Conversion of cell movement responses to Semaphorin-1 and Plexin-1 from attraction to repulsion by lowered levels of specific RAC GTPases in *C. elegans*

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Summary

Plexins are functional receptors for Semaphorin axon guidance cues. Previous studies have established that some Plexins directly bind RAC^{GTP} and RHO. Recent work in *C. elegans* showed that *semaphorin 1 (smp-1* and *smp-2)* and *plexin 1 (plx-1)* are required to prevent anterior displacement of the ray 1 cells in the male tail (Fujii et al., 2002; Ginzburg et al., 2002). We show genetically that *plx-1* is part of the same functional pathway as *smp-1* and *smp-2* for male ray positioning. RAC GTPase genes *mig-2* and *ced-10* probably function redundantly, whereas *unc-73*, which encodes a GEF for both of these GTPases, is required cell autonomously for preventing anterior displacement of ray 1 cells. RNAi analysis indicates that *rho-1*-encoded RHO GTPase, plus *let-502* and *K08B12.5*-encoded RHO-kinases, are also required to prevent anterior displacement of ray 1 cells, suggesting that different kinds of RHO-family GTPases act similarly in ray 1 positioning. At low doses of wild-type *mig-2* and *ced-10*, the Semaphorin 1 proteins no longer act through PLX-1 to prevent anterior displacements of ray 1, but have the opposite effect, acting through PLX-1 to mediate anterior displacements of ray 1. These results suggest that Plexin 1 senses levels of distinct RHO and RAC GTPases. At normal levels of RHO and RAC, Semaphorin 1 proteins and PLX-1 prevent a forward displacement of ray 1 cells, whereas at low levels of cycling RAC, Semaphorin 1 proteins and PLX-1 actively mediate their anterior displacement. Endogenously and ectopically expressed SMP-1 and SMP-2 suggest that the hook, a major source of Semaphorin 1 proteins in the male tail, normally attracts PLX-1-expressing ray 1 cells.

Key words: Plexins, Migration, RHO-GTPases, *C. elegans*

Introduction

The organization of the nervous system depends on the guidance of migrating cells and neuronal growth cones to their appropriate targets. Growth cones change their morphology in response to repulsive or attractive extracellular guidance cues. An attractive cue causes spreading and extension of the growth cone leading edge towards the cue and a repulsive cue causes growth cone stalling, deflection, or repulsion by mechanisms involving partial or total growth cone collapse. These cell shape changes and movements are clearly based on differential regulation of actin dynamics (da Silva and Dotti, 2002); however, little is known about how extracellular guidance cues signal to the guidance receptors to regulate actin polymerization and depolymerization in order to change growth cone morphology.

Good candidates for downstream effectors of guidance cues and their receptors are the small GTPases of the RHO family, RAC, RHO and CDC-42. These GTPases regulate actin cytoskeleton dynamics in neurons (Luo, 2000) and in non-neuronal cells (Hall, 1998), and they act as molecular switches cycling between a GTP-bound 'on' state and a GDP-bound 'off' state (Hall, 1998). Positive and negative regulators of RHO GTPases include guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively (Dickson, 2001).

RHO-family GTPases have been implicated in axon pathfinding and cell migration through the analysis of constitutively active or dominant-negative forms of these proteins and their effectors in cultured migrating cells (Eickholt et al., 1999; Jin and Strittmatter, 1997; Kuhn et al., 1999). However, the involvement of RAC GTPases in regulating cell movements and morphogenesis in vivo has been best demonstrated through genetic analyses of model organisms. For example, the three known RAC genes in *C. elegans*, *ced-10*, *mig-2* and *rac-2*, have been thoroughly examined for their effects on CAN cell, gonadal leader cell (e.g. distal tip cells of the hermaphrodite gonad), P cell and axon growth cone migrations, plus apoptotic cell phagocytosis (which involves aspects of cell migration), using both genetic and RNAi-induced loss-of-function approaches, as well as genetic gain-of-function approaches (Kishore and Sundaram, 2002; Lundquist et al., 2001; Spencer et al., 2001; Zipkin et al., 1997). *C. elegans* RAC GTPases clearly have shared (redundant or same pathway) and distinct (parallel pathways) functions, sometimes dependent on cell type and in other cases dependent on the aspect of migration being examined in a

particular cell type. For example *ced-10*, *mig-2* and *rac-2* have largely redundant functions in CAN and GABAergic axon guidance, and in CAN cell migration, but *mig-2* and *ced-10* have distinct functions in determining the direction of the third phase of DTC migration (Lundquist et al., 2001), with double-mutant analysis suggesting that these two genes act in the same pathway to regulate this migration. The *C. elegans* RAC GEF activity of UNC-73, previously shown to be involved in axon guidance and cell migration (Steven et al., 1998), behaves genetically as though it activates CED-10, MIG-2 and RAC-2 in vivo (Lundquist et al., 2001), and is therefore another important component of axon guidance and cell migration signaling mechanisms. The *Drosophila* and vertebrate homologs of *C. elegans* UNC-73 appear to have evolutionarily conserved functions in related signaling pathways (Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000).

In the literature it is unclear whether specific RHO-family GTPases have the same function in different cell types or in different situations. Several studies have shown that attractive guidance cues activate RAC or CDC-42 to promote cell or growth cone advance (Luo, 2000; Mueller, 1999; Suter and Forscher, 1998), whereas repulsive cues activate RHO to inhibit cell or growth cone advance, or to induce retraction (Dickson, 2001; Jalink et al., 1994; Luo, 2000; Yuan et al., 2003). However, the axon guidance receptor most directly implicated in regulating RHO and RAC activities for its output is the Semaphorin receptor Plexin. Both *Drosophila* and *C. elegans* lack the other major class of semaphorin receptors, the neuropilins, but *Drosophila* Sema-1a binds Plexin A and *C. elegans* SMP-1 (Ce-sema-1a) binds Plexin-1 (PLX-1) (Fujii et al., 2002; Winberg et al., 1998). *Drosophila* PlexB and mammalian PlexB1 directly bind the activated GTP-bound form of RAC but not its inactive GDP-bound form (Driessens et al., 2001; Hu et al., 2001; Vikis et al., 2000). *Drosophila* PlexB also binds GDP and GTP forms of RhoA (Rho1 – FlyBase) and has been proposed to stimulate RhoA (Hu et al., 2001).

In *Drosophila*, it has been proposed that semaphorin-activated Plexin B (PlexB) sequesters RAC^{GTP} and thereby downregulates its downstream serine/threonine kinase effector PAK while stimulating the RHO pathway (Hu et al., 2001; Vikis et al., 2002). According to this view, inactivation of the RAC^{GTP}-dependent growth cone spreading mechanism is a pre-requisite step for RHO-induced collapse stimulated by Plexin B (Hu et al., 2001; Vikis et al., 2002). This model is based on in vivo gain-of-function studies and has not yet been validated by loss-of-function studies. Nonetheless, these *Drosophila* studies indicate that semaphorin signaling through plexins is an excellent starting point for understanding how the activation of particular guidance receptors affect signaling through RHO family GTPases to influence cell movements and morphogenesis.

We examine genetically the function of *C. elegans* Semaphorin 1 proteins and Plexin 1 in the positioning of sensory ray 1 cells during male tail development. *C. elegans* has two plexin-related genes, *plx-1* and *plx-2*, encoding Plexin 1 (most closely related to *Drosophila* and human Plexin A) and Plexin 2, respectively. *C. elegans* also has three semaphorin genes, *smp-1*, *smp-2* and *smp-3*, encoding Sema 1A, Sema 1B and Sema 2A/MAB-20, respectively. We find that in mutants lacking *semaphorin 1* genes (i.e. *smp-1* and *smp-2)* or *plexin 1* (i.e. *plx-1*), ray 1 cells are positioned anterior to their normal position. *smp-1* and *smp-2* were shown previously to be required redundantly to prevent this anterior displacement of the ray 1 cells (Fujii et al., 2002; Ginzburg et al., 2002). We now show that *smp-1* and *smp-2* largely require *plx-1* for this function. We further show that prevention of the anterior displacement of the ray 1 cells also depends on RAC and RHO GTPases independent of *smp-1*, *smp-2* and *plx-1*; however, the relative levels of active RAC are deciphered when Semaphorin 1 activates Plexin 1 signaling. Lowered doses of specific wild-type RAC-encoding genes can cause a polarity switch in the Plexin 1-dependent positioning of ray 1 cells.

Based on expression patterns for *plx-1*, *smp-1* and *smp-2*, and on the genetic analysis of mutants in these genes in *C. elegans*, we propose that at normal cycling RHO^{GTP} and RAC^{GTP} levels, PLX-1 induces an apparent attraction to sources of SMP-1 and SMP-2, by using the known cell spreading and adhesion functions of RHO-family GTPases. By contrast, at low RAC^{GTP} levels, PLX-1 induces an apparent repulsion from the same sources of SMP-1 and SMP-2. The anterior displacement of ray 1 cells caused by *plx-1* mutations is suppressed by mutations in *unc-33*/CRMP, a known mediator of semaphorin-induced axon growth cone collapse in other animals (Goshima et al., 1995). The spatiotemporal expression patterns of *plx-1*, *smp-1* and *smp-2* reporters suggest a cell-based model for the control of anterior ray 1 displacements, which we have further examined by cell ablation and ectopic expression studies.

Materials and methods

Nematode culture

General procedures used for the culture, maintenance and storage of nematodes can be found in Wood (Wood, 1988). Mutant strains used in this study were as follows.

Linkage Group X (LGX): *mig-2(mu28)* (Zipkin et al., 1997) and *mig-2(gm103gf)* (Forrester and Garriga, 1997).

LGI: *smp-1(ev715)* (Ginzburg et al., 2002), *unc-73(ev509)* (Steven et al., 1998), *unc-73(e936)* (Desai et al., 1988), *unc-73(rh40)* (Steven et al., 1998), *smp-2(ev709)* (Ginzburg et al., 2002) and *mab-20(bx61)* (Baird et al., 1991).

LGIV: *unc-33(e1261)* (Li et al., 1992), *ced-10(n1993)* (Ellis et al., 1991), *lin-1(e1275)* (Kimble et al., 1979), *plx-1(ev724)* (this study) and *plx-1(nc37)* (Fujii et al., 2002).

LGV: *him-5(e1490)* (Hodgkin et al., 1979).

Strains not isolated in our laboratory were obtained from the *C. elegans* Genetics Center, care of T. Stiernagle (The University of Minnesota), or from G. Garriga (U. C. Berkeley).

Reverse genetics

A frozen reverse genetics library, which represents 1.7 million mutagenized haploid genomes, was screened for deletions in the *Ce-plexin-1* gene using nested PCR methods (Roy et al., 2000; Zwaal et al., 1993). Once a deletion sample was identified, sib selections were performed to isolate the homozygous deletion strain NW1391 *plx-1(ev724)*. AmpliTaq GOLDTM (Perkin-Elmer) was used in all PCR reactions. The isolated deletion allele *plx-1(ev724)* was outcrossed with N2 Bristol strain at least five times before further analysis. Defining the first nucleotide of the initiation codon as the first nucleotide in a DNA sequence, the *plx-1(ev724)* allele was deleted for nucleotide pairs 16135 to 18134.

Molecular biology

Standard molecular biology methods (Sambrook et al., 1989) were used unless otherwise noted. The λZAPII (Stratagene) cDNA clone *yk535f1* was provided by Y. Kohara and excised in vivo.

Gene specific cDNA analysis and genotyping

Total RNA was isolated using the standard Trizol (GIBCO-BRL) protocol. A standard reverse transcription (RT) protocol (Moon and Krause, 1991) was used to amplify gene specific products either using oligo dT or random primers to identify all RNA populations. RT-PCR products comprising wild-type or mutant cDNAs were cloned into *pBSK+* or *pGEMT*-easy vectors and sequenced to confirm the ORFs. *Ce-plx-1* specific primers flanking the genomic DNA deletion of *plx-1(ev724)* were used to follow the mutation during outcrossing and multiple mutant strain constructions. Primer sequences are available upon request.

Transgene constructs

A *plx-1* transcriptional *gfp* reporter was constructed by cloning the 2621 bp sequence immediately 5′ to the initiation codon into the multiple cloning site of pPD95_77 to generate plasmid pPD95_77cplx. A *plx-1(+)* rescuing construct was assembled from multiple PCR fragments encompassing the entire coding sequence of Ce-PLX-1. The 3′ portion of the construct comes from the cDNA *yk535f1* and contains 739 bp of the 3′UTR. This *plx-1(+)* cDNA minigene was cloned downstream of the promoter sequence of the pPD95_77cplx transcriptional reporter to obtain the plasmid pZH127. The *gfp* coding sequence is out of frame in pZH127. The construct contains the full-length *plx-1(+)* minigene with 2621 bp of sequence immediately 5′ to the initiation codon and 739 bp of the 3′UTR sequence.

The GFP-encoding portion of pZH127 was put in frame with the PLX-1(+) sequence by fusing it after the *Pml*I site located four amino acids before the stop codon. For this, a *Sph*I-*Kpn*I fragment was deleted from pZH127, cut with *Pml*I and re-ligated in combination with a linker sequence into the *Sph*I-*Kpn*I cut pZH127 to obtain the new *plx-1* translational reporter plasmid pZH157.

An *unc-73(+)* gene driven by the *plx-1* 5′ regulatory sequence was constructed by sub-cloning a 6340 bp *Nco*I-*Sma*I cDNA fragment of pZH63 (Steven et al., 1998), encoding the full-length UNC-73, into a modified version of pPD95_77cplx transcriptional reporter construct. For this sub-cloning, the *Pst*I site in the multiple cloning site of pPD95_77cplx was mutated to generate an *Nco*I site. The resulting plasmid (pZH163) encodes a full-length *unc-73(+)* under the control of the *plx-1* promoter.

A *smp-1* translational GFP reporter gene was obtained by ligating a GFP cassette, PCR amplified from pPD95_77, into the unique *Nhe*I site (exon 12) of the pVGS1a plasmid containing a 10 kb *Xba*I genomic fragment from the *smp-1* locus (Ginzburg et al., 2002). The resulting plasmid (pVGS1a::GFP) encodes the entire extracellular domain, the transmembrane domain and an intra-cellular GFP reporter. The original plasmid pVGS1a has the ability to completely rescue the phenotypes of *smp-1* mutant animals (Ginzburg et al., 2002).

Generation and analysis of transgenic strains

Transgenic strains were as follows:

evIs140[pPD95_77cplx plx-1::gfp; rol-6(su1006)] (*plx-1* transcriptional reporter);

evEx162 [pZH127 plx-1(+); rol-6(su1006)] (cDNA rescues *plx-1* mutant);

evIs162 [pZH127 plx-1(+); rol-6(su1006)] (cDNA rescues *plx-1* mutant);

evEx168 [pZH163 plx-1::unc-73(+); rol-6(su1006)] (*unc-73* expressed by *plx-1* promoter);

evEx169 [pZH157 plx-1(+)::GFP; rol-6(su1006)] (*plx-1* translational reporter); and

evEx170 [pVGS1a::GFP; rol-6(su1006)] (*smp-1* translational reporter).

Transgenic strains were generated by co-microinjection of the DNA mix into the distal gonad arms of N2 or *him-5(e1490)* hermaphrodites (Mello and Fire, 1995). DNA mixes consisted of a test construct at a concentration of 50 mg/μl or 30 mg/ml and a co-injection marker to create a final DNA concentration of 100 mg/μl. Transgenic extra-chromosomal arrays were integrated using a UV irradiation-based method (Mitani, 1995). Integrated alleles were backcrossed five times before phenotypic analysis.

RNA interference

RNAi constructs were made by sub-cloning a PCR fragment representing a unique sequence from the targeted gene into the multiple cloning site of L4440 (Timmons et al., 2001). The targeted genes included a *C. elegans* homolog of RHO (*Y51H4A.3*), two *C. elegans* homologs of RHO-kinases (*C10H11.9/let-502* and *K08B12.5*) and exon 8 of *unc-33* (Y37E11C.1). Plasmids were transfected into bacterial strain HT115. Bacteria were induced with IPTG using a variation of Protocol Number 2 from Kamath (Kamath et al., 2001). After induction, bacteria were immediately used to seed NGM growth plates.

Cos 7 transfection and sub-cellular localization

A DNA construct encoding full-length *C. elegans plx-1* MYC-tagged cDNA under the control of the CMV promoter (Fujii et al., 2002) was transfected into Cos7 cells using lipofectamine following the manufacturer's protocol (Gibco). Cells were grown at 37°C in 5% CO_2 for 24 hours post-transfection in RPMI media supplemented with 10% fetal bovine serum. Prior to immnunostaining, cells were fixed in 4% paraformaldehyde for 10 minutes, then permeabilized with PBS containing 0.2% Triton X-100. Cells were immunostained using a mouse monoclonal anti-MYC antibody (9E10, Santa Cruz) and an Alexa 488-conjugated anti-mouse secondary antibody. Cells were co-stained with rhodamine-conjugated phalloidin (Molecular Probes). Cell morphology was observed using DIC optics of a DMRA2 Leica microscope.

Microscopy

Male tail anterior ray 1 displacement and ray fusion events were scored by mounting 1 mM levamisole-treated animals on 2% agarose pads for observation using DIC optics. The *ajm-1::GFP* translational reporter (Simske and Hardin, 2001) was visualized with an Applied Precision Deconvolution microscope or a Leica DMRXA microscope to assess epidermal cell morphologies.

Standard errors for percentages of the anterior ray 1 phenotypes were calculated assuming a binomial distribution with the observed percentage value and the actual sample size. Statistical tests were carried out using a standard (two-tailed) comparison of two proportions (Moore and McCabe, 1998). All *P* values represent the probability that the measured penetrance of the phenotype is significantly different between two strains. A *P* value less than 0.05 was considered significant. All comparisons described as significant in the Results were based on this criterion.

Laser ablations

Laser cell ablations were performed using a Leica DMLFS confocal microscope. Briefly, *him-5(e1490)* third larval stage males were anaesthetized using 10 mM sodium azide in M9 solution and mounted on 2% agarose pads. Developing hook cells were located using the *ajm-1::GFP* reporter and ablations were assisted with the Leica confocal software (version 11.04). Worms were recovered after the ablations and allowed to grow 24 hours before scoring the male tail phenotype. Using this same protocol, two control ablations of L3 stage ray 3 cells were both successful at specifically eliminating ray 3 in the adult.

Results

Cloning of the plexin encoding gene *plx-1*

The sequence of linkage group IV, determined by the Sanger Center *C. elegans* consortium, revealed the *plx-1* gene *Y55F3AL.1*, which encodes a Plexin 1 homolog (see also Fujii et al., 2002). A full-length cDNA of 5.8 kb was constructed by RT-PCR-amplification of RNA using predicted gene-specific primers and verified by sequencing (Materials and methods). The ORF encodes a 1978 amino acid protein (Plexin 1 or PLX-1), which comprises an N-terminal hydrophobic signal sequence for secretion, followed by a semaphorin domain, three PSI domains (homology to Plexins, Semaphorins and Integrins) and four immunoglobulin-like folds (three IPT and one TIG) as revealed by SMART analysis (Fig. 1B) (Letunic et al., 2002; Schultz et al., 1998). The predicted cytoplasmic domain of PLX-1 shares the highest homology with Plexins from other species. The PLX-1 cytoplasmic domain contains a seven amino acid sequence that is almost identical to the RAC-binding domain (NTLAHYG) described for *Drosophila* Plexin B (Fig. 1C) (Hu et al., 2001). A multiple alignment of the C termini of Plexins from many species shows divergence between the RhoA-binding region defined for *Drosophila* PlexinB (Hu et al., 2001) and other Plexins, including Ce-PLX-1 (Fig. 1B,C).

The isolation and molecular analysis of a *plx-1* deletion mutant

plx-1(ev724) (isolated as described in Materials and methods) is deleted for 1200 bp of the wild-type *plx-1* genomic sequence, which removes exon 19. Splicing of exon 18 to exon 20, as confirmed by sequencing of RT-PCR products, creates a stop codon at position 3502 of the corresponding cDNA (Fig. 1A,B). Thus, this deletion is predicted to produce a truncated receptor lacking both its transmembrane and cytoplasmic domains. The mutant protein is predicted to be unable to anchor itself in the cell membrane and to have no intracellular signaling activity. The mutant allele is totally recessive to the wild-type allele in an otherwise wild-type genetic background, suggesting that it does not have a dominant-negative effect on gene function. The *plx-1(nc37)* allele from Fujii et al. (Fujii et al., 2002), is deleted for the amino-terminal part of PLX-1, but has the same penetrance of ray 1 positioning defects (Table 1; also see below). It is likely therefore that both alleles are nulls.

Plexin 1 and the Semaphorin 1 proteins function together to prevent anterior displacement of male ray 1 cells

The adult male tail possesses nine bilaterally symmetric sensory rays protruding within a spade-shaped fan made of cuticle (Fig. 2B). Baird et al. (Baird et al., 1991) visualized cell position and shape changes during male tail morphogenesis by staining an adherens junction epitope encoded by *ajm-1* with the mAb MH27 (Baird et al., 1991; Francis and Waterston, 1985). In males, starting as early as the third larval stage, the posterior seam cells (V5, V6 and T) undergo additional rounds of division, producing nine bilaterally symmetric ray/SET precursor cells [R(*n*) cells, where *n*=1-9]. V5 generates the most anterior cells, ray 1, whereas V6 and T generate the other rays. Each ray precursor divides to produce an anterior (Rn.a) and a posterior cell (Rn.p). For ray lineages 1-5, the Rn.p cells ultimately fuse to one another to form the lateral epidermal syncitium in the tail called the SET cell. For all nine ray lineages, the Rn.a cell divides to produce four cells, one of which undergoes a programmed cell death, and the other three, the ray cell cluster, ultimately form a ray sensillum containing two neurons (RnA and RnB) and a support cell (Baird et al., 1991; Sulston and Horvitz, 1977).

Using the *ajm-1::GFP* tagged protein reporter to observe adherens junctions in vivo (Simske and Hardin, 2001), we found that in wild-type animals, after the initial sorting of ray cell clusters, the ray 1 cell cluster remains at a relatively fixed distance from other landmarks such as other rays and the male hook (Figs 2, 5). However, in *plx-1(ev724)* mutant males, the ray 1 cell cluster is frequently displaced anterior to its normal position (Fig. 2E,F; Table 1). This phenotype is completely recessive and is identical to the one previously described for *smp-1(ev715)* and *smp-2(ev709)* single mutants, and for *smp-1(ev715); smp-2(ev709)* double mutants (Table 1) (Ginzburg et al., 2002). The *plx-1(nc37)* allele has been reported to share this phenotype (Fujii et al., 2002), which has two separable degrees of severity: a class 1 severe phenotype in which ray 1 is positioned anterior to the fan area; and a class 2 mild phenotype in which ray 1 is shorter and is displaced anteriorly, but is still within the fan area (Fig. 2C,D). For the severe class 1 phenotype, ray 1 is often positioned dorsal to its normal ventral lateral position and close to the adherens junction that connects the SET to the lateral epidermis (Fig. 2D,G,H).

The abnormal anterior positioning of ray 1 cells in the third larval stage is the earliest defect observed in the *plx-1(ev724)* male tail, and becomes apparent as R1.p changes shape, as it does in the wild type, by extending toward the anterior (Fig. 2E). The other Rn.p cells also change their morphology and fuse normally with each other in *plx-1* mutants.

In contrast to the wild type, *plx-1* mutant ray 1 cells often fail to detach from the SET during the late fourth larval stage (Fig. 2H, compare with Fig. 2G). This occurs almost invariably with the severe class 1 phenotype, but the milder class 2 phenotype is occasionally observed without this persistent adhesion of ray 1 to the SET (data not shown).

plx-1 normally works in the same genetic pathway as *smp-1* and *smp-2*

Single mutants for *smp-1(ev715)* and *smp-2(ev709)* have an incompletely penetrant anterior ray 1 defect. However, the *smp-1(ev715); smp-2(ev709)* double mutant is synergistically enhanced for this phenotype (Ginzburg et al., 2002). The severity of the ray 1 phenotype was re-examined in *smp-1* and *smp-2* mutants according to criteria defined above (anterior displaced ray 1 classes 1 and 2), and compared with the ray 1 phenotype observed in *plx-1(ev724)*. Interestingly, the phenotype is temperature-sensitive for *smp-1(ev715); smp-2(ev709)* double mutants and for the *plx-1(ev724)* single mutant (Table 1), suggesting the existence of an unknown temperature-sensitive process involved in preventing anterior displacement of ray 1 that is revealed when Sema-1/PLX-1 signaling is absent. At the restrictive temperature (25°C), the penetrance of the anterior class 1 and class 2 ray 1 phenotypes combined is slightly but significantly higher ($P<0.005$) in the *plx-1(ev724)* single mutant compared with in the *smp-1(ev715); smp-2(ev709)* double mutant (Table 1) (e.g. 32% versus 29%, and 50% versus 35%, for class 1 and 2 defects, respectively). This suggests that PLX-1 has some minor

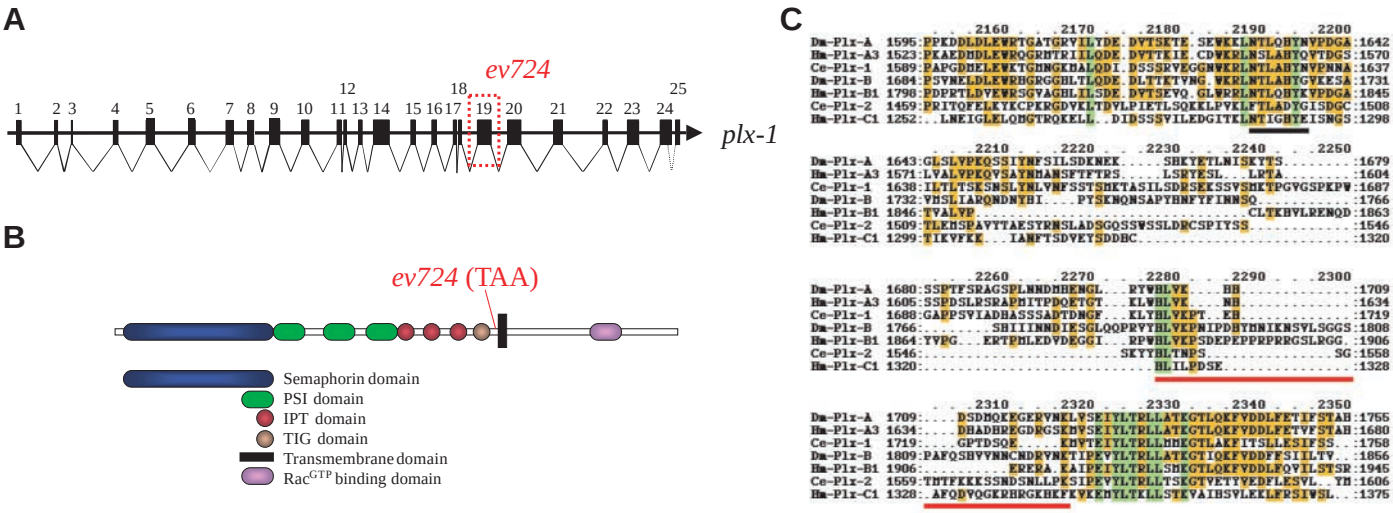

Fig. 1. The isolation of a deletion allele within the *C. elegans plexin 1* locus. (A) A deletion allele of *Ce-plx-1* was isolated from a mutagenized *C. elegans* N2 strain frozen library screened using a PCR based method (see Materials and methods). The genomic DNA deletion removes all of exon 19 (red dotted rectangle), which encodes the transmembrane domain. (B) The *plx-1* genomic DNA sequence was used to design primers to PCR amplify multiple cDNAs that were sequenced and assembled into a full-length clone. The cDNA from *plx-1(ev724)* was sequenced and revealed abnormal splicing between exons 18 and 20 resulting in a frame-shift mutation. (C) The intracellular portion of *C. elegans* PLX-1 is highly conserved with human and *Drosophila* plexins. In particular, the seven residue region (black underline) responsible for RAC^{GTP} binding in *Drosophila* Plexin B is well conserved in *Drosophila* Plexin A, *C. elegans* PLX-1 and PLX-2, and human plexins A3, B1 and C1. The RHO-binding region defined for *Drosphila* Plexin B (red underline) is less well conserved, as a large portion of it is missing in plexins from other species. However, the amino acids bordering this region are well aligned in plexins from *C. elegans*, *Drosophila* and human. The alignment includes Hm-PLX-A3 (X87852), Hm-PLX-B1 (X87904), Hm-PLX-C1 (AF030339), Dm-PLX-A (T13937), Dm-PLX-B (T13164), Ce-PLX-1 (NP_500018) and Ce-PLX-2 (NP_497001).

function in ray 1 positioning that is independent of Semaphorin 1 signaling.

To determine whether *plx-1(+)*, *smp-1(+)* and *smp-2(+)* work in the same genetic pathway, a triple mutant was constructed. At 25°C, the class 1 and class 2 ray 1 defects combined were slightly but significantly suppressed (P<0.0005) in the *plx-1(ev724); smp-1(ev715); smp-2(ev709)* triple when compared to *plx-1(ev724)* (Table 1). Despite the minor differences in penetrance and expressivity between the mutants, these results strongly suggest that *plx-1* and *smp-1* and *smp-2* function largely in the same pathway (see also Fig. 6 and Discussion).

plx-1 is expressed in the male ray 1 cells and is associated with actin filaments in membrane ruffles of Cos7 cells

To determine where *plx-1* is expressed, we constructed GFP transcriptional and translational reporters for *plx-1* (see Materials and methods). Expression of both reporters is observed in all body wall muscles, male sex specific muscles and in the lateral epidermis during post-embryonic development (data not shown). At the third larval stage, male tail hypodermal expression begins in all dividing Rn.a and Rn.p cells although predominantly in R1.a and R1.p (Fig. 3A,B). The strongest expression of the transcriptional reporters is observed in the ray 1 cells. Expression of the transcriptional reporters in other rays is weak and eventually disappears. A similar effect is observed for the translational reporter, which expresses first and most highly on the ray 1 and ray 2 cells. Although the translational reporter is found on all rays at later stages of male tail development, this expression is weak relative to the earlier expression in precursors to rays 1 and 2 (Fig. 3B-D). These results suggest that, during male tail ray development, *plx-1* is predominantly expressed in the cells that

Table 1. Ray 1 anterior phenotype in wild-type, *plexin 1* and *semaphorin 1* mutant strains

Genotype*	Ray 1 class 1 (%)†	Ray 1 class 2 (%)†	n†
Wild type 16°C	0	0	124
Wild type 20°C	0	0	120
Wild type 25°C	0	0	110
plx-1(ev724) 16°C	5±2	15±3	132
plx-1(ev724) 20°C	10±2	39±3	359
plx-1(ev724) 25°C‡	32±4	50±4	157
plx-1(nc37) 25°C	33±4	50±5	120
smp-1(ev715); smp-2(ev709) 16°C	4±2	7±2	115
smp-1(ev715); smp-2(ev709) 20°C	4±1	25±3	226
smp-1(ev715); smp-2(ev709) 25°C‡	29±3	35±4	190
smp-1(ev715); smp-2(ev709); plx-1(ev724) 25°C‡	21±2	46±3	309

*All strains have the *ajm-1::gfp* reporter gene in the *him-5(e1490)* background. Animals were grown at the indicated temperature.

†The frequency of the severe (class 1) and mild (class 2) anterior ray 1 phenotype was determined as described in Materials and methods. n represents the number of animals scored. Standard deviations were calculated assuming a binomial distribution with the observed percentage value and the actual sample size. For all comparisons described as significant in the Results, the P value was <0.05.

‡Statistical comparisons between *plx-1(ev724)* 25°C and *smp-1(ev715); smp-2(ev709)* 25°C (P<0.005), *plx-1(ev724)* 25°C and *smp-1(ev715); smp-2(ev709); plx-1(ev724)* 25°C (P<0.0005), and *smp-1(ev715); smp-2(ev709)* 25°C and *smp-1(ev715); smp-2(ev709); plx-1(ev724)* 25°C (P>0.5) take into account the sum of the ray 1 class 1 and 2 phenotypes; other comparisons described in the Results were based on the ray 1 class 1 phenotype.

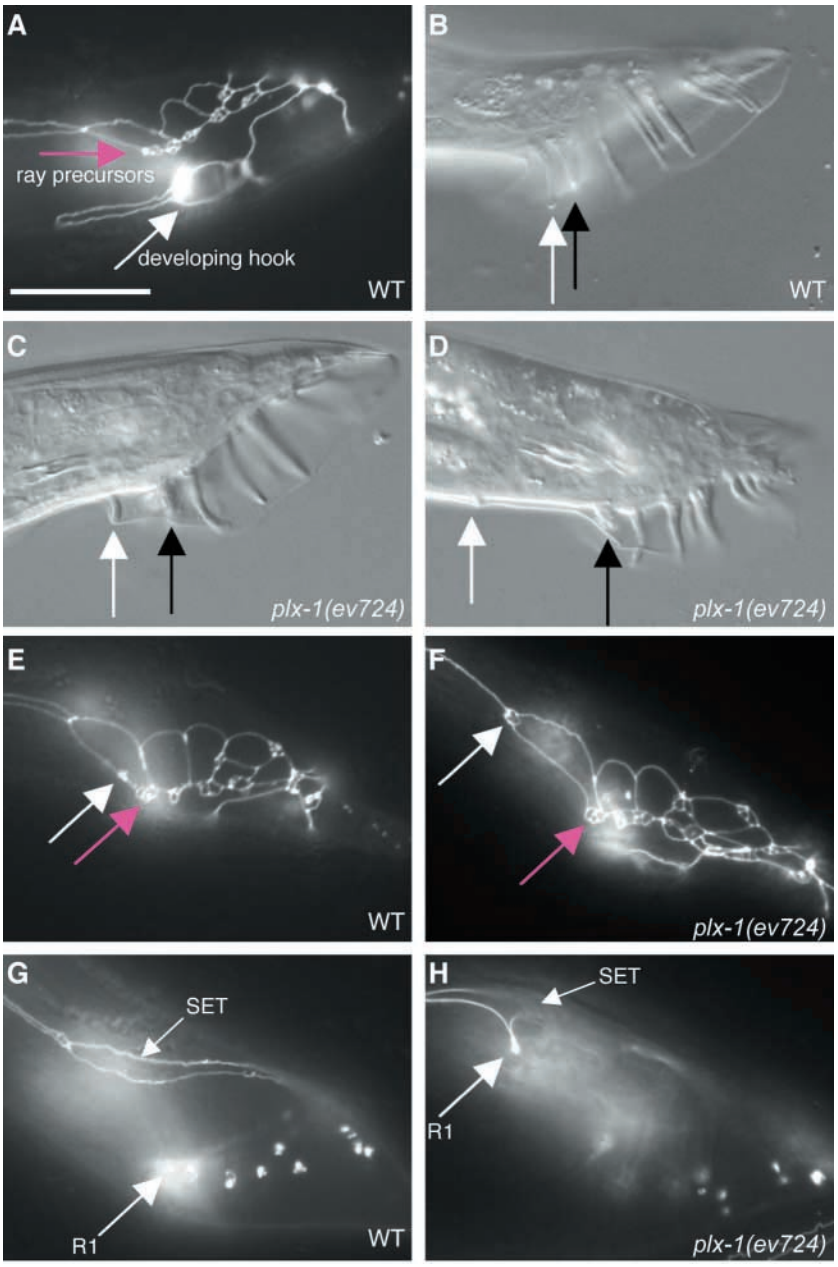

Fig. 2. Ray 1 cells and the adult ray 1 are displaced anteriorly in *plx-1(ev724)*. The position of ray 1 cells was determined by fluorescence microscopy using the *ajm-1::GFP* reporter in L3 males (Baird et al., 1991; Koppen et al., 2001). For all panels, anterior is left and ventral is bottom. (A) Male rays develop from two bilaterally symmetric ray/SET precursor cells (R*n* cells, where *n*=1-9). In the third larval stage (L3), ray 1-4 cell clusters (pink arrow) lie ventral to their corresponding R1-4.p sister cells. The developing hook (A; white arrow) is located ventral to the ray 1-2 cells. (B-D) The position of adult male rays was determined by DIC microscopy. Ray 1 is observed at an abnormal anterior position in *plx-1(ev724)* adult males (C,D) when compared with wild-type males (B). In wild-type males, ray 1 (B; white arrow) is observed in close opposition to ray 2 (B; black arrow). A mild ray 1 anterior phenotype (ray 1 class 2) is scored when ray 1 (C; white arrow) is observed just anterior to it normal position (C; black arrow), but is still within the fan structure. A severe ray 1 anterior displacement phenotype (ray 1 class 1) is defined as a ray 1 located outside the fan area (D; white arrow) even further anterior to ray 2 (D; black arrow). A ray 1 that is shorter than in wild-type males is also characteristic of both types of anterior ray 1 displacement (B-D). Ray 1 cells (white arrow) are displaced anterior in *plx-1(ev724)* L3 males (F) when compared with wild-type animals (E) of the same stage. Other ray cells, including ray 2 cells (E,F; pink arrow), are not affected in *plx-1(ev724)* L3 males. (G) A detachment of all rays [ray 1 (R1) shown by large arrow] from the male tail syncitium (SET) is always observed in adult wild-type males. (H) Anterior ray 1 (large arrow) displacements in adult *plx-1(ev724)* males is usually accompanied by a persistent adhesion to the SET. Scale bar: 25 μm.

comprise rays 1 and 2 at the stage when the defect (anterior displacement of the R1.a derived ray precursors) first manifests in mutants of *plx-1*, *unc-73*, *mig-2*, *ced-10*, *smp-1* and *smp-2*.

The *plx-1* transcriptional reporter fills cells that express it, whereas the translational reporter appears localized to the cell periphery, as expected for a transmembrane protein. To determine whether PLX-1 co-localizes with cellular structures associated with migration, we decided to express it in mammalian cells grown in culture. Cos7 cells were transfected with a Myc-tagged *plx-1(+)* gene driven by a CMV promoter and immunostained with anti-Myc antibodies (see Materials and methods). Actin filaments were visualized with phalloidin-rhodamine, and membrane ruffles were observed by D.I.C. optics. As shown in Fig. 4, *C. elegans* PLX-1 clearly co-localizes with actin filaments in membrane ruffles of Cos7 cells.

Both Semaphorin 1 family members SMP-1 and SMP-2 are expressed in the male tail hook and are suspected attractants to the ray 1 cells

We found that the male hook strongly expresses both *smp-1::gfp* and *smp-2::gfp* transcriptional reporters (*smp-2::gfp* in Fig. 5C,D; *smp-1::gfp* is not shown). For the *smp-2::gfp* transcriptional reporter, we also observed expression in rays 7, 8 and 9 (Fig. 5C,F). To confirm these results obtained with the transcriptional reporters, a GFP translational reporter for *smp-1* was produced (see *smp-1::GFP* reporter in Materials and methods). Expression was observed at the cell membrane of the developing male hook precursors (Fig. 5A,B), the adult hook (Fig. 5E), in rays 7, 8 and 9, and in the bursa of the male tail (Fig. 5G). No expression was observed in ray 1-6 lineages.

Of possible relevance, the developing hook is located close to the ray 1 cell cluster during the third larval stage at the time

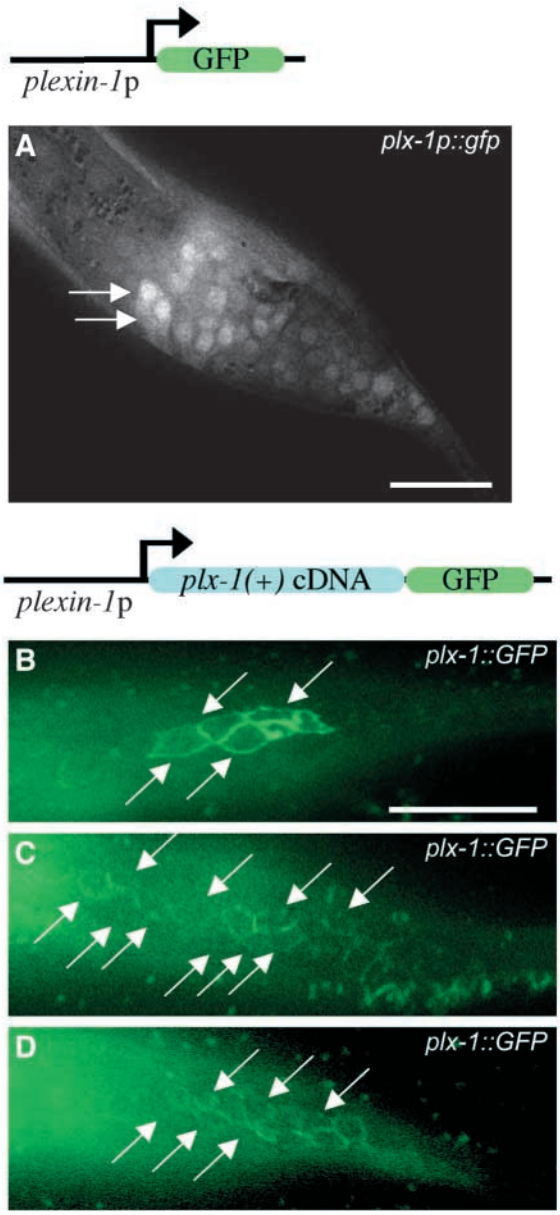

Fig. 3. Transcriptional and translational reporters for *plexin 1* are expressed in developing ray cells. For all panels anterior is left and ventral is bottom. The expression pattern of GFP reporters for *plx-1* (see Materials and methods, schematics show constructs used) was determined in transgenic males using fluorescence microscopy. A similar expression pattern was observed in transgenic animals with an extra-chromosomal translational reporter array (*evEx168*) or an integrated transcriptional reporter array (*evIs140*). (A) Expression of *evIs140* is observed in the dividing Rn.a and Rn.p cells, but predominantly in R1.a and R1.p cells (white arrows) in L3 males. (B) Expression of *evEx168* encoding the entire *plx-1(+)* coding sequence (minus four C-terminal amino acids, see Material and methods) fused to a GFP reporter. Expression is observed at the cell membrane of developing ray cells, predominantly in the 3-cell clusters for rays 1 and 2 during the early L3 stage (white arrows). (C,D) Low expression is detected in all ray precursor clusters at the late L3 stage (white arrows). No expression is detected in adult rays for either the transcriptional or the translational reporter (data not shown). Scale bars: A, 15 μm; B-D, 25 μm.

we first observe abnormal anterior ray 1 positioning in *plx-1* and *semaphorin 1* mutants (Fig. 2A,E,F, and above results). As we observed positioning of the ray 1 cell cluster anterior to the hook in *plx-1*, *smp-1* and *smp-2* single mutants, and in *smp-1; smp-2* double mutants, it is possible that SMP-1 and SMP-2 expression from the hook may normally attract the PLX-1-expressing ray 1 cells to keep them in their normal, more posterior position (i.e. closer to the ray 2 cells). To further examine this possibility, we characterized ray 1 cell positioning in *lin-1* mutants males. In *lin-1* mutant males, additional hooks are present anterior to their normal position because of a ventral epidermal cell lineage defect (Sulston and Horvitz, 1981) (Fig. 5H-L), which we have found does not affect the ray lineages. The *smp-2::gfp* transcriptional reporter is expressed in the normally positioned hook and in the anterior hooks in *lin-1(e1275)* animals (Fig. 5H). An anterior ray 1 phenotype essentially identical to the one in *plx-1(ev724)* mutant males is observed in *lin-1(e1275)* mutant males. The penetrance of anterior ray 1 defects is 3% at both 16°C and 25°C for the less severe class 2 phenotype, but 14% and 28% at 16°C and 25°C, respectively, for the more severe class 1 phenotype (n=150). In those *lin-1(e1275)* mutant animals that have a nearly normal looking ectopic hook, we find that ray 1 is often positioned much closer to this ectopic hook than to the normal hook throughout larval development (Fig. 5I-L).

The anterior displacement of ray 1 observed in *lin-1(e1275)* mutant males is considerably suppressed when *smp-1(+)* function is taken away. For example, in *smp-1(ev715); lin-1(e1275)* double mutants, the severe ray 1 anterior displacement (class 1) caused by an ectopic hook is 5% (n=58) compared with 28% (n=150) for *lin-1(e1275)* alone. These results further support the hypothesis that *semaphorin 1* expression from the hook attracts ray 1 cells.

To confirm the possible involvement of Semaphorin 1 proteins expressed from the male hook in normally attracting ray 1 cells to the posterior side, we laser ablated hook precursor cells in L3 stage males (see Materials and methods). In hook-ablated animals, anterior displacement of ray 1 cells was observed for four out of seven sides examined. However, the ray 1 cells did not differentiate into a fully developed ray, suggesting that factors expressed by the hook are also required for ray 1 cell differentiation.

mig-2, *ced-10* and *unc-73* also prevent the anterior displacement of male ray 1 cells

To determine whether RAC GTPases are involved in ray 1 positioning, we made use of mutations in two existing RAC GTPase genes in *C. elegans*, *mig-2* and *ced-10*. Cell migration and phagocytosis of apoptotic cells are affected by mutations in these genes, although no male tail defects were reported previously (Kishore and Sundaram, 2002; Lundquist et al., 2001; Zipkin et al., 1997).

mig-2(mu28) and *ced-10(n1993)* single mutants exhibit very low penetrance anterior ray 1 displacement defects (Table 2). To test for possible redundancy between the two RAC-encoding genes for ray 1 positioning, we attempted to construct a *mig-2(mu28); ced-10(n1993)* double mutant. However, double-mutant animals are sterile, and die as embryos and early larvae

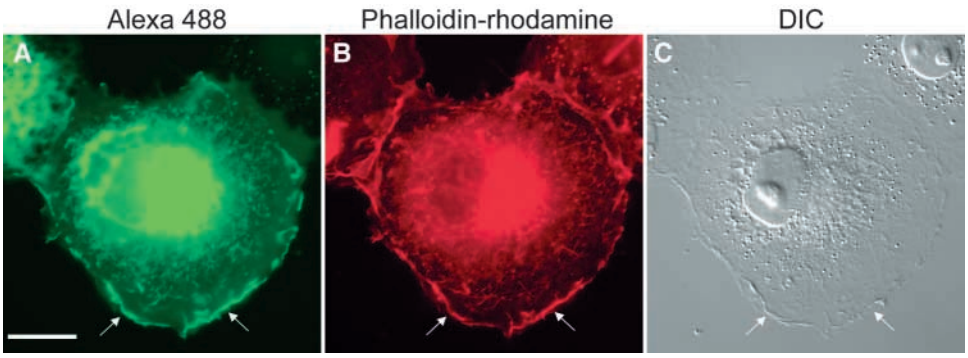

Fig. 4. Localization of *C. elegans* PLX-1 in Cos7 cells. Cos7 cells were transfected (see Materials and methods) with a DNA construct encoding the *C. elegans* PLX-1 fused to a MYC epitope (Fujii et al., 2002). Cells were fixed in 4% paraformaldehyde and stained with a mouse monoclonal anti-MYC antibody (9E10) and an Alexa 488-conjugated anti-mouse antibody. Actin microfilaments were revealed using phalloidin-rhodamine. Cell morphology was observed using DIC filters on a Leica DMRA2 microscope. Arrows indicate co-localization of PLX-1 with actin microfilaments in membrane ruffles. Scale bar in A: 20 μm for A-C.

as reported previously (Kishore and Sundaram, 2002; Lundquist et al., 2001). Nonetheless, *mig-2(mu28); ced10(n1993)/+* males survive and exhibit a severe anterior ray 1 displacement defect compared with respective single mutants (Table 2). This suggests that *mig-2* and *ced-10* normally function redundantly or in series (see Discussion) to prevent anterior displacement of ray 1 cells. We have not examined the effects of *rac-2* on ray 1 cell positioning, although it also may act redundantly or in series with *mig-2* and *ced-10* in this process.

To examine further the importance of RAC activation for ray 1 positioning we characterized an allelic series of mutations in *unc-73*, which encodes a Trio homolog (Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000) known to function in the activation of MIG-2 and CED-10 for cell movements and shape changes (Kishore and Sundaram, 2002; Lundquist et al., 2001; Spencer et al., 2001; Steven et al., 1998). *unc-73 (ev509)*, *unc-73(e936)* and *unc-73(rh40)* animals have mild, moderate and severe uncoordinated (Unc) phenotypes, respectively (R. Steven, personal communication). We find that all alleles have an anterior ray 1 positioning defect that varies in penetrance in a manner correlated with the severity of the Unc phenotype (Table 2). Notably, the strongest mutation *(rh40)* has a penetrance equal to the penetrance of *mig-2(mu28); ced10(n1993)/+* mutant animals, or to the *mig-2(gm103gf)* gain-of-function mutant animals (Table 2). Hook positioning is normal in mutants of *unc-73* and in *mig-2(mu28); ced-10(n1993)/+* mutant strains.

Like the *mig-2(mu28); ced-10(n1993)* double mutant, the *unc-73* null allele is lethal (Steven et al., 1998), preventing us from determining the ray 1 anterior phenotype in this context. However, this analysis clearly demonstrates that UNC-73 is necessary for preventing anterior displacement of ray 1 cells.

unc-73, and by implication *ced-10* and *mig-2*, act in *plx-1*-expressing cells to prevent anterior displacement of ray 1 cells

Biochemical analyses have shown that UNC-73 activates RAC GTPases, and genetic analyses show that *unc-73* requires *ced-10* and *mig-2*, which encode RAC GTPases, for its activity in cell migrations and axon guidance (Kishore and Sundaram, 2002; Lundquist et al., 2001; Spencer et al., 2001; Steven et al., 1998). A simple explanation is that UNC-73 is a GEF activator for both CED-10 and MIG-2 involved in cell migrations.

Table 2. Ray 1 anterior phenotype in Rac GTPase and *unc-73* mutant strains

Genotype*	Ray 1 class 1 (%)†	Ray 1 class 2 (%)†	*n*†
ced-10(n1993lf)	3±1	8±2	297
mig-2(mu28lf)	2±1	2±1	185
ced-10(n1993lf)/+; mig-2(mu28lf)‡	86±3	12±2	174
mig-2(gm103gf)	91±3	1±1	130
mig-2(gm103gf)/+‡	92±3	0	73
unc-73(ev509)	21±4	17±4	110
unc-73(e936)	61±5	15±3	119
unc-73(rh40)	84±4	12±3	106
unc-73(e936); evEx168[plx-1::unc-73(+)]	1±1	6±2	146

*All strains have the *ajm-1::gfp* reporter gene in the *him-5(e1490)* background except for strains marked ‡, which are heterozygous for *him-5(e1490)*.

†The frequency of the severe (class 1) and mild (class 2) ray 1 anterior phenotype was evaluated as in Table 1. *n* represents the number of animals scored. All animals were grown at 20°C. Standard deviations were calculated assuming a binomial distribution with the observed percentage value and the actual sample size. For comparisons described in the Results, a P value <0.05 was considered significant.

‡Heterozygous genotype generated by crossing.

Although *unc-73*, *ced-10* and *mig-2* prevent the anterior displacement of ray 1 cells, it is not clear whether they do so by acting in the ray cells, in the hook or, perhaps, even in a third cell type. If *unc-73* affects ray cell migrations by acting in the hook or in a cell type other than the ray cells, then we might expect a deficit in *unc-73* function to cause abnormal regulation of *smp-1* expression. However, *smp-1::GFP* expression was totally normal in *unc-73(e936)*, suggesting that *unc-73* is unlikely to affect ray cell movements by acting in the hook or a cell type other than the ray precursors.

To examine this question more directly, we also expressed *unc-73(+)* under the control of the *plx-1* promoter. This promoter was previously shown to drive expression of a *gfp* reporter primarily in the ray 1 cells of the male tail and not in the hook. If *unc-73* and *plx-1* act in the same set of cells, a *plx-1::unc-73(+)* transgene should rescue *unc-73* mutant ray 1 positioning defects, but it should not rescue them if *plx-1* and *unc-73* act in different cell types. As shown in Table 2, this rescue was nearly complete (1% class 1 and 6% class 2 defects, *n*=146), showing that UNC-73, and by implication its effectors CED-10 and MIG-2, probably function in the ray 1 cell (or its

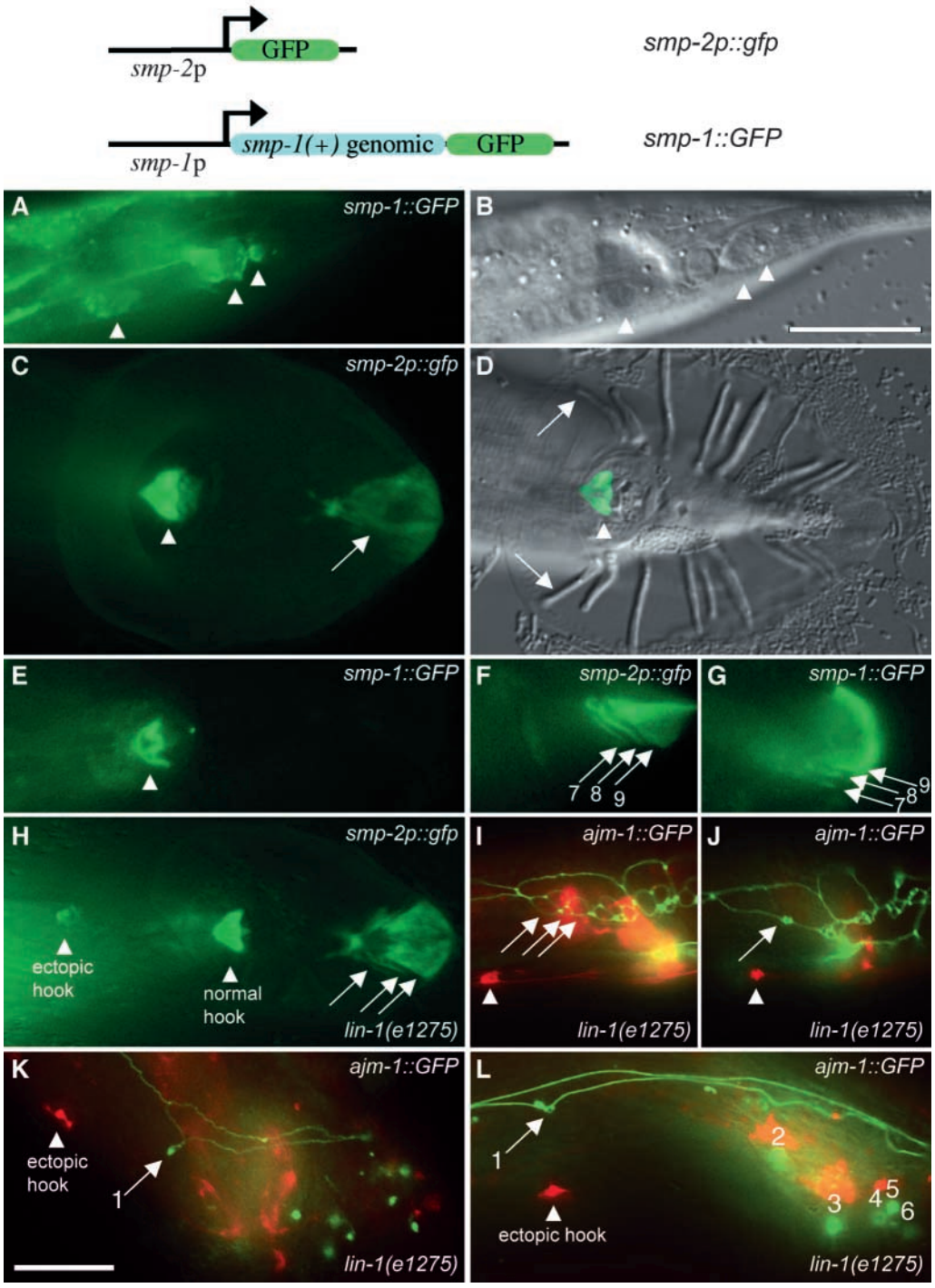

Fig. 5. GFP reporter expression (schematics show constructs used) for *smp-1* and *smp-2* relative to ray 1 cells in wild-type and *lin-1(e1275)* animals. For all panels, anterior is left. (A-E,H) Ventral views; (F,G,I-L) lateral views. (A-E) Reporter genes for *smp-1* and *smp-2* are expressed in the male tail hook. The translational reporter gene *smp-1::GFP* is expressed at the cell membrane of the developing hook (A, L3 stage male (*smp-1::GFP*); B, DIC, arrowheads) and in adult hook cells (E, *smp-1::GFP*, arrowhead) in wild-type males. The *smp-2p::gfp* transcriptional reporter is also expressed in the hook (C, *smp-2p::gfp*; D, *smp-2p::gfp*/DIC overlay; arrowhead) in close proximity to ray 1 (D, arrows show ray 1) in wild-type adult males. No expression is detected in rays 1-6 for any reporters analyzed (*smp-1p::gfp*, *smp-2p::gfp* and *smp-1::GFP*). (F,G) Both *smp-2p::gfp* and *smp-1::GFP* express in rays 7, 8 and 9 (arrows). (H) Expression of *smp-2p::gfp* is observed in both the normal and ectopic hook (arrowheads) of *lin-1 (e1275)* mutant males, and in ray 7, 8 and 9 (arrows) and in the ray tail bursa (above arrows in G). (I-L) The *ajm-1::GFP* reporter was used to determine the position of both the ectopic hook and ray 1 in *lin-1(e1275)* adult males [the hook focal plane (red) and the ray focal plane (green) are shown in overlays I-L]. In developing *lin-1(e1275)* males, the ectopic hook (arrowhead) is located anterior to the developing ray 1 cells (I, early L3 stage; J, late L3 stage; K, L4 stage). Ray lineages are normal in *lin-1(e1275)* males [I, all ray cells are present (green), ray 1 cells shown by arrows]. By the late L3 stage, ray 1 cells are already found anterior to their normal position (J, arrow) and by the L4 stage, ray 1 is still further anterior (K, arrow). In adult *lin-1(e1275)* males, ray 1 is frequently observed anterior (arrow, class I phenotype) to its normal position relative to other rays (L, numbering), and in closer proximity to the ectopic hook position (L, arrowhead). Scale bars: 20 μm; bar in B applies to A-G, bar in K applies to H-L.

descendants) to prevent displacement of ray 1 to an abnormal anterior position.

unc-73, *mig-10* and *ced-2* can position ray 1 cells without *plx-1* activity

Each of the RAC signaling genes described above (*mig-2*, *ced-10* and *unc-73*) is at least partially required for normal ray 1 positioning independent of PLX-1 signaling. Mutations in each of these genes are also enhanced by the *plx-1(ev724)* null, which on its own has an incompletely penetrant ray 1 defect even though it is predicted to be totally non-functional (Table 3). The mild ray displacement phenotype caused by *mig-2(mu28)* is enhanced by reducing *plx-1(+)* function (Table 3), the greater the reduction in *plx-1(+)* dose, the greater the

Table 3. *plx-1* and *mig-2* and *ced-10* function in parallel pathways for preventing ray 1 displacement

Genotype*				Ray 1 class 1 (%)†	Ray 1 class 2 (%)†	
mig-2	*ced-10*	*unc-73*	*plx-1*			*n*†
–/–	+/+	+/+	+/+	2±1	2±1	185¶
+/+	+/+	+/+	–/–	10±2	39±3	359§
–/–	+/+	+/+	+/–	35±3	32±3	249‡
–/–	+/+	+/+	–/–	54±3	29±3	312
+/+	–/–	+/+	+/+	3±1	8±2	297¶
+/+	–/–	+/+	–/–	70±4	24±4	128
+/+	+/+	–/–	+/+	61±5	15±3	119¶
+/+	+/+	–/–	–/–	97±2	2±1	127

*Alleles used were *mig-2(mu28)*, *ced-10(n1993)*, *unc-73(e936)* and *plx-1(ev724)*. All strains have the *ajm-1::gfp* reporter gene in the *him-5(e1490)* background except for some genotypes that are heterozygous (‡) for *him-5(e1490)*.

†The frequency of the severe (class 1) and mild (class 2) anterior ray 1 phenotype was evaluated as in Table 1. *n* represents the number of animals scored. All animals were grown at 20°C. Standard deviations were calculated assuming a binomial distribution with the observed percentage value and the actual sample size. For comparisons described in the Results, a *P* value <0.05 was considered significant.

‡Heterozygous genotype generated by crossing.

§For comparison purposes, these numbers come from Table 1.

¶For comparison purposes, these numbers come from Table 2.

enhancement. An even stronger enhancement phenotype is observed in *plx-1(ev724); ced-10(n1993)* double-mutant animals (Table 3). Thus when there is a loss of only *mig-2* function, or of only *ced-10* function, *plx-1(+)* is still required to prevent anterior displacement of ray 1 cells.

The finding that reducing the *plx-1(+)* dosage in a *mig-2(mu28)* null-mutant background results in an enhancement of the anterior ray 1 phenotype suggests that *plx-1* functions in a pathway that acts in parallel with *mig-2* (and by implication in parallel with *unc-73* and *ced-10*) to prevent anterior ray 1 displacement (Fig. 6 and Discussion). These data also indicate that in the absence of PLX-1 signaling, *unc-73*, *mig-2* and *ced-10* can still at least partially function to position ray 1 cells. By contrast, an almost completely penetrant anterior ray 1 phenotype is observed in *plx-1(ev724); unc-73(e936)* double mutants (Table 3). This suggests that the MIG-2 and CED-10 function that is PLX-1 independent is likely to require activation by UNC-73. Furthermore, the PLX-1-independent function of these RHO family GTPases, together with PLX-1 function, can, in principle, account entirely for preventing anterior displacement of ray 1 cells in *C. elegans*. The synergistic effects of the double mutant on class 1 defects suggest that *unc-73* and *plx-1* functions are also partially redundant.

RAC family genes switch the 'polarity' of Semaphorin 1 signaling that occurs through Plexin 1

The results reported above demonstrate that threshold levels of MIG-2 and CED-10, and activation of PLX-1 by SMP-1 and SMP-2, are required to prevent anterior displacement of ray 1 cells in the male tail (see above). To determine whether the

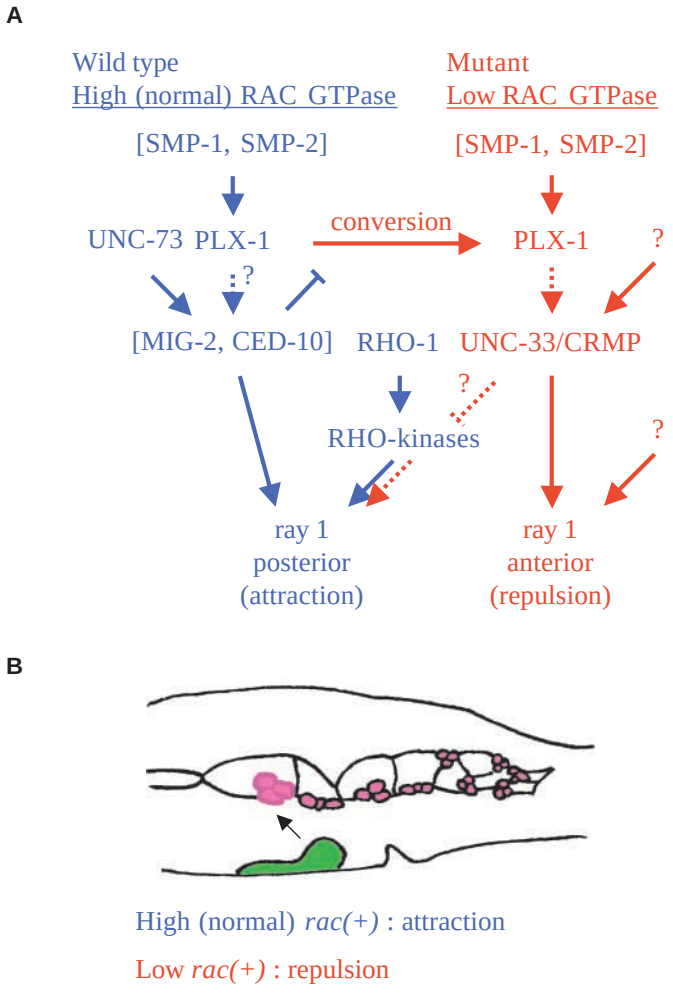

Fig. 6. Model of Semaphorin 1 and Plexin 1 signaling in male ray 1 positioning. (A) In a wild-type genetic background, we find that MIG-2 and CED-10 (RAC GTPases) are probably redundant in preventing anterior displacement of ray 1 cells (however, see Discussion). There is a requirement for UNC-73 (RAC GEF) in MIG-2 and CED-10 function. Some UNC-73 functions are required in parallel with PLX-1 for preventing this phenotype. RHO-1 GTPases, and the RHO-kinases LET-502 and K08B12.5, appear to be required in parallel with the PLX-1 and UNC-73/MIG-2/CED-10 pathways to prevent anterior ray 1 displacement, but the analyses do not rule out a possible direct feed-forward from PLX-1 signaling to RHO-family GTPase signaling (dashed arrow on left). Debilitation of UNC-73, MIG-2 and CED-10 displaces ray 1 anterior to normal, whereas debilitation of UNC-33 prevents anterior ray 1 displacement. At high (normal) levels of MIG-2 and CED-10, SMP-1 and SMP-2 signaling through PLX-1 helps to prevent anterior displacements of ray 1 (pathway in blue). However, a conversion of PLX-1 function occurs at low levels of both MIG-2 and CED-10 [genotype *mig-2(mu28); ced-10(n1993)/+*], as a stimulation of the ray 1 anterior positioning function occurs (pathway in red). This implies that high (normal) levels of RAC GTPases (MIG-2 and CED-10) prevent the switch in the polarity of PLX-1 output. Ray anterior displacements require CRMP/UNC-33, which could act as an effector of PLX-1 at low RAC levels (dashed arrow on right), or could act independently. (B) A cell migration model for positioning of ray 1 cells during male development. In a wild-type background [normal *mig-2(+)* and *ced-10(+)* (*rac*) levels], expression of Semaphorin 1 proteins from the hook primordium (green) attracts PLX-1-expressing ray 1 cells (purple) toward the posterior side. At low *mig-2(+)* and *ced-10(+)* (*rac*) levels [genotype *mig-2(mu28); ced-10(n1993)/+*], ray 1 cells are repulsed away from sources of Semaphorin 1 proteins.

Table 4. Requirement for Plexin 1 signaling in anterior displacement of ray 1 occurring at low doses of wild-type *rac* genes *mig-2* and *ced-10*

Genotype*					Ray 1 class 1 (%)†	Ray 1 class 2 (%)†	
mig-2	*ced-10*	*smp-1*	*smp-2*	*plx-1*			*n*†
–/–	+/–	+/+	+/+	+/+	86±3	12±2	174‡,§
–/–	+/–	+/+	+/+	+/–	53±5	25±4	118‡
gf/gf	+/+	+/+	+/+	+/+	91±3	1±1	130§
gf	+/+	+/+	+/+	+/+	92±3	0	73‡,§
gf	+/+	+/+	+/+	+/–	70±4	15±3	164‡
gf	+/+	+/–	+/–	+/+	61±5	15±4	101‡
gf	+/+	–/–	+/+	+/+	80±4	13±3	113

*Alleles used were *mig-2(mu28lf)*, *mig-2(gm103gf)*, *ced-10(n1993)*, *smp-1(ev715)*, *smp-2(ev709)*, and *plx-1(ev724)*. All strains have the *ajm-1::gfp* reporter gene in the *him-5(e1490)* background except for some genotypes that are heterozygous (‡) for *him-5(e1490)*. All animals were grown at 20°C.

†The frequency of the severe (class 1) and mild (class 2) ray 1 anterior phenotype was evaluated as in Table 1. *n* represents the number of animals scored. Standard deviations were calculated assuming a binomial distribution with the observed percentage value and the actual sample size. For comparisons described in the Results, a *P* value <0.05 was considered significant.

‡Heterozygous genotype generated by crossing.

§For comparison purposes, these numbers come from Table 2.

gf indicates gain-of-function allele.

severe anterior ray 1 defect caused by a strong reduction in RAC function is dependant on *plx-1(+)*, the dose of *plx-1(+)* was reduced in a strain in which RAC was already strongly compromised (as judged by the penetrance of anterior ray 1 defects). Unexpectedly, in *mig-2(mu28lf); ced-10(n1993)/+* males carrying only one copy of *plx-1(+)*, a significant suppression rather than enhancement of the severe ray anterior phenotype occurs (Table 4). This result demonstrates that at low levels of RAC activity, *plx-1(+)* is required for the anterior displacement of ray 1 cells, i.e. the opposite of the function of *plx-1(+)* at normal levels of RAC, which is to prevent anterior displacement of ray 1 cells.

As the *mig-2(gm103gf)* anterior ray 1 phenotype also behaves like a strong RAC reduction of function (Table 4), we tried to determine whether similar suppression would be observed by reducing *plx-1(+)* dosage in this background, as it does in a severe RAC loss-of-function background [*mig-2(mu28); ced-10(n1993)/+*]. In *mig-2(gm103gf)* males containing only one copy of *plx-1(+)*, a partial suppression of the severe anterior ray 1 phenotype is observed (Table 4). A similar partial suppression is observed in *mig-2(gm103gf)* males containing only one copy each of *smp-1(+)* and *smp-2(+)* (Table 4), but no significant suppression occurs when only *smp-1(+)* function is missing (Table 4). By inference, the anterior ray 1 displacement phenotype that occurs at low doses of *rac(+)*, whether it be caused by constitutively GTP-loaded MIG-2 or by simultaneous loss of *mig-2(+)* and *ced-10(+)* function [*mig-2(mu28); ced-10(n1993)/+*], depends to some extent on both PLX-1 and Semaphorin 1 function. We could not generate the *mig-2(gm103gf); plx-1(ev724)* double, the *mig-2(mu28); ced-10(n1993); plx-1(ev724)* triple, nor the *mig-2(gm103gf); smp-1(ev715); smp-2(ev709)* triple mutants as they died from a vulva rupturing phenotype similar to the one observed in *mig-2(mu28); ced-10(n1993)* double mutants.

CRMP/UNC-33 functions in anterior ray 1 positioning and opposes posterior ray 1 positioning mechanisms that function independently of PLX-1

The ray1 anterior displacement phenotype of a strong RAC loss-of-function [*mig-2(mu28); ced-10(n1993)/+* double mutant] depends to some extent on PLX-1 and its putative ligands SMP-1 and SMP-2. As it is possible that anterior ray 1 displacement results in part from a Semaphorin 1-induced repulsion of PLX-1 expressing ray 1 cells (see Discussion), we decided to examine the effects of mutations in *unc-33*, which encodes proteins related to mammalian CRMP proteins known to be required for axon growth cone repulsions induced by Semaphorins (Goshima et al., 1995). The anterior ray 1 defect is rarely observed in *unc-33(e1261)* mutant males (Table 5) suggesting that *unc-33* is not absolutely required for normal posterior positioning of ray 1. However, *unc-33(e1261)* suppresses significantly the severe (class 1) anterior ray 1 phenotype of *plx-1(ev724)* mutants (Table 5). *unc-33(+)* function is therefore at least partially required for the anterior ray 1 displacement phenotype observed in *plx-1(ev724)*.

A *plx-1(+)* multi-copy array partially rescues the *plx-1(ev724)* phenotype and induces an apparent loss-of-function phenotype

A *plx-1(+)* cDNA minigene was placed directly under the control of the 2621 bp sequence upstream of the initiation codon (see Material and methods). As an integrated array (*evIs162*) this transgene induced anterior ray 1 defects in a wild-type genetic background (Table 5). In principle, this could be the result of a co-suppression effect or of a dominant interfering effect [e.g. sequestration of a PLX-1-interacting component by the putative higher-than-normal amounts of PLX-1(+) protein]. However, we used a non-integrated (*evEx162*) and an integrated (*evIs162*) array of the *plx-1(+)* cDNA minigene to attempt transgenic rescue of the anterior ray 1 phenotype observed in *plx-1(ev724)* males. The class 1 and class 2 ray 1 phenotypes of *plx-1(ev724)* were both significantly rescued at 20°C by the *plx-1(+)* minigene (Table 5). As an extra-chromosomal array (*evEx162*) and an integrated transgene (*evIs162*), only the class 1 severe ray 1 phenotype of *plx-1(ev724)* males was significantly rescued at 25°C (Table 5). These results indicate that both integrated and non-integrated *plx-1(+)* transgene arrays produce functional wild-type PLX-1 protein. Any rescue would be unlikely if the array caused a co-suppression or a dominant interfering effect.

Some hints about the mechanism used to regulate the reversal in the ray 1 positioning function of PLX-1 at low RAC levels may also be gleaned from the *plx-1(+)* overexpressing arrays. For example, the anterior ray 1 phenotype induced by the *evIs162 [plx-1(+)]* transgene array is also enhanced by *mig-2(mu28)* (Table 5), but does not rescue the severe ray 1 displacement of *mig-2(mu28); ced-10(n1993)/+* (80% class 1 and 9% class 2 defects, *n*=110). One interpretation of this result is that the PLX-1(+) function provided by *evIs162* can mimic the enhancement of *mig-2(mu28)* by *ced-10(n1993)/+*, possibly because PLX-1(+) overexpression from the array causes a reversal in the positioning function of PLX-1, just as *mig-2(mu28); ced-10(n1993)/+* does. This could happen if, for example, the higher ratio of functional PLX-1 to functional RAC is what

Table 5. *plx-1* and *unc-33* function in ray 1 anterior displacements

Genotype*				Ray 1 class 1 (%)†	Ray 1 class 2 (%)†	
plx-1	Transgene	*mig-2*	*unc-33*			*n*†
–/– 20°C	None	+/+	+/+	10±2	39±3	359§
–/– 20°C	*evEx162[plx-1(+)]*	+/+	+/+	2±2	16±4	95
–/– 25°C	None	+/+	+/+	32±4	50±4	157§
–/– 25°C	*evEx162[plx-1(+)]*	+/+	+/+	4±2	58±6	72
–/– 25°C	*evIs162 [plx-1(+)]*	+/+	+/+	5±2	46±4	174
+/+ 25°C	None	+/+	–/–	1±1	4±2	139
–/– 25°C	None	+/+	–/–	13±2	54±3	226
+/+ 25°C	*evIs162 [plx-1(+)]*het	+/+	+/+	8±2	19±3	161‡
+/+ 20°C	*evIs162 [plx-1(+)]*	+/+	+/+	5±2	23±4	135
+/+ 20°C	*evIs162 [plx-1(+)]*	–/–	+/+	20±3	37±4	184
+/+ 25°C	*evIs162 [plx-1(+)]*	+/+	+/+	17±3	39±4	180
+/+ 25°C	*evIs162 [plx-1(+)]*	+/+	RNAi	0	18±5	50

*The *plx-1(+)* extra-chromosomal array is designated *evEx162* and the integrated transgene is designated *evIs162.* All strains have the *ajm-1::gfp* reporter gene in the *him-5(e1490)* background except for some genotypes that are heterozygous (‡) for *him-5(e1490).* Alleles used were *plx-1(ev724), mig-2(mu28)* and *unc-33(e1261).*

†The frequency of the severe (class 1) and mild (class 2) ray 1 anterior phenotype was evaluated as for Table 1. *n* represents the number of animals scored. All animals were grown at 25°C unless otherwise noted. Standard deviations were calculated assuming a binomial distribution with the observed percentage value and the actual sample size. For comparisons described in the Results, a *P* value <0.05 was considered significant.

‡Heterozygous genotype generated by crossing.

§For comparison purposes, these numbers come from Table 1.

Table 6. Effect of RNAi of *rho-1* and RHO-kinase genes on male ray 1 precursor cluster position

Genotype*		Ray 1 class 1 (%)†	Ray 1 class 2 (%)†	
Allele	RNAi			*n*†
unc-73(e936) 20°C	–	61±5	15±3	119§
	rho-1	79±4	10±3	113
	let-502	84±4	14±4	96
	rho-kinase	96±2	4±2	177
plx-1(ev724) 20°C	–	10±2	39±3	359‡
	rho-1	40±5	40±5	119
plx-1(ev724) 25°C	–	32±4	50±4	157‡
	let-502	45±5	48±5	85
	rho-kinase	38±5	58±5	89
Wild type 25°C	–	0	0	110‡
	rho-1	5±3	7±3	62
	let-502	4±2	15±3	119
	rho-kinase	2±1	4±2	119

**plx-1(ev724), unc-73(e936)* and wild-type L1 larvae were grown on HT115 bacterial lawn transfected with RNAi constructs targeting *C. elegans* homologs of *rho-1* (*Y51H4A.3*), and two predicted RHO-kinase genes, *C10H11.9* (*let-502*) and *K08B12.5*. The three RNAi constructs affect ray 1 position as indicated in the table but *rho-1* RNAi also resulted in roughly 50% of males displaying a severe disorganization of Rn.a and Rn.p cells. All animals were grown at the indicated temperature.

†The frequency of the severe (class 1) and mild (class 2) anterior ray 1 phenotype was evaluated as in Table 1. *n* represents the number of animals scored. For *rho-1* RNAi, numbers in the table come from males that could be scored. Standard deviations were calculated assuming a binomial distribution with the observed percentage value and the actual sample size. For comparisons described in the Results, a *P* value <0.05 was considered significant.

‡For comparison purposes, these numbers come from Table 1.

§For comparison purposes, these numbers come from Table 2.

determines the reversal in the ray positioning function of PLX-1(+).

We have shown that the ray 1 defects observed in *plx-1(ev724)* mutants can be suppressed by a mutation in *unc-33*, demonstrating a requirement for UNC-33/CRMP in anterior ray 1 displacement that is independent of *plx-1* function. Consistent with this, the ray 1 anterior displacement observed at 25°C in *evIs162[plx-1(+)]* (which we argue above is probably caused by overexpression of wild-type PLX-1 protein) is significantly suppressed by performing RNAi on *unc-33* (Table 5; 17% versus 0% for the ray 1 class 1 and 39% versus 18% for the ray 1 class 2 defects).

RNA interference with *rho-1*-encoded GTPase, or *let-502*- or *K08B12.5*-encoded RHO-kinases, enhances anterior displacement defects of a *plx-1* null and an *unc-73* hypomorph

Recent studies have reported that vertebrate Plexins and *Drosophila* Plexin B bind the active GTP-bound RAC GTPase (RAC^{GTP}), and both RHO^{GDP} and RHO^{GTP} (Driessens et al., 2001; Hu et al., 2001; Rohm et al., 2000; Vikis et al., 2000). To examine the possibility that RHO GTPases might be involved in preventing the anterior displacement of ray 1 cells, RNAi experiments were performed on the RHO GTPase encoded by *rho-1*, and on the putative RHO-kinase effectors encoded by *let-502* and *K08B12.5*. All RNAi experiments involved feeding larvae with bacteria designed to produce specific ds-RNAs (see Materials and methods). RNA interference with each of these three genes produced mildly penetrant ray 1 anterior displacement phenotypes (Table 6). Each of them also significantly enhanced the anterior ray 1 displacement defects of *unc-73(e936)* animals (Table 6). RNAi of *rho-1* on *plx-1(ev724)* animals also significantly enhanced the class 1 defect (Table 6). RNAi of *rho-1* at 25°C did not enhance *plx-1(ev724)* for unknown reasons (not shown); however, RNAi of RHO-kinase encoding genes marginally enhanced these defects in *plx-1(ev724)* (Table 6). These results suggest that *C. elegans* RHO GTPases, like the *C. elegans* RAC GTPases MIG-2 and CED-10, are also involved in preventing the anterior displacement of ray 1 cells in developing males.

Discussion

Ray 1 positioning and adhesion functions of *plexin 1*

We and others (Fujii et al., 2002) have used a genetic approach to characterize two molecular mechanisms that effect the normal (posterior) positioning of ray 1 and ray 1 cells during development of the male tail of *C. elegans*. One of these mechanisms appears to involve Semapohorin 1 signaling through Plexin 1, as loss-of-function mutations in *sema-1* (*smp-1* and *smp-2*) and *plx-1* each cause anterior ray 1 displacement defects, and the *smp-1; smp-2; plx-1* triple mutant is not enhanced for the penetrance of these defects relative to the semaphorin 1 double-null mutant strain (Ginzburg et al., 2002). These data show that Semaphorin 1 proteins and PLX-1 act largely in the same pathway to prevent ray 1 anterior displacements, and are most consistent with the idea that both SMP-1 and SMP-2 prevent ray 1 displacement

by acting through the PLX-1 receptor to which SMP-1 has been shown to bind in vitro (Fujii et al., 2002). However, the genetic results suggest that PLX-1 may have some function in ray 1 positioning that is independent of the Semaphorin 1 proteins.

A second mechanism for ray 1 positioning involves the RAC sub-types of the RHO family of GTPases, MIG-2, CED-10 and their putative activator UNC-73 (a RAC GEF). Loss-of-function mutations in *mig-10* or *ced-10* alone cause few, if any, effects on ray 1 positioning; however, concomitant reductions in the dosage of wild-type *mig-2* and *ced-10* genes [i.e. *mig-2(mu28); ced-10(n1993)/+*] causes significant anterior ray 1 displacements. This suggests that the RAC GTPase sub-types of the RHO family of GTPases normally act redundantly to prevent anterior ray 1 displacement; however, because *mig-2(mu28)* might not be a null allele, we cannot rule out the possibility that these RAC GTPase sub-types act in series. In either case, these results are consistent with the finding that even partial loss-of-function mutations in *unc-73* have significant ray 1 defects, as *unc-73* has been shown to be required for MIG-2, CED-10 and RAC-*2* functions in other types of cell migrations (Kishore and Sundaram, 2002; Lundquist et al., 2001). In the future, it should prove interesting to examine the effects of simultaneously reducing all RAC gene functions on ray 1 cell positioning, including the remaining known *rac-2 C. elegans* gene (Lundquist et al., 2001).

As RAC and RHO GTPases are traditionally thought to act antagonistically in guiding migrating axon growth cones (and by implication in cell positioning), we examined the effects of reducing the function of the single known *C. elegans* RHO GTPase gene *rho-1*. Although RNAi of *rho-1* in control *him-5* animals did not dramatically affect ray 1 cell positioning, it did dramatically enhance the anterior displacement of ray 1 cells of *unc-73(e936)* and *plx-1(ev724)* mutant animals. Similar results were obtained by RNAi of RHO-kinase genes *let-502* and *K08B12.5*. These results suggest that RHO-1 and putative RHO effectors act in the same sense as RAC GTPases and their putative activator UNC-73, which is to prevent the anterior displacement of ray 1 cells. This is contrary to the reported antagonistic roles for RHO and RAC functions in axon growth cone migration (Dickson, 2001; Jalink et al., 1994; Luo, 2000; Mueller, 1999; Suter and Forscher, 1998; Yuan et al., 2003), but is certainly not the only exception to this view to be found in the literature (Dickson et al., 2001; Driessens et al., 2001).

The nearly complete penetrance of *unc-73; plx-1* double mutants further suggests that UNC-73 and PLX-1 functioning together could account for all of the normal posterior positioning of ray 1. In principle, they could do this by acting in the same or in different cell types. For example, PLX-1 could act in the ray 1 cells, whereas UNC-73 and the RAC GTPases could act in some nearby tissue (e.g. the nearby hook, see below). We examined this possibility by using the *plx-1* promoter to drive expression of *unc-73(+)* in *plx-1*-expressing cells. The fact that we obtained nearly complete rescue of *unc-73(e936)* (1% class 1 and 6% class 2 defects) by an extrachromosomal transgene array carrying *plx-1::unc-73(+)* strongly indicates that UNC-73, and by implication the RAC GTPases it putatively activates, MIG-2 and CED-10, normally function cell-autonomously to position the ray 1 cells.

The fact that *unc-73*, *mig-2* or *ced-10* mutations enhance the *plx-1* null for anterior ray 1 defects, demonstrates that RAC GTPases and UNC-73 function in parallel to Semaphorin 1 protein signaling through PLX-1. At the very least, the synergistic effects of *unc-73* and *plx-1* mutations on the penetrance of anterior ray 1 defects indicate that PLX-1 function is partially redundant with the RHO family GTPases. However, it is important to note that these genetic analyses do not rule out the possibility that the RHO family GTPases also act in the same pathway as PLX-1 and may be intracellular effectors of the PLX-1 signal transduction machinery.

A switch in PLX-1 function caused by an alteration in the relative levels of RAC reverses the polarity of ray 1 cell positioning

Whether or not the RHO family of GTPases acts as PLX-1 effectors, one thing that appears fairly certain is that the RHO family GTPases can affect PLX-1 signal transduction in a profound way. This is demonstrated by our finding that at high (normal) doses of wild-type RAC genes, *plx-1* acts to prevent anterior displacements of ray 1, but at low doses of wild-type RAC genes, PLX-1 signaling is switched in the polarity of the response that is elicited by Semaphorin 1 proteins – instead of being required to prevent anterior displacements of ray 1, it is required to cause them.

MIG-2 GTPase cycling may be also required to prevent anterior ray 1 cell displacement as evidenced by the finding of anterior ray 1 positioning defects in *mig-2(gm103gf)*, which encodes a mutant form of the RAC-like MIG-2 that is constitutively stuck in a GTP-bound active state by being unable to exchange GDP for GTP (Zipkin et al., 1997). Although *mig-2(gm103gf)* appears to be a gain-of-function mutation (Zipkin et al., 1997), it mimics a loss of function for RAC activity both phenotypically (i.e. it causes significant anterior displacement defects), and with respect to its genetic interactions with mutations in genes encoding other components of the ray 1 positioning mechanism. Of most relevance, *mig-2(gm103gf)* anterior ray 1 defects are partially suppressed by loss of *plx-1(+)* dosage just as *mig-2(mu28); ced-10(n1993)/+* anterior ray 1 defects are. This is consistent with the proposed switch in PLX-1 activity observed when RAC GTPase levels are low. That *mig-2(gm103gf)* mimics the effect of low RAC activity on PLX-1 function allows an examination of the role that Semaphorin 1 proteins might play in a situation that mimics low RAC activity. In this situation, it appears that the Semaphorin 1 proteins are also required for manifestation of the proposed switch in PLX-1 function. The apparent defect in preventing anterior displacement of ray 1 in *mig-2(gm103gf)* could result partly from a requirement for GTPase cycling, from low levels of RAC^{GDP}, or from the proposed ability of constitutively GTP-loaded MIG-2 to bind and inactivate RAC GEFs [for possible functions of the *gm103gf* allele see Lundquist et al. (Lundquist et al., 2001)]. The ability of *mig-2(gm103gf)* to switch the polarity of ray cell positioning caused by Semaphorin 1 signaling distinguishes it from *unc-73* mutations. The latter presumably have increased levels of RAC^{GDP}, which in principle could account for the difference.

An intriguing corollary to the molecular mechanisms that underlie attraction versus repulsion is that the intracellular levels of small molecules such as cGMP and cAMP can

determine whether an axon guidance receptor mediates an attraction to its ligand or a repulsion away from it (Song et al., 1998). The molecular mechanisms by which cGMP and cAMP switch the polarity of receptor-mediated responses are being elucidated but are still incompletely understood (Song et al., 1998). If switches in guidance receptor activity occur in response to levels of RHO family GTPases, the study of semaphorin/plexin signal transduction mechanisms would be an excellent system with which to reveal the detailed molecular mechanisms underlying these switches, because plexins interact with both RAC and RHO, and the activation of RHO is reportedly dependent on plexin receptor stimulation by its semaphorin ligand(s) (Hu et al., 2001).

Is active migration involved in anterior ray 1 displacement?

It is distinctly possible that the switch in response to Semaphorin 1/PLX-1 signaling we have observed represents a switch from attraction to repulsion, similar to the switch from repulsion to attraction of growth cones caused by cGMP or cAMP (Song et al., 1998). Anterior ray 1 displacements could be caused by a reversal in the orientation of migration of the ray 1 cells, or it could simply represent a passive movement that results from an abnormal adherence of the ray 1 cell cluster to the lineally related elongating SET cell that it contacts. There are reasons to imagine a purely adhesive function for PLX-1 signaling in *C. elegans* (Ginzburg et al., 2002), but we favor the migration model for several reasons. First, we find normally positioned ray 1s that sometimes exhibit an abnormally persistent SET contact, suggesting that there is no causal connection between the persistent adhesion per se and anterior displacement of ray 1 in the mutants. More enlightening is the discovery by Fitch and Emmons (Fitch and Emmons, 1995) who found striking similarities of early larval ray lineages and cell-cell contacts in the developing male tail of several species of the Rhabditidae family of nematodes, which includes *C. elegans* (Fitch and Emmons, 1995). However, in spite of the developmentally early similarities, significant differences in adult ray position were observed between *C. elegans* and other Rhabditidae (Fitch and Emmons, 1995). Furthermore, species-specific ray position changes occur that are not in any obvious way correlated with a change in shape of an associated SET cell. When considered together with our results for *C. elegans* male ray 1 cells, the Semaphorin 1 and Plexin 1 guidance system is involved in what appears to be a migration of the ray 1 cell cluster on the anteroposterior axis while they contact their clonally related R1.p cell.

The finding that the male hook expresses transcriptional and translational reporters for *smp-1* and *smp-2* at the same time in development, and that ray 1 cells express *plx-1* reporters, suggests a straightforward model for how ray 1 positioning is accomplished (see Fig. 6). At normal RAC levels, the Semaphorin 1 proteins in the hook act as attractants to the PLX-1-expressing ray 1 cells, helping to keep them in a posterior position near the ray 2 cells. At low RAC activity or in the presence of non-cycling RAC^{GTP} [i.e. in *mig-2(gm103gf)*] the semaphorins can no longer act as attractants, but instead are actually actively involved with PLX-1 as repellants to the ray 1 cells, effectively pushing them to the anterior.

Consistent with the idea that semaphorins emitted by the hook attract the ray 1 cells is that, in *lin-1* mutant males harboring an ectopic anterior hook, we find anterior ray 1 cells in close proximity to the *smp-1*- and *smp-2*-expressing ectopic hook (Fig. 5H-L). Semaphorin 1 downregulation in a *lin-1* mutant background [i.e. *smp-1(ev715);lin-1(e1275)*] significantly suppresses the severe ray 1 anterior displacement toward the anterior ectopic hook. This strongly suggests that the ectopic anterior hook in *lin-1* mutants attracts ray 1 cells in a Semaphorin 1-dependant manner. These findings are also most consistent with a role for these molecules in the active migration of ray 1 cells.

Ablation of the hook precursors caused anterior displacements of ray 1 in four out of seven ray 1s that could be examined, therefore the anterior displacement defects are not fully penetrant in hook-ablated animals. This could mean that hook-independent mechanisms exist for keeping ray 1 in its normal posterior position and is consistent with the finding that even *plx-1* null mutations are not fully penetrant for this defect.

Molecular model for ray 1 positioning

Implicit in our results, which clearly show that one function of the RHO family members (MIG-2, CED-10 and RHO-1), the putative RHO effectors (RHO-kinases LET-502 and K08B12.5), the two Semaphorin 1 family members (SMP-1 and SMP-2) and their putative receptor (PLX-1) in *C. elegans* is to prevent the anterior displacement of ray 1 cells in the male tail, is the understanding that there must exist an anterior positioning mechanism for ray 1 that counteracts or antagonizes normal Semaphorin 1, PLX-1 and RHO family GTPase functions (Fig. 6). In situations where anterior ray 1 displacement occurs, such as when *plx-1(+)* levels are low (PLX-1 signaling assumed to be low) or when *plx-1(+)* is putatively overexpressed (by *evIs162*), anterior displacement appears to require UNC-33/CRMP. This is consistent with the idea that *unc-33* is required in a mechanism that normally opposes Semaphorin 1/Plexin 1- and/or RHO-family GTPase-mediated attractive signaling.

How might UNC-33/CRMP mediate what appears to be a repulsion of the ray 1 cells from the hook (see Fig. 6)? UNC-33 could oppose PLX-1 signaling by antagonizing some component of the PLX-1 signaling pathway, or it may simply be part of a mechanism providing force to counteract the force of PLX-1 signaling used to determine ray 1 positioning. One possible molecular mode of UNC-33 activity is suggested by the finding that CRMP-1 binds and inhibits mammalian RHO-kinase (Leung et al., 2002), a probable RHO effector. Therefore UNC-33/CRMP could cause anterior displacement of ray 1 cells by simply antagonizing RHO GTPase-mediated mechanisms that prevent anterior displacement (Fig. 6). Another possibility is that UNC-33 could be part of an independent, parallel acting pathway that causes repulsion. This would be the classical view of UNC-33/CRMP activity because its function appears to be directly required for growth cone collapse and repulsion in several systems (Goshima et al., 1995; Hall et al., 2001). Evidence for a direct requirement of UNC-33/CRMP in growth cone repulsion comes from the finding that the Fes/Fps tyrosine kinases upon binding of Sema3A to PlxA1, are recruited to phosphorylate the cytoplasmic portion of PlexA1 and an associated complex of the proteins CRAM and CRMP-2 (the latter is a splice variant of UNC-33/CRMP) (Mitsui et al., 2002). Sema3A-induced growth cone collapse of dorsal root ganglion neurons is suppressed in Fes kinase

negative mutants (Mitsui et al., 2002), which shows the requirement of the kinase for collapse and indicates a possible involvement of Fes/Fps-mediated phosphorylation of CRMP in the plexin collapsing pathway.

A non-exclusive possibility is that UNC-33/CRMP, by promoting tubulin hetero-dimer assembly (Fukata et al., 2002), might disturb the microtubule network and in this way cause a localized collapse. Recent evidence suggests that growth cone attraction and repulsion can be completely blocked by specifically inhibiting the dynamics of microtubule ends in the growth cone (Buck and Zheng, 2002).

Based on biochemical interactions described previously and our genetic data we propose a model (Fig. 6) in which Semaphorin 1 proteins emitted by the hook primordium bind to PLX-1 on the ray 1 cell(s) causing the conserved intracellular domains of PLX-1 to bind cycling RAC^{GTP} thereby mediating an attractive response to a Semaphorin 1 ligand. It remains unclear whether *C. elegans* PLX-1 can bind RHO because of sequence divergence in this region between the *Drosophila* PlxB and *C. elegans* PLX-1. However RHO and RHO-kinase signaling is required in parallel to or in series with MIG-2 and CED-10 for attraction. This could override a default ray 1 anterior positioning function, part of which requires CRMP/UNC-33. By inference, at relatively low levels of *rac(+)*, the ray 1 repulsion mediated by Semaphorin 1-induced activation of PLX-1 may be dependent on the recruitment of Fes/Fps tyrosine kinases and phosphorylation of the CRAM (Mitsui et al., 2002) and UNC-33/CRMP protein complex, causing cytoskeletal changes leading to ray cell repulsion (e.g. away from the hook). Another possibility is that RHO-kinase may normally be antagonized by UNC-33, reducing attraction of ray 1 to the hook and allowing an unidentified default repulsion mechanism to increase its activity. These two possibilities are not mutually exclusive, nor do they exclude other possible molecular mechanisms for UNC-33/CRMP activity, some of which were discussed above.

Implications of model

The ability of *C. elegans* PLX-1 to serve as a sensor of levels of RHO-family GTPases, and to switch its activity accordingly, indicates that PLX-1 stimulation by Semaphorin 1 proteins is instructive for cell positioning. This together with the incomplete penetrance of a null mutation in *plx-1* on ray 1 cell migration suggests that there may exist other membrane-associated receptors with similar functions in regulating ray 1 or other cell migrations. These receptors, like PLX-1, might sense relative levels of active RAC and RHO in the cell, perhaps in the same way (e.g. by direct or indirect binding of these GTPases), and thereby regulate the actin cytoskeleton associated with the cell membrane at the leading edge of migration, causing attraction (cytoskeletal growth) toward the ligand(s) of the receptor when functional cellular RAC levels are high, but causing repulsion (cytoskeletal collapse) when RAC levels are low.

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