Modulation of EGF receptor-mediated vulva development by the heterotrimeric G-protein Gαq and excitable cells in C. elegans

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Summary

The extent to which excitable cells and behavior modulate animal development has not been examined in detail. Here, we demonstrate the existence of a novel pathway for promoting vulval fates in C. elegans that involves activation of the heterotrimeric Gαq protein, EGL-30. EGL-30 acts with muscle-expressed EGL-19 L-type voltage-gated calcium channels to promote vulva development, and acts downstream or parallel to LET-60 (RAS). This pathway is not essential for vulval induction on standard Petri plates, but can be stimulated by expression of activated EGL-30 in neurons, or by an EGL-30-dependent change in behavior that occurs in a liquid environment. Our results indicate that excitable cells and animal behavior can provide modulatory inputs into the effects of growth factor signaling on cell fates, and suggest that communication between these cell populations is important for normal development to occur under certain environmental conditions.

Key words: EGF, Muscle, Neurons, Behavior, Vulva, G protein

Introduction

C. elegans vulva development is used as a model system to study the regulation of cell fate by growth factors. Normally, only three of six vulval precursor cells (VPCs) (P3.p-P8.p) adopt vulval cell fates (reviewed by Greenwald, 1997; Moghal and Sternberg, 2003b). At the end of the second larval stage of development, LIN-3 (Hill and Sternberg, 1992), an EGF-like growth factor produced by the gonadal anchor cell induces P6.p to adopt a primary vulval fate. LET-23 (Aroian et al., 1990), an EGF receptor-like tyrosine kinase, and its downstream effectors, which include LET-60 (RAS) (Beitel et al., 1990; Han and Sternberg, 1990) and MPK-1 (MAP kinase) (Lackner et al., 1994; Wu and Han, 1994), transduce the LIN-3 signal. Following LET-23 activation in P6.p, LIN-12 NOTCH-like receptors (Yochem et al., 1988) and LET-23 are stimulated on the adjacent P5.p and P7.p cells, which induces secondary vulval fates in these cells (Katz et al., 1995; Simske and Kim, 1995). A WNT signaling pathway acts parallel to the RAS pathway to promote vulval fates, but unlike the RAS pathway, WNT signaling is not absolutely essential for vulval fate specification. A null mutation in the β-catenin gene, bar-1, causes a partially penetrant vulvaless phenotype (Eisenmann et al., 1998), in which fewer than three VPCs adopt vulval fates, and a loss-of-function mutation in the axin-like gene pry-1 (Korswagen et al., 2002; Maloof et al., 1999) causes some animals to have a multivulva phenotype because of more than three VPCs adopting vulval fates (Gleason et al., 2002).

Several genes have been identified whose mutation does not affect vulval induction under standard laboratory growth conditions, but affects vulva development in sensitized backgrounds. These include ksr-1 (Kornfeld et al., 1995; Sundaram and Han, 1995), ksr-2 (Ohmachi et al., 2002), sur-8 (Sieburth et al., 1998), sur-6 (Sieburth et al., 1999), ptp-2 (Gutch et al., 1998), unc-101 (Lee et al., 1994), sli-1 (Jongeward et al., 1995; Yoon et al., 1995), gap-1 (Hajnal et al., 1997), ark-1 (Hopper et al., 2000), lip-1 (Berset et al., 2001), dpv-22/sop-1 (Moghal and Sternberg, 2003a), eor-1 and eor-2 (Howard and Sundaram, 2002), and the redundant class A and class B genes in the synthetic multivulva pathway (e.g. Ferguson and Horvitz, 1989). It is conceivable that the general absence of mutant phenotypes for these genes reflects roles under natural ecologic conditions that are not recapitulated in the laboratory. Therefore it is unclear whether vulval cell fate specification is modulated by additional pathways in the wild, and in what context this modulation might occur.

The first report of a large-scale genetic screen to identify mutations affecting vulva development provided evidence that the environment and an animal’s physiology can modulate the ability of growth factors to induce vulval fates (Ferguson and Horvitz, 1985). The severity of the vulvaless phenotypes of certain let-23, lin-2, lin-7, lin-3, lin-24 and lin-33 alleles is reduced by starvation and exit from dauer. The dauer is an arrested, alternative third stage of larval development that occurs under conditions of high population density and reduced food supply (reviewed by Riddle and Albert, 1997). Both entry and exit from dauer are controlled by chemosensory cues involving neurons (Bargmann and Horvitz, 1991; Shakir et al., 1993; Tabish et al., 1995), suggesting that excitable cells have
the capacity to modulate vulval cell fate. Because the contribution of excitable cell activity to growth factor-dependent regulation of cell fate has not been studied in detail, we sought to analyze this in *C. elegans*.

We find that activation of the heterotrimeric Gqα protein EGL-30, normally associated with regulation of animal behavior, promotes vulval cell fates. Post-embryonic muscles, and in particular, muscle-expression of the EGL-19 αL-type voltage-gated-calcium channel subunit are required, suggesting that muscle excitation can promote development of vulval tissue. This pathway is sensitive to functional levels of BAR-1 (β-catenin), and can be stimulated by activation of EGL-30 in neurons, or by the EGL-30-dependent change in behavior that occurs when worms are grown in a liquid environment. On plates, ablation of the post-embryonic muscles, or egl-19 and egl-30 loss-of-function mutations, do not affect vulval induction, suggesting that this pathway might exist to modulate development in response to certain environmental conditions.

### Materials and methods

#### Strains and genetics

*C. elegans* were cultured at 20°C using standard protocols (Brenner, 1974). Alleles used were: egl-30[tg26gf] (Doi and Iwasaki, 2002), egl-30[dad805] (Brandenburg et al., 1996), unc-13[e51] (Brenner, 1974), goa-l[n363] (Segalat et al., 1995), phn-2[ad538] (Avery, 1993), unc-54[e190] (Epstein et al., 1974) on LGI; let-23[syl] (Aroian and Sternberg, 1991), let-23[ad62gf] (Katz et al., 1996), unc-4[e120] (White et al., 1992) on LGII; dpy-17[e164] (Brenner, 1974), sur-l[kau] (Wu and Han, 1994), unc-64[e246] (Brenner, 1974), pha-l[e1236t] (Granato et al., 1994) on LGIII; unc-24[e138] (Riddle, 1978), lin-3[n378] (Ferguson and Horvitz, 1985), lin-3[e1417] (Ferguson and Horvitz, 1985); egl-19[n582] (Trent et al., 1983), egl-19[n2368gf] (Lee et al., 1997), mec-3[e1338] (Way and Chalfie, 1989), let-60[n1046gf] (Beitel et al., 1990; Ferguson and Horvitz, 1985), let-60-sy95dn (Han et al., 1990; Han and Sternberg, 1991), dpy-20[e1362], dpy-20[e1282] (Hosono et al., 1982) on LGIV; him-5[e1490] (Hodgkin et al., 1979) on LGV; syIs1 (Katz et al., 1995), bar-1[ma63] (Maloof et al., 1999), bar-1[go80] (Eisenmann et al., 1998), and lin-15[e1763] (Ferguson and Horvitz, 1985) on LGX. Genetic balancers used were: mntC1[dpy-10[e128] unc-52[e444]] (Herman, 1978; Sigurdson et al., 1984) on LGIV; nT1[let[m435]] on LGIV and LGV (Rogalski and Riddle, 1988).

let-23[sa62gf]/+; syIs1/+ worms were obtained by first crossing N2 males into hemizygous for the X chromosome were used to transfer syIs1 into homozygous let-23[sa62gf] unc-4[e120] hermaphrodites. Non-Unc F1 hermaphroditic cross progeny were scored for vulval induction during the L4 stage. Let-23[sa62gf]/+; let-60[sy95dn]/+; syIs1/+ animals were obtained by crossing syIs1-bearing males into let-23[sa62gf] unc-4[e120]; unc-24[e138] let-60[sy95dn]/ntT[let[m435]] hermaphrodites, and picking non-Unc cross progeny. Because the F1 cross progeny consisted of both let-60[sy95dn]/+ and ntT[let[m435]]/+ genotypes, we scored vulval induction in all cross progeny, recovered the individual worms and dead larvae in the F2 generation.

### Molecular biology

The plasmid pR30, used to overexpress wild-type egl-30, was constructed as follows. Full-length egl-30 genomic DNA was amplified by PCR from wild-type worms using the upstream primer 5′-ATGGCCCTGCTTATATCCGAAGAG-3′ and the downstream primer 5′-TTACACCAAGTTGACTCCTCTCAATATGCTGTA- GAAT-3′. The PCR product was blunt-end ligated into the BamHI site of the unc-119 promoter plasmid pBY103 (a gift from M. Maduro), making pR30 (unc-119::egl-30). To determine how the egl-30 introns contributed to the expression pattern of pR30, pR39 was constructed from pR30, and contained the gfp open reading frame cloned in-frame to egl-30. The same upstream egl-30 primer was used with the downstream primer 5′-CACCAAGTTGACTCCTCTCAATATGCTGTA- GAATTG-3′ that does not contain a stop codon, to amplify full-length egl-30 genomic DNA by PCR. This egl-30 coding fragment was fused in-frame to gfp by blunt-end ligating the PCR fragment into the BamHI site of the promoterless GFP plasmid pPD95.75 (a gift of A. Fire), creating the construct pR36. A 5 kbp XbaI-ApaI fragment from pR36 was swapped between the Nhel-Apal sites of pR30 to create the unc-119::egl-30::gfp plasmid pR39. The myo-3::egl-19 and myo-3::egl-19::gfp constructs were generated by injection of ligation reactions directly into worms and have been described previously (Garcia et al., 2001). The aex-3::egl-30[tg26gf] plasmid, pTG100.1, which places the egl-30 cDNA containing the tg26 mutation under the control of the aex-3 promoter, has been described previously (Doi and Iwasaki, 2002). This egl-30 cDNA contains the last intron of egl-30 to aid with expression from this vector. To determine how the last intron of egl-30 affects expression from the aex-3 promoter, pAEXYFP was constructed. This plasmid contains the yfp coding region and unc-54 3′UTR inserted downstream of the aex-3 promoter and upstream of the last 58 bp of exon 7 to the end of exon 8 (including the last intron) of egl-30. The yfp coding region and unc-54 3′UTR were amplified by PCR from pSX95.77 (courtesy of S. Xu) with the primers 5′-GCCCGGGG-ATCCAAATGATGAAGAGGAGAAGACTTTTAC-3′ and 5′-ACGTGCGCGAACAAGACATTTGATGTATAGGGAAT-3′. pSX95.77 contains the yfp coding region and unc-54 3′UTR from pPD13.64 (a gift of A. Fire) inserted into pBR322. The yfp::unc-54 PCR product was digested with BamHI and Sali, and ligated into BamHI/SalI-digested pTG100.1. pLN31EGL30 was constructed by amplifying the egl-30[tg26gf] cDNA from pTG100.1 with the primers 5′-ATAGAAATGGGCCCCAAATGCGGCTGTGGTTTTACCC-3′ and 5′-CCCTTGAACCGGCGCTTACCACAAATGCTGACTCTCAG-3′, and cloning the NotI-digested PCR product into the NotI site of the lin-31 expression vector, pBS255 (Tan et al., 1998). The unc-18::egl-30[tg26gf] plasmid, pUNC18tg26, was created as follows. First, the egl-30 cDNA containing the egl-30[3′tg26gf] mutation and the last intron was released from pTG100.1 by digestion with ApaI and BsrGI. The egl-30 fragment was made blunt, and subsequently cloned into the Smal site of pBSKS to generate pBSig26. The unc-18 promoter (extending from the unc-18 start ATG to the next 5′ gene F2TD9.8) was amplified by PCR with the primers UNC18-1 5′-AGCCCAAGTCTTTGAAGGACAA-TGAACTAGAGG GAC-3′ and UNC18-4 5′-AGCCAAACGTCTTCCCTATTTCACAAATGCTGACACTCAG-3′, digested with HindIII, and cloned into HindIII-digested pBStg26 to yield pUNC18tg26. The 5′-ATCGCCCTGCTTATATCCGAAGAG-3′ and 5′-TCCCGGGGAGATTTCAGCTGATGGG-3′ were used to PCR amplify the egl-30[tg26gf] cDNA and its 3′ UTR sequences from pTG100.1. The ~2.3 kbp PCR
fragment was then blunt-end ligated between the NsiI and SmaI sequences in the unc-4 expression vector. The cloning fuses the first egl-30(tg26gf) ATG 18 basepairs downstream of the unc-4 initiation codon.

**Microinjections and transgenic experiments**

PCR fragments containing native egl-30 upstream sequences and coding region were too unstable to be maintained in worms when injected into the gonadal syncytium at concentrations even as low as 5 pg µl⁻¹. Transgenic worms were extremely hyperactive, slow growing and had low fertility, making transmittance of the extrachromosomal arrays not efficient for strain maintenance. To circumvent this problem, we fused the egl-30 genomic coding region to the unc-119 promoter to make the pR30 hybrid construct that led to more stable expression of egl-30. pR39, which has gfp cloned in-frame to egl-30 in pR30, was used to determine the expression pattern of the pR30 hybrid construct. Injection of pR39 at a concentration of 50 ng µl⁻¹ into worms resulted in expression of GFP in the nervous system, pharyngeal muscles, sex muscles, anal depressor muscles and epidermis (data not shown). Although the unc-119 promoter drives expression mainly in neurons, sequences in the egl-30 genomic DNA (specifically from the first intron) contribute to broad expression of the transgene. Consistent with this expression pattern, injection of pR30 at 50 pg µl⁻¹ rescued every behavioral phenotype caused by the loss-of-function mutation egl-30(ad805) [data not shown, rescuing array: syEx474 [myo-2::gfp (10 ng µl⁻¹); unc-119::egl-30 (50 pg µl⁻¹)]]. The extrachromosomal array that overexpresses wild-type egl-30, syEx532, was obtained by co-injecting pR30 (750 pg µl⁻¹) and pTG96 (sur-5::gfp) (Gu et al., 1998) (10 ng µl⁻¹) into egl-30(ad805); let-23(sy1) hermaphrodites. From the GFP-positive transgenic animals, the most hyperactive transmitting line was kept and scored for vulval development.

Experiments with the egl-30 cDNA driven from the unc-119 promoter indicated that functional rescue of the egl-30(ad805) phenotypes could be obtained in the F1 generation, but could not be segregated into the F2 generation without the first intron of egl-30. Microinjection mixtures containing digested N2 genomic DNA as a source of carrier DNA were also used in an attempt to create complex arrays; however, this did not alleviate the generational silencing problem. These data suggested to us that the first intron of egl-30 prevents generational silencing of the transgene, and that high levels of EGL-30 activity in certain cell populations are toxic. Because of this problem, we were not able to achieve stable expression of the wild-type or tg26 mutation-containing egl-30 cDNA from certain heterologous promoters. To analyze the consequences of driving egl-30(tg26gf) cDNA expression in neurons, we coinjected either pTG1001 [unc-18::egl-30(tg26gf)] (50 ng µl⁻¹), or pUNC18tg26 [unc-18::egl-30(tg26gf)] (50 ng µl⁻¹), or pR1 [unc-4::egl-30(tg26gf)] (50 ng µl⁻¹) with pPD118.33 (myo-2::gfp) (10 ng µl⁻¹) and pBSSK (120 ng µl⁻¹) into lin-3(n378) animals. Transgenic F1s were identified by expression of myo-2::gfp in the pharynx. The extrachromosomal arrays containing lin-31::egl-30(tg26gf) were generated by injecting plN131EG30 [lin-31::egl-30(tg26gf)] (50 ng µl⁻¹) with pBSSK (140 ng µl⁻¹) and pPD118.33 (myo-2::gfp) (10 ng µl⁻¹) into lin-3(n378) animals. The extrachromosomal arrays syEx570 and syEx594 were generated by injecting pAEXYFP (aex-3::yfp::egl-30) (200 ng µl⁻¹) with pBX-1 (pha-1) (Granato et al., 1994) (100 ng µl⁻¹) or pUNC18YFP (unc-18::yfp::egl-30) (50 ng µl⁻¹) with pBX-1 (pha-1) (100 ng µl⁻¹) and pBSSK (30 ng µl⁻¹) into pha-1(e2123ts) animals, respectively.

**Vulval induction assay and M cell ablations**

Vulval induction was scored during the L4 stage under Nomarski optics (Sternberg and Horvitz, 1986). The number of vulval nuclei is used to extrapolate how many of the Pn,p cells were induced to adopt vulval fates. A VPC that gives rise to seven or eight great granddaughters and no hyp7 tissue is scored as 1.0 cell induction. A VPC in which one daughter fuses with hyp7, and the other daughter divides to generate three or four great granddaughter cells is scored as 0.5 cell induction. In wild-type animals, P5.p, P6.p and P7.p each undergo 1.0 cell induction, whereas the other Pn,p cells do not adopt vulval fates, resulting in a total of 3.0 cell induction. Animals displaying more than 3.0 cell induction are multivulva and animals with less than 3.0 cell induction are vulvaless. Laser ablations were conducted using a standard protocol (Bargmann and Avery, 1995). M cell ablations were done at the L1 stage and were confirmed by the loss of M-derived coelomocytes (Sulston and Horvitz, 1977).

**Results**

**Activation of EGL-30 (Gαq) promotes vulval cell fates**

EGL-30 is the ortholog of mammalian Gαq/GGα11, and is the only C. elegans member of the Gαq class of heterotrimeric Gα proteins (Brundage et al., 1996; Jansen et al., 1999). During the course of behavioral studies with the egl-30(tg26) allele (Doi and Iwasaki, 2002), we noticed that rare hermaphrodites (0.5%, n=200) developed ectopic vulval tissue. The egl-30(tg26) allele causes a glutamine for arginine substitution (R243Q) in the α3 helix region of Gαq, a region implicated in GTP hydrolysis and effector binding (Itoh and Gilman, 1991; Noel et al., 1993; Sprang, 1997). In contrast to egl-30 loss-of-function alleles that decrease motility and egg-laying (Brundage et al., 1996), the tg26 allele induces semi-dominant hyperactive locomotion and egg-laying behaviors (data not shown), suggesting that the mutation confers gain-of-function properties to the protein. The rare occurrence of ectopic vulval tissue in egl-30(tg26gf) hermaphrodites suggests that activated EGL-30 might interact with the LET-23 pathway during vulval induction. To study interactions with the LET-23 pathway, we used the same strategy used to study other modulators of LET-23 signaling, which also do not cause penetrant phenotypes when mutated in isolation. We examined the effects of activation of EGL-30 in sensitized backgrounds. We made double mutant combinations of gain-of-function egl-30(tg26gf) with loss-of-function lin-3(n378), let-23(sy1), and dominant-negative let-60(sy95dn). egl-30(tg26gf) reduces the severity of the vulvalless phenotypes caused by all three mutant alleles (Table 1; see Table 4).

Because egl-30(tg26gf) causes several behavioral phenotypes, we tested whether modulation of vulval induction was caused by an indirect physiological consequence of the activated allele. egl-30(tg26gf) worms have hyperactive muscle behaviors and feeding problems. Thus, we tested other mutations that cause similar phenotypes for suppression of the let-23(sy1) vulvalless phenotype. phm-2(ad538b) has a deletion removing the 3’ end of dys-l (dystrophin-like), and goa-1(n363) has a deletion removing goa-1 (Gtα). Although both alleles cause muscle hyperactivity and feeding defects (Avery,
To determine the site(s) of action for EGL-30, we expressed the egl-30(tg26gf) cDNA under the control of muscle, neuronal and vulval-specific promoters. In general, stable expression of the egl-30 cDNA from heterologous promoters in transgenes was difficult to achieve (see Materials and Methods). When egl-30(tg26gf) expression was driven by the myo-3 muscle-specific promoter in lin-3(n378) animals (Okkema et al., 1993), high transgene doses failed to yield viable F1 transformants, and at low doses, F1s carrying the transgene did not display any behavioral phenotypes, and were not rescued for the vulvalless phenotype (data not shown).

To drive activated EGL-30 expression in neurons, we first used the aex-3 (Iwasaki et al., 1997) and unc-18 (Gengyo-Ando et al., 1993) promoters, which drive expression in multiple neurons, including head, tail and ventral cord motor neurons. Because the egl-30 cDNA we used contained the last intron of egl-30 to promote splicing, we verified that this intron did not cause ectopic expression in muscle, the Pn.p cells or the anchor cell. When placed downstream of the yfp coding region, the last intron of egl-30 did not alter the activity of the aex-3 or unc-18 promoters. In both cases, YFP accumulated in the ventral cord and head and tail neurons, but not in muscle, the anchor cell or the Pn.p cells (Fig. 1A-F and Fig. 2). We co-injected the aex-3::egl-30(tg26gf) or unc-18::egl-30(tg26gf) plasmids with pPD118.25, which drives GFP expression in the pharynx, into lin-3(n378) vulvalless animals. We observed two types of GFP-positive transgenic F1 animals: those that displayed a hyperactive phenotype similar to that of egl-30(tg26gf) mutants, and those that looked behaviorally wild-type. None of the F1s displaying a strong behavioral phenotype transmitted this phenotype to subsequent generations, suggesting that transgenic expression of egl-30 was being lost, and that high levels of Egl-30 activity can be toxic (see Materials and Methods). Therefore, we only examined F1 animals for rescue of vulval induction defects. We found that aex-3 or unc-18-driven Egl-30 (R243Q) could only rescue the vulvalless phenotype of lin-3(n378) when expressed in cells at levels sufficient to confer a hyperactive phenotype (Table 2). Animals that were GFP-positive, but did not show a behavioral phenotype, did not have more vulval induction than uninjected worms (Table 2).

This tight correlation between the effects of the aex-3::egl-30(tg26gf) and unc-18::egl-30(tg26gf) transgenes suggests that transgenic expression of egl-30 is cell autonomous. However, the possibility that the phenotype is caused by an interaction between the transgenes and endogenous egl-30 cannot be entirely excluded. To determine whether the transcriptional fusion is acting as an enhancer, we injected animals with plasmids containing a transcriptional fusion of the lin-3 promoter to egl-30(tg26gf) (Table 2). Animals that were GFP-positive, but did not show a behavioral phenotype, did not have more vulval induction than uninjected worms (Table 2).
30(tg26gf) and unc-18::egl-30(tg26gf) transgenes on behavior and rescue of the lin-3(n378) vulvaless defect, suggests that activation of EGL-30 in motor neurons promotes vulval induction. To examine this possibility in more detail, we used the unc-4 promoter (Miller and Niemeyer, 1995) to drive expression of activated EGL-30 in the A-type motor neurons, which include the VAs and DAs in ventral cord, and the SABs in the retrovesicular ganglion. We obtained three stable transgenic lines, with one line demonstrating clear functional rescue of the lin-3(n378) vulvaless phenotype (Table 2). Together, these results suggest that activation of EGL-30 in ventral cord motor neurons promotes vulval induction.

To examine the effects of activating EGL-30 in the vulval precursor cells, we cloned the egl-30(tg26gf) cDNA downstream of the lin-31 promoter, which only drives expression in the Pn,p cells (Tan et al., 1998). When injected into lin-3(n378) vulvaless animals, this construct was not able to rescue the vulvaless phenotype in either transgenic F1s or stable lines (Table 2). Thus, activation of EGL-30 in excitable cells, rather than the vulval precursor cells, promotes vulval cell fates.

### EGL-30 (Gαq) modulation of vulval induction requires muscle-expressed EGL-19 L-type voltage-gated calcium channels

Because of the hyperactive movement phenotype displayed by egl-30(tg26gf) mutants, the correlation between hyperactive behavior and enhancement of vulval induction in the aex-3::egl-30(tg26gf) and unc-18::egl-30(tg26gf) transgenic animals, and the ability of activated EGL-30 to promote vulval development from the A-type motor neurons, we considered the possibility that muscle excitation might be necessary for EGL-30 to promote vulval cell fates. We therefore used an egl-19 loss-of-function mutation to compromise muscle excitation (Jospin et al., 2002; Lee et al., 1997). egl-19 encodes the worm homolog of the L-type voltage-gated calcium channel α1 subunit, and although it is expressed in both neurons and muscles, site of action experiments have thus far only demonstrated function in muscles (Garcia et al., 2001; Jospin et al., 2002; Lee et al., 1997). Reducing EGL-19 activity with the egl-19(n582) allele did not affect vulval induction by itself, but it strongly reduced the ability of egl-30(tg26gf) to suppress the let-23(sy1) vulvaless phenotype (Table 3). However, hyperactivation of EGL-19 by the gain-of-function egl-19(n2368gf) allele, which induces hypercontraction of muscles (Lee et al., 1997), did not rescue the vulvaless phenotype of let-23(sy1), suggesting that EGL-30 has additional targets besides EGL-19 (Table 3).

To confirm that muscle-expression of EGL-19 is required for EGL-30 to promote vulval induction, we used a transgene that expresses the genomic coding region of egl-19 from the muscle-specific myo-7 myosin promoter (Okkema et al., 1993). When the entire egl-19 genomic coding region was fused in-frame to gfp, the myo-7 promoter directed high levels of GFP expression to muscle, but not to neurons, the anchor cell or the vulval precursor cells (Fig. 1G-L), confirming the specificity of this promoter and the absence of regulatory elements in egl-19 introns. We found that wild-type EGL-19 expressed from the myo-7 promoter restores vulval induction to egl-30(tg26gf); let-23(sy1); egl-19(n582) triple mutants, indicating that EGL-30 requires muscle-expressed EGL-19 for modulating vulval induction (Table 3).

To identify muscles involved in the EGL-30-stimulated pathway, we ablated the M mesoblast in egl-30(tg26gf); let-23(sy1) L1 larvae. The M mesoblast gives rise to 14 post-embryonic body-wall muscles prior to the onset of vulval

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**Table 2. Activation of EGL-30 in neurons promotes vulva development**

<table>
<thead>
<tr>
<th>Relevant genotype*</th>
<th>Hyperactive†</th>
<th>% Muv‡</th>
<th>% Vul§</th>
<th>VPC induction¶</th>
<th>P value††</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-23(sy1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5±0.9</td>
<td>24</td>
</tr>
<tr>
<td>egl-30(tg26gf); let-23(sy1)</td>
<td>0+ ††</td>
<td>0</td>
<td>0</td>
<td>0.5±0.9</td>
<td>24</td>
</tr>
<tr>
<td>unc-13(e51)</td>
<td>8</td>
<td>65</td>
<td>2.6±1.0</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>unc-64(e246)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.0±0.0</td>
<td>23</td>
</tr>
<tr>
<td>egl-30(tg26gf) unc-13(e51); let-23(sy1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.0±0.0</td>
<td>23</td>
</tr>
<tr>
<td>egl-30(tg26gf); let-23(sy1); unc-64(e246)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.0±0.0</td>
<td>23</td>
</tr>
<tr>
<td>lin-3(n378)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.6±0.8</td>
<td>27</td>
</tr>
<tr>
<td>lin-3(n378); Ex [aex-3::egl-30(tg26gf)]</td>
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<td>0</td>
<td>0</td>
<td>0.5±0.8</td>
<td>40</td>
</tr>
<tr>
<td>lin-3(n378); Ex [aex-3::egl-30(tg26gf)]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5±0.8</td>
<td>40</td>
</tr>
<tr>
<td>lin-3(n378); Ex [unc-4::egl-30(tg26gf)]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5±0.8</td>
<td>40</td>
</tr>
<tr>
<td>lin-3(n378); Ex [unc-4::egl-30(tg26gf)]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5±0.8</td>
<td>40</td>
</tr>
<tr>
<td>lin-3(n378); Ex [aex-3::egl-30(tg26gf)]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5±0.8</td>
<td>40</td>
</tr>
<tr>
<td>lin-3(n378); Ex [aex-3::egl-30(tg26gf)]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5±0.8</td>
<td>40</td>
</tr>
<tr>
<td>lin-3(n378); Ex [aex-3::egl-30(tg26gf)]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5±0.8</td>
<td>40</td>
</tr>
<tr>
<td>lin-3(n378); Ex [unc-4::egl-30(tg26gf)]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5±0.8</td>
<td>40</td>
</tr>
<tr>
<td>lin-3(n378); Ex [unc-4::egl-30(tg26gf)]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5±0.8</td>
<td>40</td>
</tr>
<tr>
<td>F1 generations</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.9±1.2</td>
<td>40</td>
</tr>
<tr>
<td>Line 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.0±1.0</td>
<td>27</td>
</tr>
<tr>
<td>Line 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.6±1.0</td>
<td>21</td>
</tr>
<tr>
<td>Line 3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.7±1.0</td>
<td>25</td>
</tr>
<tr>
<td>Line 4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.8±1.0</td>
<td>25</td>
</tr>
</tbody>
</table>

*In the egl-30(tg26gf); let-23(sy1) strain, let-23(sy1) was linked to unc-4(e120). Ex and syEx denotes a transgene. aex-3::egl-30(tg26gf), unc-18::egl-30(tg26gf) and lin-31::egl-30(tg26gf) express the egl-30 cDNA with the tg26 mutation from the aex-3, unc-18, unc-4 and lin-31 promoters, respectively. syEx593 is unstable in long-term culture.

†Animals displaying faster locomotion and moving with deeper body bends.

‡Multivulva. Percentage of animals that have greater than three VPCs induced.

§Vulvaless. Percentage of animals that have less than three VPCs induced.

¶Average number of VPCs induced in vulval fates.

**Number of animals assayed.

††P values were calculated for VPC induction using Student’s t-test.

†‡ 8 65 2.1±1.1 29 <0.00001 versus lin-3(n378)

‡‡ 8 65 2.6±1.0 35 0.002 versus egl-30(tg26gf); let-23(sy1)

0 95 0.7±1.0 22

0 100 0.5±0.9 24

0 96 0.7±1.0 25 0.8 versus lin-3(n378)

0 93 1.0±1.0 27 0.2 versus lin-3(n378)

0 96 0.7±1.0 25 0.8 versus lin-3(n378)

0 96 0.8±1.0 25 0.4 versus lin-3(n378)

0 96 0.8±1.0 25 0.4 versus lin-3(n378)
induction, and 16 sex muscles after vulval induction has occurred (Sulston and Horvitz, 1977). Removal of the M mesoblast does not enhance the vulvaless phenotype of animals containing the weak \textit{ku1} mutation in the worm MAP kinase gene \textit{mpk-1} (Wu and Han, 1994), demonstrating that ablation of the M lineage does not generally exacerbate mutation-induced defects in vulval induction (Table 3). However, ablation of the M cell reduces \textit{egl-30(tg26gf)}-
mediated suppression of the let-23(sy1) vulvaless phenotype (Table 3). Because the sex-muscles are not formed until after the period of vulval induction, and the myo-3 promoter is not expressed in undifferentiated sex myoblasts (data not shown), the post-embryonic body-wall muscles are the muscles that probably transduce some of the EGL-30-modulating activity to the VPCs. Furthermore, these muscles only contribute to vulval induction under certain conditions, such as in the presence of activated EGL-30, which causes muscle hyperactivity.

**EGL-30 (Gq) acts downstream or parallel to LET-60 (RAS) and is sensitive to functional levels of BAR-1 (β-catenin)**

Experiments in cultured mammalian cells have demonstrated that heterotrimeric G-protein signaling can promote EGFR and MAP kinase activation (reviewed by Gschwind et al., 2001; Lowes et al., 2002). In some instances, G-proteins promote metalloprotease-dependent shedding of the EGFR ligand, HB-EGF, whereas in other cases G-proteins promote MAP kinase activation through stimulation of signaling molecules such as...
Table 3. EGL-30 requires muscle-expressed EGL-19 to promote vulva development

<table>
<thead>
<tr>
<th>Relevant genotype*</th>
<th>M cell†</th>
<th>% Muv‡</th>
<th>% Vul§</th>
<th>VPC induction¶</th>
<th>n**</th>
<th>P value††</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-23(sy1)</td>
<td>+</td>
<td>0</td>
<td>93</td>
<td>0.7±0.9</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>egl-30(tg26gf); let-23(sy1)</td>
<td>+  7.7</td>
<td>65</td>
<td>2.6±1.0</td>
<td></td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>egl-19(n852)</td>
<td>+</td>
<td>0</td>
<td>0.0</td>
<td>3.0±0.0</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>egl-30(tg26gf);let-23(sy1); egl-19(n852)</td>
<td>+  0</td>
<td>95</td>
<td>0.7±0.8</td>
<td></td>
<td>21</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>let-23(sy1); egl-19(n2368gf)</td>
<td>+  0</td>
<td>91</td>
<td>0.9±1.1</td>
<td></td>
<td>23</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>egl-30(tg26gf); let-23(sy1); egl-19(n852); syEx65[pmyo-3::egl-19]</td>
<td>+  4.3</td>
<td>65</td>
<td>1.9±1.3</td>
<td></td>
<td>47</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>egl-30(tg26gf);let-23(sy1)</td>
<td>–</td>
<td>0</td>
<td>85</td>
<td>1.1±1.2</td>
<td>34</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>sur-1(ku1)</td>
<td>+</td>
<td>0</td>
<td>29</td>
<td>2.8±0.4</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>sur-1(ku1)</td>
<td>–</td>
<td>0</td>
<td>22</td>
<td>2.8±0.4</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

*In the egl-30(tg26gf); let-23(sy1) strain, let-23(sy1) was linked to unc-4(e120). syEx denotes a transgene. syEx465 expresses egl-19 from the myo-3 promoter.
†M cell present or removed by laser ablation during the L1 larval stage.
‡Multivula. Percentage of animals that have greater than three VPCs induced.
§Vulvaless. Percentage of animals that have less than three VPCs induced.
¶Number of animals assayed.
**P values were calculated for VPC induction using Student’s t-test.

Table 4. EGL-30 acts downstream or parallel to LET-60 and is dependent on wild-type BAR-1 activity

<table>
<thead>
<tr>
<th>Relevant genotype*</th>
<th>% Muv†</th>
<th>% Vul‡</th>
<th>VPC induction¶</th>
<th>n†</th>
<th>P value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-60(sy95dn)/+</td>
<td>0</td>
<td>79</td>
<td>0.6±0.8</td>
<td>21</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>egl-30(tg26gf); let-60(sy95dn)/+</td>
<td>0  35</td>
<td>0.5±0.9</td>
<td></td>
<td>20</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>let-23(sy1); lin-3(n378)</td>
<td>0</td>
<td>100</td>
<td>0.0±0.0</td>
<td>20</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>egl-30(tg26gf); let-23(sy1); lin-3(n378)</td>
<td>0  100</td>
<td>0.2±0.6</td>
<td></td>
<td>20</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>let-23(sa62gf)</td>
<td>98</td>
<td>0</td>
<td>4.3±0.6</td>
<td>41</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>let-23(sa62gf); let-60(sy95dn)/+</td>
<td>0  95</td>
<td>0.5±0.9</td>
<td></td>
<td>20</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>let-23(sa62gf);+; syIs1/+</td>
<td>100</td>
<td>0</td>
<td>5.1±0.7</td>
<td>21</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>let-23(sa62gf)+; let-60(sy95dn)/+; syIs1/+</td>
<td>0  100</td>
<td>1.3±0.8</td>
<td></td>
<td>24</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>bar-1(ga80)</td>
<td>0  48</td>
<td>2.3±0.9</td>
<td></td>
<td>31</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>egl-30(tg26gf); bar-1(ga80)</td>
<td>0</td>
<td>41</td>
<td>2.5±0.7</td>
<td>22</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>let-23(sy1)</td>
<td>0  100</td>
<td>0.3±0.4</td>
<td></td>
<td>24</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>bar-1(mu63)</td>
<td>0</td>
<td>0</td>
<td>3.0±0.0</td>
<td>20</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>let-23(sy1); bar-1(mu63)</td>
<td>0</td>
<td>100</td>
<td>0.3±0.5</td>
<td>21</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>egl-30(tg26gf); let-23(sy1); bar-1(mu63)</td>
<td>0  16</td>
<td>48</td>
<td>2.4±1.1</td>
<td>44</td>
<td>&lt;0.000004</td>
</tr>
<tr>
<td>egl-30(tg26gf); let-23(sy1); bar-1(mu63)</td>
<td>0</td>
<td>95</td>
<td>1.1±1.0</td>
<td>21</td>
<td>&lt;0.000004</td>
</tr>
</tbody>
</table>

*let-60(sy95dn) strains carried unc-24(e138) and were balanced with nT1[let(m435)]; let-23(sa62gf) was linked to unc-4(e120). let-23(sy1) was linked to unc-4(e120) in let-23(sy1); lin-3(n378) and egl-30(tg26gf); let-23(sy1); lin-3(n378) strains. syIs1 is an integrated transgene that contains multiple copies of the lin-3 gene.
†Multivula. Percentage of animals that have greater than three VPCs induced.
‡Vulvaless. Percentage of animals that have less than three VPCs induced.
¶Number of animals assayed.
**P values were calculated for VPC induction using Student’s t-test.

protein kinase C, c-SRC and PYK2. We therefore tested whether activated EGL-30 acts by promoting LET-23 (EGFR) activation. We constructed double mutants with dominant-negative let-60(sy95dn) and either the gain-of-function let-23(sa62gf) allele or egl-30(tg26gf). let-23(sa62gf) encodes a constitutively active receptor that induces ectopic vulval fates in more than 90% of animals (Katz et al., 1996) (Table 4). Despite the ability of constitutively active LET-23 to cause a much stronger multivulva phenotype than egl-30(tg26gf), the let-23(sa62gf) allele did not suppress the let-60(sy95dn) mutation as well as egl-30(tg26gf) (Table 4). We also examined whether overexpression of LIN-3 can suppress the vulvaless phenotype conferred by dominant-negative LET-60 (RAS). In this experiment, we also included one copy of let-23(sa62gf) to further enhance the amount of pathway activation upstream of RAS. Despite the fact that multiple copies of the lin-3 gene in the form of the integrated transgenic array syIs1 (Katz et al., 1995) also confer a much stronger multivulva phenotype than egl-30(tg26gf) (Table 4), egl-30(tg26gf) is still a much better suppressor of the dominant-negative let-60(sy95dn) mutation. These data indicate that EGL-30 pathway activity is not correlated with functional levels of LIN-3 or LET-23 activation, and that EGL-30 acts either downstream or parallel to LET-60 (RAS).

To determine whether EGL-30 acts directly on some component downstream of LET-23 signaling, we made use of the observation that egl-30(tg26gf) suppresses the vulvaless phenotypes of lin-3(n378) and let-23(sy1) single mutations (Table 1). If EGL-30 acts downstream of receptor activation, then activation of EGL-30 might be expected to suppress a let-
23(sy1); lin-3(n378) double mutant (Table 4). We find that egl-30(tg26gf) cannot suppress the vulvaless phenotype of let-23(sy1); lin-3(n378) double mutants (Table 4). Taken together, our data suggest that EGL-30 might act parallel to the LET-23 pathway.

WNT signaling is the only other pathway known to act parallel to the RAS pathway during vulval induction. A null mutation in the β-catenin bar-1 results in a partially penetrant vulvaless phenotype, partly because of a reduction in lin-39 hox gene expression (Eisenmann et al., 1998), also a target of the RAS pathway (Maloof and Kenyon, 1998). Moreover, hyperactivation of WNT signaling through a mutation in ppy-1, an axin-like inhibitor of the WNT pathway, can suppress let-23 pathway vulvaless mutations (Gleason et al., 2002). Based on these observations, we explored the possibility that activated EGL-30 might promote vulval induction by elevating WNT signaling. In particular, bar-1(mu63) suppresses the ectopic mab-5 expression, and polyray and vulval phenotypes conferred by ppy-1(mu38) (Maloof et al., 1999; Moghal and Sternberg, 2003a). bar-1(mu63) also reduces the ability of egl-30(tg26gf) to suppress the vulvaless phenotype of let-23(sy1) (Table 4). The bar-1(mu63) mutation does not, however, affect the ability of a mutation in the RAS pathway component dpy-22 to comparably suppress let-23(sy1) (Moghal and Sternberg, 2003a). Thus, bar-1(mu63) reduces WNT and EGL-30 pathway activity, but not RAS pathway activity. These data are consistent with a model in which EGL-30 acts through BAR-1, rather than on RAS signaling, or a novel third pathway, to promote vulval induction.

**EGL-30 (Goαq) is required for liquid growth-mediated stimulation of vulval induction**

Because excitable cell populations can respond to changing environmental conditions, we thought the EGL-30 pathway might modulate vulval induction in response to certain environmental conditions. We therefore searched for an environmental condition that might promote vulval induction in an EGL-30-dependent manner. Previous work has shown that the vulvaless phenotypes of lin-3(n378) and let-23(n1045) can be partially suppressed by exit from dauer and starvation, respectively (Ferguson and Horvitz, 1985). We found that these conditions only weakly suppressed let-23(sy1), and did not suppress the dominant-negative let-60(sy95dn) mutation (data not shown). Besides being grown on standard NG Petri plates, *C. elegans* can be grown in liquid media. When we grew *lin-3(n378), let-23(sy1)* and *let-60(sy95dn)* single mutants in liquid M9 buffer, instead of on Petri plates, we found that in all cases, animals consistently had a higher number of VPCs adopting vulval cell fates compared with animals grown on standard NG plates (Fig. 3, Table 5). Thus, growth of worms in a liquid environment promotes vulval induction.

*C. elegans* behave differently in liquid media than on NG plates. Instead of crawling in a sinusoidal fashion, the worms vigorously thrash their bodies back and forth. Because activation of EGL-30 promotes hyperactive locomotion on plates, and loss-of-function mutations in egl-30 slow movement (Brundage et al., 1996), it is conceivable that in a liquid environment, endogenous wild-type EGL-30 is strongly activated. Because muscles and the EGL-19 calcium channel only contribute to vulval induction under conditions in which EGL-30 is strongly activated, we predicted that an egl-30 loss-of-function mutation would not affect vulval induction on standard NG plates, but would block the ability of a liquid environment to promote vulval cell fates. Consistent with this model, we found that on NG plates, the loss-of-function egl-30(ad805) allele does not cause a vulvaless phenotype on its own, nor does it suppress the multivula phenotype caused by excessive activation of the RAS or WNT pathways (Table 5). egl-30(ad805) does not suppress the gain-of-function mutations lin-15(e1763) and ppy-1(mu38). Furthermore, on NG plates, egl-30(ad805) does not enhance the vulvaless phenotype of a weak loss-of-function mutation in the MAP kinase gene *mpk-1(ku1)* (Table 5). In contrast, egl-30(ad805) blocks the ability of liquid growth to suppress let-23(sy1) (Table 5). Thus, EGL-30 specifically modulates vulval induction under certain environmental conditions, as mimicked by growth in a liquid environment.

Because the pathway stimulated by activated EGL-30 on NG plates is sensitive to functional levels of BAR-1 (Table 4), we tested whether the liquid growth-stimulated pathway displayed
the same sensitivity to WNT pathway mutations. Similar to the results with \textit{egl-30(tg26gf)} animals growing on NG plates, liquid growth was not able to suppress the vulvaless phenotype of \textit{bar-1(mu63)} (Table 5). Furthermore, as with \textit{egl-30(ad805)}, the weak loss-of-function \textit{bar-1(mu63)} mutation blocked the ability of liquid growth to suppress the \textit{let-23(sy1)} vulvaless phenotype (Table 5). This result is also similar to the sensitivity of \textit{egl-30(tg26gf)} to \textit{bar-1(mu63)} on NG plates (Table 4). Thus, liquid growth-mediated effects on vulval induction are strongly dependent on \textit{EGL-30} and \textit{BAR-1} (β-catenin) signaling.

**Discussion**

Genetic analyses performed under standard laboratory conditions have provided much information regarding the mechanisms by which growth factor signaling regulates cell fates in vivo. However, the paradigms worked out under these conditions might not fully explain the mechanisms underlying animal development, especially with regards to how development occurs in the wild. By analyzing a gain-of-function allele of the heterotrimeric Gqα gene \textit{egl-30}, we have identified a connection between the activity of excitable cells and the responsiveness of epithelial precursor cells to EGF in \textit{C. elegans}. Activation of \textit{EGL-30} by either a point mutation or overexpression causes hyperactive forward and backward locomotion and suppresses the vulvaless phenotype of loss-of-function mutations in the \textit{let-23} pathway (Table 1). \textit{egl-30} is strongly expressed in neurons (Lackner et al., 1999) (C.B., M.S. and P.W.S., unpublished), and consistent with this expression pattern, we find that transgenic expression of activated \textit{EGL-30} in neurons, especially those that regulate motor output, including the SABs, and the VA and DA ventral cord motor neurons, also drives this pathway (Table 2). These results suggest that activation of neurons that innervate body-wall muscle, and whose cell bodies are close to the vulval precursor cells, can promote vulval cell fates (Fig. 4). However, mutations in \textit{unc-64} and \textit{unc-13}, which reduce synaptic transmission, only weakly impair the ability of endogenous activated \textit{EGL-30} to promote vulval induction (Table 2). This observation could reflect the non-null nature of these alleles, or it could indicate a role for the neurons that is not heavily dependent on synaptic transmission. Although the vulval induction-promoting activity of \textit{EGL-30} can be mediated by excitation of motor neurons, hyperactive locomotion, per se, is not required. \textit{egl-30(tg26gf)}; \textit{let-23(sy1)} animals are severely paralyzed in the presence of the \textit{unc-64} and \textit{unc-13} mutations, yet still display enhanced vulval development. Despite the absence of a requirement for hyperactive locomotion for \textit{EGL-30} to promote vulva development, muscle excitation appears to be necessary. Disruption of muscle-expressed \textit{EGL-19} L-type voltage-gated calcium channel activity, which affects muscle excitation (Garcia et al., 2001; Jospin et al., 2002; Lee et al., 1997), blocks the \textit{EGL-30} pathway (Table 3). Because \textit{EGL-30} is also expressed in muscle, it is possible that \textit{EGL-30} has a second site of action, in muscle, to regulate vulva development (Fig. 4). In this scenario, the \textit{egl-30(tg26gf)} genetic mutation might cause

### Table 5. \textit{EGL-30} is required for liquid growth-mediated stimulation of vulva development

<table>
<thead>
<tr>
<th>Relevant genotype*</th>
<th>Growth condition†</th>
<th>%Muv‡</th>
<th>%Vul§</th>
<th>VPC induction¶</th>
<th>P value††</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{lin-3(n378)}</td>
<td>NG plates</td>
<td>0</td>
<td>95</td>
<td>0.5±0.9</td>
<td>41</td>
</tr>
<tr>
<td>\textit{lin-3(n378)}</td>
<td>M9</td>
<td>0</td>
<td>91</td>
<td>1.1±1.0</td>
<td>44</td>
</tr>
<tr>
<td>\textit{let-23(sy1)}</td>
<td>NG plates</td>
<td>1</td>
<td>69</td>
<td>1.7±1.2</td>
<td>80</td>
</tr>
<tr>
<td>\textit{let-23(sy1)}</td>
<td>M9</td>
<td>0</td>
<td>95</td>
<td>0.8±1.0</td>
<td>20</td>
</tr>
<tr>
<td>let-60(sy95)+</td>
<td>NG plates</td>
<td>0</td>
<td>70</td>
<td>2.0±0.9</td>
<td>20</td>
</tr>
<tr>
<td>\textit{egl-30(ad805)}; \textit{let-23(sy1)}</td>
<td>NG plates</td>
<td>0</td>
<td>95</td>
<td>0.8±1.0</td>
<td>64</td>
</tr>
<tr>
<td>\textit{egl-30(ad805)}; \textit{let-23(sy1)}</td>
<td>M9</td>
<td>0</td>
<td>86</td>
<td>0.9±1.1</td>
<td>70</td>
</tr>
<tr>
<td>\textit{let-23(sy1)}; \textit{bar-1(mu63)}</td>
<td>NG plates</td>
<td>0</td>
<td>54</td>
<td>2.5±0.7</td>
<td>25</td>
</tr>
<tr>
<td>\textit{egl-30(ad805)}; \textit{pry-1(mu38)}</td>
<td>NG plates</td>
<td>0</td>
<td>25</td>
<td>2.8±0.4</td>
<td>20</td>
</tr>
<tr>
<td>\textit{let-23(sy1)}</td>
<td>NG plates</td>
<td>90</td>
<td>0</td>
<td>3.8±0.4</td>
<td>21</td>
</tr>
<tr>
<td>\textit{egl-30(ad805)}; \textit{let-23(sy1)}</td>
<td>NG plates</td>
<td>85</td>
<td>0</td>
<td>4.5±0.8</td>
<td>20</td>
</tr>
<tr>
<td>\textit{let-60(n1046gf)}</td>
<td>NG plates</td>
<td>73</td>
<td>0</td>
<td>4.0±0.8</td>
<td>22</td>
</tr>
<tr>
<td>\textit{egl-30(ad805)}; \textit{let-60(n1046gf)}</td>
<td>NG plates</td>
<td>60</td>
<td>0</td>
<td>3.8±0.8</td>
<td>30</td>
</tr>
<tr>
<td>\textit{lin-15(e1763)}</td>
<td>NG plates</td>
<td>100</td>
<td>0</td>
<td>6.0±0.1</td>
<td>20</td>
</tr>
<tr>
<td>\textit{egl-30(ad805)}; \textit{lin-15(e1763)}</td>
<td>NG plates</td>
<td>100</td>
<td>0</td>
<td>6.0±0.2</td>
<td>20</td>
</tr>
<tr>
<td>\textit{pry-1(mu38)}</td>
<td>NG plates</td>
<td>22</td>
<td>26</td>
<td>2.9±0.5</td>
<td>23</td>
</tr>
<tr>
<td>\textit{egl-30(ad805)}; \textit{pry-1(mu38)}</td>
<td>NG plates</td>
<td>22</td>
<td>9</td>
<td>3.1±0.4</td>
<td>23</td>
</tr>
<tr>
<td>\textit{let-23(sy1)}; \textit{bar-1(mu63)}</td>
<td>NG plates</td>
<td>0</td>
<td>100</td>
<td>0.3±0.5</td>
<td>21</td>
</tr>
<tr>
<td>\textit{let-23(sy1); bar-1(mu63)}</td>
<td>NG plates</td>
<td>0</td>
<td>100</td>
<td>0.2±0.3</td>
<td>32</td>
</tr>
<tr>
<td>\textit{bar-1(ga80)}</td>
<td>NG plates</td>
<td>0</td>
<td>48</td>
<td>2.3±0.8</td>
<td>31</td>
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<tr>
<td>\textit{bar-1(ga80)}</td>
<td>M9</td>
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<td>61</td>
<td>2.3±0.7</td>
<td>31</td>
</tr>
<tr>
<td>\textit{egl-30(ad805)}</td>
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<td>0</td>
<td>3.0±0.0</td>
<td>21</td>
</tr>
<tr>
<td>\textit{egl-30(ad805)}</td>
<td>M9</td>
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<td>0</td>
<td>3.0±0.0</td>
<td>35</td>
</tr>
<tr>
<td>\textit{egl-30(tg26)}</td>
<td>NG plates</td>
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<td>0</td>
<td>3.0±0.0</td>
<td>36</td>
</tr>
<tr>
<td>\textit{egl-30(tg26)}</td>
<td>M9</td>
<td>0</td>
<td>0</td>
<td>3.0±0.0</td>
<td>22</td>
</tr>
</tbody>
</table>

*\textit{let-60(sy95dn)} was linked to \textit{unc-24(e138)} and was balanced with \textit{nT1[let(m435)]}. \textit{sur-1(ku1)} was linked to \textit{dpy-17(e164)}.

†Media on which worms were grown.

‡Muv, Multivula. Percentage of animals that have greater than three VPCs induced.

§Vulvaless. Percentage of animals that have less than three VPCs induced.

¶Average number of VPCs induced to vulval fates.

**Number of animals assayed.

††P values were calculated for VPC induction using Student’s t-test.
sufficient cell-autonomous excitation of the muscles to bypass
the requirement for UNC-64- and UNC-13-mediated synaptic
transmission. This model would be consistent with data that
L-type voltage-gated calcium channels can be positively
modulated by protein kinase C (Linn, 2000), and
diacylglycerol and/or inositol-1, 4, 5-trisphosphate, two second
messengers downstream of Gtq (Boyer et al., 1994; Jiang et
al., 1994). However, because we find that the egl-19(n2368gf)
gain-of-function allele does not phenocopy the effect of
activated EGL-30 on vulval induction (Table 3), it is possible
that EGL-30 has targets other than EGL-19. Alternatively,
because the egl-19(n2368gf) mutation does not cause the same
hyperactive behavior observed in egl-30(tg26gf) mutants, the
G365R change conferred by the egl-19(n2368gf) mutation
might not mimic the effect of activated EGL-30 on the
biophysical properties of EGL-19, and the biological properties
of muscles.

The M cell lineage is required for the EGL-30 pathway, and
myo-3-driven egl-19, which is only expressed in differentiated
muscle, can promote pathway activity (Table 3). Because the
M-derived body-wall muscles are formed during L2, prior to
vulval induction, whereas the M-derived sex muscles are not
formed until the L4 stage, after vulval induction is completed
(Sulston and Horvitz, 1977), it is probable that post-embryonic
body-wall muscles promote vulva development. These muscles
occupy positions posterior to the gonadal primordium, both
dorsally and ventrally, and are used for locomotion, similar to
their embryonically derived counterparts (Garcia et al., 2001;
Sulston and Horvitz, 1977). The most anterior of the muscles
are functionally distinct from the other body-wall muscles.
Therefore, we favor a model in which ablation of the M cell
simply reduces the number of muscles below a critical
threshold, so that the EGL-30 pathway can no longer fully
promote vulva development.

Several models could explain the form of communication
between the neurons, muscles and vulval precursor cells that
promotes vulval induction. In all cases, excitation of body-wall
muscles is crucial. In a simple model (Fig. 4), EGL-30-driven
activation of motor neurons stimulates body-wall muscle,
which in turn, signals to the VPCs. In contrast, in C. elegans,
body-wall muscles have been shown to modulate neuronal
synaptic development via the C2 domain protein AEX-1 and
the AEX-5 prohormone convertase (Doi and Iwasaki, 2002).
Therefore, it is also possible that neurons may directly
modulate vulval induction in a manner that is not strongly
dependent on synaptic transmission, but requires retrograde
signaling from the muscle to the neurons. Finally, it’s possible
that two parallel signals are sent by the neurons and body-wall
muscle to the vulval precursor cells.

Experiments with cultured mammalian cells have
demonstrated that G-protein-coupled receptor activation can
lead to metalloprotease-stimulated shedding of HB-EGF, an
EGFR ligand (Daub et al., 1996; Prenzel et al., 1999), which
can then act in an autocrine and paracrine manner. However,
we find that expression of activated EGL-30 in the vulval
precursor cells does not promote vulval induction (Table 2),
nor is the activity of EGL-30 correlated with functional levels
of LIN-3 or LET-23 activation (Table 4). Instead, we find that
activated EGL-30 acts downstream or parallel to the LET-60
(RAS) (Table 4). WNT signaling is the only other pathway
known to act parallel to RAS signaling during vulval induction,
with one convergence point being the lin-39 hox gene
(Eisenmann et al., 1998). We find that although activation of
EGL-30 can suppress RAS pathway mutations, it cannot suppress the partially penetrant vulval phenotype of the bar-1(ga80) null allele (Table 4). Moreover, although the bar-1(mu63) loss-of-function allele does not affect vulval induction on its own, it reduces the ability ofegl-30(tg26gff) to suppress the vulval phenotype oflet-23(sy1) (Table 4). This sensitivity ofegl-30(tg26gff) to bar-1(mu63) is specific because suppression oflet-23(sy1) by the RAS pathway mediator component dpy-22 is not affected bybar-1(mu63) (Moghali and Sternberg, 2003a). These data suggest that one possible model in which EGL-30 promotes vulval induction is by upregulatingbar-1 (β-catenin) signaling (Fig. 4). In support of this model, elevated bar-1 (β-catenin) signaling resulting from a loss-of-function mutation in the axin-like gene, pry-1, or from overexpression of a non-degradable form of BAR-1 suppresses the vulval phenotype oflet-23(sy1) (Gleason et al., 2002).

Mammalian cell culture experiments have suggested that Gαq may be a downstream component of WNT receptors (Liu et al., 2001; Liu et al., 1999). However, because transgenic expression of activated EGL-30 in neurons, but not in the vulval precursor cells, promotes vulval induction (Table 2), this model is unlikely to explain our results. Furthermore, BAR-1 is expressed in the vulval precursor cells, but not in muscles and neurons (Eisenmann et al., 1998), and we have shown that the latter cells mediate the effects of EGL-30.

Excitable cells can act as sensors for an animal’s environment. Thus, the existence of pathways by which excitable cells can contribute to the developmental fates of cells may be generally important in ensuring that correct developmental decisions are made under a wide range of conditions. Accordingly, we find that when animals are removed from NG plates, and are placed in a liquid environment, an EGL-30-dependent pathway is activated which promotes vulval induction (Table 5). Because theegl-30(ad805) mutation does not affect vulval induction under conditions in which the RAS and WNT pathways are hyperactivated or compromised by genetic mutation (Table 5), EGL-30-mediated regulation of vulval induction is specific to conditions affecting animal behavior. Similar to our studies with activated EGL-30 on NG plates, we find that growth in liquid media suppresses vulval mutations in the RAS pathway, but not the bar-1(ga80) null mutation, and that thebar-1(mu63) loss-of-function mutation blocks the liquid-stimulated pathway (Table 5). Thus, one model for the liquid growth enhancement of vulval induction could also involve the indirect stimulation of BAR-1 (β-catenin) in the vulval precursor cells by EGL-30 (Fig. 4).

The link between the environment and the EGL-30 modulatory pathway suggests that some of the other positive and negative regulators of vulval induction, which have no phenotypes under standard growth conditions on NG plates, might also play important roles under different environmental conditions. It has recently been reported that the G-protein-coupled receptor, SRA-13, and the heterotrimeric Gα protein, GPA-5, are inhibitors of vulva development (Battu et al., 2003). Although mutations in these genes can affect vulva development on standard NG plates, SRA-13 is also necessary for starvation-mediated inhibition of vulva development. sra-13 and gpa-5 are both expressed in chemosensory neurons, and sra-13 is additionally expressed in body-wall muscle (Battu et al., 2003; Jansen et al., 1999). Thus, in conjunction with our results, it appears that C. elegans can use its neuromuscular system to both promote and inhibit vulva development, depending on the environmental context.

Although RAS and WNT signaling are both important for inducing vulval cell fates, hypomorphic mutations in thelet-23 pathway cause more severe vulvalless phenotypes than a bar-1 null mutation. This difference raises questions as to why two different signaling pathways are used to specify vulval cell fates, and why they are used disproportionately. If a cell fate must be induced at a particular time in development, it might be best accomplished by robust activation of a pathway that is largely insensitive to environmental changes. However, should conditions arise that are deleterious to that pathway, the existence of a second pathway that is modulated by the environment would ensure that development remains invariant. Under stressful conditions, the hermaphrodite may use a behavioral response elicited by excitable cells and Gαq signaling to promote the activity of the WNT pathway, and ultimately enhance RAS-dependent vulval cell differentiation. The interplay between the environment, neurons, muscles and these signaling pathways adds a new dimension to the existing paradigms by which growth factors trigger cell fate changes during animal development.

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References


