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Transcriptional control of Notch signaling by a HOX and a PBX/EXD protein during vulval development in *C. elegans*

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Abstract

The Notch signaling pathway controls growth, differentiation and patterning in divergent animal phyla; in humans, defective Notch signaling has been implicated in cancer, stroke and neurodegenerative disorders. Despite its developmental and medical significance, little is known about the factors that render cells to become competent for Notch signaling. Here we show that during vulval development in the nematode *Caenorhabditis elegans* the HOX protein LIN-39 and its EXD/PBX-like cofactor CEH-20 are required for LIN-12/Notch-mediated lateral signaling that specifies the 2° vulval cell fate. Inactivation of either *lin-39* or *ceh-20* resulted in the misspecification of 2° vulval cells and suppressed the multivulva phenotype of *lin-12*(*n137*) gain-of-function mutant animals. Furthermore, both LIN-39 and CEH-20 are required for the expression of basal levels of the genes encoding the LIN-12/Notch receptor and one of its ligands in the vulval precursor cells, LAG-2/Delta/Serrate, rendering them competent for the subsequent *lin-12*/*Notch* induction events. Our results suggest that the transcription factors LIN-39 and CEH-20, which function at the bottom of the RTK/Ras and Wnt pathways in vulval induction, serve as major integration sites in coordinating and transmitting signals to the LIN-12/Notch cascade to regulate vulval cell fates.

Keywords: Notch signaling; *C. elegans*; LIN-12 receptor; LAG-2/Delta ligand; LIN-39/HOX; CEH-20/PBX/EXD; Vulval induction; Signaling crosstalk

Introduction

During animal development, Notch signaling mediates cell–cell interactions that specify cell fate by generating specific changes in gene expression inside the cell (Kimble and Simpson, 1997; Artavanis-Tsakonas et al., 1999). Vulval development in the nematode *Caenorhabditis elegans* has served as an important paradigm for studying how Notch signaling controls cell fate and tissue morphogenesis (Greenwald et al., 1983; Sternberg and Horvitz, 1986, 1989; Sternberg, 1988; Greenwald, 2005). The vulva of the *C. elegans* hermaphrodite develops from a subset of six multipotent epidermal cells called vulval precursor cells (VPCs, consecutively numbered P3.p–P8.p), which have the potential to adopt one of three cell fates termed 1°, 2° or 3°. Vulval cell fate specification occurs in response to the combined effect of multiple signaling pathways, including the RTK/Ras/MAPK (Receptor Tyrosine Kinase–Ras–Mitogen Activated Protein Kinase), Wingless (Wnt) and Notch cascades. At the beginning of the L3 larval stage, an inductive signal from the gonadal anchor cell (AC) activates a conserved RTK/Ras pathway in P6.p to promote the primary (1°) vulval fate specification (Sternberg and Horvitz, 1986; Yoo et al., 2004). A canonical Wnt signaling cascade acts in parallel with the inductive RTK/Ras pathway to specify the 1° fate (Gleason et al., 2002). By decreasing the activity of inductive signaling, a LIN-12/Notch-mediated lateral signal ensures that only P6.p, the induced VPC that is closest to the AC, adopts the 1° fate, while the two neighboring VPCs, P(5,7).p, adopt the secondary (2°)

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vulval fate (Greenwald et al., 1983; Ferguson et al., 1987; Sternberg and Horvitz, 1989; Berset et al., 2001; Chen and Greenwald, 2004; Yoo et al., 2004; Greenwald, 2005). The remaining VPCs, P(3,4,8).p, express the non-induced tertiary (3°) fate. Three ligands for lateral signaling, the Delta/Serrate orthologs APX-1, DSL-1 and LAG-2, have been identified, and their transcription has been shown to be initiated or upregulated in the VPCs in response to RTK/Ras signaling (Chen and Greenwald, 2004). These findings imply that direct transcriptional control of lateral signaling by the inductive pathways participates in the coordination of these cell signaling events.

The *C. elegans* HOX protein LIN-39, which is similar to *Drosophila* Antennapedia (Antp) and Deformed (Dfd), and mammalian HoxD4, plays a central role in the specification of vulval cell fates. LIN-39 functions downstream of and integrates the inputs from both the inductive Ras and Wnt signaling pathways (Clark et al., 1993; Clandinin et al., 1997; Maloof and Kenyon, 1998; Eisenmann et al., 1998; Chen and Han, 2001; Gleason et al., 2002). The expression of *lin-39* in the VPCs is negatively regulated by the ETS transcription factor LIN-1, whose activity in turn is influenced by the RTK/Ras and synMuv pathways (Maloof and Kenyon, 1998; Leight et al., 2005). LIN-39 forms a complex and acts with CEH-20 (Liu and Fire, 2000; Koh et al., 2002), a HOX cofactor that is orthologous to the human proto-oncogene PBX1–3 and the *Drosophila* Extradenticle proteins (Ryoo et al., 1999). These HOX cofactors are known to increase the DNA-binding specificity of HOX proteins. CEH-20, together with LIN-39, has been demonstrated to bind *in vitro* to a target promoter (Liu and Fire, 2000) and control several steps of vulval development (Shemer and Podbilewicz, 2002; Yang et al., 2005).

In this study, we have characterized vulval development in *ceh-20* and *lin-39* reduction-of-function mutants. We show that reduced activity of *ceh-20* and *lin-39* results in compromised LIN-12/Notch-mediated lateral signaling. Furthermore, inactivation of either *ceh-20* or *lin-39* suppresses the multivulva (Muv) phenotype of *lin-12*(*n137*) gain-of-function mutant animals. We also demonstrate that LIN-39 and CEH-20 are required for the expression of LIN-12 and its ligand LAG-2 in the VPCs prior to and during vulval induction. Our results identify a transcriptional control of Notch signaling by the HOX protein LIN-39 and its PBX/EXD-like cofactor CEH-20 during *C. elegans* vulval development.

Materials and methods

Nematode strains and mutant alleles

C. elegans strains were maintained as described (Brenner, 1974). The wild-type strain was *C. elegans* var. Bristol N2. Other strains were as follows: NH2106 *ceh-20*(*ay9*)III, NH2241 *ceh-20*(*ay34*)III, NH2285 *ceh-20*(*ay38*)*unc-36*(*e251*)III; sDp3(III;f), NH2286 *ceh-20*(*ay37*)*unc-36*(*e251*)III; sDp3(III;f), NH2332 *ceh-20*(*ay42*)*unc-36*(*e251*)III, MT4491 *lin-39*(*n1872*)III, MT4007 *lin-39*(*n1760*)III, MT2375 *lin-12*(*n137*)*dpy-19*(*e1259*)/*lin-12*(*n676n909*)*unc-32*(*e189*)III; *him-5*(*e1467*)V, SU93 *jcIs1*[*ajm-1*::*gfp*+*rol-6*(*su1006*)+*unc-29*(+)] IV, NL1008 *pkEx246*[*cdh-3*::*gfp*+*dpy*-20(+)]; *dpy-20*(*e1362*)IV, AH24 *gaEx*[*lin-3*::*gfp*+*unc-119*(+)]; *unc-119*(*e2498*)III, JK2868 *qIs56*[*lag-2*::*gfp*+*unc-119*(+)]IV or V; *unc-119*(*ed3*)III, GS956 *arIs11*[*lin-12*::*lacZ*+*rol-6*(*su1006*)]III; *smg-1*(*r861*)*unc-54*(*r293*)I, FR849 *swEx576*[*hs*::*lag-2*+*rol-6*(*su1006*)], GS2146 *arIs41*[*lin-12*::*gfp*+*rol-6*(*su1006*)], NH646 *ayIs9*[*egl-17*::*gfp*+*dpy-20*(+)]V; *dpy-20*(*e1282ts*)IV, FR826 *ceh-20*(*ay9*)*lin-12*(*n137*)*dpy-19*(*e1259*)III, FR791 *swEx543*[*ceh-20*::*gfp*+*rol-6*(*su1006*)], FR811 *swEx543*[*ceh-20*::*gfp*+*rol-6*(*su1006*)]; *ceh-20*(*ay38*)*unc-36*(*e251*)III.

Identification of mutations in ceh-20

In a screen for egg laying-defective (Egl) mutants with sex myoblast migration (SM) defects (Burdine et al., 1997), the mutations *ay9* and *ay13* were found to confer a similar type of vulval defect and failed to complement. *ay9* was mapped to a region of chromosome III within 0.05 map units of *mec-14*. Using a set of overlapping cosmid clones in this region (kindly provided by A. Coulson), we tested those neighboring the *mec-4* clone (TU#LM14) for their ability to rescue the Egl phenotype of *ay9* hermaphrodites in germ-line transformation rescue assays (Mello et al., 1991). Efficient rescue activity was observed with the cosmid F31E3. Furthermore, transformation rescue activity could be narrowed down to a 3.6 kb fragment of F31E3. This fragment contains essentially only the entire coding region of the previously identified *ceh-20* gene, along with approximately 1 kb of promoter region. *ceh-20* encodes the *C. elegans* ortholog of the *Drosophila* homeodomain-containing protein EXD (Bürglin and Ruvkun, 1992). To isolate additional alleles of *ceh-20*, a screen was performed for mutations that fail to complement the Egl phenotype of *ay9*. Since animals bearing *ceh-20*(*ay9*) in *trans* to the deficiency *nDf16* are viable, fertile and Egl, null mutations can be identified in such a non-complementation screen. From animals representing approximately 37,000 ethylmethanesulfonate-mutagenized genomes, six alleles, *ay34*, *ay35*, *ay36*, *ay37*, *ay38* and *ay42*, were isolated (Table 1). An additional *ceh-20* allele, *n2513*, was isolated by K. Kornfeld in a screen for suppressors of the multivulva (Muv) phenotype caused by an activated *let-60 ras* mutation. According to genetic and molecular criteria, seven mutations, *ay9*, *ay13*, *ay34*, *ay35*, *ay36*, *ay42* and *n2513*, are reduction-of-function mutations, while two alleles, *ay37* and *ay38*, appear to be null mutations that arrest development at the L2 larval stage. Sequence alterations corresponding to these mutations served to confirm that these are alleles of *ceh-20* and to explain the various degrees of severity of the Ceh-20 phenotype (Table 2). *ay9*, *ay35* and *ay36* are in regions upstream of the homeodomain of CEH-20, while *ay34* and *ay42* are within the homeodomain. The lesion associated with *ay9* is a missense mutation (M78I; Table 2; Fig. 1A) which changes a residue that is invariant between the CEH-20, EXD and PBX proteins (data not shown). This residue has been shown in vitro to be necessary for cooperative DNA binding between PBX-1 and its HOX dimerization partner (Lu and Kamps, 1996). The mutation *ay38* introduces a stop codon in the second exon (Q102-STOP; Table 2; Fig. 1A) and is predicted to truncate CEH-20 severely, consistent with its genetic classification as a null allele (data not shown). No sequence alterations were found in any of the *ceh-20* coding region for *n2513*, suggesting that its effects may be due to decreased levels of either CEH-20 RNA or protein.

Transgenic animals and GFP analysis

To generate a rescuing translational fusion CEH-20::GFP reporter gene, a 10 kb genomic fragment that contains 7 kb of the *ceh-20* upstream regulatory sequence and almost the complete coding sequence was amplified with the following forward and reverse primers: 5′-ACA TGC ATG CAT GTA AGA

Table 1
ceh-20 alleles define an allelic series

Alleles	Class	Phenotypes
ay9, ay35	I	SM, Egl
ay34, ay36	II	SM, Egl, weak Unc
ay13, ay42, n2513	III	SM, strong Egl, Unc, slow growth
ay37, ay38	IV	Let

SM: sex-myoblast migration-defective; Egl: egg laying-defective; Unc: uncoordinated; Let: lethal.

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Table 2
Molecular lesions associated with *ceh-20* mutations

Alleles	Amino acid alterations
ay9	Met78Ile
ay34	Arg189Cys
ay36	Leu79Ser
ay38	Glu102-STOP
ay42	Pro214Leu

GTG CGG ACG GTA GAG-3′ and 5′-TCC CCC CGG GGG GAA GCA TTG TCC ATT TGT TGT TG-3′. The PCR product was cloned into the expression vector pPD95.75. Standard techniques were used to perform germ-line transformation (Mello et al., 1991). Animals transgenic for *swEx543*[*ceh-20*::*gfp*+*rol-6*(*su1006*)] were crossed with animals of *ceh-20*(*ay38*)*unc-36*(*e251*) III; sDp3(III;f) genotype, and glowing Unc hermaphrodites were selected. For expression analysis, only those L3 larvae were assayed whose vulval lineage appeared normal.

RNA interference

ceh-20 RNA interference (RNAi) was generated by digesting the cDNA clone yk219d9 (gift from Y. Kohara) with *Hin*dIII and *Xho*I and cloning the resulting 955 bp fragment into the vector pPD129.36 (provided by A. Fire). This construct was transformed into the *Escherichia coli* strain HT115. For *lin-39* (*RNAi*), RT-PCR was performed with the following forward and reverse primers: 5′-ATG ACC ACA TCA ACA TCA CCG TCA-3′ and 5′-CTA GAA TTG ATT GAA AAG TGG GAA C-3′. The amplified 0.9 kb cDNA fragment was cloned into pPD129.36. To generate *mab-5*(*RNAi*), RT-PCR was performed with the

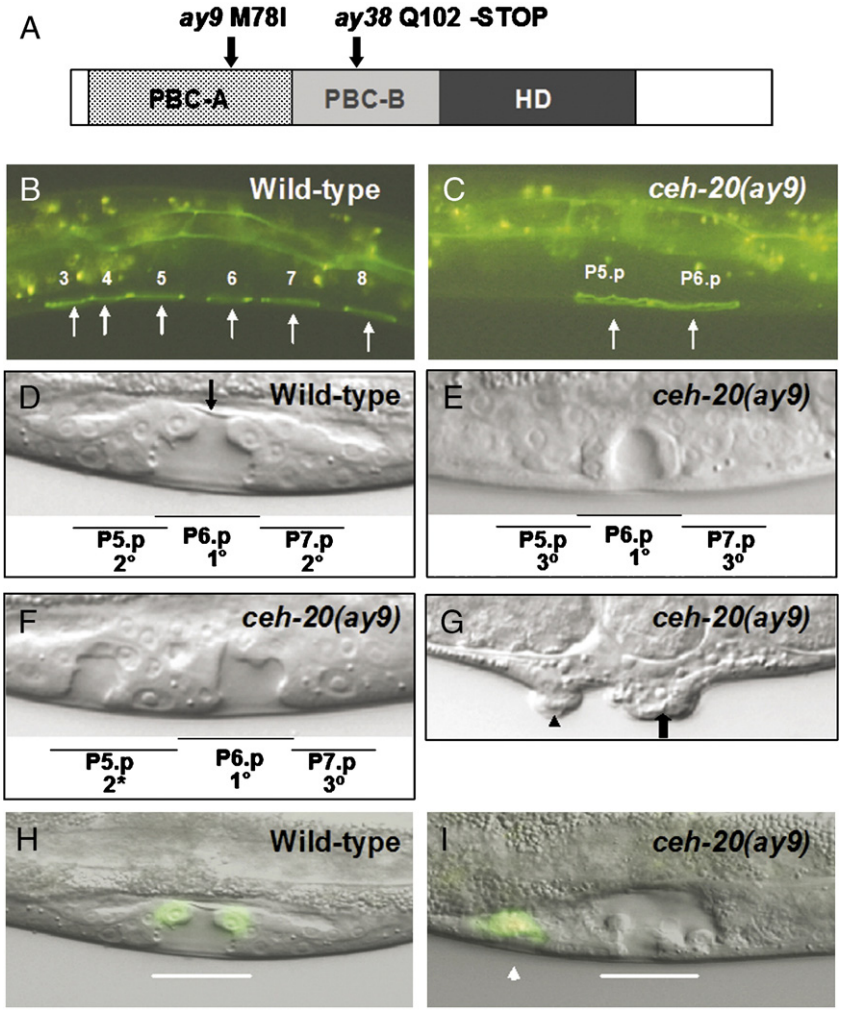

Fig. 1. Reduced activity of *ceh-20* causes various defects in vulval development. (A) The structure of CEH-20. The PBC domains (PBC-A, PBC-B and PBC-homeodomain), which are characteristic of PBC proteins including Pbx (pre-B cell homeobox), Extradenticle and CEH-20, are indicated. Arrows point to the site of amino acid alterations caused by the *ay9* and *ay38* mutations. (B) *ajm-1p*::*gfp* expression in a wild-type L1 larva. The P(3–8).p cells (VPCs) are indicated by white arrows. All VPC remained unfused. (C) Expression of *ajm-1p*::*gfp* in a *ceh-20*(*ay9*) L1 larva. In this individual, only P5.p and P6.p remained unfused, the other VPCs fused with the hypodermis. (D and E) Vulval invagination in a wild-type and a *ceh-20*(*ay9*) mutant L4 larva. In the wild-type animal, the progeny of P5.p, P6.p and P7.p express the 2°, 1° and 2° fates, respectively. The arrow points to the utse. In the *ceh-20*(*ay9*) animal, both the anterior and posterior 2° vulval cells are missing: P5.p and P7.p remained non-induced, adopting 3° fates. (F and G) Vulva in a *ceh-20*(*ay9*) L4 stage larva and in an adult hermaphrodite. P5.p displays an improperly specified 2° fate (2*). Daughter nuclei of one of the P5.ppx cells adhere to the cuticle, leading to the formation of a second vulval invagination anterior to the major invagination at the normal position. In panel G, the thick arrow points to a protruding vulva, and the arrowhead points to a pseudovulva derived from a misspecified P5.p. (H and I) Expression of the 2° cell fate marker EGL-17::GFP in a wild-type and a *ceh-20*(*ay9*) mutant L4 larva. Bars indicate the position of the normal vulval invagination, the arrowhead points to an ectopic vulval invagination resulting from a misspecified 2° P5.p. In the *ay9* mutant, vulval cells at the normal vulval invagination fail to express EGL-17::GFP.

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following forward and reverse primers: 5′-ATG AGC ATG TAT CCT GGA TGG-3′ and 5′-TCA AGA AGA ATG TTG TTC ATT TTG C-3′. The PCR fragment was cloned into pPD129.36. RNAi experiments were carried out as described (Kamath et al., 2001).

Chromatin immunoprecipitation (ChIP) analysis

ChIP experiments were performed as described by Chu et al. (2002), with some modifications. Briefly, for each reaction, 3 mg of total protein from wild-type and *lin-39*(*n1760*) mutant animals was incubated with LIN-39 antibody (kindly provided by C. Kenyon; Maloof and Kenyon, 1998) overnight at 4°C. Mixed stage worms were fixed in M9 buffer with 2% formaldehyde at room temperature for 30 min. Excess formaldehyde was quenched and removed with a 0.1 M Tris–HCl (pH 7.5). Worms were then washed with M9 buffer. Lysates were prepared by sonication in lysis buffer (Upstate) containing protease inhibitor cocktail (Roche). Cellular debris was cleared by centrifugation. Lysates were sonicated further, centrifuged and pre-cleared against Protein A agarose (Upstate) or Protein A Sepharose. Lysates were divided equally and incubated with 10 μg of affinity-purified antibodies or 10 μg of normal rabbit serum overnight. After clearing non-specific aggregates by centrifugation, the immunocomplexes were captured with Protein A agarose or Sepharose, subjected to three washes in 1 ml ChIP buffer and two washes in 1 ml TE buffer (Upstate) and finally eluted with 1% SDS, 0.01 M Tris–HCl (pH 8.0). For ChIP analysis, formaldehyde crosslinks were reversed by incubation in 0.2 M NaCl at 65°C overnight. Proteins were removed by proteinase K digestion, and DNA was purified with QIAquick PCR purification kit (QIAGEN). For input DNA control, DNA was extracted from 2% of starting lysates. PCR amplifications were carried out with 2 μl of precipitated DNA. PCR amplification cycle was generally adjusted until no or very weak signal was detected for the mock-IP DNA (no antibody). The primers were as follows: (320 bp fragment) 5′-ggt tga aga caa atg ggt gt-3′ and 5′-gcc gtt ttc caa aat ttc c-3′; (581 bp) 5′-ttg gaa aat ttg gaa atg cac-3′ and 5′-gtt tga aat tgc ccc aca ag-3′; (164 bp) 5′-ggg ctc tcg gta tct gtt cc-3′ and 5′-acc taa gcg gct gag cac at-3′. The multiplex PCR was performed using QIAGEN multiplex kit.

Results

CEH-20 and LIN-39 are required for the specification of the 2° vulval cell fate

In a previous screen for sex-myoblast-defective mutants, we isolated a number of mutations in *ceh-20*, including a putative null allele, *ay38*, and a reduction-of-function mutation, *ay9* (Tables 1 and 2; Fig. 1A; Materials and methods). *ceh-20* null mutants arrest development at the L2 larval stage, preventing assessment of the role of CEH-20 in later developmental events. However, characterization of *ceh-20*(*ay9*) mutant animals revealed a number of defects in vulval development. Consistent with this characterization of *ceh-20*(*ay9*), *ceh-20*(*RNAi*) and stronger *ceh-20* alleles, including *ay42*, caused a similar set of vulval abnormalities (Table 3 and data not shown). One of the earliest roles of CEH-20 in vulval development was in inhibiting early VPC fusion. In a set of 63 *ay9* hermaphrodites carrying a tight-junction reporter transgene (*ajm-1p*::*gfp*, Mohler et al., 1998), 5% (3/63) had at least one of the VPCs fused abnormally with the hypodermal syncytium hyp7 during the L1/L2 larval stages (Figs. 1B and C). Animals hemizygous for *ay9* (*ay9/nDf16*) showed a dramatically higher percentage of early VPC fusion (data not shown), implying that CEH-20, like LIN-39, inhibits fusion of the VPCs at the L1/L2 stage.

CEH-20 also serves a later function that preserves the competence of the VPCs to acquire induced vulval fates, with significantly more pronounced effects on 2° fates (Tables 3 and 4). Thus, in the original set of 63 animals, P5.p and P7.p adopted a non-induced 3° fate in 74% (47/63) of *ceh-20*(*ay9*) hermaphrodites, while P6.p failed to undergo induction in only 4.8% of *ceh-20*(*ay9*) animals (Figs. 1D and E; Tables 3 and 4). The absence of 2° cells could not be explained as a consequence of early cell fusion defects because the VPCs remained unfused from the hypodermis in the majority (~95%) of *ay9* animals.

ceh-20(*ay9*) mutant hermaphrodites have additional 2° fate lineage abnormalities as well. In the original set of 63 animals analyzed, 11% (7/63) had an improperly specified 2° fate in either P5.p or P7.p, accompanied by an ectopic vulval invagination (Figs. 1F, G and I). In these *ay9* mutants, the major vulval invagination at the normal position failed to express the 2° vulval cell fate marker egl-17p ::*gfp* (Burdine et al., 1998) at the L4 larval stage (Fig. 1I). RNAi-mediated inactivation of *ceh-20* could also cause an Muv phenotype. In *ceh-20*(*RNAi*) hermaphrodites, P(3,4,8).p occasionally adopted an induced fate, resulting in a low penetrance Muv phenotype (9.5%, $n=44$). Ectopic induction of P(3,4,8).p was recently observed in animals bearing a different non-null *ceh-20* allele, *mu290* (Yang et al., 2005).

The failure to execute a proper 2° fate in *ay9* hermaphrodites suggests a role for CEH-20 in this cell specification event. To test whether LIN-39 is also required, we examined vulval development in nematodes bearing the *lin-39* thermosensitive mutation *n1872ts* (Clark et al., 1993). In 16% (9/57) of *lin-39*

Table 3
Reduced activity of *ceh-20* or *lin-39* causes a failure to execute the 2° vulval cell fate

Genotype	% induction of individual VPCs						
	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	*n*
Wild-type	0	0	100	100	100	0	60
ceh-20(*ay9*)	0	0	20.7	95.2	9.5	0	63
ceh-20(*RNAi*)	4.5	9	36.3	72.7	27.3	13.6	44
lin-39(*n1872ts*) (25°C)[a]	0	0	0	0	0	0	60
lin-39(*n1872ts*) (15°C)	0	0	68.4	89.4	42	0	57

[a] The VPCs fuse with the hypodermis at the L1/L2 larval stages.

Table 4
Vulval lineage of *ceh-20*(*ay9*) mutant individuals

Genotype	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	*n*
Wild type	SS or F	SS	LLTN	TTTT	NTLL	SS	Many
ceh-20(*ay9*)	SS	SS	LOOL	TTTL	SS	SS	1
ceh-20(*ay9*)	F	SS	OTNL	OTTO	SS	SS	1
ceh-20(*ay9*)	SS	SS	SS	TTOL	SS	F	1
ceh-20(*ay9*)	F	SS	OOLN	TOTL	SS	SS	1

Pn.p lineages are indicated following the nomenclature of Sternberg and Horvitz (1989). Letters represent the type of final cell division or cell fate adopted by Pn.p descendants; T, cells divide along the transverse (left–right) axis and daughters contributed to vulval formation; L, cells divide longitudinally (anterior–posterior) and daughters contribute to vulva formation; N, cells do not divide; O, cells divide obliquely and daughters contribute to vulva formation; S, cells divide ones and daughters fuse with the hypodermis; F, cells do not divide and fuse with the hypodermis.

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(*n1872*) hermaphrodites maintained at the permissive temperature, P5.p and P7.p adopted the non-induced 3° fate (Table 3). Interestingly, ectopic vulval invagination and Muv phenotypes caused by improperly specified 2° fates were also apparent in 5.6% (10/178) of *lin-39*(*n1872*) mutants. A similar 2° cell fate misspecification was observed in genetic mosaic animals in which the function of LIN-39 was lost in some of the Pn.p lineages (Clark et al., 1993). Together, these data suggest that the transcription factor LIN-39 and its cofactor CEH-20 are required to establish 2° fates in P(5,7).p.

Table 5
Inactivation of *ceh-20* or *lin-39* suppresses the Multivulva (Muv) phenotype of *lin-12*(*n137*) gain-of-function mutants

Genotype	% Muv	Average number of vulval invaginations	*n*
Wild type	0	1	20
lin-12(*n137*)	100	5.1	60
lin-12(*n137*) *ceh-20*(*ay9*)	14.7	1.53	68
lin-12(*n137*) *lin-39*(*RNAi*)	33.3	2.73	60

The number of vulval invagination was scored at the L4 larval stage.

CEH-20 and LIN-39 are required for several aspects of lin-12 signaling

Since establishment of the 2° vulval fate, which depends on LIN-12/Notch transmembrane receptor-mediated signaling (Greenwald et al., 1983; Sternberg and Horvitz, 1986, 1989; Sternberg, 1988; Greenwald, 2005), requires normal LIN-39 and CEH-20 activities, we examined whether the activity of LIN-39 and CEH-20 is also required more generally for *lin-12* signaling. Two cell fate abnormalities in the somatic gonad are characteristic of defects in *lin-12* signaling. One of these affects the number of ACs (Greenwald et al., 1983; Wilkinson et al., 1994). We found that 14% (9/66) of *ceh-20* (*ay9*) larvae have two gonadal cells that expressed the AC marker LIN-3::GFP reporter versus the normal single cell found in wild-type animals (Hill and Sternberg, 1992; Figs. 2A and B). Similar results were obtained using another AC marker, CDH-3::GFP (Pettitt et al., 1996; Figs. 2C and D). Interestingly, a dual AC phenotype was not observed in *lin-39*(*n1872*) mutant and *lin-39*(*RNAi*) larvae, suggesting that the AC fate decision might be controlled by a *C. elegans* HOX protein other than LIN-39. Indeed, RNAi-mediated depletion of MAB-5, the closest paralog of LIN-39 (Kenyon, 1986, 1993), resulted in two CDH-3::GFP positive gonadal cells in 4.7% of the treated animals ($n=85$).

The second characteristic feature of the pleiotropic Lin-12 loss-of-function phenotype in the gonad is the uterine π cell misspecification that causes an abnormal uterine–vulval connection (Newman et al., 1995). A diaphragm called the utse, which separates the uterus from the vulva at the L4 stage, is an anatomical marker for a normal π cell lineage (Fig. 1D). The lack of utse formation was evident in both *ceh-20*(*ay9*) (72.9%, $n=85$) and *lin-39*(*n1872ts*) (5.4%, $n=55$ at 15°C) mutants (Fig. 1E).

We next assayed the effect of *ceh-20*(*ay9*) and *lin-39*(*RNAi*) on the Muv phenotype of *lin-12*(*n137*) gain-of-function mutants. *lin-12*(*n137*) animals have multiple pseudovulvae resulting from the ectopic expression of 2° fates by each VPC (Greenwald et al., 1983). In contrast, the Muv phenotype was significantly suppressed in *ceh-20*(*ay9*) *lin-12*(*n137*) double mutants (Table 5), which exhibited either a wild-type or a Lin-12-like protruded (Pvl) vulva phenotype. *lin-39*RNAi treatment similarly suppressed the Muv phenotype of *lin-12*(*n137*) mutants (Table 5), causing most of the VPCs to express a 3° fate (data not shown). Inactivation of *lin-39* and *ceh-20* may be suppressing the *lin-12* Muv phenotype by acting either upstream or downstream of *lin-12* and/or other components of lateral signaling. Thus, the activity of LIN-39 and CEH-20 is critical for *lin-12*(*n137*) signaling.

CEH-20 accumulates in the VPCs

To monitor the localization of CEH-20 in developing vulval tissue, we generated a translational fusion CEH-20::GFP reporter, *swEx543* (Fig. 3A), that is able to rescue the early larval arrest phenotype caused by the putative null allele *ay38*. CEH-20::GFP was expressed in all VPCs prior to and during vulval induction (Fig. 3B). This expression pattern is

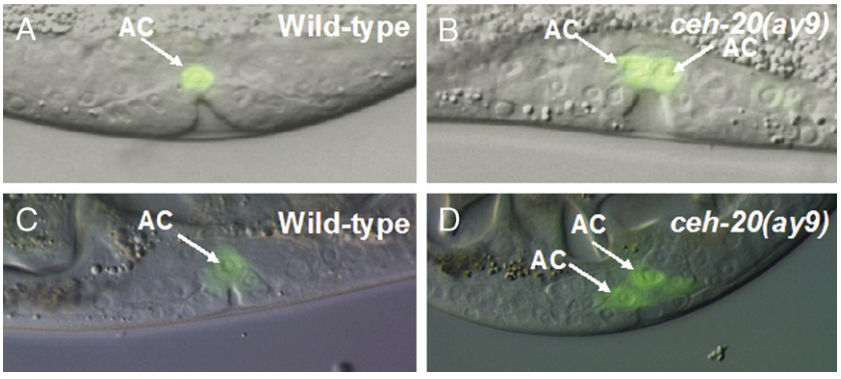

Fig. 2. *ceh-20* activity is required for *lin-12* signaling. (A and B) Expression of the gonadal anchor cell (AC) marker LIN-3::GFP in a wild-type and a *ceh-20*(*ay9*) mutant L4 larva (overlay of Nomarski and fluorescence images). Arrows indicate the ACs. (C and D) Expression of another AC marker, CDH-3::GFP, in a wild-type and a *ceh-20*(*ay9*) mutant L4 larva.

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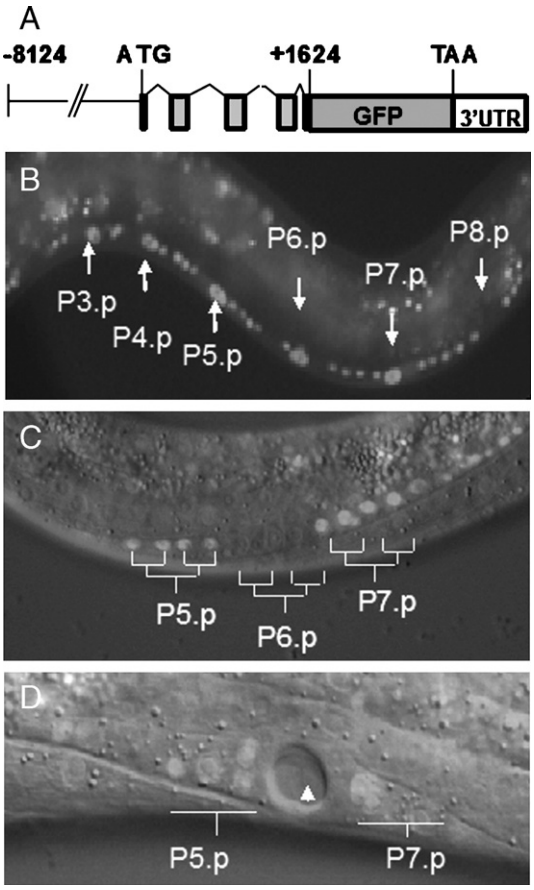

Fig. 3. CEH-20 accumulation in the vulval lineage. (A) Structure of a CEH-20::GFP translational fusion reporter, *swEx543*, that can rescue both the lethality of *ceh-20*(*ay38*) null mutants and the egg laying-defective phenotype of *ay9* mutants. The 3′ UTR is derived from the *unc-54* gene. (B) CEH-20::GFP accumulates in the VPCs (arrows) at the L3 larval stage. In this specimen, P8.p fails to express CEH-20::GFP, due to the mosaicism of the non-integrated *swEx543* transgene. (C) CEH-20::GFP accumulation in the VPC descendants at the Pn.pxx stages. GFP is detectable in the P5.p and P7.p lineages, but not in the P6.p lineage. (D) CEH-20::GFP accumulation in an L4 stage hermaphrodite (ventral view). The arrowhead indicates the vulval invagination.

consistent with that observed previously for LIN-39 and CEH-20 (Maloof and Kenyon, 1998; Yang et al., 2005). Although to date there is no direct evidence for the existence of a LIN-39/CEH-20 complex in the VPCs, the co-expression of these two proteins in these cells suggests that they function together in specifying vulval fates before and at the time of vulval induction. After VPC division, CEH-20::GFP accumulation became restricted to the P5.p and P7.p lineages, where it remained apparent until the late L4 larval stage (Figs. 3C and D). The fact that CEH-20 is not expressed in the descendants of P6.p while LIN-39 remains activated implies that LIN-39 may act independently of CEH-20 in these cells in controlling certain aspects of vulval development.

CEH-20 and LIN-39 influence the expression of lin-12 in the VPCs

LIN-12 accumulates initially in all six VPCs until the mid-L3 stage, but becomes reduced in P6.p at the time of vulval induction as a consequence of inductive signaling (Wilkinson and Greenwald, 1995; Levitan and Greenwald, 1998). To address whether LIN-39 and CEH-20 influence *lin-12* expression in the VPCs, we compared the expression of a transcriptional *lin-12*::*lacZ* reporter, *arIs11* (Wilkinson and Greenwald, 1995), in wild-type vs. *ceh-20* and *lin-39* mutant animals. In wild-type hermaphrodites, *lin-12*::*lacZ* was expressed in all VPCs prior to and at the time of vulval induction (Fig. 4A). In contrast, *lin-12*::*lacZ* expression was almost completely abolished from the VPCs in both *ceh-20* (*RNAi*) and *lin-39*(*RNAi*) L3 larvae (Fig. 4B and data not shown). Similar results were obtained by monitoring a translational fusion LIN-12::GFP reporter, *arIs41* (Fig. 4C). These results indicate that LIN-39 and CEH-20 regulate the expression of *lin-12* in the VPCs before and during vulval induction.

The expression of lag-2 in the VPCs also depends on CEH-20 and LIN-39 activity

lag-2, a *C. elegans* Delta/Serrate ortholog, has been suggested to encode a ligand for LIN-12 during 2° VPC fate specification (Chen and Greenwald, 2004). Using an integrated transcriptional *lag-2*::*gfp* reporter, *qIs56*, we studied the expression of *lag-2* in the developing vulval tissue. In wild-

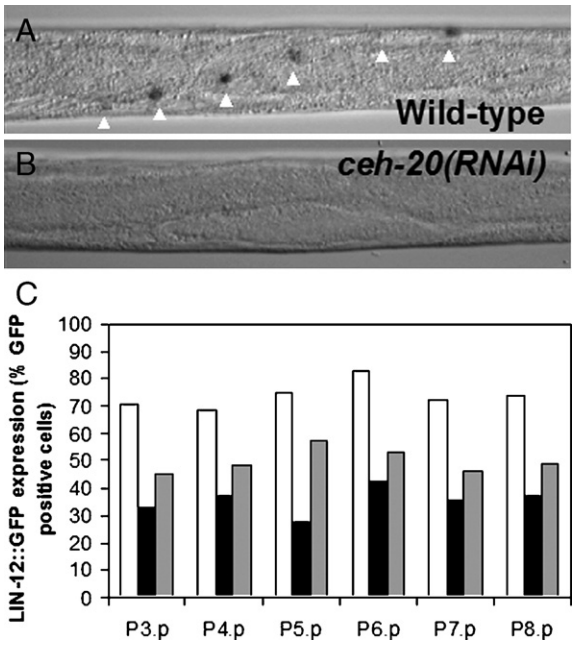

Fig. 4. Expression of *lin-12* in the VPCs requires the activity of LIN-39 and CEH-20. (A and B) Expression of a transcriptional fusion *lin-12*::*lacZ* reporter, *arIs11*, in a wild-type and a *ceh-20*(*RNAi*) L3 larva. Arrowheads indicate the VPCs. The absence of lin-12::*lacZ* expression in the VPCs was fully penetrant upon *ceh-20* dsRNA treatment. (C) LIN-12::GFP accumulation in the VPCs in wild-type (empty bars), *ceh-20*(*ay9*) mutant (black bars) and *lin-39*(*n1872*) mutant (gray bars) L3 stage hermaphrodites. *lin-39*(*n1872*) mutant animals were maintained at 20 °C. The weak signal of LIN-12::GFP fluorescence (*arIs41*) was enhanced by staining with anti-GFP antibody (Levitan and Greenwald, 1998). $n=40$ (only those animals in which at least one VPC was GFP positive were scored).

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type worms, *lag-2*::*gfp*was continuously expressed in all VPCs prior to vulval induction, but its expression became restricted to P6.p at the time of vulval induction (Figs. 5A and B; see also Chen and Greenwald, 2004). These data prompted us to investigate whether the expression of *lag-2* is also influenced by LIN-39 and/or CEH-20 activity. We found that both *ceh-20* (*ay9*) and *lin-39*(*n1872*) mutations significantly diminished the expression of *lag-2*::*gfp* in the VPCs prior to vulval induction, and also in the P6.p descendants at the time inferred to be after vulval induction (Figs. 5C, D, E and F; data not shown). This implies that LIN-39 and CEH-20 are required for the basal expression of *lag-2* in the VPCs. A similar dual control of the Notch receptor and its ligand(s) has been reported from *Drosophila*; the Ras/MAPK pathway induces both Notch and Delta expression during specification of muscle progenitors (Carmena et al., 2002).

LIN-39 binds to the lag-2 promoter in vivo

To test whether LIN-39 and CEH-20 proteins are able to bind to the regulatory sequences of *lag-2*, we first searched for canonical ANTP/EXD binding sites (TGATNNAT) (Ryoo et al., 1999; Liu and Fire, 2000) within 6 kb upstream of the ATG translation initiation site. We identified three ANTP/EXD binding sites that are located at 5505, 2924 and 1705 base pairs upstream from the ATG site (Fig. 6A). Next, we checked the ability of the LIN-39 antibody to bind to these sites by performing a chromatin immunoprecipitation (ChIP) assay. We found that two of these sites, at positions 5505 and 2924, specifically associated with LIN-39 (Fig. 6B). In addition, the two sites are conserved between *C. elegans* and *C. briggsae* (Fig. 6C). Together, our results demonstrate a physical interaction of the endogenous LIN-39 protein with the *lag-2* promoter, suggesting that these regulatory regions serve as direct targets of the transcription factor LIN-39/HOX.

Discussion

Convergent intercellular signals must be precisely coordinated in order to elicit specific biological responses. The *C. elegans* vulva provides an excellent experimental microcosm for studying how cell fate is specified according to the combined effects of different signaling pathways. Here we

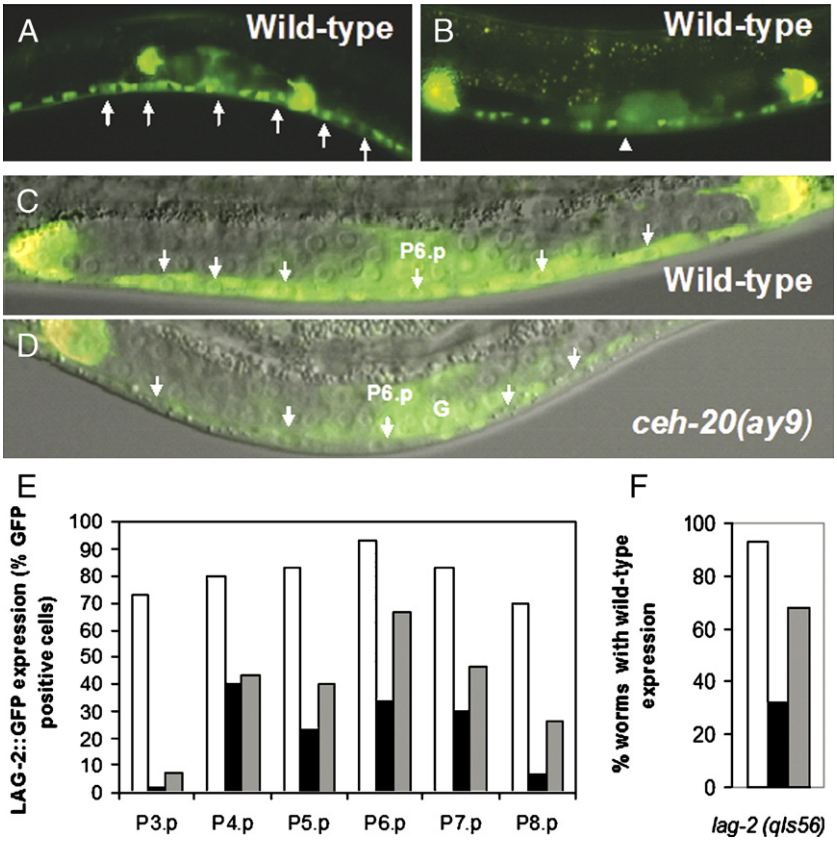

Fig. 5. The activity of CEH-20 and LIN-39 influences *lag-2* expression in the VPCs. (A and B) Expression of a *lag-2*::*gfp* reporter, *qIs56*, in the VPCs prior to and after vulval induction. Arrows point to the GFP-positive VPCs. (C and D) *lag-2*::*gfp* expression in a wild-type and a *ceh-20*(*ay9*) mutant hermaphrodite prior to vulval induction. GFP is detectable in all VPCs (indicated by white arrows) in the wild-type animal. The VPCs do not express *lag-2*::*gfp* in the *ay9* genetic background. An overall decrease in *lag-2*::*gfp* expression is also evident in the gonad (indicated by "G") and in the ventral nerve cord (overlay of Nomarski and fluorescent pictures). (E) Prior to vulval induction, *lag-2*::*gfp* expression in the VPCs is influenced by CEH-20 and LIN-39 activity. The penetrance of *lag-2*::*gfp* expression was scored in individual VPCs in wild-type (empty bars), *ceh-20*(*ay9*) mutant (black bars) and *lin-39*(*n1872ts*) mutant (gray bars) animals at the early L3 larval stage. $n=45$. (F) Expression of *lag-2* is specifically reduced in the descendants of P6.p at the Pn.px stage in *ceh-20*(*ay9*) (the black bar, $n=45$) and *lin-39*(*n1872*) (the gray bar, $n=52$) mutant backgrounds, as compared with wild-type animals (the empty bar). *lin-39*(*n1872*) mutants were maintained at 15°C.

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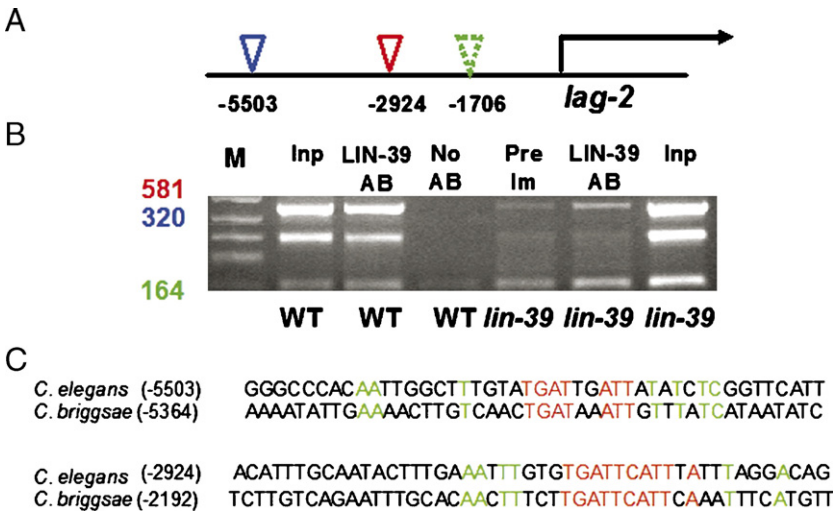

Fig. 6. LIN-39 binds to the *lag-2* promoter *in vivo*. (A) Putative Antp/EXD binding sites in the *lag-2* promoter, indicated by the colored triangles. The numbers indicate the relative positions of these sites from the ATG translational initiation site (the arrow). (B) Chromatin immunoprecipitation (ChIP) experiment using LIN-39 antibody. M: molecular weight marker, Inp: input, LIN-39 AB: LIN-39 antibody, No AB: no antibody, Pre Im: pre-immune serum, WT: wild-type, *lin-39*: *lin-39* (*n1760*) genetic null mutants. The 581- and 320-bp-long fragments from the *lag-2* regulatory region are specifically co-immunoprecipitated with LIN-39, while the 164 bp band (indicated by a green, dotted triangle) seems to be non-specific. (C) Putative ANTP/EXD binding sites at positions −5503 and −2924 positions show sequence conservation to the *C. briggsae lag-2* promoter. The putative binding sites are underlined; conserved nucleotides within the site are indicated by red coloring, conserved nucleotides near to the site are indicated by green coloring.

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have studied the role of the *Hox* gene *lin-39* and the *Exd* ortholog *ceh-20* in vulval development. We present genetic and molecular evidence that the HOX protein LIN-39 and its putative cofactor CEH-20 are required for basal expression levels of *lin-12* and *lag-2* in the VPCs prior to vulval induction; this regulation may be important to render the VPCs competent for the subsequent *lin-12/Notch* induction events at the L3 larval stage. Identifying transcriptional regulators of lateral signaling in *C. elegans* vulval development will be essential for understanding how the Notch signaling pathway specifies cell fate in divergent animal species, and how compromised Notch signaling leads to human diseases (Kimble and Simpson, 1997; Artavanis-Tsakonas et al., 1999).

We also show that LIN-39 and CEH-20 are both required at the first larval stage to prevent fusion of the VPCs to the surrounding hypodermis. Our data lead to the attractive possibility that LIN-39 and its putative cofactor CEH-20 regulate the competence of the VPCs to respond to any of the patterning signals during vulval formation. Along this line, it is challenging to speculate that, besides regulating *lin-12* and *lag-2* expression, they might also promote the expression of components of the inductive pathway (such as *let-23*) or other Notch pathway genes in the VPCs.

It has been shown that CEH-20 binds *in vitro*, together with LIN-39, to the promoter of the twist transcription factor ortholog *hlh-8* to regulate its expression in postembryonic mesodermal cells (Liu and Fire, 2000). Our ChIP experiments demonstrate that LIN-39 associates with the *lag-2*promoter, suggesting that the regulation of *lag-2* expression by LIN-39 may be direct. We propose that LIN-39 forms a heterodimer with CEH-20 to promote the basal transcription of *lag-2* and *lin-12* in the VPCs. Based on their different expression pattern in the Pn.p lineages, *ceh-20* is assumed to have some functions that are independent of *lin-39* (Yang et al., 2005; this study). Indeed, *mab-5* has been shown to be expressed in the descendants of the posterior VPCs, P7.p and P8.p, and to prevent them from adopting an induced vulval fate (Clandinin et al., 1997). Thus, it is possible that CEH-20 also interacts and functions with MAB-5 in controlling certain aspects of vulval fate specification. Furthermore, we noted that *ceh-20* (*ay9*) mutant animals sometimes displayed a dual AC phenotype, whereas *lin-39* mutants never did. RNAi-mediated depletion of *mab-5* sometimes resulted in 2 ACs, suggesting that the correct AC specification requires the combined activity of *mab-5* and *ceh-20*.

Finally, CEH-20 has been shown to be required as a cofactor for autoregulatory expression of the anterior *Hox* paralog (*labial*-like) *ceh-13* in embryonic cells (Streit et al., 2002). Because *ceh-13* is expressed all along the anteroposterior body axis in the ventral mid-line during the L1–L4 larval stages (Brunschwig et al., 1999) and a few percent of the *ceh-13*(*sw1*) mutant animals that are able to develop into fertile adults exhibit various defects in vulval formation (Vellai et al. unpublished results), it is possible that CEH-13 acts with CEH-20 to control cell fate in the anterior VPC lineages. The future analysis of a potential role of *ceh-13* in vulval development would help to establish the role of all of the major body *Hox* genes in this important process.

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