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), *dpy-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 Gαq 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 α1 L-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(ad805)* (Brundage et al., 1996), *unc-13(e51)* (Brenner, 1974), *goa-1(n363)* (Segalat et al., 1995), *phm-2(ad538)* (Avery, 1993), *unc-54(e190)* (Epstein et al., 1974) on LGI; *let-23(sy1)* (Aroian and Sternberg, 1991), *let-23(sa62gf)* (Katz et al., 1996), *unc-4(e120)* (White et al., 1992) on LGII; *dpy-17(e164)* (Brenner, 1974), *sur-1(ku1)* (Wu and Han, 1994), *unc-64(e246)* (Brenner, 1974), *pha-1(e2123ts)* (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(mu63)* (Maloof et al., 1999), *bar-1(ga80)* (Eisenmann et al., 1998), and *lin-15(e1763)* (Ferguson and Horvitz, 1985) on LGX. Genetic balancers used were: *mnC1[dpy-10(e128) unc-52(e444)]* (Herman, 1978; Sigurdson et al., 1984) on LGII*; nT1[let(m435)]* on LGIV and LGV (Rogalski and Riddle, 1988).

let-23(sa62gf)/+; syIs1/+ worms were obtained by first crossing N2 males into hermaphrodites carrying the X-linked *syIs1* transgenic array. F1 males that were hemizygous for the X chromosome were used to transfer *syIs1* into homozygous *let-23(sa62gf) unc-4(e120)* hermaphrodites. Non-Unc F1 hermaphrodite 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)/nT1[let(m435)]* hermaphrodites, and picking non-Unc cross progeny. Because the F1 cross progeny consisted of both *let-60(sy95dn)/+* and *nT1[let(m435)]/+* genotypes, we scored vulval induction in all cross progeny, recovered the individual worms and identified the F1 genotype by examining the F2 generation. The *let-60(sy95dn)/+* genotype was assigned to F1s segregating Vul animals 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′-ATGGCCTGCTGTTTATCCGAAGAG-3′ and the downstream primer 5′-TTACACCAAGTTGTACTCCTTCAGATTATGCTGTAGAAT-3′. The PCR product was blunt-end ligated into the *Bam*HI 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′-CACCAAGTTGTACTCCTTCAGATTATGCTGTAGAATTG 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 *Bam*HI site of the promoterless GFP plasmid pPD95.75 (a gift of A. Fire), creating the construct pR36. A 5 kbp *Xba*I-*Apa*I fragment from pR36 was swapped between the *Nhe*I-*Apa*I 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′-GCCGCGGATCCAAATGAGTAAAGGAGAAGAACTTTTCAC-3′ and 5′-ACGTGCTGCGACAAACAGTTATGTTTGGTATATTGGGAATG-3′. pSX95.77 contains the *yfp* coding region and *unc-54* 3′UTR from pPD136.64 (a gift of A. Fire) inserted into pBR322. The *yfp::unc-54* PCR product was digested with *Bam*HI and *Sal*I, and ligated into *Bam*HI/*Sal*I-digested pTG100.1. pLIN31EGL30 was constructed by amplifying the *egl-30(tg26gf)* cDNA from pTG100.1 with the primers 5′-ATAAGAATGCGGCCGCAAAAATGGCCTGCTGTTTATCCGAAGAG-3′ and 5′-CCTGTAAAGCGGCCGCTTACACCAAGTTGTACTCCTTCAG-3′, and cloning the *Not*I-digested PCR product into the *Not*I site of the *lin-31* expression vector, pB255 (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(tg26gf)* mutation and the last intron was released from pTG100.1 by digestion with *Apa*I and *Bsr*G1. The *egl-30* fragment was made blunt, and subsequently cloned into the *Sma*I site of pBSKS to generate pBStg26. The *unc-18* promoter (extending from the *unc-18* start ATG to the next 5′ gene F27D9.8) was amplified by PCR with the primers UNC18-3 5′-AGCCCAAGCTTTGAAGGACAATGAACTAGAGG GAC-3′ and UNC18-4 5′-AGCCCAAGCTTCCCATTTTTCAAAAATCCTCGTC GATGCACTCAC-3′, digested with *Hin*dIII, and cloned into *Hin*dIII-digested pBStg26 to yield pUNC18tg26. The *unc-18::yfp::egl-30* reporter plasmid, pUNC18YFP, was created as follows. First, the *yfp::egl-30* intron fusion from pAEXYFP was released by digestion with *Apa*I and *Bam*HI, the ends made blunt and the fragment cloned into the *Sma*I site of pBSKS to yield pBSYFPEGL30. The PCR-generated *unc-18* promoter fragment was then cloned into the *Hin*dIII site of pBSYFPEGL30 to yield pUNC18YFP. The plasmid pLR1, which was used to express the *egl-30(tg26gf)* cDNA from the *unc-4* promoter, was constructed as follows. A 3.0 kbp *Hin*dIII-*Sma* I fragment, which contains the *unc-4* promoter and extends through part of the second exon (Miller and Niemeyer, 1995), was cloned between the *Hin*dIII and *Sma*I sites in pBSKS to make the plasmid pBSUNC4 (courtesy of C. Van Buskirk). The primers 5′ ATGGCCTGCTGTTTATCCGAAGAG 3′ and 5′ TCCCCCGGGGGATTTCGAGGTTAGCTTGATGGG 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 *Nsi*I and *Sma*I 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 pTG100.1 [*aex-3::egl-30(tg26gf)*] (50 ng μl^{-1}), or pUNC18tg26 [*unc-18::egl-30(tg26gf)*] (50 ng μl^{-1}), or pLR1 [*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 pLIN31EGL30 [*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).

Liquid growth assays

A solution of commercial bleach and 4N NaOH (1:1 v/v) was applied to gravid worms on NG plates without bacteria. The next day, starved, synchronized L1 worms were transferred to liquid M9 buffer (Brenner, 1974) in 1.5 ml Eppendorf tubes. On average, tubes contained 30-40 worms in a final volume of 25 µl and *E. coli* OP50 at a concentration of A600 nm=1.0. Worms were grown in a 20°C incubator without shaking until they reached mid-L4, at which time, vulval induction was scored.

Results

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

EGL-30 is the ortholog of mammalian Gαq/Gα$_{11}$, 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 vulvaless 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)* vulvaless phenotype. *phm-2(ad538)* has a deletion removing the 3′ end of *dys-1* (dystrophin-like), and *goa-1(n363)* has a deletion removing *goa-1* (Gαo). Although both alleles cause muscle hyperactivity and feeding defects (Avery,

Table 1. Activation of EGL-30 promotes vulva development

Relevant genotype*	% Muv†	% Vul‡	VPC induction§	*n*¶	*P* value**
Wild type	0	0	3.0±0.0	31	
egl-30(tg26gf)	0	0	3.0±0.0	20	
lin-3(n378)	0	96	0.7±1.0	51	
egl-30(tg26gf); lin-3(n378)	0	35	2.8±0.7	20	<0.0005 versus *lin-3(n378)*
let-23(sy1)	0	100	0.5±0.9	24	
egl-30(tg26gf); let-23(sy1)	7.7	65	2.6±1.0	37	<0.0005 versus *let-23(sy1)*
phm-2(ad538); let-23(sy1)	0	100	0.1±0.4	20	<0.0005 versus *egl-30(tg26gf); let-23(sy1)*
goa-1(n363); let-23(sy1)	0	100	0.4±0.9	24	<0.0005 versus *egl-30(tg26gf); let-23(sy1)*
egl-30(ad805); let-23(sy1)	0	94	0.6±1.0	52	0.65 versus *let-23(sy1)*
egl-30(ad805); let-23(sy1); syEx532 [egl-30(+) XS]	3.6	71	1.9±1.2	56	0.004 versus *egl-30(ad805); let-23(sy1)*

*In the *egl-30(tg26gf); let-23(sy1)* strain, *let-23(sy1)* was linked to *unc-4(e120)*. *syEx* denotes a transgene. *syEx532* contains the wild-type *egl-30* gene.
†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 to vulval fates.
¶Number of animals assayed.
**P* values were calculated for VPC induction using Student's *t*-test.

1993; Bessou et al., 1998; Segalat et al., 1995), neither suppresses the *let-23(sy1)* vulvaless phenotype (Table 1). Therefore, modulation of vulval induction by *egl-30(tg26gf)* is unlikely to be caused by general effects on movement or worm physiology.

We next tested whether the effects on vulval cell fate caused by the R243Q change encoded by *egl-30(tg26gf)* reflected elevated EGL-30 activity or a new property of EGL-30. We injected the wild-type *egl-30* genomic coding region into an *egl-30* loss-of-function; *let-23* loss-of-function double mutant. We were able to recover one transgenic line, *egl-30(ad805); let-23(sy1); syEx532 [egl-30 XS]*, that displayed hyperactive behaviors similar to those observed in *egl-30(tg26gf)* animals (data not shown). In addition to conferring behavioral similarities with *egl-30(tg26gf)* animals, overexpression of wild-type EGL-30 also suppresses the vulvaless phenotype *let-23(sy1)* (Table 1). Thus, elevated EGL-30 activity promotes vulval induction.

Activation of EGL-30 (Gαq) in neurons promotes vulval induction

Anti-EGL-30 antibody staining indicates that EGL-30 is most strongly expressed in neurons (C.B., M.S. and P.W.S., unpublished), and *egl-30::gfp* translational fusions also reveal strong expression in neurons, as well as muscle, and the differentiated secondary cells of the mature vulva (Lackner et al., 1999) (C.B., M.S. and P.W.S., unpublished). In view of its expression in excitable cells, and the defective locomotion and egg-laying behaviors associated with loss-of-function mutations in *egl-30* (Brundage et al., 1996), we asked whether neuronal signaling is required for activated EGL-30 to promote vulval induction. We tested whether mutations in either *unc-13*, which encodes a diacyglycerol-binding protein involved in EGL-30-mediated synaptic transmission (Lackner et al., 1999; Maruyama and Brenner, 1991; Richmond et al., 1999), or *unc-64*, which encodes syntaxin 1a (Ogawa et al., 1998; Saifee et al., 1998), disrupt the ability of *egl-30(tg26gf)* to suppress the *let-23(sy1)* vulvaless phenotype. The non-null *unc-64(e246)* and *unc-13(e51)* alleles partially reduced the effect of activated EGL-30 on vulval induction, possibly indicating some involvement of neurons in this pathway (Table 2).

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 vulvaless 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)* vulvaless 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 vulvaless 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-*

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

Relevant genotype*	Hyperactive†	% Muv‡	% Vul§	VPC induction¶	*n***	*P* value††
let-23(sy1)	–	0	100	0.5±0.9	24	
egl-30(tg26gf); let-23(sy1)	–‡‡	8	65	2.6±1.0	37	
unc-13(e51)	–	0	0	3.0±0.0	23	
unc-64(e246)	–	0	0	3.0±0.0	20	
egl-30(tg26gf) unc-13(e51); let-23(sy1)	–	3	69	1.7±1.2	35	0.002 versus *egl-30(tg26gf); let-23(sy1)*
egl-30(tg26gf); let-23(sy1); unc-64(e246)	–	6.5	72	2.1±1.0	46	0.02 versus *egl-30(tg26gf); let-23(sy1)*
lin-3(n378)	–	0	100	0.6±0.8	27	
lin-3(n378); Ex [aex-3::egl-30(tg26gf)]	–	0	100	0.5±0.8	40	
lin-3(n378); Ex [aex-3::egl-30(tg26gf)]	+	0	52	2.1±1.1	29	<0.00001 versus *lin-3(n378)*
lin-3(n378); Ex [unc-18::egl-30(tg26gf)]	–	0	95	0.7±0.9	22	
lin-3(n378); Ex [unc-18::egl-30(tg26gf)]	+	0	42	2.4±1.0	12	0.00005 versus *lin-3(n378)*
lin-3(n378); syEx593 [unc-4::egl-30(tg26gf)]	–	0	73	1.7±1.2	22	0.0008 versus *lin-3(n378)*
lin-3(n378); Ex [lin-31::egl-30(tg26gf)]						
F1 generations	–	0	83	0.9±1.2	40	0.3 versus *lin-3(n378)*
Line 1	–	0	93	1.0±1.0	27	0.2 versus *lin-3(n378)*
Line 2	–	0	90	0.6±1.0	21	0.9 versus *lin-3(n378)*
Line 3	–	0	96	0.7±1.0	25	0.8 versus *lin-3(n378)*
Line 4	–	0	96	0.8±1.0	25	0.4 versus *lin-3(n378)*

*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)*, *unc-4::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 to vulval fates.

**Number of animals assayed.

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

‡‡*egl-30(tg26gf); let-23(sy1)* animals are not hyperactive because of the presence of the *unc-4(e120)* mutation.

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-3* myosin promoter (Okkema et al., 1993). When the entire *egl-19* genomic coding region was fused in-frame to *gfp*, the *myo-3* 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-3* 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

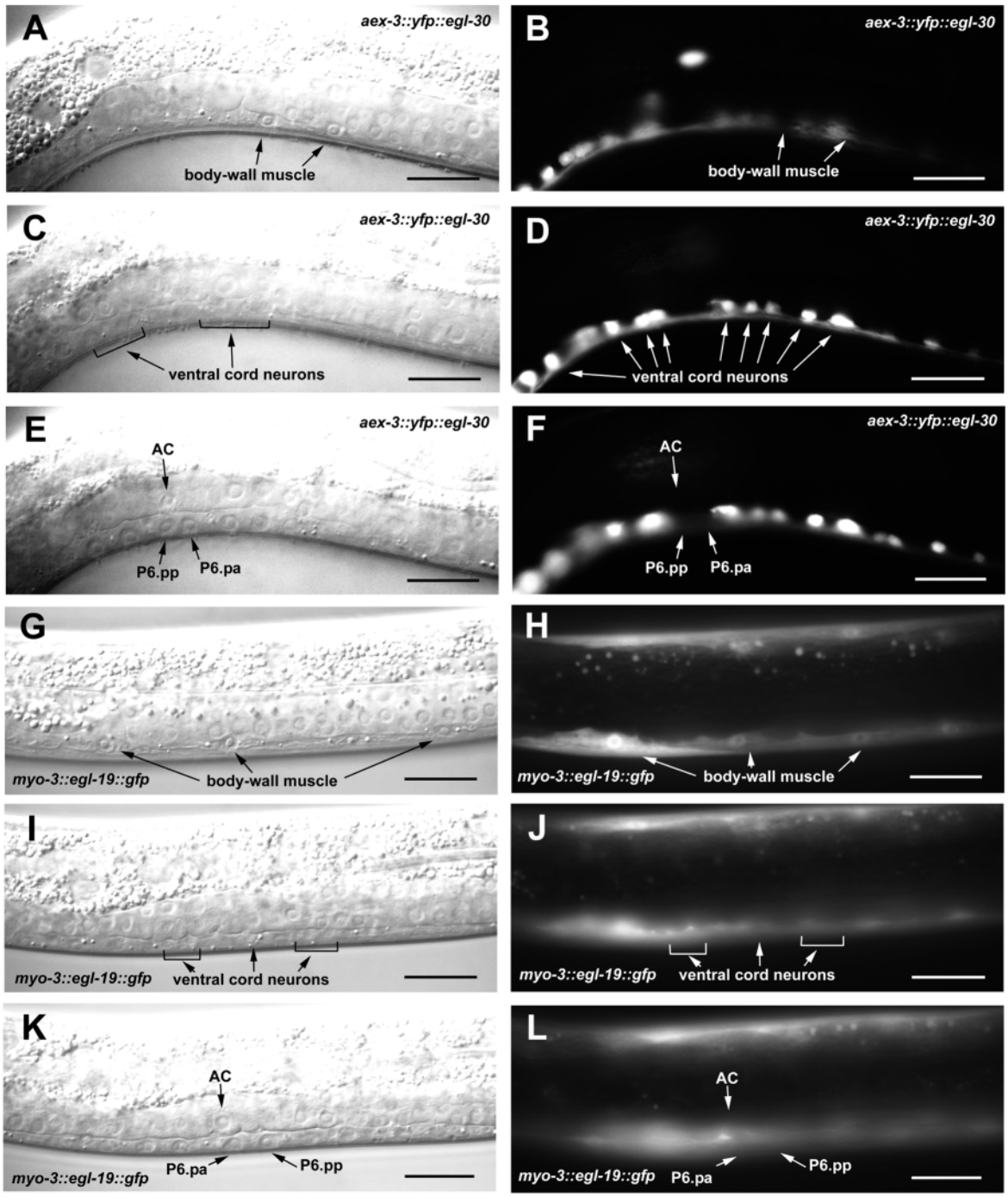

Fig. 1. Expression patterns of the regulatory elements contained in the *aex-3* and *myo-3* transgenic constructs. Left-hand images are Nomarski, and right-hand images are fluorescence. Scale bars: 20 μm. (A,B,G,H) Body-wall muscle. (C,D,I,J) Ventral cord neurons. (E,F,K,L) Vulval precursor cells. (A-F) Neuronal-specific expression of *yfp* from the *aex-3* promoter in the presence of the last intron from *egl-30*. Animal is an early L3 stage hermaphrodite containing the *pha-1(e2123ts)* mutation, and rescued by the extrachromosomal array *syEx570 [pha-1, aex-3::yfp::egl-30]*. (B,F) Background fluorescence is from neuronally expressed YFP in other focal planes. (G-L) Muscle-specific expression of the *egl-19* genomic coding region fused to *gfp* from the *myo-3* promoter. Animal is an early L3 stage hermaphrodite containing the *egl-19(n582)* mutation and the extrachromosomal array *syEx476 [myo-3::egl-19::gfp]*. (J,L) Background fluorescence is from body-wall muscle-expressing GFP in other focal planes. AC, anchor cell.

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 *ku1* mutation in the worm MAP kinase gene *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 *egl-30(tg26gf)*-

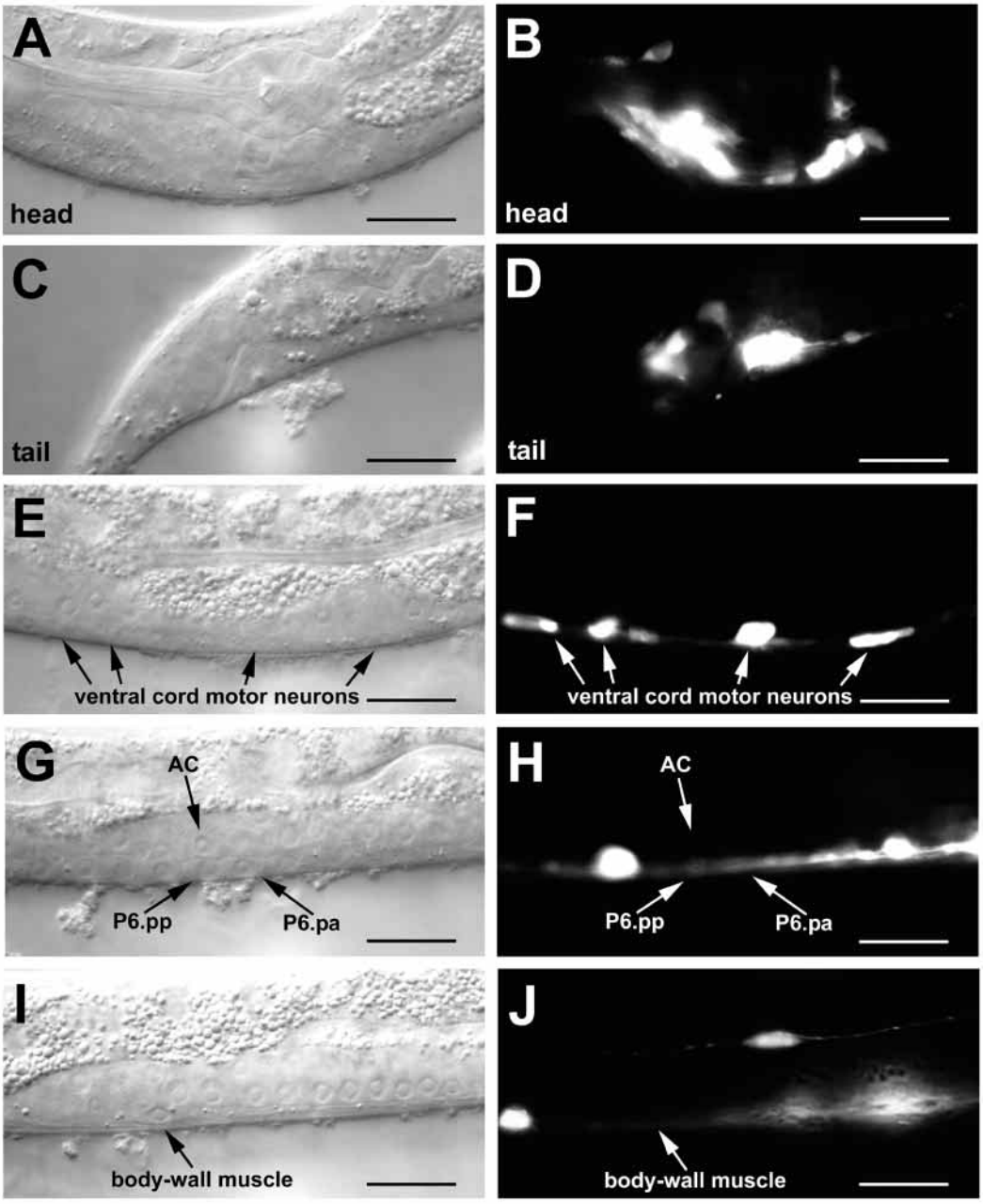

Fig. 2. Expression pattern of the *unc-18* promoter. Left-hand images are Nomarski, and right-hand images are fluorescence. Scale bars: 20 μm. Animals are *pha-1(e2123ts)* hermaphrodites at the early L3 stage, rescued by the extrachromosomal array *syEx594 [pha-1, unc-18::yfp::egl-30]*. (A,B) Head. (C,D) Tail. (E,F) Ventral cord. (G,H) Vulval precursor cells. (I,J) Body-wall muscle. Background fluorescence in H and J is from neuronal expression of the reporter. AC, anchor cell.

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 (Gαq) 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

Relevant genotype*	M cell†	% Muv‡	% Vul§	VPC induction¶	*n***	*P* value††
let-23(sy1)	+	0	93	0.7±0.9	80	
egl-30(tg26gf); let-23(sy1)	+	7.7	65	2.6±1.0	37	
egl-19(n582)	+	0	0	3.0±0.0	22	
egl-30(tg26gf);let-23(sy1); egl-19(n582)	+	0	95	0.7±0.8	21	<0.0005 versus *egl-30(tg26gf); let-23(sy1)*
let-23(sy1); egl-19(n2368gf)	+	0	91	0.9±1.1	23	0.27 versus *let-23(sy1)*
egl-30(tg26gf); let-23(sy1); egl-19(n582); syEx465 [pmyo-3::egl-19]	+	4.3	65	1.9±1.3	47	<0.0005 versus *egl-30(tg26gf); let-23(sy1); egl-19(n582)*
egl-30(tg26gf); let-23(sy1)	–	0	85	1.1±1.2	34	<0.0005 versus *egl-30(tg26gf); let-23(sy1)* 0.09 versus *let-23(sy1)*
sur-1(ku1)	+	0	29	2.8±0.4	21	
sur-1(ku1)	–	0	22	2.8±0.4	9	0.74 versus M cell intact *sur-1(ku1)*

*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.
‡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 to vulval fates.
**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

Relevant genotype*	% Muv†	% Vul‡	VPC induction§	*n*¶	*P* value**
let-60(sy95dn)/+	0	79	0.6±0.8	21	
egl-30 (tg26gf); let-60(sy95dn)/+	0	35	2.5±0.9	20	<0.0005 versus *let-60(sy95dn)/+*
let-23(sy1); lin-3(n378)	0	100	0.0±0.0	20	
egl-30(tg26gf); let-23(sy1); lin-3(n378)	0	100	0.2±0.6	20	0.16 versus *let-23(sy1); lin-3(n378)*
let-23(sa62gf)	98	0	4.3±0.6	41	
let-23(sa62gf); let-60(sy95dn)/+	0	95	0.5±0.9	20	<0.0005 versus *egl-30(tg26gf); let-60(sy95dn)/+*
let-23(sa62gf)/+; syIs1/+	100	0	5.1±0.7	21	<0.0005 versus *egl-30(tg26gf)*
let-23(sa62gf)/+; let-60(sy95dn)/+; syIs1/+	0	100	1.3±0.8	24	<0.0005 versus *egl-30 (tg26gf); let-60(sy95dn)/+*
bar-1(ga80)	0	48	2.3±0.9	31	
egl-30(tg26gf); bar-1(ga80)	0	41	2.5±0.7	22	0.3 versus *bar-1(ga80)*
let-23(sy1)	0	100	0.3±0.4	24	
bar-1(mu63)	0	0	3.0±0.0	20	
let-23(sy1); bar-1(mu63)	0	100	0.3±0.5	21	0.91 versus *let-23(sy1)* on plates
egl-30(tg26gf); let-23(sy1)	16	48	2.4±1.1	44	<0.000001 versus *let-23(sy1)*
egl-30(tg26gf); let-23(sy1); bar-1(mu63)	0	95	1.1±1.0	21	0.00004 versus *egl-30(tg26gf); let-23(sy1)*

**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.
†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 to vulval fates.
¶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 *pry-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 pathway activity. We found that unlike the effects on the RAS pathway mutations, *egl-30(tg26gf)* did not suppress the vulvaless phenotype of the *bar-1(ga80)* allele (Table 4). In addition, we examined the sensitivity of the EGL-30 pathway to *bar-1* (β-catenin) levels. Although the weak loss-of-function *bar-1*(*mu63)* allele is phenotypically wild-type by itself (Maloof et al., 1999), it reduces the output of elevated WNT signaling. In particular, *bar-1(mu63)* suppresses the ectopic *mab-5* expression, and polyray and vulval phenotypes conferred by *pry-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 (Gαq) 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

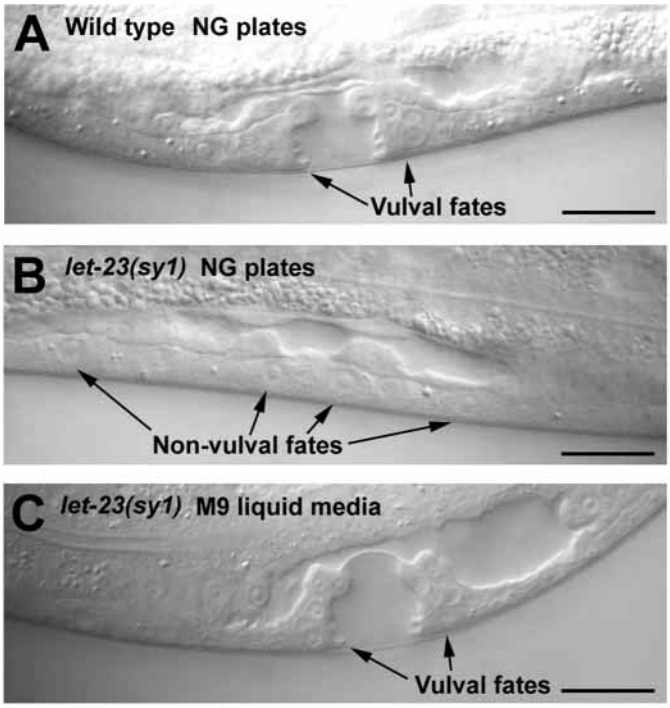

Fig. 3. Growth in a liquid environment promotes vulval induction. Scale bars: 20 μm. Animals are L4 stage hermaphrodites. (A) A wild-type animal displaying normal vulval induction that developed on standard NG plates. (B) A *let-23(sy1)* mutant animal displaying a vulvaless phenotype that developed on standard NG plates. (C) A *let-23(sy1)* mutant animal displaying normal vulval induction that developed in a liquid M9 environment.

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 multivulva phenotype caused by excessive activation of the RAS or WNT pathways (Table 5). *egl-30(ad805)* does not suppress the gain-of-function mutations *let-23(sa62)* and *let-60(n1046)*, or the loss-of-function mutations *lin-15(e1763)* and *pry-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

Table 5. EGL-30 is required for liquid growth-mediated stimulation of vulva development

Relevant genotype*	Growth condition†	%Muv‡	%Vul§	VPC induction¶	*n***	*P* value††
lin-3(n378)	NG plates	0	95	0.5±0.9	41	
lin-3(n378)	M9	0	91	1.1±1.0	44	0.005 versus *lin-3(n378)* NG plates
let-23(sy1)	NG plates	0	93	0.7±0.9	80	
let-23(sy1)	M9	1	69	1.7±1.2	80	<0.00001 versus *let-23(sy1)* NG plates
let-60(sy95)/+	NG plates	0	95	0.8±1.0	20	
let-60(sy95)/+	M9	0	70	2.0±0.9	20	0.0002 versus *let-60(sy95dn)/+* NG plates
egl-30(ad805); let-23(sy1)	NG plates	0	95	0.8±1.0	64	
egl-30(ad805); let-23(sy1)	M9	0	86	0.9±1.1	70	0.6 versus *egl-30(ad805); let-23(sy1)* NG plates
sur-1(ku1)	NG plates	0	54	2.5±0.7	25	
egl-30(ad805); sur-1(ku1)	NG plates	0	25	2.8±0.4	20	
let-23(sa62gf)	NG plates	90	0	3.8±0.4	21	
egl-30(ad805); let-23(sa62gf)	NG plates	85	0	4.5±0.8	20	
let-60(n1046gf)	NG plates	73	0	4.0±0.8	22	
egl-30(ad805); let-60(n1046gf)	NG plates	60	0	3.8±0.8	30	0.4 versus *let-60(n1046gf)*
lin-15(e1763)	NG plates	100	0	6.0±0.1	20	
egl-30(ad805); lin-15(e1763)	NG plates	100	0	6.0±0.2	20	
pry-1(mu38)	NG plates	22	26	2.9±0.5	23	
egl-30(ad805) pry-1(mu38)	NG plates	22	9	3.1±0.4	23	0.2 versus *pry-1(mu38)*
let-23(sy1); bar-1(mu63)	NG plates	0	100	0.3±0.5	21	0.91 versus *let-23(sy1)* on plates
let-23(sy1); bar-1(mu63)	M9	0	100	0.2±0.3	32	0.43 versus *let-23(sy1); bar-1(mu63)* on plates
bar-1(ga80)	NG plates	0	48	2.3±0.8	31	
bar-1(ga80)	M9	0	61	2.3±0.7	31	0.74. vs *bar-1(ga980)* on plates
egl-30(ad805)	NG plates	0	0	3.0±0.0	21	
egl-30(ad805)	M9	0	0	3.0±0.0	35	
egl-30(tg26)	NG plates	0	0	3.0±0.0	36	
egl-30(tg26)	M9	0	0	3.0±0.0	22	

**let-60(sy95dn)* was linked to *unc-24(e138)* and was balanced with *nT1[let(m435)]*. *sur-1(ku1)* was linked to *dpy-17(e164)*.
†Media on which worms were grown.
‡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 to vulval fates.
**Number of animals assayed.
††*P* values were calculated for VPC induction using Student's *t*-test.

the same sensitivity to WNT pathway mutations. Similar to the results with *egl-30(tg26gf)* animals growing on NG plates, liquid growth was not able to suppress the vulvaless phenotype of *bar-1(ga80)* (Table 5). Furthermore, as with *egl-30(ad805)*, the weak loss-of-function *bar-1(mu63)* mutation blocked the ability of liquid growth to suppress the *let-23(sy1)* vulvaless phenotype (Table 5). This result is also similar to the sensitivity of *egl-30(tg26gf)* to *bar-1(mu63)* on NG plates (Table 4). Thus, liquid growth-mediated effects on vulval induction are strongly dependent on EGL-30 and 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 Gαq gene *egl-30*, we have identified a connection between the activity of excitable cells and the responsiveness of epithelial precursor cells to EGF in *C. elegans*. Activation of 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 *let-23* pathway (Table 1). *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 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 *unc-64* and *unc-13*, which reduce synaptic transmission, only weakly impair the ability of endogenous activated 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 EGL-30 can be mediated by excitation of motor neurons, hyperactive locomotion, per se, is not required. *egl-30(tg26gf); let-23(sy1)* animals are severely paralyzed in the presence of the *unc-64* and *unc-13* mutations, yet still display enhanced vulval development.

Despite the absence of a requirement for hyperactive locomotion for EGL-30 to promote vulva development, muscle excitation appears to be necessary. Disruption of muscle-expressed 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 EGL-30 pathway (Table 3). Because EGL-30 is also expressed in muscle, it is possible that EGL-30 has a second site of action, in muscle, to regulate vulva development (Fig. 4). In this scenario, the *egl-30(tg26gf)* genetic mutation might cause

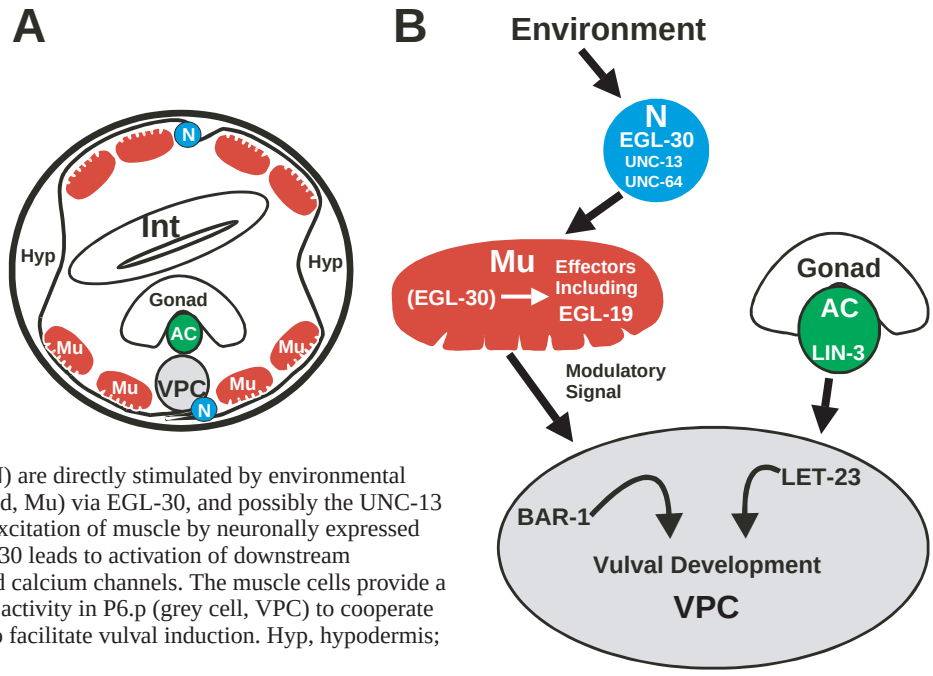

Fig. 4. Depiction of vulval induction in the context of the whole animal. (A) A schematic transverse section through a developing hermaphrodite. The dorsal side of the worm is up. Dorsal and ventral nerve cord, blue circles labeled N; body-wall muscles in the four lateral quadrants, red cells labeled Mu; gonadal tissue, inverted U-shaped tissue; anchor cell, green circle labeled AC. Representative vulval precursor cell (grey cell labeled VPC). (B) One model for EGL-30 modulation of LET-23-mediated vulva development. Neurons (blue, N) are directly stimulated by environmental conditions and transduce a signal to muscle (red, Mu) via EGL-30, and possibly the UNC-13 and UNC-64 synaptic transmission proteins. Excitation of muscle by neuronally expressed (and possibly also by muscle-expressed) EGL-30 leads to activation of downstream components that include EGL-19 voltage-gated calcium channels. The muscle cells provide a modulatory signal that may upregulate BAR-1 activity in P6.p (grey cell, VPC) to cooperate with LIN-3 from the anchor cell (green, AC) to facilitate vulval induction. Hyp, hypodermis; Int, intestine.

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 Gαq (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 is close to P7.p., which might be close enough to affect vulval induction directly (Fig. 4). However, because the *unc-4*-expressing VA and DA motor neurons innervate both ventral and dorsal body-wall muscles, respectively (White et al., 1986), it is also possible that the post-embryonic muscles can affect vulva development from a distance. At present, there is no reason to think that the post-embryonic body-wall 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 vulvaless 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 of *egl-30(tg26gf)* to suppress the vulvaless phenotype of *let-23(sy1)* (Table 4). This sensitivity of *egl-30(tg26gf)* to *bar-1(mu63)* is specific because suppression of *let-23(sy1)* by the RAS pathway mediator component *dpy-22* is not affected by *bar-1(mu63)* (Moghal and Sternberg, 2003a). These data suggest that one possible model in which EGL-30 promotes vulval induction is by upregulating *bar-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 vulvaless phenotype of *let-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 the *egl-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 vulvaless mutations in the RAS pathway, but not the *bar-1(ga80)* null mutation, and that the *bar-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 the *let-23* pathway cause more severe vulvaless 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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