A Ligand-Gated Association between Cytoplasmic Domains of UNC5 and DCC Family Receptors Converts Netrin-Induced Growth Cone Attraction to Repulsion

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Summary

Netrins are bifunctional: they attract some axons and repel others. Netrin receptors of the Deleted in Colorectal Cancer (DCC) family are implicated in attraction and those of the UNC5 family in repulsion, but genetic evidence also suggests involvement of the DCC protein UNC-40 in some cases of repulsion. To test whether these proteins form a receptor complex for repulsion, we studied the attractive responses of *Xenopus* spinal axons to netrin-1, which are mediated by DCC. We show that attraction is converted to repulsion by expression of UNC5 proteins in these cells, that this repulsion requires DCC function, that the UNC5 cytoplasmic domain is sufficient to effect the conversion, and that repulsion can be initiated by netrin-1 binding to either UNC5 or DCC. The isolated cytoplasmic domains of DCC and UNC5 proteins interact directly, but this interaction is repressed in the context of the full-length proteins. We provide evidence that netrin-1 triggers the formation of a receptor complex of DCC and UNC5 proteins and simultaneously derepresses the interaction between their cytoplasmic domains, thereby converting DCC-mediated attraction to UNC5/DCC–mediated repulsion.

Introduction

Neuronal growth cones in the developing nervous system are guided to their targets by attractive and repulsive guidance molecules, which include members of the netrin, semaphorin, ephrin, and Slit protein families. Evidence is accumulating that several of these factors are bifunctional, producing positive growth and guidance effects on some growth cone populations and negative effects on others. Thus, netrin-1 attracts commissural axons but repels trochlear motor axons (reviewed in Tessier-Lavigne and Goodman, 1996). Similarly, Slit proteins repel a variety of classes of axons but stimulate

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elongation and branching of embryonic sensory axons (reviewed in Van Vactor and Flanagan, 1999).

Several types of mechanisms could in principle underlie the bifunctionality of guidance cues. First, bifunctionality could result if a particular cue activates a single receptor mechanism but the receptor couples to different downstream effectors within different growth cones. Evidence that this type of mechanism may operate comes from in vitro experiments on the turning responses of *Xenopus laevis* spinal axons. Repulsive actions of some guidance molecules can be converted to attraction by increasing the activity of cyclic nucleotide signaling pathways (cAMP or cGMP, depending on the cue) in responsive growth cones, and conversely, attractive actions of other guidance molecules (including netrin-1) can be converted to repulsion by decreasing the activity of these signaling pathways (cAMP, in the case of netrin-1) (e.g., Ming et al., 1997; Song et al., 1998), suggesting that activation of a single receptor can elicit different responses, depending on the state of the growth cone.

Bifunctional effects of guidance cues could also result from activation of different types of receptors for attraction and repulsion. Evidence for this second type of mechanism exists in the case of the netrins, for which two families of receptors, the Deleted in Colorectal Cancer (DCC) and UNC5 families, have been defined. Attractive effects of the netrins require the function of DCC family receptors. These transmembrane proteins, which include DCC and neogenin in vertebrates, UNC-40 in *Caenorhabditis elegans*, and Frazzled in *Drosophila melanogaster*, all have similar structures, with four immunoglobulin (Ig) and six fibronectin type III domains in their extracellular portions and long (~350 amino acid) cytoplasmic domains that share several domains of homology across species. DCC is a netrin receptor (Keino-Masu et al., 1996), and loss of function of UNC-40, Frazzled, or DCC (in the appropriate species) results in misrouting of axons that are normally attracted to a netrin source (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996; Fazeli et al., 1997), implying that these proteins are receptors or components of receptor complexes that mediate attractive effects of netrins. In the case of repulsion, studies in *C. elegans* have implicated the transmembrane protein UNC-5 (Leung-Hagesteijn et al., 1992) in mediating repulsive actions of the netrin UNC-6, since (1) loss of function of *unc-5* in neurons that normally project away from an UNC-6 netrin source results in misrouting similar to that observed in *unc-6* mutants (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; McIntire et al., 1992; Wadsworth and Hedgecock, 1996), and (2) misexpression of *unc-5* in some axons that ignore an UNC-6 netrin source—and in others that grow toward the source—causes these axons to be redirected away from the source (Hamelin et al., 1993). Three vertebrate homologs of UNC-5 have been identified (UNC5H1, UNC5H2, and UNC5H3; Ackerman et al., 1997; Leonardo et al., 1997). These proteins share with UNC-5 a common structure including two
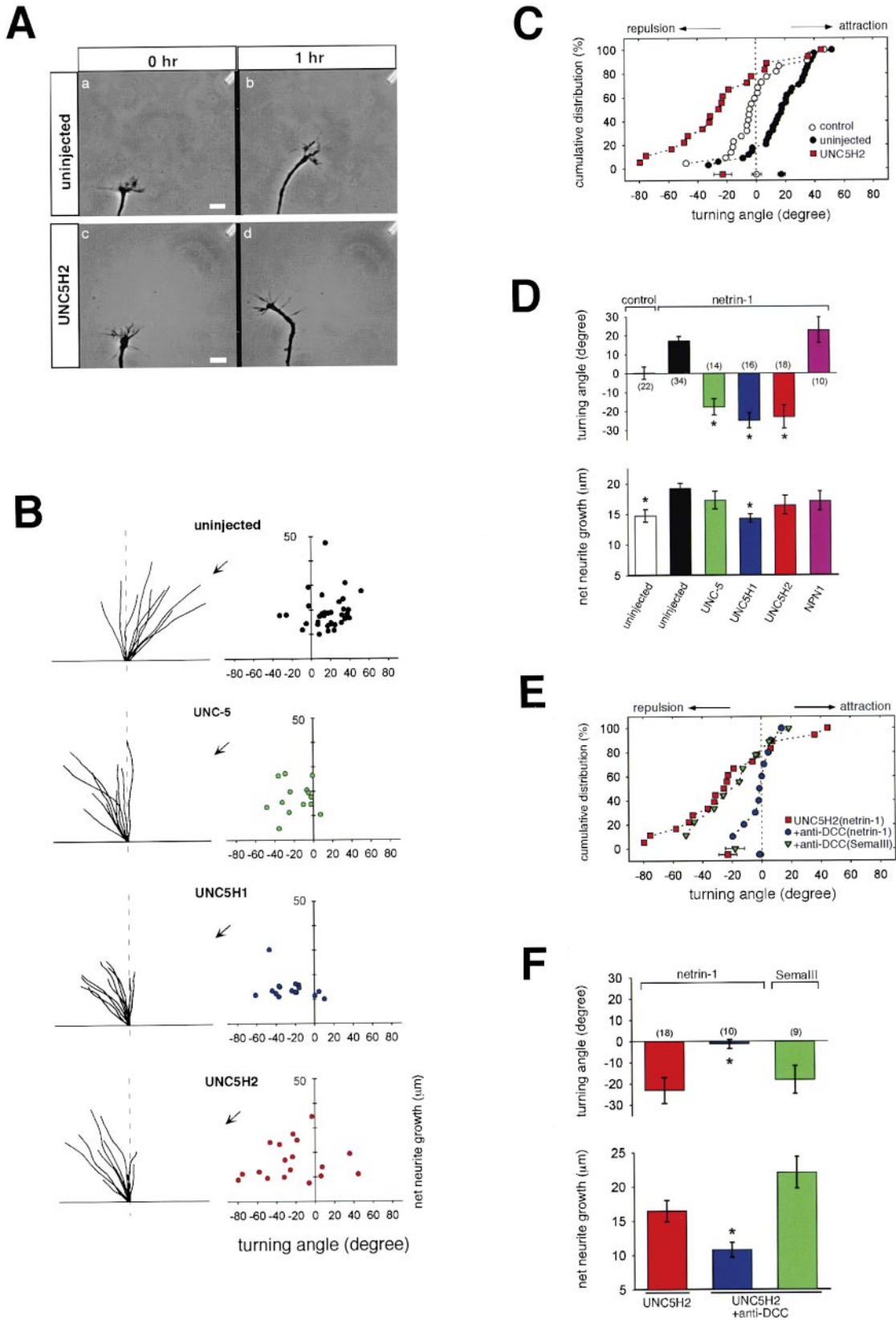
Figure 1. Heterologous Expression of UNC5 Proteins Converts Netrin-Mediated Attraction to Repulsion

(A) Images of representative growth cones at the onset (a and c) and at the end (b and d) of a 1 hr exposure to a gradient of netrin-1 (5 μg/ml in the pipette), for a control neuron derived from uninjected blastomeres (a and b), and a GFP-expressing neuron from an embryo injected with *Unc5h2* mRNA (an "UNC5H2-expressing neuron"; see text) (c and d). Scale bar, 10 μm.

Ig and two thrombospondin type 1 (TSP-1) domains extracellularly and a long cytoplasmic domain ($\sim$550 amino acids) with at least two conserved motifs: a ZU-5 domain (homologous to a portion of Zona Occludens-1; Schultz et al., 1998) and a Death Domain at the extreme carboxy terminus (Hofmann and Tschopp, 1995). All three vertebrate UNC5 proteins are netrin-binding proteins (as is expected, but not yet shown, for *C. elegans* UNC-5), and analysis of a mouse mutant in the *Unc5h3/Rcm* gene suggests that the vertebrate proteins might similarly be receptors involved in repulsion (Leonardo et al., 1997; Przyborski et al., 1998).

The simplest model for netrin bifunctionality would be that DCC receptors mediate attraction and UNC5 receptors repulsion. However, indirect evidence also suggests that the relation of netrin-mediated attraction and repulsion might be more complex. In both *C. elegans* and vertebrates, many neurons that express *unc-5* family genes also coexpress *Dcc* family genes; for example, all the neurons in *C. elegans* in which UNC-5 has been implicated in netrin-mediated repulsion also coexpress the *Dcc* family member *unc-40* (Chan et al., 1996; Leonardo et al., 1997). Furthermore, loss of function of *unc-40* also impairs migrations away from the UNC-6 netrin source (although to a lesser extent than loss of *unc-5* function) (Hedgecock et al., 1990; McIntire et al., 1992; Colavita and Culotti, 1998). It is not known whether this reflects a cell-autonomous requirement for *unc-40* within the affected neurons or a nonautonomous effect of *unc-40* in other cells; indeed, a nonautonomous guidance role for the DCC protein Frazzled in *Drosophila* has recently been described (Gong et al., 1999). If the effect is autonomous, however, this would indicate that UNC-5 and UNC-40 act in concert in individual neurons to mediate repulsion.

In this study, we demonstrate that expression of *C. elegans* or vertebrate UNC5 proteins in *Xenopus* spinal cord neurons causes their axons to be repelled by netrin-1. We also show that UNC5-dependent repulsion in this assay requires DCC protein function in individual growth cones. These findings then made it possible to address two broader issues: how is repulsion specified in the structure of UNC5 proteins, and do UNC5 and DCC

proteins actually form a receptor complex important for repulsion? We show that the cytoplasmic domain of UNC5 proteins is sufficient to specify repulsion and that netrin-1 initiates the formation of a receptor complex of UNC5 and DCC proteins, simultaneously enabling a direct association of the UNC5 cytoplasmic domain with the DCC cytoplasmic domain that converts attraction to repulsion. Furthermore, our results indicate that converting attraction to repulsion may not involve simply overwhelming one response with the other, but rather effecting a molecular switch in attractive receptor function.

Results

Expression of UNC5 Proteins Converts Netrin-Mediated Attraction to Repulsion

Growth cones of *Xenopus* spinal neurons exhibit attractive turning responses in the presence of microscopic gradients of netrin-1, an effect that requires DCC function (Ming et al., 1997). To determine the effect of expressing UNC5 family proteins in these cells, mRNAs encoding UNC-5 or its mammalian homologs UNC5H1 and UNC5H2 were injected into two blastomeres of *Xenopus* embryos at the four-cell stage, together with mRNA encoding GFP (as a marker for expression of exogenous mRNAs). Postinjection embryos were allowed to develop to stage 22–24 before the spinal neurons were isolated and cultured. After 14–20 hr in culture, responses of individual growth cones to gradients of netrin-1 were examined. Figure 1A shows examples of responses for a neuron from a control embryo and for a GFP-expressing neuron from an embryo injected with *Unc5h2* mRNA. As described (Ming et al., 1997), control neurons exhibited marked chemoattraction to netrin-1 within 1 hr (Figures 1A$_a$ and 1A$_b$). The same was true for GFP-expressing neurons from embryos injected with mRNA for a control protein, the semaphorin receptor neuropilin-1 (Figure 1D). In contrast, GFP-expressing growth cones of neurons from embryos injected with *Unc5h2* mRNA showed clear repulsive responses (Figures 1A$_c$, 1A$_d$, and 1D). Similar repulsive responses were also observed when UNC5H1 or *C. elegans* UNC-5 were

(B) Superimposed traces on the left depict trajectories of neurites over a 1 hr period. Ten randomly sampled neurites are shown for control (uninjected) neurons and for neurons expressing *C. elegans* UNC-5, UNC5H1, or UNC5H2. The arrows mark the direction of the netrin-1 gradient (5 μg/ml in the pipette). The origin is the initial position of the growth cone, and the vertical dashed line shows the initial direction of neurite extension. The scatter plots on the right depict all data obtained for each group of neurons. Each dot represents the turning angle (abscissa) and the net neurite extension (ordinate) of a growth cone.

(C) Cumulative distribution of turning angles for all data obtained from uninjected (black circles) and UNC5H2-expressing (red squares) neurons in the presence of a netrin-1 gradient and for control neurons (white circles) not exposed to the netrin-1 gradient (culture medium in the pipette). The percentage value refers to the percentage of growth cones with angular positions less than a given angle, and isolated data points along the abscissa are median values for corresponding data shown above.

(D) Top panel shows the average turning angles for control neurons not exposed to a netrin-1 gradient, as well as for uninjected neurons and neurons expressing UNC-5, UNC5H1, UNC5H2, or NPN1 after exposure to a netrin-1 gradient. The bottom panel shows the net neurite extension during a 1 hr period for the same groups of neurons. The error bar represents SEM, and the number associated with each bar represents the total number of growth cones tested. Data with asterisks were significantly different from uninjected control ($p < 0.001$, Mann-Whitney U test).

(E) Cumulative distribution plot of turning angles for neurons expressing UNC5H2 in the absence (red squares) or presence of anti-DCC antibody AF5 (1 μg/ml) (blue circles) after exposure to a netrin-1 gradient. Also shown are turning angles of UNC5H2-expressing neurons induced by a Sema III gradient (green triangles) in the presence of AF5.

(F) Top histogram panel shows the mean turning angle, and the bottom panel shows the net neurite extension for the data shown in (E). Data with asterisks were significantly different from those of UNC5H2-expressing neurons ($p < 0.05$, Mann-Whitney U test). Note that in the presence of anti-DCC, the rate of neurite extension in the presence of netrin-1 was reduced (see also Figure 6H).

used, as summarized in superimposed tracings of trajectories for neurons from control embryos and embryos injected with the three different UNC5 constructs (Figure 1B), as well as in cumulative distribution plots (Figure 1C) and histograms of average turning angles (Figure 1D). Thus, expression of UNC-5, UNC5H1, or UNC5H2 converts netrin-1-induced attraction to repulsion. The results also imply that expression of GFP in these neurons is a faithful reporter of transgene expression, so we will refer below to GFP-expressing neurons from embryos injected with mRNA for an UNC5 protein as "neurons expressing" the UNC5 protein. Netrin-1 also increased the rate of growth of neurons from uninjected embryos during the 1 hr period (Figure 1D). Interestingly, expression of UNC5H1 (but not the other two UNC5 proteins) caused a reduction in the extension rate in the presence of netrin-1 (Figures 1B and 1D).

Requirement of DCC for UNC5-Mediated Repulsion

Since vertebrate UNC5 proteins (and presumably *C. elegans* UNC-5 as well) are netrin receptors, they might mediate repulsion independently of endogenous DCC in the *Xenopus* neurons, overwhelming the attractive response mediated by DCC. Alternatively, UNC5 and DCC might act together to initiate the repulsive response. These possibilities were investigated for UNC5H2 using a monoclonal antibody (AF5) directed against the extracellular domain of the DCC receptor, which blocks netrin-induced attraction of *Xenopus* axons (Ming et al., 1997). In the presence of antibody, netrin-1 had neither attractive nor repulsive effects, with the mean turning angle close to zero (Figures 1E and 1F). The effect of the antibody was specific, since it did not impair the repulsive action of the semaphorin Sema III (Figures 1E and 1F), which is mediated by neuropilin-1 (Song et al., 1998). Thus, DCC function is required for netrin-mediated repulsion observed in the presence of UNC5 proteins.

The Cytoplasmic Domain of UNC5H2 Specifies Repulsion

To test whether the ectodomain of UNC5 proteins is required for repulsion, we examined the effect of expressing a chimeric receptor in which the transmembrane and cytoplasmic domains of UNC5H2 were fused to the extracellular domain of DCC. Neurons expressing this DCC/UNC5H2 chimera showed the same repulsive response to netrin-1 as did neurons expressing UNC5H2 (Figure 2A). To determine whether the transmembrane and cytoplasmic domains of UNC5H2 need to be fused to a netrin-binding ectodomain (as is the case for DCC), we next constructed a chimeric receptor in which the transmembrane and cytoplasmic domains of UNC5H2 were fused to the ectodomain of the NGF receptor TrkA, which does not bind netrin-1 (data not shown). *Xenopus* spinal neurons do not express TrkA endogenously and do not respond to an NGF gradient with either attraction or repulsion. Neurons expressing the TrkA/UNC5H2 chimera were repelled by netrin-1 (Figure 2B), a response that was blocked by the anti-DCC antibody (Figure 2B); NGF had no effect on these neurons (data not shown).

These results suggested that the cytoplasmic domain of UNC5H2 might be sufficient for repulsion. We tested this possibility by generating a cDNA coding for the cytoplasmic domain of UNC5H2 preceded by a myristoylation sequence that targets cytoplasmic proteins to the inner leaflet of the plasma membrane (Guy et al., 1987). Neurons expressing this myristoylated UNC5H2 cytoplasmic domain construct exhibited marked repulsive responses to netrin-1 (Figure 2C). Thus, expression of the cytoplasmic domain of UNC5H2 is sufficient to convert netrin-mediated attraction to repulsion.

A Ligand-Dependent Complex of UNC5 and DCC Proteins

The finding that UNC5-dependent repulsion is blocked by antibodies to DCC raised the possibility that these proteins form a receptor complex. To test directly whether such a complex exists, we performed coimmunoprecipitation experiments. For this, we used the 9E10 antibody directed against the myc epitope on a myc-tagged UNC5H2 construct [UNC5H2(myc)] and antibodies directed against DCC. In control experiments, each of these antibodies selectively immunoprecipitated its cognate receptor from transfected COS-1 cells without any cross-reactivity, and no background precipitation was observed in vector-transfected cells (Figure 3A).

To test whether DCC and UNC5H2 form a complex, we transiently coexpressed DCC with UNC5H2(myc) or a control myc-tagged receptor, neuropilin-2A [NPN2A (myc); Chen et al., 1997] in COS-1 cells. Cells were either exposed to control medium or to netrin-1 protein, immunoprecipitations were performed using either the anti-DCC or the anti-myc antibodies, and coprecipitating proteins were analyzed by Western blotting. As shown in Figure 3B, UNC5H2(myc) coprecipitated with DCC, but only in the presence of netrin-1 (lane 4), not in its absence (lane 3), whereas NPN2A(myc) did not coprecipitate under either condition (lanes 1 and 2). Conversely, when precipitations were performed using the anti-myc antibody, DCC coprecipitated with UNC5H2 (myc) in a netrin-dependent fashion (lanes 7 and 8) but did not coprecipitate with NPN2A(myc) under either condition (lanes 5 and 6). The cytoplasmic domains of the receptors are not necessary for this interaction, since truncated receptors comprising just the ecto- and transmembrane domains of DCC and UNC5H2 also coprecipitated in a netrin-1-dependent fashion (Figure 3C) (in this case, a small amount of interaction was also observed in the absence of netrin-1, perhaps resulting from the fact that these ectodomain constructs expressed at >10-fold higher levels than the full-length constructs; data not shown).

Thus, netrin-1 triggers the formation of a heterodimeric or heteromultimeric complex involving DCC and UNC5H2. Since netrin-1 is a heparin-binding molecule (Serafini et al., 1994), receptor aggregation may result from aggregation of netrin-1 by proteoglycans on the cell surface; alternatively, individual netrin molecules might bridge receptors directly.

Association between DCC and UNC5H2 Cytoplasmic Domains Requires P1 and DB Domains In Vivo

While these results demonstrated an interaction between DCC and UNC5H2 ectodomains mediated by netrin-1, the finding that the cytoplasmic domain of UNC5H2 is
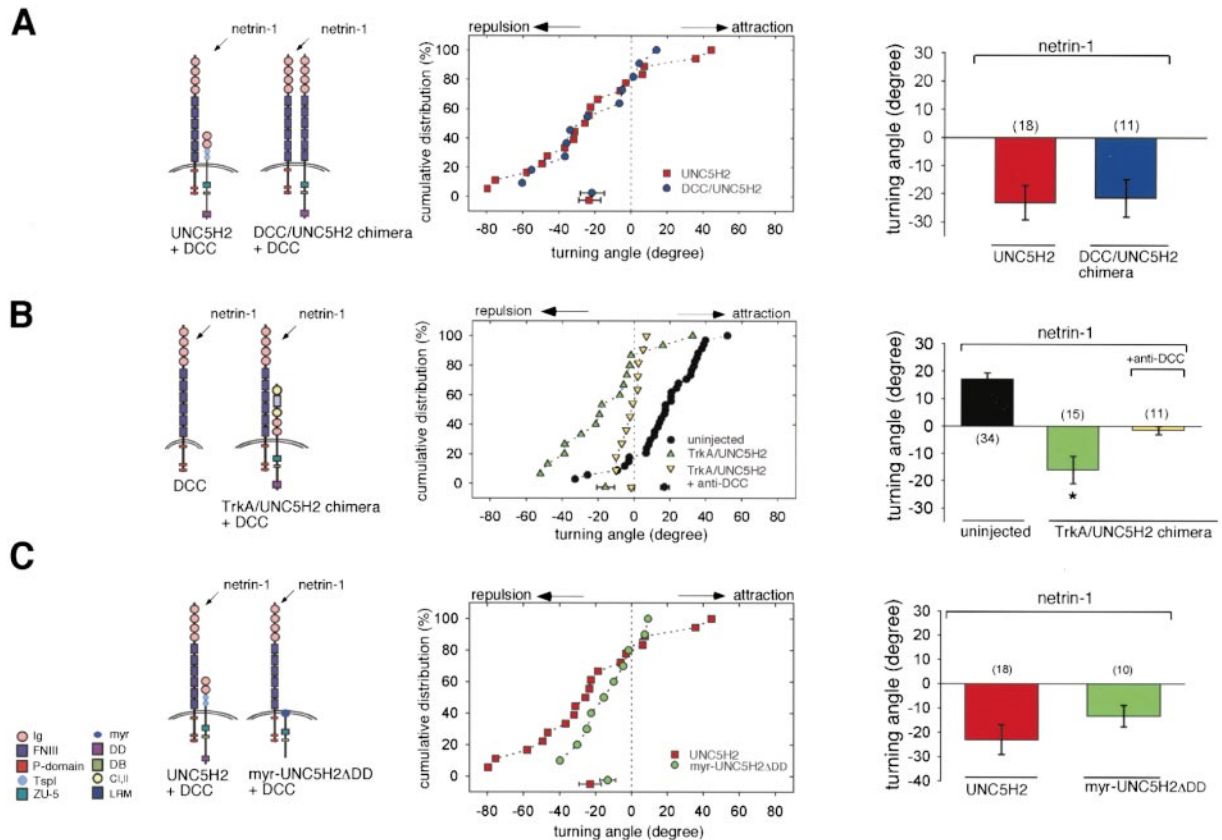
Figure 2. The Cytoplasmic Domain of UNC5H2 Is Sufficient to Convert Attraction to Repulsion

Schematic drawings on the left depict endogenous DCC, expressed UNC5H2(myc), and various chimeric receptors.

(A) Cumulative distribution plot of turning angles for neurons expressing UNC5H2 (red squares) or the DCC/UNC5H2 chimera (blue circles). Median values are indicated below by isolated data points.

(B) Cumulative distribution plot of turning angles of uninjected neurons (black circles) and neurons expressing the TrkA/UNC5H2 chimera in the absence (green triangles) or the presence of anti-DCC antibody AF5 (1 μg/ml) (yellow triangles) following exposure to a netrin-1 gradient.

(C) Cumulative distribution plot of turning angles of neurons expressing UNC5H2 (red squares) and neurons expressing myr-UNC5H2ΔDD (green circles) following exposure to a netrin-1 gradient. Data with asterisks were significantly different from those of AF5-treated neurons (p < 0.05, Mann-Whitney U test).

sufficient to convert attraction to repulsion suggested that the cytoplasmic domains of the proteins might also interact. To test this possibility, we first used the LexA yeast two-hybrid system (Vojtek et al., 1993). The yeast reporter strain L40 was cotransformed with a bait comprising the full cytoplasmic domain of DCC fused to the LexA DNA-binding domain (pBTM116-DCCcy) and a prey comprising the full cytoplasmic domain of UNC5H1, UNC5H2, or UNC5H3 fused to the VP16 activation domain (pVP16-UNC5H1, 2, and 3) or a VP16 control construct (pVP16). Whereas no growth was observed on histidine-deficient medium in transformants of the DCC cytoplasmic domain bait with the control prey alone, strong histidine-independent growth was detected with all the UNC5 cytoplasmic domain prey fusions (Figure 4A). Thus, the cytoplasmic domains of DCC and UNC5 proteins can interact in vivo in yeast.

To further dissect this interaction, we first sought to identify regions in the DCC cytoplasmic domain required for the interaction. Figure 4B displays a summary of cytoplasmic deletions of the DCCcy bait, which were cotransformed with the UNC5H2cy prey to test for histidine-independent growth. For all constructs, growth in the presence of histidine occurred under all conditions (data not shown). A construct carrying a deletion of the first 46 amino acids (aas) of the cytoplasmic domain (aas 1166–1445) did not support histidine-independent growth, but a construct encoding just the first 46 amino acids (aas 1120–1166) did (Figure 4B). Thus, the first 46 amino acids are both necessary and sufficient for the interaction. Deletion of the juxtamembrane (JM) region (aas 1120–1148) did not abolish the interaction when performed in the context of the full-length cytoplasmic domain (construct aas 1149–1445), and conversely, a construct comprising the JM domain alone (aas 1120–1149) did not support histidine-independent growth. This shows that the JM domain is neither necessary nor sufficient for the interaction and identifies amino acids 1149–1166 as a key stretch required for the interaction. These 18 amino acids comprise the P1 domain (Figure 4B), previously identified as a conserved domain among members of the DCC family (Kolodziej et al., 1996). However, a construct comprising the P1 domain alone (aas 1149–1466) was not sufficient for the interaction (Figure 4B). It is possible that the P1 domain does not fold properly in the absence of some adjacent sequences on
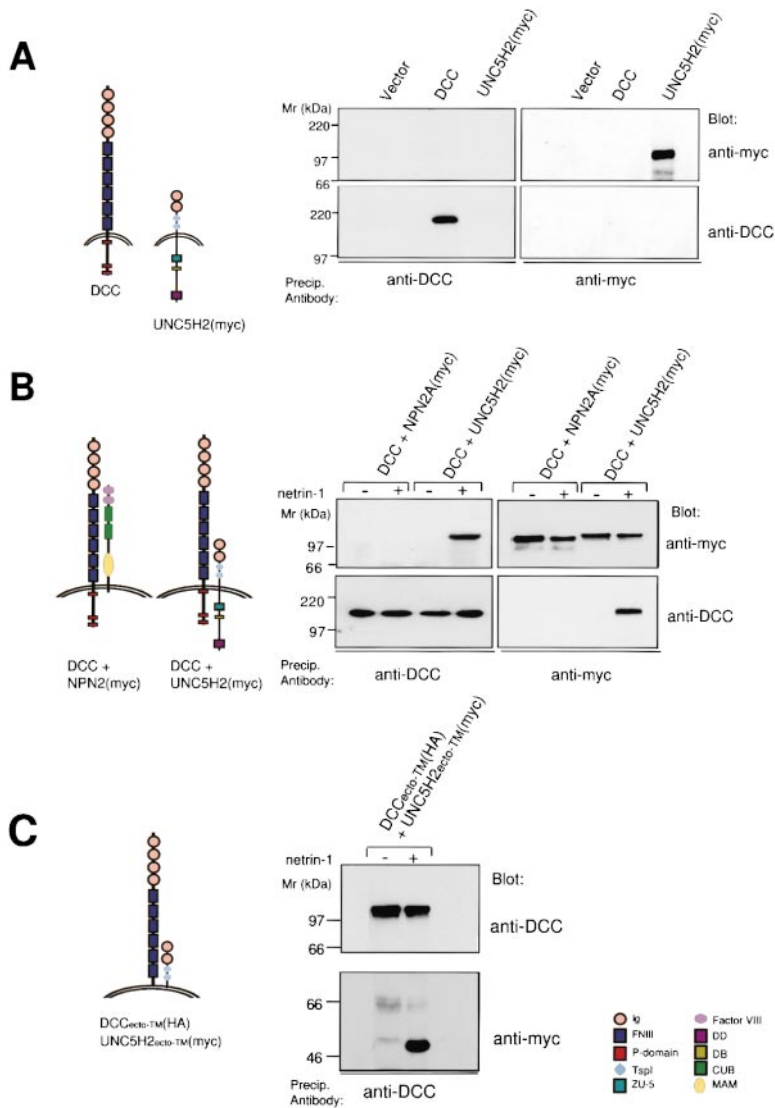
Figure 3. Ligand-Dependent Interaction of the Netrin Receptors DCC and UNC5H2

(A) Antibody characterization. COS-1 cells were transiently transfected with the indicated cDNA, and 40 hr posttransfection, cell lysates were subjected to immunoprecipitation with either an anti-myc antibody (9E10) or anti-DCC antibodies (see Experimental Procedures) and analyzed by Western blotting. 9E10 specifically recognized only the 100 kDa UNC5H2(myc) protein, whereas the anti-DCC antibodies recognized only the 160 kDa DCC protein.

(B) Ligand-dependent coimmunoprecipitation. DCC and NPN2A(myc) or UNC5H2(myc) were cotransfected in COS-1 cells. Forty hours posttransfection, cells were incubated for 20 min at 37°C with either netrin-1 or control medium, subjected to immunoprecipitation using the indicated antibodies, and analyzed by Western blotting. After exposure to netrin-1, DCC and UNC5H2 formed a coprecipitable receptor complex, but NPN2A did not associate with DCC in either case.

(C) A similar netrin-dependent coimmunoprecipitation was observed with DCC and UNC5H2 contructs comprising just the ecto- and transmembrane domains of these proteins [DCCECTO-TM(HA) and UNC5H2ECTO-TM (myc), tagged with a hemagglutinin HA epitope and a myc epitope, respectively). A small amount of interaction in the absence of ligand is observed but is greatly increased by ligand.

either its amino- or carboxy-terminal ends; alternatively, the juxtamembrane region may be redundant with some other region of the cytoplasmic domain, with either one being sufficient but at least one being necessary.

We next sought to identify the regions of UNC5 cytoplasmic domains required for DCC binding. We studied this in the context of UNC5H2 by introducing amino-terminal deletions into the VP16-UNC5H2 prey fusion and monitoring for loss of histidine-independent growth. Whereas a construct comprising UNC5H2 residues 707–946 supported histidine-independent growth, a construct comprising residues 724–946 did not, nor did constructs comprising further deletions beyond residue 724 (Figure 4C). Thus, residues 707–724 are required for binding the DCC cytoplasmic domain. These 18 residues are highly conserved among all previously described UNC5 proteins (Figure 4C), and we name this domain the DB domain (as it is required for DCC binding).

We next examined whether the interaction between DCC and UNC5 cytoplasmic domains, detected in yeast, could also be demonstrated in mammalian cells. For this, we generated cytoplasmic domain contructs for DCC (tagged with an HA epitope) and UNC5H2 (tagged with a myc epitope) that possess an N-terminal myristoylation sequence. When the constructs were cotransfected in COS-1 cells and the UNC5H2 construct precipitated with anti-myc, the DCC construct coprecipitated (Figure 4D). No coprecipitation was observed when an UNC5H2 construct was used from which residues 707–946, including the DB domain, were deleted or when a DCC construct was used from which the P1 domain was deleted (Figure 4D). These findings support the importance of the P1 and DB domains for the interaction and also indicate that the interaction between cytoplasmic domains is not due solely to the presence of myristoylation sites on the proteins, nor to their aggregation at the cell membrane.

Association between DCC and UNC5 Cytoplasmic Domains In Vitro

These studies in yeast and mammalian cells suggested that the cytoplasmic domains of DCC and UNC5 proteins might interact directly but did not exclude the possible involvement of adaptor proteins constitutively expressed in those cells. We therefore turned to an in vitro affinity-binding assay, using recombinant GST fusion
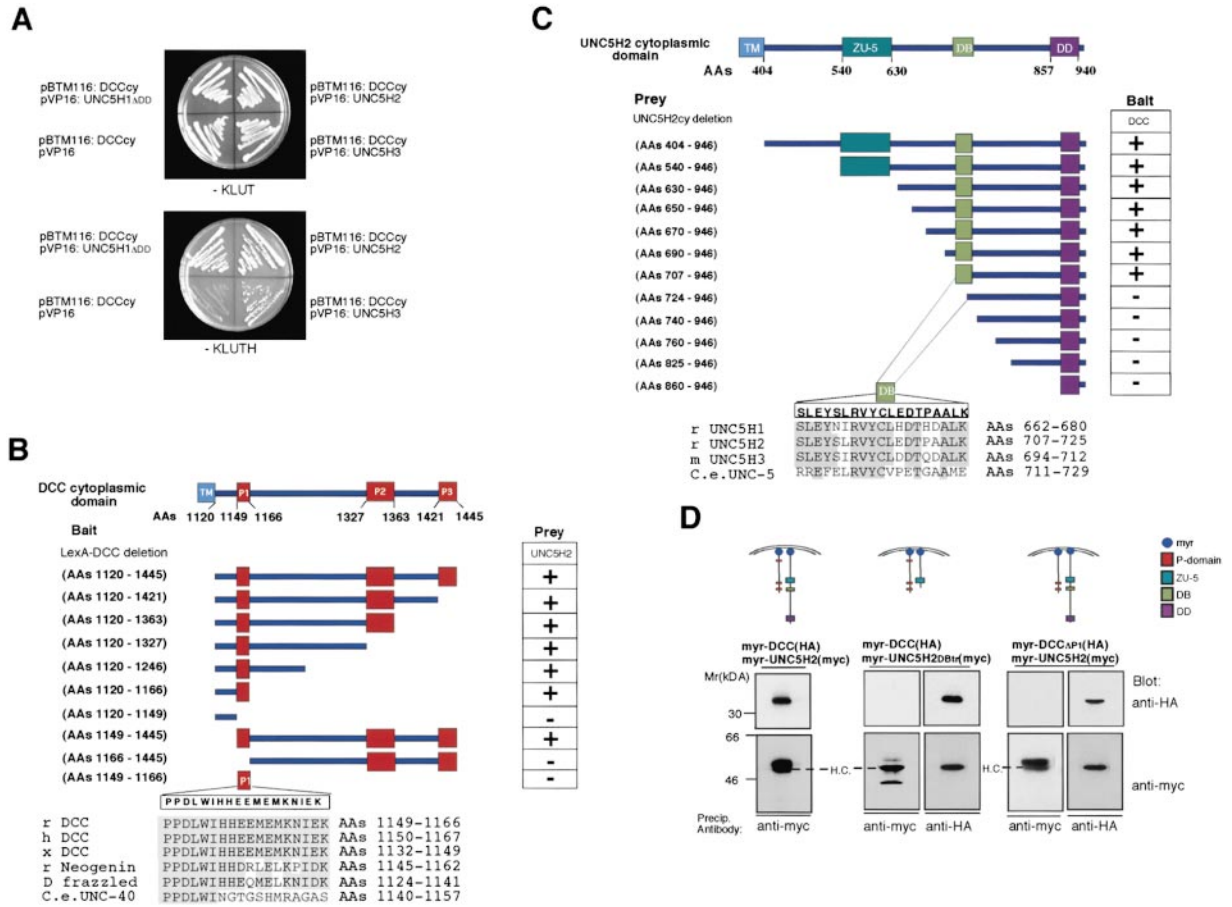
Figure 4. Interaction of the Cytoplasmic Domains of DCC and UNC-5 Homologs Detected in Yeast (Using the Two-Hybrid System) and in Mammalian Cells

(A) The cytoplasmic domains of the three vertebrate UNC5 proteins interact with the cytoplasmic domain of DCC in yeast. The reporter strain L40 was cotransformed with the indicated plasmids (see text) plated on selective medium lacking lysine, leucine, uracil, and tryptophan and grown for 3 days at 30°C. Growth of the same yeast cotransformants was compared on medium including (−KLUT) or lacking (−KLUTH) histidine. Growth was assessed and photographs taken after 3 days at 30°C.

(B) Schematic representation of DCC cytoplasmic deletion constructs and their ability to interact with the UNC5H2 cytoplasmic domain, as assessed in (A) ("+" indicates that the construct rescues, "−" that it does not). The cytoplasmic domain of DCC possesses three highly conserved domains (P1–P3) that have high homology to a similar region in the DCC family proteins UNC-40, Frazzled, and neogenin. The P1 domain is necessary for the interaction, and the P1 and juxtamembrane regions are sufficient for the interaction. An alignment of the P1 domain sequences in a variety of DCC family proteins is shown.

(C) Schematic representation of UNC5H2 cytoplasmic deletion constructs and their ability to interact with the DCC cytoplasmic domain. Truncation of UNC5H2 aas 707–724 causes loss of LexA-DCCcy bait binding. This "DB" domain is highly conserved among all UNC5 proteins previously described.

(D) An association of myristoylated cytoplasmic domain constructs of UNC5H2 and DCC can be detected by coimmunoprecipitation from transfected COS cells and does not require netrin-1 (left panel; methods as in Figure 3). Deletion of the DB motif and all sequences carboxy terminal to this motif in UNC5 (middle panels) or deletion of the P1 motif in DCC (right panels) abolished the interaction.

proteins and in vitro–translated UNC5 proteins. A recombinant GST-DCC cytoplasmic domain fusion protein (GST-DCCcy) and GST alone were expressed in Sf9 cells, immobilized on glutathione–agarose, and then incubated with in vitro–transcribed and –translated UNC5 proteins (full-length UNC5H2 and UNC5H3 and full-length UNC5H1 lacking the Death Domain [UNC5H1ΔDD]) (in the case of UNC5H1, it was necessary to delete the Death Domain in order to get sufficient expression; data not shown). Full-length NPN2A was used as a control. These proteins were labeled with ^{35}S during the in vitro translation so that bound proteins could be visualized by fluorography (Figure 5A). The three UNC5 proteins, but not NPN2A, bound beads to which GST-DCCcy had been

adsorbed but not beads to which GST alone had been adsorbed (Figure 5B, right), consistent with a direct interaction between DCC and UNC5 cytoplasmic domains.

Since the P1 motif is required for the interaction of DCC and UNC5 cytoplasmic domains in yeast, we examined whether a synthetic 18 amino acid peptide encoding this motif could compete with DCC for UNC5 binding in vitro, using a scrambled version of this peptide (C1) as a control (Figures 5C and 5D). We first identified a baseline by defining the conditions under which binding of the in vitro–translated UNC5H2 protein to GST-DCCcy was saturated. Steady-state binding was achieved when the concentrations of UNC5H2 and DCCcy protein were
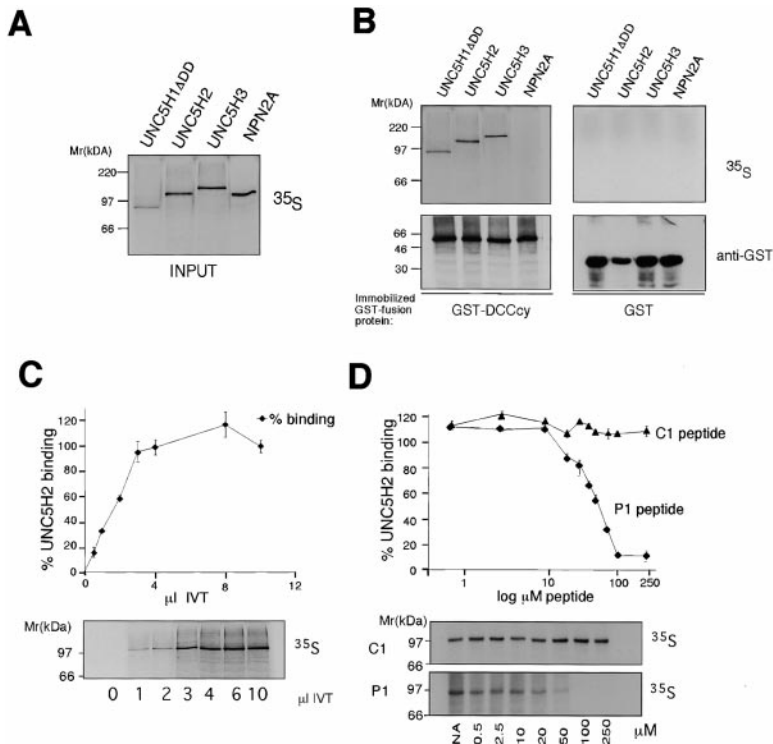
Figure 5. Interaction of the Cytoplasmic Domains of DCC and UNC5 Homologs Demonstrated In Vitro

(A) In vitro binding assay for DCC and UNC5H proteins. UNC5H1$_{\Delta DD}$, UNC5H2, UNC5H3, and neuropilin-2A (NPN2A) were transcribed and translated in vitro in the presence of [^{35}S]cysteine. An aliquot of the reaction product was separated by SDS-PAGE ("INPUT").

(B) Recombinant GST and GST-DCCcy fusion proteins were affinity purified by adsorption to GSH–agarose beads, incubated with in vitro–translated proteins for 6 hr at 4°C, and bound proteins separated by SDS-PAGE and processed by fluorography or Western blot analysis (anti-GST). UNC5H1$_{\Delta DD}$, UNC5H2, and UNC5H3 bind the DCC cytoplasmic domain, whereas NPN2A does not.

(C) Saturation binding curve for the interaction of DCCcy and UNC5H2. A given amount of immobilized GST-DCCcy fusion protein was incubated with increasing amounts of in vitro–translated UNC5H2 protein. Saturation was reached after addition of 4 μl IVT product, corresponding to approximately 5 μM UNC5H2 protein. The graph shows the fraction of the UNC5H2 IVT product that binds the GST-DCCcy fusion protein after 4 hr at 4°C and stringent washing (see Experimental Procedures). Gels at bottom show bound products (in a separate experiment).

(D) Peptide competition assay. GST-DCCcy was immobilized on GSH beads and incubated with a constant amount of in vitro–translated UNC5H2 protein and an increasing concentration of specific (P1) or scrambled (C1) peptide. Experiments were done in triplicate. One representative experiment of four is shown. Half-maximal binding of UNC5H2 was blocked in the presence of 45 μM ($\pm$ 5 μM) P1 peptide; the C1 peptide did not have any influence on binding.

about the same ($\sim$5 μM; Figure 5C). This concentration was then used for the competition experiments. Whereas the scrambled peptide had no effect, the P1 peptide competed for UNC5H2 binding with an IC50 of 45 μM, as determined by Scatchard analysis (Figure 5D and data not shown). The requirement of an $\sim$10-fold excess concentration of peptide to complete the DCC-UNC5H2 interaction could reflect the presence of additional regions in the DCC cytoplasmic domain that enhance binding or could arise if the peptide is not completely properly folded. This ratio is in the same range as that observed in other peptide competition studies (e.g., Chen et al., 1993).

Importance of Cytoplasmic Domain Interactions for Repulsion

To determine whether the interaction between DCC and UNC5 cytoplasmic domains is important for repulsion, we returned to the *Xenopus* assay and first tested the importance of the UNC5H2 DB domain through deletion analysis. mRNAs encoding various deletion constructs (Figures 6A–6C) were injected into *Xenopus* embryos and examined for their ability to mediate repulsion. Deletion of the conserved Death Domain at the carboxy terminus of UNC5H2 (construct 2, UNC5H2$_{\Delta DD}$) did not affect UNC5H2-mediated repulsion (Figures 6B and 6C). (We noted, however, that expression of the full-length construct resulted in a large amount of cell death in the cultures, and deletion of the Death Domain significantly reduced this death [see Experimental Procedures].) However, deletion of the DB domain in addition to the

Death Domain (construct 3, UNC5H2$_{\Delta DB\Delta DD}$) resulted in a dominant-negative construct that not only did not specify repulsion, but actually abolished the attractive effect of netrin-1 as well (Figures 6B and 6C). The dominant-negative action of this construct presumably arises from sequestration of endogenous DCC proteins in the growth cone into inactive complexes through netrin-mediated aggregation (see below)—a mechanism invoked to explain the dominant-negative actions of a variety of other truncated receptors (e.g., Amaya et al., 1991). This shows that the DB domain is necessary for repulsion. The DB domain was not the only domain required for repulsion, however. Deletion of both the Death Domain and 113 amino acids between the DB and the DD domains, but leaving the rest, including the DB domain, intact (construct 4, UNC5H2-$_{INBtr}$) also resulted in a dominant-negative construct. Thus, sequences between the DD and DB domains are also important for repulsion, as could arise if these sequences are important for binding adaptor proteins. Deletion of the DB domain and all sequences carboxy terminal to it (construct 5, UNC5H2-$_{DBtr}$) or deletion of all cytoplasmic domain sequences (construct 6, UNC5H2$_{ecto-TM}$) also resulted in the generation of dominant-negative constructs (Figures 6B and 6C), as expected.

The importance of the DB domain was confirmed independently using deletions of the myristoylated UNC5H2 cytoplasmic domain construct (Figure 6D). Whereas the wild-type construct (lacking the Death Domain) (construct 7, myr-UNC5H2$_{\Delta DD}$) converted attraction to repulsion (as described above), a myristoylated construct in

which the DB domain was deleted (construct 8, myr-UNC5H2ΔDBΔDD) had no effect, that is, the growth cones were attracted to netrin-1 (Figures 6E and 6F). Confocal analysis of axons expressing the full-length or the DB-deleted myristoylated constructs showed that the level of expression and distribution of the expressed proteins were indistinguishable (data not shown), suggesting that the absence of effect of the DB deletion construct is due specifically to absence of the DB domain, not to an effect on expression levels. The fact that the DB-deleted cytoplasmic domain construct does not have a dominant-negative effect is expected, since it is not anticipated to sequester DCC into inactive complexes (see below).

We also wished to examine the importance of the DCC P1 domain for repulsion. This was made more difficult by the fact that the neurons endogenously express wild-type DCC with an intact P1 domain. To circumvent this problem, we took advantage of the observation that the anti-DCC antibody blocks repulsion of axons expressing UNC5H2 (Figure 1E) and asked whether the myristoylated DCC cytoplasmic domain could rescue repulsion when coinjected with UNC5H2. As shown in Figures 6H and 6I, a construct comprising the entire cytoplasmic domain (construct 9) rescued the repulsive function of UNC5H2 in the presence of anti-DCC, whereas a construct lacking the P1 domain (construct 10) did not. This result provides evidence (1) that netrin-1 can signal via the UNC5H2 ectodomain, (2) that this signaling still requires the DCC cytoplasmic domain, and (3) that the DCC P1 domain is required for activation of the repulsive receptor complex.

Netrin-1 Derepresses the Interaction between Cytoplasmic Domains

A paradox was raised by the finding that the isolated cytoplasmic domains of DCC and UNC5 proteins can interact, yet the full-length proteins do not coprecipitate in the absence of netrin-1 (Figure 3B). This raised the possibility that the interaction between cytoplasmic domains might be repressed in the context of the full-length proteins. To explore this possibility, we coexpressed a myristoylated cytoplasmic domain of one of the receptors (DCC or UNC5H2) with the full-length version of the other and examined whether they would coprecipitate. We found that full-length DCC coprecipitated with the myristoylated UNC5H2 cytoplasmic domain, but only in the presence of netrin-1 (Figure 7A). Similarly, only a low level of interaction of full-length UNC5H2 with the myristoylated DCC cytoplasmic domain was observed constitutively, and addition of netrin-1 dramatically increased the interaction (Figure 7B). As expected, interaction of DCC with the myristoylated UNC5H2 cytoplasmic domain was abolished by deletion of the UNC5H2 DB domain, even in the presence of netrin-1 (Figure 7C), presumably explaining the lack of dominant-negative effect of the DB-deleted UNC5H2 cytoplasmic domain construct (see above). Similarly, interaction of UNC5H2 with the myristoylated DCC cytoplasmic domain was abolished by deletion of the DCC P1 domain (Figure 7D). These results imply that in the absence of ligand, the UNC5H2 and DCC cytoplasmic domains are largely inaccessible to one another and that

addition of netrin-1 causes some change in UNC5H2 and DCC that enables association of their cytoplasmic domains.
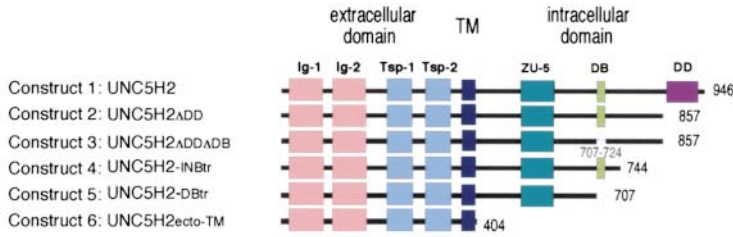
Discussion

We have shown that expression of *C. elegans* and vertebrate UNC5 proteins in *Xenopus* neurons converts netrin-mediated attraction to repulsion and that the cytoplasmic domain of UNC5 proteins is both necessary and sufficient to effect this conversion. Furthermore, UNC5 proteins appear to achieve this through formation of a receptor complex with the attractive netrin receptor DCC and direct interaction of their cytoplasmic domains, with the cytoplasmic domain of DCC being required as well. These results show that the function of UNC5 proteins—repulsion—is coded in their cytoplasmic domains, a conclusion also arrived at for the cytoplasmic domains of the attractive netrin receptor Frazzled (a DCC family member) and the repulsive Slit receptor Robo in *Drosophila* (Bashaw and Goodman, 1999 [this issue of *Cell*]). Our results also imply that the attractive and repulsive netrin receptors are designed to avoid a simple tug-of-war between attractive and repulsive responses within the same growth cone, instead favoring a model in which attraction is switched off and repulsion activated when the two types of receptors are coexpressed and their cytoplasmic domains interact.

Interactions between UNC5-Mediated Repulsion and DCC-Mediated Attraction
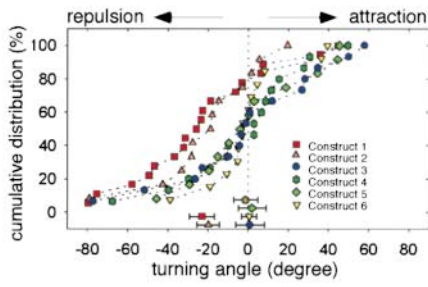
Our finding that expression of UNC5 proteins in *Xenopus* neurons converts their attractive responses to netrin-1 into repulsive responses complements and extends results obtained in vivo in *C. elegans*. UNC-5 and the netrin UNC-6 are required for migrations of cells and axons dorsally, away from the ventral midline (Hedgecock et al., 1990; McIntire et al., 1992). The findings that *unc-5* is required cell autonomously for the guidance (Leung-Hagesteijn et al., 1992) and that the ventral midline is a source of UNC-6 protein (Wadsworth and Hedgecock, 1996) have suggested a model in which UNC-5 is a component of a receptor mechanism that mediates repulsive responses to UNC-6. Furthermore, misexpression of *unc-5* in neurons whose axons normally project longitudinally (and are normally not dependent on *unc-6*) causes their axons to project dorsally in an *unc-6*-dependent fashion (Hamelin et al., 1993), consistent with the idea that UNC-5 can specify repulsion. The same effect is observed in a subset of neurons whose axons normally project ventrally in an *unc-6*-dependent fashion (Hamelin et al., 1993), suggesting that UNC-5 misexpression can convert an attractive netrin response to a repulsive response.

We have extended these observations on *C. elegans* UNC-5 to cultured *Xenopus* neurons by showing that vertebrate homologs of UNC5 also possess a repulsive function. A further parallel is found in the observation that dorsal migrations caused by *unc-5* misexpression are suppressed by loss of function of the *Dcc* family member *unc-40* (Colavita and Culotti, 1998) and our observation that antibodies to DCC block the growth
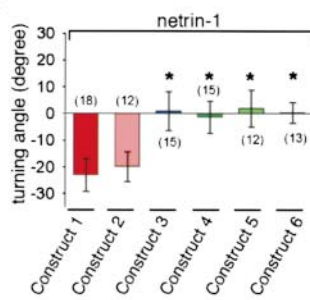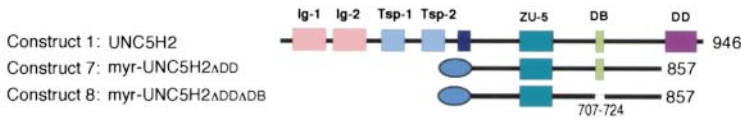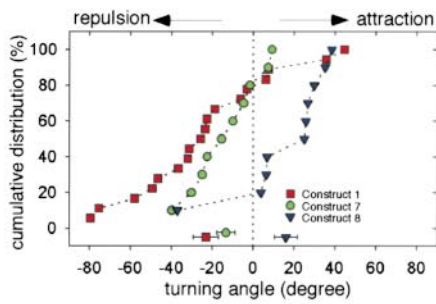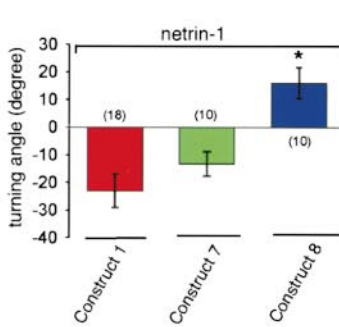
A

extracellular domain   TM   intracellular domain

Construct 1: UNC5H2
Construct 2: UNC5H2ΔDD
Construct 3: UNC5H2ΔDDΔDB
Construct 4: UNC5H2-INBtr
Construct 5: UNC5H2-DBtr
Construct 6: UNC5H2ecto-TM

B

C

D

Construct 1: UNC5H2
Construct 7: myr-UNC5H2ΔDD
Construct 8: myr-UNC5H2ΔDDΔDB

E

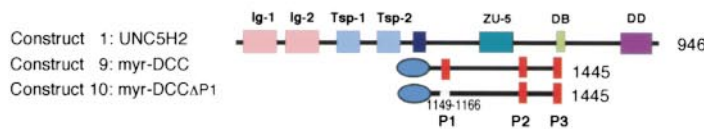**F**

G

Construct 1: UNC5H2
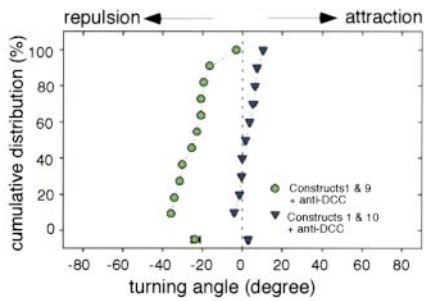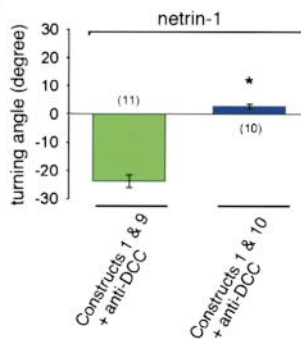Construct 9: myr-DCC
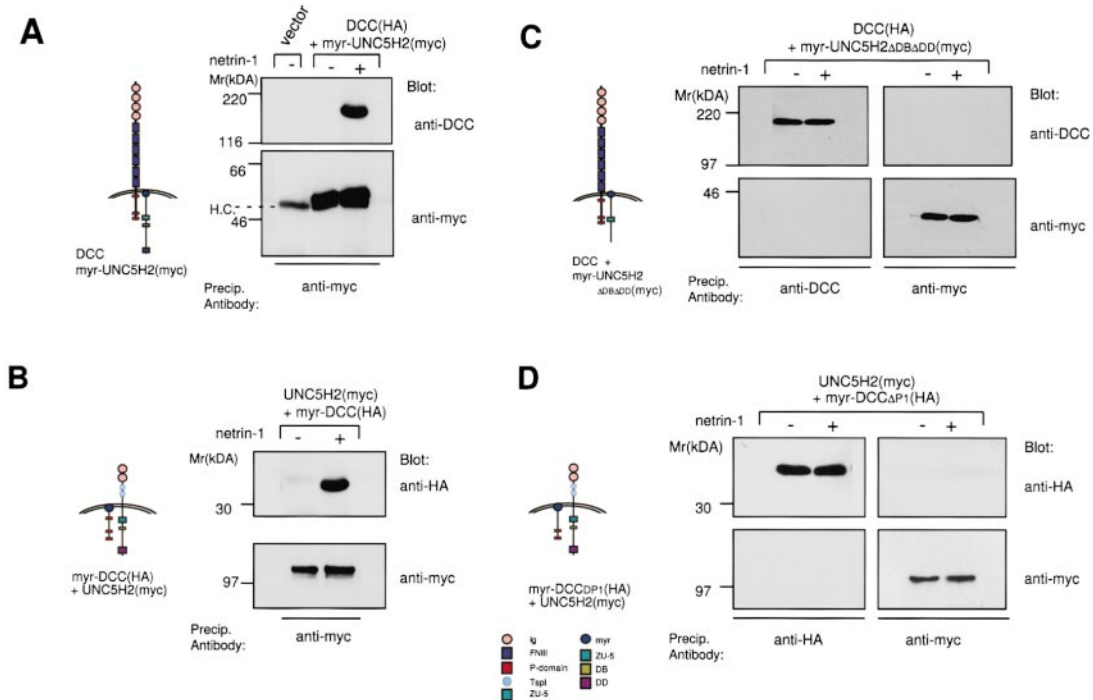Construct 10: myr-DCCΔP1

H

I

Figure 7. Netrin-Dependent Interaction of DCC and UNC5H2 Cytoplasmic Domain in the Absence of the DCC or the UNC5H2 Ectodomain

Cells were transfected with vector alone ([A], left panel), full-length DCC and myr-UNC5H2ΔDD(myc) (A), myr-DCC(HA) and full-length UNC5H2-myc (B), full-length DCC and myr-UNC5H2ΔDB(myc) (C), or full-length UNC5H2(myc) and myr-DCCΔP1(HA) (D). Thirty-six hours posttransfection, cells were incubated with netrin-1 (+) or control medium (−) for 20 min at 37°C and processed for immunoprecipitation as described earlier using the indicated antibodies. Complex formation of a myristoylated cytoplasmic and full-length receptor is a netrin-1-dependent process and is also dependent on the presence of the DB and P1 motifs of UNC5H2 and DCC.

cone repulsion observed following misexpression of *unc-5* or its vertebrate homologs in *Xenopus* embryos. Thus, in both *C. elegans* and vertebrates, the repulsive function of UNC5 proteins observed in misexpression experiments is dependent on the function of DCC proteins. Although a nonautonomous function of a DCC protein has been shown in *Drosophila* (Gong et al., 1999), our results show the requirement for DCC function in UNC5-mediated repulsion is cell autonomous in cultured *Xenopus* neurons; it is likely that the same will be true in *C. elegans* as well (Colavita and Culotti, 1998).

The requirement of DCC protein function for responses of cells and axons that normally express UNC5 proteins is less fully understood. Loss of *unc-40* function also results in impairment of dorsally directed, *unc-5*-dependent migrations, but to a lesser extent than is observed in *unc-5* mutants, suggesting that UNC-40 is only partially required for UNC-5 function to direct these normal migrations. One possibility is that these cells

and neurons express another "accessory" protein that can substitute for UNC-40 function; as the *C. elegans* genome does not show signs of a second DCC-like protein, this putative accessory protein would presumably not be a DCC family member. Another possibility is that different repulsive events may be more or less dependent on UNC-40. For instance, UNC-5 may be capable of going it alone in mediating repulsion near the UNC-6 source but need assistance from UNC-40 at a distance, where the concentration of UNC-6 is lower. This could occur if an UNC-40/UNC-5 complex has a higher sensitivity to UNC-6 than the UNC-5 receptor alone and would explain why loss of *unc-40* function results in a less severe phenotype than loss of *unc-5*.

Whatever the explanation, DCC proteins appear to be cooperating with UNC5 proteins to elicit the repulsive response both in vivo in *C. elegans* and in individual *Xenopus* neurons in vitro. If UNC5 proteins were simply eliciting a repulsive response that overwhelms attraction

Figure 6. Effects of Cytoplasmic Deletions of UNC5H2 on the Repulsive Response Induced by Netrin-1 on *Xenopus* Spinal Neurons

Responses obtained with the constructs shown in (A), (D), and (G) in the assay of Figure 1A are shown in the cumulative distribution plots and average turning angle plots in (B and C), (E and F), and (H and I), respectively (see text for details). Note that in the experiments of (G–I), cells expressing UNC5H2 and DCC cytoplasmic domain constructs showed a much reduced rate of extension. When exposed to netrin-1 in the presence of the anti-DCC antibody, the axons expressing the entire DCC cytoplasmic domain (construct 9) increased their extension rate back to control levels, whereas those expressing the P1 deletion construct (construct 10) increased their rate to a level below control but similar to that observed for cells expressing just UNC5H2 in the presence of anti-DCC antibody (Figure 1F) (data not shown).
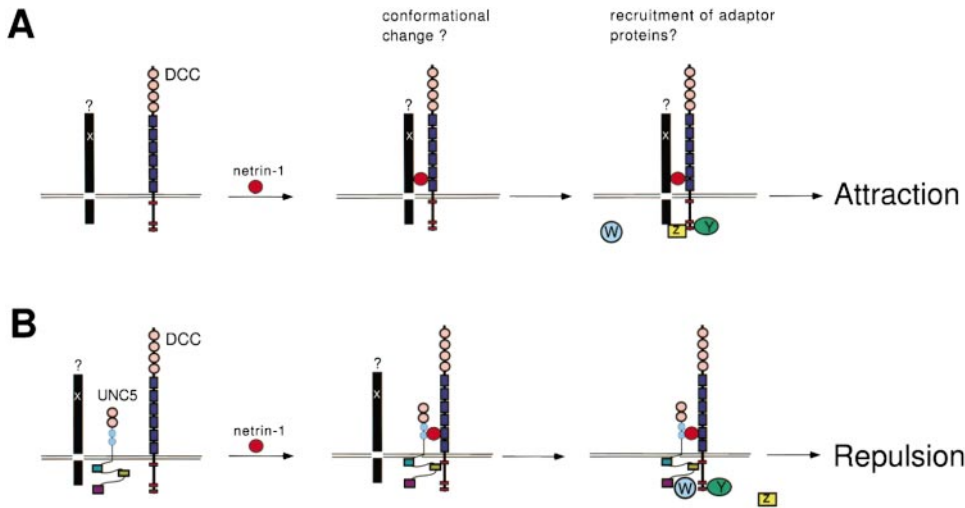
Figure 8. A Model for UNC5–DCC Interactions

(A) In the absence of UNC5 proteins, netrin-1 causes a change (perhaps conformational) in DCC that results in the recruitment of some adaptor proteins (Y and Z) but not others (W). A "coreceptor for attraction" ("X?") may be recruited and necessary as well (see text).

(B) In the presence of UNC5 proteins, changes (perhaps conformational) in both the UNC5 and DCC proteins result in the interaction of their cytoplasmic domains and consequently the recruitment of a different complement of adaptors (Y and W, not Z). It may also involve exclusion of a putative coreceptor for attraction. The diagram is drawn as if the DB and P1 domains interact directly, but this has not been shown.

mediated by DCC proteins, then loss of DCC protein function would be expected to enhance, not suppress, UNC5-mediated repulsion.

The Cytoplasmic Domain of an UNC5 Protein Specifies Repulsion: Mechanistic Implications

Our finding that fusion of the cytoplasmic domain of UNC5H2 to the DCC ectodomain yields a receptor that can still specify repulsion in response to netrin-1 parallels the observation that in *Drosophila*, fusion of the Robo cytoplasmic domain to the ectodomain of the DCC family protein Frazzled yields a repulsive netrin receptor (Bashaw and Goodman, 1999). That study also showed the reciprocal result as well, namely that the cytoplasmic domain of Frazzled is sufficient to specify attraction in the context of a Robo ectodomain. Together these findings imply that the "sign" of a guidance cue receptor—attraction or repulsion—can be specified in its cytoplasmic domain, with the ectodomain determining the specificity of ligand binding.

Remarkably, we found that the cytoplasmic domain of UNC5H2 is sufficient to effect this conversion, as repulsion is observed with a chimeric receptor comprising the ectodomain of a heterologous receptor, trkA, and even with a construct consisting simply of the UNC5H2 cytoplasmic domain fused to a myristoylation sequence. Our findings that (1) the cytoplasmic domains of UNC5H2 and DCC associate, (2) this interaction requires the DB domain in the UNC5H2 cytoplasmic domain, and (3) the DB domain is similarly necessary for the ability of the UNC5H2 cytoplasmic domain to convert attraction to repulsion all support a model in which the repulsive function of the UNC5H2 cytoplasmic domain is mediated by the interaction with the DCC cytoplasmic domain. In this model, activation of the complex can be mediated by binding of netrin-1 to DCC alone, even though UNC5 usually binds netrin as well. The converse was also

shown to hold: activation of the complex can also be triggered by binding of netrin-1 to UNC5H2 alone but requires the presence of the DCC cytoplasmic domain (with intact P1 domain), as demonstrated by showing that the blocking effect of anti-DCC antibodies on UNC5H2-mediated repulsion can be overcome by coexpression of a myristoylated DCC cytoplasmic domain.

Interestingly, we found that the Death Domain at the carboxy terminus of UNC5 proteins is not required for repulsion. This domain is, however, highly conserved across evolution, suggesting that it mediates some other conserved function, perhaps in the control of apoptosis (a function shared by many, but not all, Death Domain–containing proteins). In this context, it is of interest that expression of vertebrate UNC5 constructs was often associated with cell death in our cultures, which was alleviated by deletion of the Death Domain (see Results and Experimental Procedures; also see Experimental Procedures in Leonardo et al. [1997]). It is also of interest that DCC has recently been implicated in the control of cell death, at least in transfected cells (Mehlen et al., 1998).

Although the isolated cytoplasmic domains of UNC5 and DCC proteins interact, full-length UNC5H2 and DCC do not coprecipitate in the absence of netrin-1. These results imply that the interaction between the cytoplasmic domains of these proteins is repressed in the context of the full-length proteins. This conclusion is further supported by the finding that a myristoylated UNC5H2 cytoplasmic domain coprecipitates with full-length DCC only in the presence of netrin-1. In this case, netrin-1 cannot simply be acting as a bridge to bring together UNC5H2 and DCC. Rather, it must be causing some change in the DCC protein to derepress the interaction with the UNC5H2 cytoplasmic domain. This may involve a conformational change in the DCC cytoplasmic domain; alternatively, clustered DCC cytoplasmic domains (brought together as a result of netrin-1-mediated

aggregation) may provide a substrate to which the UNC5H2 cytoplasmic domain can bind with greater avidity. A similar conclusion applies to UNC5H2, since it coprecipitates efficiently with a myristoylated DCC construct only in the presence of netrin-1.

The picture that emerges from all of these findings is as follows. The cytoplasmic domains of DCC and UNC5 proteins can associate physically, but this interaction is repressed in the context of the full-length proteins. Netrin-1 has two effects: it brings together the proteins in a complex, and it simultaneously derepresses the interaction of the cytoplasmic domains by causing changes in both the UNC5 and the DCC proteins. The associated cytoplasmic domains mediate a signal for repulsion, whereas in the absence of the UNC5 cytoplasmic domain, the DCC cytoplasmic domain mediates a signal for attraction (Figure 8).

How the association of the UNC5 cytoplasmic domain with the DCC cytoplasmic domain causes the switch from attraction to repulsion is not known. Assuming DCC signal transduction involves the recruitment of downstream adaptor proteins (since DCC does not possess obvious catalytic motifs), interaction with the UNC5 cytoplasmic domain may lead to the recruitment of additional adaptors and/or displacement of adaptors that bind to DCC when it is expressed alone. It is also possible that the attractive function of DCC requires its interaction with an unknown transmembrane protein and that this "coreceptor for attraction" is competed away by UNC5, which functions as a "coreceptor for repulsion." Figure 8 summarizes these different possibilities.

Relation to Switching by Cyclic Nucleotides
The attractive function of netrin-1 on *Xenopus* spinal neurons can also be converted to repulsion by manipulations that result in an inhibition of the cAMP signaling pathway in growth cones (Ming et al., 1997). It is unclear at present what the relation of cAMP signaling is to UNC5 signaling. At one extreme, there could be a direct link, with activation of UNC5 proteins resulting in repression of cAMP signaling. Alternatively, cAMP may function in a parallel pathway. In fact, since manipulations of the cAMP and cGMP signaling pathways can convert attractive or repulsive responses to half a dozen different guidance cues with distinct receptors (Song et al., 1998), the targets of cyclic nucleotide function in this process could be downstream of the point where these disparate signaling pathways converge.

Bifunctionality and the Interconversion of Attraction and Repulsion
We have already commented that UNC5 proteins do not compete with DCC proteins—there is no tug-of-war between attraction and repulsion. Rather, UNC5 proteins appear to coopt DCC proteins, switching attraction to repulsion. Why should this be—why have a switching mechanism at all? The answer presumably lies in the fact that growth cones, as they navigate to their targets, change their responsiveness to guidance cues as they progress. Once a growth cone has reached a particular intermediate target, it must change its priorities in order to be able to move on to the next target. For example, commissural axons are initially attracted

to the floor plate using netrin-1, but upon crossing the midline, they lose responsiveness to netrin-1 (Shirasaki et al., 1998). Since the axons continue to express DCC (Keino-Masu et al., 1996), the switching off must involve some other change. Another switch in growth cone sensitivity at the midline is the acquisition of Slit responsiveness by upregulation of expression of the Robo receptor in *Drosophila* (Kidd et al., 1998). Although not yet demonstrated in vivo, it seems likely that there are circumstances where it is desirable not just to switch on or off responsiveness to a particular cue, but rather to convert the responsiveness from attraction to repulsion, to help move the growth cone along. The ability of one receptor to switch responses mediated by another receptor provides an economical means to achieve this end and avoid confusing the growth cone with simultaneous conflicting signals for attraction and repulsion.

Experimental Procedures

Construction of Recombinant Fusion Proteins and Baits
A detailed description of all constructs used in this study is available upon request. Briefly, constructs were made in the baculovirus expression vector pAC-GHLT-B (Pharmingen) (to generate GST fusion proteins), the yeast two-hybrid "bait" LexA fusion plasmid pBTM116 and "prey" VP16 plasmid pVP16, and the COS cell expression vectors pcDNA3 and pSEC-B (Invitrogen). Various ectodomain or cytoplasmic domain fragments were derived by PCR from the rat DCC, rat UNC5H1, rat UNC5H2, and mouse UNC5H3/RCM cDNAs (accession numbers U68725, U87305, U87306, and U72634, respectively). Overlap extension PCR was used to generate the DCC P1 deletion and UNC5H2 DB deletion constructs by the method of Ho et al. (1989). The TrkA-UNC5H2 fusion protein was generated by overlap extension PCR using the rat TrkA cDNA (Clary et al., 1994) as template. Rat TrkA ectodomain sequences aas 1–418 were fused in-frame with UNC5H2 aas 380–946, in which UNC5H2 donates the transmembrane domain. The DCC-UNC5H2 fusion was prepared using the extracellular and transmembrane domain of DCC (aas 1–1119) and cytoplasmic region of UNC5H2 (aas 404–946). A myristoylated DCC fusion construct was generated by PCR, placing a HindIII site upstream of the src-myr sequence (MGSSKSKPKDPSQR RRSLE) (Guy et al., 1987) in-frame with the DCC cytoplasmic domain (aas 1120–1445) and cloned into pCDNA3. Myristoylated UNC5H2 cytoplasmic domain (aas 404–946) constructs were generated in the same way in both pSEC-B and pcDNA3 (all experiments were done with both constructs, with identical results; data shown here used the pSEC-B construct). The myr-UNC5H2-DBtr was truncated at aa 707, and the UNC5H2ecto-TM and DCCecto-TM constructs were terminated at aa 403 or aa 1123, followed by either an in-frame myc-tag or HA tag, respectively. The GFP cDNA containing a mutation at S65T to increase the intensity of the green fluorescence was described previously (Wang and Poo, 1997). The entire coding region of the *C. elegans* UNC-5 derived from the YZ121 plasmid was PCR amplified and cloned into the PstI–XbaI site of the pSP64 Poly(A) vector. All PCR fragments and in-frame cloning sites generated were confirmed by sequence analysis. The integrity of all constructs used in the axon-turning assay was assessed by in vitro transcription and translation using the Transcend Non-Radioactive Detection System (Promega Co., Madison, WI).

Peptides
P1 (PPDLWIHHEEMEMKNIEK) and C1 (PDWHEMMNEPLIHEIKEK) peptides were synthesized, sequenced, and analyzed by mass spectrometry by the HHMI protein structure lab (UCSF).

In Vitro Transcription
Capped mRNAs were synthesized by in vitro transcription using mMESSAGE mMACHINE (Ambion, Inc., Austin, TX) as described by the manufacturer. The transcription products were purified using QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA). Integrity

of mRNAs was examined by agarose gel electrophoresis and by in vitro translation as above.

Cell Culture

Sf9 cells were grown as monolayers in Grace's insect medium (Invitrogen) supplemented with 10% FBS (fetal bovine serum). COS-1 cells were cultured as described (Stein et al., 1998).

Two-Hybrid Screen

The genotype of the *Saccharomyces cerevisiae* reporter strain L40 is MATα trp1 leu2 his3LYS2::lexA-HIS3 URA3::lexA-lacZ (Vojtek et al., 1993). Yeast strains were grown at 30°C in YPAD medium or in synthetic medium with the appropriate supplements (Rose et al., 1990). The yeast reporter strain L40 was transformed by the lithium acetate method, and transformants were grown at 30°C on synthetic medium lacking histidine, leucine, tryptophan, uracil, and lysine (−KLUTH) or on leucine, tryptophan, uracil, and lysine–depleted medium (−KLUT) and incubated at 30°C for 3 days.

Transfection and Coimmunoprecipitation

COS-1 cells were transfected with equal amounts of DCC and UNC5H2 mammalian expression constructs using Fugene transfection reagent (Boehringer Mannheim). About 40 hr posttransfection, medium was removed and cells starved for 2 to 4 hr in OPTI-MEM, incubated with either 300 ng/ml netrin-1 [(+) netrin-1] or BSA [(−) netrin-1] for 20 min at 37°C, and lysed in 2 ml buffer D. About 250 μg of precleared lysates were incubated with either anti-myc (9E10) or a mixture of DCC antibodies (AF5, Calbiochem and G92-13, Pharmingen) at a ratio of 1:1 for 8–10 hr at 4°C, and complexes were recovered and washed extensively on protein A/G–Sepharose beads. Immunoprecipitates were separated on 8% SDS-PAGE or as indicated and Western blots incubated with either anti-myc, anti-HA, or anti-DCC (Pharmingen) antibodies using the ECL reagent from Amersham. To visualize and distinguish the myr-UNC5H2ΔDB(myc) and myr-DCC(HA) proteins from heavy and light antibody chains, respectively, directly coupled myc-HRP or HA-HRP antibodies were used for Western blot analysis.

Recombinant Baculovirus Production and Affinity-Binding Assays

GST fusion proteins were generated in Baculovirus. Individual recombinant Baculovirus stocks were generated by cotransfecting a monolayer of Sf9 cells with a recombinant pAC-GST-DCC fusion construct or pAC-GST vector alone and BaculoGold Baculovirus DNA (Pharmingen). Virus stocks were amplified and cells lysed and processed as described (Stein et al., 1998). Immobilized fusion proteins were washed once with triple detergent lysate buffer, followed by two washes with buffer E (50 mM TrisCl [pH 8.0], 300 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, 50 μg/ml BSA, 1 μg/ml Aprotinin, and 1 μg/ml Leupeptin). UNC5H1-3 or NPN2A were in vitro translated as above in the presence of [^{35}S]cysteine (Amersham), diluted with buffer E, and incubated with the appropriate immobilized fusion protein at 4°C. After 4–8 hr, incubation beads were washed three times with buffer E, omitting bovine albumin from the last wash. Immobilized and bound proteins were resolved on 8% or 15% SDS-PAGE and detected by autoradiography after a 1 hr treatment with enhancer (Amersham) or by Western blot (anti-GST, Santa Cruz). For peptide competition assays, GST-affinity assays were performed as described above, except in the presence of the indicated concentration of control or competing peptide. Immobilized bound protein complexes were either subjected to SDS-PAGE or to scintillation counting.

Xenopus Embryo Microinjection and Cell Culture

In vitro fertilization was performed as described in Tabti and Poo (1991). The mRNAs were injected into two blastomeres of *Xenopus* embryos at the four cell stage. For each blastomere, we injected 4 to 8 nl of mRNA solution containing 0.125 μg/μl of the sample mRNAs and 0.25 μg/μl of GFP mRNA. GFP was used as an indicator for expression of the sample mRNA in a cell. Cultures of *Xenopus* spinal neurons were prepared from neural tube tissue of stage 22 embryos as described (Tabti and Poo, 1991). Cell death was seen in cultures from embryos injected with full-length UNC5 constructs

and was particularly extensive with UNC5H1. This death was alleviated by removing the Death Domain from these proteins. (For example, cell survival in cultures from embryos injected with full-length UNC5H2 or UNC5H2ΔDD was 46% ± 10% and 92% ± 12% of control [average of five experiments].) To avoid complications arising from cell death, most conclusions drawn here were verified using Death Domain–deleted constructs under conditions where cell death was minimized.

Growth Cone Turning Assay

Microscopic gradients of guidance molecules were produced as described (Zhang et al., 1994; Song et al., 1997). The tip of the micropipette was positioned at a distance 100 μm away from the center of the growth cone and at an angle of 45° with respect to the initial direction of neurite extension, defined by the last 10 μm segment of the neurite. Turning angle was determined by measuring the angle between the original direction of neurite extension and a straight line connecting the positions of the growth cone at the onset and the end of the 1 hr period. Net neurite extension was determined by measuring the total trajectory of the neurite at the end of the 1 hr period using a digitizer. Only those growth cones with net growth >5 μm were included for analysis. Usually only one neuron with strong GFP expression was assayed from a single culture plate, and at least three separate batches of injected embryos were tested for each experiment. Almost all turning assays were done blind: the experimenter was unaware of the nature of the mRNA expressed in the neuron or the content of solution used in filling the micropipette.

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