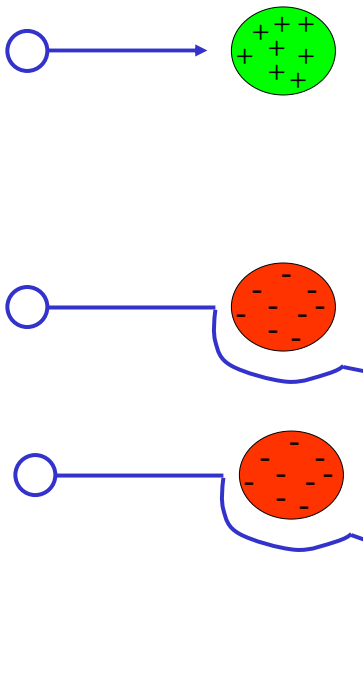
2- Slit-Robo-Netrin-DCC

Table 1. Directional Guidance cues involved in CNS neuronal migration in vivo and in vitro

Ligands	Receptors	Defects in CNS neuronal migration in vivo	Neuronal migration in vitro
Slits	Robo	—	1. Slit repels postnatal SVZa cells[37] 2. Slit repels prenatal SVZ cells of GE[43]
Netrins	DCC	1. Abnormal pontine nuclei in DCC and netrin-1 mutants[46]	1. Netrin-1 attracts pontine nuclei[11]
	Unc-5h	2. Abnormal cerebellar development in unc-5h3[64]*	2. Netrin-1 repels postnatal cerebellar granule cells and prenatal SVZ cells[48,49] 3. Anti-DCC antibody blocks directed migration of postnatal SVZa cells[47]
Semaphorins	Neuropilin Plexin	1. Abnormal GABAergic interneurons in the striatum in neuropilin-2 mutants[50]	—
Ephrins	Eph	—	1. Disruption of Eph-B/Ephrin-B system affects the migration of postnatal SVZa cells[51]

*Unc-5h3/RCM mutant mice showed abnormal development of cerebellum. However, it is still unclear that the defect is primarily caused by migration abnormality or other reasons.

1



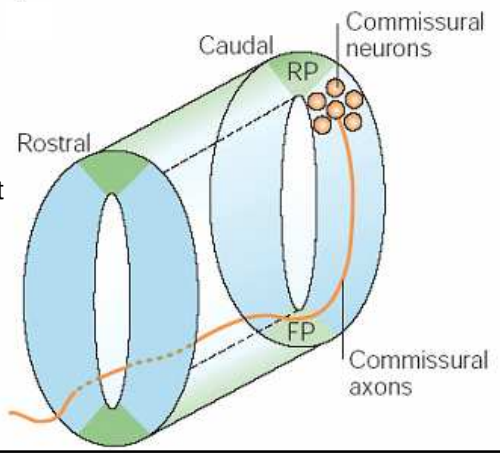
- as axons grow long distances in the developing embryo, they make use of intermediate targets to simplify their navigation into short, manageable segments
- these intermediate targets produce both attractants and repellents, which axonal growth cones must recognize in sequential order to navigate properly

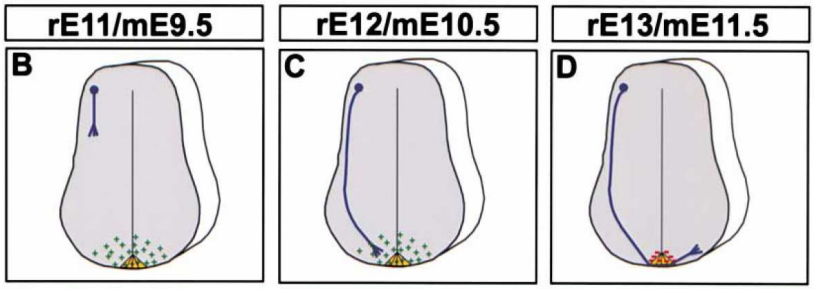
• after being initially attracted to their intermediate targets, growth cones must undergo a change in responsiveness to continue on their migratory route, losing responsiveness to the attractants that led them to their intermediate target and gaining responsiveness to repellents produced by that same target

• this change must be tightly regulated, so that growth cones can move on to the next stage in their trajectory only once they have passed through their intermediate target

• the ventral midline of the nervous system of both vertebrates and invertebrates has served as a model system for understanding the mechanisms by which axons interact with intermediate targets

• Commissural neurons, a subset of interneurons, use the ventral midline as a key intermediate target on their way to their final targets in the contralateral half of the body





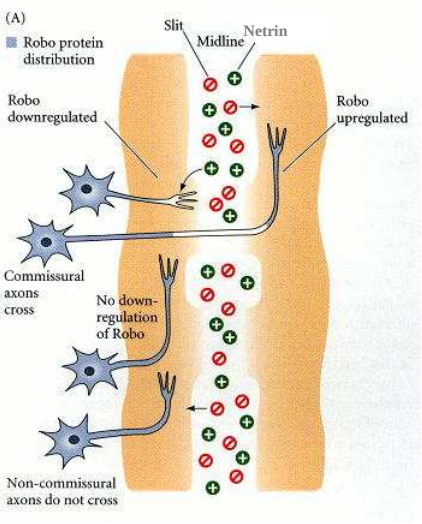
• in vertebrates and insects, commissural axons are initially drawn to the midline by attractant proteins (which include members of the netrin family)

• upon crossing the midline and reaching the contralateral side, however, these growth cones turn longitudinally, lose responsiveness to netrins and become sensitive to repellents made by midline cells (which include Slit proteins)

• this switch prevents commissural axons from recrossing the midline and allows them to move on toward their final targets

4

Simplified model for chemotactic factors directing commissural axons to cross the midline while keeping other axons on one side of the midline.



• the midline secretes netrin protein, which is stimulatory to commissural axons, and Slit protein, which is inhibitory to non-commissural axons

• when they reach the midline, commissural axons have little or no Robo protein, the receptor of Slit

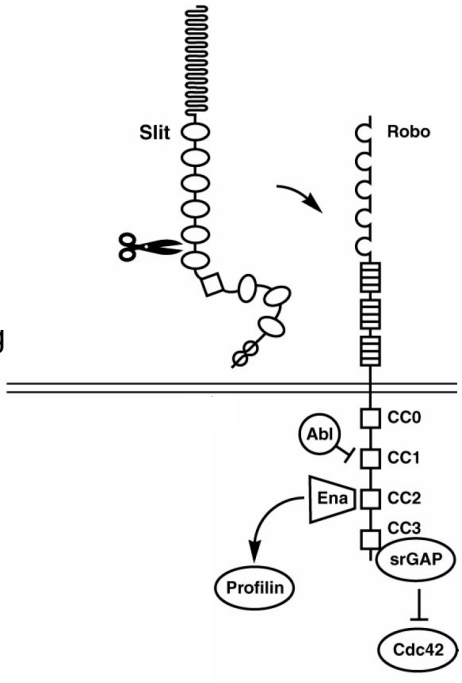
• stimulated by netrin, these axons cross the midline. Once across the midline, they re-express Robo, and therefore cannot return

• non-commissural neurons express Robo and therefore are inhibited from crossing the midline

5

SLIT (ligando)-Robo (recettore)

• axon pathfinding
• axonal and dendritic branching
• neuronal cell migration



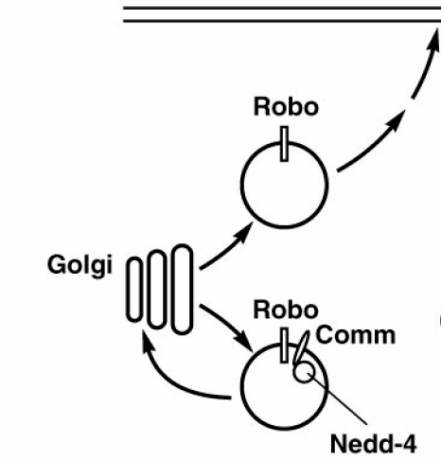
Drosophila

Robo -/-: quale fenotipo vi aspettate di trovare?

Comm: regola (down-regolandolo) il livello di espressione di Robo (interagisce con Robo sottraendolo alla superficie)

Comm -/-: quale fenotipo vi aspettate di trovare?

Comm +++: quale fenotipo vi aspettate di trovare?



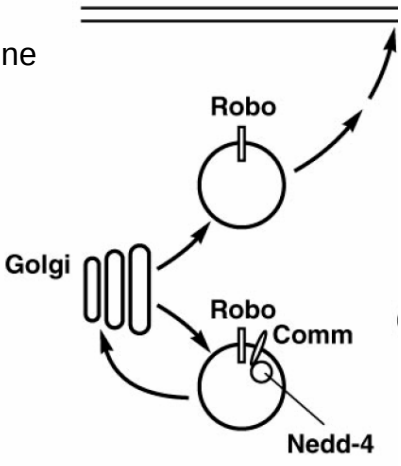
Robo: *roundabout* (*Drosophila*)

-/- attraversamenti multipli

Comm: *commissureless*: regola (down-regolandolo) il livello di espressione di Robo (interagisce con Robo sottraendolo alla superficie)

-/- no attraversamento della midline

+++ tutti gli assoni attraversano



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

Elke Stein and Marc Tessier-Lavigne*

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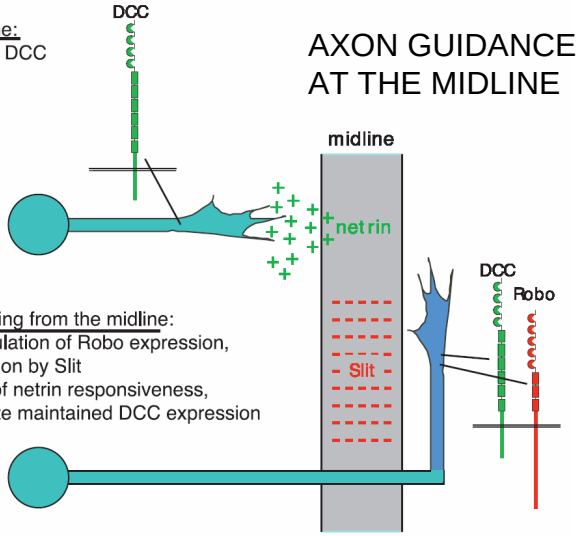
1. Attraction to midline:
netrin activation of DCC

2. Crossing and moving from the midline:
Event 1: Upregulation of Robo expression, repulsion by Slit
Event 2: Loss of netrin responsiveness, despite maintained DCC expression

AXON GUIDANCE EVENTS AT THE MIDLINE

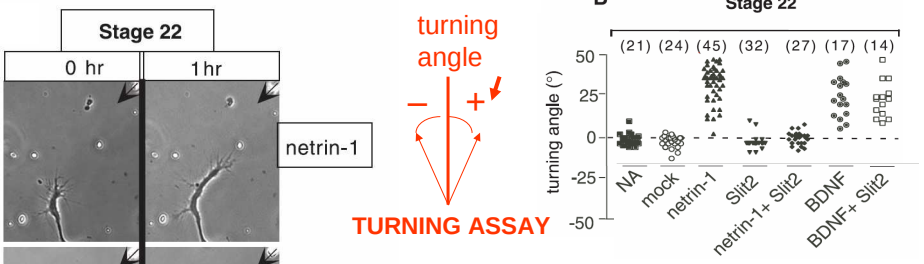


In vertebrates, insects, and nematodes, commissural axons are attracted by netrin protein(s) secreted by midline cells, which attract by activating a receptor of the DCC family on growth cones. After crossing the midline, axons change their responsiveness, such that they are repelled by the midline. 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).

10

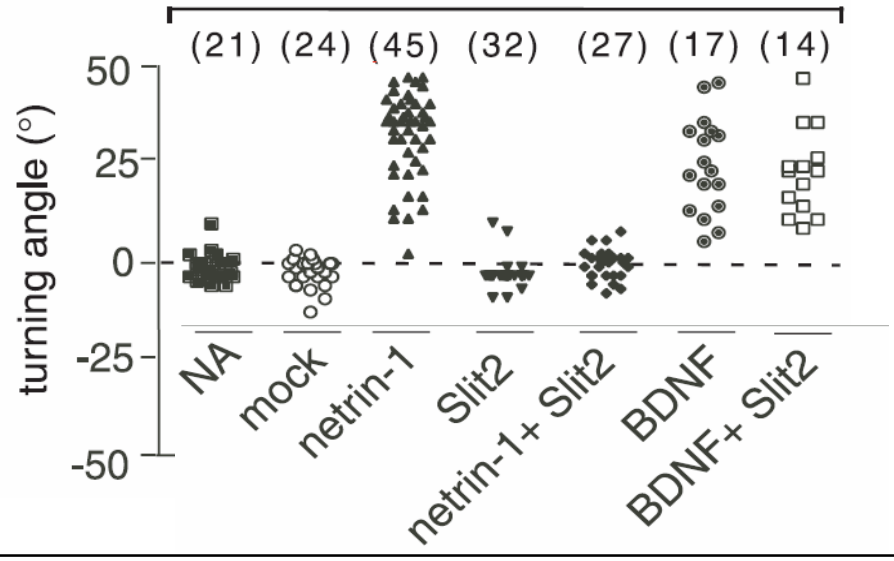
SLIT2 SILENCES ATTRACTION TO NETRIN-1 BUT NOT TO BDNF

22

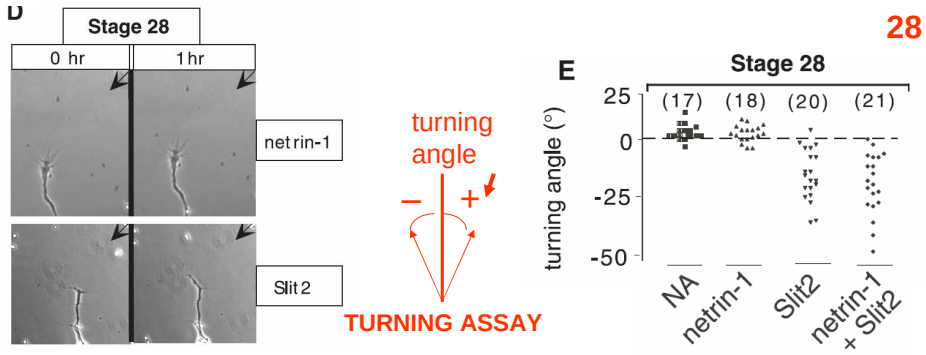


When growth cones of neurons from stage 22 Xenopus embryos are exposed to a gradient of netrin-1 for 1 hour, they turn toward the source. This attractive response requires the function of the netrin receptor DCC. In contrast, the same axons exposed to a gradient of Slit2 protein did not show a directional response. 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. 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), which attracts these axons by activating the trkB receptor in these cells.

Come si interpreta questo grafico? Quali informazioni mancano rispetto ad un classico grafico con gli istogrammi? Secondo voi, perché gli autori hanno scelto questo tipo di grafico?



•The finding that Slit2 silences netrin-1 attraction of stage 22 growth cones but does not repel them was unexpected, because Slit2 is expected to function as a repellent.



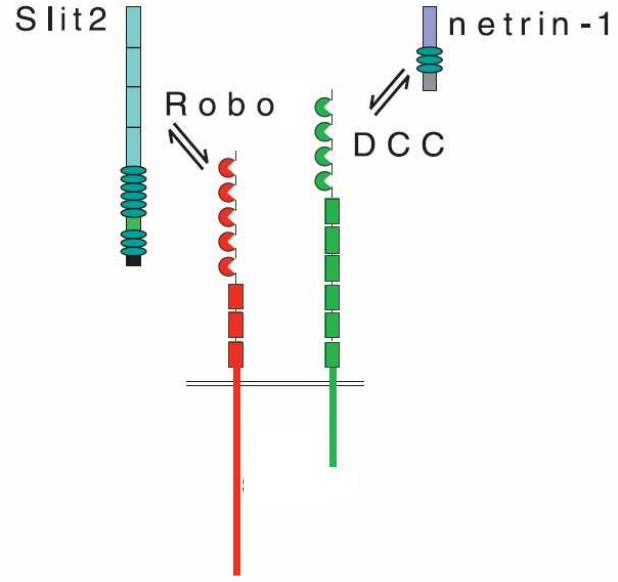
28

•The axons of older spinal neurons obtained from stage 28 embryos were consistently repelled by Slit2, but did not show any response to netrin-1, likely **because of the absence of DCC expression** in these neurons, as assessed by immunohistochemistry. So it cannot be tested whether Slit2 has a silencing function at that stage as well.

•The differences between stage 22 and stage 28 neurons suggest that the *Xenopus* spinal neurons in these cultures switch their responsiveness to netrins and Slits over time.

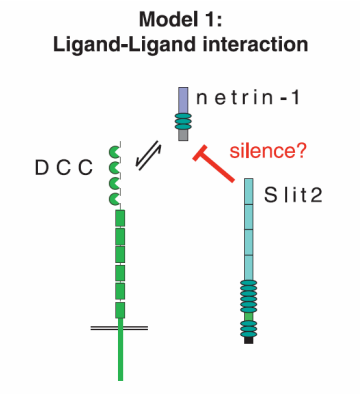
13

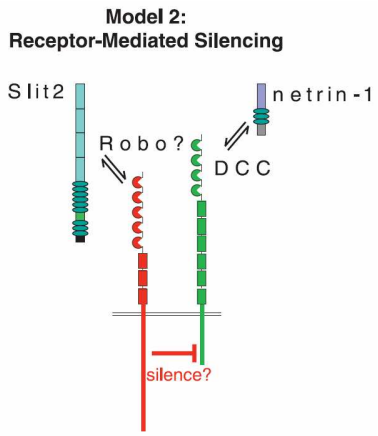
Con quali modelli si può spiegare l'effetto di silenziamento di Slit sull'attrazione mediata dalla netrina?



14

Two models could explain the silencing effect of Slit2 on netrin-mediated attraction.





1. because Slit2 can bind netrin-1 directly, silencing might be caused by binding of the two proteins, which could in principle interfere with the netrin-DCC interaction.

2. silencing might be a receptor-mediated event, with Slit2 activating a receptor (presumably a Robo receptor) on growth cones that antagonizes netrin attraction mediated by DCC.

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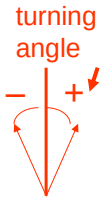
• in all subsequent **TURNING ASSAYS**, exogenous receptors were expressed by injecting in vitro transcribed mRNA encoding versions of the receptors of interest [usually tagged with a **Myc** or hemagglutinin (**HA**) epitope tag] into the second blastomere at the four-cell stage of *Xenopus* embryos, together with mRNA encoding **green fluorescent protein (GFP)** as a marker for expression of exogenous proteins

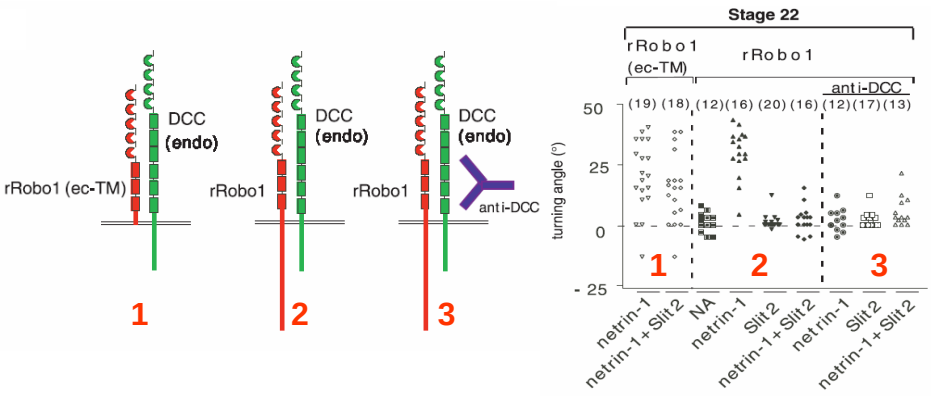
• embryos were allowed to develop to stage 22, and GFP-expressing spinal cord neurons derived from these embryos were assayed for turning responses



TURNING ASSAY

16

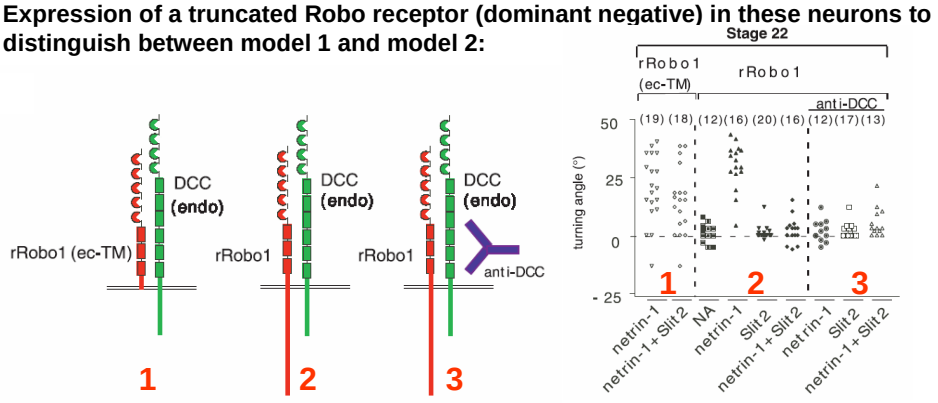
Expression of a truncated Robo receptor (dominant negative) in these neurons to distinguish between model 1 and model 2:



Osservando questi primi esperimenti, quale dei due modelli ipotizzati vi sembra il più plausibile per spiegare il silenziamento dell'attrazione?

17

Expression of a truncated Robo receptor (dominant negative) in these neurons to distinguish between model 1 and model 2:



1 Slit2 no longer silenced the attractive effect of netrin-1; this result is consistent with the involvement of a **receptor-mediated mechanism in silencing**

2 expression of full-length rRobo1 in these cells did not interfere with silencing by Slit

3 Slit2 did not repel growth cones expressing full-length rRobo1, indicating that expression of a Robo receptor is not sufficient for repulsion, which presumably requires additional signaling molecules in the growth cone

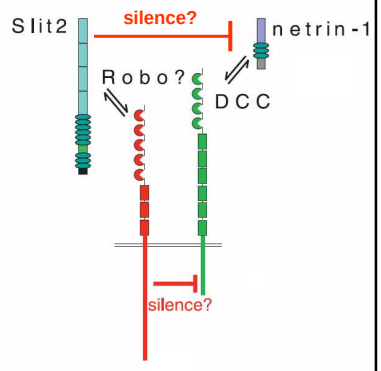
4 the attractive effect of netrin-1 observed in all experimental conditions was blocked by antibodies to DCC, consistent with the requirement of DCC for netrin-mediated attraction

18

• truncated Robo receptor can block silencing by Slit

→ **receptor - mediated mechanism but....**

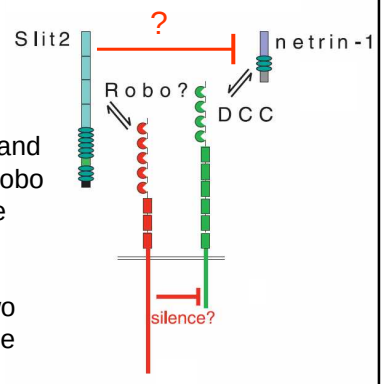
Secondo voi il modello 2 (quello dell'interazione fra recettori) è l'unico che può spiegare i dati ottenuti con il recettore privo del dominio citoplasmatico?



19

• truncated Robo receptor can block silencing by Slit

→ **receptor - mediated mechanism but....**

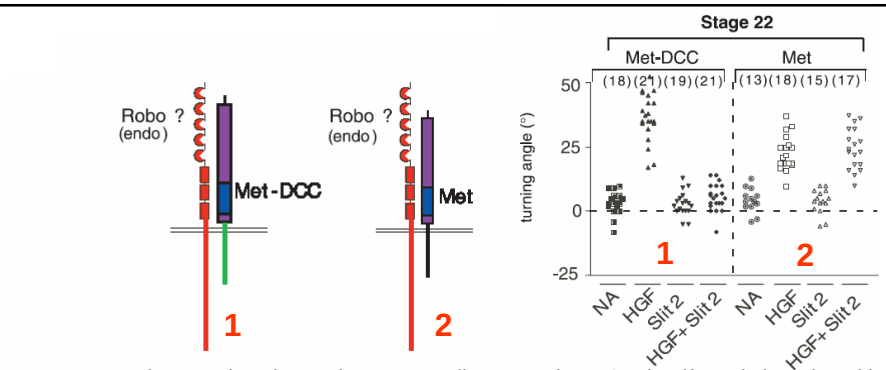


• ....this result is also compatible with a ligand-ligand interaction model of silencing if the exogenous Robo can bind and somehow locally reduce (titrate) the amount of available Slit2 protein

• to more definitively discriminate between the two models, they used chimeric receptors 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

• quali vantaggi dà un costrutto chimerico Met-DCC o Met-Robo?

20



• *Xenopus* growth cones in cultures do not normally respond to HGF, but if Met is introduced into them, they respond to HGF with attraction

• when a chimeric receptor comprising the Met ectodomain and the DCC transmembrane and cytoplasmic domain is introduced into these cells, HGF induces attractive responses

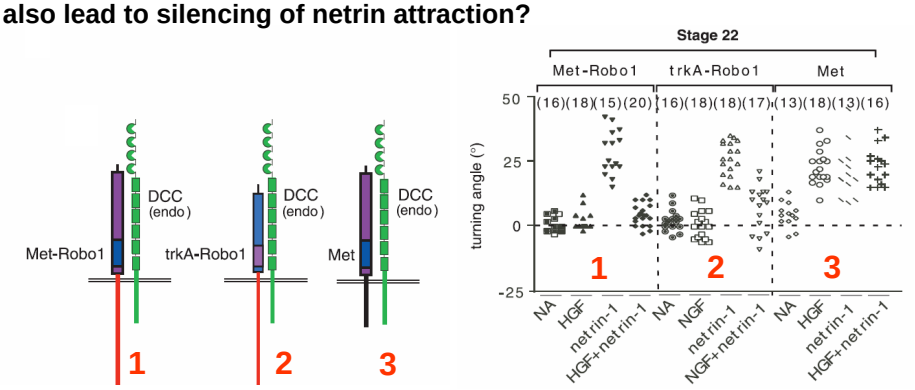
• Slit2 is as effective in silencing attractive responses elicited by HGF binding to the Met-DCC chimeric receptor as it is in silencing netrin-mediated attraction

• Slit2 does not silence attractive responses to HGF that are mediated by the wild-type Met receptor tyrosine kinase

• **silencing is observed only for attraction caused by activation of the DCC cytoplasmic domain**

21

¿Could activation of the Robo signaling pathway by a heterologous ligand also lead to silencing of netrin attraction?



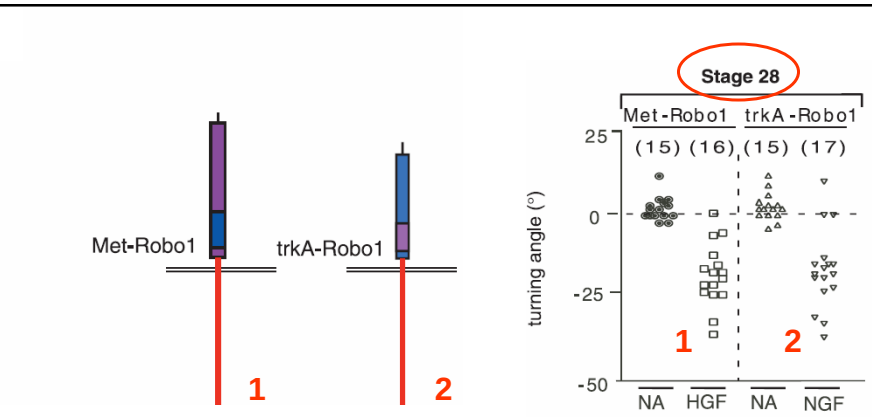
• chimeric receptors comprising the cytoplasmic domain of rRobo1 and the ectodomain of either Met or the trkA (receptor tyrosine kinase for nerve growth factor), were introduced in these cells

• in neurons expressing the Met- Robo1 chimera, as observed with Slit, HGF did not elicit directional responses, but completely silenced the attractive effect of netrin-1

• in neurons expressing the trkA-Robo1 chimera, NGF did not elicit directional responses, but completely silenced the attractive effect of netrin-1

• 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

22



• **stage 28 neurons** expressing Met-Robo1 or trkA-Robo1 showed clear repulsive responses to HGF or NGF, respectively, responses that were not observed in stage 22 neurons

• this finding supports the idea that **there are differences between stage 22 and stage 28 neurons** that determine whether only silencing or frank repulsion will be elicited by activation of the Robo signaling pathway

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• 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)

• in the absence of antibodies to *Xenopus* Robo receptors, it cannot formally be proved 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
-introduction of full-length rRobo1 into these neurons does not alter silencing
-silencing can be elicited by activating Met-Robo1 or trkA-Robo1 chimeras
-Robo mRNA is expressed in stage 22 neurons (RT-PCR data)

→ therefore, **results will be interpreted as if Slit is mediating its effect via an endogenous Robo receptor**

Quale tecnica si può utilizzare per vedere se Robo e DCC formano un complesso?

24

? Could Robo and DCC form a receptor complex in transfected cells?

RECEPTOR CO-IMMUNOPRECIPITATIONS

• HA- and Myc-tagged versions of DCC and Robo1 [**DCC(HA)** and **Robo1(Myc)**] were co-transfected into **COS cells**

• 40 hours after transfection, cells were incubated for 20 min at 37℃ with ligands (control medium, netrin-1, Slit2, HGF, NGF)

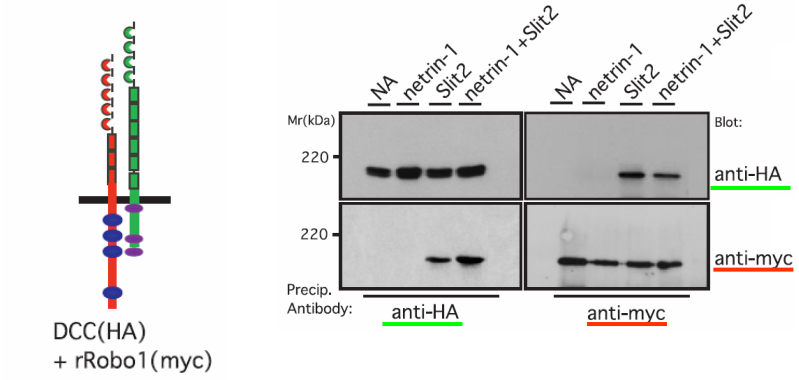
• total proteins were extracted

• proteins were subjected to immunoprecipitation, using the indicated antibodies

• proteins were analyzed by Western blotting

25

¿Could Robo and DCC form a receptor complex in transfected cells?

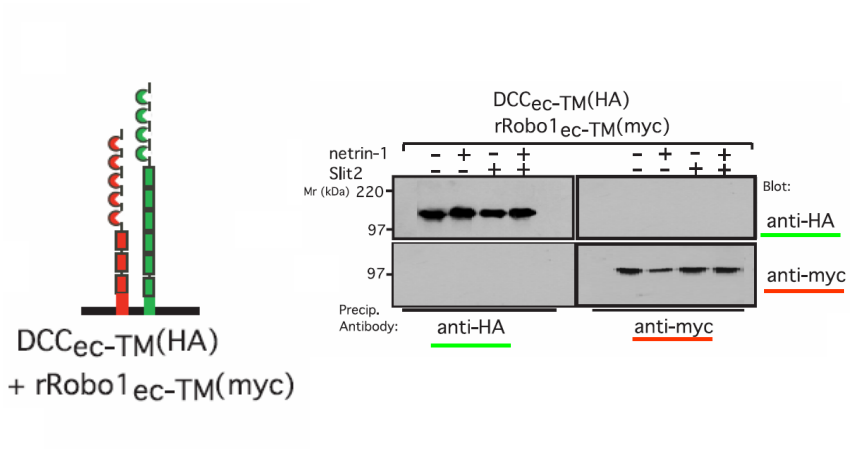


• a DCC construct tagged with an HA epitope **[DCC(HA)]** was co-expressed with a Robo1 construct tagged with a Myc epitope **[Robo1(Myc)]**

• 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

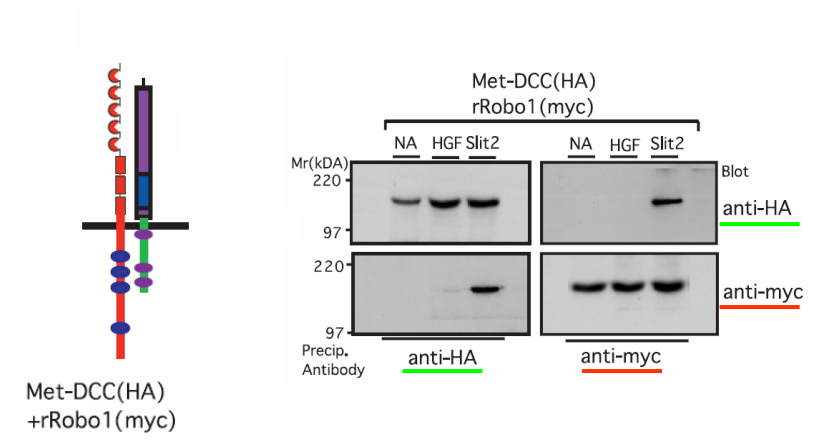
• the formation of a receptor complex of DCC and Robo1 in response to Slit2 exposure was similarly observed when the precipitations were performed with an antibody to the Myc epitope on Robo1

26



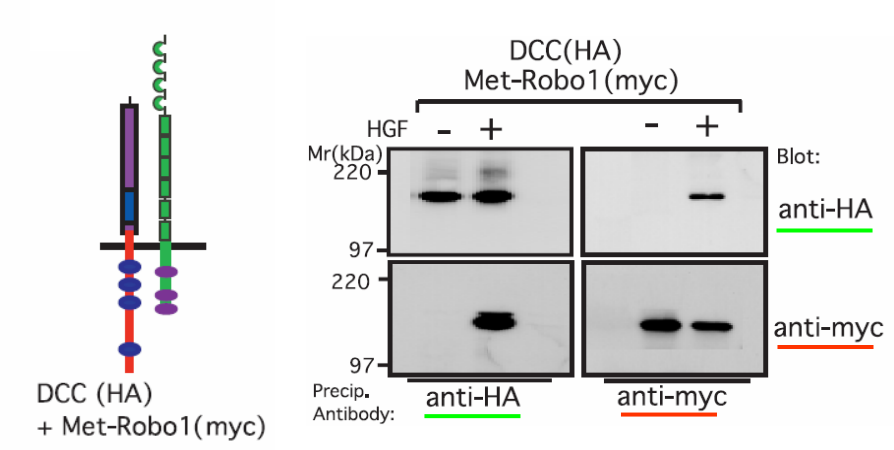
• when most of the cytoplasmic domains of the two proteins are removed, neither Slit2 nor netrin-1 induces the formation of a receptor complex

27



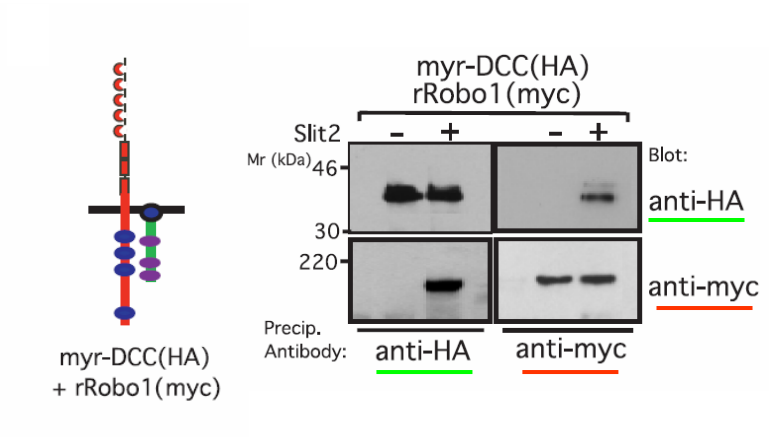
• when Robo1 was coexpressed with the Met-DCC chimera, Slit2 but not HGF induced the formation of a complex of the two receptors, as assessed by co-immunoprecipitation

28



• when Met-Robo1 was coexpressed with DCC, HGF but not netrin-1 induced the formation of a complex of the two receptors

29



• 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

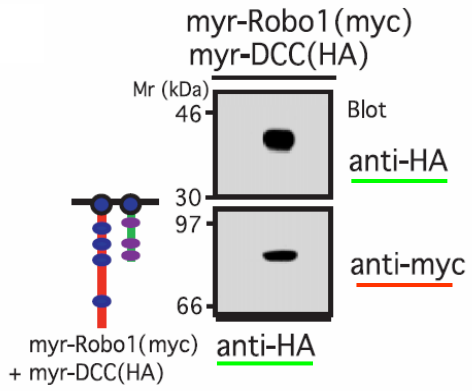
30

→ neither the Robo1 ectodomain nor the DCC ectodomain *per se* are required for the formation of a receptor complex

→ 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

31

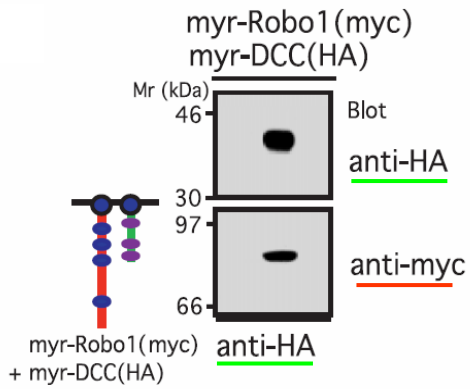


• Robo1 and DCC isolated cytoplasmic domains expressed as myristoylated proteins show a constitutive association in transfected cells although they do not associate in the absence of Slit2

• this constitutive association was also observed in yeast using the two-hybrid system

Se i domini citoplasmatici (privi della regione extracellulare) interagiscono spontaneamente, cosa accade quando il recettore intero Robo viene stimolato dal ligando Slit?

32



• Robo1 and DCC isolated cytoplasmic domains expressed as myristoylated proteins show a constitutive association in transfected cells although they do not associate in the absence of Slit2

• this constitutive association was also observed in yeast using the two-hybrid system

→ 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

33

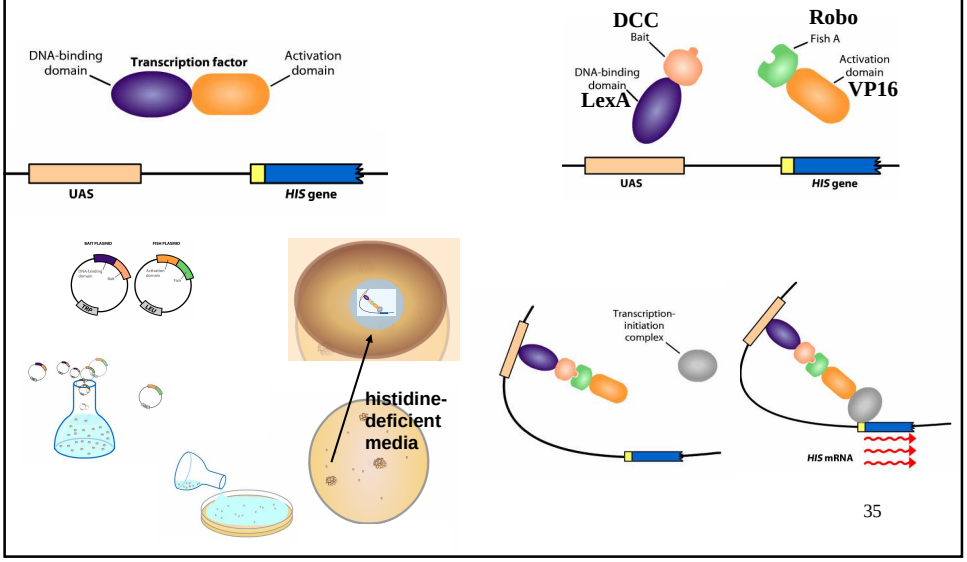
→the cytoplasmic domains can associate but this association is repressed in the context of the full-length receptors

Quale tecnica utilizzeresti per identificare i domini responsabili dell'interazione fra i due recettori?

34

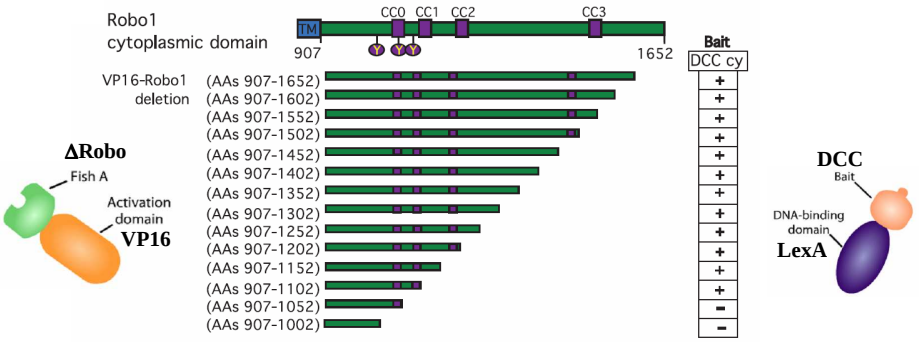
¿Interfering with the interaction, does interfere with silencing?

• to determine whether the association of cytoplasmic domains is causally involved in silencing, regions in these domains that are required for the interaction were identified through a **yeast two-hybrid analysis**



35

Yeast two-hybrid analysis of the interaction between the cytoplasmic domains of Robo1 (as a VP16 fusion fish = pesce) and DCC (as a LexA fusion bait = esca)

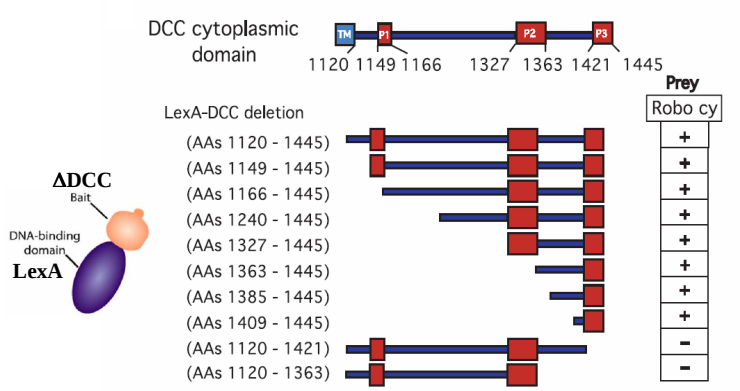


• Robo cytoplasmic domain deletion constructs and their ability to interact with the DCC cytoplasmic domain

• interactions were assessed by the ability to rescue growth on histidine-deficient plates (+, rescue; -, no rescue)

→ **deletion of the CC1 domain causes loss of interaction with DCC** 36

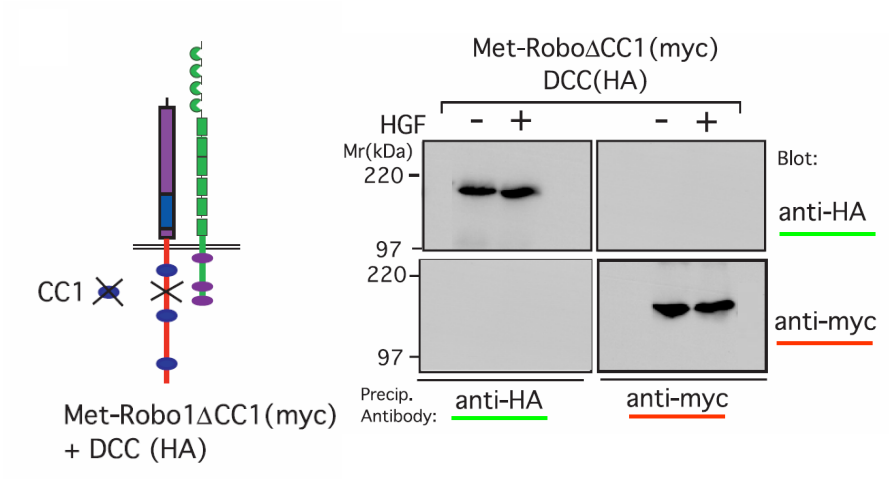
Yeast two-hybrid analysis of the interaction between the cytoplasmic domains of DCC (as a LexA fusion bait) and Robo1 (as a VP16 fusion prey)



• DCC cytoplasmic domain deletion constructs and their ability to interact with the Robo1 cytoplasmic domain prey.

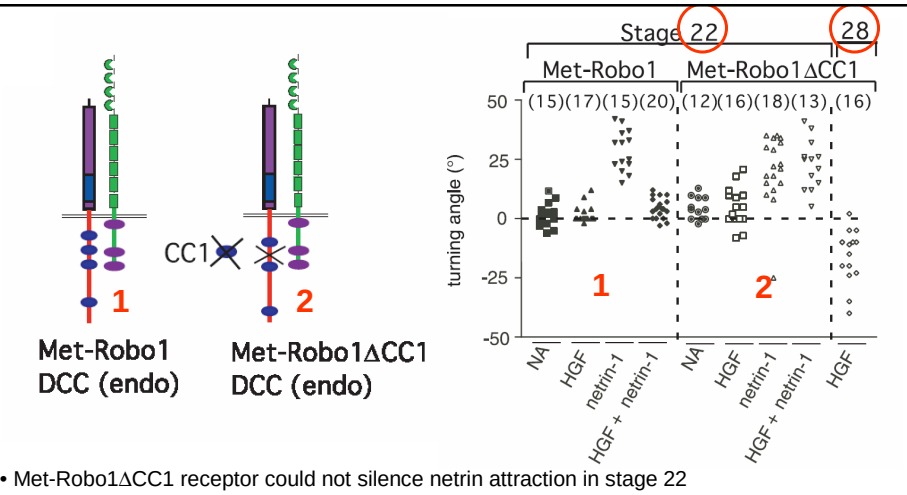
• interactions were assessed by the ability to rescue growth on histidine-deficient plates (+, rescue; -, no rescue)

→ **deletion of the P3 domain causes loss of interaction with Robo** 37



• specific deletion of CC1 abolished the association between DCC and Met-Robo1 that is induced by HGF

38



• Met-Robo1ΔCC1 receptor could not silence netrin attraction in stage 22

→ this result supports the idea that silencing requires direct cytoplasmic domain binding

• Met-Robo1ΔCC1 induces a repulsive response to HGF in stage 28 neurons

→ this result shows that the receptor functions in silencing and in repulsion can be separated

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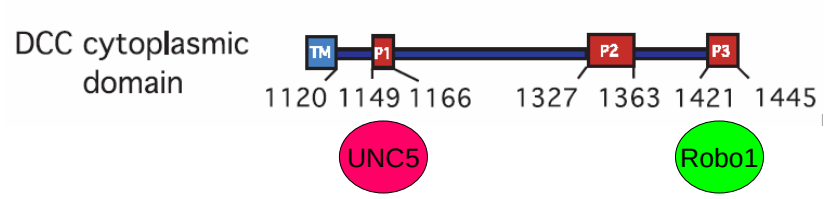

• the DCC cytoplasmic domain has three regions conserved across species, named P1, P2, and P3

• P1domain is required for the interaction of DCC and UNC5 cytoplasmic domains

• P3 domain is both necessary and sufficient for binding to the Robo1 cytoplasmic domain in yeast

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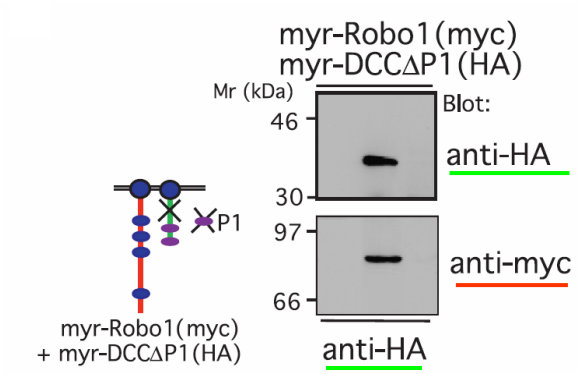

• the P1 domain is not required for the constitutive association of the DCC and Robo1 cytoplasmic domains in transfected COS cells

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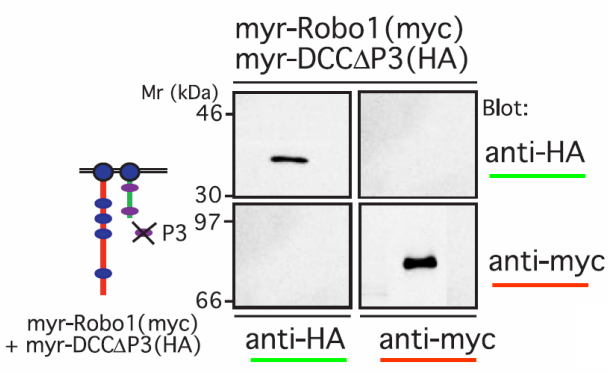
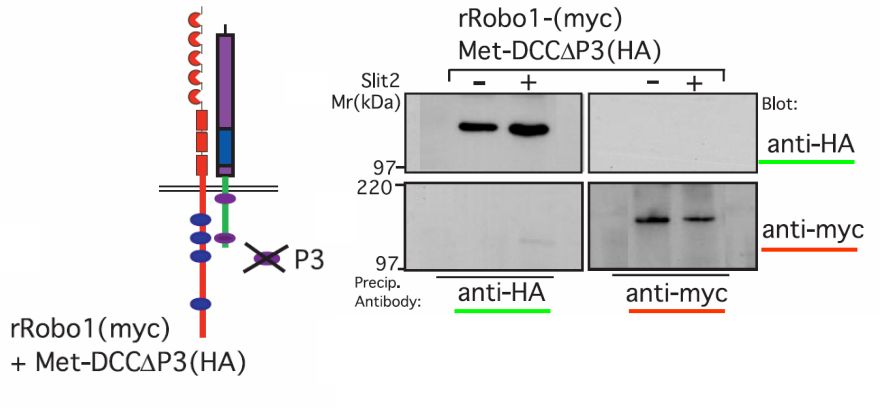


• the P3 domain of DCC is required for the constitutive association of the DCC and Robo1 cytoplasmic domains in transfected COS cells

42



• the P3 domain of DCC is required for the Slit2-induced association of Robo1 and Met-DCC

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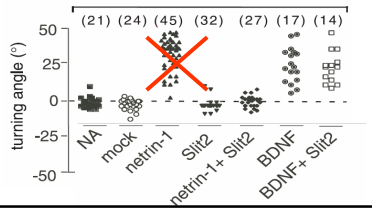
¿Does deletion of P3, by blocking the DCC-Robo interaction, also block silencing?

• one impediment to testing this is the fact that **P3 is also required for the function of DCC in attraction**
(:se elimino l'attrazione, come posso studiare il silenziamento dell'attrazione?)

• DCC and Met-DCC multimerize in response to netrin-1 or HGF, respectively, and deletion of P3 abolishes both this multimerization and the ability of Met-DCC to mediate attraction in response to HGF (previous data)

• replacing P3 with a different multimerization domain, the **SAM** domain of the EphB1 receptor, 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



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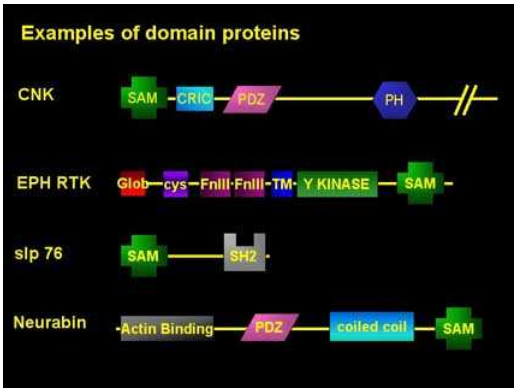
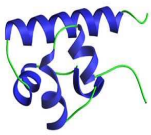
SAM Domain Binding and Function

The approximately 70 amino acid SAM (Sterile Alpha Motif) domain has been identified in over 400 different proteins with diverse cellular function, from yeast to man. SAM domains have been implicated in mediating protein-protein interaction via the formation of homo and hetero-typic oligomers. The residues at the interface of the EphA4 and EphB2 SAM domain homodimers have been mapped, but the factors that determine specificity remain to be determined.





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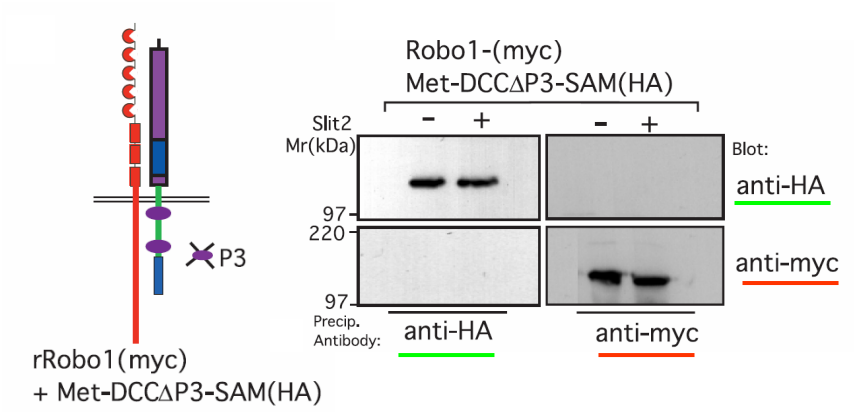

• the Met-DCC receptor in which P3 is replaced with the EphB1 SAM domain (**Met-DCCΔP3-SAM**) does not associate with Robo in response to Slit

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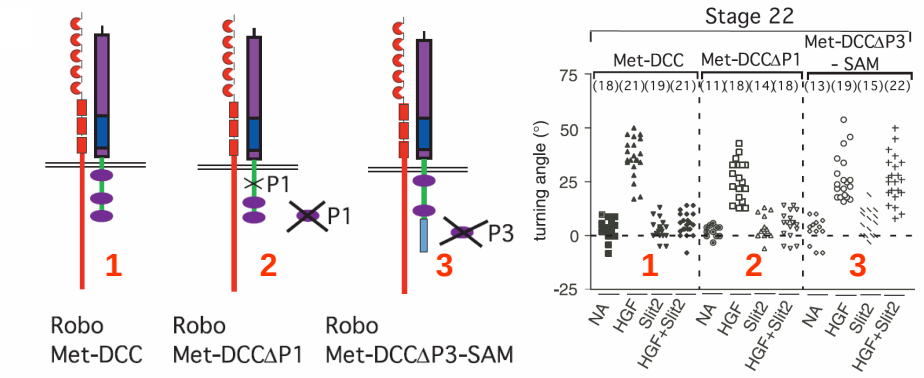

• the Met-DCC receptor in which P3 is replaced with the EphB1 SAM domain (**Met-DCCΔP3-SAM**), introduced into stage 22 neurons, can mediate attractive response to HGF but does not bind Robo1 and is not silenced by Slit2

• Slit2 silences attractive responses mediated by HGF-induced activation of **Met-DCC** or **Met-DCC ΔP1**

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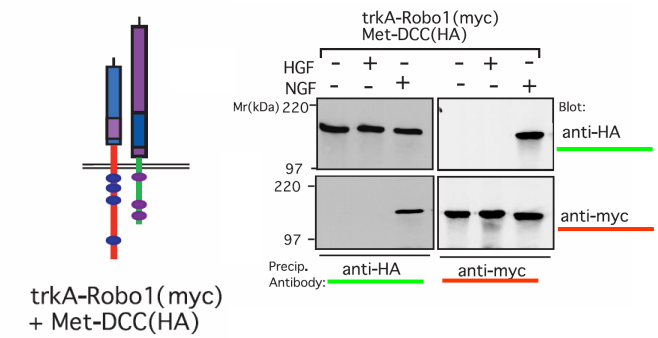
• the ability of cytoplasmic deletion mutants to interact biochemically and their ability to mediate a silencing response are strictly correlated, consistent with the hypothesis that the physical interaction mediates silencing.

¿Restoring Robo-DCC Binding Synthetically does Restore Silencing?



• 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, two chimeric receptors were used : the **trkA-Robo1** chimera and the **Met-DCC** chimera

• as expected, in transfected cells, NGF but not HGF, induced formation of a receptor complex

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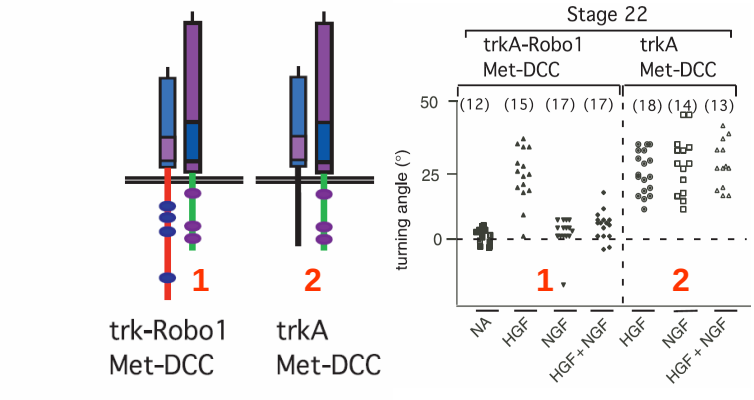

• in *Xenopus* neurons expressing the **trkA-Robo1** chimera and the **Met-DCC** chimera, HGF elicited an attractive response that was silenced by NGF

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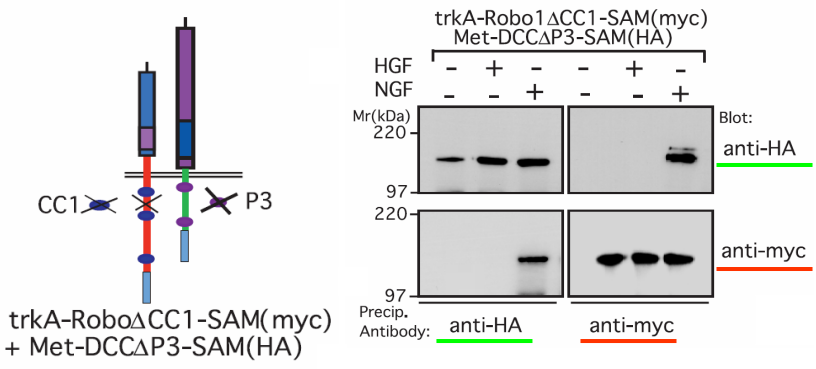

• addition of an EphB1 SAM domain to trkA-Robo1ΔCC1 might enable it to associate with Met-DCCΔP3-SAM, because of the multimerization function of the SAM domain

• NGF but not HGF induces binding of trkARobo1ΔCC1-SAM to Met-DCCΔP3-SAM

¿ Restoring the binding, would restore the silencing?

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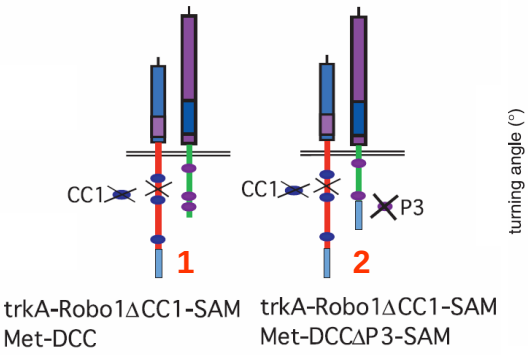
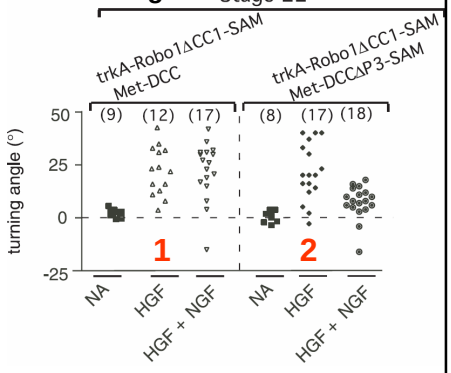
¿ Restoring the binding, would restore the silencing?



trkA-Robo1ΔCC1-SAM
Met-DCC

trkA-Robo1ΔCC1-SAM
Met-DCCΔP3-SAM



- NGF, by activating trkA-Robo1ΔCC1-SAM

  -silence the attractive effect of HGF activating Met-DCCΔP3-SAM

  -doesn't silence the attractive effect of HGF activating Met-DCC

→ synthetically restoring the physical interaction, restores silencing, consistent with silencing being mediated by the interaction

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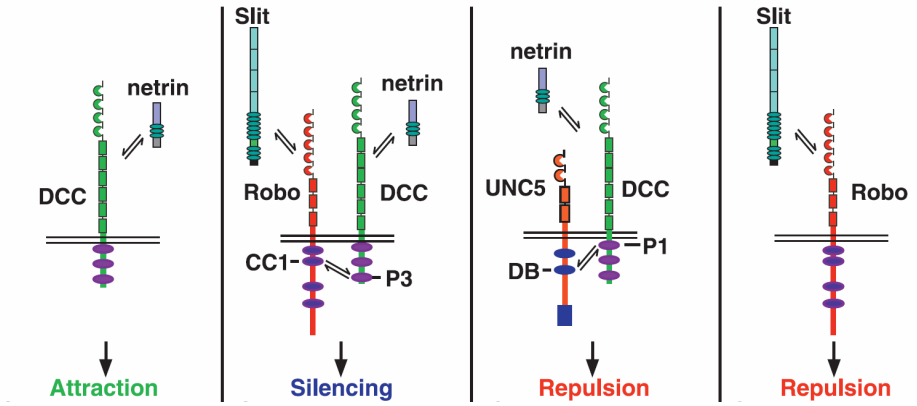

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