The plexin PLX-2 and the ephrin EFN-4 have distinct roles in MAB-20/Semaphorin 2A signaling in *C. elegans* morphogenesis

Fumi Nakao*,1, Martin L. Hudson†,1, Motoshi Suzuki*, Zachary Peckler†, Rie Kurokawa*, Zhicen Liu*, Keiko Gengyo-Ando§, Akira Nukazuka*, Takashi Fujii*, Fumikazu Suto*, Yukimasa Shibata*, Go Shioi*, Hajime Fujisawa†,1, Shohei Mitani§, Andrew D. Chisholm†,2 and Shin Takagi*,2

*Division of Biological Science, Nagoya University Graduate School of Science, Chikusa-ku, Nagoya 464-8602, Japan

†Department of Molecular, Cell and Developmental Biology, Sinsheimer Laboratories, University of California, Santa Cruz, CA 95064, USA

§Department of Physiology, Tokyo Women's Medical University School of Medicine, Tokyo 162-8666, Japan

1 These authors contributed equally to this work.

2 Corresponding authors

Shin Takagi

Division of Biological Science

Nagoya University Graduate School of Science
Andrew D. Chisholm

Department of Molecular, Cell, and Developmental Biology, 351 Sinsheimer Laboratories

University of California

Santa Cruz, CA 95064, USA

Tel: (831) 459-5720

E-mail: chisholm@biology.ucsc.edu

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Abstract

Semaphorins are extracellular proteins that regulate axon guidance and morphogenesis by interacting with a variety of cell surface receptors. Most semaphorins interact with plexin-containing receptor complexes, although some interact with non-plexin receptors. Class 2 semaphorins are secreted molecules that control axon guidance and epidermal morphogenesis in Drosophila and *C. elegans*. We show that the *C. elegans* class 2 semaphorin MAB-20 binds the plexin PLX-2. *plx-2* mutations enhance the phenotypes of hypomorphic *mab-20* alleles but not those of *mab-20* null alleles, indicating that *plx-2* and *mab-20* act in a common pathway. Both *mab-20* and *plx-2* mutations affect epidermal morphogenesis during embryonic and in post-embryonic development. In both contexts *plx-2* null mutant phenotypes are much less severe than *mab-20* null phenotypes, indicating PLX-2 is not essential for MAB-20 signaling. Mutations in the ephrin *efn-4* do not synergize with *mab-20*, indicating EFN-4 may act in MAB-20 signaling. EFN-4 and PLX-2 are coexpressed in the late embryonic epidermis where they play redundant roles in MAB-20 dependent cell sorting.
INTRODUCTION

Semaphorins are secreted molecules first identified as axon guidance cues causing growth cone collapse (FAN et al. 1993; KOLODKIN et al. 1993). Semaphorins have subsequently been shown to play a myriad of roles in neural and non-neural development, including cell migration (KRUGER et al. 2005), tissue morphogenesis (HINCK 2004), formation of the vascular system (SUCHTING et al. 2006), and regulation of the immune system (TAKEGAHARA et al. 2005). The semaphorin family comprises a large number of secreted and transmembrane proteins sharing the signature sema domain of 500 amino acid residues (Semaphorin Nomenclature Committee 1999). Semaphorins have been classified by overall architecture and by sequences of their semaphorin domains (KOLODKIN et al. 1993). Classes 1 and 2 are invertebrate-specific, whereas classes 3, 4 and 7 are found only in vertebrates; class 5 is found in both vertebrates and invertebrates. Class 2 semaphorins are composed of an N-terminal sema domain, a cysteine rich domain and a C-terminal Immunoglobulin domain; interestingly, class 2 semaphorins do not contain motifs mediating membrane attachment, yet appear to function as short-range signals. Expression of Drosophila Sema II (a.k.a. sema 2a) on specific muscles causes
them to repel axonal growth cones (Matthes et al. 1995; Winberg et al. 1998). In grasshoppers, Sema 2a is a chemorepulsive guidance cue for the axon of a pioneer neuron (Isbister et al. 2003; Isbister et al. 1999). In C. elegans the class 2 semaphorin MAB-20 promotes repulsion between adjacent epidermal cells (Roy et al. 2000). Such short range activity of the class 2 semaphorins implies that they do not diffuse freely, however the molecular basis of this is not understood.

Semaphorins interact with a remarkably diverse set of cell surface receptors. One major class of cell surface receptor are the plexins, transmembrane proteins first identified as antigens expressed in restricted regions in the nervous system (Fujisawa et al. 1997; Kameyama et al. 1996a; Kameyama et al. 1996b; Ohta et al. 1995; Ohta et al. 1992), and as molecules with a Met-related motif (Maestrini et al. 1996). Some transmembrane semaphorins, such as the invertebrate class 1 semaphorins, directly bind plexins (Winberg et al. 1998). Plexins themselves contain an N-terminal sema domain that binds the sema domain of the semaphorin. Plexins also contain cysteine rich repeats known as Met-related sequences (MRS) or PSI (plexin, semaphorin, integrin) domains, and 3 glycine-proline-rich (GP) repeats; the roles of the MRS and GP domains are not well understood. Class 3 semaphorins interact with receptor complexes composed of plexins
and neuropilins (TAMAGNONE et al. 1999). In such receptor complexes, semaphorins bind the neuropilin subunit whereas plexin is essential for signal transduction into the cell via its cytoplasmic domain. In addition to neuropilins, plexins can act in receptor complexes with a variety of membrane proteins (PASTERKAMP and KOLODKIN 2003). Finally, some semaphorins can signal independently of plexins (PASTERKAMP et al. 2003). The signaling pathways and receptors for several semaphorin classes remain to be fully elucidated. Here we report a genetic analysis of factors acting in the C. elegans class 2 semaphorin MAB-20 pathway.

_C. elegans_ encodes two class 1 transmembrane semaphorins (SMP-1/Ce-sema-1a and SMP-2/Ce-sema-1b), and one class 2 semaphorin (MAB-20/Ce-sema-2a) (ROY et al. 2000). _C. elegans_ also encodes two plexins, the Plexin A family member PLX-1, and PLX-2 described here. Compared with vertebrates, in which more than 20 semaphorins and at least 9 plexins are present, the relative simplicity of the _C. elegans_ semaphorin/plexin signaling system allows systematic tests of their ligand-receptor relationships _in vivo_. Previously we have shown that PLX-1 binds to SMP-1, but not to MAB-20 (FUJII et al. 2002). Genetic analysis showed that SMP-1 and SMP-2 play redundant roles in a pathway that involves PLX-1 (GINZBURG et al. 2002), and that PLX-1
signaling may be attractive or repulsive depending on context (DALPE et al. 2005; DALPE et al. 2004).

Mutations in *mab-20* cause abnormal embryonic epidermal morphogenesis and defects in embryonic migrations of ventral neuroblasts that may contribute to epidermal defects (CHIN-SANG et al. 2002; ROY et al. 2000). *mab-20* mutants also display aberrant morphogenesis of sensilla (rays) in the adult male tail (BAIRD et al. 1991). The neurons and structural cells of each ray form a three-cell group derived from precursors known as Rn.a cells. In *mab-20* males, adjacent rays display fusion, in which cells of one ray associate with those of a neighboring ray to form a single large ray (BAIRD et al. 1991). The ray fusion phenotypes of *mab-20* mutants have been interpreted as reflecting inappropriate adhesion between the ray cells of adjacent groups (ROY et al. 2000). It is not yet understood at the cellular level how MAB-20 regulates cell sorting. For example, as MAB-20 appears to be broadly expressed, it is not clear how it acts as an instructive cue that would prevent inappropriate cell contacts. To address these questions it is critical to define the cell surface receptors that mediate MAB-20 functions.

As MAB-20 does not bind PLX-1, an obvious candidate for the MAB-20 receptor is the other *C. elegans* plexin, PLX-2. Indeed, mutations in *plx-2* enhance ray fusion defects of weak *mab-20*
alleles (IKEGAMI et al. 2004). However, physical interactions between MAB-20 and PLX-2 have not been tested, and their interactions in embryogenesis have not been described. Here we show that PLX-2 binds MAB-20, consistent with a receptor-ligand relationship. We show that different parts of PLX-1 and PLX-2 extracellular domains are involved in binding semaphorins, suggesting that the signaling mechanisms of class 1 and class 2 semaphorins may differ. We characterize plx-2 deletion mutations isolated by reverse genetics. Surprisingly, plx-2 null mutants display very weak morphogenetic phenotypes compared to mab-20 mutants, suggesting that MAB-20 interacts with additional unknown receptors. One candidate for such an additional component of the MAB-20 pathway is the ephrin EFN-4, which functions in morphogenesis independently of the VAB-1 Eph receptor (CHIN-SANG et al. 2002). efn-4 mutants resemble mab-20 mutants in many phenotypes, and efn-4 mutations do not enhance some mab-20 mutant phenotypes (CHIN-SANG et al. 2002; IKEGAMI et al. 2004). It is unclear how an ephrin might contribute to semaphorin signaling, and further analysis of this unusual interaction has awaited the identification of the MAB-20 receptor. Our genetic analysis indicates that PLX-2 and EFN-4 have partly redundant roles in a subset of MAB-20 functions.
MATERIALS AND METHODS

Genetics

*C. elegans* culture and genetic manipulations used standard procedures (BRENNER 1974). *C. elegans* strains N2, DR466 *him-5(e1490)*, EM67 *mab-20(bx24); him-5(e1490)*, EM253 *mab-20(bx61); him-5(e1490)*, EM305 *efn-4 (bx80); him-5(e1490)* and NL2099 *rrf-3(pk1426)* were obtained from the *Caenorhabditis* Genetics Center, care of T. Stiernagle (University of Minnesota). Timelapse analysis of embryogenesis was performed on the following strains: CZ977 *efn-4(bx80)*, CZ1724 *mab-20(ev574)*, CZ4859 *plx-2(tm729)*, ST71 *tm729; bx80*, ST72 *ev574; tm729*, ST73 *ev574; bx80*, ST74 *ev574; tm729; bx80*, CZ5784 *plx-2(nc7)*. *ncIs13* is an integrant of AJM-1::GFP (LIU et al. 2005). Genotypes of all double mutants except those containing *bx61* were confirmed by PCR; primer sequences and the conditions for PCR are available on request.

To determine the penetrance of embryonic lethality, eggs that failed to hatch within 2 days after being laid were scored as embryonic arrest. All estimates of penetrance were obtained from between 5 and 17 complete broods. One-way ANOVA was used to compare differences in penetrance.

Molecular biology of *plx-2*
The cDNA clone yk21h1 encoding the C-terminal 1103 residues of PLX-2, was isolated by Y. Kohara’s group. We constructed a full length plx-2 cDNA by combining yk21h1 and a 5' RACE product amplified with a gene-specific primer and a primer corresponding to the trans spliced leader SL1. DNA sequences were determined for both strands. Our composite plx-2 cDNA sequence agrees with the Gene Summary for plx-2 in the current version of Wormbase (WS157) and does not contain “exon 1” reported by Ikegami et al. (2004).

To examine the expression of the plx-2 gene, we made transcriptional and translational GFP reporter genes. The Pplx-2-GFP transcriptional reporter SBB67 contains 10.9 kb genomic DNA 5’ to the plx-2 ATG (nt 20517-9566 of cosmid K04B12) cloned into pPD95.75 (provided by A. Fire) in two steps. A 10 kb BamHI fragment of K04B12 (20517-10500) was cloned into the BamHI site of pPD95.75; the remaining 5’ region (10500-9566) was amplified by PCR and inserted into pPD95.75. We made Pplx-2-GFP arrays with the rol-6(dm) pRF4 coinjection marker; one array underwent spontaneous chromosomal integration during strain propagation, yielding ncIs21 described here.
We made two translational fusion constructs. A full length PLX-2::GFP translational fusion construct (KKB75) was made by subcloning genomic fragments of K04B12 into pPD95.75, except for the 3’ terminal fragment (3320-2675) which was amplified and cloned into the Kpn I site of pPD95.75. We coinjected KKB75 (0.2 µg/µl) with pRF4 to generate extrachromosomal arrays that were integrated by gamma-ray mutagenesis to create ncIs30. For the partial translational fusion construct, a PCR fragment corresponding to the plx-2 genomic DNA, 20517- 6426 nt of K04B12, was cloned into BamH I-Sal I digested pFXneEGFP (S. Mitani, unpublished), resulting in the translational fusion of the N-terminal half of PLX-2 (residues 1-888) with EGFP (Living Colors Fluorescent Proteins, Clontech). All PCR amplified fragments in the reporter constructs, except for that in the partial translational fusion construct, were sequenced.

To examine GFP fluorescence, worms were mounted on 4% agarose containing 1 mM levamisole and were examined with a Zeiss Axioplan microscope using Zeiss filter set #10. Images were recorded with a camera (Coolpix 990, Nikon). We used anti-GFP immunostaining to analyze GFP reporter expression in embryos. Embryos were fixed and stained essentially as previously described (HUDSON et al. 2006) and images collected on a Zeiss Pascal confocal microscope. The full length PLX-2::GFP translational fusion did not
fluoresce at detectable levels; low levels of expression were detectable by anti-GFP immunostaining (not shown).

**Isolation of plx-2 deletion alleles**

*nc7* was isolated by Tc1 transposon-mediated deletion mutagenesis using a mutator strain MT3126 according to a protocol described previously (SHIBATA et al. 2000). First, we isolated the mutation *nc8::Tc1* in which the Tc1 transposon is inserted at the position corresponding to nt 6617/6618 of cosmid K04B12, in intron 9 of *plx-2*. Then, a deletion allele, *nc7*, which deleted the fragment corresponding to 5596-7488 nt of cosmid K04B12, with the break points of cccgagcacaccaat [*nc7*] ccatcgacaattgga, was isolated. The deletion *nc7* was confirmed by Southern blot analysis (data not shown). The deletion *tm729* was generated by chemical mutagenesis and isolated as described (GENGYO-ANDO and MITANI 2000). *tm729* deletes nucleotides 7632-9291 of cosmid K04B12, with the break points of cagagtgggtctaca [*tm729*] gagactaagcattct. Both mutations were outcrossed 10 times to N2 before analysis.

**Timelapse analysis of embryonic morphogenesis**

Four-dimensional (4D) time-lapse microscopy of embryonic development was performed as described (CHIN-SANG et al. 1999; GEORGE et al. 1998). We timed the
following morphogenetic movements: (1) gastrulation, defined as the time from ingression of the E daughter cells to ingression of the germline and D lineage; (2) closure of the ventral cleft by lateral migrations of ventral neuroblasts (Hudson et al. 2006); (3) epidermal enclosure; (4) epidermal elongation to comma stage. At least 12 embryos were analyzed for each genotype in Figure 4, although not all timepoints were scorable in each embryo.

**Analysis of male tail ray morphology**

We classified the arrangement of Rn.p epidermal cells in larval male tails into 5 classes for quantification. In class I, all Rn.ps adopted apparently wild-type morphology. In class II, R4.p adopted a triangular shape rather than wild-type rectangular shape. In class III, R1/2.ps were larger or irregular in shape compared with the wild type. In class IV, animals exhibited both class II and class III defects simultaneously. In class V, morphology of all Rn.ps was abnormal.

**RNA interference**

A *plx-2* cDNA fragment (1-789 nt), a *plx-1* cDNA fragment (1-693 nt), a *smp-1* cDNA fragment (1-566 nt), a *smp-2* cDNA fragment (1289-1951 nt), and a *mab-20* cDNA fragment (1749-2240 nt) subcloned into pBluescript SK (Stratagene) were amplified by
PCR. The PCR products were used as templates for RNA synthesis with T7 RNA polymerase (Boehringer), and double-stranded RNAs were purified with an RNeasy kit (Qiagen). RNA interference by soaking (MAEDA et al. 2001) was performed in the genetic background of rrf-3(pk1426), which causes hypersensitivity to RNAi (SIMMER et al. 2002).

**Binding analysis**

cDNAs encoding mutant plexins were generated by PCR. PLX-2ΔMRS-GP deletes D(493)-Q(1067). PLX-1ΔMRS-GP deletes L(568)-P(1108). PLX-1Δsema deletes the N-terminus up to R(496). PLX-1Δect deletes the N-terminus up to P(1108).

The wild-type and mutant plexin cDNA were inserted into pCAGGS (NIWA et al. 1991), and tagged with the c-myc epitope (GEQKLISEEDL) at the N-terminus (EVAN et al. 1985).

In all the expression constructs used in the binding assay, a native signal sequence and sequences immediately upstream of the translation initiation codon for *C. elegans* proteins were replaced with that of mouse Sema3A (1-25 aa) (PUSCHEL et al. 1995) and a vertebrate Kozak consensus sequence (CCACC), respectively (KOZAK 1992). The construction of Ce-Sema-1a-ΔC-Fc-AP and Ce-Sema-2a-Fc, containing the ecto-domain of SMP-1 and MAB-20, respectively, was described previously (FUJII et al. 2002).
To express EFN-4, we amplified cDNA corresponding to the entire coding region of *efn-4* using the cDNA clone yk449e2 as template, using primers to introduce the first 4 residues of the signal sequence and appropriate restriction sites, and cloned the resulting PCR product into the *Hind* III--*Bam* H I sites of pcDNA3 (Invitrogen) creating plasmid pCZ152.

HEK293T cells were transfected with the expression constructs using Effectene Transfection Reagent (Qiagen). Two days after transfection, culture medium containing Ce-Sema-1a-ΔC-Fc-AP or Ce-Sema-2a-Fc was collected, concentrated by ultrafiltration (Ultrafree-15 centrifugal Filter Device, Millipore) and added to transfectants expressing plexins or EFN-4. The concentration of semaphorins was 25 nM. After incubation at 37°C for 60 minutes, the cultures were washed with fresh culture medium, fixed with 4% paraformaldehyde overnight at 4°C, and rinsed with TBST [10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% Tween 20]. The cultures were then reacted with goat anti-human Ig-Fc conjugated with AP (20 μg/ml, Cappel) in TBST containing skim milk (50 mg/ml) at room temperature (RT) for 1 hour. After washing with TBST, the cultures were stained in NBT/BCIP solution (Boehringer) at RT for 5 to 30 minutes, or at 4°C overnight.
To confirm the expression of wild-type and mutant plexins on the cell surface, intact transfected cells were treated with anti-myc antibody (9E10; Evan et al., 1985). Then, the cells were fixed, reacted with AP conjugated anti-mouse Ig (Zymed) and stained similarly. Expression of EFN-4 on the cell surface was confirmed with anti-EFN-4 antibodies (CHIN-SANG et al. 2002). Immunoblot analysis was carried out as described (FUJII et al. 2002).

RESULTS

*C. elegans* PLX-2 is a divergent plexin that binds the class 2 semaphorin

MAB-20

*plx-2* corresponds to transcription unit K04B12.1, located at the right end of chromosome II (Figure 1A). The predicted PLX-2 protein is composed of 1766 amino acid residues and contains all the hallmark features of plexins: a sema domain (amino acid residues 50-479), 3 Met-related sequences (MRSs) (residues 447-479, 578-616, 708-747), 3 GP repeats (residues 750-776, 847-877, 927-956), a transmembrane region (1115-1141), and an intracellular region (1142-1766) (Figure1B).
The intracellular region of PLX-2, like that of other plexins, contains two regions of sequence conservation that together define the plexin-specific ‘sex-plex’ (SP) domains.

Overall the PLX-2 intracellular region is highly diverged relative to those of other plexins (e.g. 32% identity to Drosophila PlexB, 31% identity to mammalian Plexin Ds, whereas the C. elegans PLX-1 intracellular domain is 50% identical to mammalian PlexAs). Within the SP domain, PLX-2 contains a motif (FTLADYG, 1457-1463) similar to that in Drosophila PlexB (NTLHYG, 1722-1728) reported to be necessary for binding to Rac (Hu et al. 2001). PLX-2 also contains two arginine residues (R1303, R1607) corresponding to the invariant catalytic residues of Ras GTPase activating protein (GAP) (Rohm et al. 2000). On the other hand, the PLX-2 intracellular domain does not appear to contain other motifs found in plexins, such as the putative Cdc42/Rac interactive binding (CRIB)-like motif described in human Plexin B1 (Vikis et al. 2000), the segment in Drosophila PlexB (1617-1827) necessary for binding to Rho (Hu et al. 2001), nor the carboxyl-terminal PSD-95/Dlg/ZO-1 (PDZ) domain-binding motif, through which mammalian Plexin-B1 interacts with PDZ-RhoGEF/leukemia-associated Rho GEF (LARG) (Swercz et al. 2002).
Plexins were originally classified into 4 subfamilies, A to D (TAMAGNONE et al. 1999). A more recent analysis based on the sequences of Sema domains has classified Plexins into two main groups, the Plexin A subfamily and the B/C/D subfamilies (GERARDI et al. 2004). Whereas *C. elegans* PLX-1 consistently clusters with the Plexin A subfamily, PLX-2 appears to be a divergent member of the B/C/D group, and could not be assigned to any one of these subfamilies (Figure 1C).

We tested the physical interaction of PLX-2 with *C. elegans* semaphorins in vitro using cultured mammalian cells. We expressed the ectodomains of SMP-1/Ce-Sema-1a tagged with the Fc region of human IgG and alkaline phosphatase (Ce-Sema-1a-ΔC-Fc-AP), or MAB-20/Ce-Sema-2a tagged with Fc (Ce-Sema-2a-Fc) in HEK 293 cells, and the culture supernatants were added to cells transfected with full length PLX-2. Ce-Sema-2a-Fc bound to HEK cells expressing PLX-2, but Ce-Sema-1a-ΔC-Fc-AP did not (Fig. 2B, C). We conclude that PLX-2 specifically binds MAB-20.

*plx-2 deletion mutations do not cause overt morphogenetic abnormalities*

To analyze the function of *plx-2* we generated two deletion mutations, *nc7* and *tm729*. *plx-2(nc7)*, generated by transposon-mediated mutagenesis, is a deletion with
break points in exon 8 and exon 11, and is predicted to cause an in-frame deletion of residues D493-Q1067, corresponding to the 2\textsuperscript{nd} and 3\textsuperscript{rd} MRS and the GP rich repeats (Figure 1B). \textit{plx-2(tm729)}, generated by chemical mutagenesis, is a deletion with break points in intron 1 and exon 7, a region encoding the sema domain. The predicted structure of the message suggests that \textit{tm729} is a molecular null; if exon 1 is spliced to exon 8, the reading frame would terminate within exon 8 due to a frame-shift.

Animals homozygous for \textit{nc7} or \textit{tm729} were healthy, viable, and fertile. Their movements appeared normal, and analysis of neuronal morphology using a pan-neuronal marker failed to reveal defects in the nervous system (not shown). >99\% of \textit{nc7} and \textit{tm729} embryos hatched and reached adulthood (\(n = 1074\) for \textit{nc7}, and \(n = 878\) for \textit{tm729}). Almost all \textit{nc7} and \textit{tm729} larvae were morphologically normal (not shown), and the rays in the adult male tail were normal (Table 1, Figure 5A). These observations are in stark contrast to the highly penetrant embryonic and larval lethality and male tail ray fusion defects of \textit{mab-20} mutants (BAIRD \textit{et al.} 1991; ROY \textit{et al.} 2000).

The dramatic phenotypic difference between \textit{plx-2} and \textit{mab-20} mutants might indicate that \textit{plx-2} mutations do not eliminate PLX-2 function, although this is not plausible at least for \textit{tm729}, whose putative product should lack function. Alternatively,
loss of PLX-2 function by itself may not lead to phenotypes similar to \textit{mab-20} mutants. 

Using different alleles of \textit{plx-2}, a previous study has suggested the same possibility (IKEGAMI \textit{et al.} 2004). To further examine this, we tried to reduce the function of PLX-2 by soaking mediated RNA interference (sRNAi) in the RNAi hypersensitive mutant \textit{rrf-3(pk1426)} (SIMMER \textit{et al.} 2002). sRNAi of \textit{mab-20} partly phenocopied the ray fusions of \textit{mab-20} mutants (Table 1), and sRNAi of \textit{plx-1} and double sRNAi of \textit{smp-1} and \textit{smp-2} caused ray 1 displacement comparable to that caused by genetic null mutations (not shown), showing that sRNAi is effective during ray morphogenesis. RNAi of \textit{plx-2} did not lead to ray defects (Table 1) nor to embryonic lethality (data not shown). We conclude that loss of \textit{plx-2} function alone does not cause overt defects in morphogenesis resembling those of \textit{mab-20} mutants.

\textbf{Genetic interactions of \textit{plx-2}, \textit{mab-20} and \textit{efn-4} mutations}

To address whether PLX-2 has a cryptic role in MAB-20 signaling we constructed double mutants between \textit{plx-2} and three \textit{mab-20} alleles: the hypomorphic alleles \textit{bx61} and \textit{bx24}, and the predicted null mutation \textit{ev574} (ROY \textit{et al.} 2000). \textit{mab-20(bx61)} is a temperature-sensitive allele caused by a missense mutation, and causes weak ray fusion
defects at 20°C. \textit{mab-20(bx24)} is a hypomorphic allele of medium strength that contains an out-of-frame tandem duplication. We refer to \textit{bx61} and \textit{bx24} collectively as weak \textit{mab-20} alleles. \textit{mab-20(ev574)} is a deletion that includes the initiator methionine. \textit{ev574} is predicted to be a molecular null, and causes the most severe morphogenetic defects among the three \textit{mab-20} alleles.

First we examined whether \textit{plx-2} mutations affect the lethality of \textit{mab-20} mutants using double mutant analysis. The hypomorphic mutation \textit{mab-20(bx24)} causes incompletely penetrant (9\%) embryonic lethality (Figure 3A). Both \textit{plx-2(tm729)} and \textit{plx-2(nc7)} significantly enhanced the penetrance of embryonic lethality of \textit{bx24}: 16\% and 17\% embryos failed to hatch for \textit{bx24; tm729}, and for \textit{bx24; nc7}, respectively. \textit{plx-2(tm729)} also enhanced the penetrance of embryonic lethality of \textit{bx61}. In contrast, neither \textit{plx-2(tm729)} nor \textit{plx-2(nc7)} enhanced the embryonic lethality of the null allele \textit{mab-20(ev574)}: 22\% and 21\% embryos failed to hatch for \textit{ev574; tm729} and for \textit{ev574; nc7}, respectively. We found similar trends in larval lethality (Figure 3B). As \textit{plx-2} mutations enhanced the embryonic phenotypes of weak \textit{mab-20} alleles but not of \textit{mab-20} null alleles, we conclude that PLX-2 has a cryptic role in MAB-20 signaling revealed when MAB-20 function is compromised.
The dramatic difference in penetrance between \textit{plx-2} and \textit{mab-20} null mutants strongly argues that MAB-20 has functions independent of PLX-2. As the other plexin, PLX-1, does not appear to function in MAB-20 signaling (Fujii et al., 2002), we tested the role of another potential component of MAB-20 pathways, the ephrin EFN-4. First we examined the genetic interaction between \textit{plx-2} and \textit{efn-4} mutants. \textit{efn-4(bx80)} is a putative null allele (CHIN-SANG et al. 2002). Both \textit{plx-2(nc7)} and \textit{plx-2(tm729)} significantly enhanced the embryonic and larval lethality of \textit{efn-4(bx80)} beyond that expected for additivity of phenotypes: 10\% of \textit{bx80} embryos failed to hatch, whereas 20\% of \textit{tm729}; \textit{bx80} or 20\% of \textit{nc7}; \textit{bx80} failed to hatch (Figure 3A; \textit{P} < 0.05 by Anova). \textit{plx-2} mutations also enhanced the larval lethality of \textit{efn-4(bx80)} (Figure 3B) to a level similar to that of \textit{mab-20} null mutants. The enhancement of \textit{efn-4} null phenotypes by \textit{plx-2} suggests that PLX-2 and EFN-4 act in parallel and have partly redundant roles in morphogenesis.

We previously reported that certain \textit{mab-20}; \textit{efn-4} double mutants display subadditive interactions in embryonic and larval lethality (CHIN-SANG et al. 2002). We confirmed and extended this finding using null mutations in both genes. Interestingly, we found that \textit{efn-4(bx80)} partly suppressed the penetrance of embryonic lethality of \textit{mab-20(ev574)} (Figure 3A); the double mutants displayed an intermediate level of lethality.
compared to the single mutants. This partial genetic suppression suggests that MAB-20 and EFN-4, while having similar roles in some situations may antagonize in other contexts (see below). Although the mab-20 null allele ev574 was consistently suppressed by efn-4, the weak mab-20 alleles, bx24 and bx61, enhanced the embryonic lethality of efn-4 null mutants; the penetrance of embryonic defects in these double mutants did not exceed that of mab-20(ev574). These results from homozygous double mutants are reminiscent of the finding that bx61, but not ev574, displays dosage-sensitive interactions with efn-4(bx80) (IKEGAMI et al. 2004).

The overlap in efn-4 and mab-20 phenotypes and the enhancement of efn-4 by weak mab-20 alleles suggest that MAB-20 positively regulates EFN-4. To account for the partial suppression of mab-20(ev574) by efn-4 we propose that EFN-4 is partly activated by MAB-20/PLX-2 signals, but that MAB-20 is not essential for all PLX-2 activities. MAB-20 can be thought of as both positively and negatively regulating PLX-2. In the wild type MAB-20 binds PLX-2 and this activates a signal that promotes EFN-4 function. In mab-20 weak mutants, mutant MAB-20 products are expressed that bind PLX-2, and these mutant complexes retain a residual ability to activate EFN-4, and thus mutation of efn-4 does not suppress mab-20 weak alleles. In contrast, in the complete absence of
MAB-20, PLX-2 is now inappropriately active and can increase EFN-4 function. Elimination of EFN-4 therefore partly alleviates the mab-20 null phenotype. This model predicts that lack of plx-2 should block the suppression of mab-20(ev574) by efn-4(bx80), and we find that this is the case: triple null mutants of genotype mab-20(ev574); plx-2(tm729); efn-4(bx80) resemble mab-20(ev574) and not mab-20; efn-4 in overall penetrance (Figure 3). The significant differences in penetrance between mab-20; efn-4 and mab-20; plx-2; efn-4 strains (P < 0.05, Anova) imply that PLX-2 has some activity in the absence of either MAB-20 or EFN-4. As MAB-20 also positively regulates PLX-2, elimination of PLX-2 does not itself suppress the mab-20 null phenotype.

**PLX-2 has a minor role in embryonic ventral neuroblast movement and acts redundantly with EFN-4 in later epidermal morphogenesis**

Both mab-20 and efn-4 mutants display incompletely penetrant defects in embryonic epidermal morphogenesis. Defective epidermal morphogenesis can result from cell autonomous defects in the epidermal epithelium, or from defective formation of the underlying substrate for enclosure, which itself is formed by coordinated migrations of ventral neuroblasts (VNBs) at the end of gastrulation (CHISHOLM and HARDIN 2005). We
previously reported that during embryogenesis both *efn-4* and *mab-20* mutants display delayed VNB migrations (CHIN-SANG *et al.* 2002; HUDSON *et al.* 2006). To understand the role of *plx-2* in the MAB-20 and EFN-4 pathways in embryogenesis we quantitatively analyzed VNB migrations and morphogenetic movements in all relevant single, double and triple null mutants using time-lapse microscopy (see Materials and Methods; Figure 4A, B).

Approximately 10% of *efn-4*(bx80) mutants arrest at the epidermal enclosure stage of embryogenesis, a phenotype denoted Class I or II depending on the degree of enclosure reached (CHIN-SANG *et al.* 2002). In most *efn-4* mutants VNB migrations are slightly delayed and the epidermis encloses. In contrast, *mab-20* mutants display more variable VNB migration defects that rarely result in Class I/II arrest. The 20% embryonic lethality in *mab-20* null mutants is predominantly due to rupture of the epidermis in the elongation stage of embryogenesis (the Class III terminal phenotype; Figure 5A), suggesting MAB-20 plays a more critical role in later epidermal morphogenesis (ROY *et al.* 2000). Using quantitative analysis we found that VNB migrations of *efn-4 mab-20* double null mutants were not significantly different from those of *efn-4* single mutants (Figure 4 B). *efn-4 mab-20* double mutants displayed enclosure stage arrest (class I/II) at a frequency similar to that of *efn-4* single mutants (Figure 5C). Thus, although *mab-20* null mutants display
delayed VNB migrations they do not enhance the VNB migration or enclosure defects of efn-4 mutants, consistent with MAB-20/Sema2A and EFN-4 acting in a common process regulating VNB movement. In contrast, we found that efn-4 mab-20 double mutants displayed a reduced level of class III arrest compared to mab-20 mutants, accounting for the partial suppression of embryonic lethality (Figure 5C). These observations suggest the antagonism of MAB-20 and EFN-4 may be specific to later embryonic development.

We examined the embryonic development of plx-2 mutants. Ventral cleft closure duration in plx-2 mutants was slightly prolonged relative to the wild type (64 ± 15 min in tm729 versus 55 ± 15 min in the wild type, P = 0.04 by t test). plx-2 mutants did not show penetrant defects in epidermal enclosure or in later embryogenesis. Unlike tm729, the in-frame deletion allele plx-2(nc7) did not affect the duration of cleft closure; in nc7 mutants the onset of cleft closure was earlier than in the wild type (not shown). plx-2(tm729) did not significantly enhance the VNB migration defects of either efn-4, mab-20 or of efn-4 mab-20 double mutants (Figure 4B).

These findings suggested that PLX-2 plays a relatively minor role in VNB movement compared to that of EFN-4 or MAB-20. To test whether a cryptic role for PLX-2 in VNB movement would be revealed in a more sensitized background we
constructed double mutants between \textit{plx-2} and \textit{kal-1}, a gene required for normal VNB migration (Hudson \textit{et al.} 2006). \textit{kal-1} null mutants display delayed VNB migrations that do not typically result in embryonic lethality, but which strongly enhance VNB migration defects of \textit{efn-4} or \textit{mab-20} null alleles. We found that \textit{plx-2(tm729)} did not significantly enhance \textit{kal-1} VNB migration or other morphogenetic defects. \textit{kal-1(gb503); plx-2(tm729)} double mutants displayed a cleft duration of 74.4 minutes ($n = 21$; $P = 0.1$ compared to \textit{kal-1(gb503)} alone) and $<1\%$ embryonic lethality, consistent with a relatively minor role for PLX-2 in formation of the neuronal substrate for enclosure.

As \textit{plx-2} mutations significantly enhanced the total embryonic lethality of \textit{efn-4} mutants, these findings suggest that PLX-2 and EFN-4 have redundant roles in later embryogenesis. The enhancement of \textit{efn-4} embryonic lethality can be largely accounted for by the higher rates of elongation stage rupture compared to \textit{efn-4} alone (Figure 5C; $P < 0.05$, Fisher exact test). The frequency of late embryonic epidermal ruptures in \textit{plx-2 efn-4} strains (13.8\%) was not further enhanced in the \textit{mab-20 plx-2 efn-4} triple mutant (14.3\%), consistent with EFN-4 and PLX-2 acting redundantly in the MAB-20 dependent process that promotes later embryonic epidermal development.
PLX-2 is expressed in embryonic neuronal and epidermal cells and is partly overlapping with EFN-4 in the posterior epidermis

To address whether EFN-4 and PLX-2 might function in the same cells in MAB-20 signaling we compared in detail the embryonic expression patterns of PLX-2 transcriptional and translational reporters and a functional EFN-4::GFP reporter (juIs109). In early embryos EFN-4::GFP is widely expressed in ventral neuroblasts prior to epidermal enclosure (CHIN-SANG et al. 2002). In contrast, PLX-2 reporters were expressed in a much smaller number of ventral neuroblasts (Figure 6A), consistent with our embryological data showing that EFN-4 acts independently of PLX-2 in VNB movement. Following epidermal enclosure PLX-2::GFP reporters were expressed in a large number of neurons and in a subset of posterior lateral and ventral epidermal cells; we identified these as the lateral cell QV5 and the ventral epidermal cells P9-12 (Figure 6B-D). In addition to its widespread neuronal expression, we find that EFN-4::GFP is also consistently expressed in a subset of anterior and posterior epidermal cells, including the lateral epidermal cells H0 and QV5, the leading ventral epidermal cells of the anterior, and the posterior three pairs of ventral epidermal cells (P7-12) (Figure 6E-H). The overlap in PLX-2 and EFN-4 expression in the posterior embryonic epidermis correlates with the
defect in posterior ventral epidermal integrity of \textit{plx-2 efn-4} double mutants, and suggests that in this process EFN-4 and PLX-2 could act on the same or adjacent cells to mediate a MAB-20 cell sorting signal.

**PLX-2 and EFN-4 promote MAB-20-dependent sorting of ray cells**

In both \textit{mab-20} and \textit{efn-4} mutants, male sensory rays become aberrantly fused, a phenotype suggestive of a failure of cell sorting or repulsion between the component cells of a ray. Neither \textit{plx-2(nc7)} and \textit{plx-2(tm729)} caused overtly abnormal ray development as single mutants (Table 1; although see below for Rn.p defects). In larval male tails PLX-2 reporters were strongly expressed in neurons and muscles, and weakly in some unidentified epidermal cells. We confirmed GFP expression in the tail seam cells and R7/8/9.p (Figure 7D,F). However we could not detect translational PLX-2::GFP reporters in Rn cells, or their descendants, suggesting that this PLX-2::GFP reporter may not reflect the complete PLX-2 expression pattern.

To test whether PLX-2 had a cryptic role in ray development comparable to the embryo we examined double and triple mutants. We found that both \textit{plx-2} mutations significantly enhanced ray fusion defects of weak \textit{mab-20} alleles (Table 1 section 2; Figure
7C) but not of the null mutation \textit{mab-20(ev574)}, implying that PLX-2 promotes MAB-20 functions in ray development. Both \textit{plx-2(nc7)} and \textit{plx-2(tm729)} also significantly enhanced the ray fusion defects of \textit{efn-4(bx80)} (Table 1 section 4), consistent with EFN-4 and PLX-2 acting in parallel to promote ray cell sorting. Since ray fusion reflects aberrant aggregation of ray precursor (Rn.a-derived) cell clusters, we also analyzed the arrangement of ray precursors in larval males using the apical junction marker AJM-1::GFP (Figure 7 H,I). Using this marker we found that precursor clusters were arranged normally in \textit{plx-2(tm729)} larvae (n = 50). \textit{plx-2(tm729)} significantly enhanced the aggregation of ray precursor clusters in \textit{mab-20} weak alleles (Figure 7 J,N), consistent with enhancement of the ray fusion defects in the adult double mutants.

Our analysis of ray development suggests the role of MAB-20 in preventing inappropriate cell contacts involves partly redundant functions of EFN-4 and PLX-2. However, because \textit{mab-20(ev574)} single mutants display very highly penetrant ray fusion defects, further enhancement in a double mutant may not be easily detectable. In addition, ray precursor (Rn.a) clusters in \textit{mab-20(ev574)} and \textit{efn-4(bx80)} mutants were often so severely disorganized that individual clusters could not be scored. Since we found that the morphology of Rn.p epidermal cells adjacent to ray precursor clusters was often
abnormal in these mutants, we used it as an alternative index of arrangement defects of tail epidermal cells. We categorized Rn.ps into 5 classes, from wild type morphology (class I) to most disrupted (class V) (Figure 7 K,L,M). 81% of mab-20 mutants displayed Rn.p defects, whereas 67% of efn-4 mutants had aberrant Rn.ps. 86% of mab-20; efn-4 double mutants were defective, a proportion that is not significantly different from mab-20 single mutants (P = 0.25; Fig. 7O). The only difference we could detect between mab-20 single mutants and mab-20; efn-4 double mutants was an increase in the proportion of Class IV Rn.p morphology at the expense of class III (P = 0.03). We conclude that EFN-4 functions predominantly in MAB-20 signaling in Rn.p morphogenesis.

Despite having overtly normal ray morphology plx-2(tm729) males displayed low penetrance defects in Rn.p morphology (13% type II), consistent with previous findings that plx-2 single mutants display subtle abnormalities in the shapes and positions of lateral epidermal cells (IKEGAMI et al. 2004). Loss of plx-2 function did not significantly enhance Rn.p morphology defects in mab-20(ev574) mutants. plx-2; efn-4 double mutants displayed a slight enhancement of class V defects relative to efn-4 (32% vs. 22%) although this was not statistically significant. In general our analysis of Rn.p morphology
is consistent with both EFN-4 and PLX-2 largely functioning in a MAB-20 dependent pathway.

**The MRS and GP repeats of PLX-2 are dispensable for binding to MAB-20, whereas the same region of PLX-1 is necessary for binding to SMP-1**

The mutation *plx-2(nc7)* is an in-frame deletion predicted to create a mutant protein lacking only the 2nd and 3rd MRSs and the three GP-rich repeats. However, in several assays *nc7* behaved similarly to the presumed null mutation *plx-2(tm729)*. To examine how the *nc7* deletion might affect PLX-2 function, we analyzed its effects on protein stability and ligand binding. First, we generated *plx-2* cDNA from RNA isolated from *nc7* animals by RT-PCR, and confirmed that *plx-2(nc7)* mutants express a mRNA with a smaller size corresponding to the *nc7* deletion (data not shown). Next, we expressed PLX-2 resembling the *nc7* mutant version in HEK293 cells. We found that PLX-2∆MRS-GP, although expressed on the cell surface at lower levels compared to wild type PLX-2 (Figure 2E) was able to bind to MAB-20::Fc (Fig. 2H). These results suggest that the MRS and GP domains of PLX-2 are not directly involved in MAB-20 binding but that they promote other aspects of signal transduction by PLX-2.
To examine whether the sema domain of PLX-1 is also sufficient for its interaction with a semaphorin we generated similar deletions in the PLX-1 extracellular domain. Unexpectedly, deletion of the PLX-1 MRSs and 3 GP rich regions, PLX-1ΔMRS-GP, completely abolished its ability to bind a SMP-1 fusion protein (Ce-Sema-1a-ΔC-Fc-AP, Figure 2P). As expected, deletion of the sema domain of PLX-1, PLX-1Δsema, also abolished the binding of SMP-1 (Figure 2R). Therefore, PLX-1 and PLX-2 may interact differently with their respective semaphorin ligands.

As our genetic analyses indicated that EFN-4 acts in MAB-20 signaling, we also examined whether MAB-20 could physically interact with EFN-4 in cultured mammalian cells. EFN-4 was robustly expressed in HEK 293 cells (Figure 2V). However neither Ce-Sema-2a-Fc nor Ce-Sema-1a-ΔC-Fc-AP bound to HEK cells expressing EFN-4 (Figures 2W, X). We conclude that EFN-4 is unlikely to interact directly with MAB-20.

Discussion

The *C. elegans* semaphorin 2a MAB-20 has pleiotropic functions in cell migration and cell adhesion in development, yet the nature of the MAB-20 signaling pathway has remained elusive. In this paper we provide genetic and biochemical
evidence that the plexin PLX-2 acts in a branched MAB-20 signaling pathway. We find that plx-2 mutations enhance multiple phenotypes of weak mab-20 alleles, but not of a mab-20 null allele, suggesting that plx-2 and mab-20 act in a common genetic pathway.

We have also shown that PLX-2 binds to MAB-20, but not to another semaphorin, SMP-1. These results imply that PLX-2 is a functional receptor for MAB-20. We show that in certain contexts the atypical ephrin EFN-4 acts in parallel to PLX-2 in MAB-20-dependent processes.

**PLX-2 is not the sole receptor for MAB-20/Sema 2a**

PLX-2 can bind to MAB-20, consistent with a receptor-ligand relationship. However putative plx-2 null mutants display much weaker phenotypes when compared to mab-20 null mutants, implying that MAB-20 can signal via a second pathway. An alternative explanation of the subtle phenotype of plx-2 mutants compared to mab-20 might be that PLX-2 receives both a positive signal from MAB-20 and an antagonistic signal from some other molecule, so that deletion of PLX-2 eliminates both signals and thus results in a weak phenotype compared to the mab-20 null phenotype. However, such models predict that plx-2 should be epistatic to mab-20, and this is not so. Thus, we favor the model that MAB-20 signals via a PLX-2-dependent pathway and a parallel PLX-2-
independent pathway. The PLX-2 independent pathway might be active in the wild type or might be activated only in the absence of PLX-2. In the latter case, elimination of the parallel pathway alone would confer subtle phenotypes that are synthetic with plx-2 mutations. EFN-4 might act in the PLX-2-independent pathway, or in both pathways, as its loss of function causes phenotypes almost as strong as those of mab-20 null mutants.

The nature of the hypothesized MAB-20 receptor in the PLX-2-independent pathway remains unknown. It is unlikely that the only other C. elegans plexin, PLX-1 functions in MAB-20 signaling, as PLX-1 does not interact with MAB-20 physically or genetically (Fujii et al. 2002), and plx-1 plx-2 double mutants do not exhibit ray fusion defects (unpublished observation, S. Takagi). We infer that MAB-20 must interact with a non-plexin receptor. Using mammalian cell culture we have been unable to detect binding between MAB-20 and EFN-4; we also do not detect any effect of coexpression of EFN-4 on the ability of PLX-2 to bind MAB-20 (unpublished results). EFN-4 might act indirectly to stabilize or localize the hypothetical MAB-20 receptor, or it could perform a similar function for MAB-20 itself.

MAB-20 is a member of the ‘invertebrate specific’ class 2 semaphorins, whose signaling mechanisms remain less well studied. Drosophila expresses two class 2
semaphorins, Sema-2a and Sema-2b; Sema 2a is expressed by muscles, where it acts as a repellent in motor axon targeting in the neuromuscular system (WINBERG et al. 1998).

The Drosophila plexin PlexB has recently been shown to act as a receptor for Sema-2a (AYOOB et al. 2006). Contrary to the situation in C. elegans, in Drosophila the PlexB phenotype is much more severe than the Sema-2a phenotype, implying that PlexB interacts with additional ligands, possibly Sema-2b or itself. Thus, analysis of other class 2 semaphorins has not yet suggested comparable instances of plexin-independent signaling. However, several vertebrate semaphorins can signal independently of plexins. The transmembrane semaphorin Sema 4D interacts with two quite different receptors: in the nervous system Sema 4D interacts with a complex of PlexB1 and Met, whereas in the immune system it interacts with a lectin, CD72 (KUMANOGOH et al. 2000). The latter interaction appears to be independent of a plexin receptor. Likewise, Sema 4A functions in T cell development independently of plexins, instead binding the mucin-like protein Tim-2 (KUMANOGOH et al. 2002). The cell surface semaphorin Sema 7A binds Plexin C1, but Plex C1 is not required for Sema 7A’s function in promoting axon outgrowth, which is mediated by integrins (PASTERKAMP et al. 2003). Unlike Sema 7A, MAB-20 lacks an RGD motif and so is unlikely to interact with integrins.
Roles of plexin extracellular domains in semaphorin binding

Plexin extracellular domains contain an N-terminal sema domain and membrane proximal MRS and GP domains. Based on structure-function analyses of plexin A1, plexins are thought to exist in an autoinhibited state in the absence of ligand due to an intramolecular interaction of the sema domain with the MRS and GP domains (Takahashi and Strittmatter 2001). Binding of semaphorin ligand to the plexin sema domain by sema-sema heterodimerization releases this autoinhibition, leading to a change in the conformation of the plexin intracellular domain and activation of intracellular pathways. It is not yet known to what extent this model for Plexin A regulation applies to other plexins.

Our analysis of PLX-2/MAB-20 binding shows that as expected the PLX-2 sema domain is essential for interaction with semaphorin ligand, whereas the MRS and GP domains are essential for PLX-2 function, but not for MAB-20 binding. The plx-2(nc7) in-frame deletion of the MRS and GP domains enhances the phenotype of weak mab-20 mutants to a degree comparable to that of a plx-2 null mutation. Although the autoinhibition model suggests that deletion of MRS and GP domains should increase
plexin activity, \textit{plx-2(nc7)} behaves like a null mutation in this assay. This might be explained if the MRS and GP domains also played a role in plexin stability. In cell culture experiments both PLX-2\textDelta MRS and PLX-1\textDelta MRS proteins are expressed, albeit at lower levels than the full-length proteins. The loss of function in \textit{plx-2(nc7)} mutants therefore suggests additional roles for the MRS and GP domains beyond stabilization. However, in our analysis of VNB migrations, \textit{nc7} behaved differently from \textit{tm729}. Whereas \textit{tm729} results in a delay in VNB migrations relative to the wild type, \textit{nc7} causes the VNBs to begin migration earlier than the wild type, suggesting \textit{nc7} may indeed result in a gain of function in this context. In contrast to PLX-2, we find that PLX-1\textDelta MRS did not bind its semaphorin ligand, suggesting the roles of the MRS and GP repeats may depend on the class of plexin. We do not know of other direct tests of the roles of the MRS and GP domains in plexin-semaphorin binding. The MRS domain of Plexin B1 is not required for it to associate with Met receptor (Giordano et al. 2002); in the case of Met itself, internal deletion of the MRS domain was found to interfere with protein processing (Kong-Beltran et al. 2004), and thus the exact role of these domains remains uncertain.
MAB-20 signaling in ray development

Eph family receptor-type tyrosine kinases and their ligands, ephrins, are known to mediate both adhesive and repulsive cell-cell interactions (POLIAKOV et al. 2004).

*C. elegans* encodes a single Eph receptor, VAB-1 (GEORGE et al. 1998), and four ephrins, EFN-1 through –4. Previous genetic analysis showed that EFN-4 is unlikely to act as a ligand for VAB-1, leaving open the question of how EFN-4 signals (CHIN-SANG et al. 2002). The similarity of *mab-20* and *efn-4* phenotypes in VNB migration and male tail development, and the lack of enhancement in double mutants suggested they could affect a common process. Our analysis of the genetic interactions between *efn-4, mab-20* and *plx-2* in embryogenesis provides additional evidence that EFN-4 acts in a branched MAB-20 pathway. Some phenotypes observed in *mab-20* mutants and not in *efn-4* mutants, such as the defects in late embryonic epidermal development, can now be ascribed to PLX-2 acting in parallel to EFN-4. As *efn-4* mutants display some additional phenotypes not observed in *mab-20* mutants, EFN-4 may act independently of MAB-20 in some situations.

The interactions between MAB-20 and EFN-4 in male tail ray development have been examined in two other studies. HAHN and EMMONS found that some ray fusions (rays 2 and 6) occurred more frequently in *mab-20 efn-4* double mutants than in the single
mutants, and concluded that EFN-4 and MAB-20 had independent roles in ray development (Hahn and Emmens 2003). In contrast, Ikegami et al. concluded that EFN-4 accounted for ‘the bulk’ of MAB-20 signaling in ray sorting (Ikegami et al. 2004). Our observations are more consistent with those of Ikegami et al., as we find mab-20 efn-4 double mutants display levels of ray fusion that are not significantly different from those of mab-20 single mutants. A caveat to this interpretation is that mab-20 single mutants display very highly penetrant ray fusions, so further enhancement in double mutants may be hard to detect, or only significant with a larger sample size. Our analysis of Rn.p cell morphology, for which mab-20 and efn-4 single mutants have less penetrant defects and for which the double mutant is overall not enhanced relative to the strongest single mutant, supports the model that MAB-20 and EFN-4 have largely overlapping functions.

MAB-20 signaling pathways in VNB migration and epidermal morphogenesis may be different

In embryonic morphogenesis efn-4 and mab-20 mutants display defects in ventral neuroblast migration that are not further enhanced in the double mutant. This is striking in light of the strong synergism of efn-4 with other pathways involved in VNB movements,
such as vab-1 (Eph RTK), ptp-3 (LAR RPTP), or kal-1 (Kallmann syndrome protein/Anosmin-1) (CHIN-SANG et al. 2002; HUDSON et al. 2006). The specific lack of synergism between mab-20 and efn-4 in VNB migrations suggests that these two genes act in a common pathway to promote VNB movement. In contrast, the MAB-20 receptor PLX-2 appears to play a relatively minor role in VNB migration. Although plx-2 mutants display weak VNB migration defects, plx-2 did not significantly enhance VNB migration defects of efn-4, mab-20, or of kal-1 null mutants. We conclude that the role of MAB-20 in VNB migration is more dependent on the putative PLX-2 independent pathway that involves EFN-4, consistent with the widespread expression of EFN-4 in ventral neuroblasts compared to PLX-2.

Our analysis of embryonic morphogenesis suggests PLX-2 may have a specific, albeit redundant, function in mediating the epidermal cell sorting functions of MAB-20. MAB-20 signaling prevents ectopic contacts between ventral epidermal cells (ROY et al. 2000). Such ectopic contacts presumably lead to a weakening of the epidermal layer and the late embryonic epidermal ruptures characteristic of mab-20 mutants. In contrast, efn-4 mutants do not display ectopic P cell contacts nor do they rupture in elongation (CHIN-SANG et al. 2002). This difference can now be accounted for if PLX-2 acts redundantly
with EFN-4 in mediating the cell sorting functions of MAB-20 within the epidermis.

Whereas in ventral neuroblast migration MAB-20 and EFN-4 appear to have similar roles, in later epidermal development they appear to partly antagonize, as mab-20 epidermal rupture phenotypes are partly suppressed by efn-4. Finally, in male tail development MAB-20 and EFN-4 again appear to play similar roles in promoting ray sorting. In interpreting these interactions a central caveat is that in no case has it been demonstrated that the developmental defect results from lack of adhesion or lack of repulsion. However, defects in VNB migration are most simply explained as due to a lack of adhesion among VNB cells, whereas ectopic epidermal cell contacts and ray fusion defects are most simply explained as due to lack of repulsive interactions (Figure 8). We speculate that both MAB-20 and EFN-4, like other semaphorins and ephrins, can promote adhesion or repulsion depending on the cellular context and the level of signaling. As EFN-4 appears to promote adhesion in ventral neuroblast migration in the absence of PLX-2, PLX-2 might modulate or up-regulate EFN-4 signaling such that it promotes repulsion and not adhesion.

An important question is whether the apparent redundancy of PLX-2 in MAB-20 signaling reflects redundancy of PLX-2 with other pathways in the same cells, or redundant
roles of PLX-2 expressing and non-expressing cells. Assuming EFN-4 is a component of the redundant pathway, this may be addressed by comparing expression patterns of EFN-4 and PLX-2. EFN-4 and PLX-2 appear to be coexpressed in posterior epidermal cells in the late embryo, a context in which they have redundant roles in cell sorting, suggesting that EFN-4 and PLX-2 may act in the same cells to transduce a MAB-20 repellent or sorting signal. In other situations, such as ventral neuroblast migrations, EFN-4 appears to act largely independent of PLX-2, and is not coexpressed with PLX-2. In ray development EFN-4 and PLX-2 are coexpressed in some ray cell groups but not coexpressed in others. In conclusion, we favor the model that MAB-20 interacts with distinct receptor complexes in different processes, and that the composition of the receptor complex determines the effect of MAB-20 signaling on cell behavior. The complexity of the MAB-20 pathway and the involvement of ephrin signaling may have implications for other class 2 semaphorin pathways, and possibly for other vertebrate semaphorins for which the receptors have not been fully elucidated.
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Figure Legends

Figure 1  Structure of the plx-2 gene and PLX-2 protein

(A) Genomic structure of the plx-2 gene, location of deletions, and structure of the translational fusion construct (PLX-2::EGFP) used in the expression analysis. (B) Structures of wild-type and mutant plexin molecules (ΔMRS-GP, Δsema, Δect) used in this study. The sema domain (sema), MRS, GP and intracellular (IC) regions are shown below. (C) A phylogenetic tree of C. elegans (PLX-1, PLX-2), Drosophila [Plex A (DmA), Plex B (DmB)] and vertebrate plexins. The entire sequences of plexins were compared using the phylogeny analysis program PROPARS, as implemented in Joe Felsenstein’s program PHYLIP 3.6 (http://evolution.genetics.washington.edu/phylip.html). The vertebrate plexin sequences used are from humans except plexin A4 (mouse).

Figure 2  C. elegans semaphorin 2A/MAB-20 specifically binds PLX-2

(A-C) PLX-2 bound to Ce-Sema-2a but not to Ce-Sema-1a. HEK293T cells expressing PLX-2 were reacted with Ce-Sema-2a-Fc (B) and Ce-Sema-1a-ΔC-Fc-AP (C). (D-I) PLX-2ΔMRS-GP bound to Ce-Sema-2a. HEK293T cells expressing PLX-2 (G), PLX-
2ΔMRS-GP (H), and HEK293T cells transfected with the vector pCAGGS (I) were reacted with Ce-Sema-2a-Fc.  (J-S) Both the sema domain and the region containing MRS-GP were required for PLX-1 to bind to Ce-Sema-1a-ΔC-Fc-AP.  HEK293T cells expressing PLX-1(O), PLX-1ΔMRS-GP (P), PLX-1Δect (Q), and PLX-1vsema (R), and HEK293T cells transfected with pCAGGS (S) were reacted with Ce-Sema-1a-ΔC-Fc-AP.  The wild-type and mutant plexins were myc-tagged, and their expressions on the cell-surface were confirmed by reacting intact transfected cells with an anti-myc antibody.  HEK cells expressing PLX-2 (A, D), PLX-2ΔMRS-GP (E), PLX-1 (J), PLX-1ΔMRS-GP (K), PLX-1Δect (L), and PLX-1vsema (M), and HEK cells transfected with pCAGGS (F, N) were reacted with the anti-myc-antibody.  (T, U) Expression of full-length and mutant plexins in 293 cells.  (T) We detected immunoreactive bands of 220 kDa and 192 kDa for myc-PLX-2 (lane 1) and myc-PLX-1 (lane 2), respectively.  (U) Expression of PLX-1ΔMRS-GP, PLX-1Δsema, PLX-1Δect, and PLX-2ΔMRS-GP (lanes 1-4); we detected bands corresponding to the predicted sizes of 157 kDa, 163 kDa, 96 kDa and 133 kDa with anti-myc staining.  (V-X) HEK293 cells expressing EFN-4 were stained with an anti-EFN-4 antibody (V), Ce-Sema-2a-Fc (W), and Ce-Sema-1a-ΔC-Fc-AP (X).  Neither MAB-20 nor SMP-1 binds to EFN-4 directly.
Figure 3 Penetrance of embryonic and larval lethality and adult epidermal phenotypes in \textit{plx-2}, \textit{mab-20} and \textit{efn-4} mutant combinations

(A) Penetrance of embryonic lethality in \textit{plx-2}, \textit{mab-20} and \textit{efn-4} single mutants (white bars), \textit{mab-20; plx-2}, \textit{plx-2; efn-4}, \textit{mab-20; efn-4} double mutants (gray bars), and \textit{mab-20; plx-2; efn-4} triple mutants (black bars). Error bars indicate the SEM. \textit{plx-2} mutants rarely exhibit embryonic lethality. The \textit{plx-2} mutations significantly enhanced the penetrance of the embryonic lethality of the \textit{mab-20} weak alleles (wk), \textit{bx24} and \textit{bx61}, and the \textit{efn-4} null allele, \textit{bx80}, but not that of the \textit{mab-20} null (0) allele, \textit{ev574}. \textit{bx80} also enhanced the penetrance of embryonic lethality of the \textit{mab-20} weak alleles, but suppressed that of the \textit{mab-20} null allele slightly. Differences in penetrance were compared using ANOVA and are shown only for the relevant pair being compared; *, \textit{P}<0.05; **, \textit{P}<0.001.

(B) Penetrance of larval lethality in \textit{plx-2}, \textit{mab-20} and \textit{efn-4} mutant combinations. \textit{plx-2} mutants do not exhibit larval lethality. \textit{plx-2 (tm729)} enhances the larval lethality of \textit{efn-4(bx80)} null mutants, but not that of the \textit{mab-20(ev574)} null allele. \textit{efn-4(bx80)} does not significantly enhance the larval lethality of \textit{mab-20(ev574)}. The numbers of animals examined are shown in parentheses below bars.
Figure 4   PLX-2 plays a minor role in ventral neuroblast migration

(A) DIC micrographs of *C. elegans* embryogenesis. Gastrulation commences approximately 100 minutes after the first cell division with ingress of the Ea and Ep gut precursors (arrowheads). At 200 minutes, a transient cleft is formed on the ventral surface of the embryo (red dashed line) caused by ingress of mesodermal cells. The cleft is closed by short range lateral movements of ventral neuroblasts (VNBs) which generate a substrate for epidermis in enclosure. *plx-2* mutants show a small but significant delay in cleft closure. *mab-20* mutants show some disorganization during gastrulation which leads to an apparent delay in cleft opening (blue dotted line illustrates D grand daughters remaining on the ventral surface of the embryo) and a significant (*P* < 0.01) delay in cleft closure. *efn-4* mutants show similar but stronger defects, with D grand daughter ingression delayed by around 25 minutes. Closure of the cleft is delayed further still, with ~10% of embryos failing to close the cleft at ventral enclosure, resulting in embryonic rupture and death. *mab-20; plx-2* double mutants are similar to *efn-4* single mutants, whereas *mab-20; plx-2; efn-4* triple mutants show delayed cleft opening, but earlier cleft closure. *mab-20; efn-4* doubles resemble the triple mutant in phenotype (not shown).
Variably penetrant morphological defects in the tail are apparent in mab-20 and efn-4 mutant combinations at the 4-fold stage (arrows).

(B) Gastrulation cleft duration in mab-20, plx-2 and efn-4 strains. Each single mutant shows significantly longer cleft duration compared to wild type (P < 0.05 for plx-2 and < 0.01 for mab-20 and efn-4). Double and triple mutants are not significantly different from the strongest single mutant strain.

Figure 5. mab-20 late embryonic epidermal rupture is recapitulated in plx-2; efn-4 double mutants

(A) Late embryonic rupture phenotype (class III arrest) characteristic of mab-20 embryos. mab-20 embryos typically enclose the epidermis at the normal time, then undergo aberrant epidermal elongation in which the posterior ventral epidermis is bulged and deformed (arrow, top panel), and eventually rupture in the ventral preanal region ~4 hours after enclosure (arrow points to extruding cells, lower panel). Panels from 4D movie of mab-20(ev574). (B) Similar late embryonic rupture in the ventral posterior epidermis is seen in plx-2(tm729); efn-4(bx80) double mutants but not in the single mutants. (C) Quantitation of embryonic arrest classes. We quantified embryonic terminal phenotypes in
embryos followed at 2 h intervals using DIC microscopy (n > 50 for each genotype).

Embryonic arrest stages were classified as follows: 1, arrest at epidermal enclosure due to failure to enclose epidermis (corresponding to 4D classes I and II); 2, rupture at 2-3 fold stage of elongation; 3, rupture at 3-4 fold stage (shown in panels A and B); 4, development to hatched L1. Type 1 arrest (enclosure) is frequent in efn-4, and rare in plx-2 and mab-20. Type 3 arrest is rare in plx-2 or efn-4 single mutants but is common in efn-4; plx-2 double mutants and is the predominant mode of embryonic arrest in mab-20 single mutants.

Figure 6. Expression of PLX-2 and EFN-4 in embryonic neuronal and epidermal cells

PLX-2 and EFN-4 GFP transgene expression was detected by immunostaining with anti-GFP antibodies (green in all panels); samples are labeled with the anti-AJM-1 antibody MH27 (red) to visualize epidermal cell outlines. Panels A and B are stained with DAPI (blue) to show cell nuclei. (A) A PLX-2::GFP translational fusion protein is first expressed in a small number of ventral cells prior to epidermal enclosure. (B) In late stage embryos PLX-2::GFP was expressed in several tail epidermal cells including the preanal ventral epidermal cell pairs P11/12 and P9/10 (indicated as P with white lines).
2::GFP was also expressed in a large number of neuronal cells in the head and tail.  (C, D)

Expression of PLX-2 in the QV5 lateral epidermal cell at 1.5- and 2-fold stage, detected using the Pplx-2-GFP transcriptional reporter nclIs21; images are projections of surface focal planes from a confocal z-stack.  (E-H)  EFN-4::GFP (juIs109) is expressed in a subset of epidermal cells during and after epidermal enclosure.  During enclosure (lateral views in E,F; ventral view in G) EFN-4::GFP was detected in the lateral epidermal cells H0 and QV5, in the leading anterior epidermal cells (green ‘h’) in the head region, and in the three posterior pairs of P cells (P7/8, 9/10, 11/12) (green P cells marked in AJM-1 channel, panel F).  (H) At the two-fold stage EFN-4::GFP is seen in P9/10 and P11/12 (P, ventral view of preanal epidermis); expression also persists in head ventral epidermis.

EFN-4::GFP expression in epidermal cells persists until late embryonic stages and was not detectable in L1 larvae.  Scale: 10 µm in all panels except B (20 µm).

**Figure 7.  PLX-2 and EFN-4 promote MAB-20 function in male tail ray development**

(A-C) Ventral views of adult male tails.  All genotypes contain him-5.  Anterior is to the left.  (A) A plx-2(nc7) tail has wild-type morphology, and 9 rays can be distinguished on each side.  (B) In mab-20(bx24), rays 1 and 2 on both sides (arrowheads), and rays 3 and
4 of the left side (an asterisk) are fused. (C) In a \textit{mab-20(bx24); plx-2(nc7)} tail, rays 1 and 2 (an arrowhead), and rays 3 and 4 (a small asterisk) on the right side and rays 2, 3, 4 and 6 on the left side (a large asterisks) are fused. (D) The epidermal cells, R8.p and R9.p (arrows) express GFP in a third larval-stage tail of a male carrying the partial PLX-2::GFP translational fusion. (F) Tail seam cells (SET) express GFP (arrow) in the early fourth larval-stage male tail. The corresponding DIC images are shown in E and G, respectively. Scale, 10 \(\mu\)m. (H-M) Lateral view of the larval male tail to visualize epidermal cells with AJM-1::GFP. Anterior is to the left. (H) Wild-type epidermal cells and (I) a schematic drawing to show Rn.ps and ray precursor clusters 1, 2, 3, 4, 6, and 8 in colors. (J) In a \textit{bx61} larva, ray precursor clusters 2 and 3 (an arrowhead), and 4 and 6 (an arrow) aggregate. In animals with \textit{mab-20} and/or \textit{efn-4} mutations, in addition to the arrangement defects of ray precursor clusters, the morphology of Rn.ps is often abnormal. (K) R4.p (asterisk) adopted a triangular shape (class II) rather than a wild-type rectangular shape. (L) R1/2.p (asterisk) is larger or irregular compared with wild-type (class III). (M) The morphology of all Rn.ps is severely affected (class V). (N) Aggregation of ray precursor clusters 1 and 2, 2 and 3, 3 and 4, and 4 and 6 was scored. \textit{plx-2(tm729)} enhances the aggregation in weak \textit{mab-20} alleles, whereas no aggregation is detected in any ray precursor cluster of
*plx-2*(tm729) mutants (n = 50).  (O) Frequencies of Rn.p morphological classes in *plx-2*, *mab-20* and *efn-4* mutant combinations.  The numbers of animals examined are shown in parentheses.

**Figure 8. Model for function of PLX-2 and EFN-4 in MAB-20 signaling**

(A)  In early embryonic movements of ventral neuroblasts MAB-20 and EFN-4 may act in a common pathway that could promote adhesion between neighboring neuroblasts or between neuroblasts and unknown substrate cells.  (B)  In later embryonic development MAB-20 prevents ectopic cell contacts between epidermal cells; EFN-4 and PLX-2 have redundant functions in this process.  EFN-4 may also play an antagonistic role as the frequency of late embryonic arrest is reduced in *mab-20 efn-4* double mutants relative to *mab-20* single mutants.  (C)  In postembryonic development of male tail rays, MAB-20 and EFN-4 play non-redundant roles in preventing inappropriate adhesion of ray cells; PLX-2 plays a subliminal role that is only revealed in *mab-20* hypomorphic backgrounds.
Table 1. Male tail ray fusions in *plx-2, mab-20* and *efn-4* mutant combinations

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<th>ray 2</th>
<th>ray 3</th>
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We scored the percentage of male tail sides in which the indicated rays had fused, and the percentage of sides containing at least 3 or at least 4 rays within a single fused group. All strains contained him-5(e1490) and were maintained at 20°C. The mab-20 RNAi experiment is in the rrf-3 genetic background. We did not score ray fusions involving rays 7-9. n = number of sides. 1. Loss of plx-2 function by mutation or by soaking RNAi does not cause significant ray fusion defects. 2. plx-2 mutations enhance weak ray fusion defects of mab-20 hypomorphic alleles. bx61 at the semipermissive temperature of 20°C causes partially penetrant fusions of rays 3 and 4 that are significantly enhanced in plx-2 mutant backgrounds. bx24 causes partially penetrant fusions of ray 6 that are enhanced in plx-2 double mutants. Percentages in bold face are significantly different from the matched plx-2(+) control (P < 0.01, Fisher exact test). 3. plx-2 does not enhance mab-20(ev574). For ray 6 fusion plx-2 may slightly suppress ev574 phenotypes. 4 plx-2 enhances efn-4(bx80) for ray 6 fusion and fusion groups. 5. efn-4 does not enhance mab-20. Penetrance of ray fusions in double and triple mutants is not enhanced beyond that of the strongest single mutant.
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Figure 1

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Nakao et al. (2023). Fig. 3
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Figure 4
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Figure 5
Figure 6
A. Ventral neuroblast migrations

MAB-20 → EFN-4 cell adhesion?

B. Embryonic epidermal cell contacts

MAB-20

EFN-4

PLX-2

P cell contacts

C. Male tail ray development

MAB-20

EFN-4

PLX-2

adhesion of ray precursors

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Figure 8