Co-regulation by Notch and Fos is required for cell fate specification of intermediate precursors during *C. elegans* uterine development

Kavita S. Oommen and Anna P. Newman

The Notch pathway is the key signal for many cell fate decisions in the nematode *Caenorhabditis elegans* including the uterine π cell fate, crucial for a proper uterine-vulval connection and egg laying. Expression of the *egl-13* SOX domain transcription factor is specifically upregulated upon induction of the π lineage and not in response to other LIN-12/Notch-mediated decisions. We determined that dual regulation by LIN-12 and FOS-1 is required for *egl-13* expression at specification and for complete rescue of *egl-13* mutants. We found that fos-1 mutants exhibit uterine defects and fail to express π markers. We show that FOS-1 is expressed at π cell specification and can bind in vitro to *egl-13* upstream regulatory sequence (URS) as a heterodimer with *C. elegans* Jun.

**KEY WORDS:** LIN-12, Notch, LAG-1, CSL, FOS-1, Fos, Jun, Specification, Transcription

**INTRODUCTION**

The Notch pathway specifies numerous cell fates during development. How can diverse tissues be generated under the control of a single reiteratively utilized pathway? Understanding mechanisms that directly influence target gene expression may provide insights into how this critical pathway achieves such specificity.

The major sequence of events initiated by Notch signaling is highly conserved across evolution and ultimately converges upon a single DNA binding protein, CSL, assuming an active conformation at target gene loci. CSL factors (mammalian CBFI, *Drosophila* Suppressor of Hairless, and *C. elegans* LAG-1) are the sole terminal effectors of the pathway and form a transcription-activating complex with the Notch intracellular domain during signaling (Bailey and Posakony, 1995; Christensen et al., 1996; Jarriault et al., 1995; Lecourtois and Schweisguth, 1997; Tamura et al., 1995). Since every target gene with CSL-binding sites is not ubiquitously expressed upon signaling, transcriptional regulation must be controlled to ensure that expression is specific and yields unique cell fates.

Mechanisms fine-tuning repetitive Notch signaling to establish transcriptional selectivity and define cell fates can be addressed in *C. elegans*. The LIN-12 (a Notch ortholog; hereafter referred to as LIN-12/Notch) pathway induces three distinct postembryonic cell fates during formation of a functional uterine-vulval connection required for egg laying. First, during the AC/VU decision, reciprocal signaling between two equivalent gonadal cells results in the LIN-12 signal-receiving cell adopting a ventral uterine precursor (VU) cell fate while the other cell by default becomes the terminal anchor cell (AC) (Greenwald et al., 1983; Kimble, 1981; Seydoux and Horvitz, 1989). Despite this repeated utilization of LIN-12 signaling, target genes expressed in the uterus may not be expressed in the vulva and vice versa. We wanted to address the mechanism(s) responsible for exclusive gene expression during LIN-12-mediated induction of the uterine π cell fate.

As a culmination of Notch signaling in π cells, the genes *egl-13*, encoding a SOX domain transcription factor, and *lin-11*, encoding a LIM domain transcription factor, are upregulated and are required for maintenance and differentiation, respectively, of the π lineage (Cinar et al., 2003; Frey et al., 1990; Hanna-Rose and Han, 1999; Newman et al., 1999). Clusters of LAG-1 cis-elements within upstream regulatory sequences (URS) of LIN-12 target genes are a criterion for pursuing a candidate gene as a direct target of the pathway (Rebeiz et al., 2002; Yoo et al., 2004). Some LAG-1 binding sites present in the *lin-11* locus are sufficient to drive uterine expression, demonstrating direct regulation by LIN-12 (Gupta and Sternberg, 2002; Yoo et al., 2004). *lin-11* is also expressed in the vulva in response to Wnt activity (Gupta and Sternberg, 2002).

Unlike *lin-11*, *egl-13* is specifically expressed in the uterus and not in the vulva (Hanna-Rose and Han, 1999). We also did not detect clusters of LAG-1 binding sites in *egl-13*. Nonetheless, in this report, we establish *egl-13* as a true LIN-12 target gene. We also demonstrate the necessity of a conserved cis element for Fos and Jun transcription factors for specification stage expression of EGL-13 and rescue of mutants. Additional analyses presented here provide evidence that fos-1, the closest *C. elegans* homolog of Fos, is involved in π cell development and directly regulates *egl-13* expression.

**MATERIALS AND METHODS**

**Strains**

Nematode strains were handled and maintained at 20 °C (Brenner, 1974). The following strains were used: wild-type strain N2 (Bristol); LGIII, tsly4 [egl-13<sup>Fl：</sup>GFP], syls80[lin-11::GFP + unc-119(+)] (Gupta and Sternberg, 1989).

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2002); LG V, fos-1(ar105)pdp-11(e224) unc-42(e270), unc-76(e911); LG X, egl-13(ku194), syd123(fos-1a::YFP-TL + unc-119(+)) (Sherwood et al., 2005). The strain +DntT1 IV, fos-1(ar105)/DntT1 V, syEx679[pAC-fos-lu::YFP] (Sherwood et al., 2005) was also studied. The yls4 strain is an integrated line of egl-13^{3D}::GFP (pWH17) (Cinar et al., 2003; Hanna-Rose and Han, 1999).

**Reporter deletions**

Unique restriction endonuclease sites within the pWH17 vector were used to excise intervals of egl-13 URS. Restriction sites for deletion constructs and end point base pairs are provided in Fig. 1D. N2 hermaphrodites were injected with 20 ng/μl of each construct. The five best-transmitting extrachromosomal lines were studied.

**Bioinformatics**

The 6451 bp of sequence upstream to the translational start for *C. elegans egl-13* (clone T22B7.1) was obtained from the T22B7 cosmid sequence (bp 27,684-34,134; GenBank/EMBL accession no. U646608). An 8 kb genomic interval for *C. briggsae* CBG14721 was acquired from within the contig cb25.fpc3857 from assembly cb25.agp8 (bp 3,390,956-3,398,955; GenBank/EMBL accession no. CAAC1000068). Multiple (ClustalW 1.8) and pairwise (Sim) alignments were carried out using the Baylor College of Medicine Search Launcher (URL: http://searchlauncher.bcm.tmc.edu). TESS (Transcription Element Search Software, TRANSFAC database version 4.0) was utilized to identify candidate transcription factor binding sites (Schug and Overton, 1997). Technical Report CBIL-TR-1997-1001-v4.0, Computational Biology and Informatics Laboratory, School of Medicine, University of Pennsylvania; PA, USA (http://www.cbil.upenn.edu/tess).

**Cis-element mutagenesis**

PCR-mediated, site-directed mutagenesis using overlap extension was conducted as described by Ho et al. (Ho et al., 1989). The template for mutagenesis was the egl-13^{3D}::GFP construct. The external oligo primers were ‘A’ (forward, 5’-GGGCTGCATAGGCTTGAG-3’) and ‘D’ (reverse, 5’-CACACATACACCTGGACAAGC-3’). The egl-13^{3D}::GFP was generated using overlapping primers that removed sequence from 1325 to 2211 bp upstream of the translational start. For the LAG-1 deletion, the overlapping primer set consisted of reverse primer ‘B’ (5’-GGGTAATGTTATGTGAGAAGTTGTGAG-3’) and forward primer ‘C’ (5’-GACCGGATGTGCACATTTCCTCAAAAACACTTTCATGAC-3’). For the Fos and Jun (fos-1/Jun) deletion, the overlapping primer set consisted of reverse primer ‘B’ (5’-GGGCTGACCAA-AAAAGGCGATTCACCAAAACT-3’) and forward primer ‘C’ (5’-GGATATGTTGGAATGGTTGTCTTGTGCG-3’). For each deletion, two separate A plus B and C plus D PCR products were purified, combined, and added as template to a subsequent PCR reaction with A and D primers. The resulting overlap-extended products were digested with *Nru* and *Hin* and cloned into the egl-13^{3D}::GFP vector to make either egl-13^{3D}_{mut::GFP} or egl-13^{3D}_{null::GFP}. For the double deletion, the same mutagenesis strategy was performed for deleting the Fos/Jun binding site with egl-13^{3D}_{mut::GFP} as a template. Each construct was injected at 20 ng/μl into N2 hermaphrodites and the resulting lines used for analysis.

**cDNA-mediated rescue**

Full-length 1413 bp egl-13 cDNA with a 3’ *BanHI* site was amplified from a mixed-stage *C. elegans* cDNA library, digested with *HindIII-BanHI*, and cloned into pPD95.69yfp(–) vector (GFP was previously removed by Smal and *EcoRI* digestion and re-ligating). Then a 1.9 kb HindIII fragment containing the cDNA from this intermediate GFP-minus vector was cloned into the same sites of egl-13^{3D}::GFP to generate egl-13^{3D}::cDNA (intact). A 2.5 kb *SphI-AatII* insert from egl-13^{3D}::cDNA was cloned into the egl-13^{3D}_{mut::GFP}, egl-13^{3D}_{null::GFP}, and egl-13^{3D}_{null::loc::GFP} recipient vectors to create respective cDNA fusions. Each was co-injected at 20 ng/μl with a myo-2::GFP marker at 20 ng/μl into egl-13^{3D}_{ku194} recipients. The best transmitting lines with uniform body wall GFP were studied. In Fig. 2C, data for intact lines were collected from strains *tyls102* and *tyls101*, **ΔLAG-1** from *tyls112* and *tyls111*, **ΔFos/Jun** from *tyls121* and *tyls123*, and **ΔLAG-1** from *tyls132* and *tyls133.*

**RNAi**

We ‘blasted’ human Fos and Jun orthologs (Fos, FOSB, FRA 1 and 2, JDP 1 and 2; Jun, JUNB; retrieved from the human protein database, www.hprd.org/) to identify similar counterparts in the *C. elegans* genome. L4 stage *tyls4* animals were fed control (pie-1) or the following experimental RNAi clones from the Ahringer library (Kamath et al., 2003): F29G9.4/ fos-1, KZ0904.4, C27D6.4, F57B10.1, K08F8.2, R74.3, T24H10.7/unc-1, C34D1.5, and T04C10.2. W08E12.1 cDNA was calibrated with 5’ *EcoRI* and 3’ *BamHI* sites from a mixed-stage *C. elegans* cDNA library, cloned into the L4440 vector, and transformed into HT115(DE3) bacteria for testing.

**Co-localization and translational fusions**

We generated pPD95.69ycyan and pPD95.69yfp by *Agel-EcoRI* dropout of GFP from pPD95.69 and replacement with either CFP from L4752 plasmid or YFP from L4753 plasmid, respectively. The egl-13^{3D}_{::GFP} reporter construct was created by PCR amplification of a 4.4 kb genomic fragment with 5’ *PmeI* and 3’ *NahI* sites (forward, 5’-CTCGAGGTTTTTTAAGCGGATGGGGAGAAAACCTAAAG-3’; reverse, 5’-GGTATCCGACCATCAGATTTCACCAAAACACTTTCCATGAC-3’). For the double deletion, *EcoRI* digestion of the F29G9 cosmid and cloned into the *HindIII*-blunted-*Xhol*-treated pPD95.69yfp vector, replacing the fos-1 stop codon with an in-frame fusion to YFP.

Separate lines carrying one extrachromosomal array of either the egl-13^{3D}_{::GFP} reporter (tyEx22) or the *plastFOS-1::YFP* transgene (tyEx30 and tyEx31) were established in the unc-76 background. Non-unc (non-coordinated) unc-76; Ex[plastFOS-1::YFP](50 ng/μl) unc-76(+) (60 ng/μl) males were crossed into GFP-positive unc-76; tyEx22[egl-13^{3D}_{::GFP}::CFP 20 ng/μl] rab-3; GFP(20 ng/μl) hermaphrodites. Phenotypically non-unc, GFP-positive unc-76; tyEx30 or tyEx31[plastFOS-1::YFP unc-76(+)]/tyEx22[egl-13^{3D}_{::GFP}::CFP 20 ng/μl] cross progeny were isolated and propagated. Three independent transgenic strains carrying both arrays were generated in this manner and found to have similar fluorescence patterns.

For the *pJUN-1dc::GFP* translational reporter, T24H10 cosmid was digested with *NheI* and *PmeI*. The resulting 5293 bp (~5.3 kb) band contains 842 bp and 4266 bp of URS/intron sequence upstream of the translational starts of *jun-1d* and *jun-1c*, respectively. We cloned this fragment into *Xhol*-Snout-treated pPD95.75 to establish an in-frame GFP fusion. We observed consistent expression in three extrachromosomal lines of animals expressing this reporter transgene in the unc-76 injection system (best transmitting line is tyEx35[plpUN-1dc::GFP 20 ng/μl unc-76(+) (60 ng/μl)].

**Electrophoretic mobility shift assays**

The radiolabeled probe was generated by amplification of the 100 bp homologous region within the 1.3 kb π enhancer with 5’ *BglII* and 3’ *EcoRI* restriction sites. The fragment was digested, de-phosphorylated, and end-labeled with [γ-32P]ATP. The CDNAs for *fos-1b* and *jun-1c* engineered with 5’ *Ndel* and 3’ *BamHI* restriction sites were amplified from a mixed-stage *C. elegans* cDNA library and cloned into pCite4a (Novagen) expression vectors. The digested *fos-1* amplicon was also cloned into the pGBK7 (Clontech) to attach a 5’ Myc epitope tag, *FOS-1* and *JUN-1* proteins were then in vitro translated (Promega). For competition, forward and reverse oligonucleotides, with flanking sequences as present in egl-13 URS, containing either intact or mutated Fos/Jun binding site were annealed. The intact sequence is 5’-GGTTTGGATGAGTATGGATAAGATGTTGGG-3’ (the Fos/Jun binding site is underlined). The mutated sequence is 5’-GGTTTGGATGAGTATGGATAAGATGTTGGG-3’ (the altered Fos/Jun binding site is underlined and the mutation is in lowercase).

**RESULTS**

### A minimal region of egl-13 URS serves as a π-specific enhancer

Once specified from a population of twelve intermediate precursors, three π cells on each side of the ventral uterus undergo one dorsalmost ventral division, distinguishable from default π divisions, to generate a total of 12 π cell daughters (Newman et al., 1995) (Fig. 1A).
Fusion among eight of the 12 daughters and the AC generates the syncytial uterine seam cell (utse). The common cytoplasm and membrane shared by this utse stretches a thin laminar process dorsal to the vulva that is first visible at the mid L4 stage and ultimately permits passage of eggs to the outside. The other four daughters become the mononuclear uv1 cells (Newman et al., 1996) (Fig. 1A,B).

Following the specification event at the late L3 stage and just prior to division at L3 lethargus, fluorescence from an egl-13::GFP transcriptional reporter (pWH17) was initiated in nuclei (Fig. 1C). Expression of this reporter, which contained 6451 bp (~6.4 kb) of egl-13 URS, persisted through division, differentiation, and morphogenesis (Hanna-Rose and Han, 1999). The maintenance of expression throughout the lineage together with the robust upregulation in uv1 daughters suggest that the mechanisms governing egl-13 marker expression may also be more broadly required for distinct aspects of the cell lineage.

To resolve possible enhancers contributing to egl-13 expression and π development, we first generated large deletions (up to ~5.4 kb) within the URS of the egl-13::GFP transgene and tested the remaining sequences for potential to drive π-specific expression (Fig. 1D). For the ease of following the subsequent deletions made to this full-length transgene, we refer to the egl-13::GFP (pWH17) reporter as egl-13FL::GFP (FL for full length). In this manner, we were able to deduce a 1330 bp (~1.3 kb) region upstream of the translational start of egl-13 sufficient for expressing GFP in π cells through all relevant stages of development as in Fig. 1C.

Conserved LAG-1 and Fos/Jun binding sites in the π enhancer of egl-13 are required for expression at specification

We utilized software to predict transcription factor binding sites and performed alignments of 6.4 kb egl-13 URS in C. elegans to exactly 8 kb of URS in the Caenorhabditis briggsae ortholog (Materials and methods). We found significant conservation of discrete stretches of sequence between the minimal 1.3 kb π enhancer of C. elegans to precisely 1340 bp proximal to the translational start of the C. briggsae ortholog (Fig. 2A).

In order to determine if the conserved sequences contribute to π-specific expression, we deleted each stretch of homologous sequence independently and in combinations and observed the uterine expression pattern from an otherwise intact egl-13FL::GFP transgene. Pertinent to this report, we found that deletion of a 106 bp (~100 bp) homologous region at the 5’ end of the 1.3 kb π
Fig. 2. LAG-1 and Fos/Jun cis binding elements are required for *egl-13* expression at spermathecal cell specification and for rescue of *egl-13* mutant defects. (A) Conservation of transcription factor binding sites within the 1.3 kb π enhancer of *egl-13*. Conserved cis-elements are shown in bold. Dark gray boxes represent sequences sharing remarkable identity with *C. briggsae*: 106 bp box 1 in *elegans* shares 88.2% identity with *briggsae*, 21 bp box 2 shares 100%, 65 bp box 3 shares 96.6%, 36 bp box 4 shares 94.4%, 142 bp box 5 shares 91.7%, and 45 bp box 6 shares 93.5%. Trans factor abbreviations (in order of relative upstream positions): Hb, Hunchback; DoF, DoF domain; MBF1, Multi-protein binding factor; Y-box, Y-box binding protein; Etsr, ETS domain related; HOM, homeodomain; POU, POU domain; HES, Hairy/Enhancer of split; TFIIID, transcription factor II D; ETS domain; NBF, Nonamer binding protein; STRE, stress response element; ZnF, zinc finger; LIM, LIM domain; NF-κB or Rel homology; Sp1 or ZnF, FOX, F-box/Forkhead, GATA, GATA binding factor; BR-C, Broad complex or ZnF; E2F factor; TBP, TATA binding protein; SRY, SRY or high mobility group (HMG). (B) Effect of LAG-1 or Fos/Jun deletion on temporal uterine *egl-13FL::GFP* fluorescence. DIC images showing medial uterine-vulval development. Lateral expression of *egl-13FL::GFP* in π cells is seen here just after the cells have divided. Neither *egl-13FL/LAG-1::GFP* nor *egl-13FL/Fos/Jun::GFP* transgenic animals fluoresce at specification. *egl-13FL/LAG-1::GFP* expresses in all π descendants later, whereas later expression of *egl-13FL/Fos/Jun::GFP* is only in uv1. Brackets encompass the π cells. Dashed ovals surround uterine area where fluorescence is lost. Asterisks mark body wall expression. Scale bar: 10 μm. (C) Four constructs of *egl-13FL::cDNA* were generated with intact, singly, or doubly-deleted LAG-1 and Fos/Jun binding sites. L4 stage hermaphrodites from independent *egl-13(ku194)* transgenic lines for each construct were scored over 4 days for egg laying. Egl+ indicates healthy appearance and egg laying. Egl+/- indicates egg laying but bloated appearance and/or rupture from internal hatching. Egl– indicates no egg laying and eventual rupture. *ku194* is 100% Egl– (n=100s, not shown). The strain designations are provided in Materials and methods.
enhancer (box 1 in Fig. 2A) was the only alteration that resulted in loss of tissue-specific expression in the uterus. The egl-13FL::LAG-1::GFP showed loss of fluorescence at specification whereas later expression was retained in uv1 daughters (data not shown; Materials and methods). Expression in other tissues (body wall and neurons) was unaffected. This homologous 100 bp sequence contained two conserved binding sites: one for LAG-1 and one for basic domain leucine zipper proteins (bZIP) of the Fos and Jun (Fos/Jun) family.

In addition to the conserved, canonical LAG-1 binding site within the π enhancer, there are two additional LAG-1 binding sites, another canonical and one non-canonical, present outside the enhancer and not conserved. However, clustered LAG-1 binding sites are not apparent in the URS of either C. elegans egl-13 or the briggsae ortholog. We removed the three underlined nucleotides of the conserved LAG-1 binding site 5'-GTGGGA-3' (the consensus sequence is 5'-RTGGGAA-3') within the π enhancer of the full-length reporter transgene, egl-13FL::LAG-1::GFP (Christensen et al., 1996). This single LAG-1 deletion abolished specification stage expression of egl-13::GFP in the π lineage (5 lines, n>50 per line; Fig. 2B). Therefore, the other two LAG-1 binding sites do not play redundant roles in directing early expression. The failure of the egl-13FL::LAG-1::GFP construct to express GFP in the π cells at a stage concomitant with induction by LIN-12 signaling suggests that egl-13 is a direct target of the pathway.

 Deleting the conserved LAG-1 binding site affected early π expression; however, later expression in the differentiated uterus and uv1 daughters was resumed. We reasoned that an additional cis element in the 100 bp homologous region must be driving later uterine expression, which was absent when the entire region was removed. Thus, we deleted the Fos/Jun binding site to generate egl-13FL::LAG-1::GFP. Similar to deletion of the single LAG-1 site, deletion of the Fos/Jun site abolished expression at π specification (5 lines, n>50 per line; Fig. 2B). However, unlike the LAG-1 deletion, later uterine expression was also compromised, resembling the expression pattern observed for egl-13FL::LAG-1::GFP. We infer that the conserved Fos/Jun binding site is equally crucial to the LAG-1 site for expression of egl-13 at specification and independently required for expression in π daughters that differentiate into utse cells.

LAG-1 and Fos/Jun cis elements are required for rescue of egl-13 mutants

Homozygous egl-13(0) mutants do not lay eggs. As a consequence, the hermaphrodite is consumed by internally-hatched larva and becomes a ‘bag of worms’ (Hanna-Rose and Han, 1999; Trent et al., 1993). This egg-laying-defective phenotype is presumably caused by an earlier malformation of the uterus, such that the uterine-vulval junction is blocked with thick tissue and an unfused AC (Cinar et al., 1983). This egg-laying-defective phenotype is presumably caused at specification through later L4 stages, whereas body wall fluorescence was unaffected (Fig. 3A). The sequence alteration in fos-1(ar105) leads to a nonsense mutation truncating the fos-1a transcript; however, generation of other functional bZIP-containing transcripts, such as fos-1b, is not perturbed (Sherwood et al., 2005) (Fig. 5A). To evaluate the consequences of eliminating all isoforms, we performed RNAi to fos-1 in the tyl4 background using a sequence that specifically targets the functional leucine zipper (dimerization) domain. The L4 stage undifferentiated uterus and loss of uterine egl-13::GFP fluorescence in fos-1 RNAI-treated animals were indistinguishable from those in ar105 animals (Fig. 3B). In later adult stages, fos-1(ar105) and fos-1(RNAi) animals displayed protruding vulva (Pvul) phenotypes, typical of uterine-vulval abnormalities. In addition, both fos-1(ar105) and fos-1(RNAi) are completely penetrant for sterility (Ste). The fact that the fos-1(RNAi) uterine phenotype closely resembles but is not worse than that of fos-1(ar105) suggests that the fos-1a isoform could specifically play the earliest role in uterine development.

We also investigated whether lin-11, an additional π marker, is expressed in fos-1 mutants. Expression of lin-11 in π cells is directly regulated by LIN-12 signaling, whereas expression in the vulva is regulated by Wnt signaling (Gupta and Sternberg, 2002). We compared the dynamic expression of a lin-11::GFP reporter during uterine-vulval development in the fos-1(ar105) background with the wild type (Fig. 4). We did not detect uterine lin-11 fluorescence at π cell specification or later relevant stages in fos-1 mutants (Fig. 4C-D,L for the wild type, Fig. 4G-H,Q for ar105). Conversely, we observed appropriate lin-11 expression in the 2° vulval lineage in fos-1 mutants (Fig. 4E-F, N-R) as in the wild type (Fig. 4A-B, I-M).

The AC in fos-1(ar105) fails to invade underlying primary vulval tissue during L3 stage uterine development, a process integral in securing proper orientation of the uterine-vulval connection (Sherwood et al., 2005; Sherwood and Sternberg, 2003). By showing that specifically driving fos-1a cDNA expression in the AC was sufficient to restore AC invasion, Sherwood et al. concluded that FOS-1a probably facilitates the expression of genes required to

fos-1 mutants have uterine defects and fail to express π-specific markers

The nonconsensus bZIP binding site, 5'-TTAGTCA-3', in the π enhancer is more similar to the consensus binding site for Fos and Jun, 5'-TGA(C/G)TCA-3', than to other subclasses of bZIP transcription factors (Angel et al., 1987; Lee et al., 1987). We retrieved fos-1 and T24H10.7, sharing 33% and 41% identity, respectively, in their functional bZIP domains, as the closest C. elegans homologs to the mammalian oncogenes c-Fos and c-Jun, respectively. In this section, we address the role of fos-1 in the uterus. Later in this report, we provide the first documentation of an in vitro biological activity and tissue localization for T24H10.7, now referred to as jun-1.

We examined genetic fos-1(ar105) mutants (Seydoux et al., 1993; Sherwood et al., 2005) and found that they consistently lack an apparent uterine lumen and a utse-like process (Fig. 3A). We also noted the absence of uterine egl-13::GFP fluorescence in ar105 from specification through later L4 stages, whereas body wall fluorescence was unaffected (Fig. 3A).

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UTERINE CELL THAT REQUIRES GONADORENSES (SHERWOOD ET AL., 2005). WE OBSERVED THAT A TRANSCRIPTS ARE EXPRESSED THROUGHOUT THE UTERUS DURING REGRESSION OF A PRESUMABLY NORMAL AC (Fig. 3).

**Fig. 3. fos-1 loss-of-function causes abnormal uterine morphology and absence of uterine egl-13 expression.** (A) Specification and mid L4 stage expression of egl-13::GFP(tyIs4) in the wild type and fos-1(ar105). fos-1(ar105) do not express GFP in uterine tissue (dashed ovals indicate where fluorescence was expected; fluorescent nuclei in the wild type are bracketed). The mid L4 stage uterus in ar105 is undifferentiated by comparison with the wild type, as apparent by the absence of a uterine lumen. Arrow indicates the uterus, observed only in the wild type. Other GFP-positive cell bodies belong to the body wall, where fluorescence is not affected. (B) No uterine egl-13::GFP expression or lumen is observed after fos-1 RNAi treatment. Scale bar: 10 μm.

bestow invasive properties on the AC (Sherwood et al., 2005). We further examined a line rescued for FOS-1A activity in the AC only (Materials and methods) and found that uterine defects persisted regardless of a presumably normal AC (n=35 mid L4 stage animals, data not shown). We infer that AC invasion is not the only process impaired by loss of fos-1a function and that the AC is not the only uterine cell that requires fos-1. Rather, fos-1a appears to function independently and autonomously in uterine cells.

**FOS-1 is expressed in all precursor cells that can adopt the π cell fate**

Studies with fos-1 reporter fusions resolved that fos-1a and fos-1b transcripts are expressed throughout the uterus during gonadogenesis (Sherwood et al., 2005). We observed that a fos-1a translational fusion is expressed throughout the VU intermediate precursor cells, including π cells, a pattern consistent with loss of fos-1a in ar105 failing to give rise to π-derived tissue (data not shown). To better resolve precise uterine expression, we cloned smaller pieces of the fos-1 locus that might potentially contain a uterine enhancer separated from other gonadal enhancers (Sherwood et al., 2005). We observed uterine-specific expression from a translational fusion which we refer to as plastFOS-1c::YFP. This 4.4 kb genomic construct includes an approximately ~2 kb intron preceding the last four exons and may represent the shortest bZIP-encoding FOS-1 isoform, or fos-1c (Fig. 5A). plastFOS-1c::YFP showed expression primarily in the early dorsal and ventral uterus during L3 and L4 stages. Importantly, we observed expression in all VU intermediate precursors including π cells (Fig. 5B-D). Furthermore, we confirmed that plastFOS-1c::YFP co-localized with cells expressing an egl-13 π marker from the late L3 induction stage (Fig. 5D-F) through generation of π descendants (Fig. 5G-J).

plastFOS-1c::YFP may represent a downstream uterine enhancer directing expression of the characterized isoforms. However, our lab has documented that large introns within a locus can function as separate promoters for transcription of other often differentially expressed isoforms in *C. elegans* (Choi and Newman, 2006). Based on the presence of a predicted promoter region, conserved proximal TATA box, in-frame translational START codon (ATG), and identification of this specific isoform as the closest *C. briggsae* homolog, we suggest that fos-1c may be a unique product of the *C. elegans* fos-1 gene. Since the only genetic mutant of fos-1 specifically affects fos-1a and has broad uterine defects, we could not readily assess the relative contributions of fos-1a, b or c to the specific process of π cell induction.

**FOS-1 specifically binds in vitro to egl-13 URS as a heterodimer with JUN-1**

Fos proteins form less stable homodimers than Jun and generally function as heterodimers with Jun (O’Shea et al., 1992). Nonetheless, we tested if FOS-1 as a homodimer can directly bind target sequence in a novel manner in *C. elegans*. We performed electrophoretic mobility shift assays (EMSA) with the 100 bp homologous region of egl-13 URS (box 1, Fig. 2A). This radiolabelled probe containing the conserved Fos/Jun binding site failed to produce a shifted band in the presence of in vitro translated FOS-1 alone or JUN-1 alone (Fig. 6, first gel, lane 2 and 3, respectively).

The consensus seven base pair binding site 5’-TGA(C/G)TCA-3’ for Fos/Jun binding is palindromic from the central C or G base pair and results in two asymmetric half-sites 5’-TTAGTCA-3’. The nonconsensus binding site 5’-TGAGTCA-3’ may facilitate Fos/Jun heterodimer or Jun dimer binding (Glover and Harrison, 1995). The nonconsensus binding site 5’-TTAGTCA-3’ in egl-13, with one nonconsensus half site, 5’-TTAG-3’, and one consensus half site, 5’-TGAG-3’, may favor binding by a Fos/Jun heterodimer (Ramirez-Carrozzi and Kerppola, 2003). Thus, we tested if FOS-1 and JUN-1 together can bind egl-13 URS in vitro. Indeed, we observed a striking band shift in the presence of both FOS-1 and JUN-1 (Fig. 6A, first gel, lane 4). We also observed a prominent supershift when we added antibody against Myc to the reaction, demonstrating that the shifted complex specifically included a recombinant N-terminal Myc-tagged version of FOS-1.
Addition of intact but not mutated unlabeled templates significantly reduced the supershift (Fig. 6A, second gel). These results support *egl-13* being a direct target of FOS-1 regulation through an obligate heterodimer with JUN-1 in vitro.

We performed RNAi to *jun-1* but did not discern any phenotypes similar to *fos-1* knockdown. We then conducted blast searches of mammalian Fos and Jun proteins against the *C. elegans* genome to find additional paralogs to test (Materials and methods). After several rounds of single, and in combination with *jun-1(RNAi)*, feeding experiments, we were still not able to detect uterine phenotypes or loss of uterine *egl-13::GFP* upon performing RNAi to other candidates. Our inability to detect a loss-of-function phenotype could be due to redundancy in the system or lack of effectiveness of RNAi.

**VU intermediate precursors express JUN-1**

The above biochemical data prompted us to determine whether *jun-1* is expressed in the uterus at the appropriate time to be operative in π cell specification. First, we generated a transcriptional reporter of ~5 kb of URS ahead of the first exon fused to GFP. In five independently generated lines, we observed diffuse transgene expression throughout (Fig. 6A, first gel, lanes 5 and 6).
the animal; however, we could not detect specific uterine expression (data not shown). Therefore, as with our analysis of a more specific uterine enhancer in the fos-1 locus, we sought to determine if such tissue-specific drivers are present within the ~19 kb jun-1 locus. After testing several reporter fusions, our best inference of uterine jun-1 expression came from a genomic translational reporter which we refer to as pJUN-1d/c::GFP (Fig. 6B). We observed expression of pJUN-1d/c::GFP in the AC and surrounding VU intermediate precursors at the time of π cell specification (Fig. 6C-F). Interestingly, translational reporters of fos-1a, b and c and here jun-1 display indistinguishable expression throughout dorsal and ventral uterine cells at the late L3 stage (this study) (Sherwood et al., 2005). Taken together with the loss of differentiated uterine structures in L4 stage fos-1(ar105) mutants, we suggest that Fos/Jun heterodimeric regulation may be a plausible facet of proper uterine development.

DISCUSSION

Fos activity is required for π cell potential

We elucidated a novel regulatory interaction between LIN-12/Notch and FOS-1 to establish the uterine π cell fate and promote expression of a downstream gene. We found that independent deletions to conserved LAG-1 or Fos/Jun cis-regulatory elements compromised expression of egl-13 at specification. Deletion of both sites negated transgenic rescue of egl-13 mutants. We observed that uterine tissue of fos-1-deficient animals appeared undifferentiated and did not give rise to structural features such as a uterus or lumen. In addition, uterine expression of egl-13 and lin-11 markers was specifically lost in fos-1 mutants. We also demonstrated that fos-1 is uniformly expressed in the VU lineage including the π cells at the time of specification. Together, our results suggest that fos-1 is broadly required for uterine development, and it also functions more specifically in π cell induction and egl-13 expression.

Unlike lin-12, fos-1 had not previously been implicated in π fate specification. Prior study has shown that all VU granddaughters have an intrinsic ability to adopt π-like fates in the presence of constitutive LIN-12 activity, which bypasses the required cell-cell interactions with the signal-presenting AC (Newman et al., 1995). By contrast, LIN-12 signaling in other tissues results in other outcomes. Here we suggest that FOS-1 activity in the early ventral uterus is one mechanism by which progenitors are instilled with the unique potential to adopt the π cell fate. Specifically, we have shown that transcriptional regulation of the LIN-12 target gene egl-13

Fig. 5. FOS-1 is expressed in the intermediate precursors and co-localizes with EGL-13 during the π lineage. (A) fos-1 locus with isoform a, b, and putative c translational starts and origin of plastFOS-1c::YFP indicated. (B-F) Late L3 stage. (B) The AC (black arrowhead) is invading underlying primary vulval cells (black bracket). (D) Approximately 5 μm from B, FOS-1c is expressed in all dorsal and ventral uterine cells including the VU intermediate precursors (C, white arrowheads). (E) Three π cells express egl-13::CFP (white bracket). (F) Merged image of D and E showing that FOS-1c co-localizes with egl-13 at specification. (G-J) Mid L4 stage. (H) FOS-1c is broadly expressed in numerous uterine cells. White arrows point to six π cell daughters plus the AC. (I) π descendants continue to express egl-13::CFP. (I) Merged image of H and I showing FOS-1c also co-localizes to the π descendants. Asterisks mark non-specific fluorescence from the GFP co-transformation marker. Scale bar: 10 μm.
necessitates synergy with Fos activity. The overlap of these two pathways could be a critical link that sets specification of \( \pi \) cells apart from other Notch-mediated decisions. This conclusion is supported by our finding that loss of \( fos-1 \) did not alter specification of the 2\(^{nd} \) vulval cells, another well-characterized LIN-12-induced lineage, as evident by the appropriate expression of a 2\(^{nd} \) marker in this tissue.

We show that FOS-1 can specifically bind to \( egl-13 \) URS as an evolutionarily conserved heterodimer with JUN-1. The lack of a \( jun-1 \) RNAi phenotype suggests that perhaps FOS-1 alone could regulate \( egl-13 \) in vivo or that other Jun-like proteins could functionally substitute as competent partners for FOS-1. The latter redundancy of Jun activity has been observed in mammalian systems (Mechta-Grigoriou et al., 2001). Our expression analysis suggests a role for \( jun-1 \) in uterine development.

**EGL-13 expression relies on dual cis-regulation by LAG-1 and Fos/Jun**

Deletion of the conserved LAG-1 and Fos/Jun-binding sites indicated that both are required for \( egl-13 \) expression at specification, whereas Fos/Jun, but not LAG-1, is required later in the lineage. The Fos/Jun pathway may also regulate the development of \( \pi \) cell descendants, perhaps by promoting expression of \( egl-13 \) and other critical factors. Our studies also revealed the presence of a uv1-specific enhancer.

Mutant rescue by over-expressing \( egl-13 \) cDNA behind intact or ablated cis-elements gave results that were consistent with those above. Use of a rescue construct with a LAG-1 deletion resulted in a range of egg-laying ability – from comparable to intact to more attenuated. The presumptive \( \pi \) cells of \( egl-13 \) mutants initiate the appropriate division pattern before abnormally dividing again (Cinar

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**Fig. 6. FOS-1 can directly bind to egl-13 URS as a heterodimer with JUN-1, which is also expressed throughout the uterus at \( \pi \) cell specification.** (A) EMSA with labeled 100 bp homologous template containing the Fos/Jun binding site. Left gel: No band shift was detected with only lysate present (\( \phi \), lane 1), FOS-1 alone (lane 2), or JUN-1 alone (lane 3). A shifted complex (bottom arrow) was observed in the presence of both FOS-1 and JUN-1 (lane 4) and also with a Myc-tagged version of FOS-1 with JUN-1 (lane 5). Addition of polyclonal anti-Myc antibody to lane 6 produced a supershift due to increase in molecular mass of DNA-protein complex plus Ab (top arrow) and also completely neutralized the original band shift (bottom arrow) due to titration of protein from forming a stable complex. Right gel: The supershift was significantly reduced in the presence of unlabeled (COLD) sequence containing the \( egl-13 \) Fos/Jun binding site (lanes 4-6) but not effectively reduced by an excess of unlabeled sequence carrying a mutated Fos/Jun binding site (lanes 7-9). Gray triangles above lanes 4-6 and 7-9 represent increasing amounts (5\( \times \), 25\( \times \) and 125\( \times \), respectively) of unlabeled 30 bp competitor oligonucleotides. (B) This diagram represents the position of the isolated 5.3 kb genomic sequence in \( pJUN-d/c::GFP \) in the context of the entire \( jun-1 \) locus. The NruI site is a starting reference point for \( \sim 5 \) kb upstream of the first transcript. The translational starts for verified isoforms are indicated. (C-F) Uterine JUN-1 expression. (C,D) In the medial plane, expression of \( pJUN-d/c::GFP \) is detected in the AC (black arrowhead) as well as throughout the dorsal and ventral uterus. (E,F) In a lateral plane, VU intermediate precursors (white arrowheads) also express the translational reporter. Scale bar: 10 \( \mu \)m.
et al., 2003; Hanna-Rose and Han, 1999). For that reason, we infer that the function of EGL-13 is not required for the earliest aspects of the lineage. Thus, the extent of egg laying restored by the LAG-1 deletion construct may reflect egl-13 expression that is early enough to effectively maintain the lineage.

The more attenuated rescue by the Fos/Jun deletion construct is consistent with a more pronounced loss of egl-13 expression early and later in the π lineage. Yet, how can we account for the 20-30% of mutants completely rescued? First, weak fluorescence signals emitted by reporter fusion lines may be undetectable. Also, each single deletion rescue transgene at high-copy in a non-chromosomal context may be able to recruit transcriptional machinery and activate gene expression on its own. Therefore, cumulative, albeit low grade, permissive expression may provide sufficient EGL-13 activity to reinstate π cell development in single site-deleted rescue lines and account for the completely rescued mutants.

Nonetheless, the fact that mutant transgenic lines remained completely Egl when both LAG-1 and Fos/Jun binding sites were omitted from the rescue construct reinforces the importance of these sites. Our data suggests that transcriptional machinery cannot be recruited to activate egl-13 expression at specification of the uterine π cell fate in the absence of both conserved LAG-1 and Fos/Jun cis-elements, which are apparently not required for expression in other cells. We propose that regulation by LIN-12 and Fos/Jun largely governs whether egl-13 and perhaps other uncharacterized LIN-12/Notch target genes are expressed during uterine development.

Tissue-specific expression of egl-13 during π cell development
Clustered LAG-1 binding sites are a hallmark of many Notch-regulated genes. Previously, direct target genes of LIN-12/Notch signaling were predicted by in silico approaches scouting for numerous LAG-1 binding sites distributed throughout the genome (Rebeiz et al., 2002; Yoo et al., 2004; Yu et al., 2004). However, one or a few LAG-1 binding sites may be crucial (Kim et al., 1996), even in genes with multiple such sites (Christensen et al., 1996; Gupta and Sternberg, 2002; Wilkinson et al., 1994). Our findings highlight that one functional LAG-1 cis-element in conjunction with an additional element for another transcription factor or pathway can direct tissue-specific expression of a LIN-12 target gene. Such combinatorial control of Notch target gene regulation has been documented in Drosophila (Cave et al., 2005).

Relevant to our report, egl-43, a zinc finger transcription factor, contains multiple LAG-1 regulatory sites and is implicated in π cell development (Hwang et al., 2007; Rimann and Hajnal, 2007). Attenuated expression of π-specific markers and malformation of utse were observed in the absence of egl-43 via RNAi treatment (Rimann and Hajnal, 2007). In contrast to the LIN-12 target genes egl-13 and lin-11, which are expressed in just the π lineage, egl-43 expression was observed more broadly (Hwang et al., 2007; Rimann and Hajnal, 2007). Epistasis experiments suggested that egl-43 acted downstream of, or in parallel to, lin-12. Since egl-43 appears to be involved in π cell fate specification (Rimann and Hajnal, 2007) whereas egl-13 is required for π cell fate maintenance (Cinar et al., 2003), it is possible that egl-43 acts between lin-12 and egl-13. Our studies demonstrate that egl-13 is a direct target of lin-12 as well as of fos-1; however, egl-13 may also be regulated, either directly or indirectly, by egl-43.

Notch and Fos/Jun, an evolving relationship
The 100 bp interval containing the critical LAG-1 and Fos/Jun binding sites in the π enhancer of C. elegans egl-13 has ~90% identity to the homologous interval in C. briggsae and is located at an equivalent upstream distance in each ortholog. Such accurate conservation after 100 million years of divergence suggests the importance of this cis-regulatory module. Intriguingly, this motif may represent a transcription code mandating cooperation between activating complexes recruited by the NCID-LAG-1 complex and FOS-1 heterodimer. Such models of synergy among associated DNA binding proteins at discrete enhancers have been documented as underlying mechanisms for tissue-specific gene expression.

In a broader scope, Notch and Fos/Jun pathways are both involved in major events such as T cell development and cancer progression (Foletta et al., 1998; Radtke et al., 2004; Tulchinsky, 2000; Vogt, 2001; Weng and Aster, 2004). Many lines of evidence put Notch and Fos/Jun activity at close range, including recent studies of the egl-43 gene in C. elegans (Hwang et al., 2007; Rimann and Hajnal, 2007). However, our study is the first to expose a cooperative and compulsory interaction between them. Considering the widely implemented and highly conserved nature of Notch and Fos/Jun signaling pathways, we speculate that such synergistic communication of the two may be present in higher order systems as well.

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