ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Developmental Biology 256 (2003) 379–388

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

The roles of an ephrin and a semaphorin in patterning cell–cell contacts in *C. elegans* sensory organ development

Andrew C. Hahn and Scott W. Emmons*

Department of Molecular Genetics, Albert Einstein College of Medicine, Bronx, NY 10461, USA

Received for publication 24 July 2002, revised 10 December 2002, accepted 19 December 2002

Abstract

Ephrins and semaphorins regulate a wide variety of developmental processes, including axon guidance and cell migration. We have studied the roles of the ephrin EFN-4 and the semaphorin MAB-20 in patterning cell–cell contacts among the cells that give rise to the ray sensory organs of *Caenorhabditis elegans*. In wild-type, contacts at adherens junctions form only between cells belonging to the same ray. In *efn-4* and *mab-20* mutants, ectopic contacts form between cells belonging to different rays. Ectopic contacts also occur in mutants in regulatory genes that specify ray morphological identity. We used *efn-4* and *mab-20* reporters to investigate whether these ray identity genes function through activating expression of *efn-4* or *mab-20* in ray cells. *mab-20* reporter expression in ray cells was unaffected by mutants in the Pax6 homolog *mab-18* and the Hox genes *egl-5* and *mab-5*, suggesting that these genes do not regulate *mab-20* expression. We find that *mab-18* is necessary for activating *efn-4* reporter expression, but this activity alone is not sufficient to account for *mab-18* function in controlling cell–cell contact formation. In *egl-5* mutants, *efn-4* reporter expression in certain ray cells was increased, inconsistent with a simple repulsion model for *efn-4* action. The evidence indicates that ray identity genes primarily regulate ray morphogenesis by pathways other than through regulation of expression of semaphorin and ephrin.

Keywords: C. elegans; Neurogenesis; Organ assembly; Adherens junctions; Epidermis; Sensory neurons

Introduction

During development, cells show specificity in forming cell–cell contacts. For example, in the vertebrate hindbrain, cells belonging to a given rhombomere mix freely with each other, but not with cells of neighboring rhombomeres (Fraser et al., 1990). Similarly, the *Drosophila* wing imaginal disc is divided into dorsal/ventral and anterior/posterior compartments that form lineage-restricted boundaries (Garcia-Bellido et al., 1973). The ability of a cell to form specific cell–cell contacts according to its identity poses the question of what regulatory connections exist between cell identity-specifying genes and genes that directly control formation of cell–cell contacts. In the case of the rhombomeres, mutations in the identity-specifying genes HoxA1, HoxB1, and Krox20 disrupt ability of cells to form boundaries (Carpenter et al., 1993; Mark et al., 1993; Voiculescu et al., 2001), as does loss of Eph receptor function (Xu et al., 1995 1999). In these cells, HoxA1, HoxB1, and Krox20 directly activate expression of Eph receptors (Chen and Ruley, 1998; Theil et al., 1998). In the *Drosophila* wing disc, boundary formation requires expression by dorsal compartment cells of the homeodomain transcription factor Apterous, which contributes to boundary formation by activating expression of the transmembrane proteins *capricious* and *tartan* (Milan et al., 2001).

Here, we investigate possible links between cell identity-specifying genes and genes that directly control cell–cell contact formation in the cells that give rise to the sensory rays of *Caenorhabditis elegans*. Each of the nine rays is composed of three cells derived from a respective ray precursor cell, Rn ($n = 1$ to 9) (Sulston et al., 1980). The three cells of a given ray show specificity in cell–cell contact formation: they form contacts along adherens junctions with one another, but not with cells belonging to other rays

* Corresponding author. Fax: +1-718-430-8778.
E-mail address: emmons@aecom.yu.edu (S.W. Emmons).

0012-1606/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0012-1606(02)00129-X

(Baird et al., 1991). Loss-of-function in a class of genes known as the ray identity genes, which specify ray-specific properties, such as morphology and neurotransmitter usage, results in formation of ectopic contacts at adherens junctions between cells belonging to different rays (Baird et al., 1991; Chow and Emmons, 1994; Chow et al., 1995; Krishna et al., 1999; Savage et al., 1996; Zhang and Emmons, 1995). These contacts are permanent and lead to formation of a "ray fusion" instead of individual rays.

The ray identity genes include the Hox genes *mab-5* and *egl-5*, the Pax6 homolog *mab-18*, and components of the *dbl-1* TGF-β pathway (Chow and Emmons, 1994; Krishna et al., 1999; Savage et al., 1996; Zhang and Emmons, 1995). The fact that *mab-5*, *egl-5*, and *mab-18* encode transcription factors suggests that they function through regulating expression of genes that directly control cell–cell contact formation. The *dbl-1* pathway is also likely to function through transcriptional regulation as it includes the Smad transcription factors *sma-2* and *sma-3*. The *dbl-1* pathway has been shown through mosaic analysis to function in a cell-autonomous manner to control cell– cell contact formation (Savage et al., 1996). *mab-5*, *egl-5*, and *mab-18* are expressed in the ray cells that form ectopic contacts in the respective mutants, suggesting that they too function cell-autonomously (Ferreira et al., 1999; Salser and Kenyon, 1996; Zhang and Emmons, 1995; Zhang et al., 1998).

What genes might the ray identity genes regulate? Screens for ray fusion mutants suggest two candidates based on their phenotypes and the types of proteins they encode. *efn-4* (previously known as *mab-26*) and *mab-20* mutants have fusions affecting most rays, consistent with roles downstream of multiple ray identity genes (Baird et al., 1991; Chow and Emmons, 1994). EFN-4 encodes a homolog of GPI-linked ephrins (Chin-Sang et al., 2002), a family of protein ligands for Eph receptor tyrosine kinases that initiate a signaling cascade in the Eph-expressing cell and in some cases the ephrin-expressing cell as well (reviewed in Flanagan and Vanderhaeghen, 1998; Wilkinson, 2001; Cowan and Henkemeyer, 2001). In addition to EFN-4, the *C. elegans* genome contains three other ephrins, VAB-2/EFN-1, EFN-2, and EFN-3, which interact with the Eph receptor VAB-1 during embryogenesis to regulate epidermal cell migration (Chin-Sang et al., 1999; George et al., 1998; Wang et al., 1999). MAB-20 encodes a homolog of *Drosophila* Semaphorin-II, which is predicted to be secreted (Roy et al., 2000). Semaphorins have been shown to function through binding to the transmembrane receptors plexin and neuropilin, initiating a signaling cascade that leads to repulsion or, in some cases, attraction in axons and migrating cells (Hu et al., 2001; Liu and Strittmatter, 2001; Nakamura et al., 2000; Winberg et al., 2001). MAB-20 is one of three *C. elegans* semaphorins, and has been hypothesized to function by repelling extension of cell processes, thus preventing initiation of ectopic cell–cell contacts (Roy et al., 2000).

Here, we study cell–cell contact formation in mutants in *efn-4*, *mab-20*, and ray identity genes. We show that all mutants that result in ray fusions have similar developmental defects in cell–cell contact formation, causing formation of inappropriate adherens junctions between cells belonging to different rays. By studying expression of *efn-4* and *mab-20* reporters in ray identity mutants, we examine the possibility that ray identity genes function through activating expression of EFN-4 or MAB-20. We find that, in many cases, these effector genes are not regulated by the ray identity genes, and where they are, this does not appear to be sufficient to account for accurate ray morphogenesis.

Materials and methods

Strains

Strains were maintained according to Brenner (1974) and Wood (1988) at 20°C, except as noted. All strains carried *him-5(e1490)* to increase incidence of males. Strains containing *pha-1 (e2123)* were grown at 16°C. *pha-1* transgenic strains carrying *pha-1(+)* arrays were grown at 25°C. All alleles have been previously described and are nulls with the exception of *mab-5(e1751):* LGI, *mab-20(ev574)* (Roy et al., 2000); LGII, *sma-6(wk7)* (Krishna et al., 1999); LGIII, *mab-5(e1751)* (Salser et al., 1993), *egl-5(u202)* (Chisholm, 1991), *pha-1(e2123)* (Granato et al., 1994); LGIV, *efn-4(bx80)* (Chow and Emmons, 1994); LGV, *him-5(e1490);* X, *mab-18(bx23)* (Zhang and Emmons, 1995).

Nomarski and fluorescence microscopy

To score ray fusion, animals were mounted on 2% agarose pads and viewed by Nomarski optics at 400× or 1000×. To score fluorescence, males were mounted on agarose pads containing 0.5% phenoxypropanol as anesthetic and viewed at 1000× under UV light by using a Zeiss 487905 filter set. Digital images were obtained by using the SPOT camera system.

Transgenic lines and constructs

Transgenic lines were generated by microinjection using *pha-1* selection (Granato et al., 1994; Mello et al., 1991). All injection solutions contained 100 ng/μl *Pvu*II-digested N2-genomic DNA and 2 ng/μl *Xho*I-digested pBX1, which carries the *pha-1* gene (Granato et al., 1994).

The *jam-1::gfp*-carrying transgenic line *bxEx48*, described previously (Portman and Emmons, 2000), was crossed into *egl-5(u202)*, *mab-18(bx23)*, *mab-20(ev574)*, *efn-4(bx80)*, and *sma-6(wk7)* [strain designations: *him-5(e1490)* (EM613), *egl-5(u202)* (EM624), *mab-18(bx23)* (EM626), *mab-20(ev574)* (EM635), *efn-4(bx80)* (EM697), *sma-6(wk7)* (EM699)].

pPRII.14 (*Apa*I-digested, Klenow-blunted) and *pPRII.67* (*Fsp*I-digested) (Roy et al., 2000) were injected into *him-*

5(e1490), at 5 and 1 ng/μl, respectively, to generate transgenic lines *bxEx63* and *bxEx60*, which were crossed into *egl-5(u202)*, *mab-18(bx23)*, and *sma-6(wk7)* [strains carrying *bxEx63: him-5(e1490)* (EM701), *egl-5(u202)* (EM689), *mab-18(bx23)* (EM693), *sma-6(wk7)* (EM686); strains carrying *bxEx60: him-5(e1490)* (EM791), *egl-5(u202)* (EM792), *mab-18(bx23)* (EM793), *sma-6(wk7)* (EM795)].

efn-4::gfp (*Eco*RV-digested) (gift of S. George and A. Chisholm) was injected at 15 ng/μl into *him-5(e1490)* to generate transgenic line bxEx64, which was crossed into *egl-5(u202)*, *mab-18(bx23)*, *efn-4(bx80)*, *sma-6(wk7)* [strain designations: *him-5(e1490)* (EM702), *egl-5(u202)* (EM681), *mab-18(bx23)* (EM685), *efn-4(bx80)* (EM683), *sma-6(wk7)* (EM679)].

The construct driving *efn-4::gfp* expression from the *mab-20* promoter (EM#308) was generated by overlapping PCR combining the 2.5-kb sequence 5′ to the *mab-20* start codon with *efn-4::gfp* sequence extending from the *efn-4* start codon to 2.3-kb 3′ of the stop codon. Exons and splice junctions were confirmed by sequencing; gel-purified construct was injected at 15 ng/μl into *him-5(e1490)*, and crossed into *mab-18(bx23)* and *efn-4(bx80)* [strain designations: *him-5(e1490)* (EM797), *mab-18(bx23)* (EM798), *efn-4(bx80)* (EM799)].

Scoring efn-4::gfp expression

In wild-type and mutants, *efn-4::gfp* expression in Rn.a descendants was weak, such that, in some individuals, expression was not detectable in all Rn.a descendants that express in strongly expressing individuals. However, relative expression strength appeared to be preserved, since the frequency at which descendants of each Rn.a cell showed expression correlated with expression strength in strongly expressing individuals (see Fig. 3b and c). To assist scoring, animals were grown in conditions of reduced food (but not to the point of dauer induction), which increased expression strength without altering relative expression levels between descendants of different Rn.a cells, except for a slight decrease in expression strength in R4.a descendants.

Data analysis

Pearson Chi-square test was used to test significance ($P \leq 0.05$).

Results

Ray fusion has a similar cellular basis in all ray fusion mutants

The nine bilateral pairs of rays are derived from nine bilateral pairs of ray precursor cells (Rn, where $n = 1$ to 9), which execute a stereotyped ray sublineage to generate the three cells that comprise each ray, as well as an epidermal

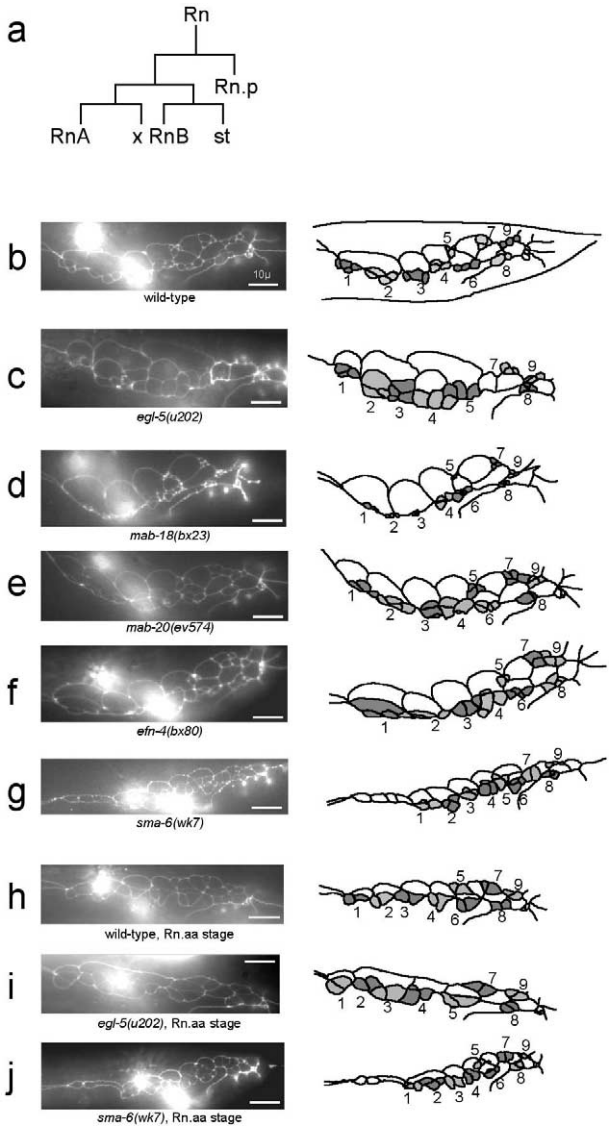

Fig. 1. The ray sublineage and *jam-1::gfp* expression in wild-type and ray fusion mutants. (b–g) Rn.aaa stage; (h–i) Rn.aa stage. Fluorescence pictures in left column, tracing diagrams in right column. Scale bar, 10 μm. (a) The ray sublineage. (b) Wild-type. Rn.a descendants form distinct three-cell groups. (c) *egl-5(u202)* showing ectopic contacts between descendants of R2.a, R3.a, R4.a, and R5.a (100% cases, $N = 30$). Unlike in wild-type, R5.a descendants lie ventral to R4.p and R5.p. (d) *mab-18(bx23)* showing ectopic contacts between R4.a and R6.a descendants. (e) *mab-20(ev574)* showing ectopic contacts between R1.a and R2.a descendants, and between R3.a, R4.a and R6.a descendants. Ectopic contacts occur in all individuals, $N > 30$. (f) *efn-4(bx80)* showing ectopic contacts between R1.a and R2.a descendants, and R3.a, R4.a, and R6.a descendants. Ectopic contacts occur in all individuals, $N > 30$. (g) *sma-6(wk7)* showing ectopic contacts between R4.a and R5.a descendants, and R6.a and R7.a descendants. In contrast to wild-type, R5.a descendants lie in contact with descendants of R4.p and R6.p, while R7.a descendants lie anterior to R7.p (7/7 sides scored). (h) Wild-type, Rn.aa stage. Descendants of different Rn.aa cells do not contact one another. (i) *egl-5(u202)* at Rn.aa stage. R5.aa and R5.ap lie ventral to R5.p (5/5 sides scored). (j) *sma-6(wk7)* at Rn.a stage. R5.aa and R5.ap are born dorsal to R4.p and R6.p (5/5 sides scored). R7.aa and R7.ap lie lateral to R7.p instead of dorsal.

Table 1
Ray fusion frequencies in mutants

Genotype	Ray							*N*
	1	2	3	4	5	6	7	
wild-type		0	0	0	0	0	0	200
wild-type; *mab-20p::efn-4gfp*	0	0	18	18	0	0	0	102
wild-type; *efn-4::gfp*	0	0	1	2	0	1	0	238
egl-5(u202)	0	100	100	100	100		0	30
egl-5(u202);efn-4(bx80)	0	100	100	100	100		0	10
mab-18(bx23)	0	0	0	83	0	83	0	123
mab-18(bx23); mab-20p::efn4gfp	0	0	0	90	0	90	0	207
mab-20(ev574)		74	95	96	0	58		222
mab-20(bx61ts) @ 25		65	65	0	0	0		300
mab-20(ev574); mab-20::gfp		2	4	3	0	0	0	222
efn-4(bx80)		69	94	96	0	31		226
efn-4(bx80)/+		0	0	25	25	0	0	300
efn-4(bx80); efn-4::gfp		36	63	56	0	7	0	206
efn-4(bx80); mab-20p::efn4gfp		53	91	79	0	8	0	149
sma-6(wk7)	0	1	1	24	24	58	58	307
mab-20(ev574); efn-4(bx80)	0	86	93	94	0	72		300
vab-1(dx31)	0	0	0	0	0	0		200

Note. Individual left and right sides were scored independently. Rays 8 and 9 are difficult to visualize and hence were not scored. In *mab-20(ev574)* and *efn-4(bx80)* mutants, ray 1 forms at an abnormally anterior position and therefore was not scored. Rays do not form in *egl-5(u202)*, so ectopic contact formation was scored with *jam-1::gfp*. Significance of differences were calculated by standard chi-square test. *mab-20::gfp* rescues *mab-20(ev574)* ray fusion in all rays ($P \leq 0.001$). *efn-4::gfp* rescues *efn-4(bx80)* ray fusion in all rays ($P \leq 0.001$). *mab-20p::efn-4gfp* rescues ray 6 fusion in *efn-4(bx80)* ($P \leq 0.001$) but not *mab-18(bx23)*. Ray fusion in *mab-20(ev574); efn-4(bx80)* is increased over both *mab-20(ev574)* and *efn-4(bx80)* in rays 2 and 6 ($P \leq 0.001$) but not rays 3 or 4.

cell (Rn.p) and a cell that undergoes apoptosis (Rn.aap) (Fig. 1a). The cell bodies of the ray cells subsequently migrate into the body but leave behind a dendritic attachment to the cuticle, thus forming an extended process that becomes the visible portion of the ray. The apical surfaces of Rn cells and their descendants, as well as neighboring epidermal cells, are surrounded by adherens junctions (Francis and Waterston, 1991; Mohler et al., 1998). By visualizing the adherens junction component JAM-1, Baird et al. (1991) found that, during the ray sublineage, cell apical surfaces undergo stereotypical changes in shape and position and form reproducible patterns of cell–cell contacts. Notably, upon completion of the ray sublineage, the three cells of a given ray form contacts with one another but not with cells of other rays (Baird et al., 1991; Fig. 1b).

In contrast to wild-type, Baird et al. (1991) and Roy et al. (2000) found that, in *mab-18* and *mab-20* mutants, cells belonging to different rays form "ectopic" contacts at adherens junctions in patterns corresponding to ray fusions (Fig. 1d and e). Using the reporter *jam-1::gfp*, we found similarly in null mutants of *egl-5(u202)*, *efn-4(bx80)*, and *sma-6(wk7)*, which encodes the type I TGF-beta receptor of the *dbl-1* pathway, that ray fusion was preceded by ectopic formation of adherens junctions between cells of different rays (Fig. 1c, f, and g) (Krishna et al., 1999). As expected, patterns of ectopic contacts correspond to the rays that fuse in the respective adults. In *egl-5(u202)*, ectopic contacts form between descendants of R2.a, R3.a, R4.a, and R5.a (Fig. 1c); in adults, rays 2, 3, 4, and 5 fuse (Chisholm, 1991). In *efn-4(bx80)*, ectopic contacts form between Rn.a descendants in variable patterns (Fig. 1f); in adults, rays fuse in variable patterns (Table 1; Chow and Emmons, 1994). In *sma-6(wk7)*, ectopic contacts form between descendants of R4.a and R5.a, R6.a and R7.a, and R8.a and R9.a (Fig. 1g); in adults, rays 4 and 5, 6 and 7, and 8 and 9 fuse (Table 1; Krishna et al., 1999).

Unlike other ray fusion mutants, *egl-5* and *sma-6* mutants show defects in positioning of apical cell surfaces of R5.a and R7.a descendants. In wild-type and in *mab-18*, *mab-20*, and *efn-4* mutants, the apical surfaces of R5.a descendants lie dorsally to R4.p and R6.p, and apical surfaces of R7.a descendants lie dorsally to R5.p and R7.p (Fig. 1b, d–f). In contrast, in *egl-5(u202)*, apical surfaces of R5.a descendants are positioned ventral to R4.p, even before ectopic contacts with R4.a descendants are formed (Fig. 1c and i). In *sma-6(wk7)*, the apical surfaces of R5.aa and R5.ap are born in their normal positions dorsal to R4.p and R5.p, but subsequently cross to the ventral side of R4.p and R5.p, where they form ectopic contacts with R4.a descendants (Fig. 1g). In addition, in *sma-6(wk7)* mutants, the apical surfaces of R7.aa and R7.ap lie lateral rather than dorsal to R6.p and R7.p (Fig. 1j). Abnormal positioning of R5.a and R7.a descendants in *egl-5* and *sma-6* mutants may be a prerequisite for ectopic contact formation, since in wild-type, R4-7.p cells separate R5.a and R7.a descendants from R4.a and R6.a descendants, respectively. We do not know whether ectopic positioning is sufficient for ectopic contact formation, and it is possible that *egl-5* and *sma-6* control cell–cell contact formation in these cells through additional functions.

mab-20::gfp expression is unaffected in egl-5 and mab-18 mutants

Consistent with a role for *mab-20* downstream of ray identity genes, descendants of most Rn.a cells form ectopic contacts in *mab-20* mutants, resulting in ray fusion, including those that form ectopic contacts in *egl-5, mab-18,* and *sma-6* mutants (Table 1; Baird et al., 1991; Roy et al., 2000). Based on its homology to semaphorins, MAB-20 was hypothesized to control cell–cell contact formation in Rn.a descendants by repelling extension of cell processes, thus preventing initiation of inappropriate cell–cell contacts (Roy et al., 2000). MAB-20 is predicted to be secreted and hence has the potential to act at a distance (Roy et al., 2000). To study *mab-20* expression in ray cells, we used two *mab-20* GFP reporters, *pPRII.14* and *pPRII.67* (gifts of P. Roy and J. Culotti) (Fig. 2a). *pPRII.14* consists of genomic sequence extending from 6 kb upstream of the predicted *mab-20* start codon to 1 kb downstream of the stop codon, with GFP inserted near the MAB-20 N terminus; *pPRII.67* consists of a 2.5 kb sequence upstream of the *mab-20* start codon driving expression of GFP containing a nuclear localization signal (Roy et al., 2000). *pPRII.14* strongly rescues ray fusion in the null mutant *mab-20(ev574),* suggesting that it drives MAB-20 expression in a wild-type pattern (Table 1).

In agreement with Roy et al. (2000), we found that *pPRII.14* and *pPRII.67* have similar expression patterns. During execution of the ray sublineage, both reporters are expressed in Rn descendants and not in other epidermal cells in the tail (Fig. 2b). Weak expression is first detected at the Rn and Rn.a stages and increases in strength with time. At the Rn.aa and Rn.aaa stages, all Rn.a descendants express the reporters (Fig. 2b; Roy et al., 2000). Expression levels are similar between descendants of a given Rn.a cell and between descendants of different Rn.a cells. Expression in Rn.p cells is usually undetectable and relatively weak when present.

The facts that *mab-20* reporters are expressed in all Rn.a descendants and that descendants of all Rn.a cells form ectopic contacts in *mab-20* mutants suggest that MAB-20 acts directly on all Rn.a descendants. We hypothesized that ray identity genes might function by activating *mab-20* expression in a subset or in all Rn.a descendants that form ectopic contacts in the respective mutants. However, we found that *pPRII.14* and *pPRII.67* expression patterns were indistinguishable from wild-type in the null mutants *egl-5(u202)* and *mab-18(bx23)* (Fig. 2c and d). Assuming that reporters accurately reflect *mab-20* expression, these results suggest that ray fusion in *egl-5* and *mab-18* mutants does not result from failure of Rn.a descendants to express *mab-20*. In contrast, in the null mutant *sma-6(wk7),* expression of both *mab-20* reporters is missing from Rn.a descendants at low frequency (10–20% sides), raising the possibility that failure to express *mab-20* may contribute to ectopic contact formation in *sma-6(wk7),* in conjunction with abnormal positioning of R5.a and R7.a descendants (Fig. 2c and d; Table 1).

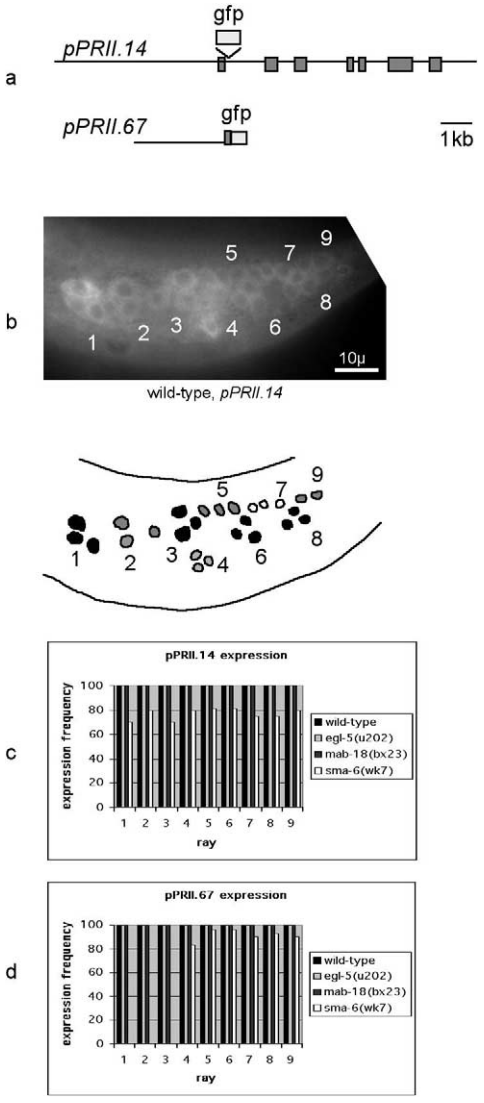

Fig. 2. *mab-20* reporter expression in Rn.a descendants in wild-type and ray identity mutants. Expression patterns at the Rn.aa and Rn.aaa stages were identical to one another in wild-type and in mutants; expression frequencies represent combined data from both stages. For each animal, one left or right side was scored. (a) *pPRII.14* consists of genomic sequence extending from 6 kb upstream of the *mab-20* start codon to approximately 1 kb downstream of the end of the final exon, with GFP inserted near the *mab-20* N terminus; *pPRII.67* consists of 2.5 kb *mab-20* promoter sequence driving expression of GFP (Roy et al., 2000). (b) *pPRII.14* expression at Rn.aaa stage. Scale bar, 10 μm. (c) *pPRII.14* expression frequency in wild-type and ray identity mutants, combined Rn.aa and Rn.aaa stages. *pPRII.14* expression in wild-type in *egl-5(u202)* and *mab-18(bx23).* Expression is lost at low frequency in Rn.a descendants in *sma-6(wk7)* ($P \leq 0.01$ for all rays). For *egl-5(u202)*, $N = 27$; for *mab-18(bx23)*, $N = 25$; for *sma- 6(wk7)*, $N \geq 10$. (d) *pPRII.67* expression is wild-type and ray identity mutants, combined Rn.aa and Rn.aaa stages. $N \geq 16$ for *egl-5(u202); N* = 39 for *mab-18(bx23), N* = 31 for *sma-6(wk7).* Expression is absent at low frequency in *sma-6(wk7)* in descendants of R4.a-R9.a ($P \leq 0.01$ for all rays); descendants of R1.a, R2.a, and R3.a were not scored due to strong background expression in underlying cells.

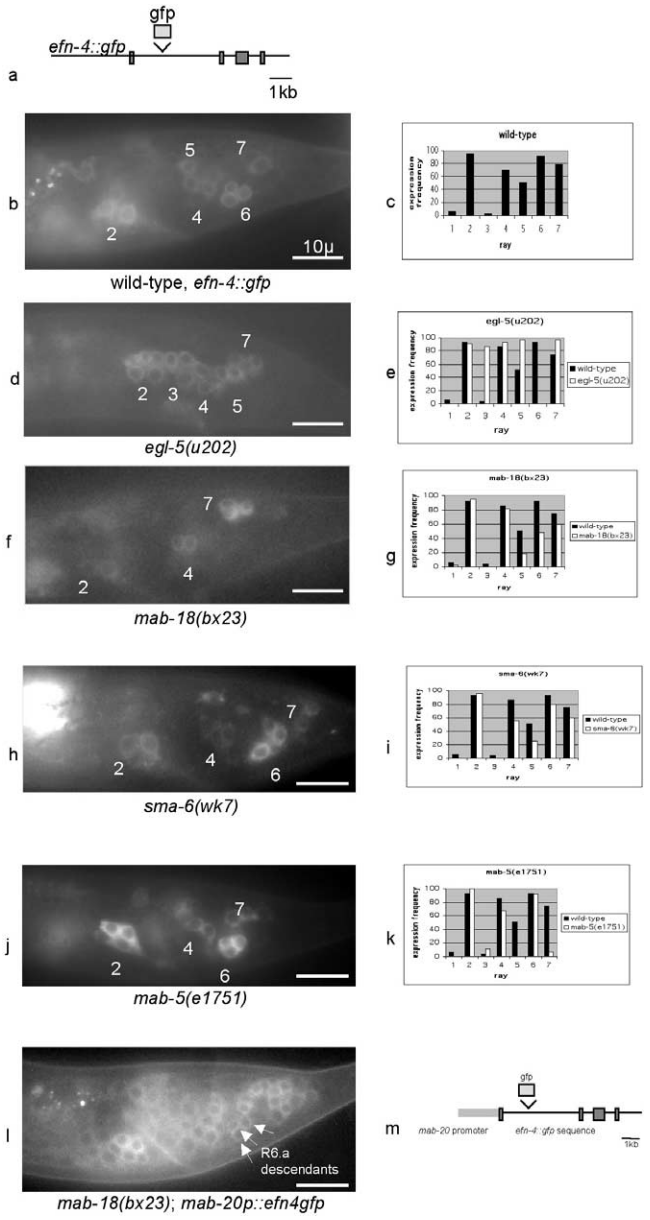

Fig. 3. *efn-4::GFP* expression in Rn.a descendants in wild-type and ray fusion mutants. All photos show Rn.aaa stage individuals. Expression patterns at the Rn.aa and Rn.aaa stages were identical to one another in wild-type and in mutants; graphs contain combined data from both stages. For each animal, one left or right side was scored. Scale bar, 10 μm. (a) *efn-4::gfp* consists of genomic sequence extending from 5.3 kb upstream of the *efn-4* start codon to 2.3 kb downstream of the stop codon. A GFP cassette is inserted into the intron following the first coding exon (S. George and A. Chisholm, personal communication). (b, c) *efn-4::gfp* expression in wild-type. Expression is strongest in R2.a and R6.a descendants, intermediate in R4.a, R5.a, and R7.a descendants, and low in R1.a and R3.a descendants. Expression was not scored in R8.a and R9.a descendants due to high variability in expression frequency between trials. Expression frequency correlates with expression strength found in strongly expressing individuals: expression frequency is highest in R2.a and R6.a descendants, intermediate in R4.a, R5.a, and R7.a descendants, and low in R1.a and R3.a descendants; $N \geq 78$. (d, e) *efn-4::gfp* expression in *egl-5(u202).* In most *egl-5(u202)* individuals, *efn-4::gfp* is expressed at a uniform level in R2.a, R3.a, R4.a, and R5.a descendants. Expression frequency in R3.a descendants and R5.a descendants is higher than wild-type ($P \leq 0.001$ and $P = 0.03$, respectively); $N \geq 27$. (f, g) *efn-4::gfp* expression in *mab-18(bx23).* Expression frequency is reduced in R6.a descendants ($P \leq 0.001$) and R5.a descendants ($P = 0.003$). $N \geq 45$. (h, i) *efn-4::gfp* expression in *sma-6(wk7).* Expression is reduced approximately twofold in R5.a descendants ($P = 0.05$), but not in other cells; $N \geq 24$. (j, k) *efn-4::gfp* expression in *mab-5(e1751). efn-4::gfp* remains off in R1.a and R3.a descendants. Expression does not differ from wild-type in R1.a and R3.a descendants ($P = 0.42$ and 0.20, respectively), but is reduced in R7.a descendants and absent from R5.a descendants ($P \leq 0.001$ and $P \leq 0.01$, respectively); $N \geq 9$. (l) *mab-20p::efn-4gfp* consistently drives expression in R6.a descendants in *mab-18(bx23)* (10/10 sides). (m) *mab-20::efn-4gfp* consists of 2.5 kb *mab-20* promoter sequence driving expression of *efn-4::gfp* sequence extending from the *efn-4* start codon to 2.3 kb downstream of the stop codon.

efn-4::gfp is expressed in Rn.a descendants at ray-specific levels

To study *efn-4* expression in ray cells, we used an *efn-4::gfp* reporter consisting of genomic sequence extending from 5.3 kb upstream of the *efn-4* start codon to 2.3 kb downstream of the stop codon, with GFP inserted after the first predicted coding exon (Fig. 3a; gift of S. George and A. Chisholm). This reporter shows some degree of rescuing ability for ray fusion in the null mutant *efn-4(bx80)* (Table 1). Among epidermal cells in the tail, *efn-4::gfp* was expressed in Rn.a descendants but not in other tail epidermal cells (Fig. 3b and c). Expression was first detected in Rn.aa and Rn.ap cells, and expression increased in strength in their descendants. Expression levels among descendants of the same Rn.a cell were similar. Expression was strongest and most frequent in descendants of R2.a and R6.a, intermediate in strength and frequency in R4.a, R5.a, and R7.a, and absent from R1.a and R3.a; expression was not scored in R8.a and R9.a descendants because it was weak and highly variable between individuals.

In other systems, ephrins have been shown to function through interaction with the Eph class of receptor tyrosine kinases, leading in most cases to repulsion (Flanagan and Vanderhaeghen, 1998; Wilkinson, 2001). The expression pattern of *efn-4* in the rays suggests a model where EFN-4 regulates cell–cell contact formation in Rn.a descendants in a contact-dependent manner through interaction with a membrane-bound protein expressed in descendants of neighboring Rn.a cells, resulting in repulsion in one or both cells.

egl-5 is necessary for expression of efn-4::gfp at differing levels in rays 2–5

egl-5 encodes an Abd-B homolog expressed in R3, R4, R5, R6, and their descendants. In *egl-5* mutants, ectopic contacts form between R2.a, R3.a, R4.a, and R5.a descendants (R6 fails to execute the ray sublineage) (Fig. 1c) (Chisholm, 1991; Ferreira et al., 1999). We hypothesized that *egl-5* might regulate cell–cell contact formation in R4.a and R5.a descendants by activating *efn-4* expression, and

therefore we might expect to find reduced or absent *efn-4::gfp* expression in *egl-5(u202)* mutants. However, we found that *efn-4::gfp* expression is actually increased in descendants of R3.a and R5.a in *egl-5(u202)* mutants (Fig. 3d and e), resulting in uniform expression in rays 2-5, and indicating that *egl-5* has a repressive effect on *efn-4::gfp* expression in rays 3 and 5. If *efn-4* functions as a repellent, ectopic *efn-4* expression would not be expected to induce ectopic contact formation. Assuming that the reporter faithfully reflects endogenous *efn-4* expression, we conclude that *egl-5* controls expression of additional genes besides *efn-4* to prevent incorrect cell–cell contact formation. An alternative possibility is that ectopic *efn-4* expression in *egl-5(u202)* mutants causes ectopic contact formation. However, we found that ectopic contacts still form at full penetrance in *egl-5(u202); efn-4(bx80)* double mutants (Table 1).

mab-18 activates efn-4::gfp expression but does not function solely through activating efn-4 expression

mab-18 encodes a Pax6 homolog expressed in R6 and its descendants (Zhang et al., 1998). In *mab-18* mutants, R6.a descendants form ectopic contacts with R4.a descendants (Zhang and Emmons, 1995) (Fig. 1d). We hypothesized that *mab-18* might function through activating *efn-4* expression in R6.a descendants, and therefore we might expect *efn-4::gfp* expression to be reduced or absent in ray 6 cells in *mab-18(bx23)*. We found that, in *mab-18(bx23)* mutants, the frequency at which *efn-4::gfp* expression was detected in R6.a descendants was approximately half that of wild-type, and when present, expression in R6.a descendants was weaker than in R4.a descendants, in contrast to wild-type, where expression in R6.a descendants was stronger (Fig. 3f and g). Expression frequency of *efn-4::gfp* was also decreased in R5.a descendants, suggesting a cell-nonautonomous effect of *mab-18*. If *efn-4::gfp* accurately reflects endogenous *efn-4* expression, then reduced *efn-4* expression in R6.a descendants in *mab-18(bx23)* would be expected to contribute to ectopic contact formation between R6.a and R4.a descendants.

These results raise the question of whether *mab-18* functions solely through activating *efn-4* expression. Therefore, we determined whether restoring *efn-4* expression to R6.a descendants is sufficient to prevent ectopic contact formation in R6.a descendants in *mab-18* mutants. Restoration of *efn-4* expression in ray 6 was achieved by driving *efn-4* expression from a transgene carrying *efn-4::gfp* under control of the *mab-20* promoter (*mab-20p::efn-4gfp*) (Fig. 3m). This construct is expressed in R6 descendants in *mab-18(bx23)* mutants (Fig. 3I) and strongly rescues fusion of ray 6 with ray 4 in *efn-4(bx80)* (Table 1). However, it has no rescuing effect on ray 6 fusion in *mab-18(bx23)* (Table 1). Therefore, ectopic contact formation in R6.a descendants in *mab-18* is not solely due to reduced *efn-4* expression in R6.a descendants, and *mab-18* must have additional targets to prevent incorrect cell–cell contact formation.

efn-4::gfp expression is slightly reduced in R5.a descendants in sma-6(wk7)

In *sma-6(wk7)* mutants, ectopic contacts form between descendants of R4.a and R5.a, and R6.a and R7.a. We hypothesized that *sma-6* controls cell–cell contact formation by activating *efn-4::gfp* expression in descendants of one or more of these Rn.a cells, in addition to its positioning functions in R5.a and R7.a descendants. We found that *efn-4::gfp* expression frequency is wild-type in descendants of R6.a and R7.a, but is reduced in R4.a and R5.a descendants, raising the possibility that *sma-6* may control cell–cell contact formation in R5.a descendants by activating *efn-4* expression (Fig. 3h and i).

Expression of mab-5 in R1.a and R3.a descendants is not sufficient to activate efn-4::gfp expression

We considered what other factors might control *efn-4::gfp* expression. Like *efn-4::gfp*, the Hox gene *mab-5* is expressed in R2.a and R4.a descendants, and not in R1.a or R3.a descendants (Salser and Kenyon, 1996). In *mab-5* heterozygotes and the weak mutant *mab-5(bx54)*, ray 4 fuses with its anterior neighbor ray 3, indicating that *mab-5* controls cell–cell contact formation in at least R4.a descendants (Chow and Emmons, 1994; Salser and Kenyon, 1996). We could not test whether *mab-5* is required in R2.a and R4.a descendants for activating *efn-4::gfp* expression by examining loss-of-function mutants because *mab-5* is also required for formation of ray precursor cells R1-6. However, we were able to test whether MAB-5 expression in R1.a and R3.a descendants is sufficient to activate *efn-4::gfp* expression using *mab-5(e1751)*, a promoter mutation which drives ectopic *mab-5* expression in P neuroblasts, seam cells, and Rn descendants (Salser et al, 1993). *mab-5(e1751)* has a ray phenotype similar to that caused by heat-shock-driven ectopic expression of *mab-5*, namely fusion of rays 1 and 3 with their respective posterior neighbors rays 2 and 4, suggesting that the *e1751* mutation drives ectopic *mab-5* expression in R1.a and R3.a descendants (Salser and Kenyon, 1996). We found that *efn-4::gfp* is absent from R1.a and R3.a descendants in *mab-5(e1751)* (Fig. 3j and k). Therefore, we conclude that MAB-5 expression in R1.a and R3.a descendants is not sufficient to activate *efn-4::gfp* expression in these rays. Furthermore, since *mab-5(e1751)* causes fusion of ray 1 to ray 2, and ray 3 to ray 4, *mab-5* may repress expression of a gene required to prevent cell–cell contact formation with R2.a and R4.a descendants. In addition, we saw loss of *efn-4::gfp* expression in R5.a and R7.a descendants, indicating that, in these cells, ectopic *mab-5* represses *efn-4::gfp* expression, but it does not cause fusion of these rays.

efn-4 and mab-20 act in independent pathways to regulate contact formation by R2.a and R6.a descendants

The similarity of the *efn-4* and *mab-20* phenotypes raised the possibility that they act in a single linear pathway. To test this hypothesis, we compared the ray phenotypes of the null mutants *mab-20(ev574)* and *efn-4(bx80)* with that of the *mab-20(ev574); efn-4(bx80)* double mutant. If *efn-4* and *mab-20* function in a single linear pathway, then the double mutant phenotype should not be more severe than *mab-20(ev574)* [which is more severe than *efn-4(bx80)*]. Rays 3 and 4 almost always fuse in *mab-20(ev574)* and *efn-4(bx80)* mutants. We found that rays 2 and 6 fuse at significantly higher frequency in the double mutant than in *mab-20* (Table 1), suggesting that *efn-4* and *mab-20* act in independent pathways in these cells, each contributing to the prevention of ray fusion.

Discussion

Pathways that directly control formation of cell–cell contacts

efn-4 and *mab-20* play important roles in regulating cell–cell contact formation during ray development. Loss of *efn-4* or *mab-20* causes most Rn.a descendants to form ectopic contacts. Despite the similarity of the *efn-4* and *mab-20* phenotypes, genetic tests suggest that they act in independent pathways. This conclusion is consistent with the molecular nature of EFN-4 and MAB-20, which are ligands in distinct signaling pathways. *mab-20* encodes a predicted secreted semaphorin; *mab-20* reporters are expressed in all Rn.a descendants, and in *mab-20* mutants, descendants of most Rn.a cells form ectopic contacts. This indicates that MAB-20 controls cell–cell contact formation in most Rn.a descendants, perhaps by inhibiting cell process formation (Roy et al., 2000). *efn-4* encodes a GPI-linked ephrin and a *efn-4::gfp* reporter is expressed in Rn.a descendants, suggesting that the simplest model for EFN-4 function is as a cell-membrane-bound protein expressed in Rn.a descendants that binds to a protein expressed in Rn.a descendants of neighboring rays to mediate repulsive signaling in either a forward or reverse (or both) manner. In *efn-4* mutants, driving *efn-4* expression in R6.a descendants using the *mab-18* promoter strongly rescued ray 6 fusion, suggesting that *efn-4* may act cell-autonomously. The alternating expression pattern of *efn-4::gfp* in R1.a, R2.a, R3.a, and R4.a descendants is reminiscent of complementary expression patterns of Eph receptors and ephrins in rhombomeres and other tissues, such as blood vasculature and spinal cord, raising the possibility that the EFN-4 binding partner is expressed in descendants of R1.a and R3.a and not R2.a or R4.a (Imondi et al., 2000; Taneja et al., 1996; Wang et al., 1998). The identity of the EFN-4 receptor in the rays remains unknown, as the null mutant *vab-1(dx31)*, the only Eph receptor in the genome identified by BLAST search, has wild-type rays 1–6 (Table 1).

Although loss of *mab-20* and *efn-4* function results in a high frequency of ectopic contact formation between ray cells, R2.a and R6.a descendants sometimes do not form ectopic cell–cell contacts even in *mab-20; efn-4* double mutants, suggesting that additional pathways or mechanisms control cell–cell contact formation. Cell–cell contact formation could potentially be regulated through a variety of mechanisms that control cell–cell adhesion or repulsion, cell shape, movement, or extension of processes.

Multiple factors control efn-4::gfp expression

In contrast to the uniform expression pattern of *mab-20* reporters in Rn.a descendants, *efn-4::gfp* has a complex expression pattern, suggesting that it may be regulated by multiple factors. We find that *egl-5* is responsible for repressing *efn-4::gfp* expression in some of the Rn.a descendants in which it is expressed, whereas *mab-18* activates expression in R6.a descendants as well as R5.a descendants, and *sma-6* may have a small activating effect in R5.a descendants. *mab-5* is expressed in a similar pattern to *efn-4::gfp* in descendants of R1.a-R4.a; however, we found that MAB-5 expression in R1.a and R3.a descendants is not sufficient to activate *efn-4::gfp* expression. It remains to be determined whether *efn-4* expression in R2.a and R4.a descendants requires MAB-5.

The roles of mab-20 and efn-4 in mediating ray identity gene function

Assuming that *mab-20::gfp* reporters accurately reflect expression of the endogenous *mab-20* gene, our results show that *mab-20* expression is wild-type in *egl-5* and *mab-18*, and hence these genes likely do not function to prevent ray fusion by activating *mab-20* expression. Consistent *mab-20* expression in Rn.a descendants appears to require the *dbl-1* pathway, raising the possibility that lack of *mab-20* expression may contribute to ectopic contact formation in *sma-6* mutants.

The observation of ectopic and increased *efn-4::gfp* expression in R3.a and R5.a descendants in *egl-5* mutants is inconsistent with a simple repulsion model for *efn-4* function. It suggests either that *egl-5* controls cell–cell contact formation through mechanisms other than control of *efn-4* expression or that lack of *efn-4* expression is somehow necessary to prevent fusion. Consistent with the latter possibility, reduced *efn-4* dosage rescues fusion of rays 3 and 4 in *egl-5* heterozygotes (Chow and Emmons, 1994). *efn-4::gfp* expression is reduced in R6.a descendants in *mab-18* mutants, which would be expected to decrease the ability of R6.a descendants to repel R4.a descendants. However, driving *efn-4* expression in R6.a descendants in *mab-18* mutants did not rescue ray 6 fusion, indicating that, like EGL-5, MAB-18 also mediates contact specificity

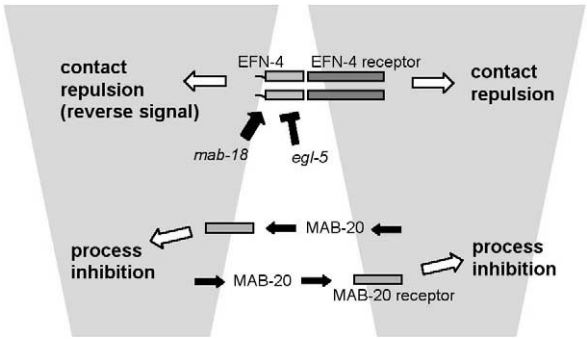

Fig. 4. Model for functions of *mab-20, efn-4,* and ray identity genes. *mab-20* is proposed to act as a secreted repellent that inhibits extension of cell processes (Roy et al., 2000). We propose that *efn-4* expressed on the surfaces of Rn.a descendants acts as a contact-dependent ligand for an unidentified receptor expressed on descendants of neighboring Rn.a cells, initiating repulsive signaling in the receptor-expressing cell and/or the *efn-4*-expressing cell through an ephrin reverse signaling pathway (Cowan and Henkemeyer, 2001; Davy et al., 1999). The ray identity genes could potentially regulate expression of any component of *mab-20* or *efn-4* pathways, or other possible unidentified pathways controlling cell–cell contact formation.

through additional mechanisms. As with *mab-20::gfp,* consistent *efn-4::gfp* expression in R5.a descendants requires *dbl-1* pathway function, raising the possibility that absent or reduced *efn-4* expression may contribute to ectopic contact formation in R5.a descendants in *dbl-1* pathway mutants.

Regulation of cell–cell contact formation by cell-identity-specifying genes

Our studies suggest that *egl-5, mab-5, mab-18* and *sma-6* control cell–cell contact formation through mechanisms other than control of *mab-20* or *efn-4* expression. They could potentially function by regulating expression of any gene in the *efn-4* and *mab-20* signaling pathways, or further unidentified pathways in cell–cell contact regulation (Fig. 4). Our results also raise the question of whether cell-identity-specifying genes generally function through more than one pathway. Formation of the *Drosophila* imaginal disc dorsal–ventral boundary requires multiple genes (Milan et al., 2001). It is not known whether Eph–ephrin signaling is sufficient for specification of cell contact by Hox genes in the rhombomeres. Further understanding of how ray identity genes control cell–cell contacts will require identifying other genes that regulate cell–cell contact formation which may not have been identified in screens for ray fusion mutants due to an essential role earlier in development.

Acknowledgments

We thank P. Roy, J. Culotti, S. George, and A. Chisholm for reporters, strains, and unpublished information; the *C. elegans* Genetic Center for strains; and members of the Emmons laboratory for assistance and guidance. This work was supported by NIH Grant RO1 GM39353. A.H. was supported by NIH Medical Scientist Training Program Grant T32 GM07288. S.W.E. is the Siegfried Ullmann Professor of Molecular Genetics.

References

Baird, S.E., Fitch, D.H., Kassem, I.A.A., Emmons, S.W., 1991. Pattern formation in the nematode epidermis: determination of the peripheral sense organs in the *C. elegans* male tail. Development 113, 515–526.

Carpenter, E.M., Goddard, J.M., Chisaka, O., Manley, N.R., Capecchi, M.R., 1993. Loss of Hox-A1 (Hox-1.6) function results in the reorganization of the murine hindbrain. Development 118, 1063–1075.

Chen, J., Ruley, H.E., 1998. An enhancer element in the EphA2 (Eck) gene sufficient for rhombomere-specific expression is activated by HOXA1 and HOXB1 homeobox proteins. J. Biol. Chem. 273, 24670–24675.

Chin-Sang, I.D., Moseley, S.L., Ding, M., Harrington, R.J., George, S.E., Chisholm, A.D., 2002. The divergent *C. elegans* ephrin EFN-4 functions in embryonic morphogenesis in a pathway independent of the VAB-1 Eph factor. Development 129, 5499–5510.

Chin-Sang, I.D., Chisholm, A.D., 2000. Form of the worm: genetics of epidermal morphogenesis in *C. elegans*. Trends Genet. 16, 544–551.

Chin-Sang, I.D., George, S.E., Ding, M., Moseley, S.L., Lynch, A.S., Chisholm, A.D., 1999. The ephrin VAB-2/EFN-1 functions in neuronal signaling to regulate epidermal morphogenesis in *C. elegans*. Cell 99, 781–790.

Chisholm, A., 1991. Control of cell fate in the tail region of *C. elegans* by the gene *egl-5*. Development 111, 921–932.

Chow, K.L., Emmons, S.W., 1994. HOM-C/Hox genes and four interacting loci determine the morphogenetic properties of single cells in the nematode male tail. Development 120, 2579–2592.

Chow, K.L., Hall, D.H., Emmons, S.W., 1995. The *mab-21* gene of *Caenorhabditis elegans* encodes a novel protein required for choice of alternate cell fates. Development 121, 3615–3626.

Cowan, C.A., Henkemeyer, M., 2001. The SH2/SH3 adaptor Grb4 transduces B-ephrin reverse signals. Nature 413, 174–179.

Davy, A., Gale, N.W., Murray, E.W., Klinghoffer, R.A., Soriano, P., Feuerstein, C., Robbins, S.M., 1999. Compartmentalized signaling by GPI-anchored ephrin-A5 requires the Fyn tyrosine kinase to regulate cellular adhesion. Genes Dev. 13, 3125–3135.

Dollé, P., Lufkin, T., Krumlauf, R., Mark, M., Duboule, D., Chambon, P., 1993. Local alterations of Krox-20 and Hox gene expression in the hindbrain suggest lack of rhombomeres 4 and 5 in homozygote null Hoxa-1 (Hox-1.6) mutant embryos. Proc. Natl. Acad. Sci. USA 90, 7666–7670.

Ferreira, H.B., Zhang, Y., Zhao, C., Emmons, S.W., 1999. Patterning of *Caenorhabditis elegans* posterior structures by the Abdominal-B homolog, *egl-5*. Dev. Biol. 207, 215–228.

Flanagan, J., Vanderhaeghen, P., 1998. The ephrins and Eph receptors in neural development. Annu. Rev. Neurosci. 21, 309–345.

Francis, R., Waterston, R.H., 1991. Muscle cell attachment in *Caenorhabditis elegans*. J. Cell Biol. 114, 465–479.

Fraser, S., Keynes, R., Lumsden, A., 1990. Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. Nature 344, 431–435.

Garcia-Bellido, A., Ripoll, P., Morata, G., 1973. Developmental compartmentalization of the wing disk of *Drosophila*. Nat. New Biol. 245, 251–253.

George, S.E., Simokat, K., Hardin, J., Chisholm, A.D., 1998. The VAB-1 Eph receptor tyrosine kinase functions in neural and epithelial morphogenesis in *C. elegans*. Cell 92, 633–643.

Granato, M., Schnabel, H., Schnabel, R., 1994. *pha-1*, a selectable marker for gene transfer in *C. elegans*. Nucleic Acids Res. 22, 1762–1763.

Hattori, M., Osterfield, M., Flanagan, J.G., 2000. Regulated cleavage of a contact-mediated axon repellent. Science 289, 1360–1365.

Holder, N., Klein, R., 1999. Eph receptors and ephrins: effectors of morphogenesis. Development 126, 2033–2044.

Holmberg, J., Clarke, D.L., Frisen, J., 2000. Regulation of repulsion versus adhesion by different splice forms of an Eph receptor. Nature 408, 203–206.

Hu, H., Marton, T.F., Goodman, C.S., 2001. Plexin-B mediates axon guidance in Drosophila by simultaneously inhibiting active Rac and enhancing RhoA signaling. Neuron 32, 39–51.

Imondi, R., Wideman, C., Kaprielian, Z., 2000. Complementary expression of transmembrane ephrins and their receptors in the mouse spinal cord: a possible role in constraining the orientation of longitudinally projecting axons. Development 127, 1397–1410.

Krishna, S., Maduzia, L.L., Padgett, R.W., 1999. Specificity of TGF-beta signaling is conferred by distinct type I receptors and their associated SMAD proteins in *Caenorhabditis elegans*. Development 126, 251–260.

Lints, R., Emmons, S.W., 1999. Patterning of dopaminergic neurotransmitter identity among *Caenorhabditis elegans* ray sensory neurons by a TGF-beta family signaling pathway and a Hox gene. Development 126, 5819–5831.

Liu, B.P., Strittmatter, S.M., 2001. Semaphorin-mediated axonal guidance via Rho-related G proteins. Curr. Opin. Cell Biol. 13, 619–626.

Mellitzer, G., Xu, Q., Wilkinson, D.G., 1999. Eph receptors and ephrins restrict cell intermingling and communication. Nature 400, 77–81.

Mello, C.C., Kramer, J.M., Stinchcomb, D., Ambros, V., 1991. Efficient gene transfer in *C. elegans:* extrachromosomal maintenance and integration of transforming sequences. EMBO J. 10, 3959–3970.

Milan, M., Weihe, U., Perez, L., Cohen, S.M., 2001. The LRR proteins Capricious and Tartan mediate cell interactions during DV boundary formation in the *Drosophila* wing. Cell 106, 785–794.

Mohler, W.A., Simske, J.S., Williams-Masson, E.M., Hardin, J.D., White, J.G., 1998. Dynamics and ultrastructure of developmental cell fusions in the *Caenorhabditis elegans* hypodermis. Curr. Biol. 8, 1087–1090.

Nakamura, F., Kalb, R.G., Strittmatter, S.M., 2000. Molecular basis of semaphorin-mediated axon guidance. J. Neurobiol. 44, 219–229.

O'Leary, D.D., Wilkinson, D.G., 1999. Eph receptors and ephrins in neural development. Curr. Opin. Neurobiol. 9, 65–73.

Roy, P.J., Zheng, H., Warren, C.E., Culotti, J.G., 2000. *mab-20* encodes Semaphorin-2a and is required to prevent ectopic cell contacts during epidermal morphogenesis in *Caenorhabditis elegans*. Development 127, 755–767.

Salser, S.J., Kenyon, C., 1996. A *C. elegans* Hox gene switches on, off, on and off again to regulate proliferation, differentiation and morphogenesis. Development 122, 1651–1661.

Salser, S.J., Loer, C.M., Kenyon, C., 1993. Multiple HOM-C gene interactions specify cell fates in the nematode central nervous system. Genes Dev. 7, 1714–1724.

Savage, C., Das, P., Finelli, A.L., Townsend, S.R., Sun, C.Y., Baird, S.E., Padgett, R.W., 1996. *Caenorhabditis elegans* genes *sma-2*, *sma-3*, and *sma-4* define a conserved family of transforming growth factor beta pathway components. Proc. Natl. Acad. Sci. USA 93, 790–794.

Seitanidou, T., Schneider-Maunoury, S., Desmarquet, C., Wilkinson, D.G., Charnay, P., 1997. Krox-20 is a key regulator of rhombomere-specific gene expression in the developing hindbrain. Mech. Dev. 65, 31–42.

Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A., Krumlauf, R., 1996. Altered segmental identity and abnormal migration of motor neurons in mice lacking Hoxb-1. Nature 384, 630–634.

Sulston, J.E., Albertson, D.G., Thomson, J.N., 1980. The *Caenorhabditis elegans* male: postembryonic development of non gonadal structures. Dev. Biol. 78, 542–576.

Taneja, R., Thisse, B., Rijli, F.M., Thisse, C., Bouillet, P., Dolle, P., Chambon, P., 1996. The expression pattern of the mouse receptor tyrosine kinase gene MDK1 is conserved through evolution and requires Hoxa-2 for rhombomere-specific expression in mouse embryos. Dev. Biol. 177, 397–412.

Wang, H.U., Chen, Z.F., Anderson, D.J., 1998. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell 93, 741–753.

Wang, X., Roy, P.J., Holland, S.J., Zhang, L.W., Culotti, J.G., Pawson, T., 1999. Multiple ephrins control cell organization in *C. elegans* using kinase-dependent and -independent functions of the VAB-1 Eph receptor. Mol. Cell 4, 903–913.

Wilkinson, D.G., 2001. Multiple roles of Eph receptors and ephrins in neural development. Nat. Rev. Neurosci. 2, 155–164.

Winberg, M.L., Tamagnone, L., Bai, J., Comoglio, P.M., Montell, D., Goodman, C.S., 2001. The transmembrane protein off-track associates with plexins and functions downstream of semaphorin signaling during axon guidance. Neuron 32, 53–62.

Xu, Q., Mellitzer, G., Robinson, V., Wilkinson, D.G., 1999. In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. Nature 399, 267–271.

Zhang, Y., Emmons, S.W., 1995. Specification of sense-organ identity by a *Caenorhabditis elegans* Pax-6 homologue. Nature 377, 55–59.

Zhang, Y., Ferreira, H.B., Greenstein, D., Chisholm, A., Emmons, S.W., 1998. Regulated nuclear entry of the *C. elegans* Pax-6 transcription factor. Mech. Dev. 78, 179–187.