Hierarchical Organization of Guidance Receptors: Silencing of Netrin Attraction by Slit Through a Robo/DCC Receptor Complex

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Axonal growth cones that cross the nervous system midline change their responsiveness to midline guidance cues: They become repelled by the repellent Slit and simultaneously lose responsiveness to the attractant netrin. These mutually reinforcing changes help to expel growth cones from the midline by making a once-attractive environment appear repulsive. Here, we provide evidence that these two changes are causally linked: In the growth cones of embryonic *Xenopus* spinal axons, activation of the Slit receptor Roundabout (Robo) silences the attractive effect of netrin-1, but not its growth-stimulatory effect, through direct binding of the cytoplasmic domain of Robo to that of the netrin receptor DCC. Biologically, this hierarchical silencing mechanism helps to prevent a tug-of-war between attractive and repulsive signals in the growth cone that might cause confusion. Molecularly, silencing is enabled by a modular and interlocking design of the cytoplasmic domains of these potentially antagonistic receptors that predetermines the outcome of their simultaneous activation.

In the developing nervous system, many axons find their final targets by navigating a series of intermediate targets. In general, axons are attracted to each successive intermediate target. This presents an apparent paradox: If the cells that form the intermediate target are initially perceived as attractive, how can the axons move on from this target to the next one? The answer appears to lie in the ability of axonal growth cones to change their response to guidance molecules presented by intermediate target cells, so that what was initially perceived as an attractive cellular environment is now interpreted as repulsive.

This changing preference is well documented for the guidance of commissural axons at the midline of the nervous system (Fig. 1). In vertebrates, insects, and nematodes, commissural axons are attracted to the midline by chemoattractants of the phylogenetically conserved netrin family, which signal attraction by activating receptors of the DCC (Deleted in Colorectal Cancer) family of guidance receptors on growth cones (*1*). Commissural axons then cross the midline and project alongside it, never recrossing. This failure to recross is explained, at least in *Drosophila*, by the fact that midline cells, in addition to expressing attractive netrin proteins, also express the repellent protein Slit, which signals repulsion by activating the Roundabout (Robo) receptor (*2*). The growth cones can cross once because they do not initially express Robo protein on their surface (even though their cell bodies express Robo mRNA), but upon crossing, through a mechanism that is still poorly understood, they up-regulate Robo protein on their surface and therefore become responsive to Slit, which prevents them from recrossing. In vertebrates as well, commissural axons that cross the spinal cord midline become responsive to a midline repulsive activity that appears to involve both vertebrate Slit proteins and semaphorin proteins; when receipt of the repulsive signal is impaired through genetic ablation of a repellent receptor, the axons stall at the midline, consistent with a failure of the axons to be expelled from the midline (*3*).

The up-regulation of growth cone responses to a midline repellent activity in vertebrates and flies can help to explain why the axons do not linger there and instead move on. However, for the growth cones to progress from the intermediate target in an efficient manner, they not only should up-regulate their responsiveness to a midline repellent, but also should lose the attractive responses to the midline that got them there in the first place. Indeed, in the mammalian hindbrain, commissural axons lose responsiveness to netrins upon crossing the midline, despite continued expression of the netrin receptor DCC on their surface (*4*) (Fig. 1).

Thus, growth cones undergo two mutually reinforcing changes upon midline crossing: loss of response to a midline attractant, and up-regulation of response to a midline repellent that helps to expel them from the midline and move them on to the next leg of their trajectory. Having both changes provides a more effective means of

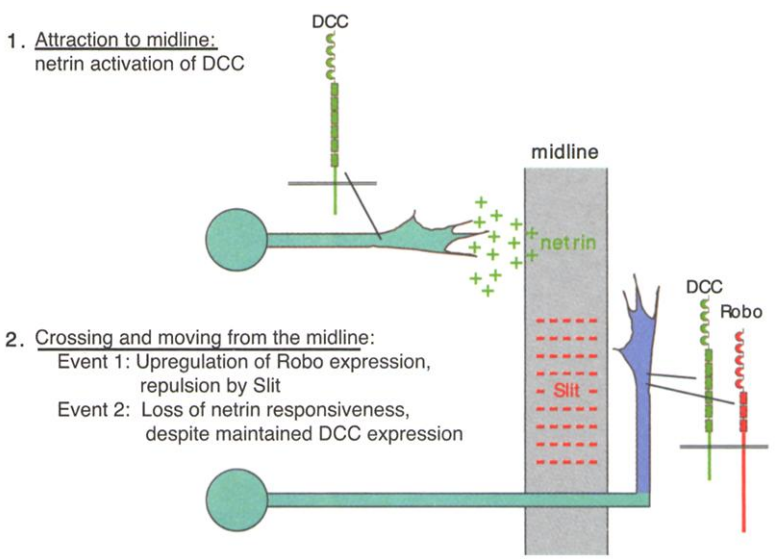

Fig. 1. Axon guidance events at the midline. In vertebrates, insects, and nematodes, commissural axons are attracted by netrin protein(s) secreted by midline cells, which attract by activating a receptor of the DCC family on growth cones. After crossing the midline, axons change their responsiveness, such that they are repelled by the midline. In *Drosophila* (and likely in vertebrates as well) this involves up-regulation of the Robo receptor on the postcrossing portions of the axons, so they become responsive to the midline repellent Slit. Axons that cross the midline also lose responsiveness to the netrin attractant, despite maintained expression of the DCC receptor (at least in vertebrates).

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ensuring that the growth cones will not stall at the midline. We now show that these two events are linked: Up-regulation of the response to the midline repellent Slit causes loss of response to the netrin attractant. This silencing effect of Slit on netrin attraction is mediated by a direct physical interaction of the cytoplasmic domains of the Slit receptor Robo with the netrin receptor DCC. We propose that this hierarchical organization of guidance receptors ensures tight temporal scheduling of repulsion and loss of attraction, and that it helps to minimize the possibility that growth cones will be confused by simultaneous activation of attractive and repulsive responses.

Silencing of Netrin Attraction, But Not Growth Stimulation, by Slit

Because commissural axons in vertebrates and insects are simultaneously exposed to netrins and to Slits at the midline in vivo, we tested how individual growth cones respond when exposed to both proteins in vitro. For this, we used a well-characterized assay in

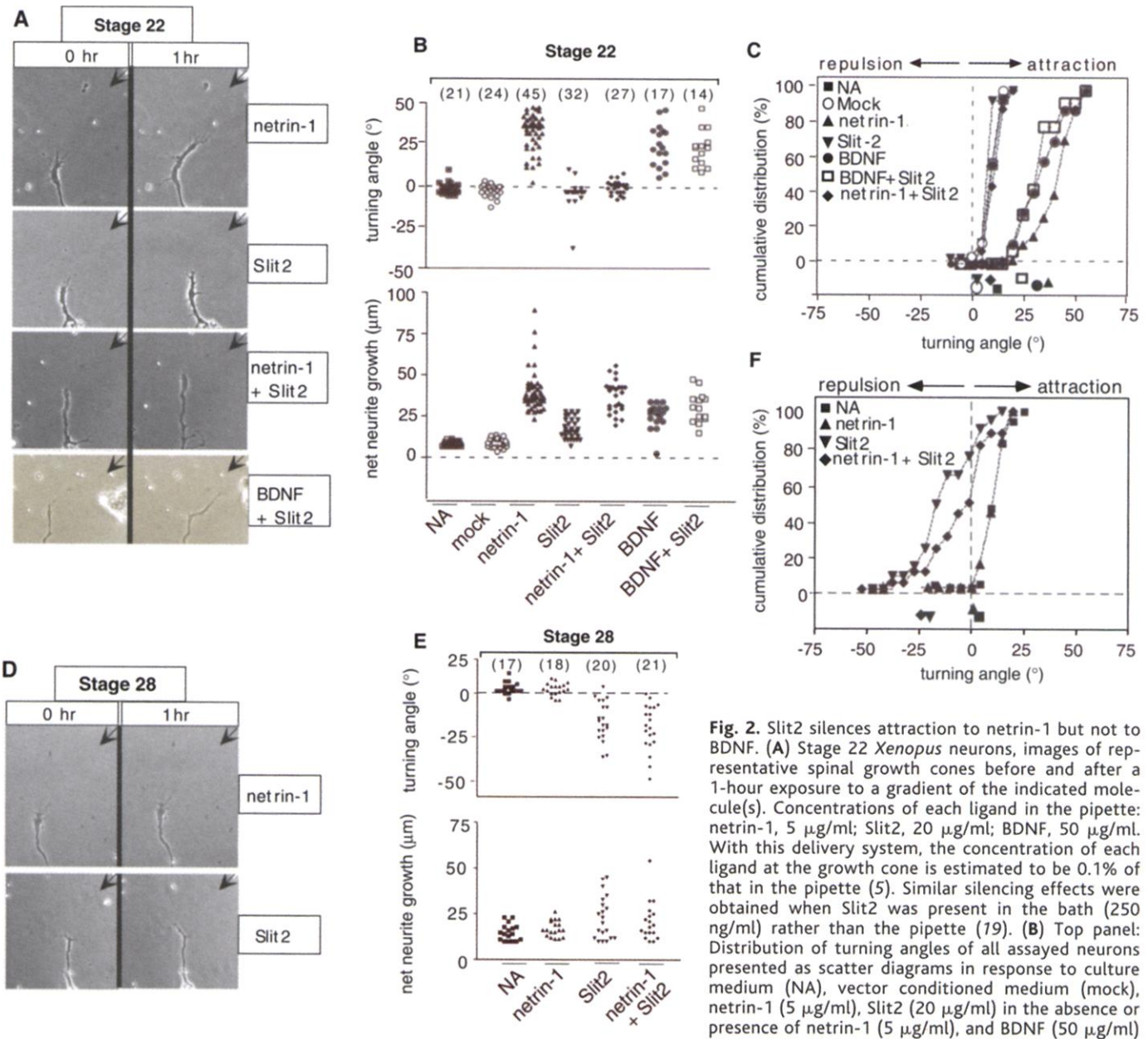


Fig. 2. Slit2 silences attraction to netrin-1 but not to BDNF. (**A**) Stage 22 *Xenopus* neurons, images of representative spinal growth cones before and after a 1-hour exposure to a gradient of the indicated molecule(s). Concentrations of each ligand in the pipette: netrin-1, 5 µg/ml; Slit2, 20 µg/ml; BDNF, 50 µg/ml. With this delivery system, the concentration of each ligand at the growth cone is estimated to be 0.1% of that in the pipette (*5*). Similar silencing effects were obtained when Slit2 was present in the bath (250 ng/ml) rather than the pipette (*19*). (**B**) Top panel: Distribution of turning angles of all assayed neurons presented as scatter diagrams in response to culture medium (NA), vector conditioned medium (mock), netrin-1 (5 µg/ml), Slit2 (20 µg/ml) in the absence or presence of netrin-1 (5 µg/ml), and BDNF (50 µg/ml) in the absence or presence of Slit2 (20 µg/ml) in the pipette. Each data point represents the turning angle of an individual growth cone. Bottom panel: Net neurite extension during the 1-hour period for the same group of neurons as in the top panel. Numbers in parentheses at the top represent the total number of growth cones tested in each condition. Slit2 did not attract or repel growth cones, but it caused a straightening of the trajectories ($P < 0.056$), probably because elongation rate was increased. The single growth cone that was repelled was likely derived from an older embryo that was inappropriately staged [see (E)]. Turning was observed with netrin-1 or BDNF alone, or with BDNF plus Slit2 ($P < 0.0001$ in each case); no turning was observed under any of the other conditions ($P > 0.18$). (**C**) Cumulative distribution plot of turning angles in (B) for growth cones exposed to the indicated ligands. Percentage value refers to proportion of growth cones with angular positions less than a given angle. Data points along abscissa are median values for corresponding data shown above. (**D** to **F**) Netrin-1 and Slit2 effects on spinal neurons derived from st. 28 *Xenopus* neurons. Netrin-1 does not affect the direction or rate of extension of these neurons ($P > 0.61$), whereas Slit2 strongly repels those neurons ($P < 0.0001$) and increases their rate of extension ($P < 0.02$). Netrin-1 does not affect either of these effects of Slit2 (turning, $P > 0.36$; growth, $P > 0.81$). All experimental conditions and concentrations were identical to those in (A) to (C).

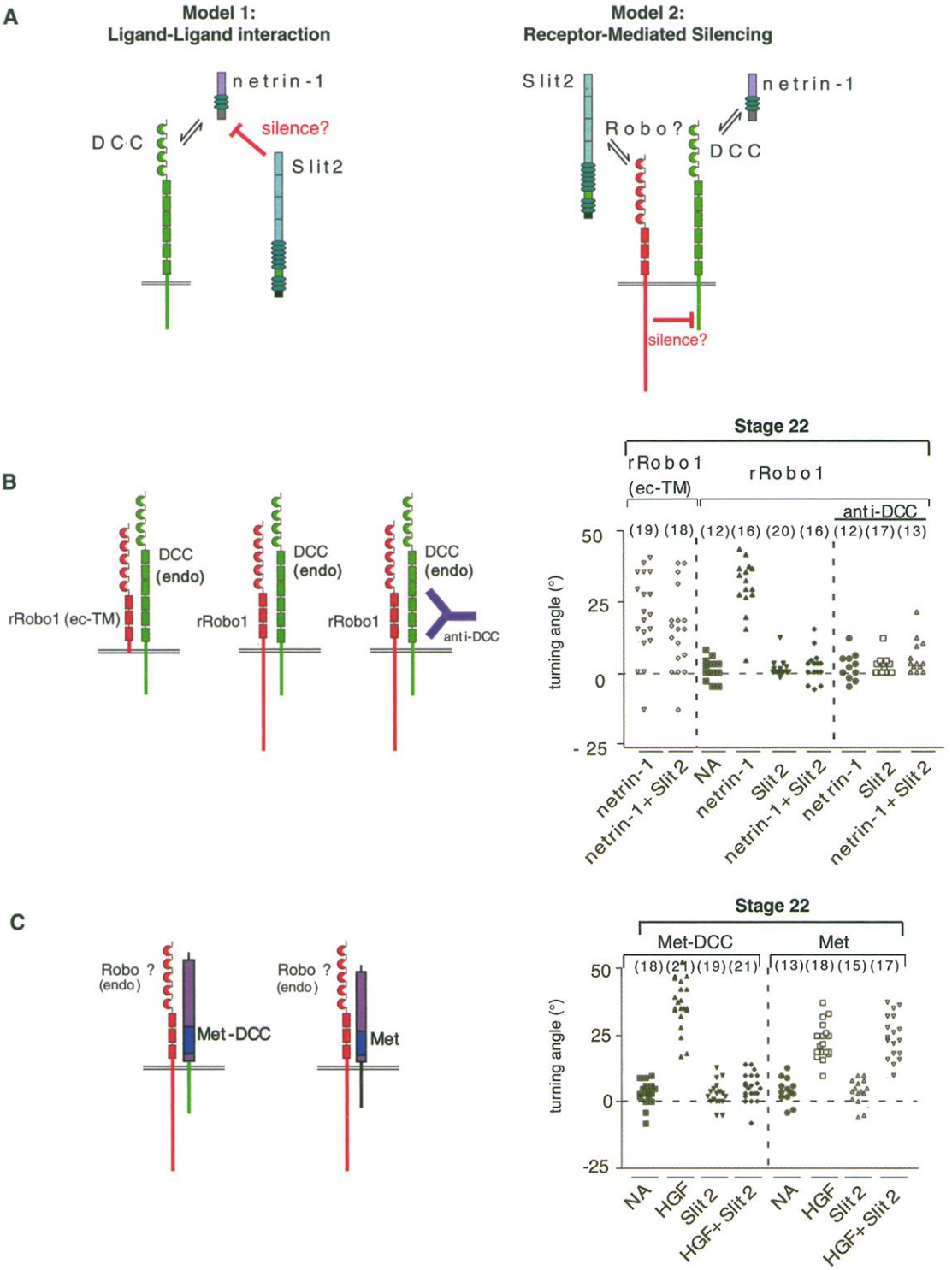


Fig. 3. Slit2 silences netrin-1 attraction through a receptor-mediated mechanism. (**A**) Two mechanisms that could account for silencing by Slit. Model 1: Silencing is caused by binding of Slit2 to netrin-1 in a ligand-ligand interaction that somehow prevents activation of the netrin receptor DCC. Model 2: Slit2 binds its receptor (presumably a Robo protein) and silences DCC-mediated attraction either through direct binding of DCC or through interference with downstream signaling. (**B** to **E**) Effects of misexpression of wild-type, truncated, or chimeric receptors on growth cone turning. The left side of each panel shows the receptors introduced into the growth cone exogenously and the relevant receptors expressed endogenously (endo). The question mark in "Robo?(endo)" indicates the lack of formal proof that Slit mediates its effect on wild-type neurons through an endogenous Robo receptor (see text). (B) Scatter diagram of turning angles of st. 22 growth cones expressing either rat Robo1 (rRobo1) or a presumed dominant negative form of rat Robo1 [rRobo1(ec-TM), comprising the ectodomain and transmembrane domain of Robo1] in the absence or presence of a functional blocking antibody to DCC (AF5, Oncogene; 1 μg/ml). Growth cones were exposed to gradients of indicated ligands as in Fig. 2, B and E. Truncated Robo1 blocks Slit2-mediated silencing of netrin-1 attraction, whereas full-length rRobo1 does not, and all attractive responses require DCC function. (C) Scatter diagram of turning angles of st. 22 growth cones expressing either a Met-DCC chimeric receptor or the wild-type Met receptor tyrosine kinase after a 1-hour exposure to control medium (NA), HGF, Slit2, or HGF in the presence of Slit2 (pipette concentrations: HGF, 10 μg/ml; Slit2, 20 μg/ml). Slit2 silences attraction caused by activation of Met-DCC but not Met. (D) Scatter diagrams of turning angles of st. 22 growth cones expressing Met-Robo1 or trkA-Robo1 chimeric receptors (*15*) after a 1-hour application to the indicated ligands (HGF, 10 μg/ml; NGF, 50 μg/ml; netrin-1, 5 μg/ml). The full-length Met kinase was used as a control. Activation of the Robo1 cytoplasmic domain by the relevant ligand for each chimeric receptor is sufficient to silence netrin-1 attraction. Activation of wild-type Met stimulates attraction and does not interfere with netrin-1 attraction. (E) The Met-Robo1 and trkA-Robo1 receptors mediate repulsive responses to their cognate ligands [concentrations as in (D)] in growth cones of st. 28 neurons, as shown by turning angles after a 1-hour exposure.

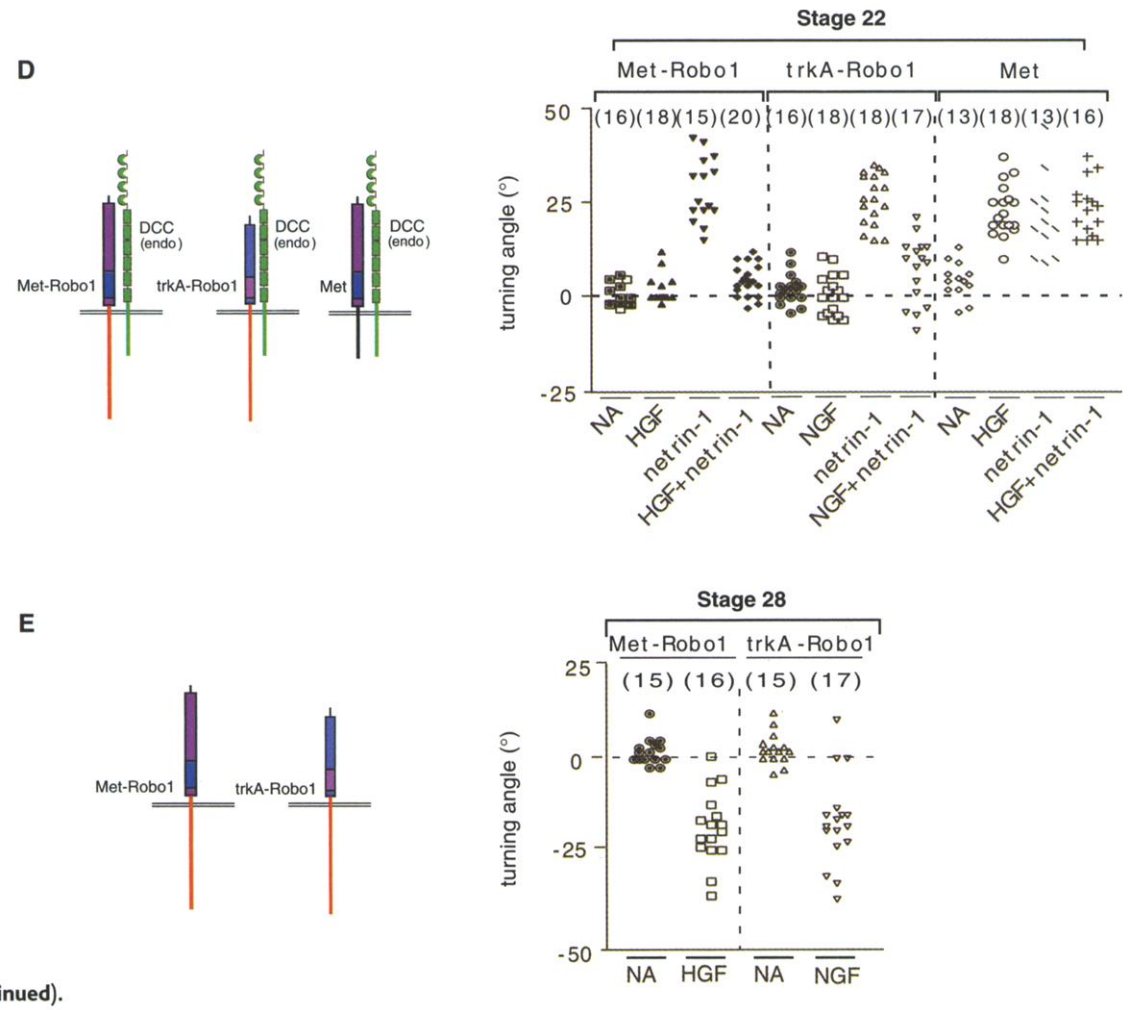


Fig. 3 (continued).

which the growth cones of individual embryonic *Xenopus* spinal axons in culture are exposed to gradients of soluble factors established by repetitive pulsatile release from a micropipette (*5, 6*). This assay has been used to characterize growth cone responses to netrin-1 (*7–9*).

When growth cones of neurons from stage 22 (st. 22) embryos are exposed to a gradient of netrin-1 for 1 hour, they turn toward the source (Fig. 2, A to C) (*7*). This attractive response requires the function of the netrin receptor DCC (*7–10*). In contrast, the same axons exposed to a gradient of Slit2 protein (*11*) did not show a directional response (Fig. 2, A to C). Nevertheless, when growth cones were exposed to a gradient of netrin-1 and simultaneously exposed to Slit2 (in the pipette or in the bath), the attractive effect of netrin-1 was completely abolished (silenced) in all cases (Fig. 2, A to C). This silencing effect of Slit2 appeared specific for attraction by netrin-1, because Slit2 did not block the attractive effect of brain-derived neurotrophic factor (BDNF) (Fig. 2, A to C), which attracts these axons by activating the trkB receptor in these cells (*12*).

In addition to attracting the axons, netrin-1 also stimulates their rate of elongation (Fig. 2B) (*7–9*). Slit2 itself also has a modest stimulatory effect on axon elongation (Fig. 2B; $P < 0.003$, Student's t test). In the presence of both netrin-1 and Slit2, the elongation rate is faster than without either factor ($P < 0.0001$) or with Slit2 alone ($P < 0.0001$). The average rate with both factors (33.5 ± 9.5 μm/hour) is slightly lower (~15%) than with netrin-1 alone (39.6 ± 12.6 μm/hour), but this change is not statistically significant ($P > 0.14$), indicating that Slit2 either does not affect the ability of netrin-1 to stimulate extension or at most reduces it only minimally. The apparent lack of effect on elongation rate contrasts with the ability of Slit2 to silence completely the directional effect of netrin-1. BDNF also stimulates axon extension, but this effect, like its directional effect, is not altered by Slit2 (Fig. 2B; $P > 0.08$).

The finding that Slit2 silences netrin-1 attraction of st. 22 growth cones but does not repel them was initially unexpected, because we were expecting Slit2 to function as a repellent. However, we found that the axons of older spinal neurons obtained from st. 28 embryos were consistently repelled by Slit2 (Fig. 2, D to F); hence, Slit2 can function as a repellent for *Xenopus* axons [as it can for mammalian axons (*13–18*)]. The older neurons did not show any response to netrin-1 (Fig. 2D) [likely because of the absence of DCC expression in these neurons, as assessed by immunohistochemistry (*19*)], so we could not test whether Slit2 has a silencing function at that stage as well. The differences between st. 22 and st. 28 neurons suggest that the *Xenopus* spinal neurons in these cultures switch their responsiveness to netrins and Slits over time. For example, if the neurons are commissural neurons, the change might reflect changes occurring in vivo upon encountering the floor plate. We cannot be certain of this, however; indeed, in the absence of any specific markers to identify these *Xenopus* neurons, we cannot even exclude the possibility that we are studying different neuronal populations at these two stages.

Receptor-Mediated Silencing by Slit

Two models could explain the silencing effect of Slit2 on netrin-mediated attraction (Fig. 3A). Because Slit2 can bind netrin-1 directly (*13*), silencing might be caused by

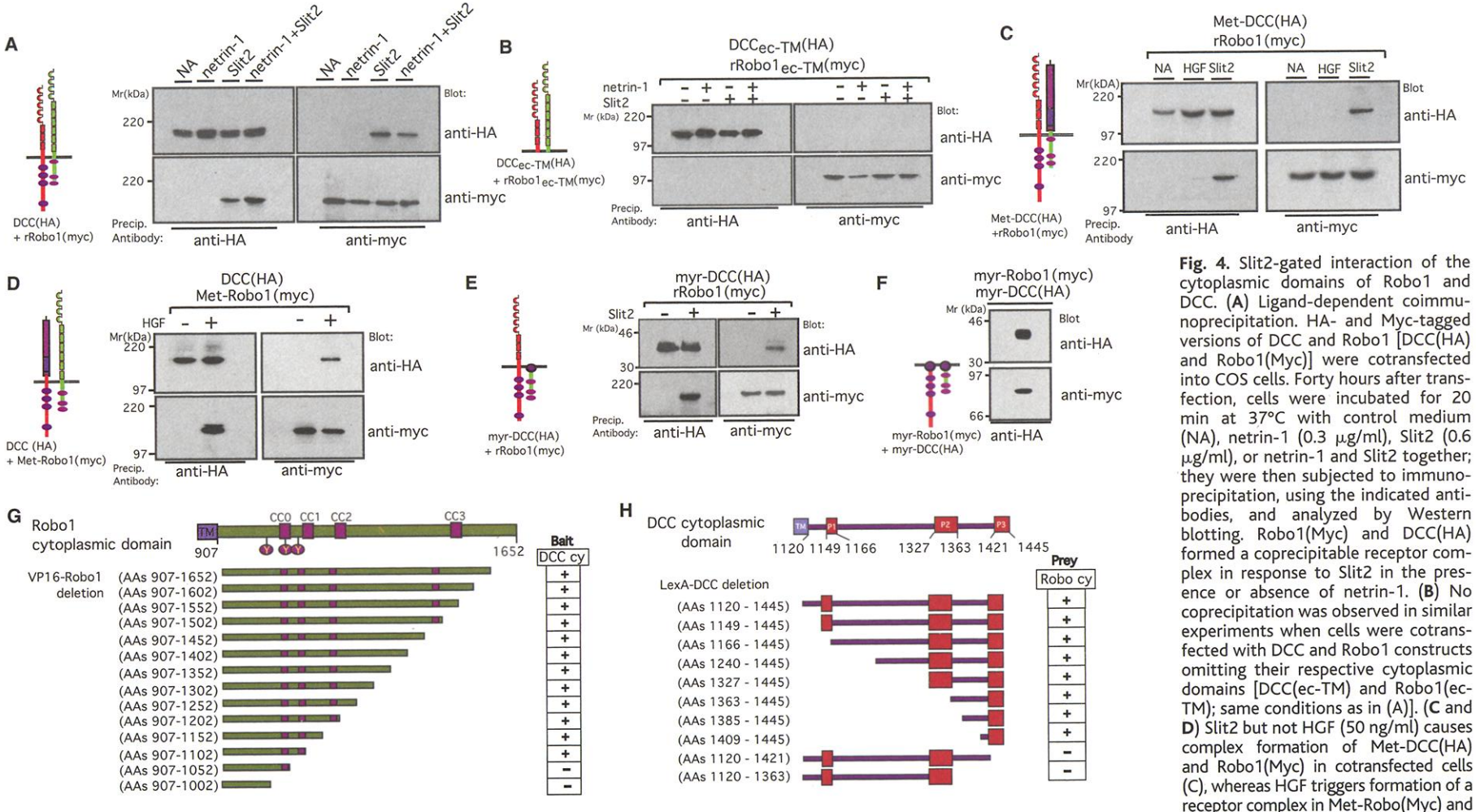


Fig. 4. Slit2-gated interaction of the cytoplasmic domains of Robo1 and DCC. (**A**) Ligand-dependent coimmunoprecipitation. HA- and Myc-tagged versions of DCC and Robo1 [DCC(HA) and Robo1(Myc)] were cotransfected into COS cells. Forty hours after transfection, cells were incubated for 20 min at 37°C with control medium (NA), netrin-1 (0.3 μg/ml), Slit2 (0.6 μg/ml), or netrin-1 and Slit2 together; they were then subjected to immunoprecipitation, using the indicated antibodies, and analyzed by Western blotting. Robo1(Myc) and DCC(HA) formed a coprecipitable receptor complex in response to Slit2 in the presence or absence of netrin-1. (**B**) No coprecipitation was observed in similar experiments when cells were cotransfected with DCC and Robo1 constructs omitting their respective cytoplasmic domains [DCC(ec-TM) and Robo1(ec-TM); same conditions as in (A)]. (**C** and **D**) Slit2 but not HGF (50 ng/ml) causes complex formation of Met-DCC(HA) and Robo1(Myc) in cotransfected cells (C), whereas HGF triggers formation of a receptor complex in Met-Robo(Myc) and DCC(HA) cotransfections (D). (**E**) Coprecipitation of a myristoylated form of the cytoplasmic domain of DCC [myr-DCC(HA)] with Robo1(Myc) in response to Slit2 exposure. (**F**) Constitutive association of myristoylated cytoplasmic domains of Robo1 [myr-Robo1(Myc)] and DCC [myr-DCC(HA)] in COS cells was observed as occurring in a ligand-independent fashion. (**G** and **H**) Yeast two-hybrid analysis of the interaction between the cytoplasmic domains of DCC (as a LexA fusion bait) and Robo1 (as a VP16 fusion prey) (*8*). Interactions were assessed by the ability to rescue growth on histidine-deficient plates. (G) Robo cytoplasmic domain deletion constructs and their ability to interact with the DCC cytoplasmic domain. Deletion of the CC1 domain causes loss of interaction with DCC. (H) DCC cytoplasmic domain deletion constructs and their ability to interact with the Robo1 cytoplasmic domain prey. Truncation of the P3 domain leads to loss of VP16–Robo cytoplasmic domain interaction (+, rescue; −, no rescue).

binding of the two proteins, which could in principle interfere with the netrin-DCC interaction. Alternatively, silencing might be a receptor-mediated event, with Slit2 activating a receptor [presumably a Robo receptor (*2, 13–18, 20*)] on growth cones that antagonizes netrin attraction mediated by DCC.

To distinguish these possibilities, we first tested whether the silencing effect of Slit2 could be blocked by expressing a truncated Robo receptor in these neurons. In this and all subsequent experiments, exogenous receptors were expressed by injecting in vitro transcribed mRNA encoding versions of the receptors of interest [usually tagged with a Myc or hemagglutinin (HA) epitope tag] into the second blastomere at the four-cell stage of *Xenopus* embryos, together with mRNA encoding green fluorescent protein (GFP) as a marker for expression of exogenous proteins. Embryos were allowed to develop to st. 22, and GFP-expressing spinal cord neurons derived from these embryos were assayed for turning responses. Expression of the receptors was always verified in control experiments using antibodies to the tag. This approach has been used previously to misexpress trk, DCC, and UNC5 receptors in these neurons (*8, 21*).

A Myc-tagged truncated version of rat Robo1 (rRobo1), comprising its ectodomain and transmembrane domain but with a truncated cytoplasmic domain, was expressed in st. 22 neurons in this way. This protein is expected to function as a dominant negative Robo receptor capable of interfering with endogenous receptors for Slit (presumably Robo proteins). In the presence of this exogenous construct, Slit2 no longer silenced the attractive effect of netrin-1 (Fig. 3B); this result is consistent with the involvement of a receptor-mediated mechanism in silencing. In a control experiment, expression of full-length rRobo1 in these cells did not interfere with silencing by Slit2 (Fig. 3B). It is of interest that Slit2 did not repel growth cones expressing full-length rRobo1 (Fig. 3B) (expression was assessed by immunohistochemistry with antibodies to the epitope tag), indicating that expression of a Robo receptor is not sufficient for repulsion, which presumably requires additional signaling molecules in the growth cone. As expected, the attractive effect of netrin-1 observed in all experimental conditions was blocked by antibodies to DCC, consistent with the requirement of DCC for netrin-mediated attraction (Fig. 3B).

The fact that a truncated Robo receptor can block silencing by Slit is consistent with a receptor-mediated mechanism. It could be argued, however, that this result is also compatible with a ligand-ligand interaction model of silencing if the exogenous Robo can bind and somehow locally reduce the amount of available Slit2 protein. To more definitively discriminate between the two models, we performed experiments that avoided using one or the other ligand. For this, we used chimeric receptors (*22*) in which the ectodomain of DCC or that of Robo1 is replaced with an exogenous ectodomain: that of the Met receptor tyrosine kinase, a receptor for hepatocyte growth factor (HGF), a soluble chemoattractant. We have shown that st. 22 *Xenopus* growth cones in our cultures do not normally respond to HGF, but if Met is introduced into them, they respond to HGF with attraction (*23*). When a chimeric receptor comprising the Met ectodomain and the DCC transmembrane and cytoplasmic domain is introduced into these cells, HGF also induces attractive responses (Fig. 3C), but this time apparently by tapping into the netrin signaling pathway (*23*).

In st. 22 neurons, Slit2 was as effective in silencing attractive responses elicited by HGF binding to the Met-DCC chimeric receptor as it was in silencing netrin-mediated attraction (Fig. 3C). Slit2 did not, however, silence attractive responses to HGF that were mediated by the wild-type Met receptor tyrosine kinase (Fig. 3C). Thus, silencing is observed even if the netrin ligand is replaced by another ligand, but only for attraction caused by activation of the DCC cytoplasmic domain.

We next asked whether, conversely, activation of the Robo signaling pathway by a heterologous ligand could also lead to silencing of netrin attraction. We constructed chimeric receptors comprising the cytoplasmic domain of rRobo1 and the ectodomain of either Met or the trkA receptor tyrosine kinase, a receptor for nerve growth factor (NGF). As observed with Slit, HGF did not elicit directional responses in the growth cones of st. 22 neurons expressing the Met-Robo1 chimera, but it did completely silence the attractive effect of netrin-1 (Fig. 3D). The same effects were observed in response to NGF in st. 22 neurons expressing the trkA-Robo1 chimera (Fig. 3D). As a control, introduction of the wild-type Met receptor into these neurons led to attractive responses to HGF, as well as to netrin-1 together with HGF (Fig. 2D).

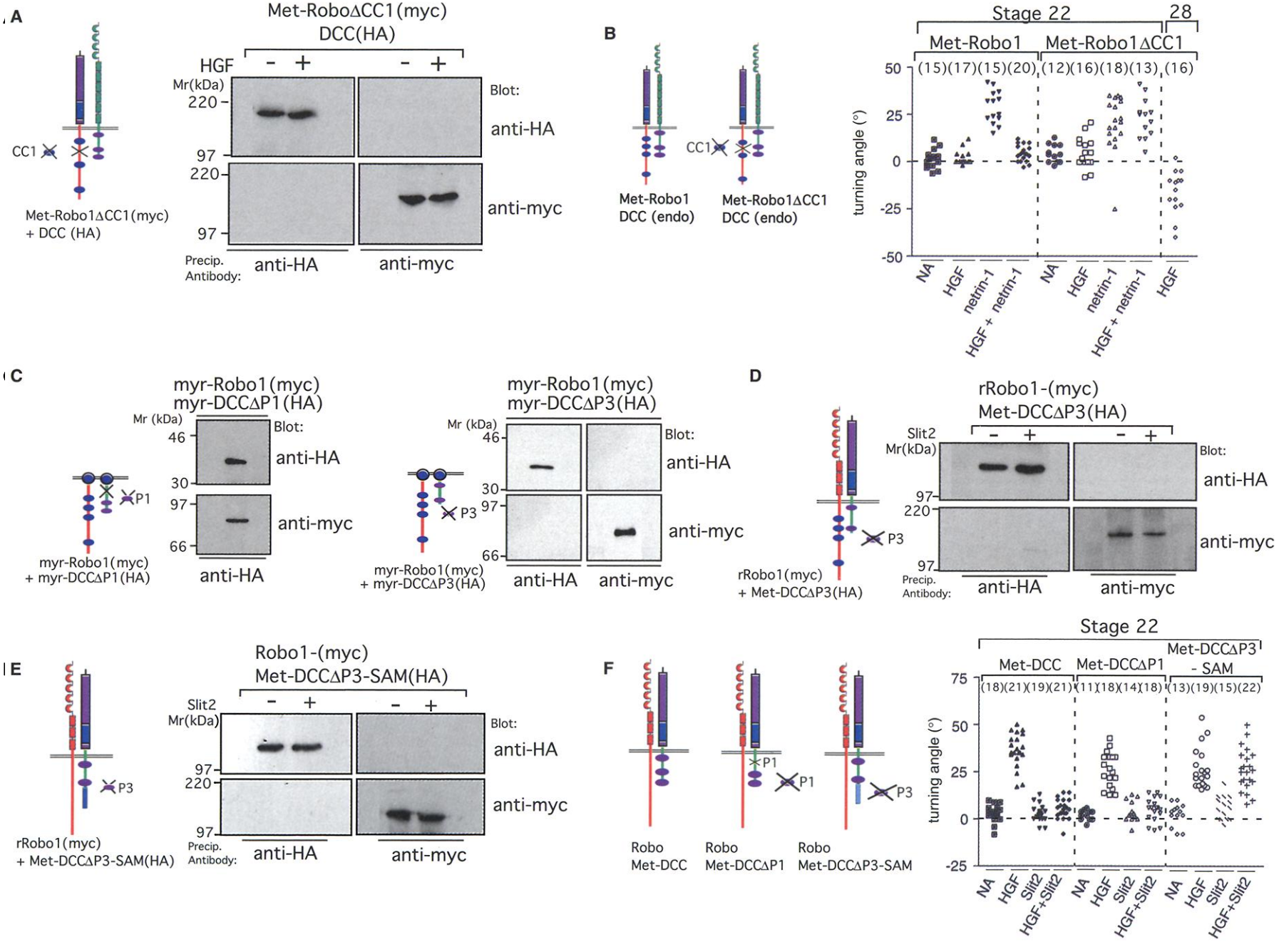
Stage 28 neurons expressing Met-Robo1 or trkA-Robo1 showed clear repulsive responses to HGF or NGF, respectively (Fig. 3E), responses that were not observed in st. 22 neurons (Fig. 3D). This finding supports the idea that there are differences between st. 22 and st. 28 neurons that determine whether only silencing or frank repulsion will be elicited by activation of the Robo signaling pathway.

Taken together, these studies strongly support the receptor-mediated silencing model by indicating that attractive responses elicited by activation of a DCC cytoplasmic domain (whether by netrin-1 or by a heterologous ligand acting on a chimeric receptor) can be silenced by activation of a Robo cytoplasmic domain (whether by Slit or by a heterologous ligand acting on a chimeric receptor). The fact that the truncated Robo blocks silencing also shows that the mere presence of Slit protein is not sufficient to block netrin attraction, arguing against an important role for the Slit-netrin interaction in silencing. In the absence of antibodies to Robo receptors in these *Xenopus* neurons, we cannot formally prove that Slit is mediating its effects on these axons through an endogenously expressed Robo receptor. Nonetheless, this assumption is supported by the findings that a truncated Robo receptor blocks silencing, that introduction of full-length rRobo1 into these neurons does not alter silencing, and that silencing can be elicited by activating Met-Robo1 or trkA-Robo1 chimeras. It is further supported by our finding of Robo mRNA expression in st. 22 spinal cord by reverse transcription polymerase chain reaction (*19*). In what follows, therefore, we will interpret results as if Slit is mediating its effect via an endogenous Robo receptor.

Association of Robo and DCC Cytoplasmic Domains

Silencing could be mediated by a direct interaction of Robo with DCC. Alternatively, activation of Robo by Slit could trigger a signaling cascade that blocks DCC signaling further downstream. To test these possibilities, we first asked whether Robo and DCC could form a receptor complex in transfected cells (*24*). A DCC construct tagged with an HA epitope [DCC(HA)] was coexpressed with a Robo1 construct tagged with a Myc epitope [Robo1(Myc)]. When DCC was immunoprecipitated with an antibody to the HA tag, Robo1 did not coimmunoprecipitate under control conditions or when the cells were exposed to netrin-1, but it did coimmunoprecipitate with DCC when the cells were incubated with Slit2, whether or not netrin-1 was present (Fig. 4A, left). The formation of a receptor complex of DCC and Robo1 in response to Slit2 exposure was similarly observed when the precipitations were performed with an antibody (9E10) to the Myc epitope on Robo1 (Fig. 4A, right).

Several experiments showed that the formation of the Robo-DCC complex is mediated by a cytoplasmic domain interaction. First, when most of the cytoplasmic domains of the two proteins are removed, neither Slit2 nor netrin-1 induces the formation of a receptor complex (Fig. 4B).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DCC(HA)
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anti-HA
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CC1
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Antibody:
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CC1
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Stage 22
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HGF
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Stage 22
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Met-DCCΔP3
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(11)(18)(14)(18)
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turning angle (°)
75
50
25
0
-25
NA
HGF
Slit2
HGF+Slit2
NA
HGF
Slit2
HGF+Slit2
NA
HGF
Slit2
HGF+Slit2

Second, when Robo1 was coexpressed with the Met-DCC chimera, Slit2 but not HGF induced the formation of a complex of the two receptors, as assessed by coimmunoprecipitation (Fig. 4C). Conversely, when Met-Robo1 was coexpressed with DCC, HGF but not netrin-1 induced the formation of a complex of the two receptors (Fig. 4D). Thus, neither the Robo1 ectodomain nor the DCC ectodomain per se are required for the formation of a receptor complex. In fact, activation of Robo1 by Slit2 even enabled it to bind the isolated cytoplasmic domain of DCC expressed as a myristoylated protein targeted to the inner leaflet of the plasma membrane (Fig. 4E). Thus, these results are consistent with the idea that activation of the Robo1 cytoplasmic domain (whether by Slit2 acting on Robo1 or by HGF acting on Met-Robo1) enables it to bind to the cytoplasmic domain of DCC (in the context of either DCC itself or Met-DCC, or expressed in isolation). The binding relation is asymmetric: Activation of Robo causes binding to DCC, but activation of DCC does not cause binding to Robo.

Although Robo1 and DCC did not associate in the absence of Slit2, their isolated cytoplasmic domains expressed as myristoylated proteins showed a constitutive association in transfected cells (Fig. 4F). This constitutive association was also observed in yeast using the two-hybrid system (Fig. 4, G and H) (*19*). Taken together, these results indicate that the cytoplasmic domains can associate but this association is repressed in the context of the full-length receptors; Slit2 functions to derepress this interaction, presumably by causing a conformational change in the cytoplasmic domain of Robo1.

Fig. 5 (opposite). Blocking the Robo-DCC cytoplasmic domain interaction by deleting the Robo CC1 domain or the DCC P3 domain blocks silencing. **(A)** Coimmunoprecipitation of Met-Robo1 and DCC in response to HGF could not be detected when Met-Robo1 lacks CC1 (Met-RoboΔCC1). **(B)** Turning angles of st. 22 growth cones expressing either Met-Robo1 or Met-RoboΔCC1 after 1 hour of exposure to the indicated ligands (concentrations as in Fig. 3). In Met-Robo1–expressing growth cones, netrin-1–induced attraction is silenced in the presence of HGF, whereas in Met-RoboΔCC1–expressing growth cones, HGF does not affect netrin-1 responses. Met-RoboΔCC1 can still function as a repulsive receptor in growth cones derived from st. 28 in a HGF gradient (right panel). **(C)** The ligand-independent interaction between the myristoylated cytoplasmic domains of Robo1 and DCC (Fig. 4E) is abolished by deletion of the P3 domain of DCC (right panels) but not by deletion of the P1 domain (left panel). **(D)** Deletion of P3 also abolishes the Slit2-induced association of Robo1(Myc) and Met-DCC (Fig. 4C). **(E)** Addition of the SAM domain of EphB1 to the extreme COOH-terminus of Met-DCCΔP3 does not restore the interaction of the two receptors. **(F)** The Met-DCCΔP3-SAM construct of (E) can mediate attraction (see text) but does not bind Robo1. Slit2 silences attractive responses mediated by HGF-induced activation of Met-DCC or Met-DCCΔP1 (lacking the P1 domain), but not that induced by activation of Met-DCCΔP3-SAM.

Blocking Robo-DCC Binding Blocks Silencing

To determine whether the association of cytoplasmic domains is causally involved in silencing, we identified regions in these domains that are required for the interaction and then tested whether interfering with the interaction also interfered with silencing. The Robo1 cytoplasmic domain has four conserved motifs termed CC0, CC1, CC2, and CC3 (*25, 26*) (Fig. 4G). By analyzing a series of deletion mutants starting at the COOH-terminus, we found that a construct including CC1 bound the DCC cytoplasmic domain in yeast, but further deletion of CC1 and the region between CC1 and CC0 abolished the binding (Fig. 4G). Although we have not narrowed the region required for interaction further in yeast, the relevant region appears to be CC1 itself, because specific deletion of CC1 abolished the association between DCC and Met-Robo1 that is induced by HGF (Fig. 5A).

Because deletion of CC1 from Met-Robo1 blocks the interaction with DCC, we tested whether this receptor, when introduced into st. 22 *Xenopus* neurons, could still silence attractive responses to netrin-1. As shown in Fig. 5B, this receptor could not silence netrin attraction (expression of the receptor was verified by immunohistochemistry with an antibody to the Myc tag). This result supports the idea that silencing requires direct cytoplasmic domain binding. Deletion of the CC1 domain in Met-Robo1 did not impair the ability of this receptor, when expressed in st. 28 *Xenopus* neurons, to induce a repulsive response to HGF (Fig. 5B); this result shows that the receptor's function in silencing and its function in repulsion can be separated, and it is consistent with the fact that deletion of CC1 in *Drosophila* Robo does not block its ability to rescue a *robo* mutant phenotype (*26*).

The DCC cytoplasmic domain has three regions conserved across species, named P1, P2, and P3 (*8, 27*) (Fig. 4H). P1 is required for the interaction of DCC and UNC5 cytoplasmic domains (*8*). In contrast, we found by deletional analysis that the P3 domain of DCC (at its extreme COOH-terminus) is both necessary and sufficient for binding to the Robo1 cytoplasmic domain in yeast (Fig. 4H). Similarly, the P3 domain of DCC (but not the P1 domain) is required for the constitutive association of the DCC and Robo1 cytoplasmic domains in transfected COS cells, and also for the Slit2-induced association of Robo1 and Met-DCC (Fig. 5, C and D).

Does deletion of P3, by blocking the DCC-Robo interaction, also block silencing? One impediment to testing this is the fact that P3 is also required for the function of DCC in attraction (*23*). Indeed, in a separate study (*23*), we found that DCC and Met-DCC multimerize in response to netrin-1 or HGF, respectively, and that deletion of P3 abolishes both this multimerization and the ability of Met-DCC to mediate attraction in response to HGF. We also found that replacing P3 with a different multimerization domain, the SAM domain of the EphB1 receptor (*28, 29*), can restore the multimerization of both DCC and Met-DCC in response to their ligands, as well as the ability of the Met-DCC receptor to induce an attractive response in neurons in response to HGF (*23*).

This Met-DCC receptor in which P3 is replaced with the EphB1 SAM domain (Met-DCCΔP3-SAM) does not associate with Robo in response to Slit (Fig. 5E). As predicted, when this receptor was introduced into st. 22 neurons, the attractive response to HGF in these neurons was not silenced by Slit2 (Fig. 5F).

Restoring Robo-DCC Binding Synthetically Restores Silencing

These experiments indicate that the ability of cytoplasmic deletion mutants to interact biochemically and their ability to mediate a silencing response are strictly correlated, consistent with the hypothesis that the physical interaction mediates silencing. A further prediction of that hypothesis is that if we could restore the physical interaction of deletion mutants synthetically, this might also restore a silencing interaction. We used the following strategy to test this possibility. First, because we wished to use deletion constructs of both the Robo and DCC cytoplasmic domains simultaneously in *Xenopus* neurons and to avoid confusion from the activities of endogenous receptors, we used two chimeric receptors: the trkA-Robo1 chimera (Fig. 3) and the Met-DCC chimera. In control experiments, the receptors behaved together as expected; that is, in transfected cells, NGF but not HGF induced formation of a receptor complex (Fig. 6A), and in *Xenopus* neurons expressing the two receptors, HGF elicited an attractive response that was silenced by NGF (Fig. 6B). As was further expected, deletion of CC1 from trkA-Robo1 blocked both the

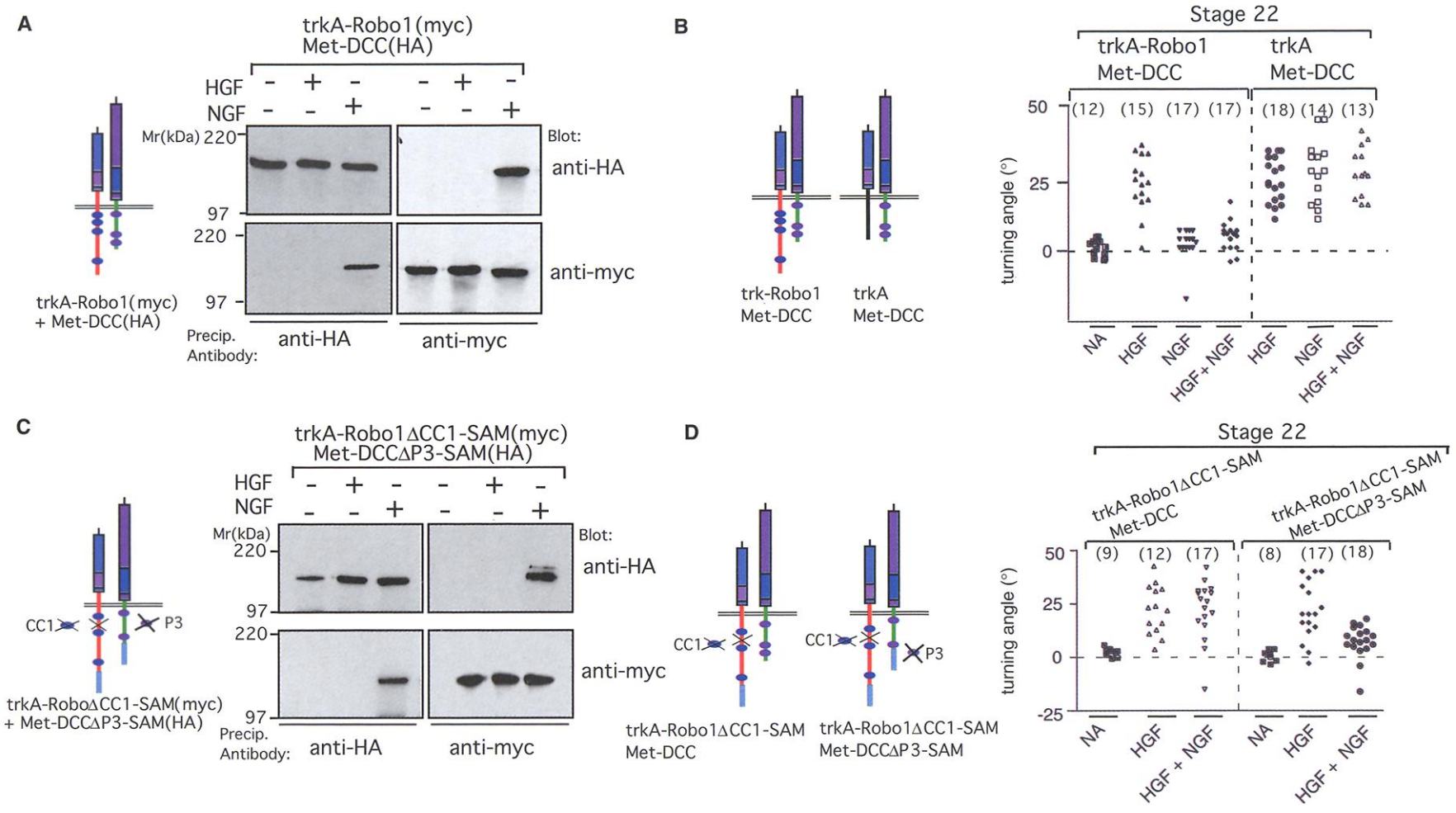

Fig. 6. Restoring the Robo1-DCC cytoplasmic domain interaction by addition of a synthetic interaction domain restores silencing. (**A** and **B**) Characterization of interactions between trkA-Robo1 and Met-DCC. (A) In transfected cells, activation of trk-Robo1 by NGF stimulates formation of a receptor complex with Met-DCC, whereas activation of Met-DCC by HGF does not. Coimmunoprecipitation conditions are as in Fig. 4, with NGF at 50 ng/ml. (B) Attraction mediated by activation of Met-DCC by HGF is silenced by activation of trkA-Robo1 by NGF, whereas attraction mediated by activation of wild-type Met by HGF is not. (**C** and **D**) The NGF-induced physical interaction of Met-DCC and trkA-Robo1 seen in (A), and the silencing of Met-DCC attraction by trkA-Robo1 activation seen in (B), are both abolished by deleting CC1 from the cytoplasmic domain of trkA-Robo1 (trkA-Robo1ΔCC1) or P3 from the cytoplasmic domain of Met-DCC (Met-DCCΔP3-SAM) (*19*). (C) Addition of the EphB1 SAM domain to the extreme COOH-termini of trkA-Robo1ΔCC1 (to generate trkA-Robo1ΔCC1-SAM) restores the NGF-induced association with Met-DCCΔP3-SAM, as assessed by coimmunoprecipitation. (D) It also restores the ability of NGF to induce silencing of attractive responses mediated by HGF-induced activation of Met-DCCΔP3-SAM, as seen in the scatter diagrams of turning angles in response to the indicated ligands.

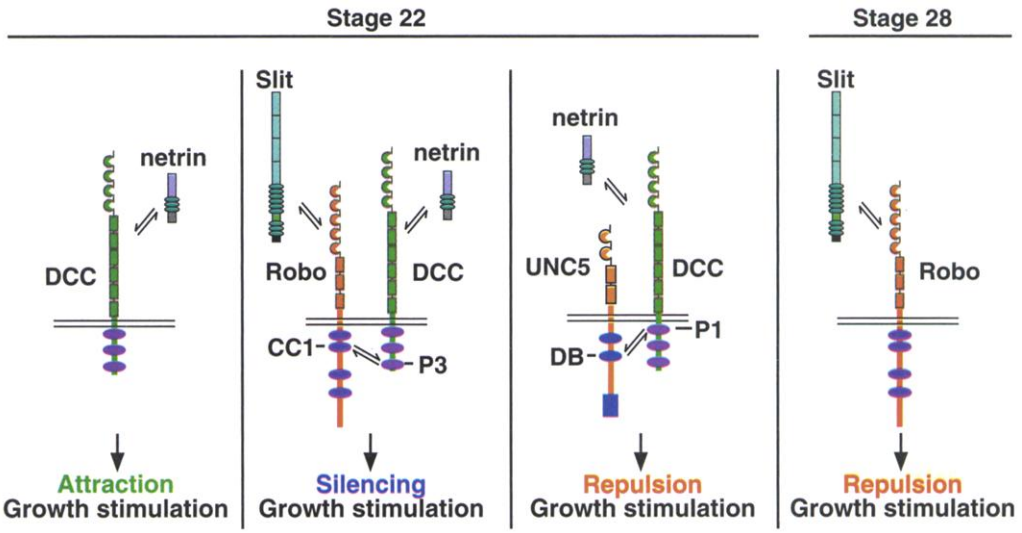

Fig. 7. Modular and interlocking design of guidance receptor cytoplasmic domains permits switching of growth cone responses. In st. 22 neurons, activation of DCC by netrin leads to attraction and a stimulation of the rate of axonal growth. Activation of Robo by Slit leads to silencing of the attractive netrin response without effect on its growth-stimulatory effect, through an interaction of the Robo and DCC cytoplasmic domains involving the CC1 (in Robo) and P3 (in DCC) domains. Expression of UNC5 proteins in neurons expressing DCC converts netrin-mediated attraction to repulsion, through an interaction of the UNC5 and DCC cytoplasmic domains involving the DB (in UNC5) and P1 (in DCC) domains. In st. 28 neurons, activation of Robo leads to repulsion.

physical association with Met-DCC and the ability of NGF to silence attraction caused by activation of Met-DCC by HGF (*19*). Similarly, this trkA-Robo1ΔCC1 construct also did not interact with or silence attractive responses elicited by activation of Met-DCCΔP3-SAM [the DCC receptor that lacks P3 but still functions in attraction (Fig. 5F)], but it did evoke a repulsive response to NGF when expressed in st. 28 neurons (*19*).

We predicted that addition of an EphB1 SAM domain to trkA-Robo1ΔCC1 might enable it to associate with Met-DCCΔP3-SAM, because of the multimerization function of the SAM domain. Indeed, NGF but not HGF induced binding of trkA-Robo1ΔCC1-SAM to Met-DCCΔP3-SAM. Having restored the binding, we could now ask whether this would restore silencing. Indeed, we found that NGF, by activating trkA-Robo1ΔCC1-SAM, could silence the attractive effect of HGF activating Met-DCCΔP3-SAM (Fig. 6D) but not the attractive effect of HGF activating Met-DCC (*19*). Thus, synthetically restoring the physical interaction restores silencing, consistent with silencing being mediated by the interaction.

Discussion

We have shown that activation of a Robo receptor by a Slit protein can silence the attractive effect of netrin-1 on cultured neurons without affecting the stimulation of extension rate by netrin-1. Thus, growth cones exposed to different guidance cues do not always simply integrate attractive and repulsive effects, as is commonly proposed; instead, cues can also interact in a hierarchical fashion, with the response to one gating the response to the other.

We have also shown that activation of Robo leads to binding of the cytoplasmic domain of Robo to that of the netrin receptor DCC and proposed that this interaction causes silencing (Fig. 7). The most conclusive evidence on this point builds on our finding that replacement of the DCC P3 domain by a SAM domain and of the Robo CC1 domain by a SAM domain generates receptors that are functional in attraction and in repulsion, respectively. When the SAM substitution is performed in only one of the two receptors, both the cytoplasmic domain interaction and the silencing effect are abolished. However, when the SAM substitution is performed simultaneously in both receptors, the interaction is restored, and so is the silencing effect. These results provide strong evidence that silencing is mediated directly by the cytoplasmic domain interaction. How the interaction causes silencing is unclear, but it presumably involves altering the complement of adaptor proteins recruited by the activated DCC receptor. Because the interaction selectively abolishes the directional effect elicited by DCC activation without abolishing its effect in stimulating extension, it is likely that only a subset of adaptors recruited by DCC activation are affected by Robo binding. An alternative possibility is that Robo interferes with DCC multimerization [which is also mediated by P3 and is required for attraction (*23*)], but this possibility seems less likely because blocking multimerization is expected also to block the stimulation of extension by netrin-1 (*23*).

We propose that this silencing effect is partly or entirely responsible for the loss of responsiveness of commissural axons to netrin-1 that occurs as they cross the midline in the vertebrate hindbrain. It remains to be determined whether loss of responsiveness to netrins upon crossing also occurs in the vertebrate spinal cord and in invertebrates, but this seems likely. In this model, as the axons cross the midline, they up-regulate the function of Robo receptors on their surface, and activation of Robo by Slit at the midline not only serves to repel the axons from the midline but also serves to switch off their attraction to the midline. Linking repulsion and loss of attraction in this way would ensure that the growth cone is never confronted with conflicting signals for attraction and repulsion, thus avoiding confusion of the growth cone as it becomes reprogrammed to move away from an environment it once perceived as attractive. Indeed, because changes in growth cone responsiveness at intermediate targets would usually involve a switch from attraction to repulsion, this hierarchical silencing relation between repellent and attractant mechanisms may be quite widespread and may involve repellent-attractant pairs other than Slits and netrins.

Our results have also documented that expression of a Robo receptor is not sufficient to predict the response of the neuron, because at st. 22 the activation of the receptor by Slit can cause silencing but does not elicit a repulsive response, whereas at st. 28 the activation results in repulsion. Repulsion and silencing are separable, because deletion of the CC1 domain abolishes the latter (in st. 22 neurons) without affecting the former (in st. 28 neurons). It remains to be determined whether the different responses at different stages reflect the presence of a different complement of adaptor proteins and/or coreceptors in the growth cone at these stages, or some post-translational modification. One interesting correlation is that st. 22 but not st. 28 growth cones express DCC, which raises

the question of whether it is the presence of DCC that prevents Robo from transducing a repulsive response.

The finding of dual silencing and repulsive functions of a Robo protein raises the possibility that some of the reported phenotypes in *robo* and *Slit* loss-of-function mutants in *Drosophila*, including lingering of the axons at the midline, may reflect not just loss of a repulsive function but also loss of a silencing function, so that the axons continue abnormally to be attracted by midline netrin. In this context, it is of interest that deletion of the CC1 domain of *Drosophila* Robo generates a receptor that can partially, but not entirely, rescue the *robo* mutant phenotype (*26*). This was interpreted, in light of the finding that binding of the Enabled adaptor was slightly reduced upon deletion of CC1, to suggest that the ability of this receptor to mediate repulsion was slightly impaired (*26*). An alternative (but not mutually exclusive) interpretation is suggested by our finding that deletion of CC1 abolishes the ability of Robo1 to silence netrin attraction (at one stage) without abolishing its ability to mediate repulsion (at another stage). If the same is true of *Drosophila* Robo, then axons expressing RoboΔCC1 might remain attracted by the midline netrin signal after they have crossed; even if they are repelled by Slit, this could result in a mild *robo*-like phenotype as the axons are simultaneously drawn back into the midline. In addition to being an interaction domain for Robo, CC1 is also the site of regulation of *Drosophila* Robo function by the Abl tyrosine kinase. Phosphorylation of a conserved tyrosine by Abl impairs Robo function, and mutation of that tyrosine to phenylalanine creates a hyperactive Robo receptor (*26*). It will be of interest to determine whether phosphorylation by Abl alters the silencing function of Robo receptors, or, conversely, whether the Robo-DCC interaction affects the ability of Abl to regulate Robo function.

It is even conceivable that silencing rather than repulsion may be the major function of Robo receptors in some situations in vivo, as it appears to be in st. 22 *Xenopus* neurons in culture. It should also be stressed, however, that there are nonetheless clear cases where Robo receptors function in repulsion, not just silencing. In the rat, the evidence indicates that commissural axons become repelled by Slit-2 immediately upon crossing the midline (*3*). Recent studies in *Drosophila* on Robo2 and Robo3 (both of which have a CC1 domain and are predicted to silence DCC receptors) have also provided clear evidence that they function to repel axons away from the midline, not just to block attraction (*30, 31*). Future studies will determine the extent to which the silencing and the repulsive functions of various Robo receptors predominate in different axon guidance events.

Our findings also provide a potential explanation for the otherwise puzzling observation that in all organisms DCC family receptors are widely expressed, even in neurons that are not responsive to netrins (as assessed either genetically or in vitro) (*4, 32*). For example, longitudinal neurons in *Drosophila* coexpress Robo and the DCC family receptor Frazzled but show no signs of attraction to the midline (*2, 32*). We propose that at least in some cases, the disjunction between DCC family receptor expression and netrin responsiveness may reflect silencing of the DCC receptor by another receptor such as Robo.

Finally, our finding of a Slit-gated interaction between the cytoplasmic domains of Robo1 and DCC is reminiscent of the netrin-gated interaction between DCC and UNC5 cytoplasmic domains that we previously described (Fig. 7) (*8*). In both cases, the isolated cytoplasmic domains can interact but this interaction is repressed in the context of the full-length proteins, and the ligands derepress the interactions (presumably by inducing a conformation change in the target receptor). In both cases as well, the cytoplasmic domain interaction alters the function of DCC: The interaction with UNC5 converts attraction to repulsion, whereas the interaction with Robo leads to silencing. Two different domains of DCC (the P1 and P3 domains, respectively) are dedicated to this interaction, as are specific domains in Robo and UNC5 (Fig. 7). Thus, these three guidance receptors (DCC, UNC5, and Robo) are designed to permit rapid and unambiguous switching of DCC function from attraction to repulsion or to silencing—a type of interlocking, modular design that may be found in other families of axon guidance receptors as well.

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6. The growth cone turning assay was performed as follows: Microscopic gradients of guidance molecules were produced as described [J. Q. Zhang, M. Felder, J. A. Connor, M.-M. Poo, *Nature* **368**, 140 (1994)]. 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-hour period. Net neurite extension was determined by measuring the total trajectory of the neurite at the end of the 1-hour period with imaging software. Only those growth cones with net growth of >10 μm were included for analysis. Usually only one neuron with strong GFP expression was assayed from a single culture plate, and at least two separate batches of injected embryos were tested for each experiment.
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