggesting that they exit from the cell cycle as they lose their competence (Wang and Sternberg, 1999). The proximal vulval cells, on the other hand, go on to divide two more times before undergoing terminal differentiation and forming a functional vulva. It is therefore possible that PUF-8 ensures that the distal vulval cells exit from the cell cycle immediately after they have been generated and then fuse with hyp7. A somewhat similar function has been proposed for the *Drosophila* PUF-8 orthologue Pumilio, which blocks the cell cycle progression of the migrating pole cells during embryogenesis by repressing *cyclin B*

Table 4. *puf-8*, *fbf-1* and *fbf-2* act in part in the vulval cells

Row	Genotype	Transgene	% Muv*	Induction†	χ^2-test‡	*n*
1	*puf-8(ga145); gap-1(ga133)*§	–	85±3	–		477
2	*puf-8(ga145); gap-1(ga133)*§	Cosmid C30G12 line 1	57±10	–	x	104
3	*puf-8(ga145); gap-1(ga133)*§	Cosmid C30G12 line 2	24±8	–	x	116
4	*puf-8(ga145); gap-1(ga133)*§	Cosmid C30G12 line 3	35±9	–	x	121
5	*puf-8(ok302); gap-1(ga133)*§	–	68±6	4.0		260
6	*puf-8(ok302); gap-1(ga133)*§	*zhEx173.1 [$P_{bar\text{-}1}$::puf-8]*	30±13	3.3	x	46
7	*puf-8(ok302); gap-1(ga133)*§	*zhEx173.2 [$P_{bar\text{-}1}$::puf-8]*	38±19	3.5	y	26
8	*puf-8(ok302); gap-1(ga133)*§	*zhEx173.3 [$P_{bar\text{-}1}$::puf-8]*	52±20	3.7		23
9	*puf-8(ok302); gap-1(ga133)*§	*zhEx170.1 [$P_{dpy\text{-}7}$::puf-8]*	71±18	4.2		24
10	*puf-8(ok302); gap-1(ga133)*§	*zhEx172.1 [$P_{dpy\text{-}7}$::puf-8]*	77±12	4.0		51
11	*puf-8(ok302); gap-1(ga133)*§	*zhEx172.2 [$P_{dpy\text{-}7}$::puf-8]*	63±18	3.9		27
12	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*¶	–	96±2	4.9		441
13	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*¶	*zhEx175.1 [P_{bar}-1::fbf-1]*	57±18	3.9	x	30
14	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*¶	*zhEx175.2 [$P_{bar\text{-}1}$::fbf-1]*	58±20	3.8	x	24
15	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*¶	*zhEx175.3 [$P_{bar\text{-}1}$::fbf-1]*	73±16	4.1	x	30
16	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*¶	*zhEx174.1 [$P_{bar\text{-}1}$::fbf-2]*	60±21	3.8		20
17	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*¶	*zhEx174.2 [$P_{bar\text{-}1}$::fbf-2]*	59±19	3.8	x	27
18	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*¶	*zhEx174.3 [$P_{bar\text{-}1}$::fbf-2]*	52±18	3.6	x	29
19	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*¶	*zhEx176.1 [$P_{dpy\text{-}7}$::fbf-2]*	81±14	4.5		32
20	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*¶	*zhEx176.2 [$P_{dpy\text{-}7}$::fbf-2]*	83±12	4.3	y	35
21	*fbf-1(ok91) fbf-2(q704); gap-1(ga133)*¶	*zhEx176.3 [$P_{dpy\text{-}7}$::fbf-2]*	94±8	4.7		35

Rows 1, 5 and 12 show the average of animals without the transgene that were counted for each genotype in parallel.
*% Muv indicates the fraction of animals with more than three induced VPCs, and the 95% confidence intervals are indicated.
†Induction indicates the average number of induced VPCs per animal.
‡For each line the χ^2 test was performed comparing the animals with and without the array from the same plate. x indicates a *P* value <0.01 and y indicates a *P* value <0.05.
§*puf-8(ga145)* and *puf-8(ok302)* were cis-linked with *unc-4(e120)*.
¶These strains were maintained balanced with *mln1* and their homozygous *fbf-1 fbf-2* double mutant F1 progeny was scored.

translation to prevent their premature differentiation (Asaoka-Taguchi et al., 1999). One could, for example, imagine that the cell cycle state of the vulval cells and the hyp7 hypodermis needs to be coordinated to allow the fusion between these two different cell types to occur at the right time.

FBF-1 and FBF-2 inhibit 1° cell fate specification

In contrast to PUF-8, the FBF proteins do not regulate the timing of vulval cell fusions, but they are more directly involved in repressing 1° vulval fate specification. In *fbf-1 fbf-2* double mutants, the expression of the 1° fate marker EGL-17::YFP is upregulated in the ectopically induced distal VPCs as well as in the proximal VPCs, P5.p and P7.p, which normally adopt the 2° cell fate. *puf-8* mutants, on the other hand, only rarely exhibit ectopic expression of the 1° fate marker. This *fbf-1 fbf-2* phenotype is reminiscent of the phenotype caused by mutations that compromise the LIN-12 Notch-mediated lateral inhibition of the 1° cell fate (Yoo et al., 2004). For example, in *ark-1* or *lip-1* mutants, P5.p and P7.p frequently express 1° cell fate marker genes. In combination with a second mutation in an inhibitory gene, *ark-1* or *lip-1* mutants show similar cell fate transformations as observed in *fbf-1 fbf-2; gap-1* animals (Berset et al., 2001; Hopper et al., 2000). Whereas ARK-1 and LIP-1 directly regulate EGFR and MAPK activity, respectively, *fbf-1* and *fbf-2* probably inhibit vulval induction indirectly by repressing the translation of specific target genes that activate the EGFR/RAS/MAPK pathway.

Ablation and rescue experiments indicated that *fbf-1* and *fbf-2* act in the vulval cells and in the germline in two distinct pathways that may involve different target genes. One established target of FBF-1 and FBF-2 in the germline is *gld-1*, which encodes a translational repressor that is required for germ cells to progress through meiosis (Crittenden et al., 2002). Another possible FBF target proposed by Thompson et al. (Thompson et al., 2006) is *lin-3 egf*, which encodes the inductive signal that is normally produced by the AC and repressed in the germ cells until oocyte maturation. In feminized *fbf-1 fbf-2* mutants, *lin-3 egf* might be de-repressed in the meiotic germ cells, leading to excess vulval induction from the oogenic germ cells. Inactivation of *gld-1* might prevent the overproduction of *lin-3 egf* because the germ cells do not enter meiosis (Thompson et al., 2006).

In the soma, *fbf-1* and *fbf-2* probably repress a different set of target genes, since we could not observe any consistent *gld-1* expression in the vulval cells, and Pn.p cell-specific RNAi against *lin-3* (Dutt et al., 2004) did not suppress the *fbf-1 fbf-2; gap-1* Muv phenotype (data not shown). The specific targets of FBF-1 and FBF-2 in the soma therefore remain to be identified.

PUF proteins are conserved from yeast to humans, suggesting that they control cell fate determination in a similar way in higher organisms (Wickens et al., 2002). It will therefore be necessary to define the exact interplay between the PUF family of translational regulators and the ubiquitous RTK/RAS/MAPK signalling cascade. Translational repressors of the PUF family may turn out to play a similar role to that of the microRNAs, in fine-tuning signalling pathways during animal development (Giraldez et al., 2005; Harfe et al., 2005).

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/17/3461/DC1

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