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***C. elegans* BED domain transcription factor BED-3 controls lineage-specific cell proliferation during organogenesis**

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Abstract

The control of cell division is critical to organogenesis, but how this control is achieved is not fully understood. We found that mutations in *bed-3*, encoding a BED Zn-finger domain transcription factor, confer a phenotype where a specific set of cell divisions during vulval organogenesis is lost. Unlike general cell cycle regulators in *C. elegans*, the function of *bed-3* is restricted to specific lineages. Transcriptional reporters suggest that *bed-3* is expressed in a limited number of cell types including vulval cells whose divisions are affected in *bed-3* mutants. A *bed-3* mutation also affects the expression pattern of the *cdh-3* cadherin gene in the vulva. The phenotype of *bed-3* mutants is similar to the phenotype caused by mutations in *cog-1* (Nkx6), a component of a gene regulatory network controlling cell type specific gene expression in the vulval lineage. These results suggest that *bed-3* is a key component linking the gene regulatory network controlling cell-type specification to control of cell division during vulval organogenesis.

Keywords

C. elegans; organogenesis; cell cycle regulation; cell division; cell proliferation; BED domain; zinc finger; transcription; gene regulatory network (GRN)

Introduction

The control of cell division is a critical part of organogenesis, and determines the size and the structure of the organ produced. This is especially apparent in *C. elegans*, where the pattern of cell division (the cell lineage) during development is nearly invariant from one animal to another, causing individual *C. elegans* worms to be identical at the cellular level (Sulston and Horvitz, 1977). Despite its importance, developmental regulation of cell division, and how it is tied to cell fate specification, is not fully understood.

To understand cell division control, we are investigating *C. elegans* vulval development, a well-studied model of organogenesis (reviewed in; Sternberg, 2006). During the initial phase of vulval development (third larval stage; L3), EGF, Wnt and Notch signaling induce three

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precursor cells (called P5.p, P6.p and P7.p, also vulval precursor cells; VPC) to adopt vulval fates. In the fourth larval (L4) stage, these three cells undergo three rounds of division in a stereotyped pattern to produce the specific number and arrangement of cells needed to form the adult vulva (Sulston and Horvitz, 1977) (Figure 1). The first two rounds of division occur along a longitudinal (anterior/posterior; a/p) axis and produce a row of twelve Pn.p granddaughter cells (Pn.pxx). In contrast, the third (final) round of division occurs in sublineage-specific orientations (Figure 1): The outermost four Pn.p granddaughters divide along a longitudinal (a/p) axis, whereas two Pn.p granddaughters, P5.ppp and P7.paa do not undergo the third round of division (see Figure 1 legend for cell nomenclature). The remaining six Pn.p granddaughters divide along a transverse (left/right; l/r) axis. Whereas the mechanism by which Pn.p cells are induced to adopt vulval fates is well understood, how division of each Pn.p granddaughter is specified is not known.

Examination of cell morphology and gene expression reveals that vulval lineages produce seven terminally differentiated cell types, vulA, vulB1, vulB2, vulC, vulD, vulE and vulF (Inoue et al., 2002; Sharma-Kishore et al., 1999). With the exception of the vulB1/B2 case, the terminal division leads to cells with identical fates, suggesting that cell-type specification could operate at the level of Pn.p granddaughters (Pn.pxx cells; Figure 1). A set of genes, including *lin-11* (Freyd et al., 1990; Gupta et al., 2003), *cog-1* (Palmer et al., 2002), *lin-29* (Inoue et al., 2002; Inoue et al., 2005; Ririe et al., 2008), *egl-38* (Chang et al., 1999; Rajakumar and Chamberlin, 2006) and *nhr-67* (Fernandes and Sternberg, 2007) establish the cell-type specific pattern of gene expression in the seven vulval cell types. These genes encode sequence-specific transcription factors and are mutually interacting, suggesting that they form a gene regulatory network (GRN; (Davidson and Levine, 2008) that specifies the cell type through regulation of cell type specific gene expression (Inoue et al., 2005; Ririe et al., 2008).

Because division of each Pn.pxx cell (presence/absence of division and orientation) correlates with the cell type produced by the Pn.pxx cell, it is reasonable to hypothesize that division and cell fate are controlled by a single mechanism. Of genes in the vulval gene regulatory network, mutations in *cog-1* and *lin-11* alter the cell division pattern (Freyd et al., 1990; Palmer et al., 2002). In contrast, mutations in *lin-29*, *egl-38* and *nhr-67* do not visibly affect cell division. General regulators of cell cycle, such as *cye-1* (cyclin E) (Fay and Han, 2000), *cul-1* (cullin) (Kipreos et al., 1996), *cdc-14* (Cdc14) (Saito et al., 2004) and *cki-1* (cyclin dependent kinase inhibitor) (Hong et al., 1998) regulate cell division in multiple cell lineages, including the vulval lineage. How these genes contribute to the specification of cell division pattern, however, is unclear.

Here we describe *bed-3*, a gene required for the terminal division of Pn.p lineages. *bed-3* encodes a protein containing a single BED Zinc-finger domain (Aravind, 2000), a sequence-specific DNA-binding domain (Hirose et al., 1993; Hirose et al., 1996; Zhao et al., 1995). Analyses of *bed-3* mutant phenotypes and expression pattern suggest that *bed-3* is not required for all cell divisions in *C. elegans*. Instead, *bed-3* mutations are similar to, and interact with mutations in *cog-1*, a component of the vulval gene regulatory network, indicating that this network controls both gene expression and the terminal divisions in vulval lineages.

Materials and Methods

Worm keeping and informatics

C. elegans was cultured and manipulated as described (Brenner, 1974; Mello et al., 1991). All strains are in the N2 Bristol background unless noted otherwise. Mutations and markers used in this study include: LGII *cog-1*(*sy275*), *cog-1*(*sy607*); LGIII *unc-119*(*ed4*), *cul-1*(*e1756*), *cye-1*(*eh10*); LGIV *unc-24*(*e138*), *egl-20*(*n578*), *dpy-20*(*e1282*), *egl-38*(*sy294*), *egl-38*

(*s1775*); LGV *kuIs29[cog-2::gfp]*, *syIs51[cdh-3::cfp]*; LGX *syIs55[ceh-2::yfp]*, *syIs59[egl-17::cfp]*; Translocations *nT1[qIs51](LGIV/LGV)*, *hT2[qIs48](LGI/LGIII)*.

All references to *C. elegans* genome resources are for the WormBase referential data freeze WS160. Accession numbers (RefSeq) of homologs discussed here are; hDREF (ZBED1), NP_004720; ZBED2, NP_078784; ZBED3, NP_115743; ZBED4, NP_055653; BED-1, NP_001024227; BED-2, NP_499474; BED-3, NP_501785; DREF, NP_523529; BEAF32, NP_523747. Locations of BED, hATC and BESS domains are based on NCBI annotations. The region of weak homology was detected by performing a PSI-BLAST search using a portion of BED-3 sequence not containing the BED domain (amino acid 200 to 599). The PSI-BLAST search was done using the NCBI website (<http://www.ncbi.nlm.nih.gov/>).

Isolation of *bed-3* mutations

bed-3(sy644) was isolated in a screen for late vulval development defects using vulval cell fate markers *cdh-3::cfp* and *ceh-2::yfp*. The PS3528 *syIs51[cdh-3::cfp]*; *syIs55[ceh-2::yfp]* strain (Inoue et al., 2002) was mutagenized with EMS (ethylmethanesulfonate) and propagated for two generations. Candidate mutants were picked in the F2 generation based on the protruding vulva (Pvl) phenotype or based on gross alteration of marker expression as scored using a dissecting microscope equipped for epifluorescence. Lines generated from these animals were later examined under high magnification. Lines that exhibited Pn.p fate changes (i.e. 1° vs. 2° vs. 3° VPC fates; Sternberg and Horvitz, 1986) were discarded, because this type of mutant has been studied extensively. Only those lines with later lineage defects or those altering marker expression were retained. From approximately 10000 mutagenized genomes, we identified six mutations that met these criteria. Based on frequencies of similar mutations from other screens, it is unlikely that all such mutations present in the 10000 mutagenized genomes were recovered. Three mutations, *sy633 I*, *sy635 I* and *sy636 III* increased the level of *ceh-2::yfp* expression in cells that normally express *ceh-2::yfp*. One mutation, *sy638*, caused a low penetrance P-Rvl (posterior reversal) phenotype indicative of anterior-posterior reversal of the P7.p lineage (Green et al., 2008; Inoue et al., 2004). One mutation, *sy634 I* was found to be a mutant allele of *lin-11* by a complementation test. *bed-3(sy644)* was initially identified as a mutation altering the numbers of *cdh-3::cfp* expressing cells.

To generate additional alleles of *bed-3*, we crossed EMS mutagenized wild-type males to *unc-24 bed-3(sy644) dpy-20 IV* hermaphrodites. Cross progeny were distinguishable from self-progeny as non-Unc non-Dpy animals, and among these, Egl animals were picked as candidate *bed-3* alleles. Although some F1 animals had mated with siblings, it was possible to establish lines of new mutants by picking Egl animals. Where possible, the new allele was made homozygous by following the segregation of *unc-24* and *dpy-20*, which were linked in *cis* to the original allele. *sy700* was maintained originally balanced by the *unc-24 bed-3(sy644) dpy-20* chromosome, and was later balanced by *nT1[qIs51]*. Mutations *bed-3(sy704)* and *bed-3(sy710)* were isolated in the same round of screening as *bed-3(sy705)*. Although *sy704*, *sy705* and *sy710* were derived from separate F1 animals, subsequent analysis showed that *sy704* and *sy710* are the same mutation as *sy705*. These lines were not analyzed further.

Mapping and cloning of *bed-3*

bed-3(sy644) was mapped to the *unc-24* to *egl-38* interval on chromosome 4 using standard methods. Mutations in this interval, *egl-38(sy294)*, *egl-38(s1775)* and *egl-20(n578)* complemented the Egl phenotype of *bed-3(sy644)*. For fine-scale mapping, an *unc-24 bed-3(sy644) dpy-20 IV* chromosome was placed in *trans* to a chromosome from the strain CB4856, which is a wild-type isolate of *C. elegans* containing numerous single nucleotide polymorphisms (snp) in comparison to N2. From the heterozygous parent, Unc non-Dpy and Dpy non-Unc recombinant progeny were picked and lines established. These lines were tested

for the presence of *bed-3(sy644)* and for N2/CB4856 polymorphisms in the region. The most informative polymorphic markers were *pkP4081* (cosmid K07F5), *snp_T13H10[3]* (cosmid T13H10) and *dbP3* (cosmid K08F4). Each of these polymorphisms alters a restriction enzyme recognition site, and was scored by PCR amplification of short DNA fragments followed by a restriction digest. Multiple recombinants placed *sy644* between *pkP4081* on the left and *dbP3* on the right (288,836 bp interval). However, among 80 recombination events scored between *unc-24* and *dpy-20*, none recombined between *bed-3(sy644)* and *snp_T13H10[3]*, suggesting that these loci are very close.

Cosmid clones of genomic DNA from the interval between *pkP4081* and *egl-38* were tested for transformation rescue of the Egl phenotype. The following cosmids did not rescue the *bed-3* mutant: W08D2, K07F5, F49C8, F32B6, F25H8, T13H10, C47E12, F44D12, T04B2, W01B6, C04G2, W01E11, W01E2. A transgenic line made by coinjecting a mixture of overlapping cosmids F26D3 and C13H3 rescued *bed-3(sy702)*, whereas multiple lines made from injection of F26D3 failed to rescue, suggesting that *bed-3* is on the part of cosmid C13H3 not overlapped by F26D3. Although exact ends of C13H3 and F26D3 clones are not known, examination of the physical map suggested that *bed-3* is near F25H8.6. Multiple lines made with cosmid F25H6 failed to rescue *bed-3(sy644)* even though the gene should be fully contained in this cosmid, possibly because of cosmid rearrangement. Microinjection of a single-gene subclone of C13H3 which contains only F25H8.6 (pTI05.37) rescued mutant F1 animals. To determine if F25H8.6 is *bed-3*, mutant alleles were sequenced. Because four mutant alleles contain four different mutations in the coding region of this gene, F25H8.6 is *bed-3*.

Lineage analysis and scoring of cell numbers

Lineage analyses of *bed-3* and *cye-1* mutants were performed as previously described by keeping live animals on agar pads on sealed slides (Sulston and Horvitz, 1977). *cye-1* and *cul-1* mutants were maintained balanced by *hT2[qIs48[myo-2::gfp]]* and non-GFP progeny were scored. For Table 2, lineages were typically observed from Pn.p daughters or granddaughters. Worms were observed for at least one hour past the last division before undivided cells were scored as "U" (undivided). We did not find a strong effect of *bed-3(sy644)* on the timing of cell divisions; however, we cannot rule out the possibility that some of the "U" cells divided later.

Vulval cell numbers were determined in the mid-L4 stage. In most animals, cell size, arrangement and morphology could be used to infer which cells were derived from which precursor. The organization of P6.p-derived cells was wild type in most *bed-3* mutants. However, because the cell arrangement was disorganized in some animals, it is possible that scoring of cells as P5.p-, P6.p- or P7.p-derived was incorrect in some animals (Supplementary Figure S1).

For *cul-1*, we scored non-GFP progeny of *cul-1/hT2[qIs48[myo-2::gfp]]* parents. We counted 38 ± 2.9 ($n = 4$) vulval cells for *cul-1(e1756)* mutants and 33 ± 6.6 ($n = 3$) vulval cells for *cul-1(e1756); bed-3(sy702)* double mutants. While these results and the small size of *cul-1; bed-3* vulval cells demonstrate that *cul-1; bed-3* mutants undergo extra rounds of cell division, the numbers for *cul-1* are smaller than previously reported by Kipreos *et al.* (1996) and are likely to be undercounts due to the difficulty in distinguishing vulval and non-vulval cells in highly disorganized *cul-1* animals. Notably, Kipreos *et al.* (1996) used a *ceh-24::gfp* marker and videomicroscopy to obtain accurate cell counts, which was not done for this study. Because of these uncertainties, it is unclear whether *cul-1; bed-3* double mutants have reduced cell numbers compared to *cul-1* single mutants.

Examination of non-vulval lineages

The number of π cell descendants was scored in the mid-L4 stage using *kuls 29[cog-2::gfp]* (Hanna-Rose and Han, 1999), which is expressed in daughters of π cells and the anchor cell. Ventral cord neurons were counted in the P5.p to P8.p interval in L2 animals using Nomarski optics. Lateral hypodermal nuclei were counted using the *syIs51[cdh-3::cfp]* marker (expressed in seam cells as well as vulval cells) in mid-L4 to young adult animals.

Molting phenotype of *bed-3* mutants

Semi-synchronized populations of N2, *bed-3(sy702)* and *bed-3(sy705)* strains were generated by overnight egg lays and scored two days later as adults. 21 of 55 *bed-3(sy705)* mutants examined had not shed the L4 cuticle completely. In contrast, no molting defect was observed in N2 (n=20) and *bed-3(sy702)* (n=57) animals.

bed-3::gfp fusion

The genomic DNA from 1.4 kb (kilobase) upstream of the *bed-3* gene was PCR amplified using primers TTAAGCATGCAGAAGCGTAGAAGGTAGGCAAGTGGG and GAGAGTCGACTCCAGGTAATGGGATACCTG. The resulting fragment was cloned into pCR-XL-TOPO (Invitrogen) using TOPO cloning. The promoter fragment was then excised from the vector using NsiI (site in vector multiple cloning site) and SalI (site in downstream primer) and cloned into 4xNLSgfp vector pPD121.83 cut with PstI and SalI to generate the promoter-gfp construct pTI06.15. To test for enhancers, intron-containing fragments were isolated from the genomic clone pTI05.37 cut with HindIII or NspI and cloned into pTI06.15 cut with HindIII and SphI respectively. This places each intron-containing fragment 5' to the promoter fragment. All reporter constructs were injected into *unc-119(ed4)* using *unc-119* (+) as a coinjection marker (Maduro and Pilgrim, 1995; Mello et al., 1991).

Results

Isolation and molecular characterization of *bed-3* mutations

We isolated the *bed-3(sy644)* mutation in a screen for mutations that affect the expression of vulval cell type markers *cdh-3::cfp* and *ceh-2::yfp* (see Materials and Methods) (Inoue et al., 2002). *cdh-3::cfp* is expressed in vulC, vulD, vulE and vulF (Pettitt et al., 1996), and *bed-3(sy644)* affected the number of *cdh-3::cfp* expressing cells. In addition, *bed-3(sy644)* caused Egl (egg-laying defective) and weak Pvl (protruding vulva) phenotypes. Mapping and complementation tests found that *bed-3* is a new genetic locus on chromosome 4. To isolate additional alleles, we carried out a genetic screen for mutations that fail to complement *bed-3(sy644)* for the Egl phenotype (see Materials and Methods). From a screen of approximately 4400 EMS mutagenized genomes, we identified three additional mutations that fail to complement *sy644* (Table 1). This frequency is consistent with loss-of-function alleles (Greenwald and Horvitz, 1980).

We cloned *bed-3* using a combination of SNP mapping and germline transformation rescue (see Materials and Methods) and found that it corresponds to the predicted gene F25H8.6 (Figure 2). The mRNA structure was determined by RT-PCR, and was identical to the structure in WormBase (release WS160). *bed-3* is predicted to encode a 599 amino acid protein (BED-3) with a single BED-type zinc finger domain (Figure 3, Supplementary Figure 2). BED Zn-finger proteins are sequence-specific DNA binding proteins (Aravind, 2000). In *C. elegans*, a BED domain protein DPY-20 regulates body shape (Clark et al., 1995). In *Drosophila melanogaster*, a BED domain protein DREF activates transcription of genes involved in cell proliferation (Hirose et al., 1993; Hirose et al., 1996; Matsukage et al., 1995), and a BED domain protein BEAF32 (boundary element associated factor) associates with elements that separate

chromatin domains (Emberly et al., 2008; Zhao et al., 1995). The human ortholog of DREF (hDREF) also regulates cell proliferation in cell culture (Ohshima et al., 2003). The sequence conservation within this family is mostly in the BED domain, although weak similarity outside the BED domain exists between the *C. elegans* BED-3 and human proteins hDREF and ZBED4 (Figure 3; see Materials and Methods). Because the level of sequence conservation is low even within the BED domain, a convincing orthology relationship was not detected between BED-3 and any other protein outside of nematodes. However, BED-3 is architecturally similar to hDREF in containing only a single BED domain and a weakly conserved C-terminal region. In contrast, some proteins (e.g. human ZBED4, *C. elegans* BED-2) contain multiple BED domains. Also, some BED domain proteins (e.g. BEAF32, human ZBED2) show no similarity to BED-3 in the C-terminal region.

To determine the nature of the mutations, we sequenced mutant alleles of *bed-3* and found *sy644*, *sy702* and *sy705* to be nonsense mutations in exons 4, 5, and 3, respectively (Figure 2, Supplementary Figure 2, Table 1). None of these completely disrupt the BED domain, and they are unlikely to be null mutations. The *bed-3(sy700)* mutation is a frame-shifting deletion. *bed-3(sy700)*, unlike other alleles, cannot be maintained in a homozygous state because of an apparent maternal effect embryonic lethality. However, the deletion occurs downstream of the nonsense mutation in homozygous-viable *bed-3(sy705)*, and *sy700* exhibits a weaker vulval lineage phenotype (see below). Based on these results, the lethal phenotype in the *sy700* strain is likely caused by a linked background mutation rather than by *sy700* itself.

We tested the effect of RNAi against *bed-3* by using a clone from a bacteria-feeding library (Open Biosystems) (Rual et al., 2004). Wild-type animals that grew on *E. coli* expressing *bed-3* dsRNA exhibited both the Egl defect and the vulval cell-lineage defect (Supplementary Figure S1), demonstrating that these phenotypes are caused by a reduction or loss of function in the *bed-3* gene. Previously, RNAi against *bed-3* (F25H8.6) was reported to cause molting defects (Frand et al., 2005), and we found that a strong *bed-3* mutation also caused molting defects (Figure 4C, Materials and Methods).

***bed-3* regulates cell division in the vulval lineages**

To determine the function of *bed-3* in vulval development, we observed the pattern of cell divisions (the lineage) in *bed-3(sy644)* and *bed-3(sy705)* mutants (Figure 1C, Figure 4A, Table 2). In both mutants, granddaughters of P5.p, P6.p and P7.p often failed to divide. Cells most affected by *bed-3(sy644)* were P5.ppa and P7.pap, the parents of vulC cells (failed to divide in 7/16 or 45% of cases). Parents of vulA and vulB cells divided normally. *bed-3(sy705)* caused a stronger defect in which most P5.p and P7.p granddaughters failed to divide (parents of vulA, vulB and vulC failed to divide in 16/18 or 89% of cases). In addition, in some *bed-3(sy705)* animals, divisions of P6.p granddaughters (3/12 P6.pxx cells) and P5.p and P7.p daughters were defective (data not shown). The penetrance of the cell division defect observed in *bed-3(sy644)* is less than the penetrance of the Egl phenotype (egg-laying defective; almost 100%), therefore the Egl phenotype is not caused solely by the cell division defect.

Since loss of cell division reduces the final number of vulval cells, we compared other *bed-3* mutations by the cell number (Figure 5; Supplementary Figure S1). We found that all mutations caused a qualitatively similar phenotype, with *sy705* exhibiting the strongest defect. The cell counts place the mutants in an allelic series with respect to the vulval cell division phenotype; *sy700* < *sy644* = *sy702* < *sy705*.

***bed-3::gfp* reporter is expressed in Pn.p granddaughters**

To study where the *bed-3* gene could be expressed, we assayed genomic sequences surrounding the *bed-3* coding region for promoter and enhancer activities (Figure 2). A 6.5kb PspOMI to

Clal genomic clone rescues the *bed-3* mutant (pTI05.37). This clone contains 1.3 kb of sequence upstream of the *bed-3* coding region, all exons and introns, and 3' sequence to the middle of the next gene in the genome, *dur-1*.

We first tested the construct in which 1.4 kb of the upstream sequence directed the expression of a nuclear localized GFP variant (Figure 2; pTI06.15; see Materials and Methods). This *bed-3::gfp* reporter was most prominently expressed in lateral hypodermal (seam) cells, and occasionally in the *hyp7* hypodermal cell. This expression was dynamic, with a notable increase in GFP expression around the time of the L3-to-L4 molt. Although occasional animals expressed GFP in vulval cells, this expression was faint and inconsistent. Genes required for molting are expressed in seam and *hyp7* cells in a dynamic pattern with expression peaks near the time of the molt (Frand et al., 2005; Kostrouchova et al., 1998; Kostrouchova et al., 2001). Thus, the expression pattern of *bed-3* is consistent with its role in molting, and suggests that *bed-3* drives expression of other molting-related genes in hypodermal cells.

We next tested for the presence of enhancers in introns. Of seven introns in the *bed-3* gene, introns 3 and 5 are exceptionally large (>500 bp) and contain sequences that are conserved between *C. elegans* and *C. briggsae* (WABA alignment, WormBase WS160). To test whether these introns contained transcriptional enhancers, we placed intron-containing fragments upstream of the 1.4 kb *bed-3* promoter in the *bed-3::gfp* (pTI06.15) construct. Two intron-containing fragments enhanced transcription from the *bed-3* promoter in two distinct patterns (Figure 2). The construct containing introns 5, 6 and 7 expressed GFP in a subset of neuronal cell bodies in the ventral cord and in HSN neurons required for egg laying (HindIII fragment; pTI06.28; see Materials and Methods). The fragment containing introns 3 and 4 increased the level of expression in the lateral hypodermis and *hyp7* cells (NspI fragment; pTI06.29; see Materials and Methods). Furthermore, this enhancer fragment caused expression in P5.p, P6.p and P7.p granddaughters (Figure 6). Although this expression was variable in that not all animals expressed GFP in all Pn.p granddaughters, it was consistently observed in multiple independently generated lines. These results suggest that *bed-3* acts autonomously in cells whose divisions are affected in *bed-3* mutants, however, the possibility that *bed-3* acts in seam or *hyp7* hypodermal cells to control vulval cell division has not been ruled out.

Comparison of *bed-3* with general cell-cycle regulators

Disruption of general cell cycle regulators can cause loss of cell division in vulval lineages. For example, a mutation in *cye-1* (encoding cyclin E) causes vulval precursor cells to undergo only two rounds of division (Fay and Han, 2000). Because this phenotype is superficially similar to that of *bed-3* mutants, we performed a detailed comparison (Figure 1C). During wild-type vulval development, the first two divisions occur along an anterior-posterior axis and the third (terminal) division occurs along sublineage-specific axes. In *cye-1(eh10)* mutants, the second (terminal) division occurs along sublineage-specific axes. Furthermore, the time delay between the first and the second division is abnormally long, so that the second (terminal) division of *cye-1(eh10)* occurs at about the same time as the third (terminal) division of the wild type. These results suggest that the *cye-1(eh10)* causes a lengthening of the interval between cell divisions, and loss of the second round of cell division (Fay and Han, 2000).

In contrast, we found that *bed-3(sy705)* caused a specific loss of the third (terminal) division (Figure 1C). Direct observation of division (n=5) and observation of cell arrangement after the second division (many) showed that the second division occurs in the anterior/posterior orientation. Furthermore, the time between cell divisions in *bed-3(sy705)* was much shorter than for *cye-1(eh10)*. The interval between the first and second division in *cye-1(eh10)* was 320 minutes (standard deviation 20 min.; n=6). The same interval was 178 minutes (s.d. 31 min.; n=12) in *bed-3(sy705)*. The second division of *bed-3* mutants also occurs earlier than the third (terminal) division of the wild type.

To further investigate the relationship of *bed-3* to general cell cycle regulators, we examined the interaction between *bed-3* and *cul-1*. *cul-1* encodes a cullin, a component of an E3 ubiquitin ligase complex (Kipreos et al., 1996). In *cul-1* mutants, many postembryonic cell lineages undergo extra rounds of division, demonstrating that *cul-1* negatively regulates cell division. Vulval lineages also undergo extra rounds of division, and consequently, the adult vulva of *cul-1* mutants contains more cells than the wild type. To determine how *bed-3* interacts genetically with *cul-1*, we examined *bed-3(sy702); cul-1(e1756)* double mutants. The vulva of *bed-3; cul-1* double mutants contained more cells than the wild type; i.e., *bed-3* does not fully suppress the *cul-1* mutant phenotype (Figure 4A, Materials and Methods). This result is consistent with the hypothesis that *bed-3* functions upstream of general cell cycle regulators like *cul-1*. The double mutant also had a smaller body size than either *bed-3(sy702)* or *cul-1(e1756)* single mutants (Figure 4B) and appeared to be slow-growing.

***bed-3* does not affect all postembryonic cell divisions**

Mutations in general regulators of cell division like *cye-1* and *cul-1* affect multiple lineages (Fay and Han, 2000; Kipreos et al., 1996; van den Heuvel, 2006). To determine whether *bed-3* is a general regulator of cell division, we looked for *bed-3* function in additional lineages. In particular, we analyzed cells that express *bed-3::gfp*, cells that undergo division at the same time as Pn.pxx cells, and cells closely related to vulval cells by lineage (Figure 5).

bed-3::gfp is expressed in the lateral hypodermal (seam) cells. In each larval stage, each seam cell undergoes one (L1, L3, L4 stages) or two (L2) rounds of division (Sulston and Horvitz, 1977). In a stem-cell-like pattern, one of two (or two of four in L2) cells produced retains the parental seam cell identity, while the other cell fuses with the terminally differentiated hyp7 hypodermal cell. Mutations in *cye-1* and *cul-1* respectively decrease and increase the number of seam cell nuclei in the adult (Fay and Han, 2000; Fujita et al., 2008; Kipreos et al., 1996). To determine whether *bed-3* regulates seam cell lineages, we examined the number of seam cell nuclei in *bed-3(sy705)* mutants (see Materials and Methods). We found the same number of seam cell nuclei in *bed-3(sy705)* mutants as in the wild type, suggesting that *bed-3* is not required in the seam cell lineage to regulate cell division.

We also looked at the function of *bed-3* in uterine lineages (including π cell lineages), which undergo final rounds of cell division near the L3 to L4 molt, contemporaneous with terminal vulval cell divisions (Kimble and Hirsh, 1979; Newman et al., 1996; Sulston and Horvitz, 1977). *cye-1* and *cul-1* mutations affect uterine lineages and cause gross morphological defects in the uterus (Fay and Han, 2000; Fujita et al., 2008; Kipreos et al., 1996). In contrast, we found that the gross morphology of the uterus was normal in the strongest *bed-3* mutant, *bed-3(sy705)*. We also examined the uterine π cell lineage using the marker *cog-2::gfp*, which is expressed in twelve π cell daughters and the anchor cell (Hanna-Rose and Han, 1999) (see Materials and Methods). We found no difference in the number of *cog-2::gfp* expressing cells in the wild type, *bed-3(sy702)* and *bed-3(sy705)* mutants, suggesting that π cell divisions are not affected by *bed-3* mutations.

Finally, we examined cells derived from Pn.a neuroblasts, which are sister cells of Pn.p cells (Sulston and Horvitz, 1977). These cells divide two to three rounds in the late L1 stage to generate neurons whose cell bodies lie in the ventral cord region. This region contains 15 neuron cell bodies at hatching, to which Pn.a lineages add 52 additional neurons during the L1 stage. To assay for defects in Pn.a lineages, we counted the number of neuron cell bodies in a specific interval in L2 animals (between P5.p to P8.p; see Materials and Methods). We found no difference in the number of neurons in this interval between N2 and the *bed-3(sy705)* mutant, suggesting that *bed-3* mutations do not affect Pn.a lineages.

bed-3* interacts genetically with *cog-1

The observation that *bed-3* mutations do not affect many lineages suggested that *bed-3* function is specific to terminal division of the vulval lineage. Previously, mutations in only one other gene, *cog-1*, was known to affect specific terminal cell divisions during vulval development. *cog-1* encodes a Nkx6 homeobox transcription factor regulating gene expression in terminal vulval cell types (Inoue et al., 2005; Palmer et al., 2002). *cog-1(sy275)* is a missense mutation in the DNA binding domain, and *cog-1(sy607)* is a deletion eliminating one of two *cog-1* transcripts. Neither allele is likely to cause a complete loss of function. In *cog-1(sy275)* mutants, granddaughters of P6.p often fail to divide. In *cog-1(sy607)* mutants, granddaughters of P5.p and P7.p often fail to divide (Table 2). Interestingly, cell divisions which are most commonly affected in *cog-1* mutants (P5.ppa, P6.pap, P6.ppa, P7.pap) are also most commonly affected in the weak *bed-3* mutation *bed-3(sy644)* (Table 2). These results suggested that *cog-1* and *bed-3* function in the same process and may interact genetically.

To look for genetic interaction between *cog-1* and *bed-3*, we examined *cog-1(sy275); bed-3(sy644)* and *cog-1(sy607); bed-3(sy644)* double mutants, and found that the cell division defect was strongly enhanced (Table 2). For example, P5.paa and P7.ppp (parents of vulA in wild-type) always divided in *bed-3(sy644)*, *cog-1(sy275)* and *cog-1(sy607)* single mutants (Table 2) (Palmer et al., 2002). However, the same cells failed to divide in *cog-1(sy275); bed-3(sy644)* (11/12 cells) and in *cog-1(sy607); bed-3(sy644)* double mutants (8/8 cells). The phenotype of double mutants (Table 2) suggest that both *cog-1* and *bed-3* function in all divisions of Pn.p granddaughters, but are not required for earlier rounds of Pn.p divisions. Because none of the mutations analyzed are null, these results are consistent with both parallel and serial function of *cog-1* and *bed-3*.

***bed-3* affects cell type specific gene expression in vulval cells**

Mutations in *cog-1* affect cell type specific pattern of gene expression as well as cell division. The pattern of alteration in gene expression is not fully understood, and is not consistent with a simple transformation of cells to the non-dividing vulD fate. Nevertheless, the co-occurrence of both cell division and cell type specific gene expression defects in *cog-1* mutants suggest that a common underlying mechanism controls both processes. We tested whether *bed-3* acts strictly in controlling cell division or like *cog-1*, *bed-3* also controls cell type specific pattern of gene expression. In particular we analyzed *cdh-3* and *egl-17* expression, which are affected by *cog-1* mutations (Inoue et al., 2005) (Table 3).

In a wild-type background, *egl-17::cfp* is expressed in vulC and vulD cells, but not in vulA and vulB cells (Inoue et al., 2002). (Expression in vulE and vulF cells was not scored in this experiment.) To determine whether this pattern is affected by the strongest *bed-3* allele *sy705*, we focused our analysis on P5.p and P7.p lineages which produced four descendant cells of similar size, since each of these cells is likely to be a Pn.p granddaughter. (In other cases, it can be ambiguous which cell belongs to which Pn.pxx sublineage.) If the correspondence between Pn.p granddaughters and gene expression is maintained, we expect two granddaughters of each Pn.p (P5.ppa/P5.ppp, or P7.paa/P7.pap) to express *egl-17::cfp*. Indeed, we found that in *bed-3(sy705)* mutants, two out of four cells expressed *egl-17::cfp* in 12 out of 12 cases (Table 3). In 11 of these, the innermost two Pn.p granddaughters expressed *egl-17::cfp* (data not shown; the one exceptional pattern could be due to rearrangement of cells).

In contrast to *egl-17::cfp*, we found that *bed-3(sy705)* affected the expression pattern of *cdh-3::cfp*. In a wild-type background, *cdh-3::cfp* is expressed in vulC, vulD, vulE and vulF (Inoue et al., 2002; Pettitt et al., 1996). If the correspondence between Pn.p granddaughters and cell type specific gene expression is maintained, we expect the inner two granddaughters

of each Pn.p (P5.ppa/P5.ppp, or P7.paa/P7.pap) to express *cdh-3::cfp*. Instead, we found that eight of 17 such Pn.p lineages expressed *cdh-3::cfp* in only one cell, always the innermost granddaughter (P5.ppp or P7.paa) (Figure 7, Table 3). Seven of 17 Pn.p lineages expressed *cdh-3::cfp* in two cells as expected, and two lineages expressed *cdh-3::cfp* in three out of four cells. In all animals examined, expression in vulD, vulE and vulF cells was intact. This phenotype is reminiscent of *cog-1(sy607)*, which causes a loss of *cdh-3::gfp* expression in vulC, vulD and vulE cells (Inoue et al., 2005). The phenotype in *bed-3(sy705)* is weaker than is observed for *cog-1(sy607)*, however, this is not simply due to the strength of the mutation, because the cell division defect is stronger in *bed-3(sy705)* than in *cog-1(sy607)*.

Discussion

During *C. elegans* vulval development, each vulval precursor cell (VPC; P3.p ~ P8.p) adopts one of three fates; 1°, 2° or 3°. Although VPC fate specification has been analyzed extensively over the last two decades, the mechanism by which VPC fates are 'executed' (i.e. how VPC descendants undergo fate-specific pattern of subsequent development) has received less attention. We found that the BED Zn-finger transcription factor BED-3 is involved in this phase of vulval development, specifically in the regulation of several cell division events in vulval cell lineages. Our analyses suggest that BED-3 is not a general regulator of cell division. Instead, similarities of *bed-3* mutant phenotypes with those of *cog-1* suggest that *bed-3* interacts with, or is an integral component of the gene regulatory network (GRN) specifying cell type specific pattern of gene expression in vulval cells. The sequence similarity of BED-3 to cell proliferation regulators DREF and hDREF is intriguing and suggests possible conservation of function, however, it is unclear whether cell division control is the primary function of BED-3. Here we discuss these possibilities.

BED-3 is not likely to be a general regulator of cell division

Loss or gain of cell division in the vulval lineage can be caused by mutations in general cell cycle regulators (van den Heuvel, 2006). In *C. elegans*, the disruption of some cell cycle regulators (e.g. *cul-1*, *cye-1* mutations) causes a relatively mild phenotype in which many lineages undergo hyper- or hypo-proliferation, but the overall pattern of development is not severely affected (i.e. mutants are viable) (Fay and Han, 2000; Kipreos et al., 1996). Some mutations such as *cye-1(eh10)* cause loss of vulval cell divisions, as do *bed-3* mutations. Because *bed-3* mutations we examined do not cause complete loss of function, we cannot rule out the possibility that *bed-3* has a widespread function as a cell division regulator. Although we found no effect of *bed-3* mutations on cell division outside of the vulva, this apparent specificity could be due to sensitivity of vulval lineages to mild reduction in *bed-3* function. Nevertheless, several lines of evidence argue against this possibility. Uterine and seam cell lineages commonly affected in cell proliferation mutants (e.g. *cul-1*, *cye-1*) are normal in *bed-3* mutants, and analysis of *bed-3::gfp* expression suggests that this gene functions in a rather limited number of tissues.

bed-3 and the vulval gene regulatory network

Our results indicate that the function of *bed-3* is closely related to that of *cog-1*, which encodes an Nkx6 homeobox transcription factor. First, *cog-1* and *bed-3* mutations affect terminal cell division in vulval lineages. Second, *cog-1* and *bed-3* mutations affect the lineage-specific expression pattern of the *cdh-3::cfp* reporter. Finally, *cog-1* and *bed-3* mutations show synergistic interaction in the double mutant. Since *cog-1* is a component of the vulval gene regulatory network, these results suggest that *bed-3* may also function in this network.

The VPC fate execution phase of vulval development is an excellent example of complex patterning events in which multiple cell types are generated in a specific spatial pattern. The

underlying mechanism that enables the organism to perform this type of task is a network of interacting genes (gene regulatory network; GRN) that functions as a complex information-processing unit (Alon, 2007; Davidson and Levine, 2008; Davidson et al., 2002). Cell-type specification occurs when such a network responds to developmental signals and adopts a particular state in which genes appropriate for that cell type are activated. During VPC fate execution, transcription factor genes *lin-11*, *lin-29*, *cog-1*, *egl-38* and *nhr-67* constitute a part of the vulval GRN, as evidenced by the fact that mutations in these genes alter the pattern of vulval cell type specific gene expression (Figure 8) (Fernandes and Sternberg, 2007; Inoue et al., 2005; Ririe et al., 2008). Our current understanding of this network is incomplete, and the model is not yet predictive. Nevertheless, we can identify network motifs commonly found in GRNs (Alon, 2007), including negative auto-regulation (*nhr-67*), positive auto-regulation (*egl-38*), a double-negative feedback loop (*cog-1* and *nhr-67*), and a feed-forward loop (*lin-29*, *lin-11* and *egl-17*), indicating that the function of this network is likely similar to other described GRNs.

Given that the cell division of a Pn.p granddaughter is correlated with the cell type, it is likely that the same gene regulatory network regulates cell division. Indeed, mutations in *cog-1* and *lin-11* alter the pattern of cell division as well as gene expression. However, although regulated by overlapping sets of genes, cell division and gene expression are not fully correlated. For example, the Pn.p granddaughter fated to become the vulD cell does not undergo the third round of VPC division. However, non-dividing Pn.p granddaughters in *cog-1* mutants are not transformed to vulD based on gene expression. Similar loss of correlation between vulval lineage cell division and gene expression is apparent in Wnt pathway mutants (see Figure 5 in Deshpande et al., 2005). From these results, we may infer that there is no single master regulator for each vulval sublineage that controls both cell division and gene expression. Instead, cell division and gene expression can be considered separate outputs of a single gene regulatory network.

Where does *bed-3* fit within this scheme? One possibility is that *bed-3* is a component of the core decision making circuitry of the vulval gene regulatory network. Alternatively, *bed-3* may be a peripheral component which connects the core decision making circuitry to specific outputs, which includes control of cell proliferation. The *bed-3(sy705)* mutation alters the pattern of *cdh-3::cfp* expression. This is perhaps suggestive of a more central function, although the latter possibility is not ruled out. Cell ablation studies indicate that regulation of cell type specific gene expression is mostly cell-autonomous after the second Pn.p division (Gupta et al., 2003). However, the possibility remains that the *cdh-3::cfp* expression defect is a secondary consequence of the cell division defect.

Are BED domain proteins conserved regulators of cell proliferation?

In *Drosophila* and human cells, BED Zn-finger transcription factors DREF (DNA replication element factor) and hDREF (human DREF) regulate cell proliferation (Matsukage et al., 2008). Promoters of many *Drosophila* genes required for cell proliferation (e.g. DNA polymerase alpha and proliferating cell nuclear antigen) contain a motif called the DNA replication-related element (DRE; TATCGATA) (Hirose et al., 1993; Matsukage et al., 1995). The DREF protein, in complex with TRF2 (TBP-related factor 2), binds to DREs and activates transcription (Hirose et al., 1993; Hirose et al., 1996; Hochheimer et al., 2002). DREF and TRF2 also interact with proteins regulating chromatin, including another BED Zn-finger domain protein BEAF-32 (Emberly et al., 2008; Hirose et al., 2001; Hochheimer et al., 2002; Nakamura et al., 2008; Zhao et al., 1995). Functional studies demonstrate that manipulation of DREF can have direct effect on cell proliferation during organogenesis (Hirose et al., 2001; Hirose et al., 1999; Hyun et al., 2005; Yoshida et al., 2001). In general, overexpression of DREF promotes cell proliferation whereas disruption of DREF inhibits cell proliferation.

Combined with the observation that promoters of genes upregulated during cell proliferation in organ primordia are highly enriched for DRE (Jasper et al., 2002), these results suggest that DREF may play a central role in regulating cell proliferation in *Drosophila* organogenesis (Matsukage et al., 2008). The human homolog of DREF (hDREF) also promotes cell proliferation in cell culture (Ohshima et al., 2003).

These results suggest that BED-3's role in regulation of cell division may be conserved. Other BED domain proteins, such as BEAF32 (Emberly et al., 2008; Zhao et al., 1995) and ZBED3 (Chen et al., 2009) are not direct regulators of cell proliferation. However, the overall domain structure of the BED-3 protein (a single N-terminal BED domain followed by a weakly conserved C-terminal region) is similar to hDREF and differs from BEAF32 or ZBED3. If the cell proliferation control is a conserved function, like hDREF, BED-3 may regulate cell division directly by activating transcription of genes that are required for cell cycle progression. Alternatively, the regulation may be indirect, and could involve transcriptional regulation of general cell cycle regulators such as *cul-1* and *cye-1*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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diagram. The time is plotted on the vertical axis, and markers on the left are at one hour intervals. Each branching of the inverted tree figure represents a cell division. The orientation of the terminal division is marked below each sublineage. Cells are referred to by their ancestry: The posterior daughter of P5.p is P5.pp. The anterior daughter of P5.pp is P5.ppa and so on. We use x(e.g. P5.ppx) to refer to an unspecified daughter cell, and Pn.p refers to P5.p, P6.p or P7.p. C. Top: Lineage diagrams of representative *bed-3(sy644)* and *bed-3(sy705)* mutant animals. Observations were initiated after the completion of the first two rounds of Pn.p divisions and continued until several hours after the last division. Bottom: Lineage diagrams of representative *cye-1(eh10)* and *bed-3(sy705)* animals. Observations were started before the first Pn.p division and stopped after the second round of division. Whether these cells had divided further was not determined. The observed portion of the lineage is marked by black lines. Grey lines represent portions of the lineage that were inferred based on cell size and position. The time of molting is indicated by a horizontal line across the lineage diagram. Molting was not scored in the *bed-3(sy644)* animal, and probably took place after all divisions were completed.

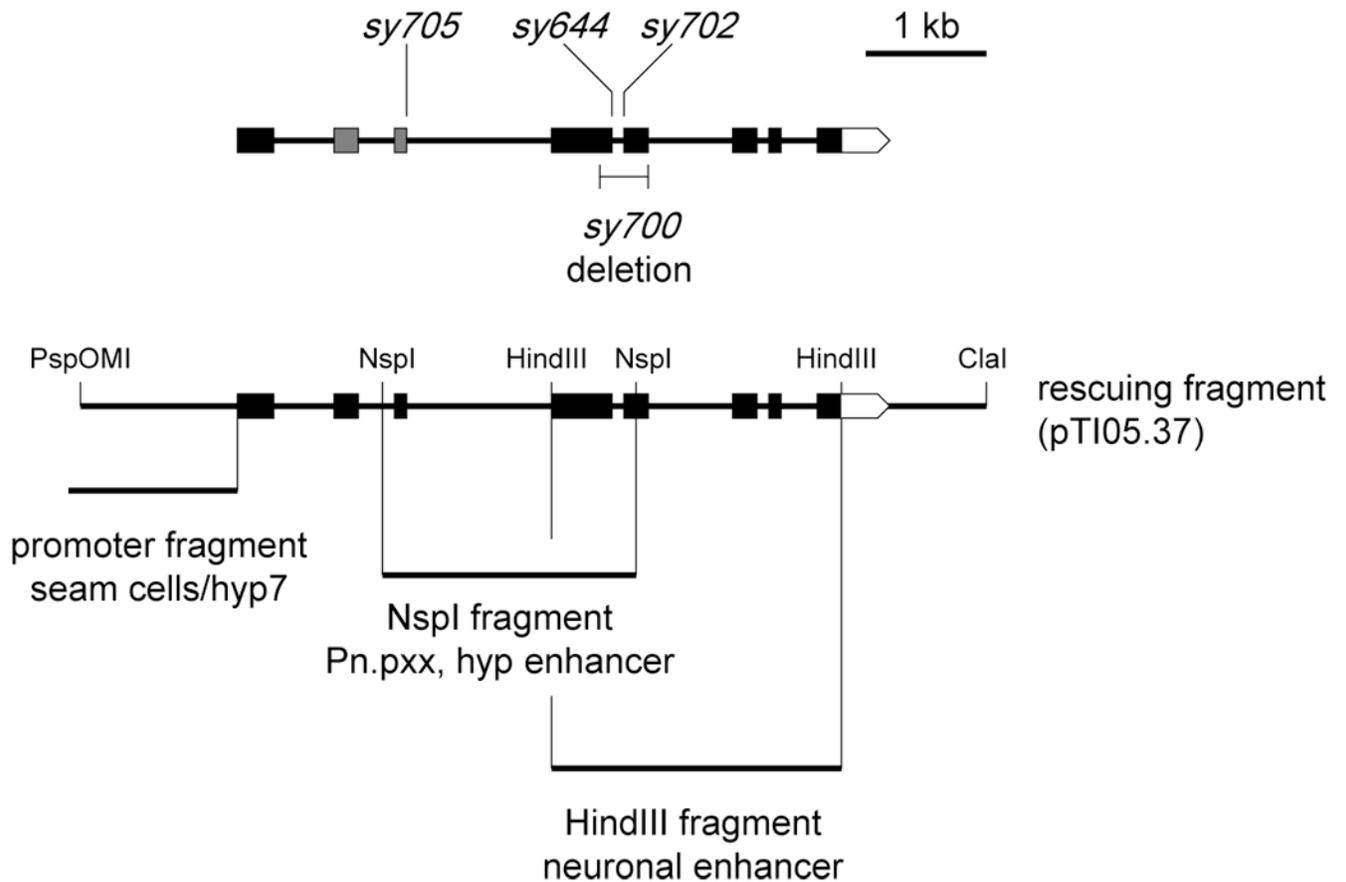
F25H8.6/*bed-3*

Figure 2. *bed-3* gene structure

Top: the intron/exon structure of *bed-3* and locations of mutations. The BED Zn-finger domain is encoded in second and third exons (gray). Bottom: genomic regions tested for promoter and enhancer activity. The promoter fragment, placed upstream of a *gfp* reporter, drives expression in seam and *hyp7* cells. When placed upstream of the promoter fragment, NspI and HindIII fragments drive additional expression, demonstrating the presence of enhancer activity.

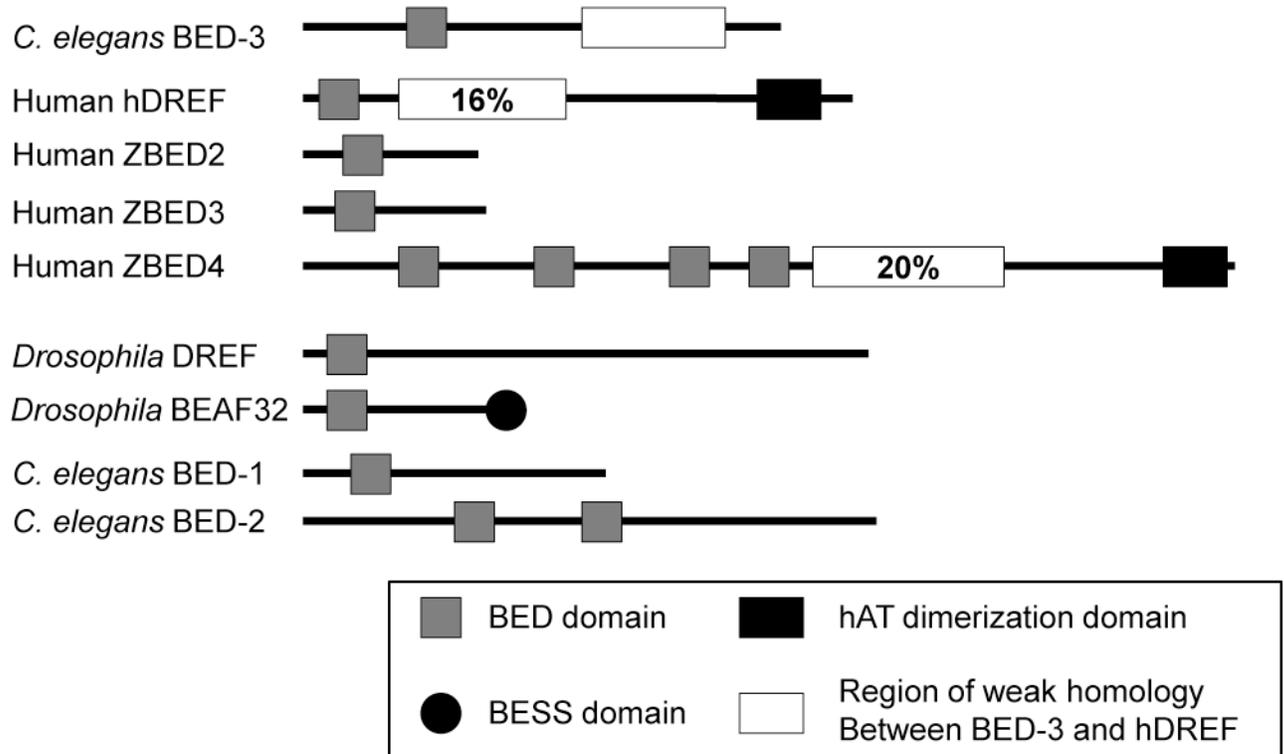


Figure 3. Domain structures of BED Zn-finger domain containing proteins
 N-terminus is to the left. Percentages indicate amino acid identity with the homologous region of BED-3.

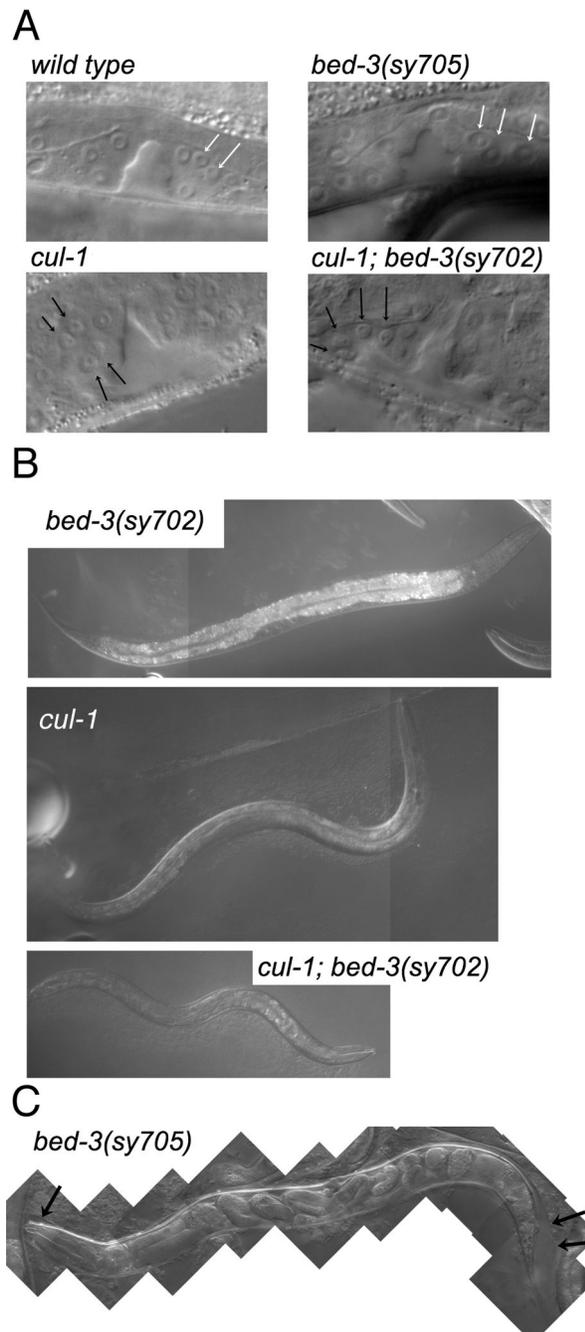


Figure 4. Phenotypes of *bed-3* mutants and interaction with *cul-1*

A. Mid-L4 stage vulva, after cell divisions had been completed. Arrows point to vulval nuclei. Not all nuclei are in the plane of focus. However, note that *bed-3* mutant nuclei are larger than in the wild type due to the lack of terminal division. In contrast, *cul-1* nuclei are smaller due to extra divisions. The *cul-1; bed-3* double mutant is similar to *cul-1*. B. Body size of *bed-3* (*sy702*), *cul-1* and *cul-1; bed-3(sy702)* mutants. All animals are in the mid-L4 stage and shown at the same scale. *bed-3(sy702)* mutants are about the same size as the wild-type (not shown). *bed-3(sy705)* mutants are slightly smaller (not shown). C. The molting defect of *bed-3* (*sy705*). This animal is an old adult, as indicated by the presence of late stage embryos in the uterus. However, because of the molting defect, the L4 cuticle has not been shed (black arrows).

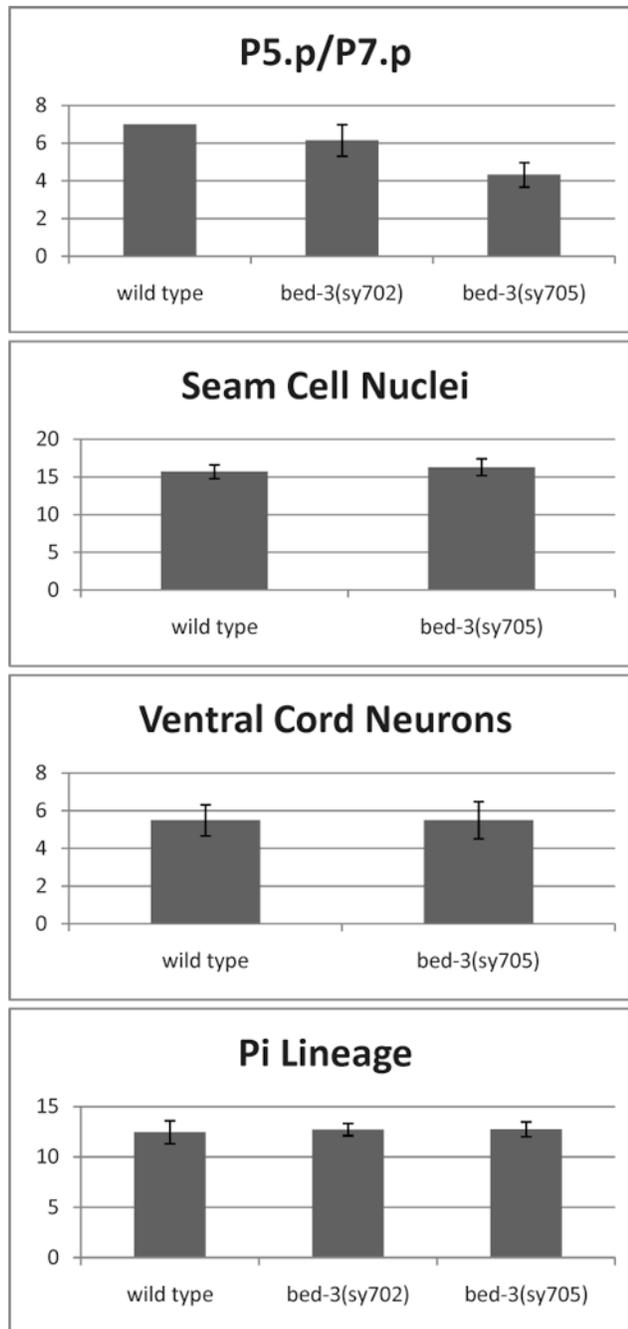


Figure 5. Cell numbers in various lineages

Numbers of cells produced by diverse lineages were assayed in the wild-type and *bed-3* mutant backgrounds. P5.p/P7.p: Descendants of the P5.p or the P7.p lineage. Seam Cell Nuclei: The total number of seam cell nuclei (per side) in late L4 or young adult stage animals. Ventral Cord Neurons: The number of neuronal cell bodies in the ventral cord, per Pn.p-to-Pn.p interval. Pi Lineage: The number of π cell descendants in the mid-L4 stage. The counts include +1 for the anchor cell, which is also labeled by the *cog-2::gfp* marker used to identify π cell descendants. Error bars indicate standard deviations.

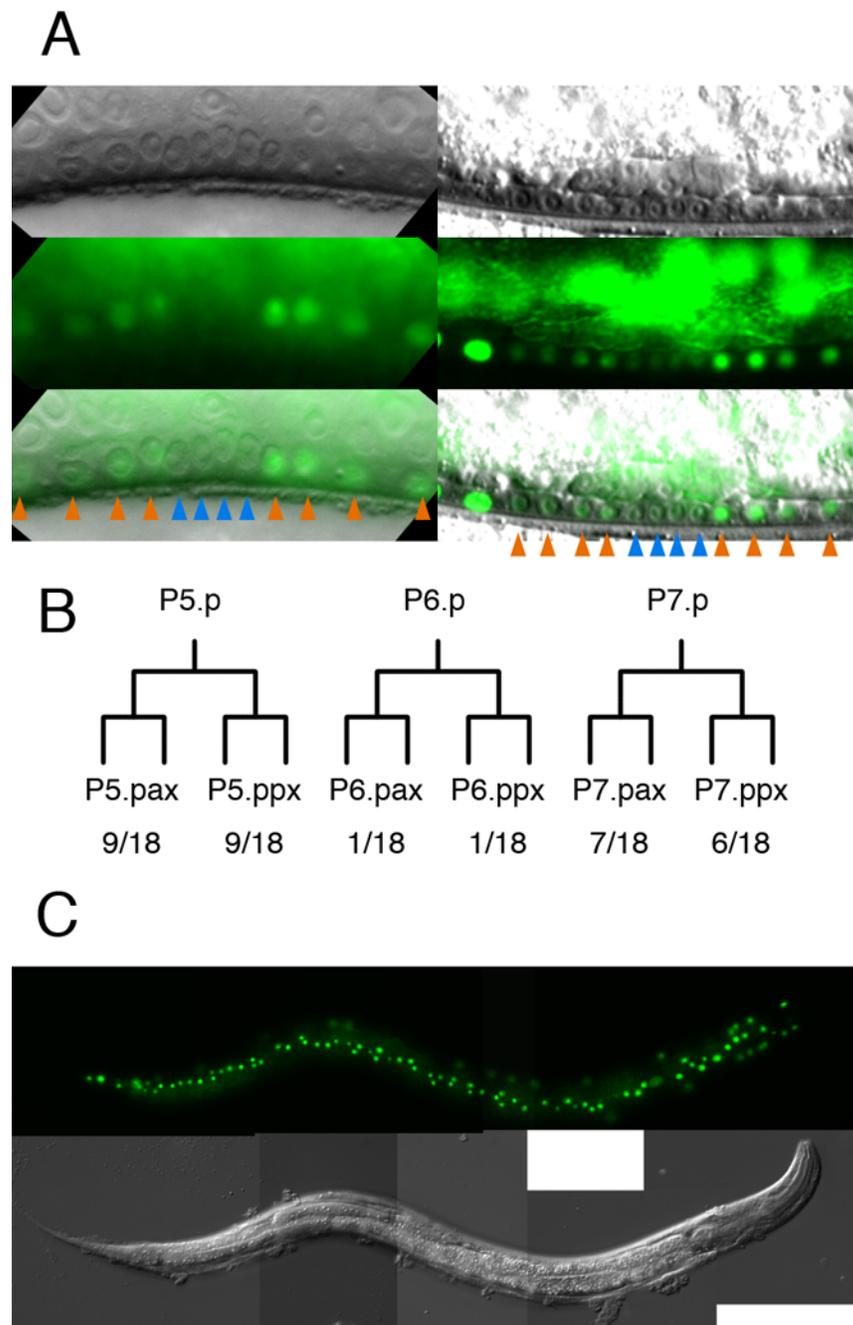
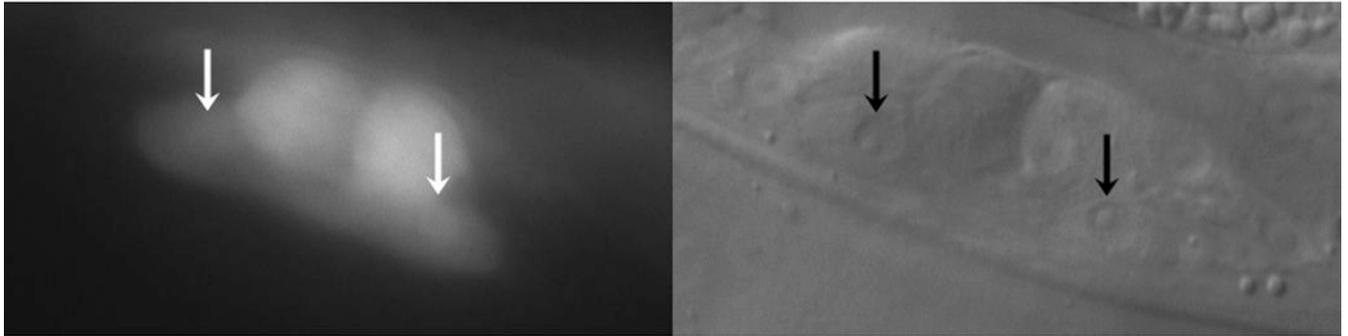
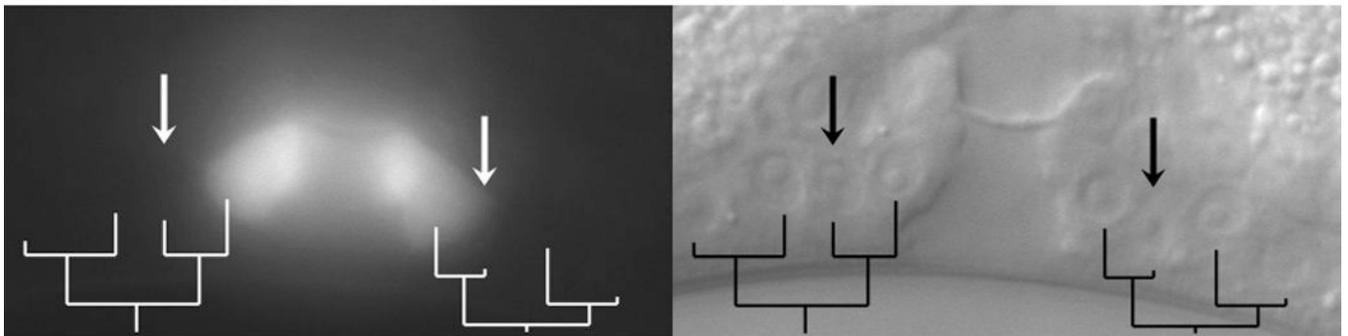


Figure 6. *bed3::gfp* expression in vulval cells

Expression of *bed-3::gfp* enhanced by the NspI fragment. The reporter shown (pTI05.29) expresses a nuclear localized variant of GFP. **A.** Nomarski, epifluorescence and combined images of two animals expressing *bed-3::gfp* in Pn.p granddaughters. Anterior is to the left. In the animal shown to the left, P5.p and P7.p granddaughters (orange arrows) express GFP. In the animal shown to the right, P6.p granddaughters (blue arrows) also express GFP. **B.** Frequencies of *bed-3::gfp* expression in individual Pn.p granddaughters. Because sister cells (e.g. P5.paa and P5.pap) always expressed similar levels of GFP, they were scored together. P5.pxx and P7.pxx often express *bed-3::gfp*, whereas expression in P6.pxx is observed less often. **C.** Expression of *bed-3::gfp* in hyp7 and seam cells.

cdh-3::cfp*bed-3(sy705); cdh-3::cfp***Figure 7. Expression of vulval cell type marker *cdh-3::cfp* in *bed-3(sy705)* background**

Corresponding Nomarski (right) and epifluorescence (left) images are shown. Top: Wild type. vulC cells are indicated by arrows. Bottom: *bed-3(sy705)*. In this animal, P5.p and P7.p lineages divided only two rounds as shown on the lineage tree below. P5.ppa and P7.pap, which give rise to vulC in the wild type, are marked by arrows. vulC expression is lost in *bed-3(sy705)*.

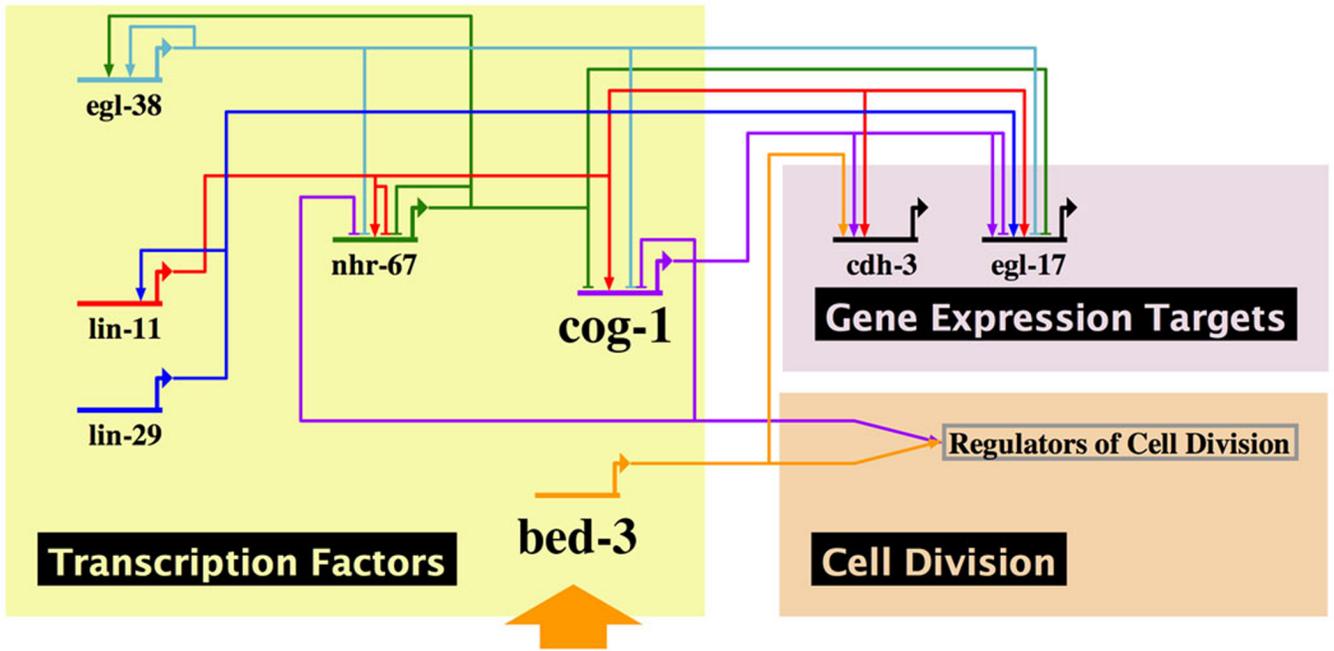


Figure 8. Vulval gene regulatory network regulates gene expression and cell division
 Only selected genes in the network are shown. The current knowledge of the vulval gene regulatory network is such that the model is not predictive. However, it can be observed that cell division and gene expression are regulated by related, but not fully linked mechanisms. (Note, since *bed-3* and *cog-1* encode transcription factors, regulation of cell division is also likely to involve expression of cell-division promoting genes.) Because mutations we analyzed are not nulls, our results are also consistent with *cog-1* and *bed-3* (orange arrow) acting in series to regulate cell division. The network was drawn using BioTapestry Editor (<http://www.biotapestry.org>) (Longabaugh et al., 2009).

Table 1*bed-3* mutations and phenotypes

allele name	nature of mutation	phenotypes
<i>sy644</i>	nonsense, exon 4	Egl, Pvl
<i>sy702</i>	nonsense, exon 5	Egl, Pvl
<i>sy705</i>	nonsense, exon 3	Egl, Pvl
<i>sy700</i>	deletion, exon 4 to 5	Egl, Maternal effect lethal

Egl = egg laying defect, Pvl = protruding vulva

Table 2Lineages of *bed-3* mutants based on observations of cell divisions

genotype	lineage (P5.p, P6.p, P7.p granddaughters)
Wild type	LLTU TTTT UTLL *
<i>cog-1(sy275)</i>	LLTU TUUT UTLL *
<i>cog-1(sy607)</i>	LLUU TTTT UULL *
<i>bed-3(sy644)</i>	LLUU TTTT UTLL LLUU TTTT UOLL LLUU TUUT UULL LLOU TTTT UTLL LLUU TTTT UTLL LLOU TTTT UTLL LLTU TTTT UTLL LLUU TTTT UULL
<i>bed-3(sy705)</i>	UUUU TTTU UULU UUUU TTTU UUUU UUUU TTUT ULUU
<i>cog-1(sy275) bed-3(sy644)</i>	UUUU TUUT UUUU UUUU TUUT UUUU UUUU TUUT UUUU UUUU TUUT UUUU ULUU TUUT UODU UUUU TUUT UULL
<i>cog-1(sy607); bed-3(sy644)</i>	UUUU TTUT UUUU UUUU TUUU UUUU UUUU UTTT UUUU UUUU UTTT UUUU

Divisions of Pn.p granddaughters are represented by "T" = transverse, "L" = longitudinal, "O" = oblique, or "D" = divided with axis undetermined, based on the orientation of the division axis. The failure of a Pn.p granddaughter to divide is represented by "U" = undivided.

* Idealized lineages. Penetrances of division defects in *cog-1* mutants are less than 100%. See Palmer et al. for details. For *bed-3* mutants, each line represents a single animal.

Table 3Alteration of cell type specific expression in *bed-3* mutants

Genotype	Expression pattern in P5.p and P7.p*	Number of Pn.p lineages
<i>cdh-3::cfp</i>	vulC and vulD (2/4 Pn.pxx sublineages)	30
<i>bed-3(sy705); cdh-3::cfp</i> [†]	1/4 Pn.pxx	8
	2/4 Pn.pxx	7
	3/4 Pn.pxx	2
<i>egl-17::cfp</i>	vulC and vulD (2/4 Pn.pxx sublineages)	16
	1/4 Pn.pxx	0
<i>bed-3(sy705); egl-17::cfp</i> [†]	2/4 Pn.pxx	12
	3/4 Pn.pxx	0

* No obvious difference was observed between P5.p and P7.p lineages. The numbers are combined tally for P5.p and P7.p.

[†] Only lineages in which P5.p or P7.p produced four descendants were analyzed.