LIN-12/Notch Regulates *lag-1* and *lin-12* Expression during Anchor Cell/Ventral Uterine Precursor Cell Fate Specification

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During *Caenorhabditis elegans* gonadal development, a stochastic interaction between the LIN-12/Notch receptor and the LAG-2/Delta ligand initiates cell fate specification of two equivalent pre-anchor cell (AC)/pre-ventral uterine (VU) precursor cells. Both cells express *lin-12* and *lag-2* before specification, and a small difference in LIN-12 activity leads to the exclusive expression of *lin-12* in VUs and *lag-2* in the AC through an unknown feedback mechanism. In this Notch signaling process, the cleaved LIN-12/Notch intracellular domain (NICD) binds to the LAG-1/CSL transcriptional repressor, forming a transcriptional activator complex containing LAG-1 and NICD. Here we show that clustered LAG-1 binding sites in *lin-12* and *lag-1* are involved in regulating *lin-12* and *lag-1* expression during AC/VU cell fate specification. Both genes are expressed in VU cells, but not the AC, after specification. We also show that *lin-12* is necessary for *lag-1* expression in VU cells. Interestingly, *lin-12* (*null*) animals express *lag-1* in the AC, suggesting that LIN-12 signaling is necessary for the suppression of *lag-1* expression in the AC. Ectopic expression of *lag-1* cDNA in the AC causes a defect in the vulval-uterine (V-U) connection; therefore, LAG-1 should be eliminated in the AC to form a normal V-U connection at a later developmental stage in wild-type animals.

INTRODUCTION

Notch signaling mediates cell-cell interactions of neighboring cells through the interaction between membrane-bound ligand and receptor proteins (Artavanis-Tsakonas et al., 1999). Thus, it is often involved in specification of fates of cells that are adjacent to each other and originate from common ancestral cells. In some cases, a stochastic difference or any small increase in Notch activity is amplified through feedback loops, which establishes stable states from which cell fate cannot be reverted (Xiong and Ferrell, 2003).

The binding of the Delta/Serrate ligand to the Notch receptor induces the cleavage of the Notch transmembrane domain by a membrane-bound γ (gamma)-secretase (Greenwald, 2005). The

cleaved Notch intracellular domain (NICD) binds to CSL, a sequence-specific DNA-binding protein that, alone, acts as a transcriptional repressor and, upon NICD binding, becomes an activator (Hsieh et al., 1996; Jarriault et al., 1995). The fact that CSL can repress or activate the transcription of target genes suggests a model by which Notch signaling achieves cell fate specification through lateral inhibition. In this model, cells expressing Notch at a higher level than neighboring cells will produce more NICDs and, thus, more transcription activator complexes, eventually increasing the expression of CSL target genes. In contrast, neighboring cells expressing less or no Notch will decrease or repress target gene expression. A few CSL target genes involved in cell fate specification have been identified; however, a sufficient number have not been identified to suggest a mechanism for the production of exclusive cell fates by lateral inhibition, or one for the exclusive expression of Notch by one cell and Delta/Serrate by the other (Johnston and Desplan, 2010).

Because the fate specification of the anchor cell (AC) and a ventral uterine (VU) precursor cell is initiated by a stochastic interaction between LIN-12/Notch and LAG-2/Delta in two neighboring cells in the *Caenorhabditis elegans* somatic gonad, it has been a good model system to understand the molecular mechanism of Notch-dependent lateral inhibition through which a small difference in Notch activity establishes two exclusive cell fate states that cannot be reverted (Kimble and Simpson, 1997; Seydoux and Greenwald, 1989; Wilkinson et al., 1994). In wild-type animals, two somatic gonadal cells (Z1.ppp and Z4.aaa) have equal potential to become the AC or a VU and the two cells express both LAG-2 and LIN-12 before specification (Seydoux and Greenwald, 1989; Wilkinson et al., 1994). After AC/VU specification, one of the cells expresses only LAG-2 and adopts the AC fate, and the other expresses only LIN-12 and becomes a VU cell (Seydoux and Greenwald, 1989; Wilkinson et al., 1994).

The AC and VU cells have been used not only to study the mechanisms underlying Notch-dependent cell fate specification but also to understand cellular differentiation events. The VU cells undergo four rounds of cell division (Kimble and Hirsh, 1979; Newman and Sternberg, 1996; Newman et al., 1996),

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whereas the AC remains a single cell in the gonad and expresses LIN-3, a member of the epidermal growth factor (EGF) family, that induces patterned proliferation of vulval precursor cells (VPCs) (Hill and Sternberg, 1992). The AC also invades the vulval epithelium by extending a process toward the 1° lineage (P6.p) of vulval cells (Sherwood and Sternberg, 2003). *fos-1*, a *C. elegans* homolog of the proto-oncogene *Fos*, is involved in the destruction of the underlying basement membranes during AC invasion (Sherwood et al., 2005). After the AC has invaded the vulval epithelium, it is involved in the formation of a structure that connects vulva and uterus (Newman and Sternberg, 1996; Newman et al., 1996). Here, the AC fuses with some of the VU descendents to form a H-shaped structure (utse) in which a thin laminar cytoplasmic bridge separates the upper uterine and lower vulval lumens.

A genome-wide computational search to identify genes co-expressed from a common regulatory element identified *egl-43* as a gene co-expressed with *lin-3* during AC/VU specification as well as a LIN-12 downstream target (Hwang and Sternberg, 2004; Hwang et al., 2007). Here, an enhancer element (anchor cell element of *lin-3*, ACEL) in both *lin-3* and *egl-43* was sufficient to activate these genes in pre-AC/pre-VU cells and the AC, but not in VU cells. This element consists of two E-boxes and one FTZ-F1 nuclear hormone receptor- (NHR-) binding site (Hwang and Sternberg, 2004). HLH-2, *C. elegans* ortholog of mammalian E12/E47 and *Drosophila melanogaster* Daughterless involved in the exclusive expression of LAG-2 in the AC (Karp and Greenwald, 2003; 2004), binds to the E-boxes and is necessary for *lin-3* transcription in the AC (Hwang and Sternberg, 2004). Thus, a similar element could activate *lag-2* transcription during AC/VU specification. Expression of *egl-43* appears to be regulated by several distinct regulatory elements during AC/VU specification and later cellular processes that involve the AC and VU cells (Hwang et al., 2007). In addition to ACEL, an element that involves clustered LAG-1 binding sites supports *egl-43* expression in VU cells immediately after AC/VU specification. This element is also expressed in the VU cells and their descendents as a downstream of the transcription factor FOS-1. Another element supports *egl-43* expression in the AC when vulval-uterine connection takes place at early L4 stage (Hwang et al., 2007).

In previous experiments to dissect the *egl-43* regulatory elements, the role of a regulatory element that contains clustered LAG-1 binding sites was not clear due to the presence of an ACEL element that strongly supports gene expression during AC/VU specification (Hwang et al., 2007). Here, in an attempt to study the role of regulatory elements containing clustered LAG-1 binding sites, we addressed whether clustered LAG-1 binding sites in *lag-1* and *lin-12* are involved in the expression of these genes during AC/VU specification. Studies using transgenic animals and site-directed mutagenesis clearly show that LAG-1 binding sites in both genes are involved in eliminating their expression in the AC and supporting expression in the pre-AC/pre-VU and VU cells, as well as their descendents. Thus, LAG-1 appears to act as a transcriptional repressor in the AC and as an activator in the VU cells during AC/VU specification. We also show that LIN-12 signaling is necessary for the suppression of *lag-1* expression in the AC. To address the significance of the elimination of LAG-1 in the AC, we ectopically expressed *lag-1* cDNA in the AC, which caused a defect in V-U connection. Thus *lag-1* expression in the AC should be repressed during AC/VU specification for the proper differentiation event to occur at a later developmental stage.

MATERIALS AND METHODS

General methods and strains

C. elegans strains were handled, maintained at 20°C, and crossed following standard protocols (Brenner, 1974). Experiments were conducted at 20°C unless otherwise indicated. Cell anatomy was observed with Nomarski optics, and cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) expression was detected using a Zeiss Axioplan compound microscope. Micrographs were taken with a Hamamatsu ORCA-ER digital camera and Improvision Openlab software, and images were overlaid using Adobe Photoshop CS.

Transgenic lines were generated using a standard microinjection protocol (Mello et al., 1991). The *lag-1::YFP* or *lin-12::YFP* construct (25 or 50 ug/ml) was co-injected with the plasmid *myo-2::YFP* plasmid (5 ug/ml) and pBluescript (170 ug/ml) into *unc-119(ed4); syls57[cdh-3::CFP + pDP#MM016B]* or *lin-12(n676n909) unc-32(e189)/qC1* animals. The *cdh-3 (AC element)::YFP* or *cdh-3 (AC element)::lag-1 cDNA* construct (50 ug/ml) was co-injected with *myo-2::YFP* plasmid (5 ug/ml) and pBluescript (150 ug/ml), into N2 and *kuls29[unc-119(+)] + pWH17(cog-2::gfp)* animals. After injection, animals expressing *myo-2::YFP* in the pharynx were identified and maintained as transgenic lines.

Constructs

lag-1(wild-type)::YFP-PEST and *lag-1(mutated LAG-1 binding sites)::YFP-PEST* constructs were prepared as follows. The *lag-1(wild-type)::YFP-PEST* construct, containing wild-type LAG-1 binding sites, was prepared by cloning part of the first intron region of *lag-1* into the plasmid *pPD122.53(YFP-PEST)*, which contains *NLS₄::YFP-PEST*. The 1758-bp genomic fragment (aagettcccatcctagttttccacacq --- tqggaaccccccaactqggaqaaqct) of *lag-1* was PCR-amplified from N2 genomic DNA and cloned into a *HindIII* site in *pPD122.53(YFP-PEST)*, which contains a PEST sequence from the mouse ornithine decarboxylase gene (amino acids 820-942) downstream of YFP. In the *lag-1(mutated LAG-1 binding sites)::YFP-PEST* transcriptional fusion construct, a PCR fragment containing mutations in all 13 putative LAG-1-binding sites was cloned into *pPD122.53(YFP-PEST)*.

The *lin-12(wild-type)::YFP* transcriptional fusion construct was prepared by cloning a PCR-amplified genomic DNA fragment into the *pPD122.53(YFP)* plasmid, which contains *NLS₄::YFP*. The fragment (cgaatactggaaatgatg --- tttttqcccaattccata) contains the 5' upstream region, 1st exon, 1st intron, 2nd exon, and part of the 2nd intron of *lin-12*. The genomic region contains 17 putative LAG-1-binding sites [seven in the 5' regulatory region (3,400 bp), eight in the 1st intron (1,519 bp), and two in the 5' end of the 2nd intron (115 bp)]. All 17 candidate binding sites were mutated in the *lin-12(mutated LAG-1 binding sites)::YFP* transcriptional fusion construct.

Site-directed mutagenesis of 13 LAG-1 binding sites in *lag-1* and 17 sites in *lin-12* was performed by sequentially ligating PCR fragments containing mutations in the sequences 'TCCCA' and 'TGGGA'. In *lag-1*, 13 LAG-1 binding sites were changed to 'TCACT', 'TACCT', 'TGCCT', 'TCACT', 'TCGTA', 'TCACT', 'TGGAT', 'TCACT', 'TCCAT', 'TCCTC', 'AGTGA', 'TGTGA', and 'AGTGA' sequences. In *lin-12*, 17 sites were to 'TGATC', 'TAGTA', 'ACCCA', 'TCATA', 'CGCGA', 'TGTGT', 'TGACT', 'TGCCT', 'TGTGT', 'TGAGA', 'AACCA', 'TCTCA', 'TTCGA', 'CCAGA', 'TGCAT', 'CTCGA', and 'GTCCA'.

The *lag-1* cDNA or YFP was cloned after a *cdh-3* AC regulatory element (known as mk 62-63), which is known to drive

expression of *cdh-3* in the AC from mid-L2 to L3 molt (Kirouac and Sternberg, 2003).

Vulval induction, AC invasion, and vulva-uterine connection analysis

Vulval induction, anchor cell invasion, and vulval uterine connection were assayed as described previously (Hill and Sternberg, 1992; Hwang et al., 2007; Newman and Sternberg, 1996; Sherwood and Sternberg, 2003). In a wild-type hermaphrodite, three of the six VPCs adopt 1° and 2° cell fates to divide into 22 descendants during L3, which was measured in the transgenic lines expressing *lag-1* cDNA in the AC from the AC-specific *cdh-3* promoter (Kirouac and Sternberg, 2003). AC invasion was measured at mid-L3 and the L3 molt using Nomarski optics. In wild-type animals, the basement membranes are degraded beneath the AC, and the basolateral side of the AC crosses through a hole in the basement membranes and penetrates between central 1°-fated vulval cells beginning in mid-L3, resulting in the overlap of AC cytoplasm with that of the vulval cells at early L4 (Sherwood and Sternberg, 2003). The ventral uterine cells form the connection between uterus and vulva through which eggs pass to exit the worm. V-U connection was measured by the formation of a thin laminar cytoplasmic bridge that separates the forming uterine and vulval lumens (Newman and Sternberg, 1996; Newman et al., 1996).

RESULTS AND DISCUSSION

Clustered LAG-1 binding sites in *lin-12* are involved in regulation of *lin-12* expression during AC/VU specification

Because the role of clustered LAG-1 binding sites in *egl-43* could not be determined due to the presence of an ACEL element that strongly supports gene expression during AC/VU specification (Hwang et al., 2007), we investigated the role of clustered LAG-1 binding sites in *lag-1* and *lin-12* (Christensen et al., 1996), which appear to be similar in structure to those in *egl-43*, in AC/VU cell fate specification. The expected frequency of LAG-1 binding sites ('RTGGGAA') in the *C. elegans* genome is approximately 1 out of 8 kb, and eight binding sites are located within one 1590-bp sequence of *egl-43* (-197 to +1393). Thirteen LAG-1 binding sites are present in the 5' region of the *lag-1* 1st intron (1,758 bp) and 10 binding sites are in the 1st intron of *lin-12* (1,519 bp). Thus, the LAG-1 binding sites are highly clustered in all three genes. All predicted LAG-1 binding sites in these regions fall into two classes; 21 have the sequence 'RTGGGAA' and 10 have the sequence 'YTGGGAA'. LAG-1 appears to bind to most of the predicted binding sites because 14 of 15 double-stranded competitor oligonucleotides that contain the 'RTGGGAA' motif and three of six that contain the 'YTGGGAA' motif competed with LAG-1 binding to a DNA probe *in vitro* (Hwang et al., 2007) (data not shown). Interestingly, these LAG-1 binding sites are clustered in the 1st intron of each gene.

In the L2 stage in the *C. elegans* hermaphrodite, one of the somatic gonadal cells (Z1.ppp or Z4.aaa) moves into the central position on the ventral surface of the gonad to become the AC that does not divide at all (Hirsh et al., 1976; Kimble and Hirsh, 1979). In early L2, the fate of neither the Z1.ppp nor the Z4.aaa cell has been determined and neither has moved to the central position on the ventral surface of the gonad. In mid-L2, either Z1.ppp or Z4.aaa has moved to the central position to become the AC. In late L2, *cdh-3::CFP* signal starts being detected only in the AC (Fig. 1).

To test whether clustered LAG-1 binding sites in *lin-12* are

Fig. 1. Expression pattern of *lin-12::YFP* in *C. elegans* gonad during AC/VU specification. (A-D) Both *lin-12* (wt)::YFP and *lin-12* (mut)::YFP (green) are expressed in the pre-AC/pre-VU cells in mid L2, before AC/VU specification. (E-H) Either the Z1.ppp or the Z4.aaa cell moves to the central position on the ventral surface of the gonad to become the AC that expresses *cdh-3::CFP* from late L2 (red). AC is labeled yellow due to the co-localization of *cdh-3::CFP* (red) and *lin-12* (wt)::YFP or *lin-12* (mut)::YFP (green) at late L2. (I, J) *lin-12* (wt)::YFP, but not *cdh-3::CFP* (red), disappears in the AC at early L3 stage, which labeled the AC in red. (K, L) At early L3, *lin-12* (mut)::YFP (green) is retained in the AC, which also express *cdh-3::CFP* (red) and, therefore, AC is labeled yellow. *lin-12* (mut)::YFP expression disappears in VU cells from late L2 after AC/VU specification. (M, N) *lin-12* (wt)::YFP expression (green) in a VU focal plane. The YFP signal is seen in the VU cells at early L3. (O, P) The *lin-12* (mut)::YFP signal disappears from VU cells. The green signal in the middle is from the AC located in a different focal plane. Fluorescence images are shown in (B, D, F, H, J, L, N, and P). Overlays of fluorescence and Nomarski images are shown in (A, C, E, G, I, K, M, and O).

involved in its expression in the hermaphrodite somatic gonad during AC/VU specification, we examined transgenic animals containing a transcriptional reporter construct that places the region of clustered binding sites before *Δpes-10 promoter::NLS₄::YFP*. As shown in Fig. 1, *lin-12::YFP* expression was first detected in the pre-AC/pre-VU cells at early to mid L2 (Figs. 1A-1D). Site-directed mutagenesis of all 17 putative LAG-1 binding sites in *lin-12::YFP* did not affect YFP expression, suggesting the presence of a regulatory element, independent of

Table 1. Effect of LIN-12 activity on the *lag-1::YFP* expression during AC/VU specification

	<i>lag-1</i> (LAG-1, wt or mut)::YFP-PEST expression ¹		<i>lag-1</i> (LAG-1, wt)::YFP-PEST expression	
	wt (wild-type) LAG-1 binding sites	mut (mutated) LAG-1 binding sites	+/ <i>n676n909</i> ²	<i>n676n909/n676n909</i> ⁵
AC ³	0/31	16/91	0/34	13/29
VU cells and their descendants	34/35	4/95 ⁴	26/32	8/32 ⁵

¹Expression was measured in transgenic animals carrying the wild *lin-12* gene.

²Expression was measured in PS1771 [*lin-12(n676n909)unc-32(e189)/qC1*].

³*lag-1::YFP* expression was measured in animals at mid-L3 (2-cell VPC).

⁴1-2 VU cells next to the AC of mid-L3 (2 to 4-cell VPC) express *lag-1::YFP* very weakly.

⁵2-4 VU descendants next to the ACs of early L4 animals express *lag-1::YFP* very weakly.

⁶VU cells were transformed into 2-4 Acs.

Fig. 2. Schematic diagram of *lin-12::YFP* expression patterns. An unknown enhancer element (factor Y-binding site) is responsible for *lin-12::YFP* expression in the Z1.ppp and Z4.aaa cells before AC/VU specification. Expression of *lin-12::YFP* appears to be supported by clustered LAG-1 binding sites to be bound by the LAG-1/NICD transcriptional activator complexes at early L2. While AC and VU cell fates are specified, *lin-12::YFP* expression is suppressed by the LAG-1 transcriptional repressor in the AC, eventually eliminating expression of *lin-12* and factor Y in the AC. LAG-1/NICD transcriptional activator complexes support *lin-12* expression in VU cells.

the LAG-1 binding sites, that supports *lin-12* expression in the pre-AC/pre-VU cells (Z1.ppp, Z1.ppa, Z4.aaa, and Z4.aap) before AC/VU specification.

Expression of *lin-12(wt)::YFP* is maintained in VU cells after specification (Figs. 1M and 1N) but the signal disappears in the animals carrying the *lin-12(mut)::YFP* transgene (Figs. 1O and 1P). Thus, LAG-1 binding sites in *lin-12* appear to be necessary for *lin-12* expression in VU cells after AC/VU specification. Both *lin-12(wt)::YFP* and *lin-12(mut)::YFP* were expressed in the AC immediately after cell fate specification in mid to late L2 stage animals, when expression of *cdh-3::CFP* began in the AC (Figs. 1E-1L; *lin-12::YFPs* (in green) and *cdh-3::CFP* (in red) signals are merged yellow). However, the YFP signal in the AC disappeared at late L2 and early L3 in the transgenic animals carrying *lin-12(wt)::YFP*, but not in those carrying *lin-12(mut)::YFP* (Figs. 1E-1L). Thus, LAG-1 binding sites in *lin-12* appear to be involved in the disappearance of LIN-12 in the AC, possibly through the suppression of *lin-12* transcription by LAG-1 transcriptional repressor activity (Fig. 2).

LIN-12 activity is necessary for regulation of *lag-1* expression

We previously showed that *lag-1::YFP* is expressed in the pre-AC/pre-VU cells before specification. It is also expressed in VU cells, but not the AC, after specification. We also showed that clustered LAG-1 binding sites in *lag-1* are necessary for the transcriptional activation of *lag-1* in these somatic gonadal cells and for the transcriptional repression of *lag-1* in the AC. Here, we examined whether LIN-12 activity is necessary for expres-

sion of *lag-1::YFP* during AC/VU specification. We used an unstable YFP to measure the dynamic change in *lag-1::YFP* expression. As shown in Table 1, transgenic animals carrying *lag-1(wt)::YFP-PEST* express YFP in VU cells and their descendants, not in the AC. In contrast, *lag-1(mut)::YFP-PEST*, which lacks all LAG-1 binding sites, is expressed in the AC at mid L3 stage, consistent with the previous observation that clustered LAG-1 binding sites in *lag-1* are necessary for repression of *lag-1* expression in the AC. In the *lag-1(wt)::YFP-PEST* transgenic animals with *lin-12 null* (*n676n909*) genetic background, in which some VU cells transformed into the AC, YFP signal is present in the ACs but absent in other somatic gonadal cells, including VU descendants (Table 1). Thus, LIN-12 activity is necessary for the expression of LAG-1 in the VU cells and for the suppression of *lag-1* expression in the AC.

Ectopic expression of *lag-1* in the AC causes a defect in the vulval-uterine connection

After the AC is created by the AC/VU cell fate specification process, it expresses LIN-3, which induces a patterned proliferation of VPCs (Hill and Sternberg, 1992). The AC also invades the vulval epithelium during the VPC proliferation period (Sherwood and Sternberg, 2003). After the AC has induced the proliferation of VPCs and invaded the vulval epithelium, it fuses with some of the VU descendants to form an H-shaped structure (utse) in which component nuclei on both sides are connected by a thin laminar cytoplasmic bridge that separates the upper uterine and lower vulval lumens (Newman and Sternberg, 1996; Newman et al., 1996).

Fig. 3. Ectopic expression of *lag-1* cDNA in the AC causes a defect in vulval-uterine connection. (A) YFP expression from the *cdh-3* AC regulatory element (mk 62-63) at mid-L3. Fluorescence (lower) and overlay of fluorescence and Nomarski images (upper). (B) V-U connection is normal as determined by the formation of a thin laminar cytoplasmic bridge that separates the forming uterine and vulval lumens. (C, D) Two different forms of defective V-U connection and failure to form a cytoplasmic bridge. (E) V-U connection was measured in several transgenic lines that express YFP or *lag-1* cDNA in the AC.

Fig. 4. A model for the regulation of genes involved in AC/VU specification. Two exclusive cell fates, AC and VU, are established by feedback loops initiated by a stochastic difference in LIN-12/Notch activity in two neighboring cells, Z1.ppp and Z4.aaa. Before specification, both Z1.ppp and Z4.aaa cells express LIN-12/Notch and LAG-2/Delta at the same level. Lateral inhibition mediated by Notch signaling leads to exclusive expression of LIN-12 in the VU and of LAG-2 in the AC. HLH-2 is necessary for the expression of LAG-2 and LIN-3/EGF in pre-AC/pre-VU cells, as well as in the AC. At least three different regulatory elements are involved in the expressions of *lag-1*, *lag-2*, *lin-3* and *lin-12* during AC/VU specification: ACEL, an element containing clustered LAG-1 binding sites, and another element (factor Y binding site) necessary for expression of *lin-12* in both Z1.ppp and Z4.aaa cells. It is not clear yet how factor Y expression is initiated and terminated in the pre-AC/pre-VU cells, but LIN-12 activity could be involved in terminating the factor Y activity. Transcriptional

repressor activity in LAG-1 itself eliminates *lag-1* and *lin-12* expression in the AC, but the transcriptional activator activity in the LAG-1/NICD complex maintains the expression of both genes in VU cells. The ACEL element in *lin-3*, and possibly an ACEL-like element in *lag-2*, appears to support the expression of these genes in the AC.

Here, we tested whether the disappearance of LAG-1 in the AC during AC/VU specification is necessary for the later-stage cellular processes that involve the AC, i.e., VPC proliferation, invasion into the vulval epithelium, and connection of vulva and uterus. We ectopically expressed *lag-1* cDNA in the AC using the *cdh-3* AC regulatory element (mk 62-63), which drives expression of *cdh-3* in the AC from late L2 to early L4 (Kirouac and Sternberg, 2003), when all three AC-dependent cellular processes take place. VPC induction and AC invasion were normal in transgenic animals ectopically expressing *lag-1* cDNA in the AC (data not shown) but vulval-uterine connection was abnormal in all transgenic lines examined (Fig. 3). This is consistent with the fact that pi cell fates of some VU descendants that are involved in utse formation are determined by their interaction with the AC through LIN-12 signaling (Newman and Sternberg, 1996). Thus, ectopic expression of *lag-1* in the AC could affect the fate decision of phi cells, resulting in a defective cell fusion between pi cells and the AC.

A model for regulation of *lag-1* and *lin-12* expression during AC/VU specification

In this study we showed that *lin-12::YFP* expression overlaps with *lag-1::YFP* expression during AC/VU specification. Both YFP signals are detected in the pre-AC/pre-VU cells before specification. The signals are present in VU cells and their descendants, but disappear in the AC after specification. Regulatory elements that contain LAG-1 binding sites appear to be involved in the disappearance of *lag-1::YFP* and *lin-12::YFP* in the AC, presumably through the transcriptional repressor activity of LAG-1 itself. The same elements could be responsible for the expression of both genes in the VU cells and their descendants. In this case, the transcriptional activator complex containing LAG-1 and NICD could be involved. It is not clear yet why *lin-12*/Notch and *lag-1*/CSL are regulated together, but the co-regulation could promote a more rapid increase in LIN-12 activity in pre-VU and a decrease in pre-AC cells. Thus, the fates of both cells become specified in a very short period of time.

The *lin-12* gene appears to contain an element responsible

for its expression in the pre-AC/pre-VU cell, independent of the LAG-1 binding sites, as in *egl-43* (Hwang et al., 2007). Unlike *egl-43* and *lin-12*, *lag-1* expression in the pre-AC/pre-VU cells appears to be supported by an element that contains LAG-1 binding sites because site-directed mutagenesis of LAG-1 binding sites in *lag-1* eliminated *lag-1* expression in those cells. Lack of an additional element to drive *lag-1* expression in the pre-AC/pre-VU cells supports our finding that LIN-12 signaling is necessary for the suppression of *lag-1* in the AC. LAG-1 should be expressed in the pre-AC/pre-VU cells from an enhancer element containing LAG-1 binding sites to suppress *lag-1* expression in the AC after AC/VU specification.

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