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*/*Notch* 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/EXD/PBX; 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°)
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, DSI-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 (Müller and Ruvkun, 2000). LIN-39 and CEH-20 are required for the expression of LIN-12 and its ligand LAG-2 in animals. We also demonstrate that LIN-39 and CEH-20 are identified a transcriptional control of Notch signaling by the HOX 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 EXD phenotype of *ay9*. Since animals bearing *ceh-20*(+*ay9*) in trans to the deficiency *n216* are viable, fertile and Egl, null mutations can be identified in such a non-complementation screen. From animals representing approximately 37,000 ethylmethane-sulfonate-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. Komfeld 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, *ay17* 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 (MT81; 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 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 regions 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 1 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>
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<tr>
<th>Alleles</th>
<th>Class</th>
<th>Phenotypes</th>
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<tr>
<td><em>ay9</em>, <em>ay35</em></td>
<td>I</td>
<td>SM, Egl</td>
</tr>
<tr>
<td><em>ay34</em>, <em>ay36</em></td>
<td>II</td>
<td>SM, Egl, weak Unc</td>
</tr>
<tr>
<td><em>ay13</em>, <em>ay42</em>, <em>n2513</em></td>
<td>III</td>
<td>SM, strong Egl, Unc, slow growth</td>
</tr>
<tr>
<td><em>ay37</em>, <em>ay38</em></td>
<td>IV</td>
<td>Let</td>
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**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*(ay49)III, NH2241 *ceh-20*(ay34)III, NH2285 *ceh-20*(ay38)unc-36(e25)*35); d*3p3(III); NH2286 *ceh-20*(ay37)unc-36(e25)*31); d*3p3(III); NH2332 *ceh-20*(ay42)unc-36(e25)*35); MT4491 lin-39(n1872)III, MT4007 lin-39(n1760)III, MT2375 lin-12(n137)pvp-19(e1259)lin-12(n676909)unc-32(e189); him-5(e1467)N, SV93 jcs1[ajm-1::gfp+rol-6(+)](+); unc-29(su1006)+; unc-119(+)]; unc-119(e2498)III, JK2868 q*56[:ag-2::gfp+unc-119

(+)] IV

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<th>Alleles</th>
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<th>Phenotypes</th>
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<tr>
<td><em>ay9</em>, <em>ay35</em></td>
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<tr>
<td><em>ay37</em>, <em>ay38</em></td>
<td>IV</td>
<td>Let</td>
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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; Dp(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 HindIII and XhoI 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

<table>
<thead>
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<tr>
<td>ay9</td>
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</tr>
<tr>
<td>ay34</td>
<td>Arg189Cys</td>
</tr>
<tr>
<td>ay36</td>
<td>Leu79Ser</td>
</tr>
<tr>
<td>ay38</td>
<td>Glu102-STOP</td>
</tr>
<tr>
<td>ay42</td>
<td>Pro214Leu</td>
</tr>
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</table>

Table 2

Molecular lesions associated with ceh-20 mutations

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.p 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° fate. In the ay9 mutant, vulval cells at the normal vulval invagination fail to express EGL-17::GFP.
following forward and reverse primers: `5’-ATG AGC ATG TAT CCT GGA TGG-3’` and `5’-TCA AGA AGA ATG TTT TTC ATT ATT GCC-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; Malof 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 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 μM each of forward and reverse primers: 5′-gtg tga aat tgc ccc aca ag-3′ and 5′-acc taa gcg gct gag cac at-3′. The PCR fragment was cloned into pPD129.36. RNAi experiments were carried out as described (Kamath et al., 2001).

**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, `ay9`, 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 `hpy7` 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, `P3(4,8)` occasionally adopted an induced fate, resulting in a low penetrance Muv phenotype (9.5%, `n=44`). Ectopic induction of `P(3,4,8)` 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`
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 marker LIN-3::GFP reporter (Pettitt et al., 1996; Figs. 2C and D). Interestingly, a dual AC phenotype was not observed 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 wild-type animals (Hill and Sternberg, 1992; Figs. 2A and B). The lack of AC 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 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

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Table 5

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Muv</th>
<th>Average number of vulval invaginations</th>
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<tr>
<td>Wild type</td>
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<td>20</td>
</tr>
<tr>
<td>lin-12(n137)</td>
<td>100</td>
<td>5.1</td>
<td>60</td>
</tr>
<tr>
<td>lin-12(n137) ceh-20(ay9)</td>
<td>14.7</td>
<td>1.53</td>
<td>68</td>
</tr>
<tr>
<td>lin-12(n137) lin-39(RNAi)</td>
<td>33.3</td>
<td>2.73</td>
<td>60</td>
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The number of vulval invagination was scored at the L4 larval stage.

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.
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-

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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.

**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).
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...
have studied the role of the Hox gene \textit{lin-39} and the Exd ortholog \textit{ceh-20} in vulval development. We present genetic and molecular evidence that the HOX protein \textit{lin-39} and its putative cofactor \textit{ceh-20} are required for basal expression levels of \textit{lin-12} and \textit{lag-2} in the VPCs prior to vulval induction; this regulation may be important to render the VPCs competent for the subsequent \textit{lin-12}/Notch induction events at the L3 larval stage. Identifying transcriptional regulators of lateral signaling in \textit{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 \textit{lin-39} and \textit{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 \textit{lin-39} and \textit{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 \textit{lin-12} and \textit{lag-2} expression, they might also promote the expression of components of the inductive pathway (such as \textit{let-23}) or other Notch pathway genes in the VPCs.

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

Finally, \textit{ceh-20} has been shown to be required as a cofactor for autoregulatory expression of the anterior \textit{Hox} paralog (\textit{labial-like}) \textit{ceh-13} in embryonic cells (Streit et al., 2002). Because \textit{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 \textit{ceh-13}(\textit{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 \textit{ceh-13} in vulval development would help to establish the role of all of the major body \textit{Hox} genes in this important process.

Acknowledgments

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Fig. 6. \textit{Lin-39} binds to the \textit{lag-2} promoter \textit{in vivo}. (A) Putative Antp/EXD binding sites in the \textit{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 \textit{lin-39} antibody. M: molecular weight marker, Inp: input, \textit{lin-39} AB: \textit{lin-39} antibody, No AB: no antibody, Pre Im: pre-immune serum, WT: wild-type, \textit{lin-39}: \textit{lin-39} (\textit{n1760}) genetic null mutants. The 581- and 320-bp-long fragments from the \textit{lag-2} regulatory region are specifically co-immunoprecipitated with \textit{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 \textit{C. briggsae} \textit{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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