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A *C. elegans* Myc-like network cooperates with semaphorin and Wnt signaling pathways to control cell migration

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Abstract

Myc and Mondo proteins are key regulators of cell growth, proliferation, and energy metabolism, yet often overlooked is their vital role in cell migration. Complex networks of protein-protein and protein-DNA interactions control the transcriptional activity of Myc and MondoA confounding their functional analysis in higher eukaryotes. Here we report the identification of the transcriptional activation arm of a simplified Myc-like network in *C. elegans*. This network comprises an Mlx ortholog, named MXL-2 for Max-like 2, and a protein that has sequence features of both Myc and Mondo proteins, named MML-1 for Myc and Mondo-like 1. MML-1/MXL-2 complexes have a primary function in regulating migration of the ray 1 precursor cells in the male tail. MML-1/MXL-2 complexes control expression of ECM components in the non-migratory epidermis, which we propose contributes to the substratum required for migration of the neighboring ray 1 precursor cells. Furthermore, we show that promigratory Wnt/β-catenin and semaphorin signaling pathways interact genetically with MML-1/MXL-2 to determine ray 1 position. This first functional analysis of the Myc superfamily in *C. elegans* suggests that MondoA and Myc may have more predominant roles in cell migration than previously appreciated, and their cooperation with other pro-migratory pathways provides a more integrated view of their role in cell migration.

Keywords

Myc; Mondo; semaphorin; plexin; Wnt; β-catenin; cell migration; ECM

Introduction

Cell migration is a complex process involving extracellular signaling molecules and their respective intracellular pathways, remodeling of the actin cytoskeleton, modulation of integrin signaling, and the ever-changing composition of the extracellular matrix (ECM) (Yamaguchi et al., 2005). Not surprisingly, misregulation of different cell migration pathways, often at the level of transcription, drives angiogenesis, invasion, and metastasis, which are characteristics of high-grade aggressive tumors (Hanahan and Weinberg, 2000). Understanding the transcriptional networks that control cell migration will provide important insight into the molecular mechanisms that regulate this critical cell process in both physiological and pathological settings.

Myc proteins are basic helix-loop-helix leucine zipper (bHLHZip) proteins, potent oncogenes, and are misregulated in approximately 15% of human malignancies (Dang, 1999). Myc proteins are DNA-binding transcription factors that require dimerization with another

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bHLHZip protein called Max to bind CACGTG E-boxes in the regulatory regions of their targets (Cole and Nikiforov, 2006). Studies in vertebrate and invertebrate systems indicate that Myc/Max complexes regulate a large number of target genes representing multiple functional classes (Fernandez et al., 2003; Lee and Dang, 2006; Orian et al., 2003). For example, Myc/Max complexes regulate multiple genes that drive cell growth and proliferation, which likely accounts for their predominant functions in these processes. Myc/Max complexes also regulate targets involved in the angiogenic switch, tumor invasion, and metastasis, suggesting these proteins have additional vital roles in cell migration (Coller et al., 2000; Frye et al., 2003; Watnick et al., 2003; Zhang et al., 2005). Genes regulated by Myc/Max complexes are involved in cell adhesion and migration including lymphocyte function-associated antigen 1 (LFA-1) (Inghirami et al., 1990), matrix metalloproteinase 2 (MMP-2) (Noujaim et al., 2002), metastasis-associated protein 1 (MTA1) (Zhang et al., 2005), thrombospondin 1 (Watnick et al., 2003), and many others (Coller et al., 2000; Frye et al., 2003). This collection of targets suggests that Myc/Max complexes regulate cell migration by a variety of mechanisms.

In addition to Myc, Max interacts with multiple bHLHZip transcriptional repressors, e.g. the Mad family and Mnt (Rottmann and Luscher, 2006). As such, Max is the center of a transcription network that has transcriptional activation and repression arms (Fig. 1A). We discovered an analogous transcription factor network with the Max-like protein Mlx at its center (Billin et al., 1999). Interactions between Mlx and Mad1 or Mad4 comprise the repression arm of the Mlx network, yet Mlx does not interact with Myc family members. Rather, interactions with the Mondo family comprise an activation arm of the Mlx network (Billin et al., 2000). The two Mondo proteins, MondoA and MondoB/WBSCR14/ChREBP, are also members of the bHLHZip family, but at nearly 1000 amino acids are among the largest members in this class of transcription factors (Billin et al., 2000; de Luis et al., 2000; Yamashita et al., 2001). Compared to the Myc family, the greater size of Mondo proteins can be attributed to five blocks of conserved sequence in their N-termini, known as the Mondo Conserved Regions (MCRs) and to a conserved block in their C-termini located just after their leucine zippers called the dimerization and cytoplasmic localization domain (DCD) (Eilers et al., 2002). The MCRs and DCD are highly conserved across species and are defining sequence features of the Mondo family. Current studies indicate that the MCRs have at least two functions: regulation of subcellular localization and regulation of a glucose-dependent transcription domain (C.W. Peterson, C.A. Stoltzman, and D.E.A., unpublished) (Eilers et al., 2002; Li et al., 2006). The DCD has only been studied in MondoA where it has a critical role in dictating subcellular localization (Eilers et al., 2002).

By several criteria, MondoA/Mlx complexes function analogously to Myc/Max complexes. MondoA and Mlx dimerize via their bHLHZip motifs, bind CACGTG E-boxes (Billin et al., 2000), and activate expression of a transcriptome that is largely overlapping with that regulated by Myc/Max (Sans et al., 2006). We observed synthetic genetic interactions between hypomorphic alleles of *D. melanogaster* Myc and MondoA orthologs (Billin and Ayer, 2006), consistent with Myc and MondoA having overlapping functions.

Our data demonstrate that MondoA/Mlx complexes are highly regulated members of the Myc-like family of bHLHZip proteins, and like the other members of this family, may have broad functions in controlling cell physiology. MondoA/Mlx complexes localize to the outer mitochondrial membrane (Sans et al., 2006), and they shuttle between the mitochondria and the nucleus suggesting that they facilitate communication between these two essential organelles. One proximal nuclear function of MondoA/Mlx complexes is the direct regulation of glycolytic target genes and consequently glycolysis (Sans et al., 2006). Therefore, we have hypothesized that one primary role of MondoA/Mlx complexes is the maintenance of cellular bioenergetics. Our gene profiling experiments, however, suggest that MondoA/Mlx complexes likely have pleiotropic roles, including one in facilitating cell migration. For example, MondoA

regulates a number of genes whose products are found in the ECM including collagen type IV, thrombospondin 2, and fibulin 2 (Sans et al., 2006).

Several issues confound study of the Myc superfamily in higher eukaryotes. There is functional redundancy between the paralogous family members, i.e. there are three Myc proteins, two Mondo proteins, four Mad proteins and the functionally related repressor Mnt (Billin and Ayer, 2006; Cole and Nikiforov, 2006; Rottmann and Luscher, 2006). Furthermore, Myc/Max and MondoA/Mlx complexes appear to function in overlapping pathways (Billin and Ayer, 2006; Sans et al., 2006). Finally, it seems likely that there is significant crosstalk between the Max- and Mlx-centered networks. Thus, the activity of the Myc superfamily and its ultimate phenotypic output is dictated by large interwoven networks of transcription factors with overlapping physical interactions among family members (Fig. 1A).

To overcome the barriers to uncovering the essential functions of the Myc superfamily in higher eukaryotes, we have begun to study a much simpler Myc-like network in the nematode *C. elegans*. Orthologs of Max and Mad, called MXL-1 and MDL-1, respectively, were previously identified in *C. elegans* and appear to constitute the transcriptional repression arm of the worm Myc-like network (Yuan et al., 1998). Here we report the identification and functional characterization of the transcriptional activation arm of the *C. elegans* Myc-like network and its primary role regulating migration of ray 1 precursor cells in the male tail. A Myc and Mondo-like factor, MML-1, and an Mlx-like factor, MXL-2 dimerize, activate transcription, and are expressed in tissues surrounding the migrating cells. MML-1/MXL-2 dimers regulate a number of ECM genes, mostly collagens and C-type lectins, some of which are required for migration of ray 1 precursor cells. Furthermore, MML-1/MXL-2 act coordinately with Wnt and semaphorin signaling to regulate cell migration.

Materials & Methods

C. elegans Strains & Manipulation

Strains were maintained as described (Brenner, 1974) and analyzed at 20°C unless specified. Bristol N2 was the wild type strain. The mutant alleles used were: LG3—*pha-1(e2123ts)* (Schnabel, 1990); LG4—*him-8(e1489)* (Hodgkin, 1979), jcIs1[*rol-6(su1006); ajm-1::GFP*] (Mohler et al., 1998), *plx-1(nc37)* (Fujii et al., 2002); LGX—*bar-1(ga80)* (Eisenmann et al., 1998), *lin-15(n765ts)* (Ferguson and Horvitz, 1989). *mxl-2(tm1516)* and *mxl-1(tm1530)* were obtained from the International *C. elegans* Gene Knockout Consortium at NBP-Japan, and worms were outcrossed at least six times prior to use. L4 hermaphrodites were heat shocked to generate males. *bar-1(ga80) X* males were generated by crossing N2 males with *bar-1(ga80) X* hermaphrodites.

RNAi clones from the Ahringer collection were grown and spotted on NGM plates with 1 mM IPTG and 50 μg/ml ampicillin (Kamath et al., 2003). Two days later, mated hermaphrodites were placed on RNAi plates at 24°C and progeny were observed 2–3 days later. The *pop-1* RNAi construct was generated by amplifying residues 1-1314 (*pop-1*) from cDNA and cloning into the *Eco*RI sites of pL4440. Bacteria carrying a *pop-1* RNAi construct were diluted with bacteria carrying empty pL4440 at a 1:4 ratio to overcome the embryonic lethality associated with *pop-1(RNAi)*.

We scored ray 1 position and migration defects by mounting males on 4% agar pads and anesthetizing in 30 mM sodium azide. Nomarski and fluorescence microscopy were conducted on a Zeiss Axioskop 2 MOT platform. We classified ray 1 displacement defects as previously described (Fujii et al., 2002). Briefly, ray 1 is anteriorly displaced and outside (severe; class I) or within (mild; class II) the fan containing the other rays. Only tails with observable rays 1–6 were assayed for ray 1 position. Error bars for ray 1 position represent the standard deviation

calculated assuming a binomial distribution given the observed percentage of tail defects and the sample size. A two-tailed t-test was used to compare the total affected tails (class I + class II) to determine the probability that two proportions were equivalent. Relevant comparisons and p values are listed in each figure.

Extracts were prepared by washing worms off two 3.5 cm plates, resuspending them in 100 μl 2X SDS loading buffer, and boiling for 15 min. Extracts were separated by SDS-PAGE, and MML-1 was detected by Western blot using α-MML-1 566 (1:500) followed by α-rabbit HRP (1:5000). *C. elegans* ACT-1 was recognized by α-β-actin (1:5000) (AbCam) followed by α-mouse HRP (1:5000) (Amersham).

Transgenic Animals

We cloned the *mml-1*/Mondo and *mxl-2*/Mlx loci by amplifying the region between the respective flanking genes. GFP was inserted in frame into an *Xho*I site (*mml-1*) or a *Sal*I site (*mxl-2*) in the final exon of each gene. Tissue specific rescue experiments utilized either ~2 kb of the *cdh-3* promoter (Pettitt et al., 1996) directly upstream of the initiating ATG or residues 122–282 of the *dpy-7* promoter (Gilleard et al., 1997) cloned into the *Hind*III/*Bam*HI sites of pPD49.83. All cDNAs were amplified from a mixed stage library. Primer sequences are available upon request.

lin-15(n765ts) worms were microinjected with 1 ng/μl of genomic *mml-1::gfp* or genomic *mxl-2::gfp*, 40 ng/μl pECK15/*lin-15+* and 60 ng/μl herring sperm DNA (Mello et al., 1991). Worms were screened at 24°C, and non-Muv transgenic lines were isolated. *mxl-2(tm1516) pha-1(e2123ts)* III; *him-8(e1489)* IV worms were injected with 30 ng *pha-1*$^{+}$, 10 ng *myo-3::dsred2*, and 60 ng of the appropriate *mxl-2* or *bar-1* construct (Miller et al., 1983). Transgenic animals were selected and maintained at 24°C (Schnabel, 1990). Transgenic males were analyzed directly or crossed into the *bar-1(ga80)* background. Male cross-progeny expressing DsRed2 were scored.

Quantitative RT-PCR and Microarray Analysis

We compared a population of mixed-stage wild type males and hermaphrodites to a similar population of *mxl-2(tm1516)* worms. Total RNA was isolated from all strains by phenol/chloroform extraction, purified with an RNeasy kit with DNaseI treatment (Qiagen), and then reverse transcribed (SuperScript III-Invitrogen). Levels of cDNAs from at least two independent preparations were obtained by quantitative PCR per the manufacturer's instructions (iCycler-BioRad). Levels of all transcripts were normalized to *act-1* prior to comparison. Fold change was determined by dividing the gene/*act-1* ratio of one strain by the same ratio from N2 worms. Error bars are the standard deviation in the fold change from 3–4 independent qPCR experiments.

For microarray analysis, total RNA was prepared as above and reverse transcribed. The resulting cDNA was used to probe *C. elegans* 22K gene arrays (Agilent). Data was analyzed with the GeneSifter software (vizX Labs) using a fold-change cutoff of 1.5.

Physical Interaction and Transcription Assays

pVP16 and pBTM116 were used to make VP16 activation domain and LexA DNA-binding-domain fusion constructs, respectively (Bhoite et al., 2001; Hollenberg et al., 1995). Plasmids were transformed into L40 yeast, and selected on YP -Trp -Leu. LacZ activity was measured by a β-galactosidase filter assay (Ausubel, 1995).

For transcription assays, 293T cells were transfected in triplicate with 200 ng M4-Luciferase (E-box assays) (Billin et al., 1999) or G4-14D Luciferase (Gal4 assays) (Ayer et al., 1996;

Sterneck et al., 1992), 25 ng CMV β-Gal, 1 μg of each expression construct, and carrier to 2 μg DNA per dish using calcium phosphate (Ausubel, 1995). Luciferase assays were conducted as previously reported (Fleischer et al., 2003).

Antibodies

MML-1 (aa566-760) or full-length MXL-2 was expressed as His-tagged fusions in BL21 *E. coli* (Stratagene). Cultures were induced with 1 mM IPTG and proteins purified using Ni-NTA resin as per the manufacturer's protocol (Qiagen). Proteins were dialyzed against PBS and used for immunizing rabbits (Covance Research Products).

Results

Identification of the Myc-like bHLHZip family in *C. elegans*

Database searches identified four *C. elegans* orthologs of the mammalian Myc-like bHLHZip family. R03E9.1 (*mdl-1*/Mad) and T19B10.11 (*mxl-1*/Max) were previously characterized (Yuan et al., 1998). F40G9.11 and T20B12.6 were related, but uncharacterized, bHLHZip proteins. F40G9.11 is 25% identical to Mlx, but is also similar to Max and MXL-1, and we named it *Max-like* 2, or *mxl-2*. A region upstream of and encompassing the bHLHZip domain of T20B12.6 is 26% identical to roughly the C-terminal half of human c-Myc; however, T20B12.6 does not contain easily identifiable Myc signature sequences such as Myc Box II in its N-terminus. T20B12.6 is also 25% identical to MondoA (Billin et al., 2000) and is the only *C. elegans* protein similar to MondoA across the entire protein. Therefore, we named T20B12.6 *M*yc and *M*ondo-*l*ike 1 or *mml-1*. MML-1/Myc & Mondo (hereafter MML-1/Mondo) has a highly conserved N-terminus corresponding to the Mondo Conserved Regions (MCRs; Fig. 1B), a central non-conserved region, and a C-terminal bHLHZip motif that has residues required for heterodimerization. Furthermore, the residues that dictate binding of the various heterodimer pairs of the Myc-superfamily to CACGTG E-boxes are absolutely conserved in MML-1 and MXL-2 (Fig. 1C, Supplemental Fig. 1) (Ferre-D'Amare et al., 1993; Nair and Burley, 2003). MML-1/Mondo is unique among Mondo family members as it harbors two nuclear localization signals (Fig. 1B) and lacks the C-terminal dimerization and cytoplasmic localization domain (DCD) (data not shown).

For MML-1, MDL-1, MXL-1, and MXL-2 to be functionally similar to the mammalian Myc-like family, they must dimerize and regulate transcription. To determine their dimerization properties, we conducted yeast two-hybrid assays. MML-1/Mondo interacted with MXL-2/Mlx but not MXL-1/Max (Fig. 2A). By contrast, MDL-1/Mad interacted with MXL-1/Max but not MXL-2/Mlx. MML-1/Mondo and MDL-1/Mad did not dimerize with one another nor did they homodimerize. To determine the transcriptional properties of the MML-1/MXL-2 complex, we assayed its activity on a CACGTG E-box-responsive luciferase reporter (Billin et al., 1999). The combination of MML-1/Mondo and MXL-2/Mlx activated transcription of the reporter in a dose-dependent fashion yet failed to activate transcription from a promoter lacking CACGTG E-boxes (Fig. 2B) despite robust expression of each protein (data not shown). MML-1/Mondo alone activated transcription, albeit not to the level of MML-1/MXL-2. As MML-1/Mondo does not homodimerize, this activation may be due to interactions with endogenous Mlx or other bHLHZip proteins. To determine the intrinsic transcriptional capacity of each protein, we fused them individually to the Gal4 DNA-binding domain and measured their activity on a Gal 4-responsive luciferase reporter. Gal4-MML-1 activated transcription ~130 fold over Gal4 alone (Fig. 2C), whereas Gal4-MDL-1 and Gal4-MXL-1 repressed transcription (Fig. 2D). Gal4-MXL-2 was inert in this assay. Therefore, MML-1/Mondo and MXL-2/Mlx heterodimerize and activate transcription forming the activation arm of a *C. elegans* version of the mammalian Myc-like bHLHZip network. These proteins do not interact with MDL-1/Mad or MXL-1/Max, the repressive arm of this network, suggesting the

C. elegans Myc-like bHLHZip network is comprised of two physically distinct dimeric complexes (Fig. 1A).

We next characterized the *mxl-2(tm1516)* allele. The *tm1516* deletion eliminates exons 2 and 3 of *mxl-2*, it introduces a premature termination codon after 64 residues (Fig. 3A), it does not retain any known functional domains, and the *tm1516* protein product was not detected by Western blot (data not shown). The phenotype of *mxl-2(tm1516)* homozygous worms (described below) was not observed in heterozygous worms suggesting this is a recessive allele (Fig. 3C; Table 1). Furthermore, RNA interference of *mxl-2* in wild type worms phenocopied *mxl-2(tm1516)* whereas *mxl-2(RNAi)* in *mxl-2(tm1516)* worms did not enhance the phenotype (data not shown). These data suggest that *mxl-2(tm1516)* is a recessive strong loss-of-function, possibly null allele.

To determine the effect of MXL-2/Mlx loss on MML-1/Mondo, we determined MML-1/Mondo levels by Western blot. MML-1/Mondo was undetectable in *mxl-2(tm1516)* worms (Fig. 3B), but expression was rescued in *mxl-2(tm1516)* worms expressing MXL-2::GFP (data not shown). *mml-1*/Mondo mRNA levels were not altered in *mxl-2(tm1516)* worms (data not shown) nor were protein levels affected in worms with a deletion of *mxl-1* (Fig. 3B). Therefore, MML-1/Mondo stability depends on MXL-2/Mlx suggesting that MML-1/Mondo and MXL-2/Mlx function as a complex in vivo. Given our extensive analysis showing MondoA and Mlx are obligate partners in DNA binding and transcription activation in mammalian cells, we conclude that the phenotype identified in *mxl-2(tm1516)* animals is primarily attributable to loss of the MML-1/MXL-2 heterocomplex.

MML-1/MXL-2 dimers regulate cell migration in the male tail

To understand the physiological role of MML-1/MXL-2 complexes, we examined *mxl-2 (tm1516)* worms. The organs and structures of males and hermaphrodites appeared wild type with one exception—the tails of male *mxl-2(tm1516)* worms exhibited significant anterior displacement of the first sensory ray (18%, $p<0.01$; Fig 3C, Fig. 4A–C). The adult male tail is a cuticular, spade-shaped fan that houses nine bilaterally symmetric sensory rays (Fig. 4A) (Emmons, 2005). The cells that generate the rays are derived from three lateral seam cells—V5 generates ray 1 while V6 and T generate rays 2–9. Properly differentiated rays open to the environment on specific surfaces of the fan, and the position of the rays is essential for their function in sensing and responding to hermaphrodites (Emmons, 2005). There are two classes of ray 1 anterior displacement defects —the severe class I defect where ray 1 is outside of the fan (Fig. 4B) and the milder class II defect where ray 1 is anteriorly displaced but still within the fan (Fig. 4C) (Fujii et al., 2002). The 18% of *mxl-2(tm1516)* males that exhibited an anteriorly displaced ray 1 were evenly split between class I and II (Fig. 3C, Table 1). The displaced ray 1 in *mxl-2(tm1516)* males contained the appropriate number of cells and opened dorsally, identical to wild type ray 1 (Fig. 4A–C). In addition to ray 1, the V5 seam cell also gives rise to the postdeirid neurons and alae producing cells. These cells were present in their wild type positions in *mxl-2(tm1516)* males (data not shown) suggesting that MML-1/MXL-2 dimers do not regulate the cell fate specification of any V5-derived structures. Finally, the spicules, fan, and rays 2–9 were normal in *mxl-2(tm1516)* worms (Fig. 4A–C; data not shown); therefore, MML-1/MXL-2 complexes appear to act exclusively in ray 1 positioning.

Each ray is composed of a three-cell cluster composed of two neurons, termed RnA and RnB, and a structural cell, Rnst, where “n” denotes the number of the ray. During ray development, only ray 1 precursor cells migrate posteriorly. The three ray 1 precursor cells migrate as a cluster, and the final position of this ray is determined by the attachment point between R1st and the epidermis (Baird et al., 1991). To determine if MML-1/MXL-2 plays a role in migration of the ray 1 precursor cluster, we analyzed worms carrying the *ajm-1::gfp* reporter which outlines all ray precursor cells (Simske and Hardin, 2001). At an intermediate stage of tail

development in wild type males, R1st is positioned close to R2st (Fig. 4D). In *mxl-2(RNAi)* males, R1st was displaced anterior of its normal position and was not associated with R2st (Fig. 4E). Earlier in ray development, R1A, R1B, and R1st were present yet displaced anteriorly indicating the entire precursor cluster fails to migrate (data not shown). Unmigrated ray 1 precursors occurred at essentially the same frequency as anteriorly displaced rays (~16% vs ~18%, respectively); therefore, we conclude that failed precursor cell migration accounts for ray 1 displacement in *mxl-2(tm1516)* males. Furthermore, migration of gonadal distal tip cells and neurons and axon pathfinding were normal in *mxl-2(tm1516)* males and hermaphrodites (data not shown), suggesting the effects of MML-1/MXL-2 on cell migration is restricted to ray 1 precursor cells.

MML-1/Mondo and MXL-2/Mlx are expressed in the epidermis and the intestine

Cell migration is a multifaceted process requiring the reception of migratory cues and remodeling the actin cytoskeleton of the migrating cell, while surrounding cells give directional and positional information through proteins found in the extracellular space. To investigate whether MML-1/MXL-2 complexes function in ray 1 precursors or in the surrounding epidermis, we first determined their expression patterns using transgenic worms expressing *mml-1::gfp* or *mxl-2::gfp* translational fusions under the control of at least 1kb of their respective upstream sequence. MML-1::GFP was observed in epidermal cells as early as the 50–100 cell stage of embryogenesis and in intestinal cells at the 4E stage (Fig. 5A, B). Expression persisted in these two cell types through all larval stages and adulthood (Fig. 5C, D, E; data not shown). Consistent with MML-1/MXL-2 functioning as a heterodimeric complex, MXL-2::GFP was also expressed in epidermal and intestinal cells (data not shown). The expression pattern of a recently published *mxl-2::gfp* transcriptional fusion corroborates our translational fusion expression data (Deplancke et al., 2006). Consistent with their transcriptional function, MML-1::GFP and MXL-2::GFP were nuclear at all stages (Fig. 5).

MML-1::GFP and MXL-2::GFP were observed in the non-migratory, syncytial epidermis, but never in the ray generating lateral seam cells (Fig. 5E). These data support a model where MML-1/MXL-2 dimers act in the stationary, syncytial epidermis to facilitate migration of ray 1 precursor cells. Supporting this model, genomic *mxl-2::gfp* was only expressed in the syncytial epidermis and rescued the ray 1 defects of *mxl-2(tm1516)* males (5 of 6 lines rescue; Table 1). These data confirm that the ray 1 defects of *mxl-2(tm1516)* worms are due to loss of MML-1/MXL-2 activity and support a role for the heterodimer in the syncytial epidermis. Nevertheless, it is formally possible that MML-1/MXL-2 complexes act in the ray 1 precursor cells, but were expressed below the level of detection in this experiment. In fact, the modest nature of the rescue (from ~18% to ~10%; Table 1) may reflect that our *mxl-2* construct does not contain all of the promoter elements necessary to recapitulate expression of the endogenous protein.

To confirm the site of MML-1/MXL-2 action in ray 1 precursor cell migration, we performed tissue-specific rescue experiments using the *dpy-7* promoter (P*dpy-7*), which is active in the syncytial epidermis and not the seam, and the *cdh-3* promoter (P*cdh-3*), which is active in seam cells and their descendants, including ray precursor cells, but not the syncytial epidermis (Gilleard et al., 1997; Pettitt et al., 1996). P*dpy-7::mxl-2::gfp* rescued *mxl-2(tm1516)* ray 1 position defects (5 of 5 lines rescue) as did P*cdh-3::mxl-2::gfp* (2 of 2 lines rescue). These data confirm that MML-1/MXL-2 complexes can act in the surrounding, syncytial epidermis to facilitate ray 1 precursor cell migration. However, the P*cdh-3::mxl-2::gfp* rescue in the migratory ray 1 precursor cells suggests a model that MML-1/MXL-2 complexes regulate the expression of secreted proteins involved in migration, i.e. MML-1/MXL-2 activity in either migratory or non-migratory cells is functionally equivalent because both cell types border the same extracellular space.

Wnt/BAR-1 signaling regulates ray 1 precursor cell migration

The combined effects of extracellular proteins and receptor-mediated signaling pathways promote migration of nearly every cell type. One of the best-defined, broadly-acting, pro-migratory pathways is Wnt signaling. Wnt cascades have been implicated in cell migration and pathfinding in a number of organisms including *C. elegans* (Hilliard and Bargmann, 2006; Pan et al., 2006; Prasad and Clark, 2006). BAR-1/β-catenin is an effector of Wnt signaling, promotes a V5-like differentiation program in lateral seam cells, and is essential for Wnt-mediated cell migration of specific neurons (Korswagen, 2002; Maloof et al., 1999). Additionally, c-Myc is essential for β-catenin-induced cell proliferation and migration in mammals (Peifer, 2002), and this genetic interaction may be conserved in *C. elegans*. Therefore, we hypothesized Wnt signaling through BAR-1/β-catenin regulates ray 1 precursor cell migration in concert with MML-1/MXL-2 complexes.

We first determined whether Wnt signaling positioned ray 1. RNAi of the Wnt and Frizzled receptors, *cwn-2*/Wnt, *egl-20*/Wnt, *mig-1*/Fz, and *cfz-2*/Fz resulted in ray 1 displacement that did not reach statistical significance (Supplemental Fig. 2). However, as in other tissues, these factors may act cooperatively (Hilliard and Bargmann, 2006; Pan et al., 2006; Prasad and Clark, 2006). By contrast, roughly 40% of *bar-1(ga80)* males exhibited an anteriorly displaced ray 1 ($p<0.01$; Fig. 6A; Table 1). No other ray defects were observed and ray 1 was properly differentiated (data not shown). BAR-1/β-catenin typically interacts with POP-1/Lef to activate transcription (Korswagen et al., 2000). Consistent with this, RNAi of *pop-1*/*Lef* resulted in significant ray 1 displacement (46%, $p<0.01$; Supplemental Fig. 2). Together, these data suggest Wnt signaling contributes to ray 1 positioning via a canonical pathway that requires the transcriptional activity of BAR-1/POP-1 complexes.

To determine if BAR-1/β-catenin affects migration of the ray 1 precursor cells, we conducted *bar-1* RNAi in *ajm-1::gfp* worms. R1st was displaced anteriorly and was not associated with R2st (Fig. 6B), demonstrating BAR-1/β-catenin facilitates ray 1 precursor cell migration. BAR-1/β-catenin is expressed in many cell types including the lateral seam cells (Eisenmann et al., 1998;Natarajan et al., 2004). To determine which cells require BAR-1/β-catenin activity, we again utilized the seam-cell expressed *cdh-3* promoter and the epidermally expressed *dpy-7* promoter. P*cdh-3::bar-1*, but not P*dpy-7::bar-1*, rescued ray 1 defects in *bar-1(ga80)* males (2 of 2 and 0 of 4 lines rescue, respectively; Table 1). Therefore, Wnt signaling through BAR-1/POP-1 acts in seam-cell derived ray 1 precursors to regulate their migration.

Our data suggest that ray 1 precursor cell migration is controlled by MML-1/MXL-2 activity in the non-migratory epidermis and Wnt signaling in ray 1 precursor cells. To determine if these pathways interact genetically, we constructed *mxl-2(tm1516); bar-1(ga80)* double-mutant worms. Total anterior displacement of ray 1 was enhanced to ~80% in these worms, well above either single mutant, due exclusively to a dramatic increase in the severe class I defects (Fig. 6A; Table 1). Therefore, the synergistic activity of MML-1/MXL-2 complexes and Wnt signaling in adjacent cells mediate migration of ray 1 precursor cells.

MML-1/MXL-2 and Wnt/BAR-1 signaling cooperate with semaphorin signaling to regulate ray 1 precursor cell migration

Prior to this study, only semaphorin signaling had been shown to regulate migration of ray 1 precursor cells. Briefly, SMP-1/Semaphorin1a and SMP-2/Semaphorin1b signal from the hook to activate a PLX-1/PlexinA pathway in ray 1 precursor cells (Fujii et al., 2002; Ginzburg et al., 2002). Activated PLX-1 signals through the RhoGEF UNC-73/Trio and the Rho GTPases CED-10/Rac1 and MIG-2/RhoG to modulate the actin cytoskeleton and mediate ray 1 precursor cell migration (Dalpe et al., 2004). To determine whether there is a functional relationship between MML-1/MXL-2 complexes and semaphorin signaling in ray 1 precursor

cell migration, we constructed *mxl-2(tm1516); plx-1(nc37)* double mutant worms. Anterior displacement of ray 1 was >95% in these worms, much greater than either single mutant ($p<0.02$; Fig. 6A; Table 1). The class I defects of *mxl-2(tm1516); plx-1(nc37)* males are significantly increased over either single mutant suggesting that ray 1 precursor cell migration is severely compromised ($p<0.01$; Fig. 6A; Table 1). Quantitative RT-PCR revealed that MML-1/MXL-2 complexes did not control transcription of semaphorin signaling components (Supplemental Table 1). Therefore, MML-1/MXL-2 complexes and semaphorin signaling act in parallel pathways in adjacent cells and synergize to determine ray 1 position.

Semaphorin signaling through PLX-1 (Dalpe et al., 2004) and Wnt signaling through BAR-1/β-catenin (Fig. 6; Table 1; Supplemental Fig. 2) are required in ray 1 precursor cells to facilitate their migration. However, loss of either pathway does not result in complete anterior displacement of ray 1, yet both pathways cooperate with MML-1/MXL-2 complexes to facilitate ray 1 precursor cell migration (Fig 6A). To determine whether these signaling pathways act serially or in parallel, we generated *plx-1(nc37); bar-1(ga80)* worms. Ray 1 was observed in less than 3% of *plx-1(nc37); bar-1(ga80)* males. Furthermore, other rays were either missing or misplaced in these males (Fig. 6C) which made analyzing ray 1 position unfeasible, yet suggests PLX-1 and BAR-1 cooperate in multiple aspects of male tail development. *plx-1(RNAi)* in wild type males produced a less severe ray 1 phenotype than *plx-1(nc37)* (Fig 6A, D). Therefore to determine interactions between *bar-1* and *plx-1*, we conducted *plx-1(RNAi)* in *bar-1(ga80)* males. Compared to the 10% effect in N2 males, *plx-1 (RNAi)* resulted in anterior displacement of ray 1 in 65% of *bar-1(ga80)* males ($p<0.01$, Fig 6D; Table 1). Transcript levels of semaphorin signaling components were normal in *bar-1 (ga80)* worms, with the exception of *smp-2* and *unc-73*, which were each down two-fold (Supplemental Table 1). Previous studies demonstrated that a two-fold decrease in *smp-2* or *unc-73* is not sufficient to alter ray 1 migration (Dalpe et al., 2004). Therefore, we conclude that the Wnt/BAR-1 and semaphorin/plexin signaling cascades act in distinct, parallel pathways to modulate ray 1 precursor cell migration.

MML-1/MXL-2 regulates transcription of genes encoding ECM components

To identify the MML-1/MXL-2 targets responsible for regulating ray 1 precursor cell migration, we conducted microarray experiments. Male tail development occurs through multiple larval stages (Emmons, 2005), and the time when MML-1/MXL-2 plays a role in this process is not known. Therefore, we compared mixed-stage wild type males and hermaphrodites to mixed-stage *mxl-2(tm1516)* males and hermaphrodites. We found several classes of genes were downregulated in *mxl-2(tm1516)* worms such as metabolic genes, receptors/transporters, ECM components, and transcription factors (data not shown). Signaling molecules, such as Wnts or semaphorins, their cognate receptors, and subsequent signaling components were not regulated by MML-1/MXL-2 complexes (Supplemental Table 1, data not shown). Therefore, ECM components were the most likely class of genes to contribute directly to migration of ray 1 precursor cells. Genes in the MXL-2-regulated ECM class encoded primarily lectins and collagens, which may modulate integrin signaling or the integrity of the ECM, respectively (Chen et al., 1999; Giblin et al., 1997; Kern et al., 1993; Levy et al., 2003). To determine if these ECM components participated in ray 1 precursor cell migration, we selected seven representative collagen and lectin genes downregulated in *mxl-2(tm1516)* worms for a more detailed analysis (Supplemental Table 2). RNAi of Y19D10A.9 (a C-type lectin) and *col-77* caused slight ray 1 position defects (Fig. 7). However, RNAi against these two genes together caused a significant displacement of ray 1 in wild type but not *mxl-2 (tm1516)* males (Fig. 7) strongly suggesting MML-1/MXL-2 complexes act in the same pathway as Y19D10A.9 and *col-77* to facilitate ray 1 precursor cell migration. Furthermore, RNAi of Y19D10A.9 and *col-77* did not cause other phenotypes suggesting that their loss did not grossly affect migration in other cell types (data not shown). These data suggest that

MML-1/MXL-2 complexes regulate ray 1 precursor cell migration by regulating the composition of the extracellular environment.

Discussion

In higher eukaryotes the Myc-superfamily plays predominant roles in growth, proliferation, differentiation and cell death (Billin and Ayer, 2006; Lee and Dang, 2006; Rottmann and Luscher, 2006). Here we present the first functional characterization of the Myc-superfamily in *C. elegans*. In worms there are single proteins representing each subclass of Myc-related factors. These proteins are split into distinct transcription activation and repression complexes comprised of MML-1/MXL-2 and MDL-1/MXL-1, respectively. The lack of functional redundancy and crosstalk between these complexes overcomes these significant hurdles to our understanding of this essential family of transcriptional regulators. MDL-1/MXL-1 (Yuan et al., 1998) and MML-1/MXL-2 complexes are co-expressed in both intestinal and epithelial cells suggesting reciprocal regulation of shared targets in these cell types. MDL-1/MXL-1 complexes are expressed independently of MML-1/MXL-2 complexes in the pharynx (Deplancke et al., 2006), suggesting their repressive activities are unopposed under some circumstances.

MML-1 is the only protein in *C. elegans* that is physically and functionally similar to both Myc and Mondo. MML-1 is similar to MondoA across its entire open reading frame but lacks sequences that are defining features of the Myc family, such as Myc box II. As such, MML-1 appears more closely related to MondoA than to Myc, raising the possibility that there is no bona fide Myc ortholog in *C. elegans*. Given the similarity of MML-1 to Myc and MondoA, however, it is somewhat surprising that loss of MML-1/MXL-2 complexes in *mxl-2(tm1516)* animals has a primary defect in cell migration, rather than defects in growth, lifespan, fat content or dauer formation (data not shown) as would be expected from studies in higher eukaryotes (Iizuka et al., 2004; Pirity et al., 2006; Sans et al., 2006). It may be that MML-1/MXL-2 complexes have fundamentally different activities than MondoA/Mlx or Myc/Max complexes. Alternatively, it is possible that MML-1/MXL-2 complexes have additional activities not revealed under standard laboratory growth conditions. Given the predominant role of MondoA/Mlx complexes in sensing intracellular bioenergetics (Sans et al., 2006), we are currently evaluating cooperation between MML-1/MXL-2 complexes and the major metabolic pathways of *C. elegans*.

MML-1/MXL-2 regulation of cell adhesion and migration is consistent with a Myc-like function and broadens the role of Mondo-like proteins to include these processes. The implication of a Myc and Mondo-like factor, Wnt/β-catenin, and semaphorin/plexin signaling in cell migration leads to a model that incorporates extracellular and intracellular components (Fig. 8). MML-1/MXL-2 complexes function in the non-migratory epidermis to activate expression of lectins and collagens. Meanwhile, the combined activity of a PLX-1 pathway and BAR-1/POP-1 complexes is required in ray 1 precursors, converging on UNC-73, CED-10, and MIG-2 to promote their migratory potential. Finally, we propose that activation of all three pathways results in modulation of integrin-mediated adhesion leading to ray 1 precursor cell migration. This multilayered regulation of ray 1 precursor cell migration strongly suggests ray 1 has critical functions. However, rays 1 and 5 appear to act redundantly in responding to hermaphrodite touch (Liu and Sternberg, 1995). Supporting this, *mxl-2(tm1516)* (data not shown) and *plx-1(nc37)* (Fujii et al, 2002) males mate as well as wild type. *bar-1(ga80)* males had mating defects, but, due to the pleiotropic phenotypes of these worms, it is not possible to attribute the mating defect to ray 1 displacement. These data suggest that ray 1 has additional, but as yet, unknown functions outside of mating.

Our experiments suggest that MML-1/MXL-2 complexes activate expression of lectins and collagens in the syncytial epidermis which contribute to the ECM upon which ray 1 precursor cells migrate. The fact that seam cell expression of MXL-2 rescued the ray 1 defects of *mxl-2 (tm1516)* animals suggests that MML-1/MXL-2 complexes can also function in the migratory ray 1 precursor cells, perhaps because the syncytial epidermis and ray 1 precursor cells have equivalent access to the surrounding ECM. We detect no seam cell expression of MML-1::GFP or MXL-2::GFP when expressed from their genomic promoters; therefore, we propose that in wild type animals MML-1/MXL-2 complexes act predominantly in the non-migratory, syncytial epidermis. However, our data do not rule out the possibility that seam cell expression of MML-1 and MXL-2 may also facilitate ray 1 precursor cell migration.

MML-1/MXL-2 targets were identified using mixed-stage males and hermaphrodites; nevertheless, these experiments identified two MML-1/MXL-2 targets, the C-type lectin Y19D10A.9 and the collagen *col-77*, that, when knocked down in wild type worms, affects cell migration to a degree similar to that observed in *mxl-2(tm1516)* animals. Furthermore, RNAi of Y19D10A.9 and *col-77* did not significantly enhance anterior ray 1 displacement in *mxl-2(tm1516)* worms suggesting these ECM components are the primary effectors of MML-1/MXL-2 transcriptional activity in controlling ray 1 precursor cell migration. Furthermore, Y19D10A.9 and *col-77* were downregulated in *mxl-2(tm1516)* worms suggesting that MML-1/MXL-2 acts as a transcriptional activator to facilitate ray 1 precursor cell migration. The Y19D10A.9 and *col-77* promoters have multiple CACGTG binding sites, and may be direct transcriptional targets of MML-1/MXL-2 complexes—a hypothesis we are currently testing. Collagens are ligands for integrins, and C-type lectins can regulate the strength of interactions between integrins and collagens (Chen et al., 1999; Giblin et al., 1997; Kern et al., 1993; Levy et al., 2003). Therefore, we propose that regulation of collagens and C-type lectins by MML-1/MXL-2 complexes modulates integrin activity and facilitates migration of ray 1 precursors. Consistent with this, RNAi of *ina-1*/α-integrin almost completely blocked ray 1 precursor cell migration (data not shown). Another possibility is that MML-1/MXL-2 regulated collagens and lectins facilitate the proper diffusion of signaling molecules or modulate the activity of their receptors. These ECM molecules may affect SMP-1 and SMP-2 dispersal from the hook or PLX-1 activity in the ray 1 precursor cells, but lack affects on members of Wnt signaling as *mxl-2(tm1516)* worms do not exhibit phenotypes associated with this pathway. Experiments to determine the time at which MML-1/MXL-2 affects ray 1 precursor cell migration relative to semaphorin signaling may help determine which of these models is correct. Furthermore, microarray experiments with a single-stage, same-sex population may uncover additional targets of MML-1/MXL-2 involved in this process.

Our data also indicate that cell autonomous Wnt/BAR-1 and semaphorin/plexin signaling pathways function in parallel to control ray 1 precursor cell migration. Knockdown of *bar-1* or *pop-1* produced ray 1 defects, suggesting that target genes regulated by the BAR-1/POP-1 complex are required for cell migration. Loss of either BAR-1/β-catenin activity or plexin signaling causes a partial ray 1 displacement defect but combined loss of these pathways results in a highly penetrant defect. A strong hypomorphic allele of the RhoGEF *unc-73*/Trio also causes an almost completely penetrant ray 1 defect (Dalpe et al., 2004), suggesting that BAR-1/POP-1 and semaphorin signaling converge on UNC-73 to regulate ray 1 precursor cell migration. UNC-73 integrates multiple signaling events to mediate cell polarity and migration (Honigberg and Kenyon, 2000), and may play a similar role in ray 1 precursor cells. Transcription of *unc-73* is only modestly regulated by BAR-1/POP-1 (Supplemental Table 1). Furthermore Hox genes, which are often targets of BAR-1/POP-1, are not expressed in ray 1 precursor cells (Emmons, 2005). Therefore, it is likely that a novel BAR-1/POP-1 target gene regulates UNC-73 activity.

To our knowledge, this is the first report of cooperation between Wnt and semaphorin signaling. However, both pathways are necessary for the development of many of the same tissues. A BAR-1-dependent Wnt signaling pathway is necessary for cell fate specification in the *C. elegans* vulva whereas SMP-1, SMP-2, and PLX-1 are involved in vulval morphogenesis (Eisenmann et al. 1998; Dalpe et al. 2005; Liu et al. 2005). Similarly, Sema3A, Sema3C, Wnt5a, and Wnt7b are each involved in branching morphogenesis of the mouse lung, yet single knockouts of these genes produce mild defects restricted to specific tissues or yield no phenotype at all (Kagoshima and Ito 2001; Li et al. 2002; Shu et al. 2002). Thus, cooperation and redundancy between Wnt and semaphorin signaling may account for the weak phenotypes associated with loss-of-function mutations in pathway components. These data, combined with our results, suggest redundancy between Wnt and semaphorin signaling is conserved and critical for development of a variety of tissues. Furthermore, the broad expression patterns of Myc and Mondo genes in mammalian development (Billin et al., 2000; Pirity et al., 2006) and their prior implication in cell migration (Coller et al., 2000; Frye et al., 2003; Watnick et al., 2003; Zhang et al., 2005; Sans et al., 2006) suggest that cooperation between these factors and Wnt and semaphorin signaling may not be restricted to *C. elegans*.

Myc family members, Wnts, and semaphorins are important angiogenic, invasive, and metastatic factors (Billin and Ayer, 2006; Nelson and Nusse, 2004; Tamagnone and Comoglio, 2004; Wade and Wahl, 2006). Our data suggest Myc and Mondo proteins, either in the tumor or the stroma, may generate an extracellular milieu that is permissive for migration, thereby facilitating angiogenesis and tumor metastasis. Studies of stromal regulation of cell migration have focused primarily on the regulation of matrix metalloproteinases required for breakdown of the ECM (Kryczek et al., 2006; McCawley and Matrisian, 2001); however, our data suggest some collagens and lectins are pro-migratory, and this facet of cell migration requires further study. Further, Myc and Mondo proteins activate transcription of key glycolytic enzymes (Lee and Dang, 2006; Sans et al., 2006; Uyeda and Repa, 2006), and increased glycolysis is strongly correlated with migratory potential (Beckner et al., 1990; Funasaka et al., 2005; Gatenby et al., 2006). Therefore, while Myc and Mondo have well-established roles in cell growth and proliferation, our data demonstrate that they also promote migration by possibly several distinct mechanisms. Finally, the cooperation we have observed between MML-1/MXL-2 complexes, Wnt, and semaphorin signaling in *C. elegans*, suggests that deregulation of the orthologous pathways in humans also cooperate to control cell migration-dependent events during tumorigenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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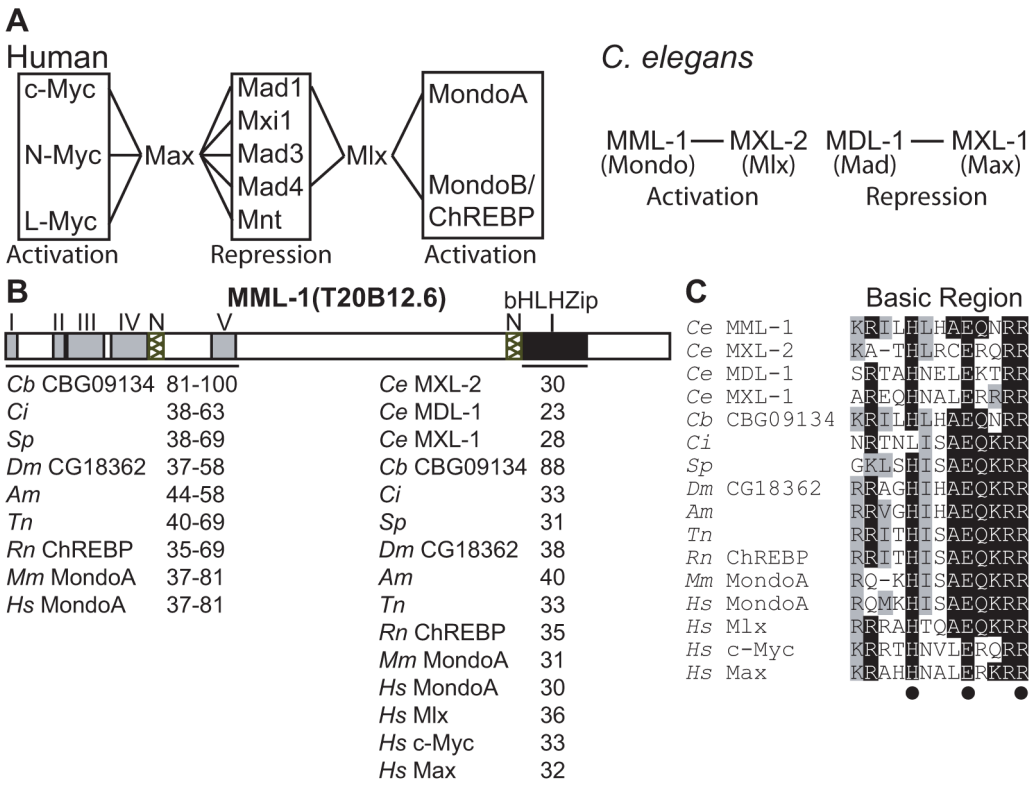

Figure 1.

MML-1 and MXL-2 are members of the Myc superfamily of bHLHZip proteins. (A) Schematic representation of the mammalian and *C. elegans* Myc-like networks of bHLHZip proteins. Lines denote dimeric partners. (B) Diagram of MML-1. Gray boxes—Mondo Conserved Regions (MCRs I-V), hatched boxes—Nuclear Localization Sequences (N), black box—bHLHZip motif. The numbers below each region denote the percentage of sequence identity shared between *C. elegans* MML-1 and the other proteins listed as determined by BLAST. *Ce—Caenorhabditis elegans*, *Cb—Caenorhabditis briggsae*, *Ci—Ciona intestinalis* (sea squirt), *Sp—Strongylocentrotus purpuratus* (sea urchin), *Dm—Drosophila melanogaster*, *Am—Apis mellifera* (honeybee), *Tn—Tetraodon nigroviridis* (freshwater pufferfish), *Rn—Rattus norvegicus*, *Hs—Homo sapiens*. (C) Alignment of the basic region of the *C. elegans* bHLHZip family, MML-1 orthologs, human c-Myc, and Max. Dots denote residues required for sequence-specific binding to CACGTG E-boxes (Ferre-D'Amare et al., 1993; Nair and Burley, 2003).

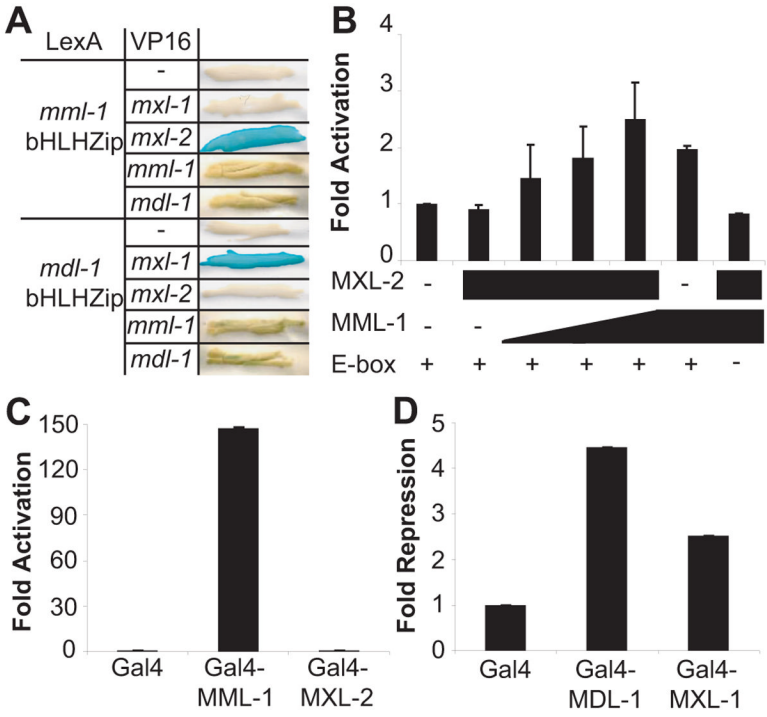

Figure 2.

MML-1 and MXL-2 interact and activate transcription. (A) Interactions between *C. elegans* bHLHZip proteins were assayed by yeast two-hybrid. Constructs were fused to either the LexA DNA-binding domain or the VP16 activation domain as indicated and screened for interaction. 293T cells were transfected with the indicated constructs and either (B) a CACGTG E-box-responsive reporter or (C, D) a Gal4-responsive promoter. (Relative luciferase units)/(β-Gal) was calculated for each construct and is represented as fold activation or fold repression relative to Gal4 alone.

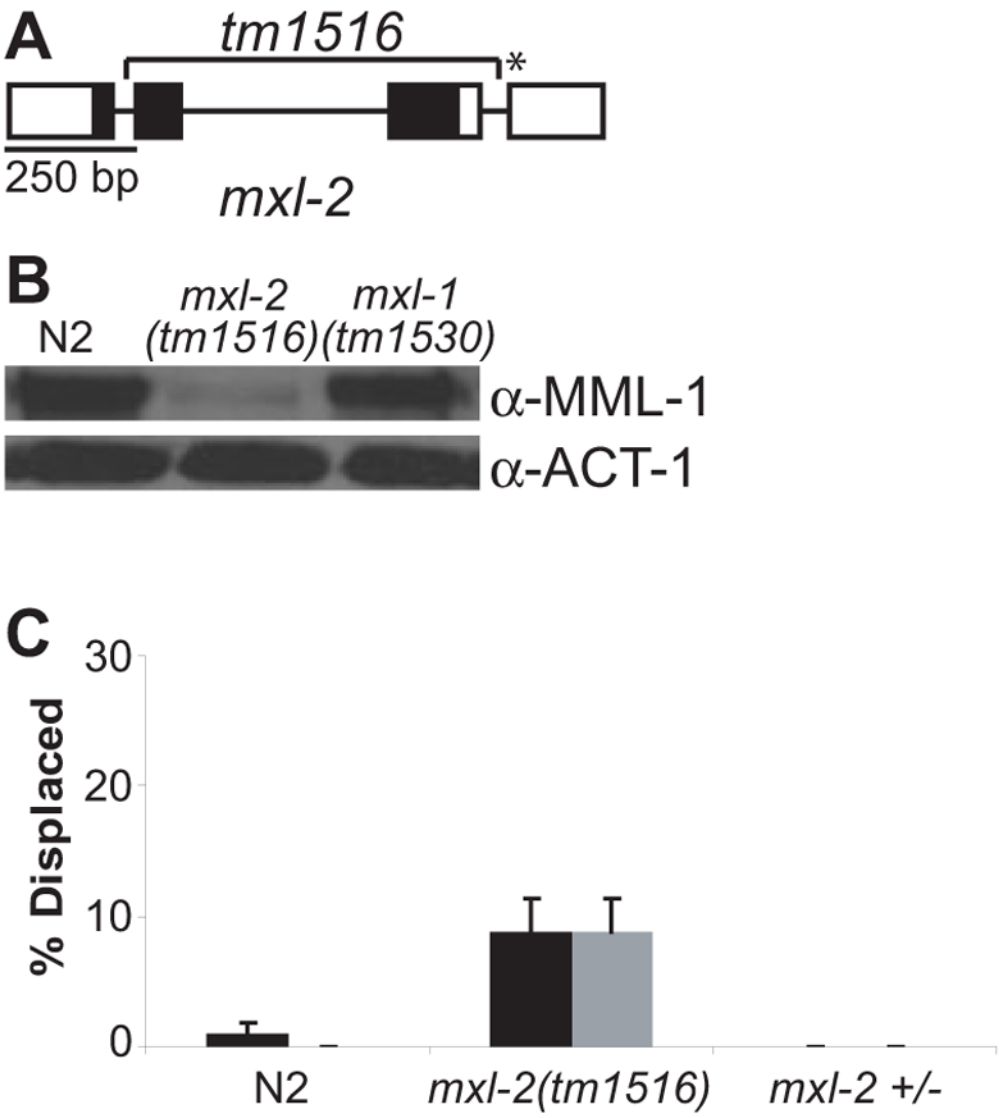

Figure 3.

mxl-2(tm1516) worms lack MML-1 and exhibit anteriorly displaced ray 1. (A) Diagram of the *mxl-2* locus. Boxes denote exons and the shaded regions encode the bHLHZip motif. The bracketed region is deleted in *tm1516*. Asterisk represents the premature termination codon. (B) Levels of MML-1 and an ACT-1 loading control were determined by Western blot from worm extracts prepared from the indicated strains. (C) Ray 1 anterior displacement in worms heterozygous (+/−) and homozygous for *mxl-2(tm1516)*. Black and gray bars indicate class I and II defects, respectively (see Materials and Methods). Ray 1 defects in *mxl-2(tm1516)* worms were significantly different from wild type or *mxl-2* heterozygotes ($p<0.01$). N>90 for each strain.

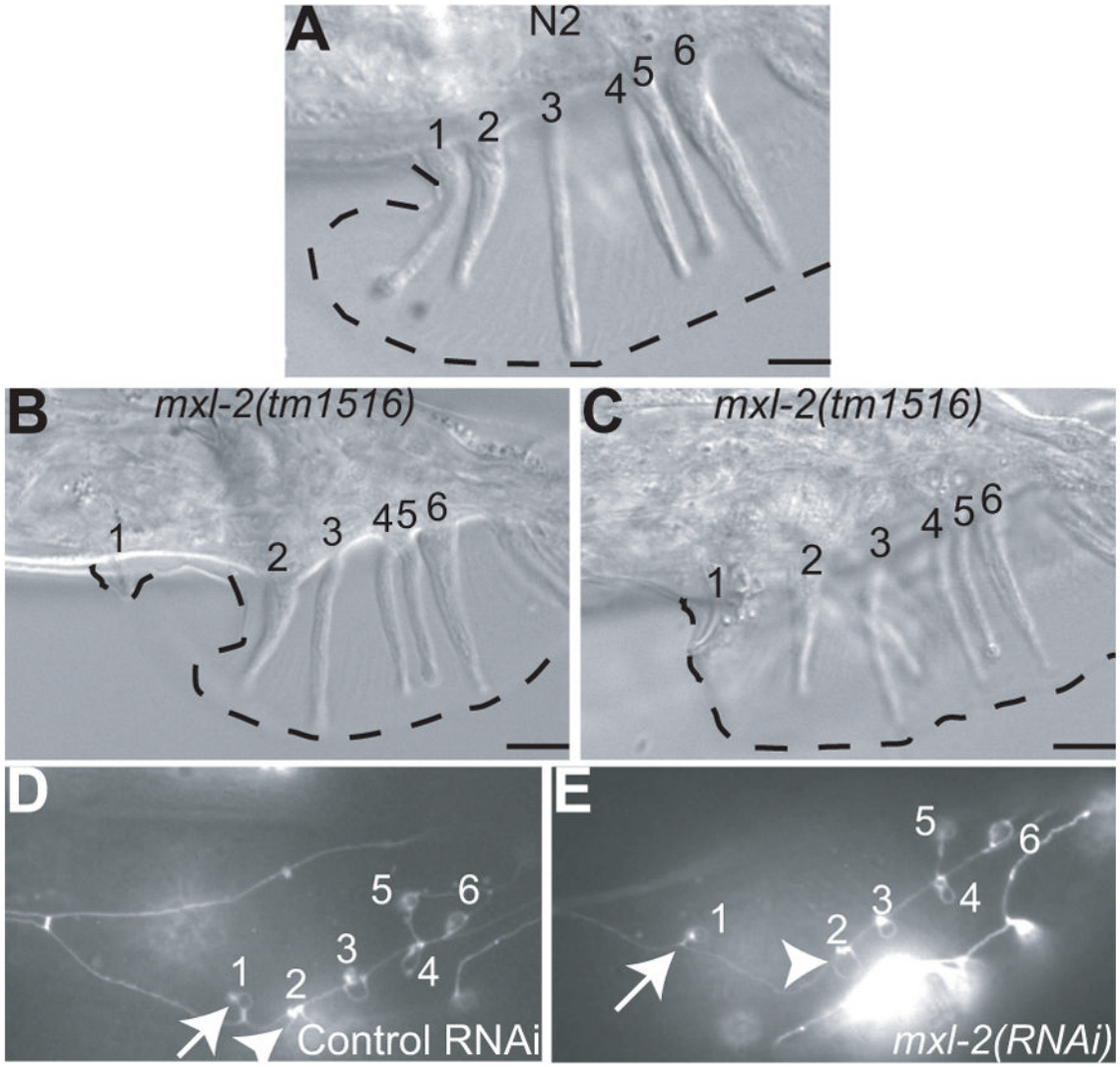

Figure 4.

MML-1/MXL-2 complexes control ray 1 precursor cell migration. Examples of (A) wild type, (B) class I, and (C) class II defects in adult males. The cuticular fan is outlined. Scale bar is 3μm. Wild type L4 males expressing AJM-1::GFP were fed (D) vector control RNAi or (E) *mxl-2* RNAi. Precursors are numbered according to the rays they generate. Only Rnst cells are visible at this stage in ray development. Arrow—R1st; arrowhead—R2st. For all images, anterior is left and dorsal is up.

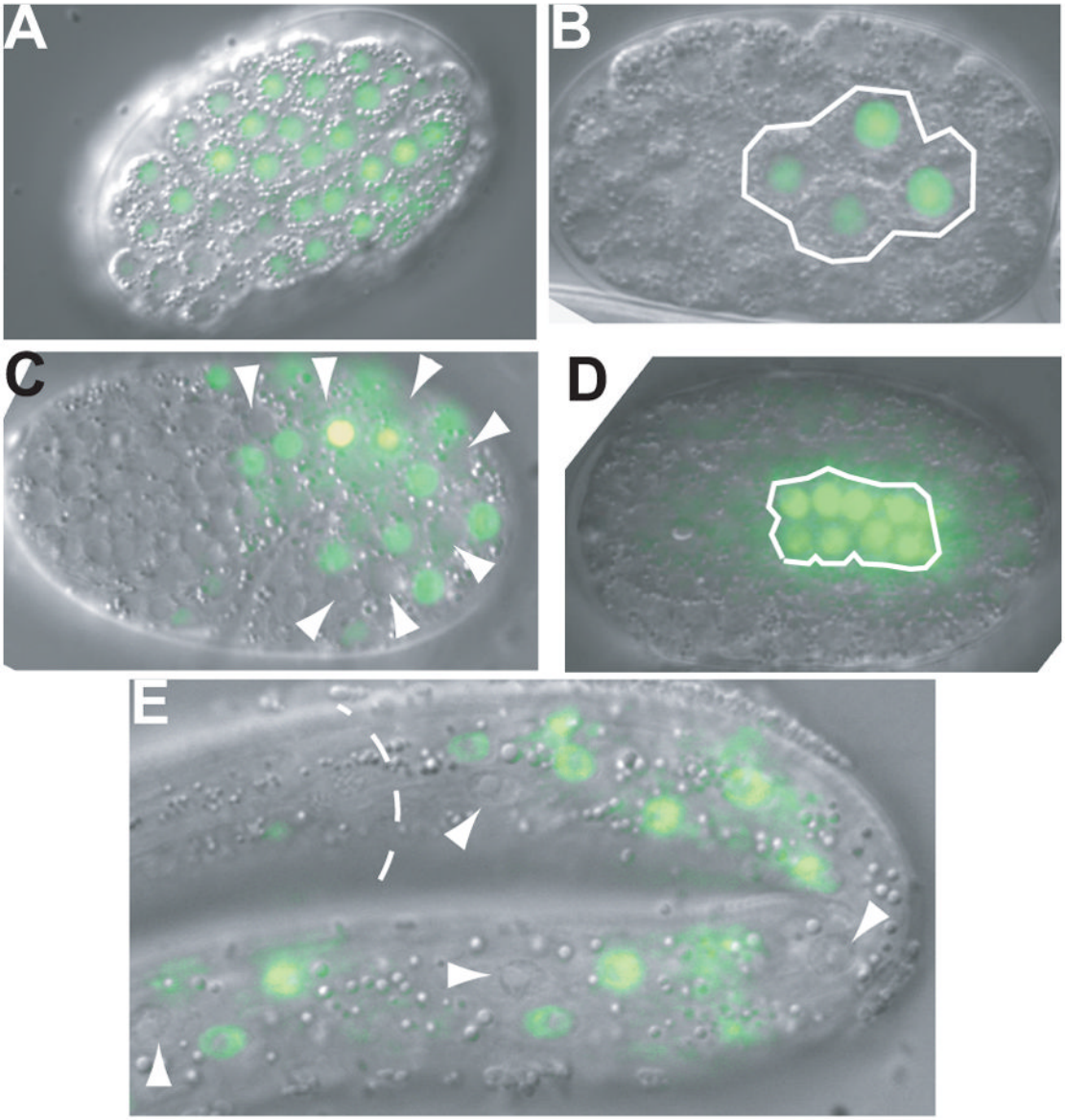

Figure 5.
MML-1 is expressed in the hypodermis and intestine. Transgenic worms expressed MML-1::GFP in the nuclei of epidermal cells (A) at 260 min and (C) at the 1.5 fold stage, in intestinal cells (B) at the 4E and (D) 8E stages and in (E) syncytial epidermal nuclei of L1 worms. White arrowheads denote seam cell nuclei, the white dashed line marks the pharynx/intestine boundary, and the solid white lines outline intestinal cells. For all images, anterior is left and dorsal is up.

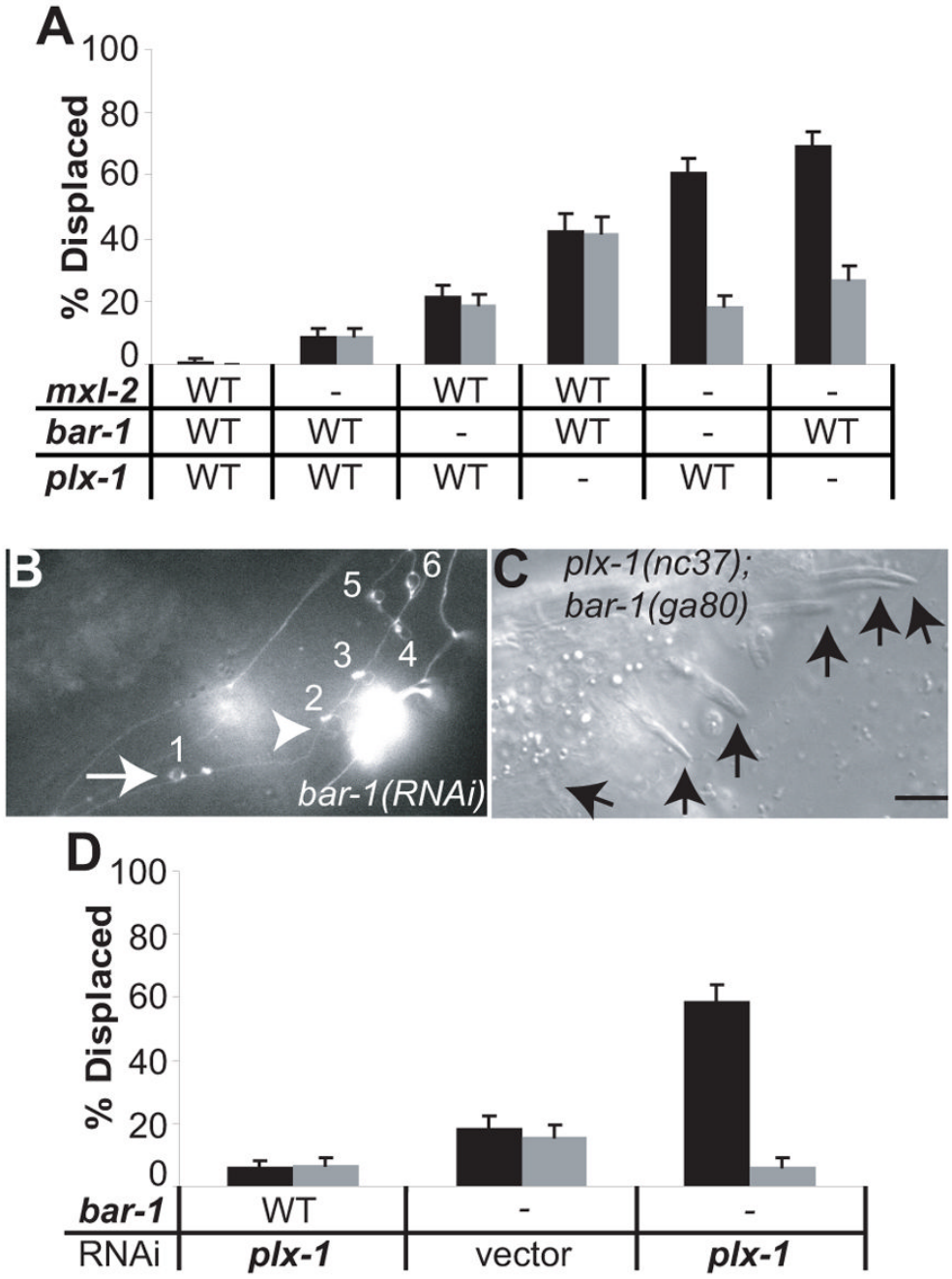

Figure 6.

mxl-2, *bar-1*, and *plx-1* have non-redundant roles in ray 1 positioning. (A) Ray defects were quantified in the indicated strains at 20°C. Strains are wild type (WT) or null (-) for alleles of the indicated genes. (B) Wild type L4 males expressing AJM-1::GFP were fed *bar-1* RNAi. Precursors are numbered according to the rays they generate. Only Rnst cells are visible at this stage in ray development. Arrow—R1st; arrowhead—R2st. (C) Male *plx-1(nc37); bar-1 (ga80)* worms have ray defects beyond ray 1 displacement. Arrows denote all observable rays. Scale bar is 3μm. (D) RNAi against *plx-1* in wild type N2 (WT) or *bar-1(ga80)* (-) males at 24°C. Data is presented as the increase in ray 1 displacement relative to wild type males fed empty vector RNAi. Black and gray bars indicate class I and II defects, respectively (see

Materials and Methods). P values were calculated by comparing each single mutant to wild type or each double mutant to the respective single mutants. P<0.02 for *mxl-2(tm1516); plx-1 (nc37)* compared to *plx-1(nc37)*. For all other comparisons, p<0.01. N>90 for each experiment. Anterior is left and dorsal is up.

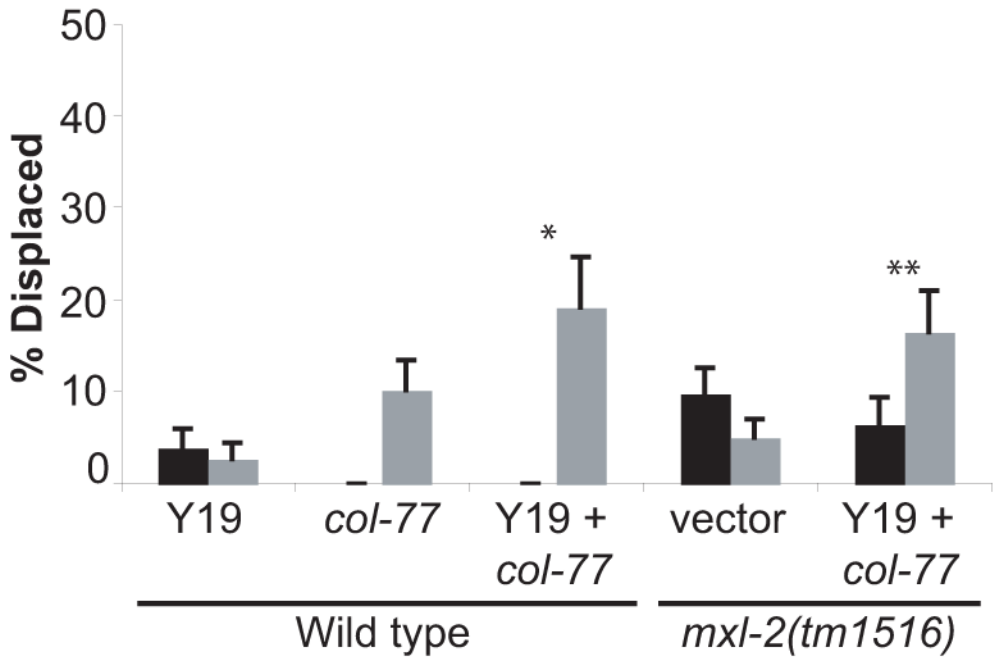

Figure 7.

Extracellular matrix components regulate ray 1 position. RNAi of the indicated constructs was conducted in the indicated strains. Y19 is short for Y19D10A.9. Data is presented as the increase in ray 1 displacement relative to wild type males fed empty vector RNAi. Black and gray bars indicate class I and II defects, respectively (see Materials and Methods). *-$p<0.02$ when compared to wild type worms with empty vector RNAi. **-$p>0.1$ when compared to *mxl-2(tm1516)* worms with empty vector RNAi. N>50 for each experiment.

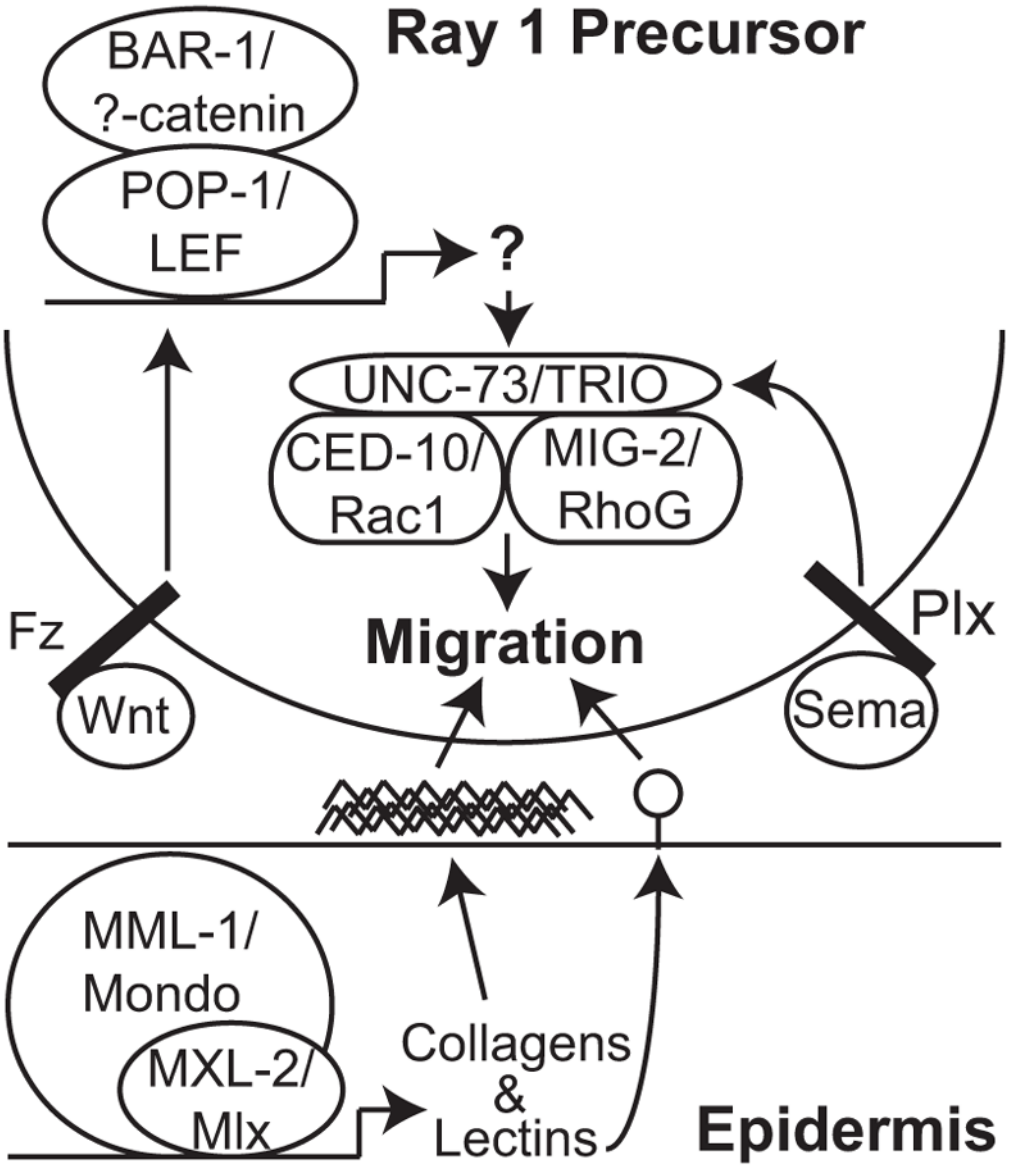

Figure 8.
Model of ray 1 precursor cell migration. See text for details.

Table 1

Ray 1 Anterior Displacement Defects

Strain	RNAi	Class I[a]	Class II[a]	Total[b]	N[c]
N2	none	0.95±0.948	0	0.95±0.948	105
mxl-2(tm1516)	none	8.8±2.65	8.8±2.65	17.5±3.56	114
mxl-2(tm1516) +/−	none	0	0	0	91
bar-1(ga80)	none	21.6±3.33	19.0±3.17	40.5±3.97	153
plx-1(nc37)	none	42.7±4.57	41.9±4.56	84.6±3.34	117
mxl-2(tm1516); bar-1(ga80)	none	61.1±4.26	18.3±3.38	79.4±3.53	131
mxl-2(tm1516); plx-1(nc37)	none	69.4±4.43	26.9±4.26	96.3±1.82	108
mxl-2(tm1516) [P*mxl-2::mxl-2::gfp*][d]	none	6.6±1.62	3.1±1.14	9.7±1.94	235
mxl-2(tm1516) [P*dpy-7::mxl-2::gfp*][d]	none	2.8±1.33	2.9±1.35	5.6±2.01	152
mxl-2(tm1516) [P*cdh-3::mxl-2::gfp*][d]	none	2.6±2.06	7.6±3.44	10.1±3.94	59
bar-1(ga80) [P*dpy-7::bar-1::gfp*][d]	none	20±8.94	5.0±4.87	25.0±9.68	20
bar-1(ga80) [P*cdh-3::bar-1*][d]	none	0	0	0	30
N2	*plx-1*	6.0±2.13[e]	6.7±2.25[e]	12.7±2.99[e]	124
bar-1(ga80)	vector	18.4±3.83[e]	15.7±3.61[e]	34.1±4.69[e]	102
bar-1(ga80)	*plx-1*	58.9±5.13[e]	6.3±2.54[e]	65.3±4.96[e]	92
N2	Y19D10A.9	4.0±2.01[e]	2.7±1.68[e]	6.7±2.57[e]	95
N2	*col-77*	0[e]	10.1±3.21[e]	10.2±3.21[e]	89
N2	Y19D10A.9 + *col-77*	0[e]	19.1±5.46[e]	19.1±5.46[e]	52
mxl-2(tm1516)	vector	9.8±2.75[e]	5±2.02[e]	14.9±3.29[e]	117
mxl-2(tm1516)	Y19D10A.9 + *col-77*	6.5±2.94[e]	16.5±4.43[e]	23±5.03[e]	70

[a]-Percentage of animals exhibiting either class I or class II ray 1 defects. P values were calculated by comparing each single mutant to N2 or by comparing each double mutant to the respective single mutant worms. For rescue experiments, transgenic lines were compared to their parental strain. P<0.02 for *mxl-2(tm1516); plx-1(nc37)* compared to *plx-1(nc37)*; for all other comparisons p<0.01. *mxl-2*+/− worms were not significantly different from N2; *bar-1(ga80)* [P*dpy-7::bar-1::gfp*] worms were not significantly different than *bar-1(ga80)*.

[b]-Class I + Class II

[c]-Total number of animals examined.

[d]-Worms carrying rescue constructs were grown at 24°C whereas the parental strains were grown at 20°C. We observed no diffence in ray 1 displacement between worms grown on OP50 at either temperature (data not shown).

[e]-These data are the ray defects beyond that caused by empty vector RNAi in N2 worms.