The Pax2/5/8 gene egl-38 coordinates organogenesis of the C. elegans egg-laying system

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Abstract

Organogenesis requires coordinated development between different tissues and cells. The Pax family of transcription factors coordinates multiple developmental events in organs including the kidney, thyroid and the eye. Studying Pax factors in different organisms should identify unifying characteristics of organ development with implications to both development and disease. Here we investigate the function of the Pax2/5/8 transcription factor EGL-38 in coordinating development of the C. elegans egg-laying system. A functional egg-laying system requires cell fate specification events in the epithelial cells of the vulva as well as the mesodermal cells in the uterus of the somatic gonad. Using gene expression studies, genetic mutant analysis and genetic mosaics, we show that egl-38 has functions in both tissues of the organ to promote its development. We incorporate these results together with previous results to propose that EGL-38 plays multiple roles in the development of the egg-laying system, acting to both promote cell fate and to coordinate the development between different cell types. As the Pax2 gene performs similar roles in the development of the mammalian kidney, we show that coordinating organogenesis is a conserved function for Pax2/5/8 transcription factors.

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Introduction

An organ is made up of different tissues and cells that must work together. Normal organ development requires coordination of a range of cellular processes, including cell division, differentiation, migration and morphogenesis. During organogenesis there is communication between cells of one tissue and with those of other tissues, to ensure the final organ can function as a whole. We are interested in understanding how cell signaling and cell fate specification act together during organ development. To understand these processes at the cellular level, we are studying the developing C. elegans egg-laying system as a simple model for organogenesis.

The developing egg-laying system comprises two tissues, the somatic gonad that forms the uterus where fertilized eggs are stored and the vulva that forms the opening through which eggs and sperm can pass. During the development of the vulva, there is reciprocal signaling between the vulva and the somatic gonad (Fig. 1). Initially, in the third larval (L3) stage, the Anchor Cell (AC) of the somatic gonad sends an Epidermal Growth Factor (EGF) signal, LIN-3, to its receptor LET-23 in three of the six Vulval Precursor Cells (VPCs) that lie along the ventral cuticle. LIN-3 induces the VPCs to adopt the vulval fate (reviewed by Greenwald, 1997). The VPC closest to the AC (P6.p) adopts the 1° fate while the other two (P5.p and P7.p) adopt the 2° fate. P6.p divides to produce eight descendents, whereas P5.p and P7.p each produce seven. These cells undergo invagination to form a stack of vulval cells. During the mid fourth larval (mL4) stage, the eight P6.p descendents lie at the apex of this stack of cells. The four P6.p descendents closest to the somatic gonad are called the vulF cells, and they express the EGF signal LIN-3 and reciprocally signal to the somatic gonad to specify a subset of π uterine cells that lie above the vulF cells called uv1 cells (Chang et al., 1999). Following specification, the uv1 cells migrate to flank the forming vulva. The other π uterine cells, along with the AC, move away from the vulva and fuse to form a syncytium called the uterine seam cell (utse). Meanwhile, the vulF cell nuclei separate along the vulva–uterine border to leave only a thin membrane of tissue between the vulva and the uterus.
at the prospective opening. The vulval cells then fuse to form torroidal rings that line the vulva cavity connecting the uterus and the external cuticle. Finally, the vulva undergoes eversion (Sharma-Kishore et al., 1999). Therefore proper organ development of the egg-laying system requires cell fate specification in both vulva and uterus, signaling between the two tissues, and cellular migration and morphogenesis.

This work focuses on the role of Pax2/5/8 genes in coordinating organogenesis. Pax genes encode transcription factors that are grouped into sub-classes based on structural similarity (reviewed by Chi and Epstein, 2002). Pax2/5/8 genes have been shown to play an important role in the development of organ systems. For example, during the development of the metanephric kidney in mouse, Pax2 is expressed in both the metanephric mesenchyme and the uretic bud. It is also required for reciprocal inductive interactions in both these tissues that promote and coordinate organogenesis (Majumdar et al., 2003; Torres et al., 1995; reviewed by Dressler, 1996). In addition, within the metanephric mesenchyme, Pax2 promotes the mesenchymal-to-epithelial transition and subsequently promotes formation of the nephrons (Dressler et al., 1993; Grote et al., 2006; Rothenpieler and Dressler, 1993; reviewed by Schedl and Hastie, 2000). These results suggest that Pax proteins can play multiple roles in different tissues of an organ to coordinate organogenesis. Previous studies have shown that a C. elegans Pax2/5/8 gene called egl-38 also affects the development of cells in both the vulva and the somatic gonad during organogenesis of the egg-laying system (Chamberlin et al., 1997). However, the function of this Pax gene has been interpreted to be limited to cells in the vulva, with the impact on the uterine cells resulting from a disruption of signaling (Chang et al., 1999). Thus although Pax genes are important for organ development in both mouse and worm, it was not clear whether the underlying cellular and developmental logic is conserved.

This research is focused on understanding the role of egl-38 in the egg-laying system. As previous studies utilized a single egl-38 mutant allele, we have characterized a range of different egl-38 mutant alleles to assess their impact on different events.

Fig. 1. The egg-laying system in C. elegans. In the early third larval stage, six Vulval Precursor Cells (VPC; P3.p–P8.p) lie in the ventral mid-section of the body below the Anchor Cell (AC). The AC induces three of the six VPC to adopt 1° and 2° vulval fates. These cells undergo cell division, producing cells that will form the vulva. In the L4 stage, four of the cells produced from the 1° lineage lie in each lateral plane. They include the dorsal vulF cells (F) and the ventral vulE cells (E). The vulF cells separate in the mL4 stage to reveal a thin membrane between the uterus and the vulva. The uterine cells dorsal to the vulva adopt a π cell fate. The π cells closest to the vulva are specified as uv1 cells (‘uv1’). In the mL4 stage, these cells move to either side of the vulva. Two uv1 cells can be seen in each lateral plane lying adjacent to vulF cells. Meanwhile the other π cells and the AC move away from the vulva and ultimately fuse to form the utse syncitium (‘utse’). By the IL4 stage, there is a syncitium of four π nuclei in each lateral plane. In one of the lateral planes, however, the nucleus of the AC can also be seen. Therefore in this plane, there are five nuclei in the utse syncitium.

during egg-laying system development. In addition, we use genetic mosaic analysis to determine in which cells and tissues egl-38 function is required. Taken together, these results support a model in which egl-38 functions in both the somatic gonad and the vulval cells to coordinate egg-laying system development. In particular, we find that functional egl-38 in the vulva is not sufficient for normal uterine development, indicating that it influences non-autonomous as well as autonomous developmental functions within the organ. We propose that a general property for Pax2/5/8 transcription factors may be to act broadly in the different tissues of an organ to coordinate its development.

Materials and methods

Genetic strains

The C. elegans strains were cultured using standard techniques ( Sulston and Hodgkin, 1988 ). Mutations are described in wormbase (http://www.wormbase.org/), or as listed below. The following mutations were used: Linkage Group II ( LGII ); let-23(sa62) ( Katz et al., 1996 ); LGIII: unc-119 ( e2498 ); LGIV: dpy-20(e1282), egl-38(sy294), egl-38(sy287), egl-38(n578), egl-38(ga22) ( Zhang et al., 2005, and references therein); LGV: him-5(e1490); LGX: lin-2(e1309).

The following transgenes were used: inc179 ( pida-1::gfp, Zahn et al., 2001), syEx98 ( lin-1::gfp, Gupta and Sternberg, 2002), syEx724 ( bam-2::gfp, Colavita and Tessier-Lavigne, 2003; Inoue et al., 2005), syis67 ( zmp-1::pes-10::gfp, Inoue et al., 2005), syEx234 ( let-23::gfp, Chang et al., 1999), kds29(evl-13::gfp, Hanna-Rose and Han, 1999), keEx44 ( ajm-1::gfp, Koppen et al., 2001), gudu1000 ( lin-3::gfp, Hwang and Sternberg, 2004), the transgene within strain UL1251 ( egl-38::gfp, John Reece-Hoyes and Ian Hope, personal communication; Dupuy et al., 2004).

Phenotypic analyses

Developmental stages: Hermaphrodite animals were observed at the adult (A) stage or at different fourth larval stages (L4) : early L4 (EL4), mid L4 (ML4) and late L4 (IL4). These developmental stages were defined on the basis of the vulval morphology in the mid-plane under a compound microscope ( Fig. 1; reviewed by Greenwald, 1997 ). During the L4 stage, the anchor cell is positioned between the vulF cells. During the ML4 stage, the vulF cells separate, leaving a thin membrane between them. During the IL4 stage, the vulva begins to evert, and the thin membrane is no longer seen between the vulF cells. Finally, in an adult worm, the vulva has undergone complete eversion.

Adherens junctions ( AJs ): AJs were visualized in the vulval tissue using an ajm-1::gfp transgene ( Koppen et al., 2001 ). The AJs between cells appear as spots at cell boundaries.

Egg-laying ability: ML4 stage worms were placed on separate plates. These plates were checked for presence of eggs laid by the worm once a day for 3 days or until the worm burst. Worms which laid any eggs at all on the plate were categorized as Egl + and those that did not were classified as Egl −.

Vulval morphology: ML4 larvae were observed under Nomarski optics to determine whether the vulF nuclei are positioned anterior and posterior, leaving behind a thin laminar process between the vulva and uterus. In egl-38(n578) mutants, the vulval nuclei separate to a lesser extent, and the tissue between the vulva and uterus remains thick (Chamberlin et al., 1997).

Uterine morphology: To assess uterine cell morphology, individual ML4 animals were selected using Nomarski optics, recovered for 3 hours and observed again during the IL4 stage. The uterine morphology was scored according to previous studies ( Newman et al., 1996 ). In wild-type animals during the ML4 stage, the π cell progeny that become the uv1 cells move to flank the vulF cells of the vulva ( Fig. 1 ). The other π cell progeny migrate with the Anchor Cell ( AC ) to either the anterior or the posterior end of the uterus. In the IL4 stage, the uv1 cells continue to remain close to the vulva and now lie to the anterior and posterior side of the vulf cells. At this stage, the other π cell progeny move near the extreme ends of the uterus and fuse to become the syncytial uterine seam cell ( utse ). In egl-38 ( n578 ) mutants, the uv1 cells lie above the vulf cells in the EL4 stage as in wild-type animals. However, in the ML4 stage, the uv1 cells migrate distally with the other π cell progeny and the AC ( Chamberlin et al., 1997).

Assay for uv1 specification using egl-13::gfp: egl-13::gfp is localized to the nucleus of all π cells in the uterus ( Hanna-Rose and Han, 1999 ). Thus, it serves as a useful marker to determine the position and thus the fate of the different π cells during the mid and late L4 stages. In wild-type animals, two uv1 cells per side ( left/right ) are positioned next to the vulf cells, while the other four π cells position away from the vulva ( Newman et al., 1996 ). In one of the lateral planes, the Anchor Cell ( AC ) also migrates with the four π cells that move away from the vulva. Thus, per side, wild-type animals have two proximal and 4 or 5 distal egl-13::gfp-positive cells, whereas in egl-38(n578) mutants all cells are distal.

LET-23 expression pattern in the uterine cells: LET-23 expression was examined using a LET-23::GFP reporter transgene ( Chang et al., 1999 ). Animals were observed at three time points in the L4 (early, mid and late), and in the adult.

Analysis of egl-38::gfp expression

Two reporter genes were used to visualize egl-38::gfp expression: pXW124 which expresses in the vulval cells, and the unnamed reporter gene contained in strain UL1251, which expresses in the uterus. pXW124 includes ~ 5 kb sequence upstream of egl-38 cloned into the GFP reporter vector pD95.69 (a gift from A. Fire). Transgenic strains of the pXW124 plasmid were generated using standard methods for germline transformation ( Mello et al., 1991 ). The plasmid pXW124 ( 76 ng/μl ) was injected using unc-119(+)( 15 ng/μl ) as a transformation marker into unc-119(2498); him-5(e1490) animals. The UL1251 strain was obtained from Ian Hope ( J. Reece-Hoyes and I. Hope, personal communication). This clone includes 457 bp of sequences from the upstream region of egl-38. It is notable that all of the sequences contained within the UL1251 reporter transgene are present within pXW124. Thus it is not clear why pXW124 promotes expression only in the vulva, and not both in the vulva and the uterus. We only conclude that sequences upstream of egl-38 include enhancers that can promote expression in the uterus, and in the vulva.

Table 1 Molecular markers used to characterize the egg-laying system in egl-38 mutants

<table>
<thead>
<tr>
<th>Reporter</th>
<th>Description</th>
<th>Transgene</th>
<th>Expression in egg-laying system</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ajm-1::gfp</td>
<td>Apical junction molecule</td>
<td>jEx44</td>
<td>At adherens junctions</td>
<td>Koppen et al., 2001</td>
</tr>
<tr>
<td>lin-3::gfp</td>
<td>Epidermal growth factor molecule</td>
<td>guEx1000</td>
<td>vulF cells</td>
<td>Hwang and Sternberg, 2004</td>
</tr>
<tr>
<td>bam-2::gfp</td>
<td>Concanavalin A-like lectin/glucanase molecule</td>
<td>syEx724</td>
<td>vulE and vulf cells</td>
<td>Colavita and Tessier-Lavigne, 2004 and Inoue et al., 2005</td>
</tr>
<tr>
<td>zmp-1::gfp</td>
<td>Zinc-protease molecule</td>
<td>syIs67</td>
<td>vulE cells</td>
<td>Inoue et al., 2005</td>
</tr>
<tr>
<td>lin-11::gfp</td>
<td>LIM homeodomain transcription factor</td>
<td>syEx598</td>
<td>Cells</td>
<td>Gupta and Sternberg, 2002</td>
</tr>
<tr>
<td>ida-1::gfp</td>
<td>Tyrosine phosphatase-like receptor</td>
<td>ins179</td>
<td>uv1 cells</td>
<td>Zahn et al., 2001</td>
</tr>
</tbody>
</table>
Mosaic analysis

egl-38(n578) animals were injected with a mixture of 10 ng/μl of C04G2 cosmid (egl-38 rescuing cosmid, Chamberlin et al., 1997), 15 ng/μl of a cytoplasmically localized transformation marker (myo-2::gfp, Okkema et al., 1993) and 100 ng/μl of a ubiquitously expressed nuclear marker (sur-5::gfp, Gu et al., 1998; Yochem et al., 1998). Mosaic analysis for egl-38 was done by selecting under the dissecting microscope mL4 stage transgenic animals that express myo-2::gfp in some cells of the pharynx. These animals were then assessed for mosaicism by examining for presence or absence of sur-5::gfp expression in vulval nuclei using a compound microscope. Simultaneously, the vulval morphology was examined under Nomarski optics for vulF cell separation. All the animals listed in Fig. 6 as vulF(+) contained the transgene in all vulval cells (derived from P5.p–P7.p). Thirty-five of 37 animals listed as vulF(−) contained the transgene in no vulval cells. As Pn cells derive alternately from ABpl and ABpr, the most parsimonious interpretation of these animals is that that transgene was lost in ABp or AB, although two separate losses are also possible. Two of 37 retained the transgene in the P5.p and P7.p-derived cells, but lacked the transgene in P6.p-derived cells (vulF and vulE). We interpret that the transgene was lost from only one cell (ABpl or ABpr). These were both in mosaic category 2 (vulF(−) and vulE(−)), and were phenotypically the same in the egg-laying system as other category 2 animals. Scoring all the vulval cells confirms that the transgene was absent in the P6.p precursor to the vulF cells. Disruption of the vulF separation function of egl-38 in vulF(−) animals also supports the idea that wild-type egl-38 activity is absent from these cells. These animals were recovered and examined again during IL4 stage for either presence or absence of sur-5::gfp in uterine nuclei and the specification of uv1 cells. All the animals listed in Fig. 6 as uv1(+) contained the transgene in all four uv1 cells, and those listed as uv1(−) lacked the transgene in all cells.

We selected the hypomorphic but non-null genotype egl-38(n578) as the genetic background for mosaic analysis. This allele choice ensured that all mosaic categories would be viable, whereas use of a null allele would limit our mosaic animals to those that retain the transgene in cells required for any egl-38 essential function(s). However, a consequence of this allele choice is that, in mosaic animals, cells lacking wild-type egl-38 retain any egl-38 activity retained by egl-38(n578).

Results

Molecular markers reveal defects in the egg-laying system of egl-38(n578) animals

Previous studies showed that egl-38 mutants fail to specify the uv1 cells of the uterus, and are abnormal for morphogenesis and expression of LIN-3 in the vulF cells of the vulva (Chamberlin et al., 1997; Chang et al., 1999). To further characterize the functions of egl-38, we have assessed the expression of additional molecular markers for the vulval and uterine cells of the egg-laying system in mutants (Table 1). We find that although egl-38 mutants have defects in the uv1 and the vulF cells, these cells are not completely abnormal.

The vulval cells derive from the ventral hypodermal cells, P5.p–P7.p (Fig. 1). These cells divide to produce 22 cells that invaginate during the L4 stage and form an inverted ‘U’ shaped structure (Sulston and Horvitz, 1977). The cells at the top (the vulF cells) move apart to form an open “apex” in the mid L4 stage, leaving behind only a thin membrane necessary to form a functional uterine–vulval connection (Hanna-Rose and Han, 2002). Subsequently, cells of similar type fuse, forming rings that line the vulval cavity (Sharma-Kishore et al., 1999). At this stage the number of cells and the architecture of the uterine–vulval connection can be visualized using the adherens junction marker AJM-1::GFP (Koppen et al., 2001). When observed in the mid-plane, AJM-1::GFP appears as dots between cells. In both egl-38(n578) animals and their wild-type counterparts, there are eight dots corresponding to seven distinct vulval cells (Fig. 2, line 1). This result suggests that although vulF is abnormal, there are no fusions between the vulE and vulF cells. However, the four vulval nuclei closest to the uterus are mis-positioned (Chamberlin et al., 1997), and this leads to an abnormal arrangement of the AJM-1::GFP dots. Consequently the apex of the vulval cavity near the uterus is essentially blocked.

There are two vulval cell types that derive from P6.p: the vulF cells, which lie closest to the uterus and the vulE cells which are found ventral to the vulF cells and thus away from the uterus (Fig. 1). These two cell types are related by lineage, and also overlap in developmental potential, since if the vulF precursors are ablated then the vulE cells can adopt features of the vulF cell fate (Chang et al., 1999). vulF and vulE cells differ with respect to expression of several cell markers (Inoue et al., 2002). In particular, LIN-3/EGF is expressed by vulF to signal to the somatic gonad, but it also serves as a vulF-specific cell marker which is not expressed in egl-38(n578) animals (Fig. 2, line 2; Chang et al., 1999). We wanted to determine whether the presumptive vulF cells in egl-38 mutants are simply abnormal, or instead adopt a fate similar to vulE. Our experiments with AJM-1::GFP (above) showed that the vulE and vulF cells do not fuse together in egl-38 mutants. This provides evidence that the two cell types retain some differences, and that the presumptive vulF cells are not transformed into vulE cells. We used additional reporter transgenes to better characterize the vulF defects in egl-38 mutants. The marker bam-2::gfp is normally expressed brightly in vulF cells and dimly in vulE cells in wild-type animals (Inoue et al., 2005) and this expression remains unaltered in egl-38(n578) mutants (Fig. 2, line 3). Similarly, zmp-1::gfp expression is normally restricted to vulF cells (Inoue et al., 2002), and we find that this pattern is largely retained in egl-38(n578) mutants (Fig. 2, line 4). This result is in contrast to that obtained by Inoue et al. (2005), who report that zmp-1::gfp is expressed ectopically in the vulF cells of egl-38(n578) mutants. However, the experiments utilize transgenes containing a different extent of gene regulatory sequences. An interpretation of the different results is that the transgene utilized by Inoue et al. (syIS49) includes negative regulatory elements absent from the transgene used in this study (syIS67). The two transgenes dissect the behavior of zmp-1::gfp in response to egl-38, but our result with syIS67 indicates that vulF and vulE cells retain differences in egl-38 mutants. Altogether, we find that the vulF cells in egl-38(n578) animals are distinct from vulE cells, and that all cell-specific gene expression is not altered. Thus egl-38 affects a subset of genes important for normal vulF cell fate.

We next utilized molecular markers to examine the role of egl-38 in the other tissue of the egg-laying system, the uterus. In the mid-L4 stage, the π cells in the uterus that lie closest to the vulva become the uv1 cells. The remaining π cells migrate away and fuse to form the utse syncytium. In egl-38(n578) animals, the uv1 nuclei fail to remain proximal to the vulva. Instead they lie close to the distal π cell nuclei, and have been
interpreted to adopt the utse fate (Chamberlin et al., 1997). To confirm this interpretation, we used lin-11::gfp to study π cell migration and fusion (Newman et al., 1999). lin-11::gfp is expressed in all π cells, but expression in uv1 cells persists longer and more intensely than that in the utse. Using this marker, we found that the presumptive uv1 cells still express lin-11::gfp as in wild-type, but migrate and physically fuse with the distal utse syncytium resulting in a fluorescent arch in the egl-38(n578) mutant animals (Fig. 3). We also confirmed that π cell nuclei are present in their normal numbers in egl-38(n578) mutants using a different π cell fate marker, egl-13::gfp (see Fig. 7; Cinar et al., 2003). Finally, we found that expression of ida-1::gfp (Zahn et al., 2001) in uv1 cells is disrupted in egl-38 mutants (Fig. 3). These studies confirm that π cell number and fate are maintained in egl-38(n578) mutants, but that there are specific defects in the uv1 cells. As a consequence, the presumptive uv1 cells fuse with the other π cell nuclei to form the utse.

Effects of hypomorphic egl-38 alleles on the egg-laying system

The function of egl-38 in the egg-laying system has been characterized primarily using a single allele, egl-38(n578). egl-38(n578) was originally identified in a screen for egg-laying defective or Egl animals (Trent et al., 1983). Subsequently, several additional alleles of egl-38 have been identified based on other defects (Fig. 4A). Genetic studies show that the different homozygous viable alleles disrupt different egl-38 functions in a tissue-preferential manner (Zhang et al., 2005; Fig. 4B). Of all the egl-38 alleles, the n578 allele confers the greatest egg-laying defect, and the functions of egl-38 in the egg-laying system have been interpreted based on analysis of

Fig. 2. Expression of molecular markers in the vulva of egl-38(n578) mutants. Each image set has Nomarski images to the left and its corresponding epi-fluorescence image to the right. At least 20 animals were observed for each genotype and transgene. (1) Adherens junctions (AJs) at the uterine–vulval junction were observed in mL4 animals as dots between cells in the mid-plane using AJM-1::GFP. The AJs in the vulva are numbered 1 to 8. The first AJ is between the vulA cell and hyp7, the next six are AJs between vulval cells, while the eighth lies between the vulF cell and the uterine uv1 cell. The number of AJs is normal in egl-38(n578) mutants. The opening between AJs of the vulF cell and the uv1 cell is called the apex (denoted by the dotted line). The apex is wide in wild-type animals, but narrow in egl-38(n578) animals. (2) lin-3::gfp is expressed in vulF cells of wild-type animals and absent in egl-38(n578) mutants. (3) bam-2::gfp is expressed brightly in the vulF cells and dimly in the vulE cells in both wild-type and egl-38(n578) mutants. (4) zmp-1::gfp is expressed in the vulE cells in both wild-type and egl-38(n578) mutants. However, we did observe some variability in the expression in egl-38 mutants. Fifteen of fifteen wild-type animals showed expression in all four vulE cells, and absence in all four vulF cells. In egl-38(n578), we observed reduced expression in vulE cells (4 of 21 showed no expression, 11 of 21 showed expression in two cells, and 6 of 21 showed expression in four cells) as well as rare expression in vulF (18 of 21 showed no expression, and 3 of 21 showed expression in four cells).
Fig. 3. Expression of molecular markers in the uterus of egl-38(n578) mutants. Each image set has Nomarski images to the left and its corresponding epi-fluorescence image to the right. Lin-11::GFP is expressed brightly in uv1 cells, and less brightly in utse cells. In the eL4 stage, all the π cells are present close to and above the vulva in both the wild-type and the egl-38(n578) animals. In the wild-type animals, the uv1 cells are close to the vulva (within the brackets) throughout the L4 stage. The other utse cells migrate distally and fuse to form a syncitium. In egl-38(n578) animals the uv1 cells are close to vulva in eL4. By the mL4 stage they have moved away from the vulva. In IL4 stage, uv1 cells are not seen near the vulva. Instead the utse syncitium appears brighter, suggesting fusion of the presumptive uv1 cells to the utse. Fifteen animals were examined in each stage. Ida-1::GFP is expressed in uv1 cells. In wild-type animals, the uv1 cells express Ida-1::GFP and lie close to the vulva (within the brackets) in both the mL4 and the IL4 stages. In egl-38(n578) animals, there is no detected Ida-1::GFP expression in the presumptive uv1 cells in the mL4 stage. In the IL4 stage, faint GFP is detected in the utse. In both wild-type and mutant, Ida-1::GFP is also expressed in the Hermaphrodite Specific Neuron (HSN, indicated with arrowhead), which is positioned at the vulva. Ten animals were examined in each stage. Exposure time and photo conditions are the same for both wild-type and mutant.
egl-38(n578) mutants. However, egl-38(n578) retains significant levels of activity in other tissues that require egl-38 function. Therefore, we wanted to assess the egg-laying system defects associated with different egl-38 mutant alleles to determine whether the egl-38(n578) phenotypes reflect the full range of egl-38 functions in this organ. In contrast, the other hypomorphic alleles confer varying levels of defect for each (Fig. 4C). Notably, we found that, in egl-38(sy294) mutants, the vulF cells do not express detectable lin-3::gfp. However, in a majority of animals, the vulF cells do separate as well as in the wild type. This result suggests that different egl-38 alleles can preferentially disrupt different functions within the same cells of a tissue. However, since no mutant shows the reciprocal defect (significant disruption of the vulval separation but not the lin-3::gfp expression), it is possible that the phenotype reflects differential sensitivity of the vulF functions to overall egl-38 activity.

egl-38(n578) mutants fail to express LIN-3 in the vulF cells in the vulva and lack uv1 cells in the uterus. Several previous results suggested that the uterine cell defect in egl-38 mutants is a secondary effect of the LIN-3 expression defect. In particular, killing the vulF precursor cells results in failure of uv1 cells to be specified, and mutants in the lin-5/let-23 pathway are disrupted for uv1 cell fate specification (Chang et al., 1999). A prediction of this model is that LIN-3 expression should correlate with uv1 cell specification, vulval separation and egg-laying ability of the different egl-38 mutant alleles. To test this prediction, we assessed lin-3::gfp (to report LIN-3 expression) and lin-11::gfp (to track uv1 cell fate) in the different egl-38 mutants. Our analysis of different egl-38 mutants to other egl-38 mutants with greater levels of egg-laying ability than egl-38(n578). To test this prediction, we assessed lin-3::gfp (to report LIN-3 expression) and lin-11::gfp (to track uv1 cell fate) in the different egl-38 mutants. Our results show that although there is a correlation between these two functions in most of the egl-38 mutants, it is not universal. Interestingly, in egl-38(sy294) mutants, almost all the animals have normal uv1 cell specification even though no lin-3::gfp expression is detected in the vulF cells (Fig. 4C). This result may reflect the possibility that the reporter gene does not adequately report lin-3 expression, or that limited amounts of LIN-3 are adequate for normal uv1 fate (Dutt et al., 2004). However, lin-3::gfp expression and uv1 cell fate are well correlated in egl-38(sy287) and egl-38(gu22) animals. For example, egl-38(sy287) mutants exhibit a greater defect in uv1 cell specification than egl-38(sy294), but have detectable levels of lin-3::gfp. These results suggested that egl-38 may have direct functions in the uterus, a possibility we tested below using genetic mosaic analysis.

egl-38(n578) mutants have a defective connection between the vulva and the uterus, and therefore fertilized eggs hatch within the animal. We examined both the uterine and vulval functions in the egl-38 mutants to learn which of these functions is critical to form a functional egg-laying system and thereby confer egg-laying ability. Based on the correlation of different egg-laying system phenotypes (Fig. 4C), we suggest that vulval morphogenesis leading to vulF cell separation plays a major role in enabling an animal to lay eggs. For example, in the egl-38(sy287) mutants, all the animals examined lay eggs, but only about 60% of them express LIN-3 or specify uterine uv1 cells. Almost 90% of these animals do show vulF cell separation. We interpret that the separation of the vulF cells may relieve a physical block in the formation of the uterine vulval connection and enable eggs to pass through the vulva.

EGL-38 is expressed and functions in both vulval and uterine cells

Our analysis of different egl-38 mutants suggested that the gene may have roles both in the vulva and the somatic gonad to mediate development of the egg-laying system. If this is the case, then the gene should be expressed and should function in both tissues. To characterize the expression pattern of egl-38, we utilized egl-38::gfp reporter transgenes. Consistent with this model, we found that these transgenes express in the vulF cells of the vulva, and the uv1 cells of the uterus (Fig. 5).

To more directly test in which cells egl-38 functions, we performed genetic mosaic analysis. We introduced an extra-chromosomal transgene with egl-38(+) rescuing DNA and sur-5::gfp which marks transgene-bearing cells with GFP (see Materials and methods; Yochem et al., 1998) into an egl-38(n578) mutant strain. This transgene can be lost in cell division, resulting in genetically mosaic animals. For these animals, cells retaining the transgene have functional egl-38 and express SUR-5::GFP, whereas cells that lack the transgene are mutant for egl-38 and lack SUR-5::GFP. We selected mid-L4 animals...
that contained the transgene in at least some cells, and observed
the vulva for presence or absence of SUR-5::GFP. At the same
time, we verified whether vulF separation was normal or
defective. These same animals were recovered and examined in
the late L4 stage for the expression of SUR-5::GFP in their
uterine cells, and for whether the uv1 cells remained at the
vulva, or had migrated distally with the utse cells.

We found that the mosaic animals fell into four categories
based on the deduced presence or the absence of egl-38(+) in
the vulval and the uterine tissue (Fig. 6). The first category
comprised of animals that had wild-type egl-38 in both tissues.
As expected, these animals were found to have wild-type
development. In a second category, egl-38 was absent in both
the tissues, and development was abnormal in both. The final
EGL-38 and LET-23 in the uterus

Our genetic mosaic results prompted us to revisit the functional relationship between EGL-38 and the LIN-3(EGF)/LET-23(EGFR) signaling pathway. Earlier studies by Chang et al. (1999) showed that expression of LIN-3 in the vulva requires egl-38 (see Fig. 2, line 2). In addition, a gain-of-function mutation, let-23(sa62), suppresses the egl-38(n578) uv1 defect, indicating that activated let-23 can bypass the requirement for egl-38 in uv1 cell development. These data are consistent with a model in which egl-38 acts in the vulva to signal to the uterus to specify uv1 cell fate. In contrast, our genetic mosaic analyses suggest that egl-38 activity in the uterus is necessary and sufficient to specify the uv1 cells. To reconcile the results obtained from these different experimental approaches, we carried out additional analysis of the relationship between let-23 and egl-38 in the uv1 cells.

In the uterus there are 12 π cells (Newman et al., 1996). These cells initially lie above the vulva with six cells in each lateral plane. Subsequently, the four central π cells that lie adjacent to the vulva are specified to be uv1 cells. The remaining cells distal to the vulva fuse to form a syncytium called utse (Fig. 1). To replicate the analysis of Chang et al. (1999), we used a reporter, egl-13::gfp, to visualize the π nuclei. In wild-type animals, the uv1 cells are specified and these animals have two proximal and 4–5 distal egl-13::gfp-positive nuclei on each side, corresponding to two proximal uv1 nuclei and four to five distal utse nuclei (Fig. 7). In contrast, in egl-38(n578) animals, all nuclei lie distal to the vulva because the presumptive uv1 cells fuse with the other π cells to form the utse cell. We constructed let-23(sa62); egl-38(n578) double mutants with the egl-13::gfp reporter and found that these animals exhibit the wild-type arrangement of π nuclei, indicating that the gain-of-function let-23 allele can bypass the egl-38 defect. These experiments using a molecular marker replicate the morphological analysis of Chang et al. (1999), supporting the idea that activated let-23 can bypass the egl-38(n578) uv1 cell development defect. Bypass of egl-38(n578) also occurs in response to induced expression of lin-3, indicating that the result does not reflect a neomorphic property of let-23(sa62) (Huang and Hanna-Rose, in press).

Since activated let-23 can bypass egl-38 to promote uv1 cell fate, we wanted to test whether egl-38 might influence the abundance or localization of LET-23 in the uv1 cells. For example, our combined results might be obtained if egl-38 influences the expression levels of both the ligand, LIN-3, in the signaling cell and the receptor, LET-23, in the responding cell. We used a LET-23::GFP reporter previously shown to rescue the loss-of-function defects of let-23 alleles (Chang et al., 1999). We found that normally LET-23 expression in the uv1 cells begins in the third larval stage and persists until early adulthood (Fig. 7B–E). Although during the fourth larval stage LET-23::GFP expression is found to surround the entire uv1 cell, it is concentrated between the uv1 cell and the vulF cell. In the egl-38(n578) mutants, we found that the LET-23::GFP pattern is normal until the middle of the fourth larval (mL4) stage. At this stage, LET-23::GFP is localized around the presumptive uv1 cell. However, instead of staying adjacent to the vulF cells, these uv1 cells begin to move away from the vulva. Finally

Fig. 5. egl-38::gfp is expressed in both the uterus and in the vulva. Transgenes that include different enhancers from the egl-38 gene are expressed in the uv1 cells of the uterus (A and B) and the vulF cells of the vulva (C and D). At least 20 animals were observed for each transgene.
during the late fourth larval stage, there is no LET-23::GFP expression around the presumptive uv1 cells (data not shown). Overall, we do not observe obvious changes in the expression dynamics both with respect to localization and in terms of levels of expression of LET-23 between wild-type and egl-38(n578) mutant animals. Consequently, we conclude

Fig. 6. Mosaic analysis for egl-38 function. (A) A cell lineage chart of the early cell divisions in C. elegans, indicating the embryonic source of the vulval and uterine cells (after Sulston et al., 1983). Genetically mosaic animals result when an extrachromosomal transgene is lost following cell division. Losses in the first division can result in animals that retain the transgene in vulval cells but not the uterus, or vice versa. (B) The mosaic animals were divided into four categories based on transgene presence or absence and phenotype. In category 1, egl-38(+) activity is present both in the vulva and in the uterine uv1 cells, the vulval and uterine phenotypes are wild-type. Therefore, the vulF cells separate and reveal a thin membrane (shown within circular brackets) while in the uterus, the uterine uv1 cells are specified and lie close to the vulva (shown within square brackets). In category 2, egl-38(+) activity is absent in both the vulF cells and the uv1 cells, and vulval and uterine functions of egl-38 are both mutant. As a result, the vulF cells fail to separate and the uv1 cells move away from the vulva and fuse with other uterine cells. In category 3, egl-38(+) activity is present in the vulva but absent in the uv1 cells, and there is wild-type vulF cell separation but no uv1 cell specification. In category 4, egl-38(+) activity is absent from the vulva and present in the uterus, and the vulF separation is defective whereas uv1 cells are normal. We conclude that egl-38 has autonomous functions in the developing egg-laying system, and that its presence in the vulva is not sufficient to specify uv1 cell fate. ’N’ is the number of animals observed in each category.
that **egl-38** does not significantly influence the expression or localization of **LET-23**.

**Discussion**

**EGL-38 plays multiple roles in the development of the egg-laying system**

**EGL-38** plays a direct role in the normal development of both the vulva and uterus of the *C. elegans* egg-laying system. Previous experiments suggested a model in which **EGL-38** mediated development of both tissues by acting solely in the vulval cells, where it ensures proper vulval morphogenesis and promotes expression of the EGF signal, **LIN-3**. **LIN-3** then signals to the uterus to specify the **uv1** cells (Chang et al., 1999). Our studies demonstrate a role for **EGL-38** in both the vulva and the uterus in coordinating the development of the egg-laying system. We find that reporter genes for **egl-38** are expressed in both uterine and vulval cells, and that in genetically mosaic animals **egl-38** can act cell-autonomously. Thus we propose a model in which **egl-38** plays a role in cells of both tissues to coordinate development of the organ (Fig. 8). In the vulva, **egl-38** acts to both promote normal morphogenesis and to promote expression of **LIN-3**, which signals to the uterus. **egl-38** also acts in the uterus, and is necessary for prospective uv1 cells to respond to the **LIN-3** signal, or to otherwise develop as uv1. We favor this model as it integrates our results with the earlier studies.

In the context of the model, we will address two apparent contradictions between the mutant analyses and the genetic mosaic analysis. First, the genetic studies indicate that activated **let-23** can bypass the uv1 defect in **egl-38(n578)** animals, whereas the mosaic result indicates that presence of **egl-38(+)** (and thus expression of the **LET-23** ligand **LIN-3**) in the vulva is not sufficient for uv1 cell fate. We suggest that this may reflect a difference between the sensitivity of uv1 cells to different levels of **let-23** activity. In this case, the cells normally require input from both **let-23** and **egl-38**, but increased or unregulated activation of **let-23** is sufficient to bypass the **egl-38** role. Second, the genetic studies indicate that **egl-38** is necessary for **LIN-3** expression in the vulva, and that the **LIN-3/LET-23**
This suggests that let-23 can somehow influence the activity of let-23 within the uv1 cell, but our results cannot rule out other possibilities. For example, egl-38 could act within the uv1 cells to somehow influence the activity of let-23. In either situation, our results demonstrate that the same Pax transcription factor can act in different cells to coordinate signaling and responding during development.

The nature of uv1 and vulF cell fate

During the development of the uterine–vulval connection in C. elegans, the vulF cells of the vulva and the uv1 cells of the uterus form the connection between the two tissues of the organ, and cell signaling establishes the fate of each. Our studies of the development of these two cells in egl-38 mutant animals dissect features that underlie each cell fate. In the egl-38(n578) mutants, the correct number of presumptive vulF cells is produced and they lie in their appropriate positions. They also acquire a cell fate distinct from those of the other cell type derived from the 1° VPC (the vulE cells), as they express bam-2::gfp at higher levels than vulE cells, as do wild-type vulF cells. However, the presumptive vulF cells are abnormal in egl-38(n578) mutants, as they fail to express lin-3::gfp and fail to undergo proper morphogenesis. Therefore, only a subset of features that define the vulF cell fate are dependent on EGL-38.

In the uterus, the uv1 cells are defined both by the expression of markers and cell position. We have found that, in egl-38 mutants, the presumptive uv1 cell expresses π cell markers like egl-13::gfp and lin-11::gfp, but not the uv1 marker, ida-1::gfp. The presumptive uv1 cells also migrate near the other π cells, and fuse with them to form the utse. Although visual markers support the idea that the presumptive uv1 cells are completely transformed to the utse cell fate in egl-38 mutants, prior to migration in the early and mid L4 stage, the cells remain adjacent to the vulva and express LET-23::GFP in a pattern and with the abundance similar to that seen in wild-type. Thus the cells retain these uv1-related features. We speculate that normal uv1 cell fate involves cell–cell adhesion or another process that anchors the cells at the vulva, and that egl-38 is normally required for this function.

Pax transcription factors in organogenesis

EGL-38 belongs to the Pax2/5/8 family of transcription factors. Members of this family play important roles to coordinate development of different cell types and organs. For example, during the development of the metanephric kidney in mouse, Pax2 is expressed multiple times, and performs different functions in the different tissues of the system to promote and coordinate organ development (reviewed by Schedel and Hastie, 2000). Our work demonstrates similarities between the role played by Pax2 in mammalian kidney formation and that of its orthologue EGL-38 during the development of the egg-laying system in C. elegans. EGL-38, like Pax2, is expressed in both epithelial and mesodermal cells of the organ. It also influences organ development in both autonomous and non-autonomous ways, as it affects the development of the cells in which it is expressed, as well as expression of signaling molecules that influence the development of other cells. We conclude that coordinating the development of cells and tissues within an organ is a conserved function for Pax2/5/8 transcription factors.
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