



EGL-38/Pax coordinates development in the *Caenorhabditis elegans* egg-laying system through EGF pathway dependent and independent functions



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ARTICLE INFO

Keywords:

EGF signaling

C. elegans

Pax transcription factor

Organogenesis

ABSTRACT

Paired box (Pax) proteins function as regulators of coordinated development in organogenesis by controlling factors such as cell growth and differentiation necessary to organize multiple cell types into a single, cohesive organ. Previous work has suggested that Pax transcription factors may regulate diverse cell types through participation in inductive cell-to-cell signaling, which has not been well explored. Here we show that EGL-38, a Pax2/5/8 ortholog, coordinates differentiation of the *C. elegans* egg-laying system through separate autonomous and non-autonomous functions synchronized by the EGF pathway. We find that EGL-38 protein is expressed at the correct times to both participate in and respond to the EGF pathway specifying uterine ventral (uv1) cell fate, and that EGL-38 is required for uv1 expression of *nlp-2* and *nlp-7*, which are both markers of and participants in uv1 identity. Additionally, we have separated uv1 cell placement and gene expression as distinct hallmarks of uv1 identity and specification, with different dependencies on EGL-38. The parallels between EGL-38 participation in cell signaling events and previous Pax studies argue that coordination of signaling and response to an inductive pathway may be a common feature of Pax protein function.

1. Introduction

Paired box (Pax) transcription factors are necessary for coordinated development and organogenesis of many mammalian systems, such as thyroid (Pax8), kidney (Pax2/8), central nervous system (Pax2/3/5/6/7/8), B lymphocytes (Pax5), pancreas (Pax4/6), eye (Pax6), and ear (Pax2/8) (Blake and Ziman, 2014; Dressler et al., 1990; Lang et al., 2007; Macchia et al., 1998; Magliano et al., 2000; Noll, 1993; Torres et al., 1995). These transcription factors contribute to the differentiation and maintenance of cell fate, as well as the initiation of cellular programs required for proper cell activity (Mansouri et al., 1998; Miccadei et al., 2002). It is known that Pax proteins are necessary for initiating complex developmental programs, and previous work has suggested that Pax proteins are capable of coordinating differentiation of diverse cell types due to their involvement in inductive cell-to-cell signaling (Lang et al., 2007; Püschel et al., 1992). For example, research on Pax2, Pax6, and Pax8 has shown these proteins are primarily expressed in cells requiring inductive cell-to-cell signaling for development, arguing that it is likely that Pax2/6/8 are targets of, or participants in, these signaling events (Dressler et al., 1990; Püschel et al., 1992). Involvement in cell-to-cell signaling would provide a clear mechanism for Pax proteins to be able to coordinate cell fate and

development among distinct, neighboring cell types; however, as of yet, Pax participation in signaling has not been well characterized in humans or mice.

To investigate the interaction of a Pax protein with signaling, and of Pax coordination of cell fate and developmental programs, we are studying the *egl-38* gene in *Caenorhabditis elegans*. EGL-38 is most similar in sequence to mammalian Pax2/5/8, and similarly possesses the paired-DNA binding domain and octapeptide sequence (Chamberlin et al., 1997). *egl-38* functions in the development of the hermaphrodite egg-laying system, hindgut, and male spicule (Chamberlin et al., 1997). In particular, the development of the egg-laying system is ideal for examining the interaction of EGL-38/Pax with an inductive signaling pathway for establishment of cell fate and of execution of cellular programs, as this system is dependent on both EGL-38 and the Epidermal Growth Factor (EGF) pathway for differentiation (Chang et al., 1999; Katz et al., 1995)

The *C. elegans* egg-laying system results from coordinated development between vulval cells in the epidermis and mesodermal cells in the somatic gonad (Fig. 1). First, vulval cell identity is established in response to EGF signaling. The *C. elegans* EGF signal, LIN-3, is sent from the uterine anchor cell during the L3 larval stage to vulval precursor cells, specifying a cell (P6.p) as the 1^o vulval cell (Fig. 1A) (Hill and

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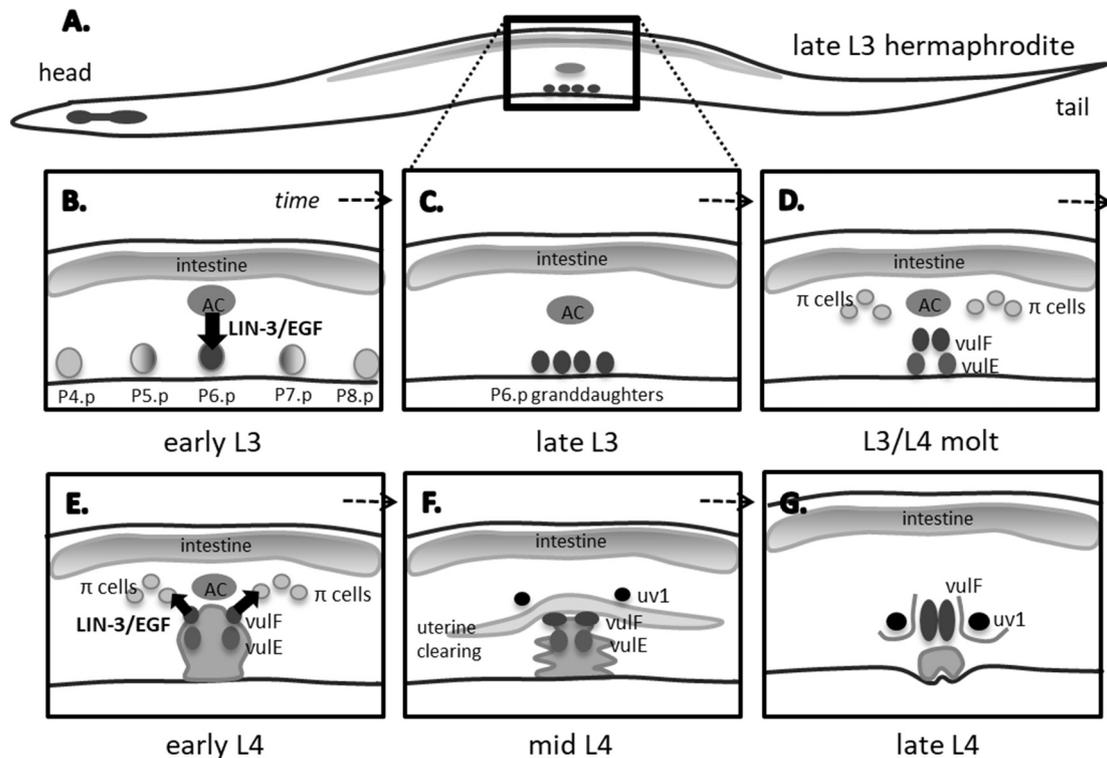


Fig. 1. (A) A representative schematic of the *C. elegans* hermaphrodite at the late L3 stage of development. The developing egg-laying system is found on the ventral side of the worm, mid-way down the body. (B–G) A brief representation of egg-laying system development through time. (B) early L3. The LIN-3/EGF signal is sent from the Anchor Cell (AC) to the P6.p cell to specify it as the 1° cell. (C) late L3. P6.p divides, forming 4 granddaughters by late L3. (D) L3/L4 molt. The 1° cell descendants have divided, and (on each side of the animal) there are two vulF and two vulE cells. The π cells have been specified surrounding the AC. (E) early L4. The vulF cells at the apex of the vulva send a reciprocal LIN-3/EGF signal back to the nearest π cells. (F) mid L4. The now-specified uv1 cells move closer to the vulva. (G) late L4. The uv1 cells are in position right next to the vulval cells to serve as an anchor of vulva to uterus.

Sternberg, 1992; Katz et al., 1996). The 1° cell then signals to neighboring cells (to produce the 2° vulval cells), and divides to produce eight cells that form the upper portion of the hermaphrodite vulva. Four of these cells at the apex of the vulva (the vulF cells) then express the *lin-3/egf* signal gene, which is used for reciprocal EGF signaling back to the uterus during the L4 larval stage (Fig. 1E) (Chang et al., 1999). This reciprocal EGF signal is sent from the vulF cells to a subset of uterine cells surrounding the anchor cell to induce these cells to assume a uterine ventral (uv1) cell identity (Chang et al., 1999). The uv1 cells will move into position closer to the vulva to serve as an anchor of the vulva to the uterus, allowing the vulF cells to separate from one another to form the passage through which eggs are laid (Fig. 1F–G) (Newman and Sternberg, 1996). Additionally, the uv1 cells are neurosecretory, and serve an inhibitory role during egg-laying to help control the periodicity and set inactive periods (Banerjee et al., 2017; Collins et al., 2016; Jose et al., 2007).

egl-38 plays a critical role in coordinating the connection between the vulva and the uterus. In an *egl-38(lf)* mutant, the *lin-3/egf* signal is not expressed in vulF, and the uv1 cells are not specified (Chang et al., 1999; Rajakumar and Chamberlin, 2007). Instead, the presumptive uv1 cells retain their original identity and migrate away with their sister cells to fuse with the anchor cell into the uterine seam cell syncytium (Newman et al., 1996). *egl-38(lf)* mutants also lack vulF cell separation, preventing the laying of eggs (Rajakumar and Chamberlin, 2007). Previous work has shown that *egl-38* has functions in both the epidermal vulF cells and the mesodermal uv1 cells to contribute to uv1 cell specification and vulF cell separation (Rajakumar and Chamberlin, 2007), but its role in coordinating development between these cell types was not clarified.

Here we show how EGL-38 functions in both autonomous (independent of the EGF pathway) and non-autonomous (dependent on

the EGF pathway) processes to influence uv1 cell fate and cellular programs necessary for egg-laying function. We have tagged the endogenous *egl-38* locus with *gfp*, which allows for clear examination of EGL-38::GFP localization and timing of expression. We have determined that *egl-38* uv1 expression is dependent on the same LIN-3/EGF signal that EGL-38 activates in the vulF cells. Furthermore, our work shows that *egl-38* is necessary for the expression in uv1 of two neuropeptide-encoding genes which may have roles in controlling egg-laying and thereby contributing to the functions of differentiated uv1 cells. We have discovered that the uv1 cell traits of positioning above the vulva and of expression of uv1-specific genes are separable activities, with the former being dependent on EGF signaling (and therefore indirectly on EGL-38 in the vulF cell), and the latter requiring direct, autonomous EGL-38 activity within the uv1 cell. EGL-38 coordination of cell fate, development, and its own expression through an inductive cell-to-cell signaling pathway represents a novel Pax function which may represent a concise, direct mechanism for Pax to organize disparate cells into a single, cohesive organ system.

2. Results

2.1. EGL-38 protein is expressed during the development of the *C. elegans* egg-laying system

Genetic experiments suggest that *egl-38* functions in both the vulva and the uterus of *C. elegans* (Rajakumar and Chamberlin, 2007). Previously, however, *egl-38* expression was evaluated only with transcriptional reporter transgenes, which do not inform us about protein expression and localization. Therefore, we utilized CRISPR-mediated genome editing to tag the endogenous locus so that the EGL-38 protein is tagged on the C-terminus with GFP. We find that in this strain, we

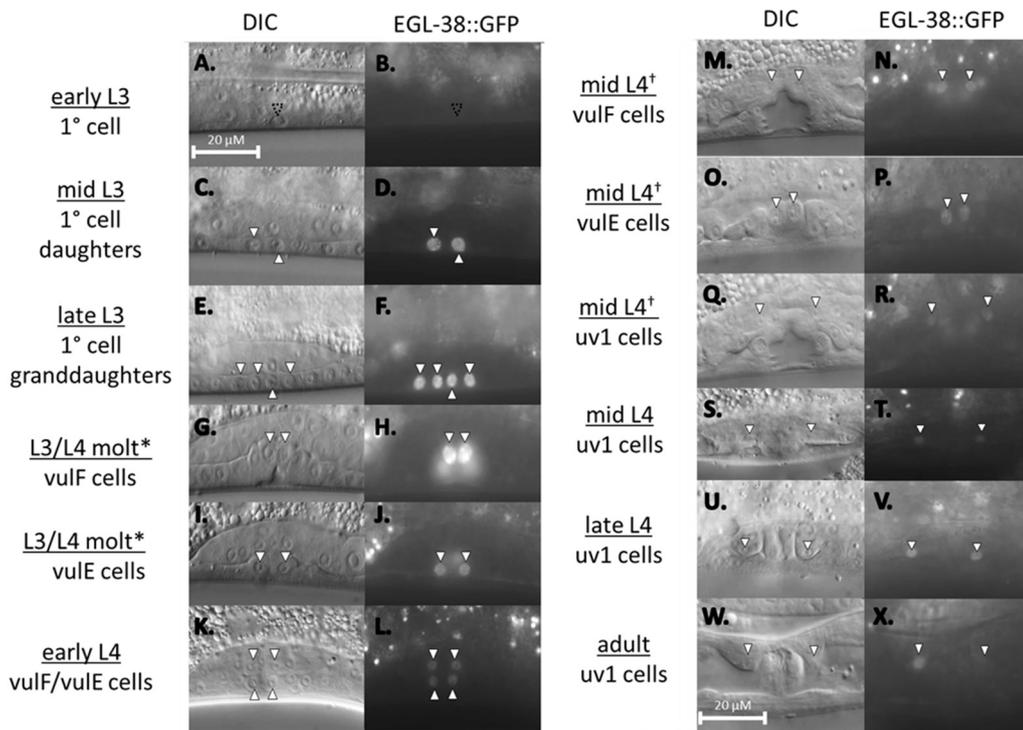
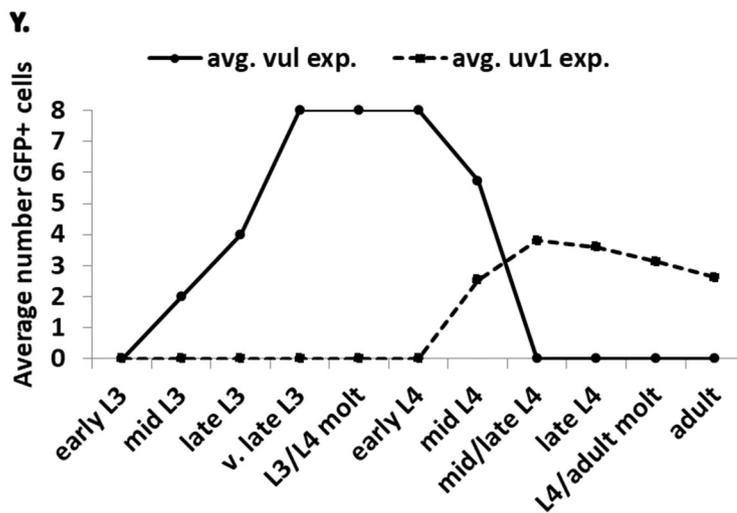


Fig. 2. EGL-38::GFP is expressed during *C. elegans* egg-laying system development. (A-X) Expression of tagged EGL-38::GFP in the mid-plane view of the developing hermaphrodite egg-laying system. Individual worms were staged and screened for a single time point. Cells of interest are indicated with an arrowhead in both DIC and fluorescent panels. (A–B) Early L3. The P6.p cell lacks expression (empty arrowhead). (C–D) Mid-L3. The two P6.p daughter cells strongly express EGL-38::GFP. (E–F) Late L3. The four P6.p granddaughters all accumulate EGL-38::GFP (third arrowhead rotated to allow visualization of the AC immediately above). (G–J*) L3-L4 molt. Same animal, image plane shifted. vulF (G-H) and vulE (I–J) cell expression. (K–L) Early L4. Presence in vulF and vulE continues. (M–R*) Mid-L4. Same animal, image plane shifted. EGL-38::GFP continues in vulF (M–N) and vulE (O–P) as expression begins in the presumptive uv1 cells prior to positioning (Q–R). (S–T) Mid-L4. By the end of the mid-L4 stage, uv1 expression is apparent while fluorescence in vulF and vulE has diminished to undetectable levels. (U–V) Late L4. uv1 cell expression continues. (W–X) Adult. EGL-38::GFP persists in uv1 cells into young adulthood, but is not as consistent as the worms age. (Y) Time-course trend of EGL-38::GFP expression. Values represent average number of GFP-positive cells, with each time point corresponding to 30 animals. To limit photo bleaching, data were collected from only one side of each worm, but are extrapolated to include cells on the other side of the developing vulva to represent all of the cells of each structure (a full eight vulval cells by late L3, and four uv1 cells by mid-L4).



detect EGL-38::GFP protein fluorescence in all of the expected hermaphrodite tissues, including the vulva, uterus, and hindgut (not shown) and that the protein localizes to the nuclei of cells as predicted for a transcription factor protein. We evaluated protein expression at different time points, beginning in the early L3 stage prior to vulval precursor cell (VPC) division (Fig. 2). During early L3, we observed that EGL-38::GFP is absent from egg-laying system cells, but is first detected in both P6.px daughters and all four P6.pxx granddaughters following the first and second divisions of the VPCs, respectively (Fig. 2A–F). After the final VPC divisions, the cells vulF and vulE (derived from P6.p) express EGL-38::GFP; this expression persists through early L4 into mid-L4 (Fig. 2G–P). At the mid-L4 time point, expression in the uv1 cells can be detected concurrent with vulF and vulE expression (Fig. 2M–R). EGL-38::GFP expression in vulF and vulE diminishes during mid-L4 so that by the transition to late L4, only uv1 cell expression remains (Fig. 2S–T). This timing is consistent with the idea that this expression participates in, or is responsive to, the developmental coordination between the two tissues. The uv1 cells continue to express EGL-38::GFP through the adult molt into adulthood (Fig. 2U–X). Thus

EGL-38 protein is expressed in both vulval and uterine cells consistent with the genetic functions of *egl-38*. EGL-38::GFP expression continues into adulthood in the uv1 cells, but not the vulval cells, indicating that EGL-38 may have a role in the function of mature uv1 cells.

2.2. EGL-38 expression in uv1 is dependent on EGF signaling

egl-38 mutants exhibit defects in both vulF and uv1 cell specification. In particular, *egl-38* mutants are defective in vulF expression of *lin-3/egf*, which is used to signal to presumptive uv1 cells to promote their development (Chang et al., 1999; Rajakumar and Chamberlin, 2007). LIN-3/EGF-initiated uv1 cell specification is marked by two features: proper placement of the uv1 cells near the vulva, and expression of uv1-specific genes. If this EGF signaling is disrupted, then the presumptive uv1 cells migrate away from the vulval region and fuse with the uterine seam cell as do the other neighboring uterine (π) cells (Newman and Sternberg, 1996; Rajakumar and Chamberlin, 2007). We have demonstrated that EGL-38::GFP is expressed in both the signaling and responding cells of this developmental event. To investigate the

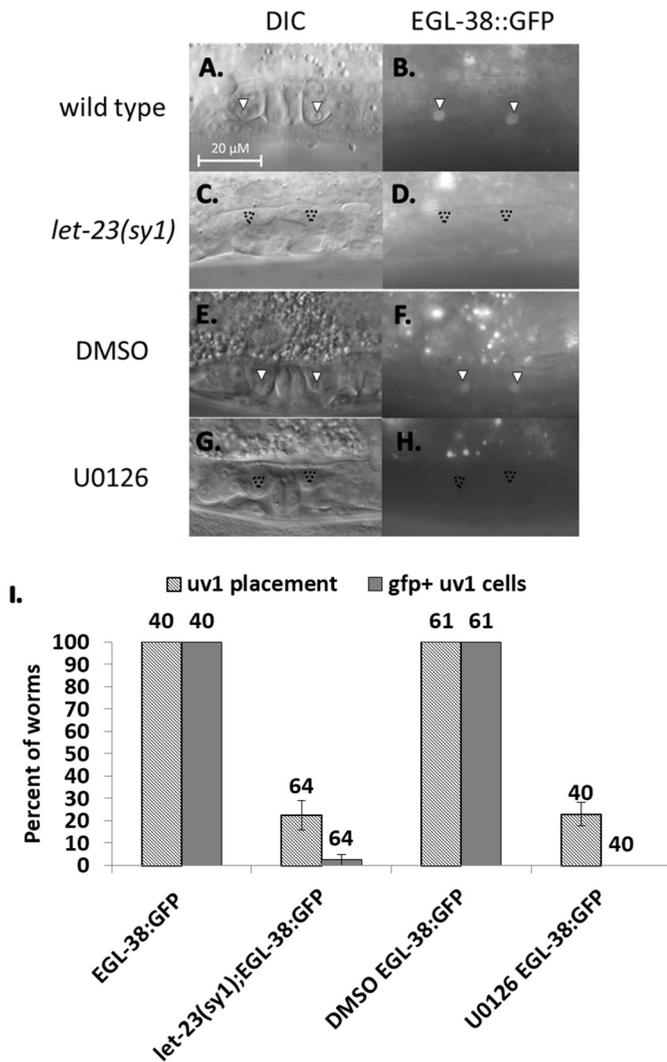


Fig. 3. EGL-38::GFP expression is dependent on EGF signaling. (A–B) Wild type late L4. EGL-38::GFP is present in uv1 cells (solid arrowhead). (C–D) *let-23(sy1)*. uv1 cells are absent in the loss-of-function *let-23* background, and EGL-38::GFP expression is also absent in this background (approximate position marked with an empty arrowhead. uv1 positioning can vary between worms). (E–F) DMSO control treatment on wild-type animals. uv1 placement and expression of EGL-38::GFP match wild type in the control treatment. (G–H) U0126 treatment. Blocking the EGF pathway in mid L3 stage results in a lack of uv1 cells, and absent EGL-38::GFP expression. (I) Percent of worms with correctly placed uv1 cells and with expression of EGL-38::GFP in the uv1 cells. Above each bar is shown the number of worms screened. Error bars are shown for the standard error of proportion. For each pairwise comparison of control to mutant/treatment (wild type to *let-23(sy1)*; DMSO to U0126), the difference is significant at a $p < 0.05$ for each pairwise comparison of experiment to control (2-tailed Z-test).

relationship between EGL-38::GFP expression and EGF signaling, we utilized both genetic disruption and chemical inhibition. For an initial genetic approach, we evaluated EGL-38::GFP expression in animals mutant for the EGF receptor gene, *let-23(sy1)*. As this is a non-null allele, we focused solely on vulvaless *let-23(sy1); egl-38::GFP* worms, to ensure that vulF cells are absent and unable to communicate with the uterine cells. These worms also lack vulval EGL-38::GFP, as there are no induced vulval cells. We find that placement of presumptive uv1 cells is eliminated in the majority of vulvaless *let-23(sy1)* worms, and that all lack any uv1 EGL-38::GFP expression (Fig. 3). Thus expression of EGL-38::GFP in the uv1 cells is dependent on *let-23/EGFR* activity in the egg-laying system. However, since these animals are both mutant for *let-23/*

EGFR and vulvaless, we sought to more directly test whether EGL-38::GFP is dependent on vulval cell signaling using the MEK inhibitor U0126 (Duncia et al., 1998). Specifically timed application of U0126 allows blocking of the vulF to uv1 EGF signal without affecting the original EGF signal from the anchor cell that triggers the VPCs to divide and form the vulva. We treated late L3 EGL-38::GFP worms with U0126, and find that these animals have morphologically normal vulvas while lacking uv1 cell specification. Similar to the *let-23(sy1)* mutants, these animals are disrupted for uv1 cell placement, and lack uv1 EGL-38::GFP expression (Fig. 3). We note that the effect of U0126 is slightly stronger than the effect of *let-23(sy1)* in this experiment and the ones below. This may reflect the fact that *let-23(sy1)* is a non-null allele, and retains some activity and that there are non-vulval sources of diffusible *lin-3/egf* that may compensate for the missing vulF signaling in *let-23(sy1)* mutants (Aroian et al., 1990; Aroian and Sternberg, 1991; Hwang and Sternberg, 2004). Nevertheless, in both *let-23(sy1)* mutants and U0126-treated animals, uv1 cell placement near the vulva was disrupted, and EGL-38::GFP expression was absent. Together, these data argue that EGL-38 expression in uv1 is dependent on EGF signaling from the vulF cells.

2.3. Expression of neuropeptide genes *nlp-2* and *nlp-7* in the uv1 cells is dependent on EGF signaling and *egl-38*

In the vulF cells, a critical function for *egl-38* is to promote expression of *lin-3/egf*; however, EGL-38 targets and functions in the uv1 cells are unknown (Chang et al., 1999). The uv1 cells are neurosecretory cells and inhibit egg-laying in response to the passage of eggs out of the uterus (Banerjee et al., 2017; Collins et al., 2016; Jose et al., 2007). To determine if EGL-38 has any direct targets within the uv1 cells, we sought to identify genes which are expressed in the mature uv1 cells. Previous work led us to focus on Neuropeptide-like proteins (*nlp*s), as one such *nlp*, *nlp-7*, has been identified as necessary for uv1 inhibition of egg-laying activity (Banerjee et al., 2017). *nlp*s encode non-insulin and non-FMRFamide related peptides, with little known about most members of this family (Nathoo et al., 2001). To determine if any additional *nlp*s are expressed in uv1 cells, therefore representing a possible EGL-38 activation target, we tested five genes, *nlp-2*, 6, 7, 9, and 12. We constructed a reporter with ~2 kb of upstream promoter sequence driving the expression of *gfp*. Only *Pnlp-2::gfp* and *Pnlp-7::gfp* were found to express in uv1 beginning in mid-L4, and persisting into the adult stages. The study which identified *nlp-7* involvement in egg-laying also found that the FMRF-like peptide-encoding gene, *f1p-11*, functions with *nlp-7* in the uv1 cell. However, we were not able to detect expression of *Pf1p-11::gfp* in uv1 cells using available reporters. Consequently, we focused our studies on *nlp-2* and *nlp-7*.

We first sought to determine if *Pnlp-2::gfp* and *Pnlp-7::gfp* expression is dependent on uv1 cell specification and if they are markers of mature uv1 cell fate. To do so, we examined the interaction of *Pnlp-2::gfp* and *Pnlp-7::gfp* with the EGF pathway by first utilizing the *let-23(sy1)* allele (Fig. 4A–Q). In *let-23(sy1); Pnlp-2::gfp* and *let-23(sy1); Pnlp-7::gfp* vulvaless animals, expression of the reporter was reduced (to 50% and 25%, respectively). As with EGL-38::GFP, we also examined reporter expression when late L3 animals were treated with U0126 to specifically block signaling from vulF to the uv1 cells. For both *Pnlp-2::gfp* and *Pnlp-7::gfp* U0126-treated animals, none of the worms showed reporter expression. Neither reporter exhibits expression in non-uv1 uterine cells in any background, while expression in neuronal cells outside the egg-laying system continued unabated in all altered conditions. Thus, *nlp-2* and *nlp-7* exhibit a similar dependence as EGL-38 on the EGF pathway for uv1 cell expression. Given that expression from both reporters in the egg-laying system occurs only in the differentiated uv1 cells, it is suggestive that both *nlp*s function in adult uv1 cell activity.

We next investigated the dependence of *nlp-2* and *nlp-7* expression on EGL-38 activity in the mature uv1 cells. We crossed the reporter transgenes into two *egl-38* loss-of-function backgrounds, *egl-38(n578)*

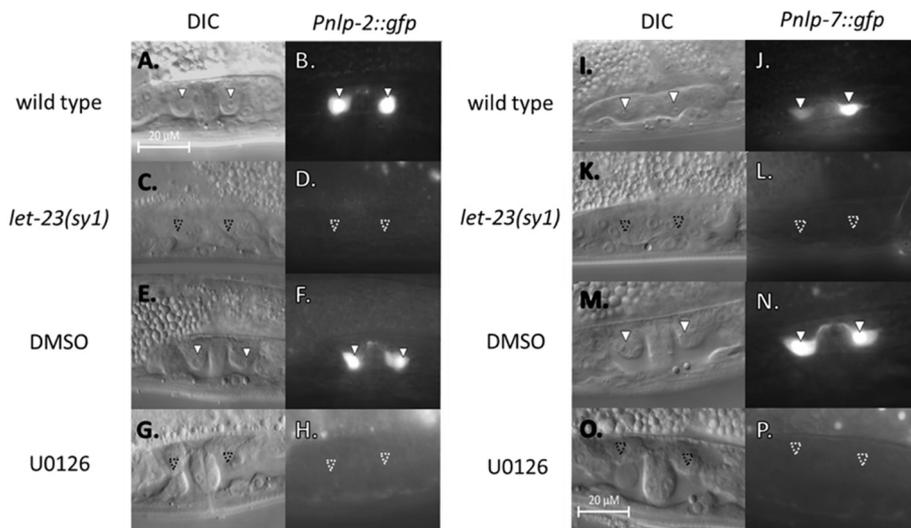
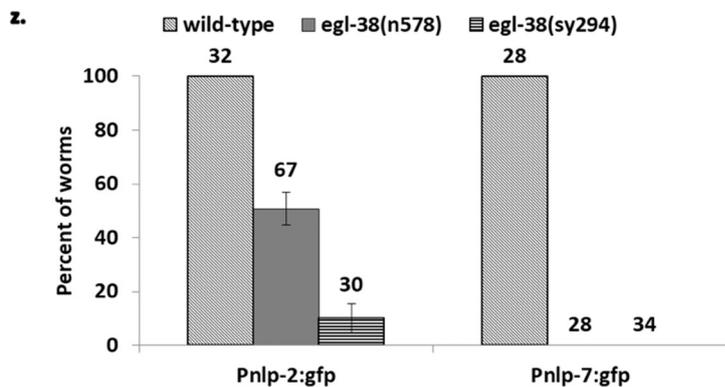
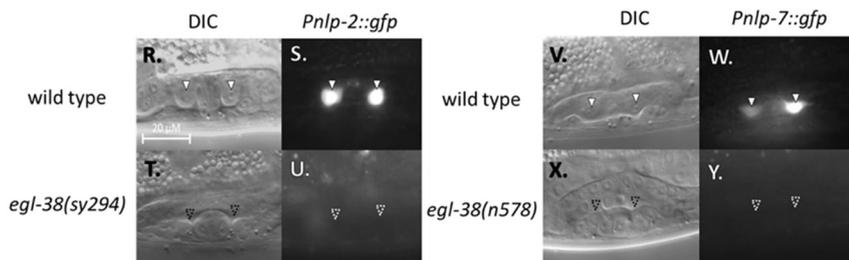
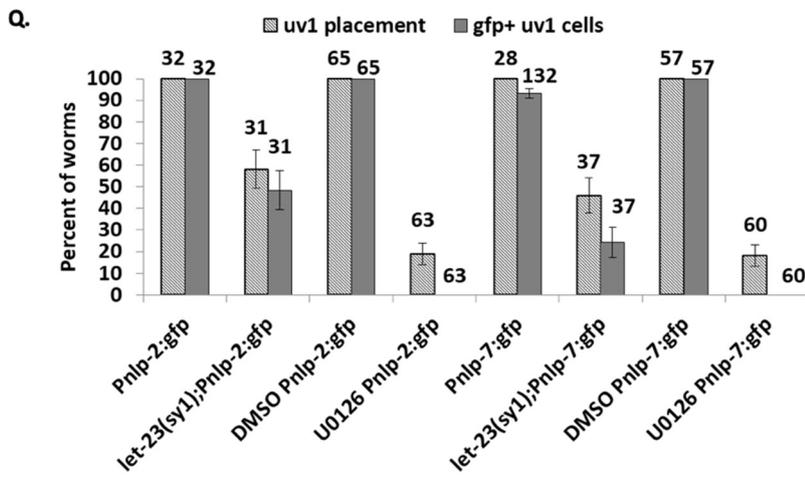


Fig. 4. Expression of *nlp-2* and *nlp-7* in uv1 cells is dependent on EGF signaling and *egl-38*. (A–H) *Pnlp-2::gfp* expression. (A–B) Wild type. *Pnlp-2::gfp* expression in mid-L4 (solid arrowhead). (C–D) *let-23(sy1)* background. Worms lack uv1 placement and *Pnlp-2::gfp* expression (approximate position of a normal uv1 cell marked with an empty arrowhead). (E–F) DMSO control treatment. uv1 cell placement and expression matches wild type in late L4. (G–H) U0126 treatment. uv1 cells are absent, as is *Pnlp-2::gfp* expression. (I–P) *Pnlp-7::gfp* expression. (I–J) Wild type. *Pnlp-7::gfp* expression in mid-L4. (K–L) *let-23(sy1)* background. uv1 cell placement and *Pnlp-7::gfp* are both absent. (M–N) DMSO control treatment. Worms in late L4 have uv1 placement and expression as in wild type. (O–P) U0126 treatment. uv1 cells and expression are absent. (Q) Percent of worms with correctly placed uv1 cells or with expression of *Pnlp-2::gfp* or *Pnlp-7::gfp* in the uv1 cells. Above each bar is shown the number of worms screened. Error bars are shown for the standard error of proportion. For each pairwise comparison of control to mutant/treatment (wild type to *let-23(sy1)*; DMSO to U0126), the difference is significant at a $p < 0.05$ for all pairwise comparisons (2-tailed Z-test). (R–Y) *Pnlp-2::gfp* and *Pnlp-7::gfp* expression in the mid-plane of L4 hermaphrodites in wild type or loss-of-function *egl-38* backgrounds. (R–U) *Pnlp-2::gfp* expression. (R–S) Wild type. uv1 cell placement and *Pnlp-2::gfp* expression in mid/late L4. (T–U) *egl-38(sy294)* background. Worms lack uv1 cells and expression from the reporter in loss-of-function *egl-38*. (V–Y) *Pnlp-7::gfp* expression. (V–W) Wild type. uv1 placement and *Pnlp-7::gfp* expression in mid/late L4. (X–Y) *egl-38(n578)* background. uv1 cells and *Pnlp-7::gfp* expression are both absent in the *egl-38* mutant. (Z) Percent of worms with correctly placed uv1 cells or with expression of *Pnlp-2::gfp* or *Pnlp-7::gfp* in the uv1 cells in wild type or loss-of-function *egl-38* background. Above each bar is shown the number of worms screened. Error bars are shown for the standard error of proportion. For each pairwise comparison of control to mutant (wild type to *egl-38(n578)* or *sy294*), the difference is significant at a $p < 0.05$ for all pairwise comparisons (2-tailed Z-test).



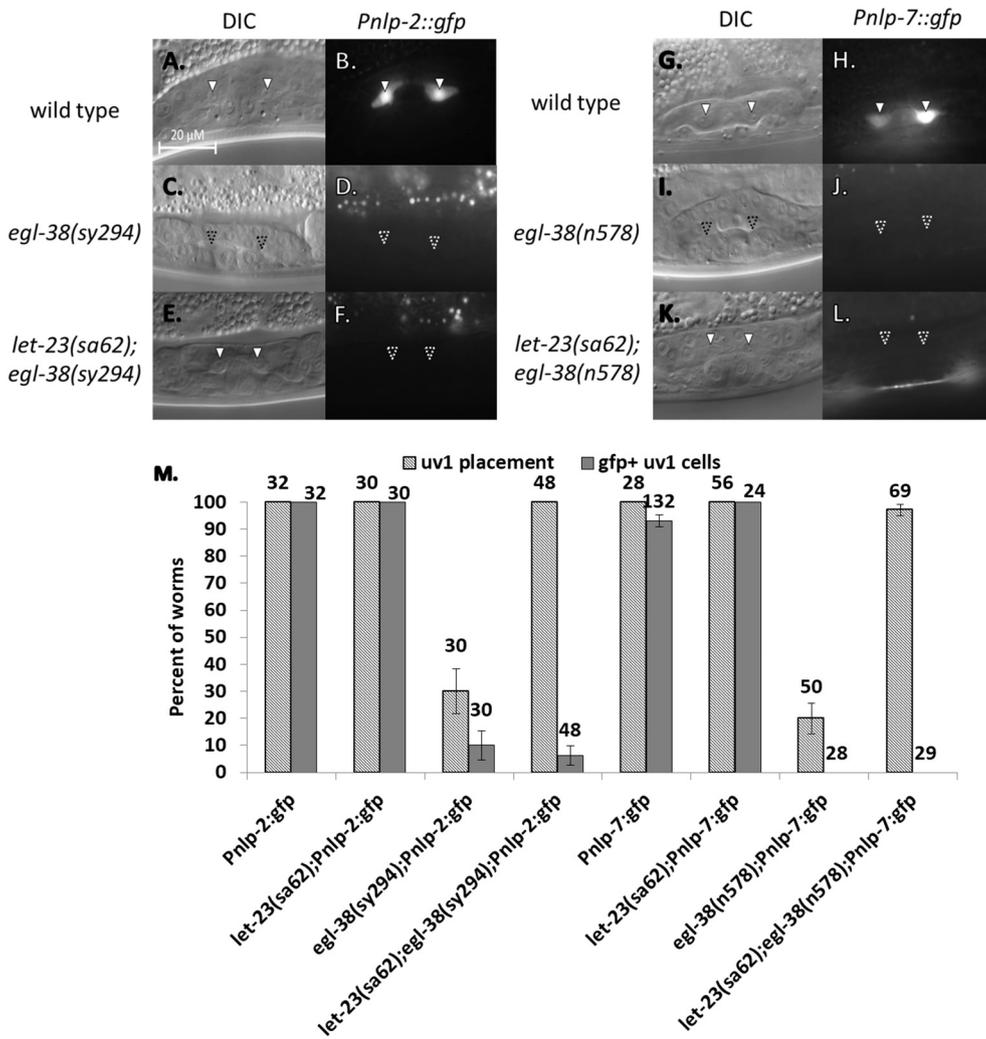


Fig. 5. Activated *let-23/EGFR* can bypass the *egl-38* defect in *uv1* placement, but not *nlp* gene expression. *Pnlp-2::gfp* and *Pnlp-7::gfp* expression in the mid-plane of L4 hermaphrodites in wild type, *egl-38(lf)*, and *let-23(gf);egl-38(lf)* backgrounds. *uv1* cells with accurate placement are indicated with a solid arrowhead; absent *uv1* cells are marked at the approximate position with an empty arrowhead. *uv1* positioning can vary between worms. (A–F) *Pnlp-2::gfp* expression. (A–B) Wild type. *uv1* cells are correctly placed and expressing *Pnlp-2::gfp*. (C–D) *egl-38(sy294)* background. Worms lack *uv1* cells and expression. (E–F) *let-23(sa62); egl-38(sy294)* background. *uv1* cell placement has been restored, but *Pnlp-2::gfp* expression remains absent. (G–L) *Pnlp-7::gfp* expression. (G–H) Wild type. Worms have *uv1* cell placement and expression of reporter. (I–J) *egl-38(n578)* background. Both *uv1* cell placement and *Pnlp-7::gfp* expression are absent. (K–L) *let-23(sa62);egl-38(n578)* background. *uv1* cell placement has been restored, but *Pnlp-7::gfp* expression is absent. (M) Percent of worms with correctly placed *uv1* cells or with expression of *Pnlp-2::gfp* or *Pnlp-7::gfp* in the *uv1* cells in wild-type or mutant background. Above each bar is shown the number of worms screened. Error bars are shown for the standard error of proportion. For each pairwise comparison of control to mutant the difference is significant at a $p < 0.05$ for all comparisons (2-tailed Z-test).

and *egl-38(sy294)* (Fig. 4R–Z). These alleles both affect egg-laying system development (Rajakumar and Chamberlin, 2007). Expression from the reporter transgenes for both genes is affected. *Pnlp-7::gfp* expression is eliminated in both *egl-38(n578)* and *egl-38(sy294)* backgrounds while *Pnlp-2::gfp* exhibited significantly reduced expression. These results demonstrate that both *nlp-2* and *nlp-7* expression is additionally dependent on *egl-38* for expression in the *uv1* cells.

2.4. EGFR activation bypasses the requirement for *egl-38* to promote *uv1* cell placement, but not neuropeptide gene expression

Our EGL-38::GFP expression results and previous genetic mosaic results indicate that EGL-38 is present and can act in both vulF and *uv1* cells (Rajakumar and Chamberlin, 2007). However, since *egl-38* is required for expression of *lin-3/egf* in vulF cells, it is possible that expression of *nlp-2* and *nlp-7* is due indirectly to EGL-38 activity in vulF and not directly to its activity in *uv1*. To separate these possibilities, we examined the epistatic relationship between *let-23/egfr* and *egl-38* in regard to *nlp-2* and *nlp-7*. Utilizing a *let-23(sa62)* gain-of-function allele, which remains constitutively active in the absence of the *lin-3/egf* signal, we created double mutants with *egl-38* (Fig. 5). For the *Pnlp-2::gfp* strain, we utilized *egl-38(sy294)*, as this mutation had a stronger effect on *Pnlp-2::gfp* expression, whereas for *Pnlp-7::gfp*, we employed *egl-38(n578)*.

For both reporters, *let-23(sa62)* background predictably resulted in wild-type levels of *uv1* cell placement and in reporter expression. There was no over-production of *uv1*-defined cells or other extraneous cells in

the egg-laying system. We constructed double mutants *let-23(sa62); egl-38(sy294); Pnlp-2::gfp* and *let-23(sa62); egl-38(n578); Pnlp-7::gfp*. For both strains, the *uv1* cell placement defect found in *egl-38(lf)* worms was rescued by *let-23(sa62)*, whereas the expression of *Pnlp-2::gfp* and *Pnlp-7::gfp* was not. Importantly, our data show that placement of the *uv1* cells near the vulva and expression of *nlp-2* and *nlp-7* in the *uv1* cells are distinct events with separate dependencies on *egl-38* activity. Consequently, we interpret that *nlp-2* and *nlp-7* expression in the *uv1* cells is dependent on *egl-38* autonomous activity from within the *uv1* cells. Because both *nlp-2* and *nlp-7* are expressed only in the differentiated *uv1* cells, their dependence on EGL-38 indicates that a function for EGL-38 within *uv1* is to activate developmental programs necessary for egg-laying.

2.5. *nlp-2* is a direct target for EGL-38 in *uv1* cells

We chose *nlp-2* to further examine the relationship of EGL-38 with the *uv1* cell neuropeptide genes. The sequence included in the *Pnlp-2* reporter consists of around 2.6 kb of upstream sequence. To identify the regulatory element responsible for *uv1* cell expression, truncations were introduced beginning at the 5' upstream end of the original 2.6 kb fragment to create successively smaller reporter genes, and the expression profile of the subsequent reporter was analyzed (Fig. 6). Due to the abrupt loss of *uv1* expression upon deletion, we interpret that a *uv1* cell enhancer element exists between 0.34 and 0.38 kb from the start codon of *nlp-2*. We evaluated this sequence for prospective Pax binding sites using the IRE2 sequence from *lin-48*, a known target for EGL-38 in

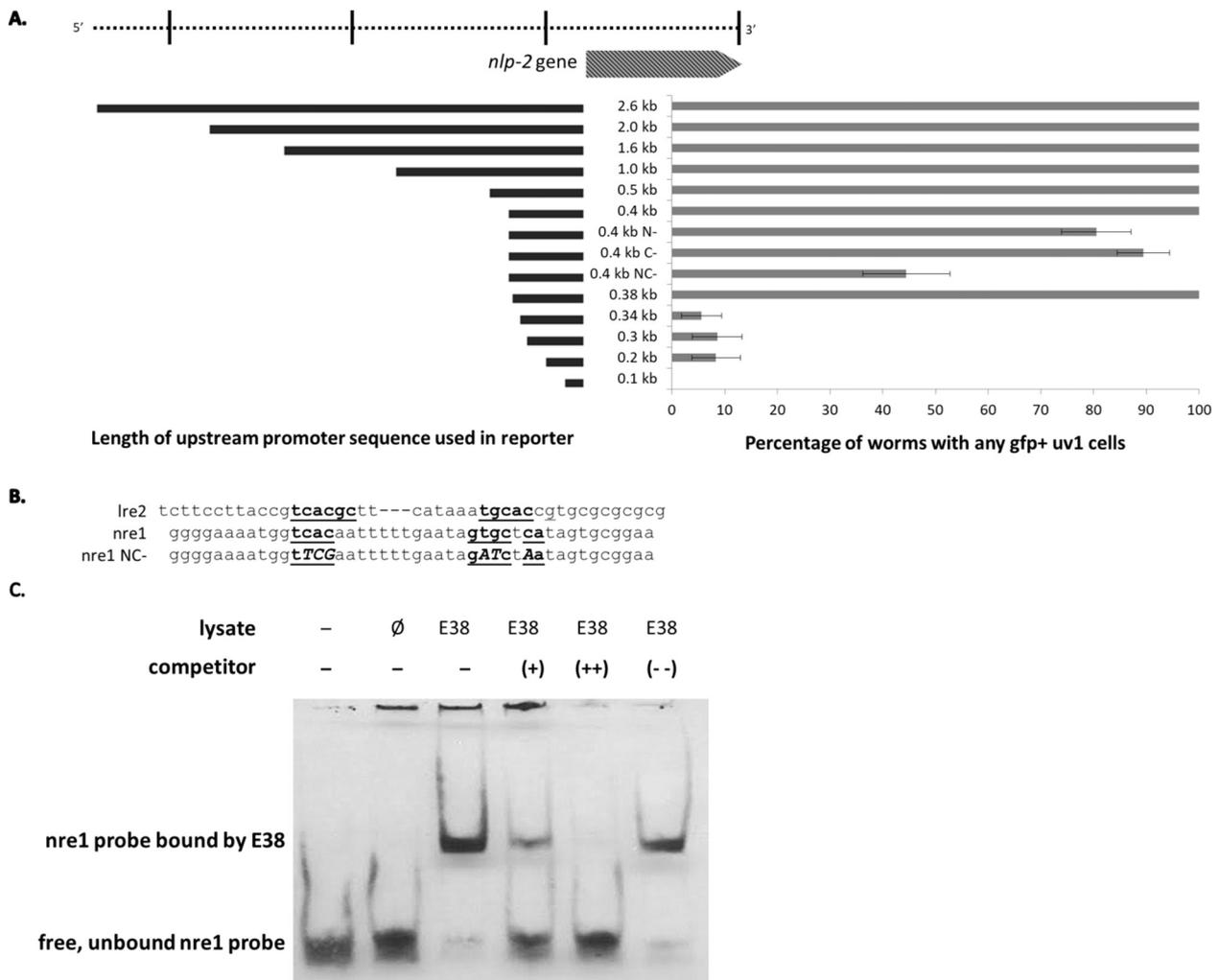


Fig. 6. *nlp-2* is a direct target for EGL-38. (A) Percent of worms with expression of *Pnlp-2::gfp* or its deletion derivatives. A full-length reporter (shown at top) was constructed and then deletions taken from the reporter to isolate a uv1 cell enhancer region. On the left is shown the length of upstream promoter sequence which was included in the reporters. Worms were screened in mid-late L4 for uv1 expression of the deletion reporter. Deletion of between 0.34 and 0.38 kb upstream from the start codon significantly reduces reporter expression. We define this 40 bp region as the uv1 cell enhancer for *nlp-2*. Point mutations in this enhancer made to the sequences hypothesized to bind the N- and both termini (NC-) of EGL-38 significantly decrease reporter expression. Residual reporter fluorescence in the mutant construct may be due to the degenerate Pax DNA-binding domain consensus sequence (Czerny et al., 1993), or failure to identify the key nucleotides. Data are recovered from > 40 worms per reporter; error bars are shown for the standard error of proportion. For each pairwise comparison of control to mutant the difference is significant at a $p < 0.05$ (comparing 0.4 kb N-, 0.4 kb NC-, 0.34 kb, 0.3 kb, 0.2 kb, and 0.1 kb to wild type). (B) Comparison of the Pax binding enhancer regions from *lin-48* (Ire2; Johnson et al., 2001) and *nlp-2* (nre1; this work). The mutations introduced in the 0.4 kb reporter are indicated under nre1 (NC-). (C) EMSA showing the EGL-38 DNA-binding Paired domain interaction with the nre1 probe containing the *nlp-2* uv1 cell enhancer. In lane 1, no lysate is added. Lane 2 is lysate from bacteria bearing the empty vector. In lane 3, lysate from bacteria expressing the paired domain of EGL-38 (indicated as E38) can bind to the nre1 probe and shift mobility. In lanes 4 and 5, this binding is competed away by increasing amounts of unlabeled Ire2, a sequence known to bind specifically to EGL-38. In lane 6, an unlabeled negative probe that corresponds to *nlp-2* sequences that partially overlap Ire2 is unable to compete away E38 binding to nre1.

the hindgut cells (Fig. 6B) (Johnson et al., 2001). We identified one site with some similarity, which we term *nlp-2* response element 1 (nre1). We introduced mutations into this sequence to alter the potential binding sites for the N-, the C-, or both termini of the EGL-38 DNA-binding domain. Only transgenes with both termini mutations exhibited a strong reduction of *Pnlp-2::gfp* expression, although not to the extent observed when the nre1 enhancer region is deleted (Fig. 6A). We utilized EMSA to ask whether the DNA binding domain of EGL-38 protein can directly bind the genetically required regulatory sequence, nre1, *in vitro*. We found that a bacterially expressed Paired Domain of EGL-38 (EGL-38 PD) caused a mobility shift in labelled nre1 probe migration (Fig. 6C). This shift was specific to lysates expressing the EGL-38 PD, as lysates expressing the empty vector failed to cause a similar shift. Further, the binding of EGL-38 PD to nre1 was competed away in a dose-dependent manner by the known EGL-38 target sequence *Ire2*, but

not by a probe with partially overlapping sequence to nre1 (Johnson et al., 2001). Altogether, these data argue that *nlp-2* is a direct target for EGL-38 in the uv1 cells.

3. Discussion

egl-38 has previously been shown to participate in the development of vulval and uterine cells necessary for egg-laying in *C. elegans* (Chamberlin et al., 1997; Chang et al., 1999; Rajakumar and Chamberlin, 2007). However, past work focused on its functions in the vulF cells. Here, we have explored more in depth the interaction of *egl-38* with the signaling end of the EGF pathway (non-autonomous functions) and the activity of *egl-38* within the responding uv1 cells for execution of cellular programs (autonomous functions). EGL-38::GFP protein is present in both the vulF and uv1 cells at developmental times

when it can be functionally responsible for, and responsive to, the EGF pathway. In fact, EGL-38::GFP expression in uv1 is dependent on the EGF pathway, and *egl-38* is also required for expression of the neuropeptide genes *nlp-2* and *nlp-7*, which have a similar dependence on the EGF pathway as EGL-38. In addition, activated LET-23/EGFR can bypass the requirement for *egl-38* in uv1 cell placement, but not in promoting *Pnlp-2::gfp* and *Pnlp-7::gfp* expression, showing that uv1 cell positioning and gene expression are two unique, separate aspects of uv1 cell fate. Ultimately, EGL-38 activity in uv1 is coordinated to EGL-38 activity in vulF through the EGF signaling pathway. EGL-38 activates *lin-3/egf* in the vulF cells; EGF signaling from vulF directs the expression of EGL-38 in the uv1 cells; EGL-38 in uv1 activates transcription of *nlp-2* and *nlp-7* to mediate cellular functions.

Consistent with an active role for *egl-38* in uv1 cells, our results argue that *nlp-2* is a direct target for EGL-38, as EGL-38 is capable of binding *in vitro* to a uv1 cell specific enhancer of *nlp-2*. We find that *nlp-7* expression likewise exhibits dependence on *egl-38*, although a direct interaction has not been established. *nlp-2* and *nlp-7* likely both act during egg-laying. The uv1 cells are generally inhibitory, releasing neuropeptides to control the periodicity of egg-laying following mechano-sensory deformation (Alkema et al., 2005; Collins et al., 2016; Jose et al., 2007). uv1 cells synapse with the hermaphrodite specific neurons; the lack of a direct junction indicates that uv1 activity must be through processes such as neuropeptide vesicle release (Banerjee et al., 2017). Consistent with this idea, *nlp-7* (with *flp-11*) has been shown genetically to inhibit egg-laying (Banerjee et al., 2017). Thus we find that EGL-38 has uv1 cell targets important for execution of a differentiated fate, in addition to its roles in coordinating development of egg-laying system cells.

Pax proteins orthologous to EGL-38 are necessary components of cellular development and differentiation for numerous systems, such as the kidney (Pax2/8), thyroid (Pax8), and B cells (Pax5) (Dressler et al., 1990; Lang et al., 2007; Macchia et al., 1998; Magliano, Lauro, and Zannini Magliano et al., 2000; Torres et al., 1995). In most organs, such as the kidneys, Pax protein expression is significantly downregulated in the differentiated tissue (Blake and Ziman, 2014; Czerny et al., 1993; Dressler et al., 1990; Lang et al., 2007; Rothenpieler and Dressler, 1993; Terzić et al., 1998). However, in some systems, such as the thyroid, Pax activity is necessary after cell specification for expression of terminally differentiated genes required for cellular function. Pax8 is necessary for establishment of the thyroid follicular cells, as well as for expression of thyroid genes including thyroglobulin and thyroperoxidase (Tg and TPO) (Magliano, Lauro, and Zannini Magliano et al., 2000; Mansouri et al., 1998; Miccadei et al., 2002). Interestingly, a study found that in thyroid-derived cell lines, re-introduction of Pax8 resulted in expression of endogenous targets without fully rescuing the differentiated follicular cell phenotype, indicating that while Pax8 is necessary for both processes, only expression of terminally differentiated targets directly involves Pax8 (Fabbro et al., 1994; Magliano et al., 2000). Similarly, we have shown in *C. elegans* that EGL-38 is present both in developing cells of the vulva and in the differentiating uv1 cells, but that EGL-38 also persists in uv1 cells into adulthood. EGL-38 protein becomes abruptly undetectable in the vulF and vulE cells at a time point following expression of the *lin-3/egf* signal in early/mid-L4 larval stage. In the uv1 cells, expression tapers off more gradually as the worms are in the adult stage, presumably accommodating the EGL-38 role in activating *nlp-2* and *nlp-7*, which are involved in the adult egg-laying function. Both the thyroid and the uv1 cells are neurosecretory organs with important physiological signaling functions in the body (Collins et al., 2016; Jose et al., 2007). Thus a common theme is that Pax2/5/8 activity participates in both the differentiation and function of these neurosecretory organs.

An interesting facet of Pax2/5/8 gene function is that their role in cellular differentiation, combined with temporal and spatial expression patterns, indicates that participating in inductive cell-to-cell signaling may be a common theme for proteins in this group (Dressler et al.,

1990; Püschel et al., 1992). For example, in HeLa, CaSki, and renal proximal tubule cell cultures, PAX2 expression was increased by exposure to EGF (de Graaff et al., 2012; Liu et al., 1997). Similarly, in both eutopic and ectopic tissue of endometriosis patients, the expression of PAX2 and EGFR was tightly correlated, indicating that PAX2 expression may be dependent on EGFR activity (de Graaff et al., 2012). Aberrant expression of PAX proteins in cancer tissue is concomitant with high levels of EGF and hallmarks of increased EGF activity, such as the maintenance of tumor growth, proliferation, and survivability (Brand et al., 2011; Herbst, 2004; Konecny et al., 2009; Muratovska et al., 2003; Wang et al., 2018; Wang et al., 2008). Knockdown of PAX2 in endometrial cancer lines significantly decreases cancer cell viability (Jia et al., 2016; Zhang et al., 2011). The relationship of these Pax2/5/8 proteins with inductive signaling pathways has not yet been examined during mammalian development, given the lethal or significant loss-of-function phenotypes that result from loss of either the Pax2/5/8 gene or of signaling activity. However, our study has observed parallels between Pax2/5/8 and the EGF pathway that were highlighted by these adult disease state and cell culture experiments. Therefore, coordination of signaling and responding within a developing organ may be a common developmental function for Pax transcription factors.

4. Experimental procedures

4.1. Genetic strains and worm culture

Strains were cultured under standard conditions for *C. elegans* (Brenner, 1974; Stiernagle, 2006). All experiments were performed at 20 °C unless otherwise noted. The reference wild type *C. elegans* strain is N2; additional *C. elegans* strains used were:

CM2762 *egl-38(gu253[egl-38::gfp])*
 CM2238 *unc-119(e2498); guEx1372 (Pnlp-2(0.4kb)::gfp)*
 CM2694 *unc-119(e2498); guEx1554 (Pnlp-7:gfp)*
 NY2040 *unc-119(e2498); ynl540(Pflp-11:gfp)*
 CM2763 *let-23(sy1); egl-38(gu253[egl-38::gfp])*
 CM2766 *let-23(sy1); unc-119(e2498); guEx1372 (Pnlp-2(0.4kb)::gfp)*
 CM2767 *let-23(sy1); unc-119(e2498); guEx1554 (Pnlp-7:gfp)*
 CM2760 *unc-119(e2498); egl-38(sy294); guEx1372 (Pnlp-2(0.4kb)::gfp)*
 CM2768 *unc-119(e2498); egl-38(n578); guEx1372 (Pnlp-2(0.4kb)::gfp)*
 CM2427 *unc-119(e2498); let-23(sa62); guEx1372 (Pnlp-2(0.4kb)::gfp)*
 CM2444 *let-23(sa62); unc-119(e2498); egl-38(sy294); guEx1372 (Pnlp-2(0.4kb)::gfp)*
 CM2769 *egl-38(sy294); unc-119(e2498); guEx1554 (Pnlp-7:gfp)*
 CM2770 *egl-38(n578); unc-119(e2498); guEx1554 (Pnlp-7:gfp)*
 CM2740 *let-23(sa62); unc-119(e2498); guEx1554 (Pnlp-7:gfp)*
 CM2739 *let-23(sa62); unc-119(e2498); egl-38(n578); guEx1554 (Pnlp-7:gfp)*

4.2. Generating tagged EGL-38:GFP

We tagged the endogenous *egl-38* locus with *gfp* sequences at the 3' end using methods of (Dickinson and Goldstein, 2016). Briefly, we used synthesized sgRNA and purified Cas9 (Prior et al., 2017) combined with a repair template that introduces *gfp* sequences and selection markers removed via self-excising cassette (Dickinson and Goldstein, 2016). A 20-bp guide RNA targeting the desired region of genomic *egl-38* was chosen using the MIT CRISPR design tool (Zhang lab, MIT, <http://crispr.mit.edu>). The guide RNA was ordered and harnessed to the Synthego EZ Scaffold, providing a 100-mer guide RNA (Synthego, Inc.). PCR-generated homology arms, with a 3-point mutated PAM sequence (primers are in Supplemental File 1) were assembled into the GFP^{SEC}3xFlag vector pDD282 (NEBuilder HiFi DNA Assembly Cloning Kit E5520S) and confirmed by PCR and sequencing (Dickinson

et al., 2013). Injection mixes contained 50 ng/μL repair template, 20 ng/μL *myo-2::mCherry* (pCFJ90), 300 mM KCl, and 20 mM HEPES, mixed with 5 μM guide RNA and 5 μM Cas9 enzyme (Frøkjær-Jensen et al., 2008; Prior et al., 2017). This mix was injected into the gonads of N2 adult worms; three days later, 250 μg/mL hygromycin was added to each plate to select for transformed F1 offspring. On days six-eight surviving Roller, non-*myo-2::mCherry* expressing F1 adults were singled out and allowed to self-cross. Multiple offspring of Rollers were selected and allowed to self-cross, and plates with a homozygous parent were identified. These animals were heat-shocked to remove the self-excising cassette, and screened for wild-type movement and GFP expression. Correct insertion of the GFP was confirmed by PCR and sequencing with flanking genomic primers. The tagged strain is viable, fertile, and appears wild type.

4.3. Generating *nlp-2* and *nlp-7* reporters and deletion transgenes

Reporter strains for *nlp-2* and *nlp-7* were constructed using pDD95.69 from the Fire Vector kit (Addgene.org). For *nlp-2*, 2.6 kb of upstream genomic promoter sequence was amplified using a 5' *SphI*-tagged primer and a 3' *Sall*-tagged primer; the 2.6 kb amplicon includes only the start codon from the coding sequence. Similarly, for *nlp-7*, 2.7 kb of upstream promoter (including the start codon) was amplified using a 5' *Sal*-tagged primer and a 3' *MscI*-tagged primer. The amplicons of both genes were ligated into the promoter-less pPD95.69 plasmid and transformed into DH5α bacteria. *nlp-2* deletion clones were generated by designing upstream primers tagged with *SphI* that were more proximal to the translational start site, and paired with the same 3' primer. Site-directed mutagenesis of the *nlp-2* promoter sequence in pRJ124 (0.4 kb) were performed using standard reagents, according to the Stratagene protocol (Stratagene Quikchange II Site-Directed Mutagenesis Kit). Positive clones were verified by restriction enzyme digest and sequencing. Injection mixes containing 75 ng/μL of confirmed plasmid and 15 ng/μL of the rescue plasmid *unc-119(pTJ1043)* were injected into RH10 (*unc-119(e2498)*) adult hermaphrodites. Non-Unc F1 offspring were selected and allowed to self to identify transgenic lines. At least three lines were obtained and screened for fluorescence. The reported data represent an individual line with the most consistent expression pattern.

4.4. Microscopy

Morphological and fluorescent phenotypes were evaluated using a Zeiss Axioplan microscope, under 100× magnification. Hermaphrodite animals were observed at mid-L3 larval stage through adult, depending on the experiment. Larval stage was defined on the basis of the vulval morphology in the mid-plane. Comparably-staged adult animals were evaluated by selecting L4 animals to fresh plates, and evaluating them 24 h later. Images were taken at auto-calculated DIC and epi-fluorescent exposures, varying from 0.5 to 0.8 ms and 1.2–1.9 ms respectively. Worms were immobilized on agar pads consisting of 3.5% noble agar in water with a 10 mM sodium azide solution. Screening for vulval cell and uv1 phenotypes involved locating the vulval or uv1 cells in the uppermost plane of the worm; expression in uv1 cells on the side where the worm was laying was not evaluated due to variability in the data due to photo-bleaching of the GFP.

4.5. U0126 experiments

U0126 was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mM. U0126 and DMSO control plates were made by adding 15 μM of U0126 or DMSO to the top of 5 mL NGM in 35 mm petri plates, drying overnight, and spotting with OP50 bacteria. Gravid worms were floated from plates using M9, dissolved with a bleach solution, and the remaining eggs were rinsed three times with M9. The eggs were hatched overnight (~18 h) with rotation. Synchronized L1

larvae were plated onto standard NGM plates and placed at 20 °C. Thirty-two hours later, the L3 larvae were removed and plated to the U0126 or DMSO plates, and returned to 20 °C. Screening was performed 8 h later, when larvae are mid-L4.

4.6. Protein expression and EMSA

The EGL-38 Paired DNA-binding domain was expressed using a pET23a vector in *E. coli* BL21 cells (strain MC702) (G. Zhang et al., 2005). Expression and lysis were performed as previously described, with empty-vector cultures (strain MC706) as a negative control (Fitzsimmons et al., 2001). In summary, 400 μL of bacterial culture were added to 40 mL LB with 50 μg/mL Carbenicillin. This was grown at 37 °C to an OD600 of ~4, before IPTG was added (final concentration 1 mM) and cultures shook at room temperature overnight. Cells were pelleted and re-suspended in 1 mL cold buffer Z (25 mM HEPES, pH 7.0; 100 mM KCl; 12.5 mM MgCl₂; 20% glycerol; 0.1% NP-40; 1 mM DTT). Cells were sonicated, lysed, and the supernatants stored on ice for use in EMSA. EMSA was carried out as described (Roche DIG Gel Shift Kit, 2nd Generation, #3353591910). 50 mM MgCl₂ was included in the binding (Johnson et al., 2001). Protein lysates were diluted 1:50 in Buffer Z, and incubated with 2 ng labelled probes, and 50×/250× un-labelled competitors. Samples were separated on a 6% non-denaturing gel, with 0.5× TBE buffer, transferred to nylon membrane in 0.5× TBE, and performed detection following the DIG Gel Shift protocol.

4.7. Key resources table

Reagent or resource	Source	Identifier
Antibodies		
None		
Bacterial and virus strains		
OP50 <i>E. coli</i>	Caenorhabditis Genetics Center	Wormbase ID: OP50
Biological samples		
None		
Chemicals, peptides, and recombinant proteins		
None		
Critical commercial assays		
None		
Deposited data		
None		
Experimental models: cell lines		
None		
Experimental models: organisms/strains		
CM2762 <i>egl-38(gu253[egl-38::gfp])</i>	This paper	N/A
CM2238 <i>unc-119(e2498); guEx1372 (Pnlp-2(0.4 kb)::gfp)</i>	This paper	N/A
CM2694 <i>unc-119(e2498); guEx1554 (Pnlp-7::gfp)</i>	This paper	N/A
NY2040 <i>unc-119(e2498); ynIs40(Pflp-11::gfp)</i>	Caenorhabditis Genetics Center	Wormbase ID: NY2040
CM2763 <i>let-23(sy1); egl-38(gu253[egl-38::gfp])</i>	This paper	N/A
CM2766 <i>let-23(sy1); unc-119(e2498); guEx1372 (Pnlp-2(0.4 kb)::gfp)</i>	This paper	N/A
CM2767 <i>let-23(sy1); unc-119(e2498); guEx1554 (Pnlp-7::gfp)</i>	This paper	N/A
CM2760 <i>egl-38(sy294); unc-119(e2498); guEx1372 (Pnlp-2(0.4 kb)::gfp)</i>	This paper	N/A
CM2768 <i>egl-38(n578); unc-119(e2498); guEx1372 (Pnlp-2(0.4 kb)::gfp)</i>	This paper	N/A
CM2427 <i>let-23(sa62); unc-119(e2498); guEx1372 (Pnlp-2(0.4 kb)::gfp)</i>	This paper	N/A
CM2444 <i>let-23(sa62); unc-119(e2498); egl-38(sy294); guEx1372 (Pnlp-2(0.4 kb)::gfp)</i>	This paper	N/A
CM2769 <i>egl-38(sy294); unc-119(e2498); guEx1554 (Pnlp-7::gfp)</i>	This paper	N/A
CM2770 <i>egl-38(n578); unc-119(e2498); guEx1554 (Pnlp-7::gfp)</i>	This paper	N/A
CM2740 <i>let-23(sa62); unc-119(e2498); guEx1554 (Pnlp-7::gfp)</i>	This paper	N/A

CM2739 let-23(sa62); unc-119(e2498); egl-38(n578); guEx1554 (Pnlp-7:gfp)	This paper	N/A
Oligonucleotides		
Primers are listed in Supplemental Table 1	This paper	N/A
Recombinant DNA		
pDD282	Dickinson et al., 2013	Addgene.org/66823/
pDD95.69	Dr A. Fire.	Addgene.org/1654
pRJ124	This paper	N/A
Software and algorithms		
None		
Other		
None		

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mod.2019.103566>.

Acknowledgements

We thank Adriana Dawes, David Ignacio, Maxine Ignacio, and Kristen Navarro for critical reading of the manuscript. We also thank Ahmed Abokor, Abdulrahman Jama, and Benjamin Kaumeyer for assistance in creating plasmids. Some *C. elegans* strains were supplied by the Caenorhabditis Genetics Center, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). Some plasmids were provided by Addgene.org.

Funding

This work was supported by the National Science Foundation (DMS-1361251). Allison Webb Chasser was a Cellular, Molecular and Biochemical Sciences Program Graduate Fellow (5T32GM086252) and Pelotonia Cancer Research Graduate Fellow.

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