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Roles for Netrin Signaling Outside of Axon Guidance: A View from the Worm

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The Netrin family of extracellular ligands and their receptors were the first identified signaling pathway regulating axon guidance. Subsequent work across model systems has begun to reveal the interactions that take place downstream of Netrin reception to facilitate growth cone migration. Though intensely studied, many aspects of this signaling system remain unclear. Even less understood are the growing number of contexts in which Netrin signaling influences cells beyond axon guidance and even outside the nervous system. Genetic and cell-biological studies in *C. elegans* have played an instrumental role in identifying critical functions for Netrin ligands in setting up specialized and potentially adhesive membrane-associated domains within a broad range of cell types. Here we review recent literature implicating Netrin or its receptors in morphogenetic processes outside of growth cone regulation with a special focus on studies in *C. elegans* that suggest cell biological mechanisms for Netrin signaling. *Developmental Dynamics 239:1296–1305, 2010.* © 2010 Wiley-Liss, Inc.

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INTRODUCTION

Because a major impetus for developing *C. elegans* as a model system was the potential to identify the genetic control of neuronal development, it is perhaps not surprising that the first mutations affecting genes in the Netrin pathway were identified through their Uncoordinated (Unc) loss-of-function phenotype in the worm (Brenner, 1974). Loss-of-function mutations in *unc-6* resulted in a failure of axonal growth cones to follow their normal dorsal and ventral migratory paths during development. A similar spectrum of growth cone migration defects were observed in the sum of the phenotypes caused by mutations in two other genes, *unc-40* and *unc-5* (Hedgecock et al., 1990). Cloning of these genes in *C. elegans* along with genetic and biochemical characterization of counterparts in *Drosophila* and vertebrates revealed the UNC-6 protein to be the *C. elegans* orthologue of Netrin ligands (Ishii et al., 1992; Serafini et al., 1994; Harris et al., 1996; Mitchell et al., 1996). In turn, UNC-40 and UNC-5 were found to be members of two distinct classes of single-pass transmembrane receptors for Netrin ligands (Leung-Hagesteijn et al., 1992; Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996; Leonardo et al., 1997; Keleman and Dickson, 2001). Recent work in *Drosophila* and in vertebrates has shown that some members of the Down Syndrome Cell Adhesion Molecule (DSCAM) family of cell-surface proteins are able to function as Netrin receptors (Andrews et al., 2008; Ly et al., 2008). However, the *C. elegans* genome does not encode a clear DSCAM orthologue, suggesting that *C. elegans* has lost this component of Netrin signaling (Vogel et al., 2003).

NETRIN SIGNALING: THE BASICS

Netrin and its receptors guide neuronal trajectories by regulating specialized structures at the tip of extending axons known as growth cones (de la Torre et al., 1997). In *C. elegans*, UNC-6/Netrin is produced by multiple cell types, but most notably by neurons within the ventral nerve cord, which runs between the basement membranes of the hypodermis and body-wall muscles on the ventral side of the animal (Wadsworth et al., 1996). Presumably UNC-6/Netrin secretion from the ventral nerve cord

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establishes a ventral-to-dorsal concentration gradient that can be sensed by emerging axons. Receipt of Netrin ligands by growth cones can cause axons to turn either up or down the Netrin protein gradient (Hong et al., 1999). Attraction towards, or repulsion from, sources of Netrin is determined by the compliment of cell-surface receptors expressed by a particular neuron (Fig. 1a) (Hamelin et al., 1993; Hong et al., 1999). Growth cones that express DCC alone track towards Netrin sources. In contrast, growth cones expressing UNC-5 family members or those expressing both UNC-5 and DCC turn away from high concentrations of Netrin ligands. It is not known whether DSCAM, normally an attractive Netrin receptor, participates in repulsive signaling like DCC (Andrews et al., 2008; Ly et al., 2008).

Work conducted in vertebrate neurons suggests that Netrin ligands regulate growth cone signaling by controlling the formation of ligand-gated receptor complexes composed of DCC or UNC-5 and DCC (Hong et al., 1999; Stein et al., 2001). Ligand-receptor dimers or multimers are then thought to bind intracellular effectors to conduct downstream signaling. Whether Netrin binding to DCC is an obligate component of signal activation in all cases, however, is unclear because in *C. elegans* UNC-40/DCC signals without a requirement for UNC-6/Netrin in some cellular contexts (Honigberg and Kenyon, 2000; Yu et al., 2002; Alexander et al., 2009). Moreover, experiments conducted in mammalian cells indicate that Netrin ligands may in some cases work through Integrin heterodimers, perhaps indicating a vertebrate-specific variation on Netrin signaling (Yebra et al., 2003). In terms of cellular behavior, experimental evidence both in vivo and in vitro has shown that Netrin signaling regulates the actin cytoskeleton and that actin-based filopodia are crucial in Netrin-mediated axon guidance (Lebrand et al., 2004; Barallobre et al., 2005). Below, we focus on the molecular mechanisms implicated downstream of DCC-family receptors, which have largely been elucidated in the context of axon guidance.

DOWNSTREAM OF DCC: INTRACELLULAR MECHANISMS AND PATHWAYS

On the intracellular domain of DCC-family receptors, three regions with higher levels of amino acid sequence conservation, referred to as P1, P2, and P3, have been identified (Fig. 1b,c) (Kolodziej et al., 1996). Of these domains, the P3 region appears to be the most well conserved and contains a leucine-rich motif known as an LD domain (Fig. 1c) (Brown et al., 1998; Ren et al., 2004). In Paxillin, a focal adhesion-associated adaptor protein, LD domains mediate interactions with actin-binding proteins, consistent with the possibility that DCC family receptors use a similar mechanism to communicate with the actin cytoskeleton (Schaller, 2001). In vertebrates, the P3 domain may mediate ligand-dependent dimerization of DCC, which is thought to be critical for initiation of the DCC signaling cascade (Stein et al., 2001). In addition, the P3 domain and an adjacent "PXXP" motif are required to recruit focal adhesion kinase (FAK) and src-family kinases in vertebrate cells, intracellular signaling components implicated in regulating actin dynamics and cell adhesion (Li et al., 2004; Liu et al., 2004a; Meriane et al., 2004; Ren et al., 2004). These interactions lead to phosphorylation on a critical tyrosine residue (Y1420) within the DCC intracellular domain that is necessary for axon guidance in culture (Fig. 1b, c). Intriguingly, in *Drosophila* and *C. elegans* neither the tyrosine nor the PXXP motif is conserved in Frazzled/DCC or UNC-40/DCC, respectively (Fig. 1c). In fact, while the cytoplasmic domain of UNC-40/DCC contains seven tyrosine residues and may be regulated by tyrosine-phosphorylation, none of these tyrosine residues are absolutely conserved in vertebrate orthologues (Chang et al., 2004). In *Drosophila*, Frazzled/DCC physically and genetically interacts with the Abelson tyrosine kinase (Abl), indicating that tyrosine phosphorylation downstream of ligand reception may occur by multiple mechanisms (Forsthoefel et al., 2005; Hsouna and VanBerkum, 2008). Moreover, *Drosophila* Frazzled/DCC may multimerize in response to Netrin ligands, but does not require the P3 domain for self-association (Garbe et al., 2007). However, the Frazzled/DCC P3 domain is important for signaling, as it controls growth cone attraction in a subset of commissural neurons (Garbe et al., 2007). In other *Drosophila* neurons, mutated Frazzled/DCC receptors lacking either the P1, P2, or P3 domains, respectively, can partially compliment *frazzled* mutants but the magnitude of rescue is lower than that achieved by expression of wild-type Frazzled (Dorsten and VanBerkum, 2008). Thus, each of these regions appears to be important for optimal signaling in at least some contexts.

Experiments conducted using a gain-of-function genetic assay in *C. elegans* are consistent with a multimodal signaling mechanism for UNC-40/DCC (Fig. 1c). In the worm, signaling through the P1 domain in neurons is mediated by UNC-34, the *C. elegans* Ena/VASP ortholog (Gitai et al., 2003). This actin regulatory protein is thought to promote filopodium formation and prevent the capping of microfilaments (Krause et al., 2003). While the P2 domain does not appear to be highly conserved in *C. elegans* at the level of primary amino acid sequence, the corresponding region of UNC-40/DCC is important for signaling in neurons. The activity of this region depends on CED-10/Rac and a downstream effector UNC-115/AbLIM (Gitai et al., 2003). Consistent with these findings, DCC is capable of activating both Rac and Cdc42 in vertebrate cells (Shekarabi and Kennedy, 2002).

The combined genetic and biochemical studies on the intracellular domain of DCC indicate a complex picture of signaling mechanisms that lie downstream of this receptor. Multi-species alignment of the cytoplasmic regions of DCC receptors mirrors this complexity, revealing that the cytoplasmic domain has undergone profound changes over evolutionary time (Fig. 1c). For example, in planaria (*S. mediterranea*) where NetR/DCC has been documented to function within neuronal cells, both the P1 and P2 domains appear to have been lost (Cebria and Newmark, 2005). In *C. elegans*, though the region

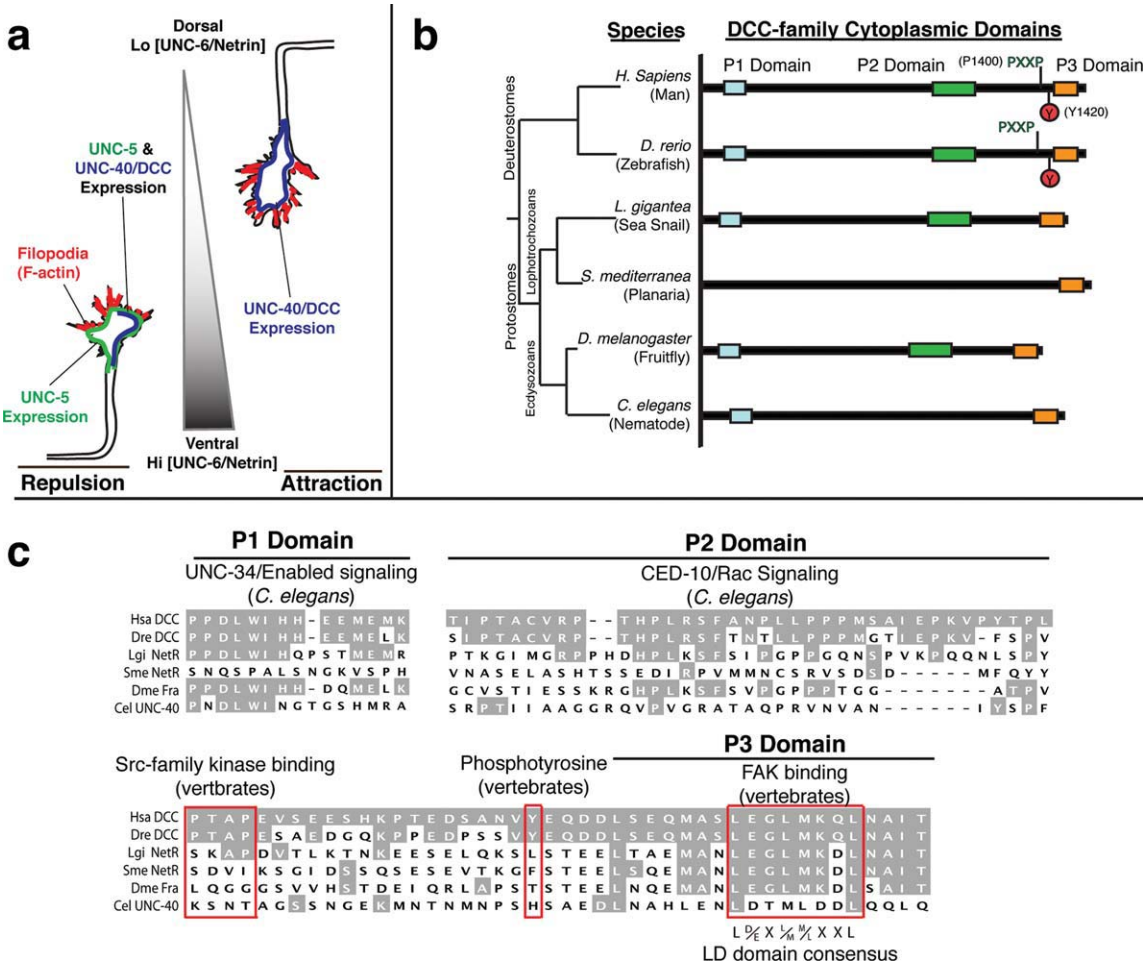

Fig. 1.

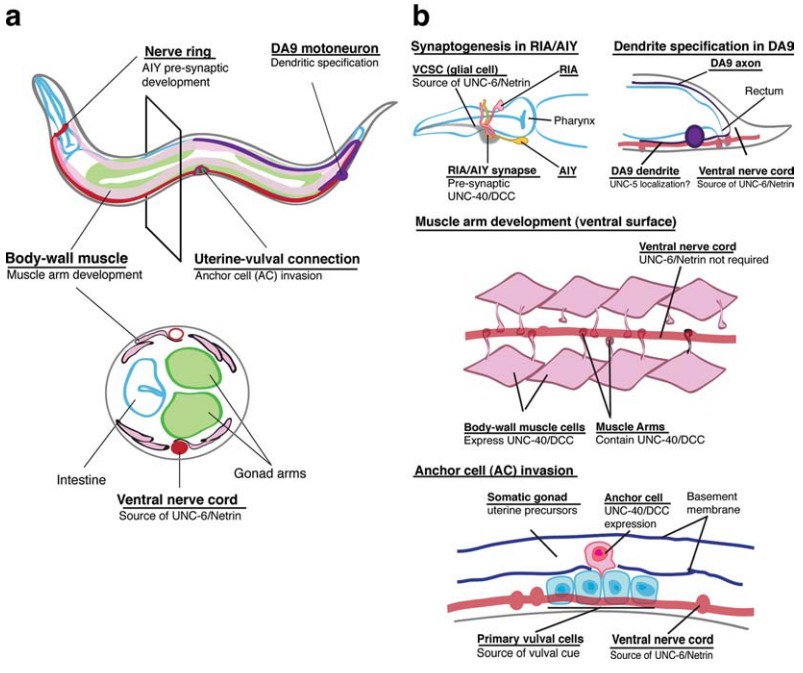

Fig. 2.

corresponding to the P2 domain is important for signaling through CED-10/Rac, it lacks sequence similarity to the P2 domain of DCC receptors in other species (Fig. 1c). One possibility is that a similar core set of effector proteins interacts with DCC family receptors in all species, but utilizes

Fig. 2. **a**: Schematic of a *C. elegans* hermaphrodite in profile (top) and cross-section (bottom) detailing the locations of non-traditional UNC-6/Netrin signaling events. These include pre-synaptic development in AIY, dendrite specification in DA9, muscle arm development in the body-wall muscle cells, as well as anchor cell (AC) invasion through basement membrane during uterine vulval connection. **b**: Schematic representation of the cells and signals in each of these non-traditional locations for UNC-6/Netrin signaling in the worm. Notably, AIY, the AC and muscle cells express UNC-40/DCC, where it is localized to specific membrane domains. DA9 utilizes UNC-6/Netrin signaling through UNC-5 to specify dendrite identity. It is not known if UNC-5 is specifically localized to that region.

Fig. 1. **a**: Neurons expressing UNC-40/DCC (purple) alone will be attracted to UNC-6/Netrin, while those expressing UNC-5 (green) or UNC-5 and UNC-40/DCC will be repulsed. In *C. elegans*, UNC-6/Netrin is thought to be distributed in a ventral-to-dorsal concentration gradient due to its secretion from the ventral cord neurons. **b**: Signaling by DCC receptors is thought to involve three cytoplasmic regions (P1, P2, and P3) with higher levels of sequence conservation across evolutionary time. The P1 domain has been lost in *S. mediterranea*, while the P2 domain has been lost or extensively altered in both *C. elegans* and *S. mediterranea*. **c**: ClustalW2 alignments of the cytoplasmic P domains and associated signaling motifs. Residues highlighted in grey are conserved relative to human DCC. Notably, neither the binding site for Src-family kinases, nor a critical phosphotyrosine residue, both thought to be important for DCC signaling in humans, is conserved in the non-vertebrate species. While the P2 domain is poorly conserved, the corresponding region in *C. elegans* signals through CED-10/Rac in neurons. The P3 domain in *C. elegans* is divergent but it still contains a recognizable LD domain when compared to a consensus sequence for LD domains.

flexible biochemical mechanisms for their interaction. Alternatively, the changes in the cytoplasmic domains of UNC-40/DCC and NetR/DCC might reflect changes in the suite of intracellular effectors engaged by the receptor in each species. Whichever of these possibilities is correct, one persistent theme is the strong connection of UNC-40/DCC to regulators of the actin cytoskeleton and adhesion.

ROLES FOR NETRIN SIGNALING BEYOND AXON GUIDANCE IN VERTEBRATES

While Netrin signaling has been most intensely studied in relation to axon guidance, a growing number of studies have documented Netrin function outside of neuronal guidance. In human patients, deletion of DCC was detected in colorectal cancers before its role in the nervous system was known (Fearon et al., 1990). While the functional significance of DCC deletion or Netrin signaling in colorectal cancer pathogenesis remains unclear, it may relate to evidence suggesting that Netrin receptors can function as dependence receptors in vertebrate cells, triggering apoptosis in the absence of Netrin ligand (Mehlen et al., 1998; Mehlen and Furne, 2005). Along with these findings, it has been shown that autocrine Netrin-1 signaling confers a survival advantage upon some metastatic mammary cancer cell lines indicating the potential importance of this pathway in regulating cell growth and survival during cancer progression (Fitamant et al., 2008). Functions for Netrin as a survival factor may be specific to vertebrates, as Netrin signaling does not seem to regulate apoptosis in the worm or fly.

Netrin ligands are important for morphogenesis in several vertebrate tissues. For example, in the vascular system and during branching morphogenesis of the lung and mammary gland, Netrin signaling controls the development of complex tubular organs (Srinivasan et al., 2003; Liu et al., 2004b; Lu et al., 2004; Navankasattusas et al., 2008). In these cases, phenotypic analysis indicates that Netrin signals directly regulate the cell or cells that are important for organizing tubular outgrowth. Despite functions for Netrin activity in various locations outside of the nervous system, it remains largely unclear how Netrin ligands function to control morphogenesis in non-traditional contexts. In part, this lack of understanding is due to the experimental complexity of vertebrate developmental models.

NON-TRADITIONAL ROLES FOR NETRIN SIGNALING IN *C. ELEGANS*

Genetic studies in *C elegans* were among the first to define roles for Netrin signaling outside of growth cone guidance. In the worm, early reports highlighted the control of many non-neuronal cell migrations by UNC-6/Netrin, or its receptors UNC-40/DCC and UNC-5 (Hedgecock et al., 1987; Ishii et al., 1992). Perhaps the best characterized of these functions is the requirement for UNC-6, UNC-5, and UNC-40 during distal tip cell (DTC) migration and guidance (Hedgecock et al., 1990). During hermaphrodite development two specialized leader cells located at the end of each gonad arm known as DTCs guide elongation of the gonad during larval stages (Hedgecock et al., 1987). Initially, the gonad extends anteriorly and posteriorly along the body wall but at a precise time during the third larval stage, executes a 90-degree turn dorsally. The gonad turn at this stage is guided in large part by UNC-6-mediated repulsion through UNC-5 and, to a lesser extent, by UNC-40 (Ishii et al., 1992; Chan et al., 1996; Su et al., 2000). The precise temporal control of the dorsal turn in response to UNC-6 involves the transcriptional regulation of UNC-5 by the steroid hormone receptor DAF-12. This receptor responds to ligand produced by a biochemical pathway involving DAF-9 that coordinates growth, development, and lifespan of the worm (Su et al., 2000; Mak and Ruvkun, 2004). As DAF-9 and DAF-12 are required for proper regulation of the dauer state and gonad development arrests in dauer larvae, these data demonstrate one mechanism by which organogenesis can be contingent upon environmental cues.

Neither the precise interplay between UNC-5 and UNC-40 during DTC turning nor their downstream effectors are well understood (Merz et al., 2001). Cytoskeletal signaling proteins including the *C. elegans* Rac orthologues MIG-2 and CED-10 and the RacGEFs, UNC-73/Trio and CED-5/DOCK180, have been shown to regulate DTC guidance; however, their mutant phenotypes are somewhat different from those seen in *unc-6, unc-40*, or *unc-5* mutants. Therefore, none of these proteins are dedicated solely to the UNC-6/Netrin pathway during DTC development, though it is still possible that they are regulated in some way by Netrin signaling (Zipkin et al., 1997; Wu and Horvitz, 1998; Lundquist et al., 2001). Perhaps more specific is the function of SRC-1, one of two *C. elegans* src family kinases, during DTC guidance. *Src-1* zygotic mutants have a gonad-turning defect similar to UNC-6/Netrin pathway mutants and can suppress the precocious gonad turning caused by forced early expression of UNC-5 within the DTCs (Itoh et al., 2005). While these data are exciting, additional work is required to determine the precise mechanism of UNC-5 signaling through SRC-1 during DTC turning.

More recent work, encompassing both genetic and cell-biological approaches, has revealed functions for Netrin signaling outside of cellular motility in both neuronal and non-neuronal cells. These roles include neuronal regionalization, neuromuscular junction formation, cell polarity, and even cell invasion through basement membrane (Fig. 2a, b) (Adler et al., 2006; Colon-Ramos et al., 2007; Poon et al., 2008; Alexander et al., 2009; Ziel et al., 2009a). Because these represent fundamental cell biological processes, functions for Netrin signaling in these contexts may be conserved in other organisms.

One novel role for Netrin signaling in *C. elegans* was discovered through a visual genetic screen to identify genes controlling synapse formation between two interneurons, AIY and RIA (Colon-Ramos et al., 2007). In wild-type worms, AIY synapses onto RIA at a precise and reproducible location in the nerve ring, near the pharynx (Fig. 2b). Mutations in *unc-40/DCC* cause mislocalization of pre-synaptic components (e.g., RAB-3, ELKS-1, and SYD-1) within the AIY axon. Specification of the pre-synaptic zone by UNC-40/DCC occurs cell-autonomously and UNC-40/DCC protein is enriched in the pre-synaptic zone. Proper localization of UNC-40/DCC to the pre-synaptic region requires local UNC-6/Netrin secretion from ventral cephalic sheath cells, glia-like cells that extend cellular processes contacting AIY synaptic sites (White et al., 1986; Shaham, 2006).

While Netrin signaling through UNC-40/DCC is an important mechanism for organizing pre-synaptic regions in the AIY neuron, mutations in *unc-5* cause defects in specifying dendritic regions in other neuronal cells (Poon et al., 2008). DA9 is a motor neuron whose cell body is located at the posterior of the worm and produces a functionally distinct dorsal axon and ventrally located dendrite (Fig. 2b). Because UNC-6/Netrin is secreted from the ventral nerve cord, it is likely that dendritic regions experience a much higher level of UNC-6/Netrin protein than axonal sites (Wadsworth et al., 1996). This presumably leads to enhanced activation of UNC-5 receptors within dendritic zones. Thus, as in axon guidance, localized UNC-6/Netrin secretion provides spatial information that can inform the selection of both dendritic and synaptic sites, depending upon the cell-surface receptor available for signaling. UNC-5 protein is expressed in both the dendrite and ventral axon of DA9. Whether it is enriched at one of these positions analogous to the enrichment of UNC-40/DCC at pre-synaptic sites in AIY is not clear. It is also not known whether UNC-5 localization or presentation at the plasma membrane is regulated by UNC-6/Netrin in DA9. Thus, additional studies are required to determine how the UNC-6/Netrin protein gradient is interpreted by UNC-5.

Recent work has shown that components of the Netrin signaling pathway also regulate post-synaptic development at the neuromuscular junction (Alexander et al., 2009). Formation of neuromuscular connections in animals is mediated in part by membrane extensions from muscle cells known as myopodia (Ritzenthaler et al., 2000). In *C. elegans*, similar, but exaggerated structures referred to as muscle arms extend from body-wall muscles towards pre-specified synaptic sites on motoneuron axons in the ventral and dorsal nerve cords to form neuromuscular junctions (Fig. 2b) (Dixon and Roy, 2005). Loss-of-function mutations in *unc-40/DCC* were isolated in forward genetic screens for Muscle arm development defective (Madd) mutants, which caused a severe reduction in the number of muscle arms projecting from body wall muscles. UNC-40/DCC functions within the muscle cells to direct muscle arm extension and is enriched in the muscle arm projections. Strangely enough, UNC-40/DCC function in muscle arm extension does not depend upon UNC-6/Netrin. While this may reflect binding of UNC-40/DCC to a cryptic ligand, an interesting alternative explanation suggested by these authors is that UNC-40/DCC might respond to other internal polarity cues and then signal in a ligand-independent fashion (Alexander et al., 2009). Additional work is required to distinguish between these possibilities; however, in *C. elegans at* least two other developmental events (elongation of the AVM axon along the anterior-posterior axis and posterior migration of the Q_L neuroblast) require UNC-40/DCC signaling without a similar requirement for UNC-6/Netrin (Honigberg and Kenyon, 2000; Yu et al., 2002). These studies suggest the intriguing possibility that DCC receptors have the capacity to signal independent of ligand.

While the previous instances highlight examples of non-traditional Netrin signaling in the regulation of neuronal cells and neuronal connectivity, work from our own laboratory illustrates functions of Netrin signaling in a non-neuronal context during cell invasion through basement membranes (Ziel et al., 2009a). In *C. elegans*, passage of fertilized eggs from the hermaphrodite into the external environment requires a functional connection between the somatic gonad and the vulva, which initially form as separate epithelia enveloped by basement membranes during early larval development (Newman and Sternberg, 1996). During late larval development, a specialized invasive cell within the somatic gonad known as the anchor cell (AC) invades through the basement membranes and connects these tissues (Fig. 2b). Initiation of invasion at the appropriate time depends upon a diffusible chemotactic cue secreted by the vulval cells (Sherwood and Sternberg, 2003). With the goal of identifying genes in the vulval cue pathway, we took a candidate approach by examining mutations in *C. elegans* genes important for cellular motility or morphogenesis. Mutations in *unc-6/Netrin* or *unc-40/DCC* cause penetrant delays in AC invasion, in some cases leading to a complete failure of invasion (Ziel et al., 2009a). UNC-40/DCC is required in the AC, and polarizes to the invasive basal plasma membrane in contact with the basement membrane, where it establishes a specialized invasive membrane domain (see below and Fig. 3a). Polarization of UNC-40/DCC to the invasive membrane depends upon UNC-6/Netrin, which, as in vertebrate tissues, accumulates within the basement membranes separating these tissues (Serafini et al., 1994; Manitt et al., 2001; Ziel et al., 2009a). Surprisingly, UNC-6/Netrin is not produced by the vulval

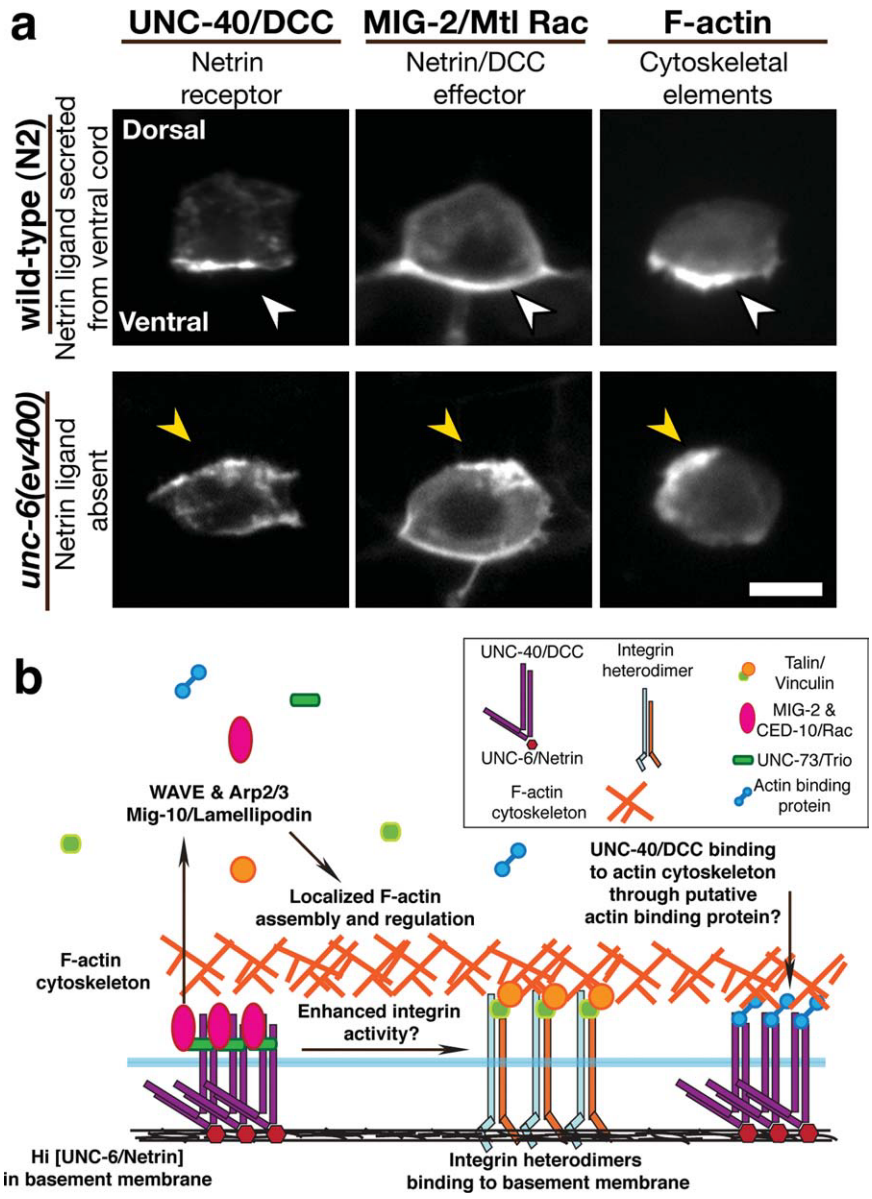

Fig. 3. **a**: Confocal micrographs of anchor cells expressing various fluorescent markers in *C. elegans*. UNC-6/Netrin signaling from the ventral nerve cord polarizes UNC-40/DCC ventrally (white arrowhead) towards the basement membrane and vulval tissue. In turn, the Netrin signaling recruits cytoplasmic signaling effectors such as the MIG-2/Mtl Rac. Through this signaling pathway, the actin cytoskeleton is oriented towards the basement membrane for invasion. In *unc-6* mutants, polarity of UNC-40/DCC is lost and F-actin and MIG-2/Mtl Rac can accumulate on apical and lateral membranes (yellow arrowheads). Scale bar at bottom left = 5 μm. **b**: In one possible model for UNC-6/Netrin signaling through UNC-40/DCC receptors to polarize a cell, binding of the UNC-40/DCC to UNC-6/Netrin on neighboring cells or basement membranes concentrates the receptor. This leads to local recruitment and activation of the Rac-like proteins MIG-2/Mtl Rac and/or CED-10/Rac, perhaps by UNC-73/Trio, which can bind to the intracellular domains of UNC-40/DCC, Frazzled/DCC, and Human DCC. Downstream of Rac activation, the pathway may diverge, utilizing context-specific regulators and effectors such as MIG-10/Lamellipodin, a known Rac effector and cytoskeletal regulator.

cells but rather originates from the ventral nerve cord to promote invasion (Fig. 2b). Thus UNC-6/Netrin is not a component of the vulval cue but rather a second extracellular signal regulating invasion.

MECHANISMS OF NETRIN SIGNALING: PUTTING THE RECEPTOR IN ITS PLACE

A common function of these Netrin pathways is to establish specialized membrane-associated domains within cells that will be dedicated to specific cellular functions such as invasion or synaptogenesis. While it is not clear from current data whether UNC-5 receptors are concentrated by Netrin signaling, signal transduction through UNC-40/DCC clearly involves receptor polarization to specific regions of cells. Localized UNC-40/DCC receptors then specialize these regions for particular cellular functions. During synaptogenesis and AC invasion, proper localization of UNC-40/DCC depends upon a localized source of UNC-6/Netrin (from ventral cephalic sheath cell contacts or in the basement membrane, respectively; Fig. 2b) (Colon-Ramos et al., 2007; Ziel et al., 2009a). How localization of UNC-40/DCC is regulated by UNC-6/Netrin is not clear based upon current data. One simple possibility is that binding to UNC-6 physically anchors the receptor, stabilizing it within a specific region of the plasma membrane. However, UNC-40/DCC is localized in muscle cells during muscle arm development without a requirement for UNC-6 (Alexander et al., 2009). Thus, other mechanisms must also control UNC-40/DCC distribution, perhaps by influencing its insertion and removal at the plasma membrane. Consistent with this idea, in the AC, targeting of UNC-40/DCC to the plasma membrane depends upon the Integrin heterodimer INA-1/PAT-3, though the mechanism underlying this regulation is unclear (Hagedorn et al., 2009). In vertebrate neurons, presentation of DCC receptors at the plasma membrane is highly regulated (Bouchard et al., 2004, 2008; Moore et al., 2008). There, Netrin-1 can promote plasma membrane localization of DCC from intracellular vesicle pools, an effect that is potentiated by protein kinase A (PKA) activation (Bouchard et al., 2004). PKA signaling to promote DCC plasma membrane delivery requires both phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC). Importantly, all of these proteins have been implicated in modulating Netrin-mediated axon guidance pathways (Barallobre et al., 2005; Bouchard et al., 2008; Moore et al., 2008). One potential mechanism linking all of

these observations is that Netrin signaling or other cues (e.g., Integrin signaling) activate PKA, PI3K, and PKC in a limited sub-cellular domain, leading to local delivery and concentration of DCC receptors.

RECEPTOR LOCALIZATION FOR SUB-CELLULAR SPECIALIZATION

The cellular effectors downstream of Netrin signaling during synaptogenesis and dendrite specification are unknown. Fortunately, work in both the AC and muscle cells have begun to shed light on the polarizing activity of localized UNC-40/DCC. In the AC, localization of UNC-40/DCC to the invasive plasma membrane leads to a profound orientation of actin regulatory activities towards the basement membrane (Ziel et al., 2009a). During invasion, Netrin signaling concentrates known effector proteins, UNC-34/Enabled, CED-10/Rac and MIG-2/Mtl Rac, to the invasive plasma membrane (Fig. 3a). UNC-34/Enabled is required for normal invasion, while MIG-2/Mtl Rac and CED-10/Rac function redundantly during invasion. In response to the activities of these proteins, the cortical region of the invasive plasma membrane becomes enriched in F-actin (Fig. 3a), while the plasma membrane accumulates the critical signaling lipid, phosphatidylinositol 4,5-bisphosphate (PIP2).

In muscle cells, UNC-40/DCC is important for stimulating outgrowth of the muscle arms, and UNC-40/DCC is specifically enriched within the muscle arms (Alexander et al., 2009). By examining worms expressing very high levels of UNC-40/DCC within the muscle cells (transgenic arrays were created with 25-fold more *unc-40::GFP* sequence than used for rescue), Alexander et al. (2009) found that UNC-40/DCC could trigger the formation of ectopic myopodia from muscle cell membrane. These ectopic myopodia can be suppressed by mutations in UNC-73/Trio, a guanine nucleotide exchange factor (GEF) for Rac and Rho proteins, as well as by RNA interference targeting components of the WAVE complex (*gex-2, gex-3*, and *wve-1*), which is known to function downstream of Rac (Miki et al., 1998). These results indicate that as in the AC, Rac signaling is a major mechanism downstream of UNC-40/DCC to direct muscle arm development. While the GEF functioning downstream of UNC-40/DCC in the AC is not known, UNC-73/Trio is expressed during invasion and point mutations in the RacGEF domain cause defects in AC invasion (J.W.Z. and D.R.S., unpublished observations; Ziel et al., 2009b).

NETRIN SIGNALING AND THE COORDINATION OF MULTIPLE PATHWAYS FOR DEVELOPMENT

Examining how Netrin signaling promotes AC invasion provides a paradigm for understanding the function of Netrin signaling in the context of other signaling pathways. Loss of Netrin signaling, which disrupts AC polarity, attenuates the AC's responsiveness to the vulval cue that stimulates invasion (Ziel et al., 2009a). These results indicate that UNC-40/DCC, localized to the invasive plasma membrane, prepares the AC for signaling by the vulva, perhaps by concentrating actin regulators or the receptor for the vulval cue. Furthermore, both Netrin signaling and the vulval cue pathway promote AC adhesion to the basement membrane during invasion. In the absence of UNC-6/Netrin and vulval cells, the AC detaches from the basement membrane in most worms (Ziel et al., 2009a). These results suggest that signaling by the vulval cue and UNC-6/Netrin feed back onto the Integrin pathway, the primary mode of cell-basement membrane attachment. Consistent with this idea, there are strong genetic interactions between mutations in *unc-40/DCC* and alpha Integrin *ina-1* alleles (Hagedorn et al., 2009). In part, this probably reflects Integrin function in targeting UNC-40/DCC to the plasma membrane. It is also likely that UNC-40/DCC activity regulates Integrin signaling through intracellular mechanisms. Interestingly, like AC invasion, Integrin signaling is required for normal muscle arm extension (Dixon et al., 2006). Whether a similar relationship between the UNC-40/DCC and Integrin signaling pathways operates during muscle arm development and cell invasion is an intriguing possibility that future work should address. These results highlight how Netrin signaling can coordinate multiple genetic pathways though its function in establishing a specialized subcellular domain.

PERSPECTIVES

In *C. elegans*, examination of Netrin signaling outside of neurons has shown critical roles for this pathway in regionalizing and polarizing a variety of cell types. Much is unknown about the mechanisms underlying this activity, though studies of Netrin signaling during AC invasion, neuronal regionalization, and muscle arm formation have suggested a few general principles (Fig. 3b). First, regulation of UNC-40/DCC localization is a consistent and likely important component of regionalizing membrane-associated domains within cells. Subcellular localization of the receptor is usually regulated by the extracellular UNC-6/Netrin gradient. However, this need not be the case, as muscle arm extension does not require Netrin ligands. Second, because UNC-40/DCC signaling can trigger regionalization in variety of cell types, we assume that this requires local activation of a core signaling mechanism that at some point triggers or recruits cell type–specific effectors. During AC invasion, muscle arm extension and in vertebrate neurons localization or activation of Rac signaling is a consistent event downstream of DCC receptors. Because the RacGEF Trio can bind DCC receptors in vertebrates, *C. elegans*, and *Drosophila*, a pathway leading from DCC to Trio to the activation of Rac may be one example of a core mechanism utilized by Netrin signaling (Forsthoefel et al., 2005; Watari-Goshima et al., 2007; Briancon-Marjollet et al., 2008). Finally, specific sub-cellular localization of UNC-40/DCC and its core signaling mechanisms leads to specialization of the cell membrane and cortex by recruiting proteins important for a specific cellular process. For example, during AIY pre-synaptic development these include SYD-1 and RAB-3, proteins important for synaptogenesis and

synaptic signaling (Nonet et al., 1997; Hallam et al., 2002).

Rac GTPases are critical regulators of the actin cytoskeleton, often through regulation of the WAVE complex and the Arp2/3 actin nucleator (Soderling and Scott, 2006). Nucleation of F-actin drives muscle arm extension and is associated with AC invasion (Dixon and Roy, 2005; Alexander et al., 2009; Ziel et al., 2009a). Actin cytoskeletal elements and regulation of the actin cytoskeleton play a key role in polarizing multiple cell types, for example by capturing microtubules to form oriented tubulin arrays or by influencing plasma membrane contractility (Li and Gundersen, 2008). Thus, formation of an F-actin network in the vicinity of DCC receptors is a consistent and perhaps critical feature of cellular polarization by Netrin ligands (Fig. 3b). Because Integrin heterodimers bind to F-actin to form cell-matrix adhesions, it is possible that regulation and generation of actin networks is also a mechanism for cross-talk between the Netrin pathway and Integrin signaling (Vicente-Manzanares et al., 2009). In addition, DCC receptors might function as bona-fide adhesion molecules by binding to the F-actin network, possibly through an unidentified actin-binding protein. Consistent with this possibility, a recent report has shown that commissural neurons, utilizing DCC, experience traction forces on immobilized Netrin-1, indicating a functional link between Netrin signaling and adhesion (Moore et al., 2009).

In the hermaphrodite-specific neurons (HSNs), which innervate the egg-laying muscles in *C. elegans*, UNC-40/DCC is critical for polarizing ventral axon outgrowth in response to UNC-6/Netrin. Here, UNC-40/DCC becomes ventrally enriched, where it presages the site of axonogenesis (Adler et al., 2006). At that site, UNC-40/DCC signaling acts through molecules that alter plasma membrane lipid composition (AGE-1/phosphatidylinositol 3-kinase) and the cytoskeleton (CED-10/Rac and the Rac effector MIG-10/Lamellipodin) (Quinn et al., 2008). This neuronal model of Netrin regulation reinforces the importance of UNC-40/DCC enrichment as a general mechanism to polarize cells. Also similar to AC invasion and muscle arm development, ventrally localized UNC-40/DCC engages core signaling components including the Rac GTPase CED-10. Because we have not detected a strong requirement for MIG-10/Lamellipodin during AC invasion and one has not been reported in muscle arm extension, this may be an example of a cell type–specific effector protein that is utilized by Netrin signaling downstream of Rac activation to promote axonogenesis. These results indicate that UNC-40/DCC signaling outside of neuronal cells has clear parallels to events early in axon guidance.

We are not aware of evidence demonstrating asymmetric localization of UNC-40/DCC within growth cones themselves. However, a model for growth cone guidance involving the asymmetric distribution of UNC-40/DCC in the growth cone plasma membrane in response to UNC-6/Netrin gradients has recently been proposed (Quinn and Wadsworth, 2008). It is tempting to speculate that a polarity-based mechanism may extend to signaling through UNC-5 as well. Repulsive signaling through UNC-5 and its relationship to DCC signaling pathways are poorly understood. For example, it is not known whether UNC-5 is localized by Netrin ligands, or if *unc-5*, like *unc-40/DCC*, has strong genetic interactions with pathways important for cell-matrix adhesion. Answers to these questions will have clear importance across species as UNC-5 family receptors are critical for mediating Netrin signaling during angiogenesis in vertebrates (Lu et al., 2004; Navankasattusas et al., 2008). Moreover, recent work has shown that expression of Unc5b by leukocytes prevents tissue invasion by these cells until inflammatory stimuli down-regulate Netrin expression in the endothelium (Ly et al., 2005). These data suggest that Netrin ligands may function as bi-directional cues in cell invasion, similar to their role in axon guidance (Muller, 2009). An attractive, though speculative, possibility is that UNC-5 family receptors may work by antagonizing those pathways activated by UNC-40 or DCC. Cell biological work investigating these questions will be important for a unified picture of the mechanisms used by Netrin signaling to regulate cellular behavior across species and tissues. Given the ever-expanding list of cell-biological implements in the worm-pickers tool-kit, this is an area in which *C. elegans* research is likely to play a central role.

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