Distinct roles of transcription factors EGL-46 and DAF-19 in specifying the functionality of a polycystin-expressing sensory neuron necessary for *C. elegans* male vulva location behavior

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Summary

Caenorhabditis elegans **polycystins LOV-1 and PKD-2 are expressed in the male-specific HOB neuron, and are necessary for sensation of the hermaphrodite vulva during mating. We demonstrate that male vulva location behavior and expression of *lov-1* and *pkd-2* in the ciliated sensory neuron HOB require the activities of transcription factor EGL-46 and to some extent also EGL-44. This EGL-46-regulated program is specific to HOB and is distinct from a general ciliogenic pathway functioning in all ciliated neurons. The ciliogenic pathway regulator DAF-19 affects downstream components of the HOB-specific program indirectly and is independent of EGL-46 activity. The sensory function of HOB requires the combined action of these two distinct regulatory pathways.**

Key words: Transcriptional regulation, Cell specification, Zinc finger proteins, TEF, RFX factors, Polycystins

Introduction

Because of its simple nervous system with invariant cell lineage and position, *C. elegans* provides an excellent model to study how diverse neuronal subtypes are specified (Sulston and Horvitz, 1977; Sulston, 1983). The anatomy and interconnectivity of all 118 hermaphrodite neuron types are known (White, 1986), as are the molecular details of many neuronal subtypes (Chalfie, 1995). The *C. elegans* male has 79 additional neurons, falling into 37 classes (Sulston et al., 1980). Most of those male-specific neurons are located in the tail region and contribute to specific motor output during mating behavior (Sulston and White, 1980; Loer and Kenyon, 1993; Liu and Sternberg, 1995; Garcia et al., 2001; Garcia and Sternberg, 2003).

During mating, the *C. elegans* male scans for the vulva by touching the hermaphrodite with the ventral side of his tail and backing along her body. If the vulva is not found, he turns at the hermaphrodite head or tail and scans the other side (Liu and Sternberg, 1995). The male hook sensillum is a copulatory structure that is located just anterior to the cloaca and mediates vulval location behavior (Liu and Sternberg, 1995). Intact wild-type males usually stop at their first or second vulval encounter. When the hook sensillum is ablated, operated males circle the hermaphrodite multiple times and fail to stop at the vulva (Liu and Sternberg, 1995). This defect is referred to as the Lov (location of the vulva defective) phenotype (Barr and Sternberg, 1999). The hook sensillum consists of five cells, including a structural cell and two ciliated sensory neurons HOA and HOB (Sulston et al., 1980). The two hook neurons have large nuclei and send dendrites into the hook structure; however, their anatomy can be distinguished by cell morphology and synaptic contacts (Sulston et al., 1980). Ablation of either HOA or HOB results in a Lov phenotype, indicating that HOA and HOB have non-redundant functions (Liu and Sternberg, 1995).

The *C. elegans* homologues of human autosomal dominant polycystic kidney disease genes *PKD1* (*lov-1*) and *PKD2* (*pkd-2*) are expressed in the HOB hook neuron (Barr and Sternberg, 1999; Barr et al., 2001; Kaletta et al., 2003). Human PKD genes, which encode divergent members of the TRP family of cation channels, possibly act in signal transduction important for renal epithelial differentiation, as mutations in PKD1 and PKD2 are associated with pathogenic renal cyst formation (reviewed by Wu, 2001). In *C. elegans*, *lov-1* and *pkd-2* mutations disrupt vulva location behavior, consistent with a defect in HOB sensory function (Barr and Sternberg, 1999; Barr et al., 2001). Although LOV-1 and PKD-2 are localized in sensory cilia and cell bodies, the ultrastructure of cilia and dendrites appears normal in *lov-1* and *pkd-2* mutants (Barr et al., 2001).

Another class of genes required for vulva location affects the formation of ciliated endings in sensory neurons. This class includes *che-3*, *daf-10*, *osm-5* and *osm-6* (Barr and Sternberg, 1999). *che-3*, *osm-5* and *osm-6* are required for most or all sensory cilia (Lewis and Hodgkin, 1977; Perkins et al., 1986), while *daf-10* only functions in a subset of ciliated sensory neurons (Albert et al., 1981). The hermaphrodite expression of *osm-5*, a homolog of the mouse autosomal recessive polycystic

kidney disease (ARPKD) gene (Haycraft et al., 2001; Qin et al., 2001), and *osm-6* has been shown to be regulated by a RFX transcription factor DAF-19, which plays a critical role in general sensory cilium differentiation (Swoboda et al., 2000; Haycraft et al., 2001).

We report the isolation of an allele of *egl-46*, a putative zinc-finger transcription factor, in a screen for loci required for fate specification of *C. elegans* hook neuron HOB. *egl-46* was previously characterized as a gene when mutated affecting the development of two mechanosensory neurons (FLP cells) (Wu et al., 2001), as well as having defects in the hermaphrodite HSN egg-laying motoneurons (Desai et al., 1988; Desai and Horvitz, 1989). We demonstrate that EGL-46 and the transcription enhancer factor (TEF) homolog EGL-44 are expressed in the HOB hook neuron and are required for expression of genes encoding polycystins LOV-1 and PKD-2, homeodomain protein CEH-26, and neuropeptide-like protein NLP-8. *egl-44* and *egl-46* mutants are defective in vulva location behavior during mating, suggesting compromised normal HOB function. This HOB-specific pathway is distinct from the DAF-19-regulated general cilia formation pathway in sensory neurons. We found that *daf-19* acts independently of *egl-44* and *egl-46* to affect expression of downstream genes in the HOB-specific program, indicating that general and cell-specific regulatory factors work in concert to establish cell-specific features crucial for HOB neuronal function in sensory behavior.

Materials and methods

Strains

Nematodes were cultured at 20°C as described (Brenner, 1974). All strains used contain *him-5(e1490)* V to obtain males, except for *egl-46(n1127)*, in which case we used *him-8(e1489)* IV (Hodgkin et al., 1979). The following alleles were used in this study: *daf-19(m86)* II (Perkins et al., 1986); *egl-44(n1080)* II, *egl-46(n1127)* V (Desai and Horvitz, 1989); *pha-1(e2123ts)* III (Schnabel and Schnabel, 1990); *unc-119(ed4)* III (Maduro and Pilgrim, 1995); *unc-31(e169)* IV, *unc-46(e177)* V, *dpy-11(e224)* V (Brenner, 1974); *unc-68(e540)* V (Lewis et al., 1980); *unc-42(e270)* V (Riddle and Brenner, 1978); *osm-5(p813)* X (Dusenbery, 1980); and *lin-15(n765ts)* X (Ferguson and Horvitz, 1985). Integrated GFP fusions or extrachromosomal GFP arrays were: *nIs133 (pkd-2::gfp)* I, *nIs128 (pkd-2::gfp)* II (H. Schwartz and H. R. Horvitz, personal communication); *mnIs17 (osm-6::gfp)* V (Collet et al., 1998); *syEx301 (lov-1::gfp)* (Barr and Sternberg, 1999); *myEx256 (osm-5::gfp)* (Qin et al., 2001); *rtEx227 (nlp-8::gfp)* (Nathoo et al., 2001); and *saEx490 (daf-19::gfp)* (Swoboda et al., 2000).

ceh-26 gfp construct

A 6.4 kb fragment of *ceh-26* containing 5277 bp 5′ flanking sequence plus coding sequence to the fourth exon was amplified by long-range PCR using primers P26-22 (GTCCTTTGGCCAATCC-CGGGGATCCAGAGCTACTGTTACTTTCAGGGC) and P26-23 (GCCTGCAGAACATTGGCATGTGGCGTCACGGG). *Bam*HI-digested pPD95.77 was joined to the *ceh-26* fragment by primer extension and linear amplification (Cassata et al., 1998). The product was cut with *Pst*I and circularized to give plasmid pRFP7. pRFP7 (100 ng/μl) was co-injected with *dpy-20* (20 ng/μl) into *dpy-20(e2017)* hermaphrodites as described (Mello et al., 1991). Integration of a transgenic line yielded strain TB1200 with *ceh-26::gfp* integrated transgene *chIs1200* linked to chromosome III. *chIs1200* was crossed into *him-5(e1490)* to yield strain TB1225.

Mapping, cloning, and complementation test

The *sy628* allele was generated by mutagenizing the strain TB1225 carrying the HOB marker *ceh-26::gfp* with EMS using standard protocols (Rosenbluth et al., 1983). In particular, we picked males descended from each single hermaphrodite daughter of mutagenized parents and examined them under a conventional epi-fluorescence microscope for GFP expression. Three-factor mapping of *sy628* on linkage group V used alleles of *unc-46*, *dpy-11*, *unc-68* and *unc-42*: *unc-46* (16/16 recombinants) *dpy-11* (0/16 recombinants) *sy628*; *dpy-11* (0/44) *sy628* (44/44) *unc-42*; *dpy-11* (4/10) *sy628* (6/10) *unc-68*. During the mapping experiments, the presence of *sy628* mutation was determined by loss of *ceh-26::gfp* expression in HOB.

The ~0.6 map unit interval between *dpy-11* and *unc-68* was covered by 17 cosmids, including 97 identified genes or predicted coding sequences (www.wormbase.org, version WS74). The *sy628* hermaphrodites had a mild egg-laying defective (Egl) phenotype. A previously identified gene associated with an Egl phenotype, *egl-46*, is located in the middle of that interval. Cosmid K11G9, which contains the entire *egl-46* locus, was injected into the strain PS3568 *ceh-26::gfp*; *egl-46(sy628) him-5(e1490)* at 40 ng/μl using *pmyo-2::gfp* plasmid pPD118.33 (5.5 ng/μl) as co-transformation marker (Mello et al., 1991). Three stable lines were obtained from individual F1 progeny that expressed *myo-2::gfp* in pharynx. Injection of cosmid K11G9 restored the *ceh-26::gfp* expression in HOB in 76/81 males from three independent transgenic lines. Injection of another cosmid in the same interval, F44C4, which contains a different predicted zinc-finger transcription factor, showed no rescue of HOB expression of *ceh-26::gfp* in fourteen stable transgenic lines (n=172). Those transgenic lines had a non-sex-specific ectopic expression of *ceh-26::gfp* in a neuron anterior to HOB, most likely PVT. It is not clear that this ectopic expression is due to injected F44C4 cosmid or interaction between *pmyo-2::gfp* plasmid and F44C4 cosmid. Cosmids were obtained from the Sanger Institute (Cambridge, UK).

To test for complementation, PS3568 *ceh-26::gfp*; *egl-46(sy628) him-5(e1490)* males were crossed to MT2316 *egl-46(n1127)* hermaphrodites. F1 hermaphrodites with CEH-26::GFP expression were cross progeny, and were examined for an Egl phenotype. F1 males were analyzed by HOB expression of *ceh-26::gfp*. All 79 *sy628/n1127* heterozygous males examined lacked *ceh-26::gfp* expression in HOB, and heterozygous hermaphrodites were Egl. Thus, *sy628* and *n1127* fail to complement.

PCR and sequencing

A 2318 bp genomic DNA fragment containing the entire *egl-46* coding region was PCR amplified from *sy628* mutant DNA using the pair of primers 5′-CTCCCCTTCTTGTAAGGTGTCTT-3′ and 5′-AATTCACTCAGCAATTTGGAAAA-3′. The PCR products from six independent PCR reactions were separately purified using QIAquick PCR purification kit and were pooled together for direct sequencing. Two nested primers, 5′-TTTCGTTCACATCTACCGTAACC-3′ at the 5′ end of the gene and 5′-CGGGGGAAATTGTAAAGAGTTAG-3′ at the 3′ end, and two internal primers, the reverse primer 5′-CCTCTTATGTGCCTTCGTTTTG-3′ at 109-131 bp of the intron 2 and the forward primer 5′-GCTAATGACACCGAGAAAACGAAC-3′ at 274-297 bp of the same intron, were used for sequencing. This sequencing therefore did not cover the 189 bp gap in the intron 2 between reverse and forward primers. The PCR primers and two outside sequencing primers were picked by an oligo design program in the *C. elegans* genome project at the Sanger Institute (www.sanger.ac.uk/Projects/C_elegans/). The two internal sequencing primers were obtained using Macvector software (Oxford Molecular Group). The G-to-A lesion site at nucleotide 165 of the first exon was observed in both strands.

Transgenics

The N-terminal *cfp::egl-46* translational fusion plasmid TU#627 and *yfp::egl-44* fusion plasmid TU#628 were kindly provided by Ji Wu and Martin Chalfie. Plasmid DNAs of TU#627 and TU#628 were

Table 1. HOB gene expression in wild type, *egl-46* and *egl-44* males

Genotype	Marker gene	GFP expression in the HOB neuron			*n*
		Normal	Decreased	Absent	
Wild type	*ceh-26::gfp**	100%			>1000
egl-46(sy628)				100%	>1000
egl-46(n1127)			2%	98%	118
egl-44(n1080)			51%	49%	99
Wild type	*lov-1::gfp*†	93%		7%	113
egl-46(sy628)		1%		99%	92
egl-44(n1080)		21%		79%	75
Wild type	*pkd-2::gfp(nIs128)**	100%			>200
egl-46(sy628)		4%	1%	95%	81
Wild type	*pkd-2::gfp(nIs133)**	100%			>200
egl-46(sy628)		8%	5%	87%	123
egl-46(n1127)		2%	2%	96%	131
egl-44(n1080)		87%	11%	2%	89
Wild type	*nlp-8::gfp*†	96%		4%	98
egl-46(sy628)			49%	51%	97
egl-44(n1080)		77%	16%	7%	87
egl-44(n1080); egl-46(sy628)		17%	59%	24%	93
Wild type	*osm-6::gfp**	100%			>200
egl-46(sy628)		100%			79
egl-44(n1080)		100%			104
Wild type	*osm-5::gfp*†	96%		4%	80
egl-46(sy628)		100%			69
egl-46(n1127)		94%		6%	83
egl-44(n1080)		98%		2%	102

*Integrated transgenes
†Extrachromosomal arrays; a few animals lack expression in HOB because of mosaicism.

injected separately into the strain *unc-119(ed4); him-5(e1490)* at 49 ng/µl. We used 50 ng/µl of pDP#MM016B, a plasmid containing a wild-type copy of the *unc-119* gene, as the co-injection marker. Transgenic animals were recognized by rescue of the Unc phenotype of *unc-119* (Maduro and Pilgrim, 1995). Three independent lines were obtained for each construct and the male expression pattern in those lines was characterized. Transgenic animals generated with the same CFP and YFP plasmids but with *myo-2::gfp* as a transformation marker had similar expression patterns in the male tail.

Mating assay (Vulva location behavior)

The mating behavior of mutant or control males was observed with sluggish *unc-31* adult hermaphrodites. All males were isolated from hermaphrodites at the L4 stage and were kept on fresh plates in groups of ~30 animals before observation. For the mating assay, a virgin adult male (12-36 hr post L4 lethargus) was placed on a 0.5 cm bacterial lawn with five 24-hour-old *unc-31* hermaphrodite adults (Barr and Sternberg, 1999; Garcia et al., 2001). Each individual male was watched under a Zeiss Stemi SV11 or Wild M420 'Macroscope' for ten vulva encounters or until he stopped at the vulva (pausing for more than 1 second or inserting his spicules), whichever came first. The vulva location efficiency of individuals for a population was calculated as described by Barr and Sternberg (Barr and Sternberg, 1999). To facilitate calculation, the vulva location efficiency of males with more than 10 vulva encounters (pass all ten vulva encounters) was considered to be 0 (actual value≤1/11). The Wilcoxon (Mann-Whitney) test was used to determine statistical significance.

Microscopy

GFP expression was analyzed by conventional fluorescence microscopy (Zeiss Axioskop) using a Chroma Technology High Q GFP long-pass filter set (450 nm excitation, 505 nm emission). CFP and YFP were visualized using a Chroma Technology CFP filter set '31044v2' (exciter D436/20, emitter D480/40, beamsplitter 455dclp) and an YFP set '41029' (exciter HQ 500/20, emitter HQ520lp, beamsplitter Q515lp).

Results

Mutations of *egl-46* affect gene expression in the hook neuron and disrupt vulva location behavior in male mating

The male tail is remodeled during the L4 stage, undergoing a series of changes in cell shape and position (Sulston et al., 1980). By the late L4 stage, most of the cells that function in adults reach their final locations, and initiate morphological changes to form the adult tail structures. At this stage, a homeodomain-containing putative transcription factor *ceh-26* (Bürglin, 1994) begins to be expressed and perdures through the adulthood in the HOB hook neuron (Fig. 1A1,A2). Therefore, the presence of CEH-26::GFP indicates a differentiated neuronal fate of HOB. Non-sex-specific expression of *ceh-26::gfp* is mostly in nuclei of the head (R. F. P. and T. R. B., unpublished). To identify genes involved in HOB fate specification, we performed a screen for mutants with altered expression of *ceh-26::gfp* in the HOB cell. This pattern allows for a rapid visual inspection of GFP fluorescence under a compound microscope in the male tail.

One of the mutants recovered from this screen, *sy628*, failed to express *ceh-26::gfp* in the HOB neuron of homozygous males with complete penetrance (Fig. 1A3; Table 1). No effect on non-sex-specific *ceh-26::gfp* expression (e.g., the head nuclei) was observed in *sy628* animals, suggesting that the *sy628* mutation does not cause a general defect in expression of GFP transgenes or of *ceh-26* (data not shown). Anatomical examination of *sy628* males at the third and the fourth larval stages showed that P10.ppap, the presumptive HOB neuron in wild-type animals, was present and occupied its normal position in *sy628* mutants. In addition, the hook structure and overall tail morphology appeared normal under Nomarski

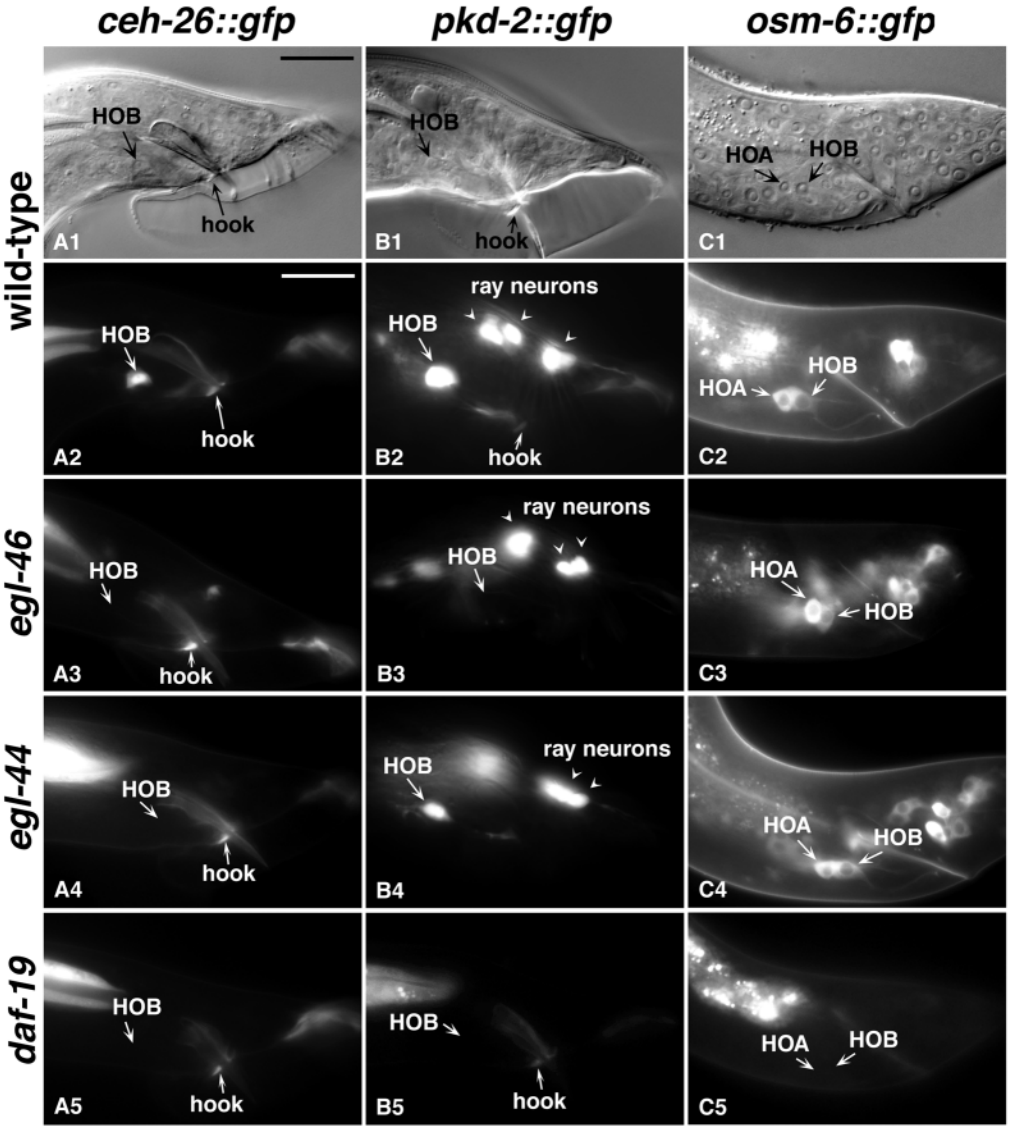

Fig. 1. HOB gene expression in wild-type and mutant males. Left lateral views (anterior leftwards, ventral downwards). Scale bar: 20 μm. (A1,A2) Expression of *ceh-26::gfp* in the HOB neuron of a wild-type adult male. Absence of fluorescence in an *egl-46(sy628)* mutant (A3), an *egl-44(n1080)* mutant (A4) and a *daf-19(m86)* mutant (A5). (B1,B2) HOB and ray expression of *pkd-2::gfp* was observed in a wild-type adult male. (B3) An *egl-46(sy628)* male with ray but not HOB expression. (B4) An *egl-44(n1080)* male with expression in both HOB and ray cells. (B5) No visible expression in both HOB and rays of a *daf-19* mutant. (C1,C2) Normal *osm-6::gfp* expression in HOA and HOB at the L4 stage. Expression was not affected in *egl-46(sy628)* (C3) and *egl-44(n1080)* mutants (C4). (C5) No expression was observed in HOA and HOB cells of a *daf-19(m86)* mutant male. Cell positions of HOB in A3,A4,A5,B3,B5, and HOA and HOB in C5 were located by overlaying with the Nomarski pictures of the same animal. Hook structure autofluorescence is indicated by small arrows. The original *osm-6::gfp* strain has a *ncl-1(–)* background. *ncl-1(–)* was still present in the *him-5* strain of *osm-6::gfp* integrant (C1,C2) and an *egl-46(sy628)* mutant background (C3), but was crossed out in *egl-44(n1080)* (C4) and *daf-19(m86)* mutants (C5). *egl-46(sy628)* mutant also had a *dpy-11* mutation in the background (C3). No effect on *osm-6::gfp* expression was detected for *ncl-1(–)* and *dpy-11* mutations.

optics. Initial observations indicated that *sy628* males had a decreased mating efficiency. Analysis of their mating behavior determined that *sy628* males were deficient in vulval location (the Lov phenotype), but had no obvious defect in other steps of mating, such as response, turning, spicule insertion, or sperm transfer. About 97% of wild-type control males stopped at the vulva during the first two vulva encounters (88% vulva location efficiency), as opposed to only 39% of *sy628* males (Fig. 2A). On average, *sy628* males required more than five encounters to find the vulva, with an overall vulva location efficiency of 36%.

We mapped *sy628* to linkage group V between *dpy-11* and *unc-68*, and identified it as an allele of *egl-46* (see Materials and methods). *egl-46* encodes a putative C2H2-type zinc-finger transcription factor homologous to human and mouse IA1 protein, mouse MLT1 protein and *Drosophila* Nerfin 1 protein (Wu et al., 2001). The lesion in *sy628* mutants was a G-to-A transition at position 165 of the first exon (161-TCTGGAACCCAACGC-175), which changes a tryptophan codon UGG to an UGA opal stop codon. This residue is located at position 55 out of 286 of the inferred EGL-46 protein, before the putative glutamine-rich transcriptional activation domain (residues 61 to 75) and other conserved domains (Wu et al., 2001). This early stop is not necessarily a null allele.

We confirmed the male phenotypes of the *egl-46* mutant using a different allele, *n1127*, which alters the splicing donor of intron 2, located before the region encoding the three zinc fingers of EGL-46 protein (Wu et al., 2001). *n1127* and *sy628* failed to complement (see Materials and methods). Desai and Horvitz (Desai and Horvitz, 1989) found that *n1127* has a decreased male mating efficiency (~50%). We observed that *n1127* males had a Lov phenotype similar to *sy628* mutants (Fig. 2B). The vulva location efficiency of *n1127* males was 39% (n=17), compared with 94% (n=16) for the control males.

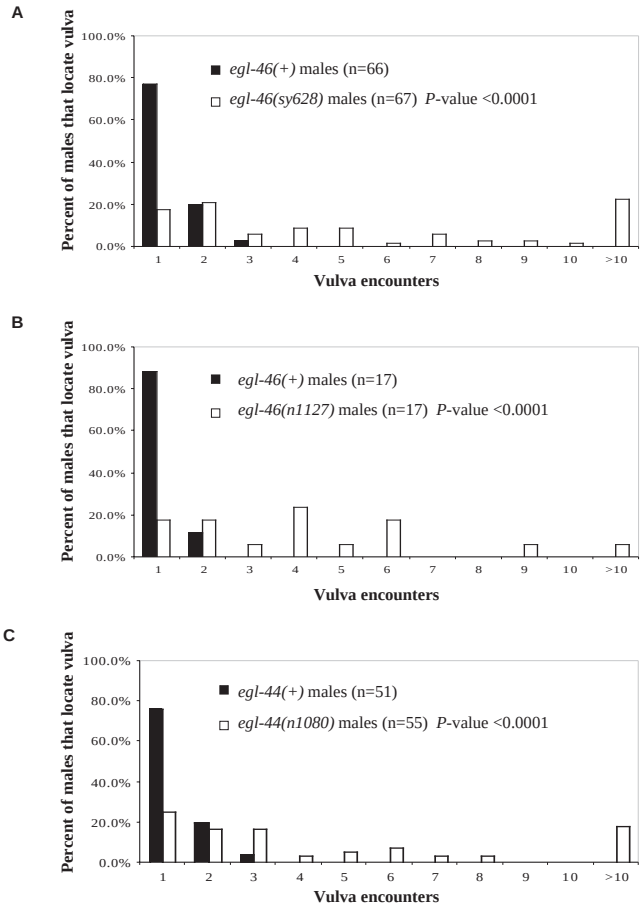

Fig. 2. Vulval location behavior. The *x*-axis represents the number of vulva encounters measured until a tested male stopped at the hermaphrodite vulva. The *y*-axis represents the distribution of males in the tested group that located the vulva at each vulva encounter. (A) *egl-46(+)* versus *egl-46(sy628)*. Both strains have *ceh-26::gfp* III; *him-5(e1490)* V in the background. (B) *egl-46(+)* versus *egl-46(n1127)*. Strains in B contain *him-8(e1489)* IV. (C) *egl-44(+) vs. egl-44(n1080)*. Animals in C are all with *him-5(e1490)* V. In each assay, similar number of wild-type control males and mutant males were examined at same time using the same microscope.

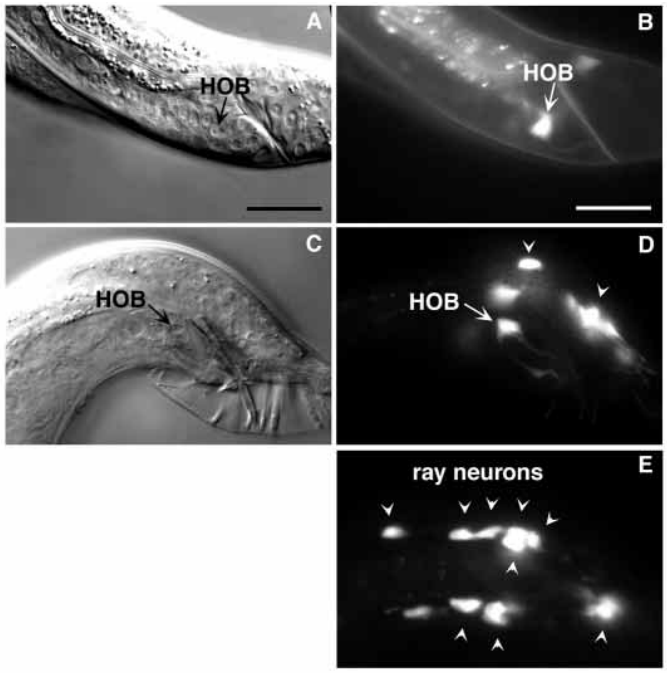

Fig. 3. *egl-46::cfp* expression in HOB and ray neurons of the male tail. Nomarski (A) and fluorescence (B) images of an L4 male with expression in HOB (arrows). Nomarski (C) and fluorescence (D) images of an adult male with CFP expression in HOB (arrows) and ray neurons (arrowheads). (E) Ventral view (left side upwards) of an adult male tail with CFP expression in some ray neurons of both sides (arrowheads). Not all the ray neurons are in the same focal plane. Scale bars: 20 μm. Left lateral views.

There was a marked decrease of *ceh-26::gfp* expression in *n1127* HOB neurons (Table 1). Only two out of 118 *n1127* homozygous males examined retained a faint GFP expression in HOB. No altered expression of *ceh-26::gfp* was detected in cells other than HOB in *n1127* mutants.

egl-46 regulates cell-specific expression of *lov-1* and *pkd-2* to specify the behavioral function of the HOB neuron

The hermaphrodite expression pattern of *egl-46* has been described by Wu et al. (Wu et al., 2001). Using an *egl-46::cfp* construct, we analyzed its expression in males and found a similar pattern for non-sex-specific expression (such as the FLP cells, ventral cord neurons and PVD). Both HOA and HOB are born from a single precursor cell (P10.p) at the late L3 stage, and they differentiate into their neuronal fates during the L4 stage. *egl-46::cfp* was expressed in the HOB neuron beginning at the L4 stage and continuing throughout adulthood (Fig. 3A-D), consistent with the timing of HOB differentiation, and a potential role in the maintenance of HOB function. No detectable expression was seen in the HOA hook neuron. The *egl-46* mating defect is reminiscent of ablation of a hook neuron (Liu and Sternberg, 1995). Based on expression of *egl-46* gene in a single hook neuron, we infer that the Lov phenotype of *egl-46* mutant males is probably due to impaired HOB function.

The dependence of *ceh-26::gfp* expression on EGL-46 activity suggested that the defective HOB sensory behavior caused by an *egl-46* mutation could result from loss of HOB-specific gene expression. The *C. elegans* polycystin genes *lov-1* and *pkd-2* are expressed in HOB and are required for vulva location (Barr and Sternberg, 1999) (Table 1; Fig. 1B1,B2). To test whether *egl-46* regulates these two genes, we used GFP transgenes to visualize their expression in an *egl-46(sy628)* mutant background. *sy628* mutants lacked expression of *lov-1::gfp* in the HOB neuron (Table 1): only one out of 92 animals examined had detectable expression. The expression of *pkd-2::gfp* in HOB was also greatly reduced by the *sy628* and *n1127* mutations of *egl-46* (Table 1; Fig. 1B3). A neuropeptide-like protein-encoding gene, *nlp-8*, is also expressed in HOB as well as in the non-sex-specific neuron PVT in the tail (Nathoo et al., 2001). The PVT expression of *nlp-8::gfp* was not affected by *egl-46* mutations; however the HOB expression of *nlp-8::gfp* was absent in about half of *egl-46(sy628)* males and was decreased in the remainder (Table 1). Therefore, EGL-46 activity is necessary for the HOB expression of all three genes,

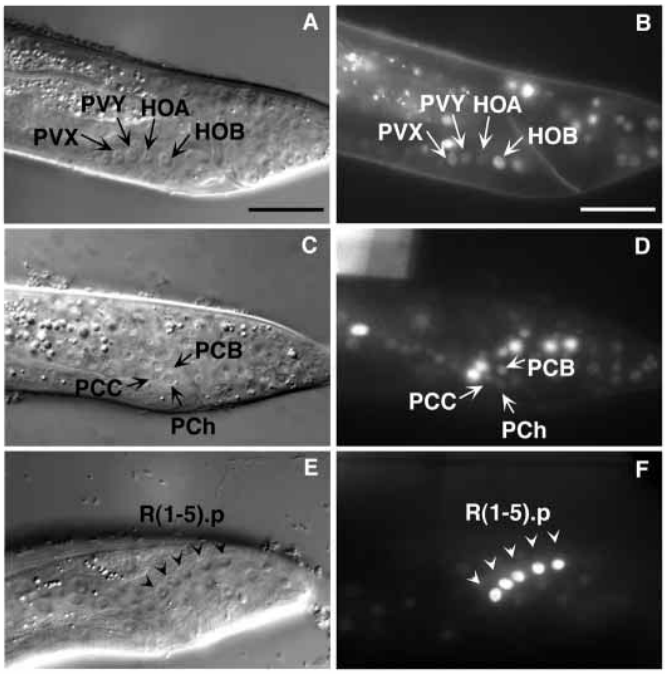

Fig. 4. *egl-44::yfp* expression in the male tail. Left lateral view. Scale bars: 20 μm. (A,B) Different levels of YFP expression in PVX, PVY, HOA and HOB (arrows). In this particular animal, HOA has extremely faint YFP fluorescence (in most cases, YFP expression is undetectable in the HOA hook neuron; data not shown). (C,D) An L4 male with faint YFP expression in the PCB, PCC and PCh cells of the left postcloacal sensilla, in addition to cells from ray lineage in the background. (E,F) Bright expression in hypodermal R1.p, R2.p, R3.p, R4.p, and R5.p at the left side (arrowheads).

and the lack of *lov-1* and *pkd-2* expression could account for the mating defect of *egl-46* mutants.

We also observed male-specific *egl-46::cfp* expression in ciliated ray neurons. The *C. elegans* male has nine pairs of rays (ray 1-9 for both the left and right sides), each associated with an A-type neuron and a B-type neuron (RnA and RnB, *n*=1-9) (Sulston et al., 1980). *egl-46::cfp* was observed in one of the two ray neurons for each ray (Fig. 3D,E); this neuron is probably a B-type neuron because of its co-localization with *pkd-2::gfp* (data not shown), which is known to be expressed in these neurons (Barr and Sternberg, 1999). However, *egl-46* regulation was not necessary for *lov-1* and *pkd-2* expression in ray neurons (Fig. 1B3). *lov-1* and *pkd-2* mutants show deficiencies in both response and vulva location during mating, correlating with their expression in the B-type ray neurons (except ray 6) and the HOB hook neuron (Barr and Sternberg, 1999; Barr et al., 2001). By contrast, despite *egl-46* expression in ray neurons, no obvious defect in either ray neuron expression of PKD genes or response behavior of the mating was detected in *egl-46* mutant males. *egl-46* might play a major role in HOB sensory specification, but some other factors function in ray neurons.

egl-44 exhibits a similar Lov defect for male mutants and may regulate gene expression in HOB

Wu et al. (Wu et al., 2001) reported that *egl-46* acts with *egl-44* to specify subtypes of mechanosensory neurons, and for HSN development in hermaphrodites (Desai and Horvitz, 1989). *egl-44* encodes a transcription enhancer factor of the TEA domain class (Bürglin, 1991) and is orthologous to the mammalian TEF factors (Wu et al., 2001). We therefore examined the behavior of *egl-44(n1080)* males, and found that this *egl-44* mutation reduced vulva location behavior (Fig. 2C). Similar to *egl-46* mutants, *egl-44* mutant males passed the vulva frequently and it took an *egl-44(n1080)* male about five encounters on average to locate the vulva. Specifically, *egl-44* mutant males had an overall 43% vulva location efficiency, while control males (wild-type for the *egl-44* locus) had an 88% vulva location efficiency.

We determined the male tail expression pattern of the *egl-44* gene with the *yfp* construct described by Wu et al. (Wu et al., 2001). Expression of *egl-44* overlapped with but was not identical to that of *egl-46*. At the L4 stage, the four neurons PVX, PVY, HOA and HOB are positioned in a signature anterior-to-posterior row at the middle left side (Sulston et al., 1980). *egl-44::yfp* fluorescence was obvious in HOB, PVX and PVY, with HOB usually the brightest, but was barely visible in HOA (Fig. 4A,B). As stated above, *egl-46::cfp* was only present in HOB. A few neurons anterior to PVX (e.g. PVV) had faint *egl-44::yfp* expression, as did several cells from the B and Y lineage, including PCB, PCC and PCh (Fig. 4C,D). These cells did not express *egl-46::cfp*. In addition, almost all the descendants of the ray precursor cells (Rn) expressed *egl-44::yfp*, including the ray neurons (RnA and RnB) and the ray structure cells (Rnst), all of which are derived from the anterior daughter Rn.a, as well as posterior daughter Rn.p hypodermal cells (Fig. 4E,F; data not shown). EGL-46 showed a more limited expression in the ray lineage. In adults, *egl-44::yfp* was still expressed in HOB, RnA, RnB and Rnst cells. Hypodermal Rn.p cells no longer displayed bright YFP expression in adults, possibly because of their fusion with the tail hypodermal syncytium. Owing to dramatic changes in cell shapes and positions during the extensive male tail remodeling at the L4-adult transition, the faint *egl-44::yfp* expression in PCB, PCC and PCh was hard to trace in adults. Overall, *egl-44::yfp* was expressed more extensively in the male tail than was *egl-46*. However, a mutation in *egl-44* did not result in broader defects in male mating behavior than did an *egl-46* mutation.

Based on its behavioral phenotype and its expression in HOB, *egl-44* might regulate HOB fate specification, similar to *egl-46*. We therefore examined HOB-specific gene expression in an *egl-44(n1080)* mutant background, and found that *egl-44* mutants displayed a significant decrease in HOB-specific expression of *ceh-26::gfp*. 50% (49/99) of *egl-44(n1080)* males lacked *ceh-26::gfp* in HOB, while the remaining 50% (50/99) had weak HOB expression (Fig. 1A4; Table 1). Reduction of *lov-1::gf*p expression in HOB by an *egl-44* mutation was striking, but only a small effect on *pkd-2* and *nlp-8* expression was observed (Table 1; Fig. 1B4). The lesion in *egl-44(n1080)* allele is a missense mutation. It is possible that the residual EGL-44 activity in *n1080* mutants leaded to an incomplete reduction of HOB gene expression. *egl-44* has six differently spliced isoforms (www.wormbase.org, version WS74). The *n1040* mutation affects four of them. Currently, we have no information about which isoform might be dominate in the HOB neuron. Expression of the ‘c’ form *egl-44* cDNA under control of the 3.1kb *egl-46* promoter gave an ambiguous result, with only about 10% restoration of *ceh-26::gfp* expression in

HOB in each of three transgenic lines (data not shown). *egl-44* mutants were not defective in ray B neuron expression of *lov-1* and *pkd-2*.

Even though the *egl-46* mutations caused a more severe defect in HOB gene expression than did an *egl-44* mutation, the Lov phenotypes are similar in male mutants. One possibility is that incomplete decrease of gene expression in the HOB neuron by the *egl-44* mutation could reduce the HOB function enough to display a comparable Lov phenotype; however, we cannot rule out the possibility that EGL-44 and EGL-46 might have some distinct targets in HOB. In addition, the faint EGL-44 expression in the HOA hook neuron, as well as in the PCB and PCC neurons of the postcloacal sensilla, might also contribute to the vulva location activity (Liu and Sternberg, 1995). The Lov phenotype is not synergistic in the *egl-44; egl-46* double mutant, and there was no observable difference in the efficiency of vulva location compared with single mutants (data not shown). By contrast, *C. elegans* males with HOB ablated have a 0% vulva location efficiency (Liu and Sternberg, 1995). Both *egl-44* and *egl-46* mutants had an incomplete loss of *nlp-8::gfp* expression, but no further elimination of *nlp-8::gfp* expression was seen in an *egl-44; egl-46* double mutant background (Table 1). This lack of enhancement for the Lov phenotype and a defect in *nlp-8* expression indicates that *egl-44* and *egl-46* act at least partially in a common pathway for HOB specification. The *egl-44; egl-46* double mutant males seemed less active than each of the single mutants and took longer to initiate mating behavior, which might be due to insufficient function of the ray neurons in the double mutant.

egl-44 and *egl-46* do not regulate each other's expression in the HOB neuron

In the non-sex-specific FLP cells, wild-type *egl-44* is required for normal *egl-46* expression (Wu et al., 2001). To determine whether *egl-44* and *egl-46* regulate each other's expression in the HOB neuron, we introduced an extrachromosomal *egl-46::cfp* array into an *egl-44* mutant, and an *egl-44::yfp* array into an *egl-46* mutant. The timing and relative brightness of *egl-46::cfp* expression in HOB was not affected in an *egl-44(n1080)* mutant background compared with a wild-type background, but CFP expression in FLP neurons was reduced. Similarly, no change in the HOB expression of *egl-44::yfp* was observed in *egl-46(sy628)* males. We infer that there is no interdependence of *egl-44* and *egl-46* expression in HOB.

The *daf-19* general cilium formation pathway is required for cell-specific features of HOB

Genes that are expressed in HOB and mutate to a Lov phenotype can be grouped into two separate pathways (Barr and Sternberg, 1999) (this work). *osm-5* and *osm-6* belong to a general ciliogenic pathway common to all ciliated neurons, including HOA and HOB (Collet et al., 1998; Qin et al., 2001). The other genes discussed above, including *egl-44*, *egl-46*, *lov-1* and *pkd-2*, define a program specific for HOB differentiation. We thus asked if there are any interactions between these two pathways; i.e., whether regulators in the cell-specific pathway, *egl-44* and *egl-46*, affect the HOB expression of the general cilium structure genes (*osm-5* and *osm-6*), and whether ciliogenesis might be a prerequisite for execution of an HOB-specific program.

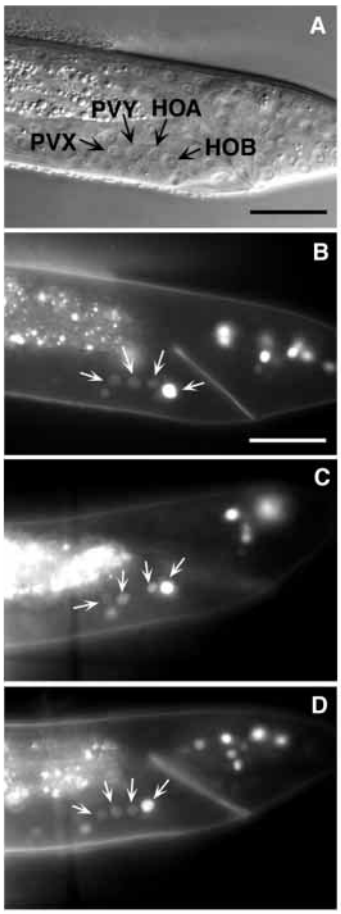

Fig. 5. *daf-19::gfp* expression in the hook neurons. Left lateral view. Scale bars: 20 μm. Nomarski (A) and fluorescence (B) images of a wild-type male tail at the fourth larval lethargus. *daf-19::gfp* expression in HOB is significantly stronger than that in HOA, PVX and PVY. The same expression pattern was present in *egl-46(sy628)* (C) and *egl-44(n1080)* mutant males (D) (arrows).

In wild-type males, OSM-5::GFP and OSM-6::GFP are expressed in the cell bodies and dendrites of HOA and HOB at the late L4 stage; their then expression decreases, which is coincident with the formation of ciliated sensory endings in these two neurons (Collet et al., 1998; Qin et al., 2001). Using an integrated *osm-6::gfp* line (*mnIs17*) and an extrachromosomal array carrying *osm-5::gfp*, we found that the HOB expression of these two GFPs at the L4 stage in *egl-44(n1080)* and *egl-46(sy628)* mutants was comparable with wild-type (Table 1; Fig. 1C1-C4). *osm-5::gfp* expression in HOA and HOB was also not affected by *egl-46(n1127)* (Table 1). In these *egl-44* and *egl-46* mutant males, the HOB dendritic process, visualized by *osm-5::gfp* or *osm-6::gfp*, was extended correctly into the male hook. Neither *egl-44(n1080)* nor *egl-46(sy628)* mutants had dye-filling defects (data not shown). We conclude that mutation of either *egl-44* or *egl-46* impedes neither gross cell morphology nor the ultimate neuronal outgrowth and wiring of HOB.

Qin et al. (Qin et al., 2001) showed that an *osm-5* mutation affects subcellular localization of LOV-1 and PKD-2, but not their expression. We found that *ceh-26::gfp* expression was not affected in *osm-5(p813)* animals. Therefore, it is unlikely that establishment of the HOB-specific program depends on the activities of downstream structure genes (such as OSM-5) in the ciliogenic pathway. The RFX transcription factor DAF-19 is a key upstream regulator of general ciliogenesis (Swoboda et al., 2000; Haycraft et al., 2001). In the male tail, we observed exclusively nuclear-localized GFP expression of *daf-19* in

male-specific ciliated sensory neurons, including the two hook neurons (Fig. 5A,B) and the 36 ray neurons. The fluorescence in HOA was usually fainter than in HOB. We observed no difference in the HOB expression of *daf-19::gfp* in *egl-44* or *egl-46* mutants compared with wild type (Fig. 5C,D). We then analyzed *egl-44::yfp* and *egl-46::cfp* in *daf-19(m86)* mutant males, and found that the timing and relative brightness of expression in HOB was similar to *daf-19(+)* animals. We infer that, during HOB differentiation, *egl-44* and *egl-46* are expressed independently of a general cilium formation pathway governed by *daf-19*.

We next examined the expression of three HOB-specific genes (*ceh-26*, *pkd-2* and *nlp-8*) in *daf-19* mutants. Swoboda et al. (Swoboda et al., 2000) have shown that *daf-19* is required for general cilium formation, but not for cell-specific properties. Surprisingly, *daf-19(m86)* mutants lacked *ceh-26* HOB expression (*n*=87) (Fig. 1A5). Non-sex-specific expression of *ceh-26::gfp* in some head neurons was also substantially reduced by the *daf-19* mutation. All male-specific expression of *pkd-2* was diminished in the *daf-19* mutant background, including the four ciliated CEM neurons in the head, and the HOB and B-type ray neurons in the tail (*n*=97) (Fig. 1B5). Only the faint non-sex-specific *pkd-2::gfp* expression in a few neurons posterior to the nerve ring was retained in *daf-19* mutant animals. Similarly, expression of *nlp-8::gfp* in *daf-19(m86)* males was only observed in the non-sex-specific PVT neuron and was totally absent in the HOB neuron (*n*=91). Therefore, complete execution of the HOB-specific program requires DAF-19 activity.

DAF-19 has been proposed to act on the X-box motifs in the *cis*-regulatory regions of downstream target genes to regulate their transcription (Swoboda et al., 2000). So far, 5′ regions of demonstrated DAF-19 target genes all harbor the X boxes in close proximity to the coding region (the typical spacing is within less than 200 nucleotides upstream). As expected from this hypothesis, expression of X-box-containing *osm-6::gfp* in the hook and ray neurons was not detected in *daf-19* mutants (*n*=68) (Fig. 1C5). A single X-box sequence is located at about 1.3 kb upstream of the ATG start codon of *egl-46*. This relatively upstream X box in *egl-46* promoter was apparently not a functional target site, as *egl-46::cfp* expression was not altered in *daf-19* mutants. We found no matches to *C. elegans* X-box consensus sequences in the 5′ regions, introns and immediate 3′ regions of *ceh-26*, *lov-1*, *pkd-2* and *nlp-8*. Regulation of *ceh-26*, *pkd-2* and *nlp-8* by *daf-19* is thus likely to be indirect and mediated by some unknown factor(s), which is probably cell-type specific.

Discussion

Specification of the HOB neuron

We have found that *egl-46* is necessary for vulva location behavior, and for gene expression during HOB differentiation. HOB is a ciliated neuron required for *C. elegans* males to sense the vulva during mating (Sulston et al., 1980; Liu and Sternberg, 1995; Barr and Sternberg, 1999; Barr et al., 2001). The regulatory relationships among *egl-46*, another transcription factor, *egl-44*, HOB-specific genes and a ciliogenic pathway support a model involving coordinate contributions of general and cell-specific factors to specify a functional HOB sensory neuron (Fig. 6).

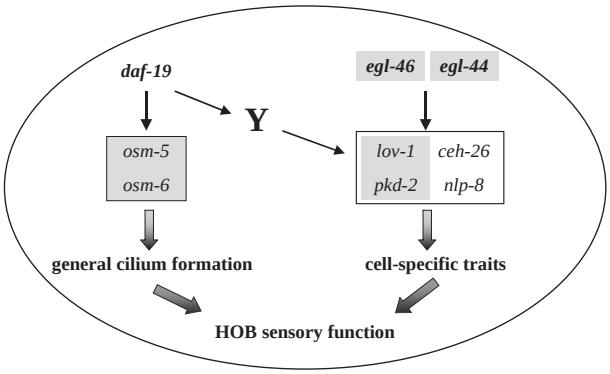

Fig. 6. Distinct pathways involved in HOB gene regulation. Transcriptional regulation by *egl-44* and *egl-46* directs a cell-specific pathway necessary for HOB function in vulva location behavior. In the general ciliogenic pathway, the RFX-transcription factor DAF-19 controls expression of cilium structural genes to provide functional compartment common for all ciliated sensory neurons. DAF-19 has an additional influence on HOB neuronal function by affecting expression of downstream genes in the HOB-specific pathway through some unknown factor(s), indicated by Y. Genes in the shadowed box are the ones in which the Lov phenotype were analyzed in mutants (Barr and Sternberg, 1999) (this work). There are no existing mutants for *ceh-26* and *nlp-8*.

To fulfill its sensory function, HOB must build specific structures and express appropriate molecules to receive and transduce signals. In our model, the general cilium formation pathway governed by *daf-19* programs HOB to have sensory cilia, and *egl-46*, partly with *egl-44*, regulates expression of genes in HOB involved in signal transduction cascades. These two pathways are distinct. Formation of the cilium structures is not necessary for HOB-specific gene expression, and regulators in the cell-specific pathway, *egl-44* and *egl-46*, showed no obvious effect on the HOB expression of the cilium structure genes *osm-5* and *osm-6*. However, these two pathways do interact: not only are they both necessary for HOB function; but the ciliogenic pathway regulator *daf-19* has an effect on downstream components of the HOB-specific program without affecting *egl-44* or *egl-46* expression.

Previous studies suggested that *daf-19* is only required for genes functioning in common aspects of cilium formation (Swoboda et al., 2000). We provide the first evidence that *daf-19* is required for the expression of some cell-type-specific factors. We propose that *daf-19* acts through some unknown factor(s) [which could be an X-box containing gene(s)] to modify HOB-specific gene expression. We observed stronger *daf-19::gfp* expression in HOB than in HOA, but whether it is associated with additional *daf-19* regulation of HOB-specific gene expression is not known. This *daf-19* regulation is not limited to the HOB neuron as *daf-19* also affects *pkd-2* expression in the ray neurons and CEM neurons, indicating some general features are common in this subtype of ciliated sensory neurons. Coupled regulation of general neuronal features and cell-specific identities by multiple transcriptional factors has been found in several different organisms, such as specification of the *C. elegans* AIY interneuron (Altun-Gultekin et al., 2001), *C. elegans* olfactory neurons (Troemel et al., 1997) and vertebrate motoneurons (Novitch et al., 2001;

Zhou and Anderson, 2002), and thus might be a general aspect of the logic of neuronal cell type specification.

Both male hook neurons, HOA and HOB, play a role in vulva location behavior. They both detect the presence of a hermaphrodite vulva, and then produce a distinctive output. This output causes the male to stop at the vulva and to proceed to the next step of mating (Liu and Sternberg, 1995) (M. M. Barr and P.W.S., unpublished). One possible explanation for the functional non-redundancy of HOA and HOB is that they possess different sensory specificity, and hence respond to different cues from the vulva. Another possibility is HOA and HOB might receive the same cues at different times. *egl-44* is broadly expressed in many cells of the male tail, but its expression is almost undetectable in HOA. None of the other genes, including *egl-46* and its downstream targets in the HOB-specific program described here, is expressed in HOA. The unequal expression of those genes in the two hook neurons provides molecular evidence supporting distinct roles for HOA and HOB in mating.

EGL-46 and EGL-44 regulation in HOB sensory function

egl-46 mutations result in an extra cell division in the terminal differentiation of the *C. elegans* Q neuroblast lineage (Desai and Horvitz, 1989). Loss of either *egl-44* or *egl-46* function does not cause a cell division defect or a failure in establishment of primary ciliated neural fate during HOB specification. This was determined by anatomical examination and by expression of the cilium structure genes, *osm-5* and *osm-6*. In the non-sex-specific FLP cells, it has been shown that *egl-44* and *egl-46* act as transcriptional repressors (Wu et al., 2001). They promote the correct subtype of mechanosensory neurons by suppressing expression of genes dedicated to another subtype. Possible positive roles in gene transcription are implicated for *egl-44* and *egl-46* in the HSN neurons, but no target has been identified (Desai and Horvitz, 1989; Wu et al., 2001). Our data suggest a positive effect of *egl-44* and *egl-46* on the expression of downstream HOB-specific genes. However, we have not ruled out that EGL-44 and EGL-46 activate gene expression in HOB by repression of a repressor of HOB-specific genes.

We propose that the sensory abilities of the HOB neuron are established by individual cell-specific components regulated by *egl-44* and *egl-46*. One of these components, *ceh-26*, is the *C. elegans* ortholog of *Drosophila prospero* (*pros*) gene (Bürglin, 1994). *pros* is involved in the initiation of differentiation in specific neurons following asymmetric cell division (Hirata et al., 1995; Broadus et al., 1998; Manning and Doe, 1999). However, expression of *ceh-26* in HOB is not coupled with cell division. Instead, it is expressed at a much later stage, after basic features of cell fate have been established. Similar to HOB, ray B neurons express both *egl-44* and *egl-46*, but unlike HOB, these neurons do not express *ceh-26::gfp*. Therefore, we think that co-expression of *egl-44* and *egl-46* is not sufficient to activate *ceh-26::gfp* in HOB and additional co-factors are also required. The other downstream components, *lov-1*, *pkd-2* and *nlp-8*, encode proteins that are probably involved in HOB sensory input and output. LOV-1 and PKD-2 accumulate in the sensory cilia and have been proposed to act in a complex; a working model is that LOV-1 is a sensory receptor and PKD-2 is a channel protein (Barr et al., 2001; Koulen et al., 2002). Neuropeptide-like protein NLP-8 might act as a neurotransmitter or neuromodulator released by HOB to mediate the response to the stimuli from the hermaphrodite vulva.

Potential mechanosensory and chemosensory interactions between the male and the hermaphrodite during mating is implied by the vulva location behavior itself, as well as by the requirement of functional ciliated sensory endings in the two hook neurons. Whether HOB is a mechanical sensor or a chemical sensor or both, as is the case for the polymodal ASH neuron (Kaplan and Horvitz, 1993), is not known. Because *egl-44* and *egl-46* distinguish between mechanosensory neuron subtypes during FLP fate specification, it is possible that these two genes regulate downstream targets that confer mechanosensory ability to the HOB neuron. If so, as members of TRP protein gene family, *lov-1* and *pkd-2* might be such targets. Known examples of TRP proteins that play a role in mechanotransduction include a *C. elegans* TRP protein OSM-9 and the *Drosophila* TRP-like NOMPC protein (Colbert et al., 1997; Walker et al., 2000). Both of these TRP proteins are expressed in mechanosensory neurons and are involved in mechanosensory response.

Transcriptional regulation of polycystins and polycystic kidney disease

Human PKD1 and PKD2 were identified as two loci responsible for the autosomal dominant polycystic kidney disease (ADPKD), a genetic disorder that causes renal failure at various ages of adulthood (reviewed by Gabow, 1993; Wu, 2001). Relatively little is known about the regulation of these PKD genes and possible alterations during the disease process. In this work, we showed that expression of *C. elegans* PKD gene homologs, *lov-1* and *pkd-2*, is affected by transcription factors *egl-44* and *egl-46*. The mammalian TEF proteins, homologous to *egl-44*, have been implicated in multiple developmental processes (Chen et al., 1994; Jacquemin et al., 1996). Specific expression in kidney was reported for multiple members of TEF proteins (Jacquemin et al., 1996; Kaneko et al., 1997; Jacquemin et al., 1998). *C. elegans* EGL-46 belongs to a novel zinc-finger protein subfamily. Identified close mammalian homologs of *egl-46* includes insulinoma associated (IA) proteins, implicated in islet differentiation of the pancreas, and murine MLT 1 protein, silenced in the liver tumors (Goto et al., 1992; Tateno et al., 2001), but their possible roles in the kidney have not been investigated. Progressive cyst formation in ADPKD is not restricted to kidney: involvement of the liver and the pancreas occurs, indicating that those organs suffer similar pathogenesis during progression of the disease (Gabow, 1993; Chauveau et al., 2000). The demonstrated gene regulation network in HOB might reveal important insights into the regulation of human polycystin gene expression.

The dependence of ciliogenesis for the function of PKD-2 may be even more relevant to renal development in mammals. In *C. elegans*, the ARPKD homolog *osm-5* is a direct target of the RFX factor DAF-19 (Haycraft et al., 2001), making the requirement of DAF-19 activity for *pkd-2* expression particularly interesting with regard to the link between ADPKD and ARPKD. Mammalian polycystins and the cilia of the kidney cells might participate in a common signaling pathway crucial for renal differentiation and function. This

hypothesis implies that RFX factor(s) might play a role in the renal development.

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