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Rimann, I; Hajnal, A
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REGULATION OF ANCHOR CELL INVASION AND UTERINE CELL FATES BY THE EGL-43 EVI-1 PROTO-ONCOGENE IN C. ELEGANS

Ivo Rimann and Alex Hajnal

Institute of Zoology, University of Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland

Corresponding author

Email: ahajnal@zool.uzh.ch

Tel. +41-44-6354854, FAX +41-44-6356878

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Abstract

Cell invasion is a tightly controlled process occurring during development and tumor progression. The nematode *C. elegans* serves as a genetic model to study cell invasion during normal development. In the third larval stage, the anchor cell in the somatic gonad first induces and then invades the adjacent epidermal vulval precursor cells. The homolog of the *Evi-1* oncogene, *egl-43*, is necessary for basement membrane destruction and anchor cell invasion. *egl-43* is part of a regulatory network mediating cell invasion downstream of the *fos-1* proto-oncogene. In addition, EGL-43 is required to specify the cell fates of ventral uterus cells downstream of or in parallel with LIN-12 NOTCH. Comparison with mammalian *Evi-1* suggests a conserved pathway controlling cell invasion and cell fate specification.
Introduction

Invasion of cells across a basal lamina into another tissue is a tightly regulated process occurring during diverse developmental processes such as trophoblast implantation, gastrulation or neural crest cell migration (Ozanne et al., 2006; Sherwood, 2006). Activation of cell invasion is also thought to be the first step during the progression of a benign sessile into a malignant metastasizing tumor (Yamaguchi et al., 2005). Cell invasion involves the activation of a specific transcriptional program (Ozanne et al., 2006). The AP-1 transcription factor complex plays a key role during the initiation of the invasion program by integrating the input from different extracellular signaling pathways. AP-1 usually exists as a heterodimer consisting of one fos and one jun family member. Activation of AP-1 correlates well with the invasive phenotype and metastasizing potential of different solid tumors in humans. Moreover, several genes up-regulated in v-fos transformed fibroblasts regulate cell motility and adhesion (Ozanne et al., 2006).

Recently, the vulva of the nematode C. elegans has been established as a model to study cell invasion during normal development (Sherwood, 2006; Sherwood and Sternberg, 2003). During vulval development, the anchor cell (AC) in the somatic gonad induces the primary (1°) vulval cell fate in the nearest vulval precursor cell (VPC) P6.p by secreting an epidermal growth factor (LIN-3) (Sternberg, 2005). After induction of the 1° fate at the beginning of the third larval stage (L3), the AC crosses the two basal laminae separating the somatic gonad from the vulva and invades the vulval tissue in between the 1° descendants of P6.p (Sherwood and Sternberg, 2003). The C. elegans fos-1 gene is at the top of a genetic pathway activating the expression of specific invasion effectors in the invading AC (Sherwood et al., 2005).
Here, we report the identification of egl-43, the C. elegans homolog of the mammalian Evi-1 proto-oncogene (Garriga et al., 1993; Mitani, 2004), as a component of the regulatory network controlling AC invasion downstream of fos-1. The egl-43 Evi-1 locus encodes two Zn-finger transcription factors, of which the shorter isoform has been shown to be required for HSN neuron migration (Garriga et al. 1993). We find that the longer of the two isoforms encoded by the egl-43 locus (egl-43L) specifically promotes AC invasion as a transcriptional target of fos-1, while the short isoform (egl-43S) is expressed independently of fos-1. In addition to its function during AC invasion, we describe a second function of egl-43 in the ventral uterine cells where egl-43 acts downstream of the Notch signaling pathway during the specification of the π-cell fate (Newman et al., 1995). Comparison with mammalian Evi-1 suggests that egl-43 may be part of a conserved gene network regulating cell invasion and fate specification.

**Results**

egl-43 is required for anchor cell invasion

We have identified the C. elegans Evi-1 homolog egl-43 in an RNA interference (RNAi) based screen for genes affecting vulval cell fate execution or vulval morphogenesis (Berset, 2005). Since a loss-of-function mutation in egl-43 (tm1802, fig. 2 A, gift of S. Mitani and colleagues) causes an L1 larval arrest phenotype, we first used egl-43 RNAi (egl-43i in fig. 2 A) to bypass the early larval arrest, allowing us to characterize the function of egl-43 during vulval development in L3 and L4 larvae. Since egl-43 RNAi animals showed no obvious change in the vulval cell lineage, egl-43 is probably not required for vulval cell fate specification or execution (data not shown). However, egl-43i animals exhibited defects in vulval
morphogenesis, resulting in a 52 % (n=65) penetrant protruding vulva (Pvl) phenotype. In particular, we noticed that in egl-43i animals the AC was unable to invade the vulval tissue across the two basal laminae separating the somatic gonad from the epidermis (fig. 1 A and C). By the Pn.pxx stage, the basal laminae under the AC were dissolved in all control RNAi animals examined (i.e. animals treated with an RNAi feeding vector lacking an insert, n=8), resulting in a broken line of mitotracker staining between the two tissues (fig. 1 B). However, in 75% (n=8) of egl-43i animals mitotracker staining revealed intact basal laminae, pointing at a defect in AC invasion (fig. 1 D). Similarly, HIM-4::GFP, a hemicentin that is localized to the extracellular matrix (Sherwood et al., 2005), showed a punctate staining near the AC during vulval invagination in all 23 control RNAi animals, but no signs of degradation in 39% (n=31) of egl-43i animals (fig. 1 E to H). In addition, we noticed that during vulval invagination the AC in egl-43i animals was often not centered above the forming cavity (fig. 1 G). The mispositioning of the AC is likely due to the failure of the AC to invade and connect to the vulval cells. In wild-type L4 larvae, the uterine and vulval lumen are separated by a thin laminar process formed by the uterus seam cell (utse) syncytium (fig. 1 I, n=26)). In 29% of egl-43i animals (n=21), no utse was visible, and a thick layer of tissue remained between the uterus and vulval tissue (fig. 1 J). As a consequence of these defects in vulval morphogenesis, the vulva of egl-43i animals did not invaginate properly, resulting in a strong Pvl phenotype in the adult animals (data not shown).

The egl-43 locus encodes two isoforms, termed egl-43S and egl-43L, that are transcribed using two different promoters (Garriga et al. 1993 and fig. 2 A). Similar AC invasion defects as in egl-43i animals were observed in egl-43(tm1802) mutants, in which the early larval arrest phenotype had been rescued by an egl-43 transgene
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encoding both egl-43 isoforms (-6-egl-43, fig. 2 A). In 55% (n=20) of egl-43(tm1802) animals carrying the -6-egl-43 construct, the basal laminae were removed and AC invasion occurred. Due to incomplete rescue by the extrachromosomal array, 45% of the transgenic animals exhibited no AC invasion. Furthermore, a minigene construct encoding only the long egl-43L isoform rescued the egl-43(tm1802) larval arrest phenotype and AC invasion defects (-6-egl-43L in fig. 2 A). In -6-egl-43L animals in which the L1 larval arrest had been rescued, approximately 70% of the animals also showed rescue of the AC invasion defect. (Three independent lines carrying extrachromosomal arrays with -6-egl-43L were analyzed; n=10 to 12.) In contrast, transgenes encoding only the short egl-43S isoform failed to rescue the larval arrest (-1.3-egl-43S in fig. 2 A). Thus, the egl-43L transcript is sufficient to promote AC invasion and uterus development during larval development.

**fos-1 activates expression of the long egl-43L transcript in the anchor cell**

The shorter egl-43S transcript was previously found to be required for HSN neuron migration and to be expressed in HSN neurons (Garriga et al., 1993), but the expression pattern of the longer egl-43L transcript has not yet been described. We therefore determined the expression pattern of both egl-43 isoforms by constructing GFP reporter transgenes containing either of the two promoters. We focused our analysis of the expression pattern on the ventral uterine and vulval cells. A transcriptional reporter consisting of 1.7 kb of 5’ regulatory sequences upstream of the egl-43L start codon (-1.7- Lp::gfp, fig. 2 A) showed GFP expression first in the ventral uterine (VU) cells of mid L2 larvae (Pn.p stage) and then in the AC beginning in mid L3 larvae, after the first round of vulval cell divisions had occurred (fig. 2 B, Pn.px stage). AC expression of -1.7-Lp::gfp increased during AC invasion in late L3
larvae, but remained constant in the VU descendants. -1.7-Lp::gfp expression was also observed in the gut and a set of neurons in the head region (data not shown). However, no expression could be detected in the vulval cells during vulval cell fate specification and AC invasion (fig. 2 B and C).

Since egl-43i causes essentially the same AC invasion defects as observed in fos-1(ar105) mutants (Sherwood et al. 2005), we tested whether egl-43L expression in the AC is regulated by FOS-1. In fos-1(ar105) homozygous L3 larvae carrying the -1.7-Lp::gfp reporter, no GFP expression could be detected in the AC, and GFP expression in the VU cells was also strongly reduced (fig. 2 C, D and data not shown). To further narrow down the FOS-responsive element (FRE) in the egl-43L promoter we constructed a series of reporters and progressively shortened the promoters from the 5′end. This analysis identified a 139 bp fragment sufficient to drive expression of GFP in the AC (-0.14-Lp::gfp, fig. 2 A and E). The canonical binding site for mammalian Fos proteins is TGA^G/C TCA (Nakabeppu et al., 1988). Sequence alignment of the 139 bp minimal FRE with the corresponding 5′ regulatory regions of C. briggsae and C. remanei egl-43L identified a conserved but imperfect putative FOS-1 binding site TTACTCA at position -83 relative to the ATG of egl-43L (suppl. fig. s1). Further deletion of the reporter beyond this site resulted in a complete loss of AC expression (data not shown), and a point mutation in the putative fos-1 binding site (changing the site from TTACTCA to ATACTCA) introduced into the -0.14-Lp::gfp construct caused a strong reduction of GFP expression in the AC, while expression in other tissues such as in the gut was unaffected (Fig. 2 E, F and G). Thus, fos-1 likely activates egl-43L transcription in the AC possibly through a non-canonical binding site.
A reporter for the short egl-43S isoform (-5.1-Sp::gfp, fig. 2 A) showed similar AC expression as the egl-43L reporter. However, AC expression of egl-43S was already detected at the Pn.p stage in mid L2 larvae, soon after the AC had been specified and before egl-43L expression was observed in the AC (fig. 2 H). In contrast to egl-43L, egl-43S expression decreased during invasion in L3 larvae, and AC expression of egl-43S remained unchanged in fos-1(ar105) mutants (fig. 2 I and J). Further dissection of the AC-specific element driving egl-43S expression led to the identification of a 1.6 kb fragment containing the first intron that was capable of driving GFP expression in the AC from the minimal Δpes-10 promoter (fig. 2 A and K). It thus appears that the fos-1-independent AC element in the first intron activates transcription of egl-43S and also egl-43L before AC invasion. Activation of fos-1 during AC invasion in L3 larvae then shifts the balance in favor of egl-43L.

egl-43L is necessary for AC invasion

The distinct regulation of the two egl-43 isoforms suggested that egl-43L might promote AC invasion downstream of fos-1. To test this hypothesis, we specifically down-regulated egl-43L expression by feeding dsRNA derived of a DNA fragment unique to egl-43L (egl-43Li, fig 2 A). egl-43Li treated animals showed the same AC invasion defects as observed after down-regulation of both isoforms (egl-43i), indicating that egl-43L is necessary for AC invasion (fig. 1 K and L). The specificity of the egl-43L dsRNA treatment was tested in transgenic animals carrying the -5.1-Sp::gfp construct, a transcriptional reporter specific for egl-43S that includes the target region of the egl-43L specific RNAi clone (fig. 2 A). In egl-43Li treated animals, GFP expression in the AC was only slightly reduced compared to control RNAi animals treated with an empty RNAi feeding vector. The observed change in
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reporter expression points at an indirect effect on EGL-43S levels, possibly through auto-regulation of egl-43. Thus, the AC invasion defects induced by egl-43Li are unlikely to be caused by the mild reduction of egl-43S expression. Furthermore, this experiment also excluded the possibility of an off-target effect, as RNAi using two non-overlapping fragments of the egl-43 open reading frame induced an identical phenotype. Since the entire egl-43S open reading frame and 3’UTR is also contained in egl-43L, we were unable to specifically down-regulate egl-43S without affecting egl-43L expression.

egl-43 promotes anchor cell expression of the invasion effectors zmp-1 and cdh-3

fos-1 positively regulates expression of the metalloprotease zmp-1, the FAT-like protocadherin cdh-3 and the hemicentin him-4 during AC invasion (Sherwood et al., 2005). In contrast to egl-43i, single mutants in zmp-1, cdh-3 or him-4 exhibit only weak AC invasion defects, indicating that these genes are genetically redundant. Moreover, it has been unknown whether zmp-1, cdh-3 and him-4 are direct or indirect fos-1 targets. We therefore asked whether egl-43 acts upstream of or in parallel with these previously identified fos-1 targets by examining the AC expression of zmp-1p::cfp and cdh-3p::cfp transcriptional reporters in egl-43i animals at the Pn.pxx stage. Expression of zmp-1p::cfp was reduced in around 50% of egl-43i animals below the levels detected in any of the control RNAi animals (fig. 3 A to C). The loss of zmp-1p::cfp expression in 50% of the animals is likely due to the fact that only around half of the animals were strongly affected by the RNAi treatment in the particular experiment shown. Loss of fos-1 function only weakly reduces cdh-3 expression (Sherwood et al., 2005), and egl-43i animals exhibited a partial reduction in cdh-3p::cfp expression to a similar extent as reported for fos-1 (fig. 3. D to F).
Interestingly, in the strongly affected *egl-43i* animals at the Pn.pxx stage also the neighboring VU cells expressed *cdh-3p::cfp*, although in mid-L2 larvae just after the AC had been specified, expression of *cdh-3p::cfp* and other AC-specific genes was restricted to the AC (data not shown). Thus, the ectopic expression of *cdh-3p::cfp* in VU cells at the Pn.pxx stage points at an additional defect in π-cell fate specification, which takes place during the L3 stage (see below). Taken together, our data indicate that *fos-1* initiates AC invasion by up-regulating *egl-43L* expression in the AC, which in turn activates effector genes such as *zmp-1* and *cdh-3* that are executing the invasion step (fig. 4 H).

*egl-43 is required to specify the π-cell fate in the ventral uterine cells*

The AC is not only required to induce vulval development and form a connection between the uterus and the vulva but also to specify the fates of the adjacent VU cells (Newman et al. 1995). During the late L3 stage, the AC produces an instructive LAG-2 DELTA signal that activates the LIN-12 NOTCH pathway in the neighboring VU cells and induces the π-cell fate (Greenwald, 1998; Newman et al., 1995). Most of the π-cell descendants then fuse with the AC to form the utse syncytium, which forms a connection between the uterus and the lateral seam cells (fig. 1 I). The zinc-finger transcription factor *lin-29* has been shown to be necessary for LAG-2 expression in the AC (Newman et al., 2000). Thus, in *lin-29* mutants π-cells are not formed and as a consequence a thick layer of tissue separates the uterine and vulval lumen. Since we noticed that also in *egl-43i* L4 larvae a proper utse was often not formed (fig. 1 J), we tested if *egl-43* plays a role in π-cell fate specification besides its function during AC invasion. For this purpose, we used transcriptional GFP reporters for *lin-11* and *egl-13/cog-2* that are expressed in the π-cells (Gupta and Sternberg, 2002; Hanna-Rose
and Han, 1999). In contrast to lin-29 mutants, π-cells are specified in mutants of egl-13 or lin-11, but the execution of a proper π-cell fate is impaired (Cinar et al., 2003; Newman et al., 1999). In most control animals, there were 12 VU cells expressing lin-11p::gfp and egl-13p::gfp (fig. 4 A, C, E and G). In egl-43i animals, the number of lin-11p::gfp and egl-13p::gfp positive cells was reduced to fewer than four gfp expressing VU cells, although there was no obvious change in the total number of cells in the uterus visible under Nomarski optics (fig. 4 B, C, F and G). Therefore, egl-43 is not only required to mediate AC invasion but also to specify the π-cell fate of the VU cells. Since the defect in π-cell fate specification in egl-43i animals resembles the defect in lin-29 mutants, egl-43 might regulate in the AC the production of the instructive lag-2 delta signal in order to activate the lin-12 notch pathway in the adjacent VU cells. Alternatively, egl-43 might act in the VU cells and be required to specify the π-fate downstream of lin-12 notch. To distinguish between these two possibilities, we tested if egl-43i suppresses the formation of extra π-cells in lin-12(n137gf) mutants, in which the Notch pathway is activated independently of the lag-2 delta signal (Greenwald, 1998; Newman et al., 1995). The average number of egl-13p::gfp expressing VU cells per animal was significantly reduced in egl-43i treated lin-12(gf) animals (fig. 4 D). Moreover, egl-43i did not reduce expression of a lag-2::gfp reporter in the AC, indicating that egl-43 is not required for the transcription of lag-2 delta in the AC (data not shown). We therefore conclude that egl-43 acts downstream of or in parallel with lin-12 notch to specify the π-cell fate in the ventral uterus (fig. 4 H).
**Discussion**

In summary, our data indicate that the *C. elegans Evi-1* homolog *egl-43* performs two distinct functions during ventral uterus development. In the AC, *egl-43* acts downstream of the *fos-1* proto-oncogene to promote the invasion of the AC into the vulval tissue (fig. 4 H). While this work was under review, a similar function of *egl-43* during AC invasion and gonad patterning has been reported by Hwang et al. (2007). Extending the findings by Hwang et al. (2007), we show that the long *egl-43L* isoform is directly regulated by *fos-1* through a conserved Fos-responsive element immediately upstream of the *egl-43L* transcriptional start site and that EGL-43L is both necessary and sufficient to mediate AC invasion. During AC invasion in L3 larvae, *egl-43L* up-regulates the expression of the effector genes *zmp-1* and *cdh-3* that are required to carry out the invasion step. *egl-43S*, on the other hand, is already expressed prior to AC invasion in L2 larvae, and is not regulated by *fos-1*. Therefore, the specific up-regulation of the longer *egl-43L* isoform by *fos-1* may lead to the initiation of the invasion program, while the constitutive *egl-43S* expression in the AC is not sufficient to induce AC invasion. One possible model is that EGL-43S competes with EGL-43L by binding to the promoters of the same target genes and repressing their transcription. Remarkably, the human *egl-43* ortholog *Evi-1* also encodes two factors that exhibit antagonistic activities (Mitani, 2004). The short *Evi-1a* isoform can form hetero-oligomers, and it interacts with the transcriptional co-repressor CtBP to recruit histone deacetylases and represses TGF-β induced gene expression (Izutsu et al., 2001; Kurokawa et al., 1998; Nitta et al., 2005). The long *Evi-1c* isoform does not oligomerize due to the presence of a PR domain at the N-terminus and cannot interact with CtBP. Thus, *Evi-1c* is thought to act as
transcriptional activator. Oncogenic activation of Evi-1 is usually achieved by overexpression or heterologous fusion of the short Evi-1a isoform (Mitani 2004).

The fos oncogene, originally identified in a retrovirus causing osteosarcoma, encodes a component of the AP-1 transcription factor. Elevated activity of the AP-1 complex correlates well with increased motility and invasiveness of different types of human tumors (Ozanne et al., 2006; Sherwood, 2006). Moreover, the locus of the human Evi-1 ortholog MEL1 is frequently amplified in osteosarcomas (Man et al., 2004) (MEL1 is termed PRDM16 in this reference). It will therefore be interesting to determine if mammalian Evi-1 is regulated by AP-1 in a similar manner and if the same regulatory network formed in C. elegans by fos-1 and egl-43 has been conserved in mammalian cells.

In addition to controlling AC invasion, egl-43 plays a distinct role in the patterning of the VU cells (fig. 4 H). Epistasis analysis indicates that egl-43 acts downstream of or in parallel with lin-12 notch to specify the π-cell fate in the VU cells. egl-43 may render the VU cells competent to respond to the instructive LAG-2 Delta signal or alternatively, egl-43 activity may be modulated by lin-12 notch signaling. The 5’ regulatory region as well as the first intron of egl-43L contains multiple putative Notch responsive CSL binding sites (Berset, 2005, Hwang et al., 2007), suggesting that lin-12 notch signaling may directly activate egl-43 transcription in the VU cells. The question whether egl-43 expression in VU cells is directly regulated by lin-12 notch signaling proved to be difficult to answer experimentally, as in lin-12 notch(lf) mutants one or more VU cells are transformed into ACs that will express egl-43 independently of the lin-12 signal. Moreover, we observed no obvious up-regulation in VU cells or ectopic expression of the different egl-43L reporter transgenes in lin-12(gf) mutants, indicating that lin-12 notch signaling is not sufficient to induce egl-43
expression (data not shown). However, it should be noted that activation of mammalian Evi-1 is involved in various malignancies of the hematopoetic system, and activating mutations in the mammalian notch genes cause T-cell-derived leukemia. It is therefore possible that Evi-1 plays a conserved role in a mammalian Notch signaling pathway that controls the homeostasis of hematopoietic stem cells.

**Material and Methods**

**General methods and strains used**

Standard methods were used for maintaining and manipulating *Caenorhabditis elegans* (Brenner, 1974). The *C. elegans Bristol* strain, variety N2, was used as the wild-type reference strain in all experiments. Unless noted otherwise, the mutations used have been described previously (Riddle and National Center for Biotechnology Information (U.S.), 2001) and are listed below by their linkage group. LGII: egl-43(tm1802)/mIn1[mIs14 dpy-10(e128)] (this study), LGIII: lin-12(n137), lin-12(n137n720), LGV: fos-1(ar105)/nT1[qIs51] (IV;V) (Sherwood et al., 2005).

Extrachromosomal arrays (all this study) and integrated arrays: *rhIs23(him-4::gfp) III* (Vogel and Hedgecock, 2001), *syIs52[cdh-3p::cfp; unc-119(+)] X* (Inoue et al., 2002), *syIs80[lin-11p::gfp, unc-119(+)] III* (Gupta and Sternberg, 2002), *syIs76[zmp-1::pes-10::cfp, unc-119(+)] IV* (Inoue et al., 2002), *qIs56[lag-2::gfp, unc-119(+)] IV* or V (gift of L. Mathies and J. Kimble), *kuIs29[egl-13p::gfp, unc-119(+)] V* (Hanna-Rose and Han, 1999), *zhEx182.1-3[-1.3-egl-43S, sur-5p::gfp], zhEx187.1-5[W02B7, sur-5p::gfp], zhEx201.11,13&.14[-6-egl-43, sur-5p::gfp], zhEx216.2[-1.7-Lp::gfp, unc-119(+)], zhEx217.4[intron1-Δpes-10::gfp, unc-119(+)], zhEx218.7[-5.1-Sp::gfp,
Transgenic lines were generated by injecting the DNA at a concentration of 5-50 ng/µl into both arms of the syncytial gonad as described (Mello et al., 1991). punc-119 (10 ng/µl), plin-48p::gfp (75ng/µl) or psur-5p::gfp (80-100ng/µl) were used as transformation markers (Maduro and Pilgrim, 1995; Yochem et al., 1997). egl-43 RNAi experiments were done by feeding worms with double-stranded RNA producing bacteria as described (Kamath et al., 2001).

The RNAi feeding vector targeting egl-43L, was generated by amplification of a genomic fragment from cosmid W02B7 (Suppl. tables 1 and 2), cloning into the SacII and PstI sites of pPD129.36 (gift from A. Fire) and transformation of the E. coli strain HT115. All RNAi experiments were conducted at 20°C on NGM plates containing 5mM IPTG.

**GFP reporter and rescue constructs**

All constructs were generated by PCR fusion (Hobert, 2002). The primers used are listed in suppl. table 1. The primer combinations used for each PCR reaction are listed in suppl. table 2. For generating the gfp reporter constructs, the nls::gfp::lacz sequence was amplified from pPD96.04 or pTB11, a derivate of pPD96.04 where the minimal Δpes-10 promoter from pPD95.21 has been cloned into the BamHI site. cDNA was prepared from total RNA preparations of N2 worms using oligodT primers (RevertAid Hminus, Fermentas).
MitoTracker staining and microscopy of *C. elegans*

The MitoTracker staining protocol was adapted from (Sherwood et al., 2005). RNAi treated worms were incubated for 2h at RT in a 10µM solution of MitoTracker Red CMXRos (Molecular probes) in M9. The initial volume was diluted 5 times by addition of M9 and pipetted on NGM plates with food. Animals were allowed to recover for 1h at RT. For observation under Nomarski optics, animals of the indicated stages were mounted on 4% agarose pads. Fluorescent images were acquired on a Leica DMRA wide-field microscope equipped with a cooled CCD camera (Hamamatsu ORCA-ER) controlled by the Openlab 3.0 software package (Improvision). For quantification of GFP intensity in the AC, all gfp images were acquired with the same microscopy and software settings. The mean intensity of gfp expression in the nucleus of the anchor cell was measured by using the measurement tool in the Openlab 3.0 software package (Improvision). Each measurement was standardized to the background intensity in the same picture.

Quantification of basal lamina defects

Synchronized animals were observed at the Pn.pxx or Pn.pxxx stage. Basal lamina removal was analyzed based on MitoTracker staining or expression of a translational *him-4::gfp* reporter (RNAi experiments) or based on appearance with Nomarski optics (*fos-1* rescue experiments). Animals were judged as defective if there was no clear gap in the labeled basal laminae (RNAi experiments) or the vulval and gonadal tissues were not connected (*fos-1* rescue experiments) in the proximity to the AC.
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References


Figure legends

Figure 1. *egl-43* is required for AC invasion during vulval development.

(A) Wild-type larva during AC invasion at the Pn.pxx stage and (B) mitotracker-red staining of the basal lamina in the same animal as shown in (A). (C) Absent AC invasion and (D) no degradation of the basal lamina in an *egl-43i* animal at the Pn.pxx stage. (E) and (F) Degradation of HIM-4::GFP during AC invasion in a wild-type larva at the Pn.pxxx stage. (G) Mispositioning of the AC and (H) no degradation of HIM-4::GFP in an *egl-43i* larva at the Pn.pxxx stage. The arrowheads in (A) to (H) point at the AC. (I) Normal invagination and utse (arrow) formation in a wild-type L4 larva. (J) Lack of the utse and invagination defect in an *egl-43i* L4 larva. A thick layer of tissue remains between the uterine and the vulval lumen (bordered by a dotted line). (K) and (L) Specific RNAi against *egl-43L* induces the same AC invasion defects as shown in (G) and (H). Scale bars in (J) and (K) are 10µm.

Figure 2. *egl-43L* is a *fos-1* target in the AC

(A) Structure of the *egl-43* locus, the rescue and the gfp reporter constructs used. The target region of the RNAi constructs used and the region deleted in *egl-43(tm1802)* mutants is indicated. The arrowhead indicates the position of the *fos*-responsive element (FRE). The star marks a point mutation in the FRE (T→A TACTCA). The numbers next to the rescue constructs indicate how many transgenic lines showed rescue of the early larval arrest in *egl-43(tm1802)* mutants relative to the total number of lines analyzed. The lengths of all parts in this scheme are drawn to scale except for the boxes that represent the *egl-43L* cDNA, the nls::gfp::lacz and the Δpes-10 coding sequences. (B) Expression of the *egl-43L* reporter in the AC and VUs of an L3 larva.
at the Pn.px stage. The arrows indicate the VU cells visible in this focal plane. (C) egl-43L reporter expression during AC invasion at the Pn.pxx stage in a heterozygous fos-1(ar105)/+ larva. (D) No AC expression detectable in a homozygous fos-1(ar105) larva at the Pn.pxx stage. The inset in (D) shows an overexposed image of the region around the AC. (E) A 139 bp upstream element is sufficient to activate AC and VU expression of egl-43L at the Pn.pxx stage. See also suppl. fig. s1. (F) A point mutation in the FRE located in the 139 bp upstream element strongly reduces AC and VU specific expression, while expression in (G) gut cells remains similar to the wild-type reporter. (H) Expression of the egl-43S reporter in a L2 larva at the Pn.p stage prior to invasion. (I) egl-43S expression during AC invasion at the Pn.pxx stage in a heterozygous fos-1(ar105)/+ larva. (J) Persisting egl-43S expression in a homozygous fos-1(ar105) larva during AC invasion. (K) The first intron contains an AC-specific enhancer element activating the heterologous minimal ∆pes-10 promoter. The filled arrowheads in (B) to (K) point at the AC. Open arrowheads in (G) indicate GFP-positive gut cells. The scale bar in (K) represents 10µm.

Figure 3. egl-43 positively regulates zmp-1 and cdh-3 expression in the AC

(A) zmp-1p::cfp expression in a control RNAi and (B) egl-43i larva at the Pn.pxx stage. (D) cdh-3p::cfp expression in a control RNAi and (E) egl-43i larva at the Pn.pxx stage. (C) Quantification of relative zmp-1p::cfp and (F) cdh-3p::cfp expression levels in control and egl-43i larvae. Scale bar in (E) is 10µm.
Figure 4. *egl-43* is necessary for the specification of the π-fate in the ventral uterine cells

(A) *egl-13p::gfp* expression in a control RNAi and (B) *egl-43i* L4 larva at the Pn.pxxx stage. (C) Number of *egl-13p::gfp* expressing VU cells in control RNAi and *egl-43i* larvae and (D) control RNAi treated *lin-12(n137gf)* and *lin-12(n137gf); egl-43i* L4 larvae. (E) *lin-11p::gfp* expression in a control RNAi and (F) *egl-43i* L4 larva at the Pn.pxxx stage. Arrowheads point at VU cells expressing GFP. Body wall muscles expressing *egl-13p::gfp* are marked by an asterisk in (A) and (B). Scale bar in (F) is 10µm. (G) Number of *lin-11p::gfp* expressing VU cells in control RNAi and *egl-43i* L4 larvae. (H) Model for the dual role of *egl-43* during AC invasion and VU patterning.
Rimann_Fig3

A  vector RNAi
  zmp-1p::cfp

B  egl-43i
  zmp-1p::cfp

C  zmp-1p::cfp
  relative fluorescence
  • vector RNAi (n=38) • egl-43 RNAi (n=40)

D  vector RNAi
  cdh-3p::cfp

E  egl-43i
  cdh-3p::cfp

F  cdh-3p::cfp
  relative fluorescence
  • vector RNAi (n=22) • egl-43 RNAi (n=27)
Rimann_Fig4

A vector RNAi
egl-13p::gfp

B egl-43i
egl-13p::gfp

E vector RNAi
lin-11p::gfp

F egl-43i
lin-11p::gfp

C egl-43i (n=16) vector (n=20)

D lin-12(gf); vector (n=29)
lin-12(gf); egl-43i (n=27)

G egl-43i (n=20) vector (n=20)

H AC

VUs

Invasion

fos-1 lag-2
egl-43L
zmp-1 cdh-3
lin-11 egl-13

n-fate
Suppl. Fig. s1. Sequence alignment of the FOS-responsive element in the *C. elegans egl-43L* promoter/enhancer with *C. briggsae* and *C. remanei*

The corresponding 5’ regulatory sequences of *C. briggsae* (Cbg) and *C. remanei* (Cre) *egl-43L* were aligned with 139 bp upstream of the initiation codon of *C. elegans* (Cel) *egl-43L* using the ClustalX algorithm. Conserved blocks are highlighted in yellow, and the putative FOS-responsive element is boxed in red. The red arrow points at the site of divergence from the canonical Fos binding site T\(\text{CAc/gTCA}\). The letter A indicates the position of the T\(\rightarrow\)A mutation that is present in the \(-0.14\)-\textit{MUTp::gfp}\) reporter.
**Supp. table 1: Oligonucleotide sequence of primers used for PCR**

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<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-&gt;3’)</th>
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<tbody>
<tr>
<td>DEL18</td>
<td>CTTCTCCGTCACTACAGCTTCC</td>
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<tr>
<td>OUTREV</td>
<td></td>
</tr>
<tr>
<td>FireC</td>
<td>AGCTTGATGCTGCAGGTCGACT</td>
</tr>
<tr>
<td>FireD</td>
<td>AAGGGGCCGTACGGCGACCTAGTGG</td>
</tr>
<tr>
<td>FireD*</td>
<td>GGAACAGTTATGTTGTGATATGG</td>
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<tr>
<td>OIR75</td>
<td>TTTCTGCAGGCCACATCATGTGTCAGTGTAG</td>
</tr>
<tr>
<td>OIR83</td>
<td>CTACCGCTTCTGGATGAC</td>
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<tr>
<td>OIR93</td>
<td>GCCGAAACCAGTGTCTCCAG</td>
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<td>OIR94</td>
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<tr>
<td>OIR98</td>
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<td>OIR132</td>
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<tr>
<td>OIR142</td>
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<tr>
<td>OIR171</td>
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<td>OIR172</td>
<td>AAACGTCAAGCTCAGCAGACGATGACAA</td>
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<td>OIR208</td>
<td>TATGACCTTCTGGAGAGAACATTCG</td>
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<td>OIR209</td>
<td>GCACATCATGTGCTGATG</td>
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<td>OIR225</td>
<td>CGAAAGATGATACATCTTGTG</td>
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<td>OIR233</td>
<td>GCTGACCGAAGCGCCGCTC</td>
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Suppl. table 2: Primer combinations used to generate the RNAi vector insert, gfp reporter and rescue constructs.

<table>
<thead>
<tr>
<th>RNAi Construct</th>
<th>PCR</th>
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<tr>
<td>egl-43Li</td>
<td>OIR171 &amp; 172</td>
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<table>
<thead>
<tr>
<th>GFP Reporters</th>
<th>PCR 1</th>
<th>PCR 2</th>
<th>PCR 3</th>
<th>PCR Fusion</th>
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<tbody>
<tr>
<td>-1.7-Lp::gfp</td>
<td>OIR75 &amp; 137</td>
<td>FireC, FireD</td>
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<td>OIR75, FireD*</td>
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<tr>
<td>intron1-Δpes-10::gfp</td>
<td>OIR128 &amp; 139</td>
<td>FireC, FireD</td>
<td>-</td>
<td>OIR138, FireD*</td>
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<td>FireC, FireD</td>
<td>-</td>
<td>OIR138, FireD*</td>
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<tr>
<td>-0.14-Lp::gfp</td>
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<td>FireC, FireD</td>
<td>-</td>
<td>OIR 215, FireD*</td>
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<tr>
<td>-0.14-L-MUTp::gfp</td>
<td>OIR208 &amp; 225</td>
<td>OIR212 &amp; 137</td>
<td>FireC, FireD</td>
<td>OIR 215, FireD*</td>
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<table>
<thead>
<tr>
<th>Rescue Constructs</th>
<th>PCR 1</th>
<th>PCR 2</th>
<th>PCR 3</th>
<th>PCR Fusion</th>
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<td>OIR126 &amp; DEL18OUTREV</td>
<td>OIR98 &amp; 107</td>
<td>OIR 83 &amp; 99</td>
<td>OIR126 &amp; 99</td>
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<tr>
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<td>OIR126 &amp; DEL18OUTREV</td>
<td>OIR135 &amp; 233</td>
<td>OIR94 &amp; 132</td>
<td>OIR99 &amp;127</td>
</tr>
</tbody>
</table>

Templates for PCR:  
1) Cosmid W02B7  
2) pPD96.04  
3) pTB11  
4) N2 cDNA