Advanced Cell Biology and Biotechnology

ACBB 2021/22

…the lecture of December 3rd is about to begin…

CELL-CELL COMMUNICATION

- methods to study cell-cell communication:
  - chemotaxis & chemokinesis
  - attraction & repulsion
  - substrate preference
  - bidirectional signalling

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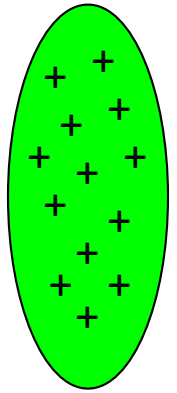
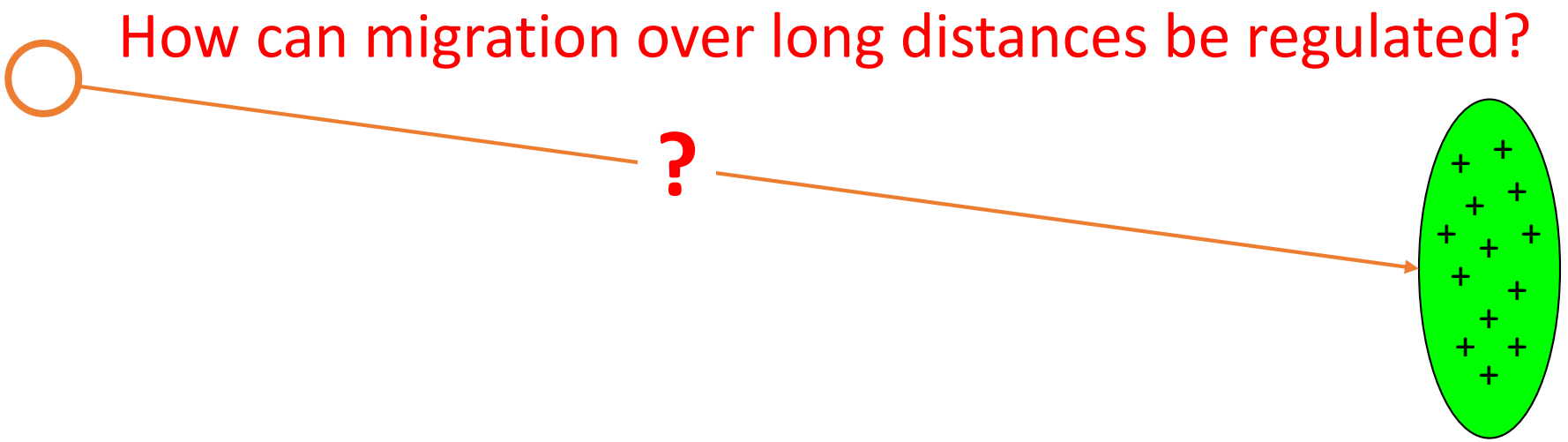
Attraction & repulsion

How can migration over long distances be regulated?

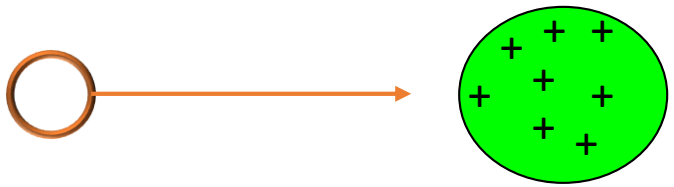


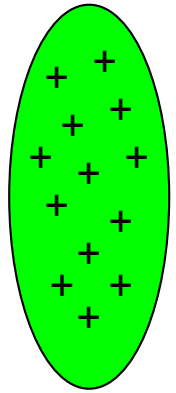
Attraction & repulsion



• as axons grow long distances in the developing embryo, they make use of intermediate targets to simplify their navigation into short segments

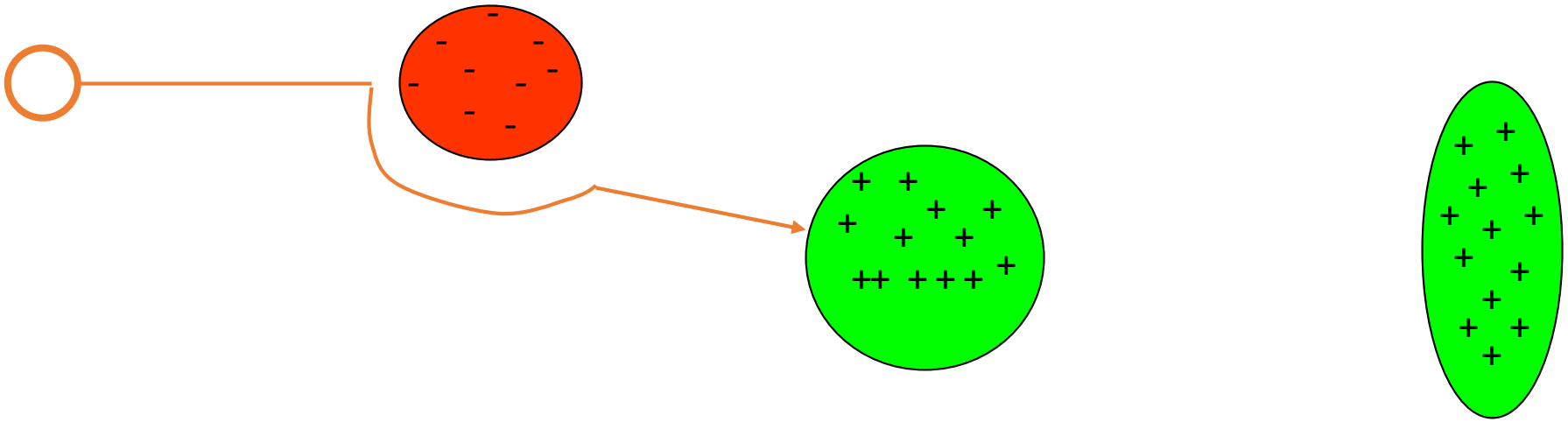
Attraction & repulsion





• these intermediate targets produce both **attractants** and **repellents**, that axonal growth cones must recognize in sequential order to navigate properly

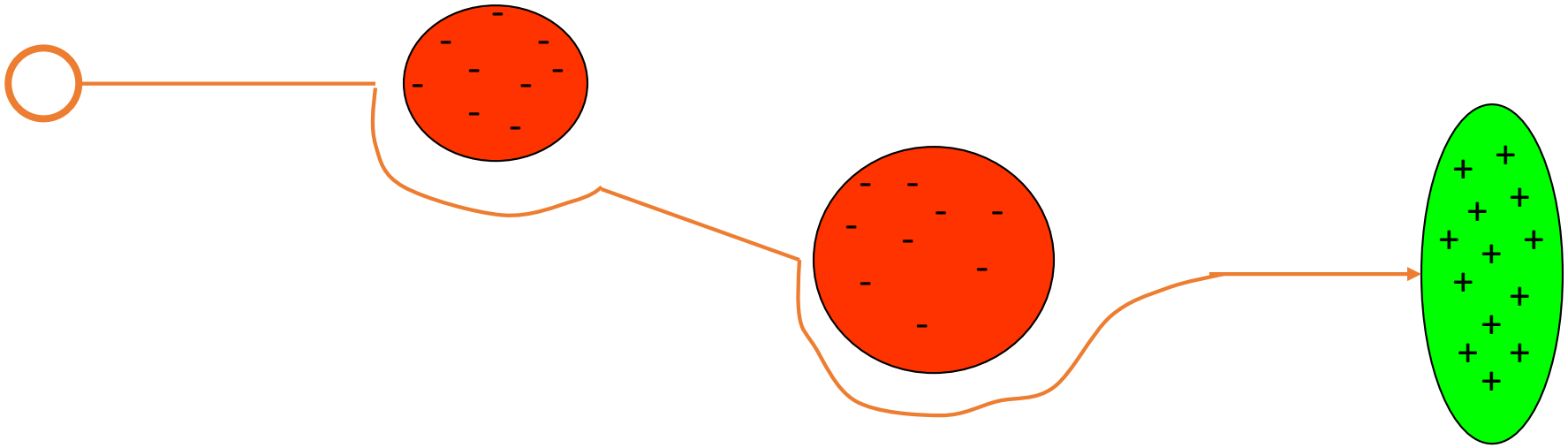
Attraction & repulsion



• 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

Attraction & repulsion

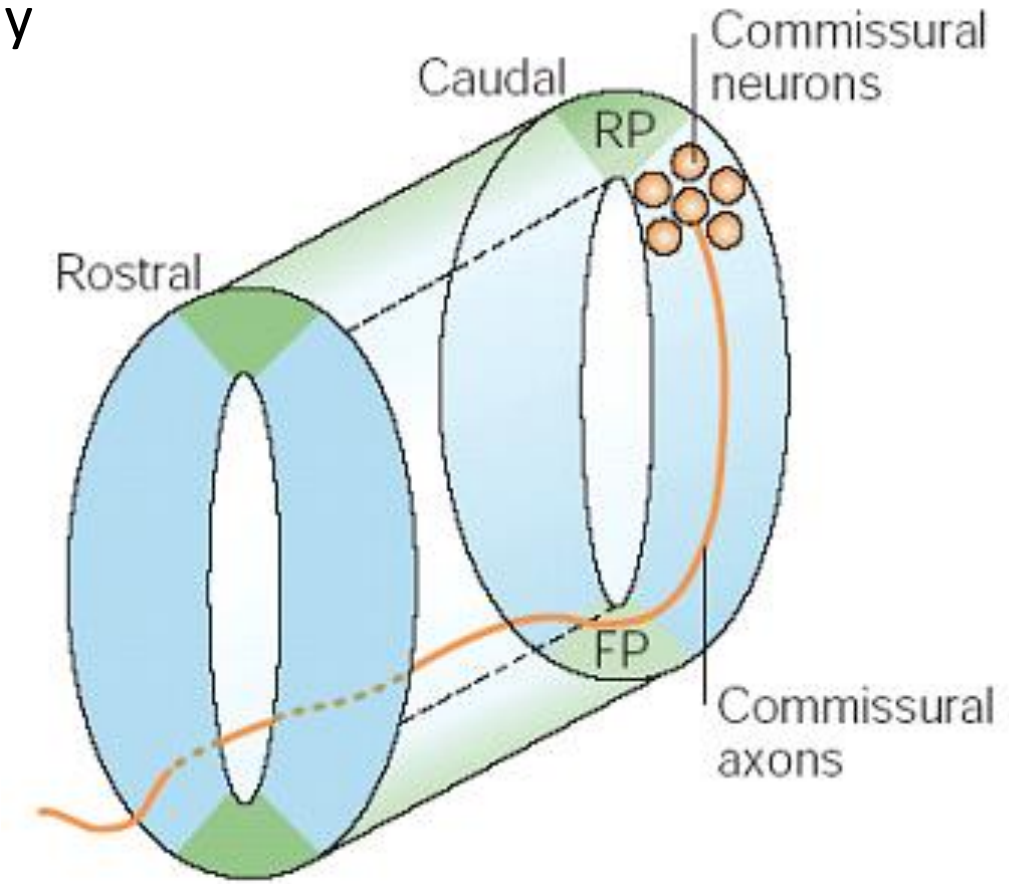


- how an attracting signal can turn to a repulsive signal?

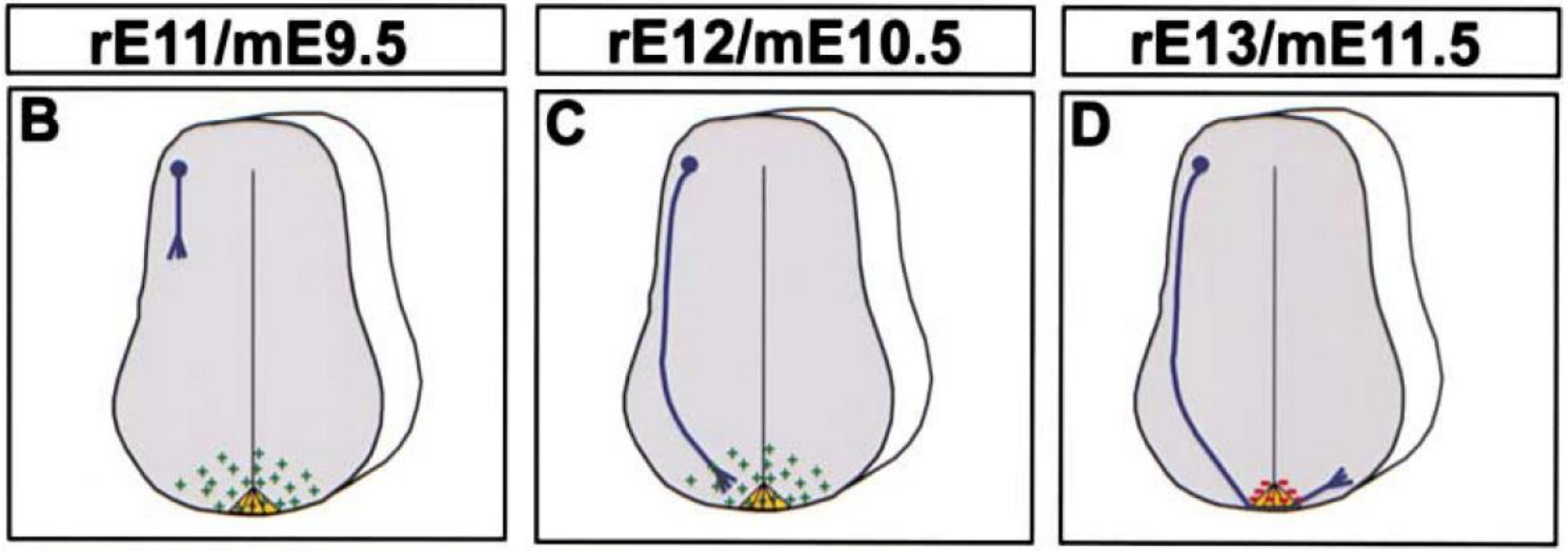
Attraction & repulsion

• the ventral midline of the nervous system of both vertebrates and invertebrates is a good model to study 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



Commissural axons

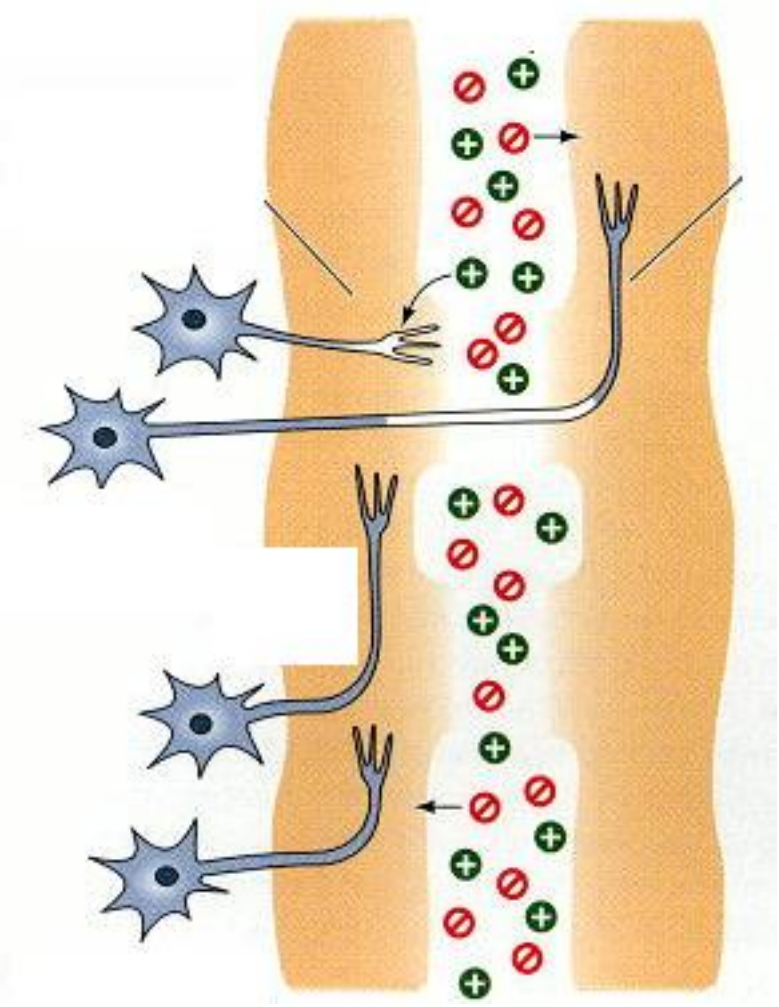


- in vertebrates and insects, commissural axons are initially drawn to the midline by attractant proteins
- upon crossing the midline and reaching the contralateral side, however, these growth cones turn longitudinally and become sensitive to repellents made by midline cells
- this switch prevents commissural axons from re-crossing the midline and allows them to move on toward their final targets

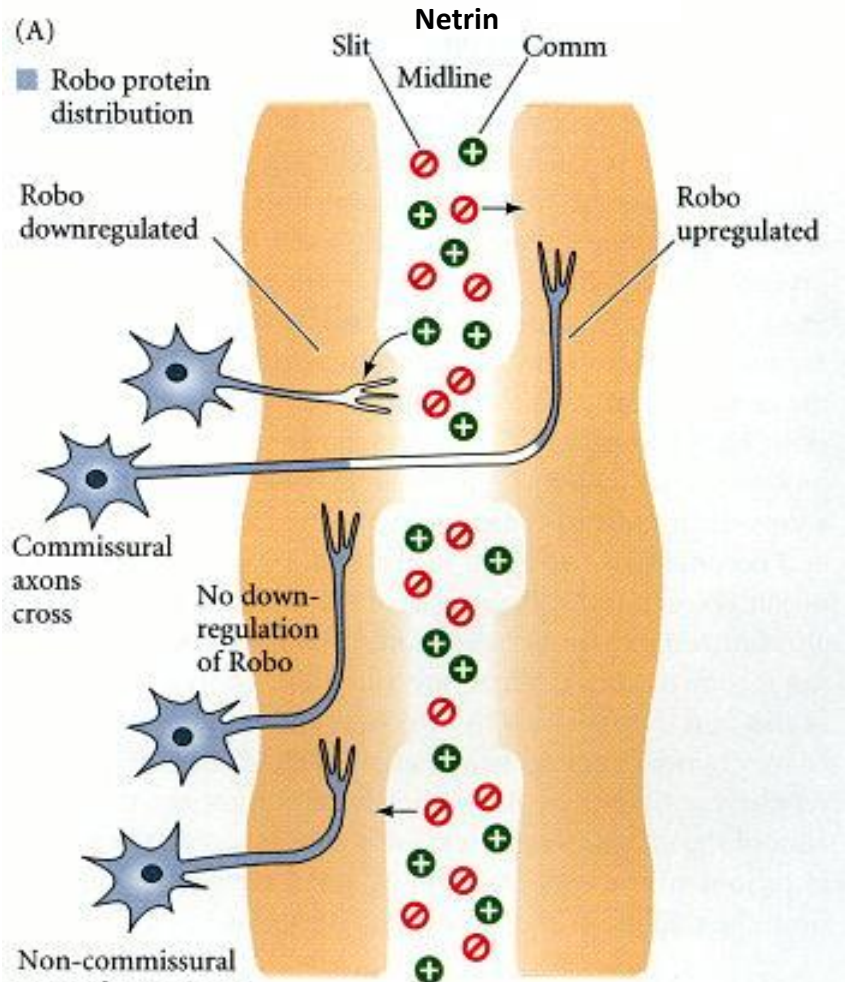
Commissural axons

The midline secretes **netrin** protein, which is stimulatory to commissural axons, and **Slit** protein, which is inhibitory to non-commissural axons.

How can a cell be sensitive to **attraction** or **repulsion?**



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

Silencing of attraction

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

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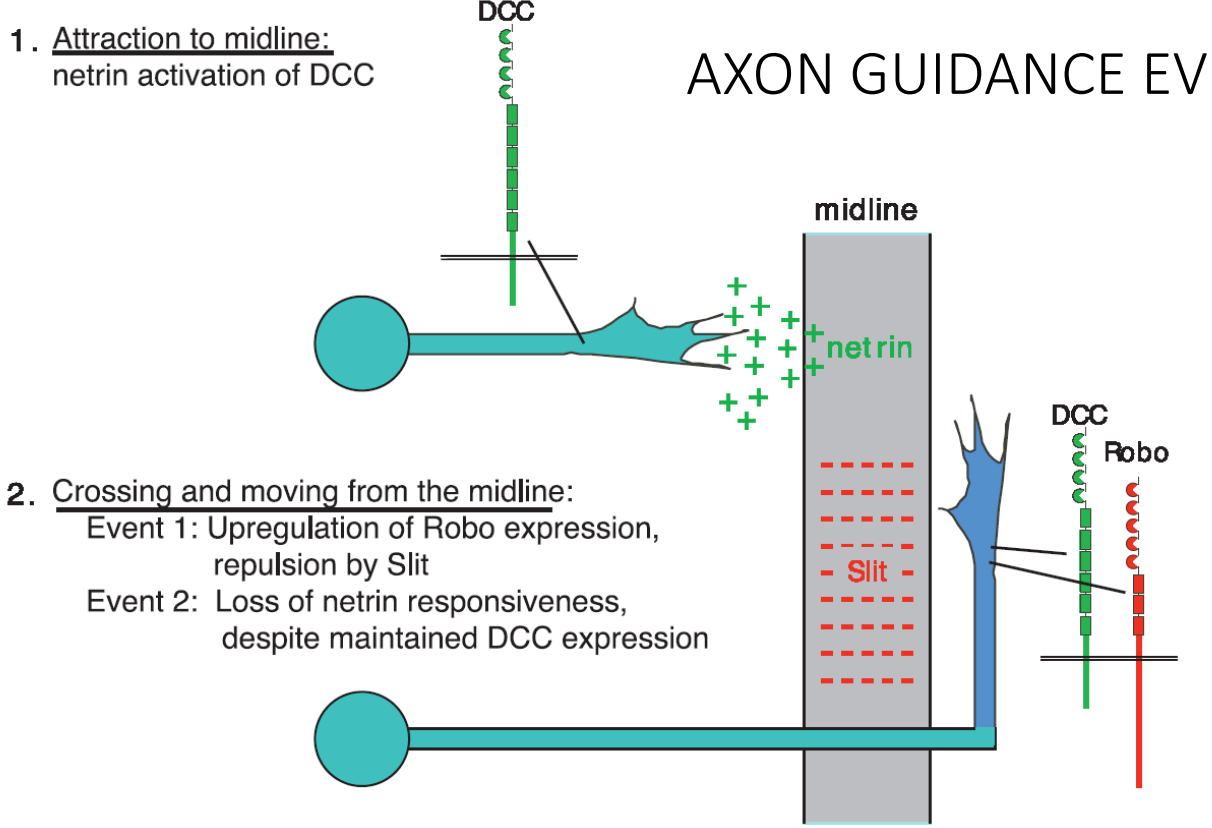
Department of Anatomy and Department of Biochemistry and Biophysics, Howard Hughes Medical Institute, University of California, San Francisco, CA 94143, USA.

AXON GUIDANCE EVENTS AT THE MIDLINE



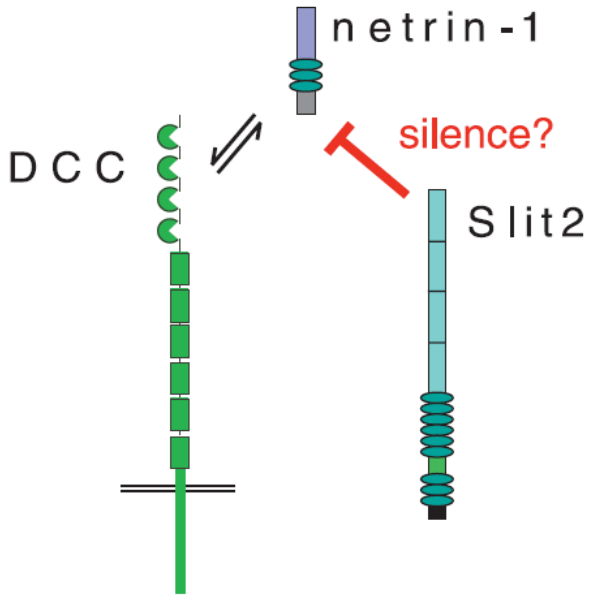
- commissural axons are attracted by **netrin** secreted by midline cells, which activates a receptor of the **DCC** family on growth cones;

- after crossing the midline, axons change their responsiveness, such that they are repelled by the midline. This involves **up-regulation of the Robo receptor** on the post-crossing portions of the axons, so they become responsive to the midline repellent **Slit**;

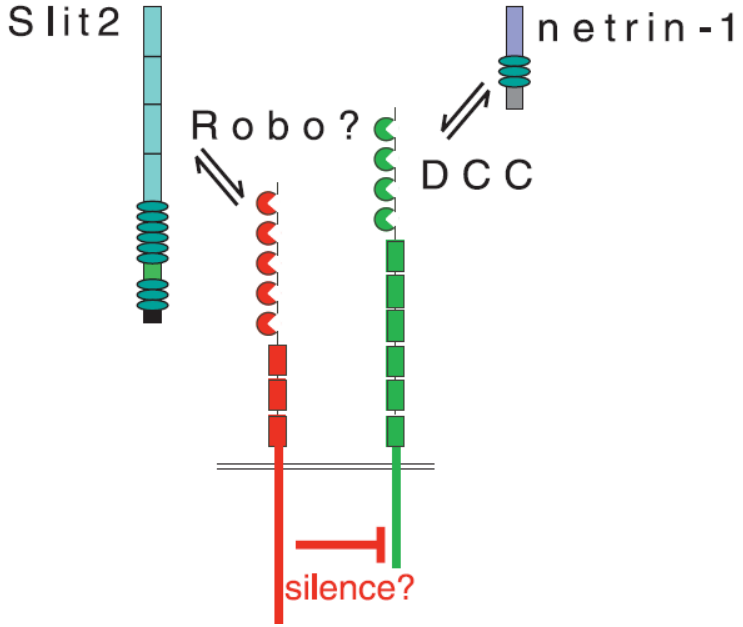
- axons that cross the midline **lose responsiveness to the netrin attractant**, despite maintained expression of the DCC receptor.

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

Model 1:
Ligand-Ligand interaction

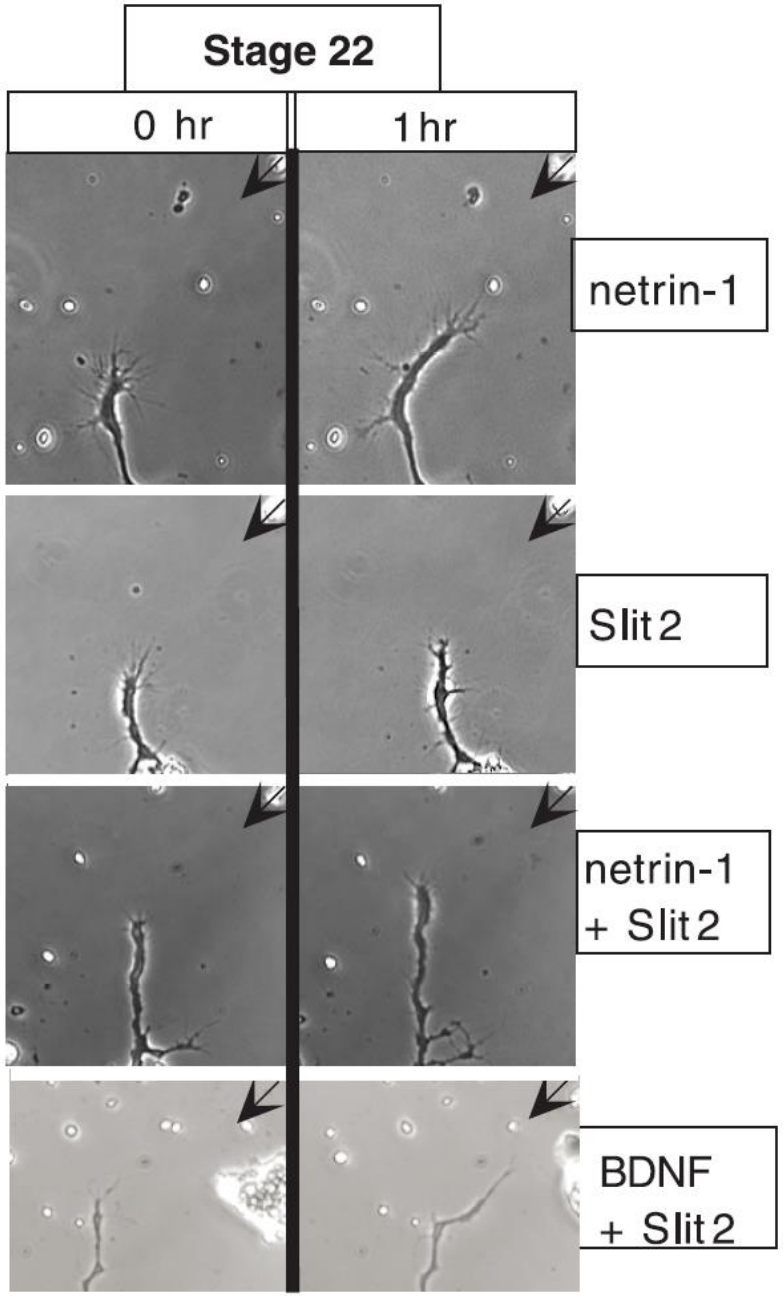


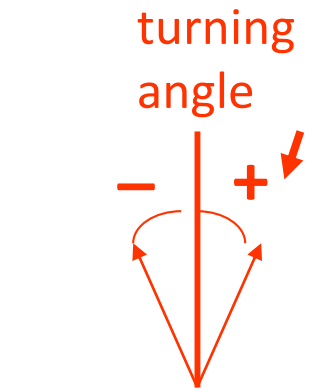
Model 2:
Receptor-Mediated Silencing



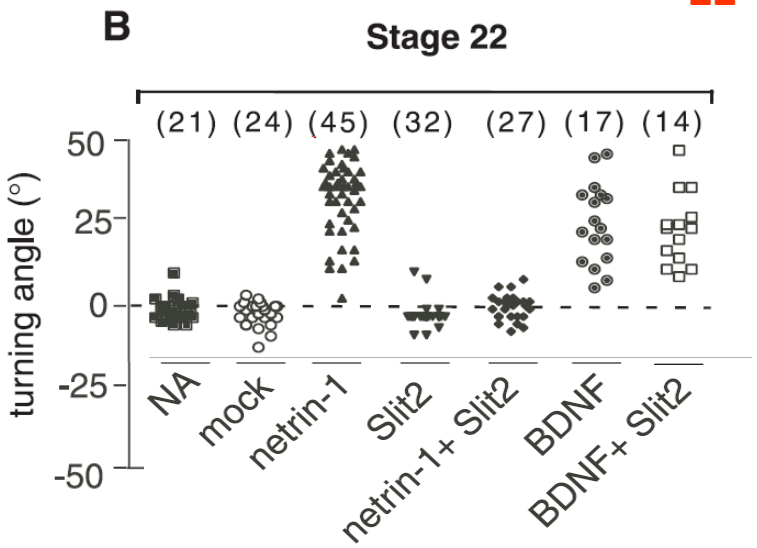
1. because Slit2 can bind netrin-1 directly, silencing might be caused by binding of the two proteins, which could in principle interfere with the netrin-DCC interaction.

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.

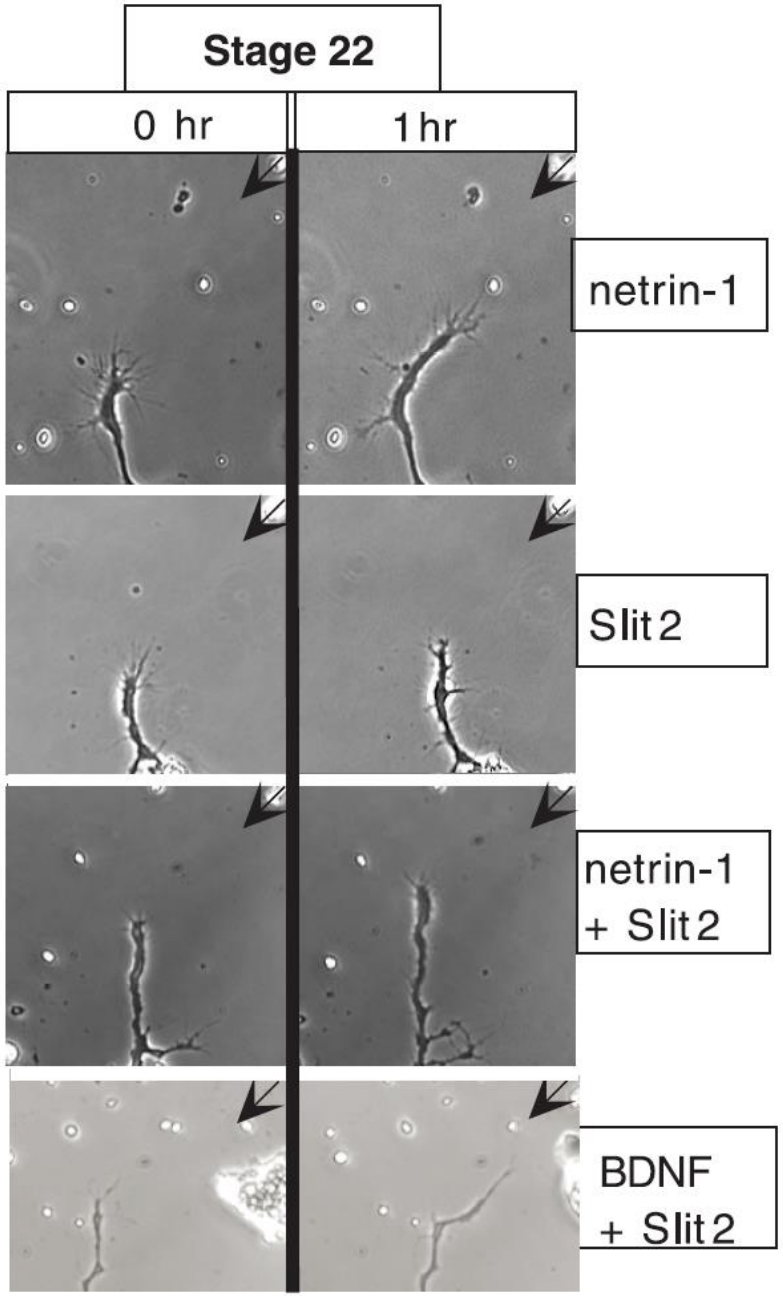


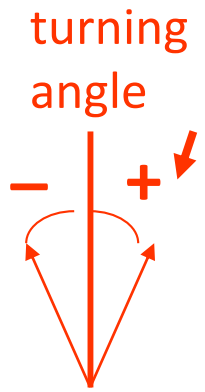


TURNING ASSAY

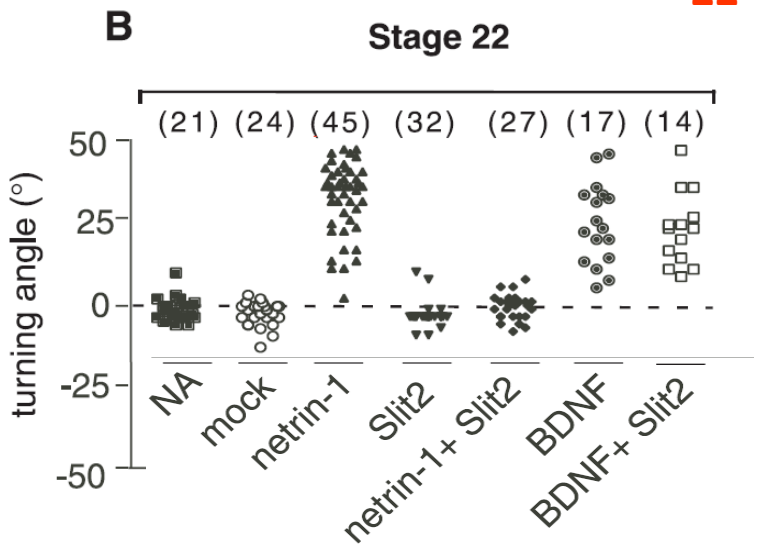


SLIT2 SILENCES ATTRACTION TO NETRIN-1 BUT NOT TO BDNF



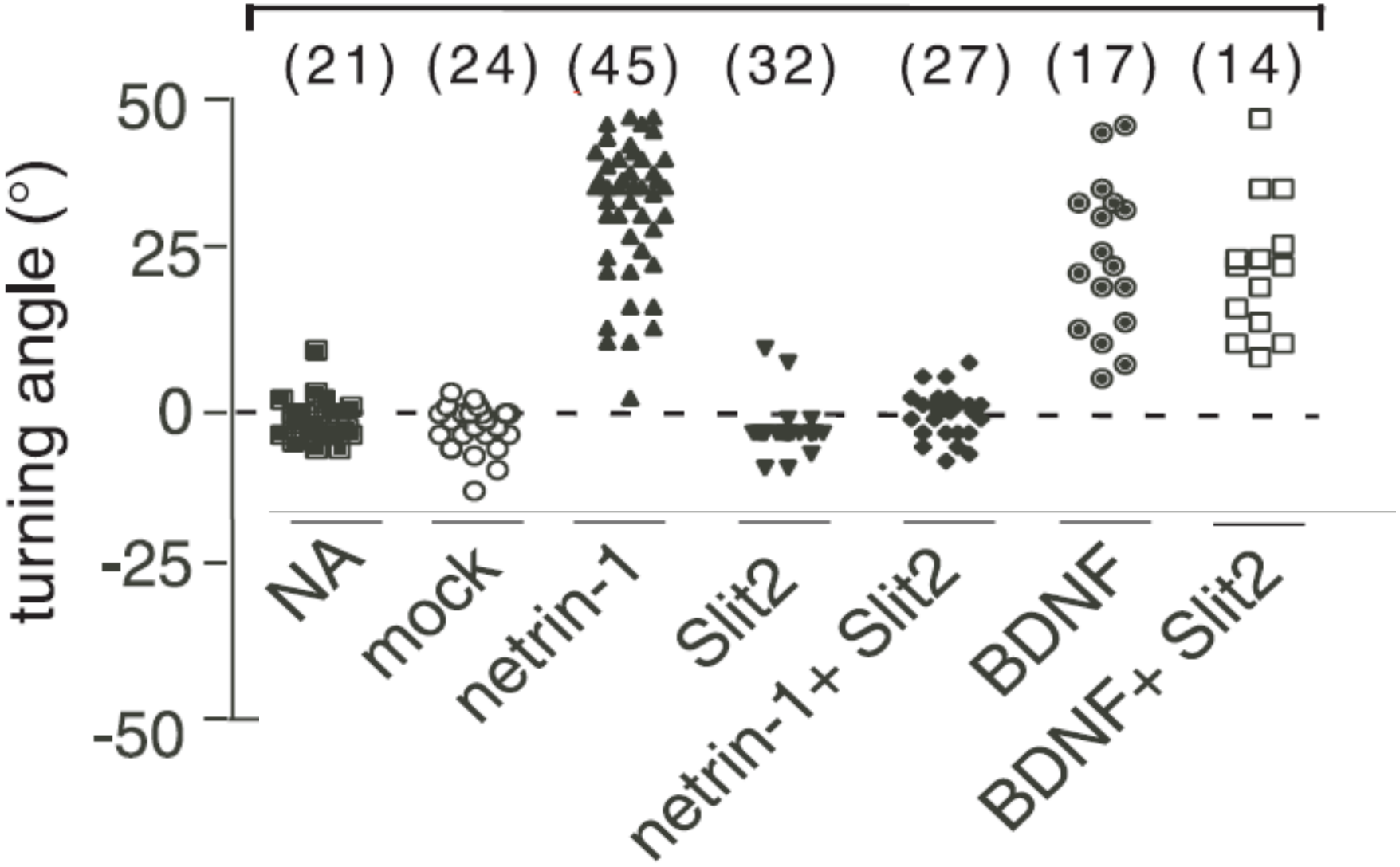


TURNING ASSAY

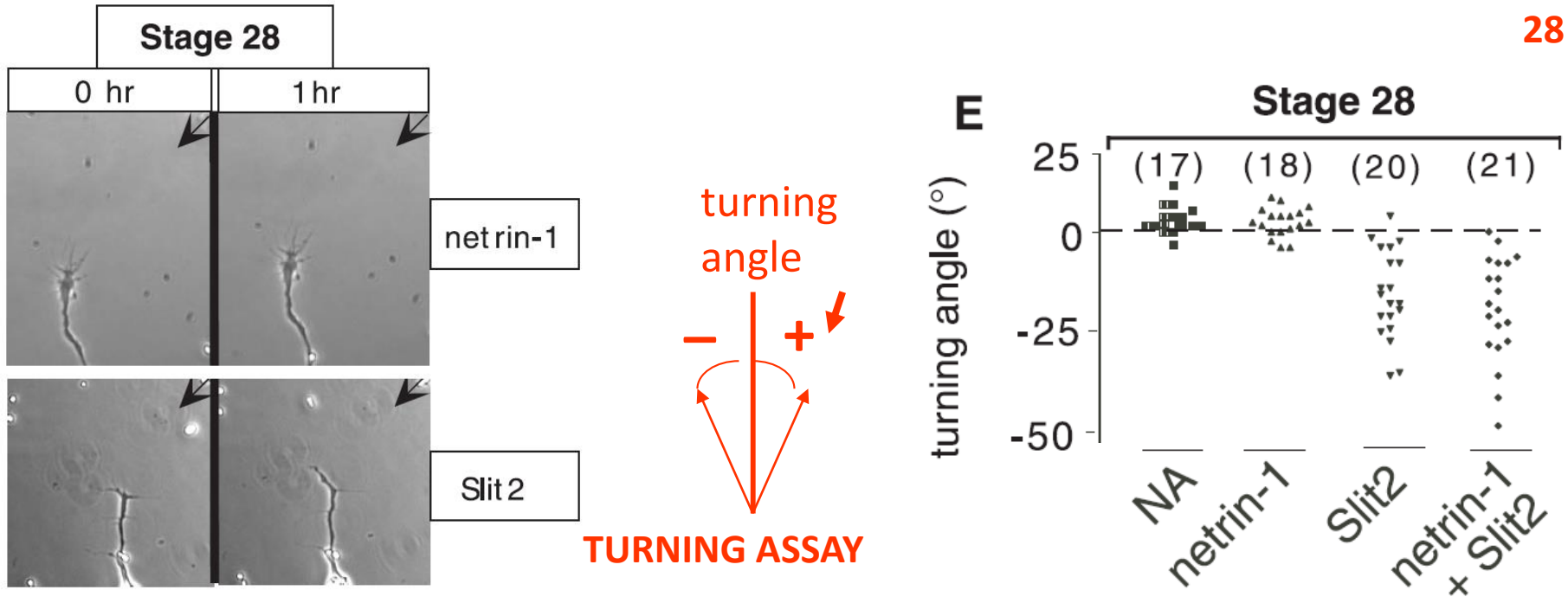


Growth cones of neurons from stage 22 Xenopus embryos exposed to a gradient of netrin-1, turn toward the source.
This response requires the function of the netrin receptor DCC.
The same axons exposed to a gradient of Slit2 protein did not show a directional response. When growth cones were exposed to a gradient of netrin-1 and 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.

How can you describe this graphic?
Which information is missing?



• 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.

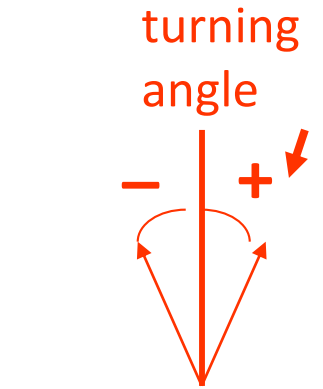


• The axons of older spinal neurons obtained from **stage 28** embryos were consistently repelled by Slit2, but did not show any response to netrin-1, likely **because of the absence of DCC expression** in these neurons, as assessed by immunohistochemistry. So it cannot be tested whether Slit2 has a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

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

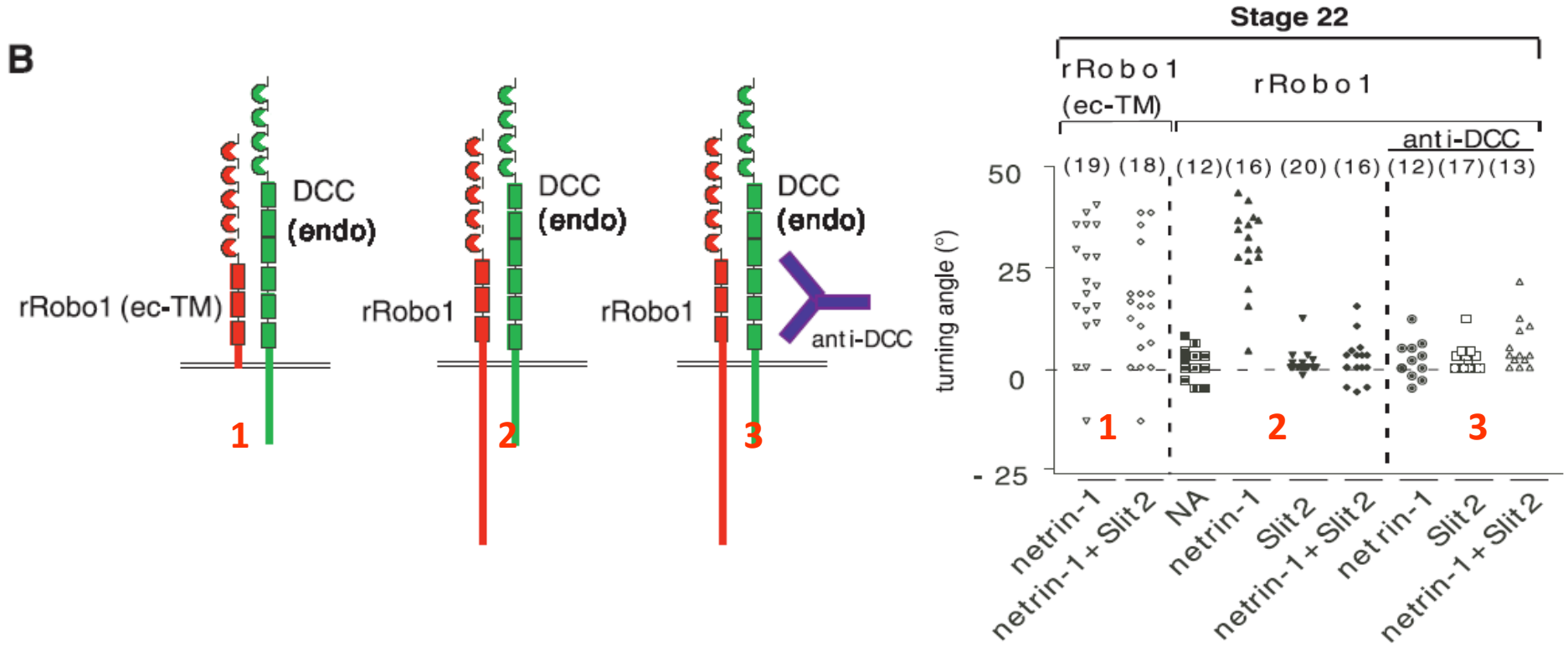
• in all subsequent **TURNING ASSAYS**, exogenous receptors were expressed by injecting *in vitro* transcribed mRNA encoding versions of the receptors of interest [usually tagged with a **Myc** or hemagglutinin (**HA**) epitope tag] into the second blastomere at the four-cell stage of *Xenopus* embryos,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

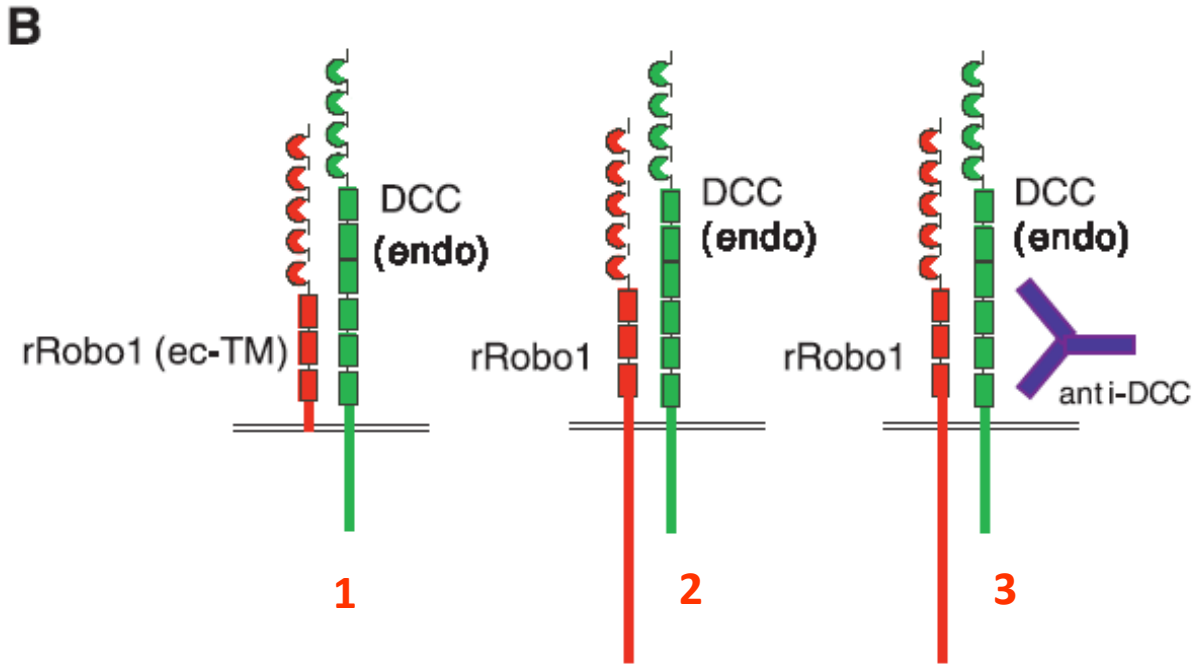


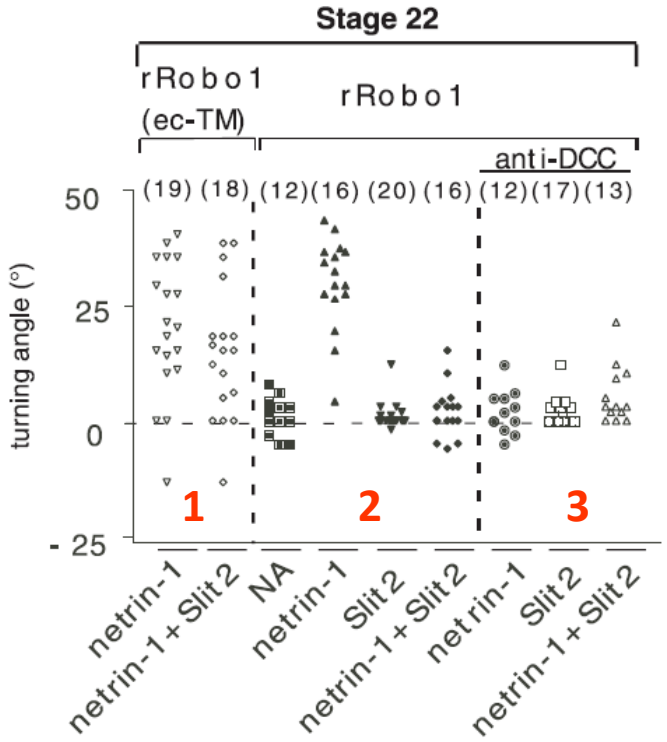
TURNING ASSAY

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



According to these results, which is the model explaining the silencing of attraction?





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

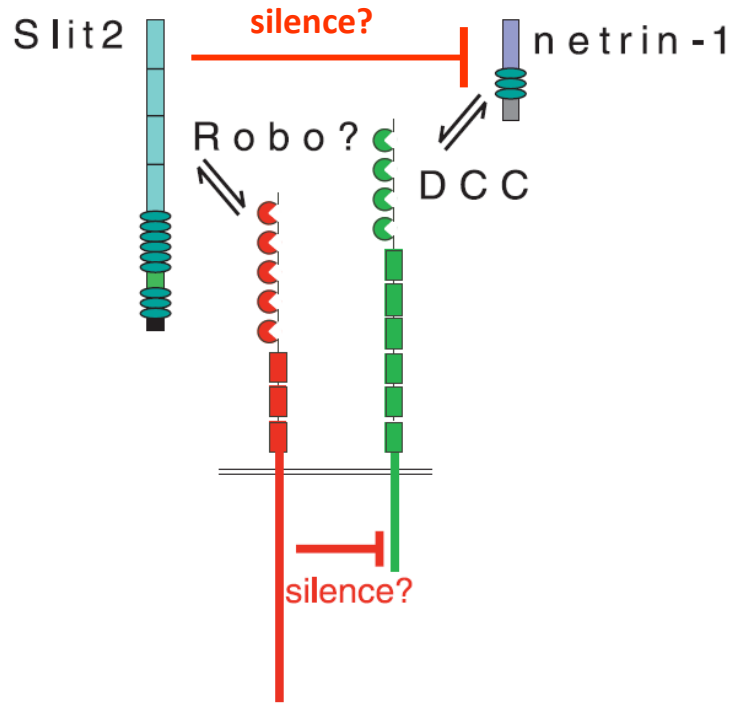
3- Slit2 did not repel growth cones expressing full-length rRobo1, indicating that expression of a Robo receptor is not sufficient for repulsion, which presumably requires additional signaling molecules in the growth cone

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

- truncated Robo receptor can block silencing by Slit

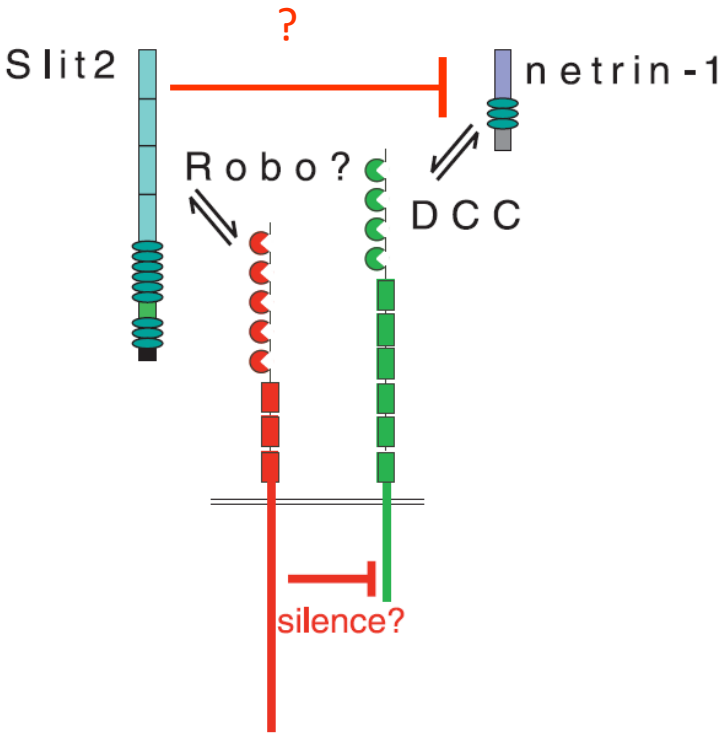
→ **receptor - mediated mechanism but....**

Is the model 2 (interaction between receptors) the only one explaining these data?



• truncated Robo receptor can block silencing by Slit

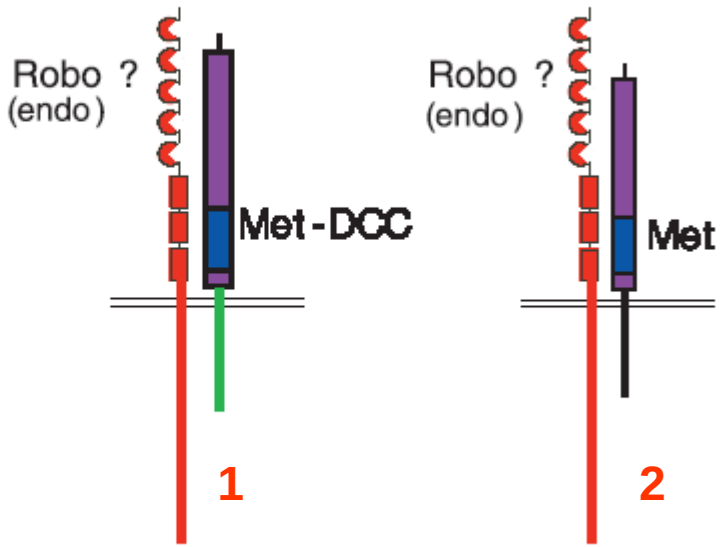
→ **receptor - mediated mechanism but....**

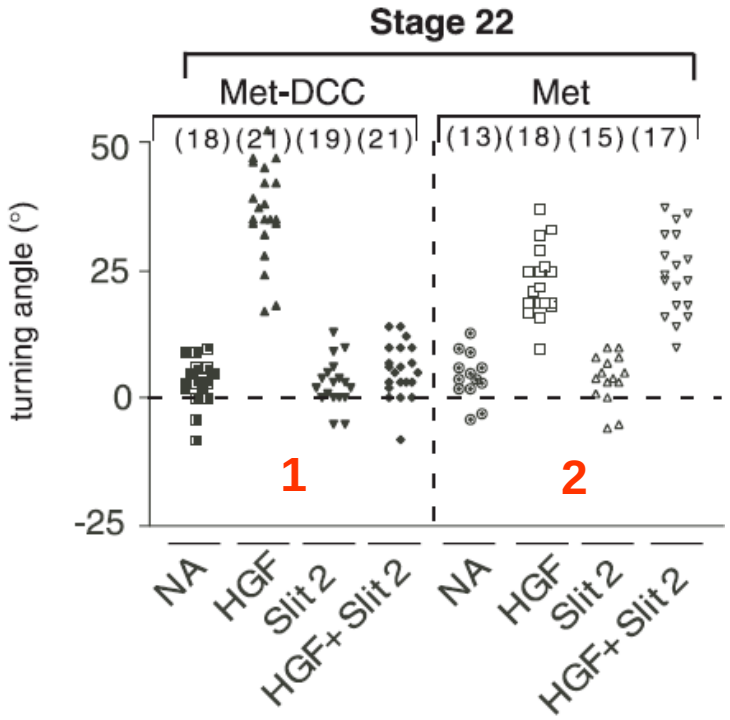


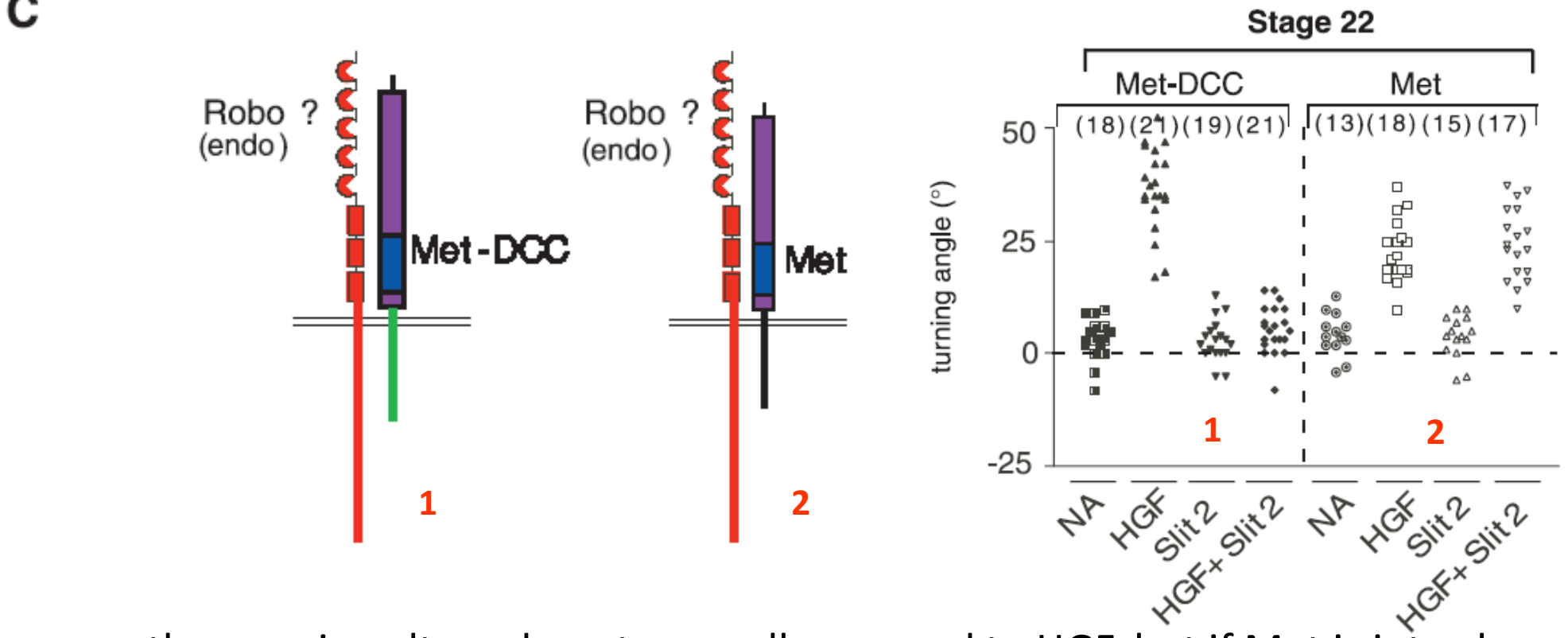
• this result is also compatible with a ligand-ligand interaction model of silencing if the exogenous Robo can bind and somehow locally reduce (titrate) the amount of available Slit2 protein

• 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

• which advantages do you have with Met-DCC or Met-Robo?

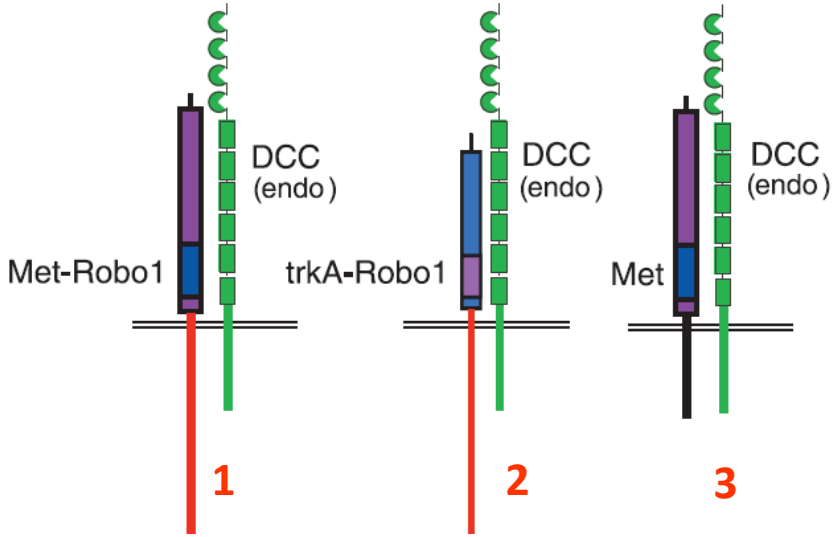


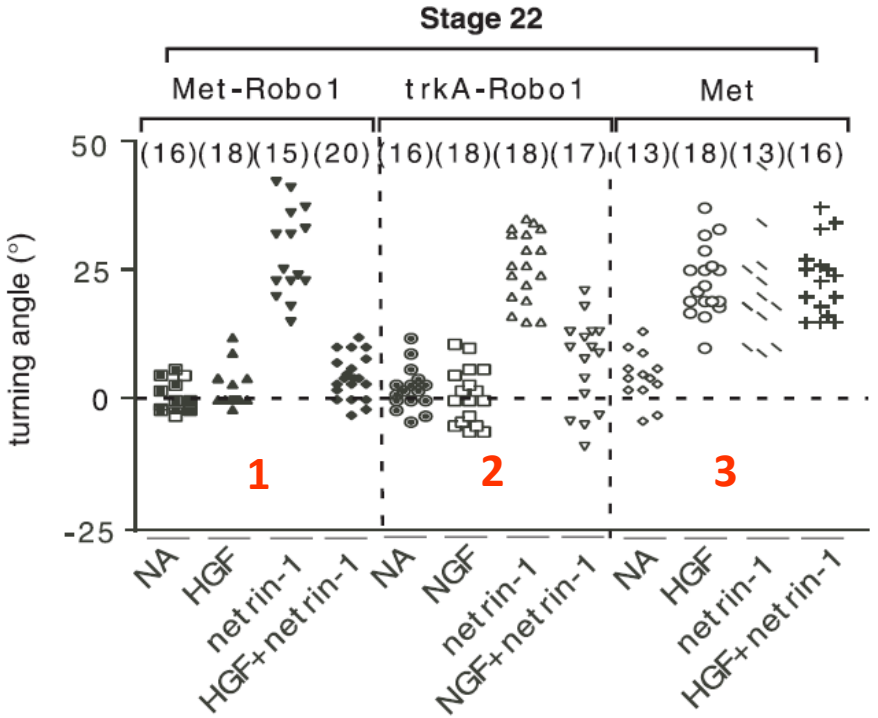




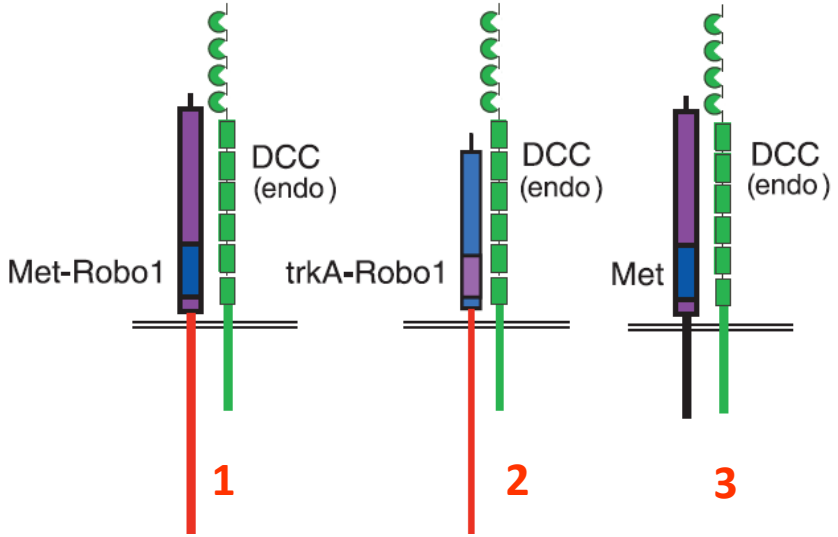
- *Xenopus* growth cones in culture do not normally respond to HGF, but if Met is introduced into them, they respond to HGF with attraction
- when a chimeric receptor comprising the Met ectodomain and the DCC transmembrane and cytoplasmic domain is introduced into these cells, HGF induces attractive 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**

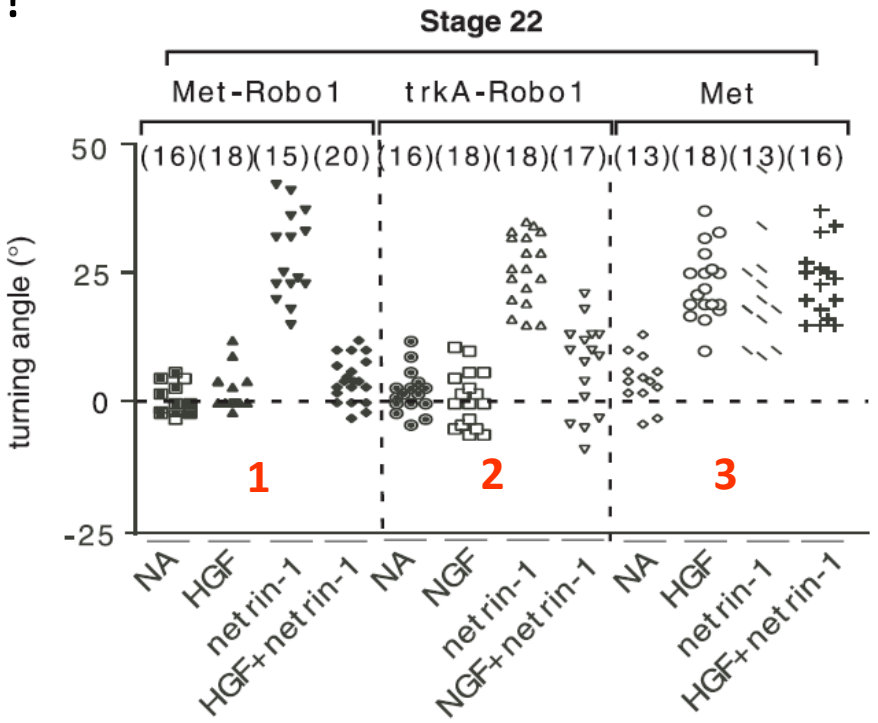
Could activation of the Robo signaling pathway by a heterologous ligand also lead to silencing of netrin attraction?



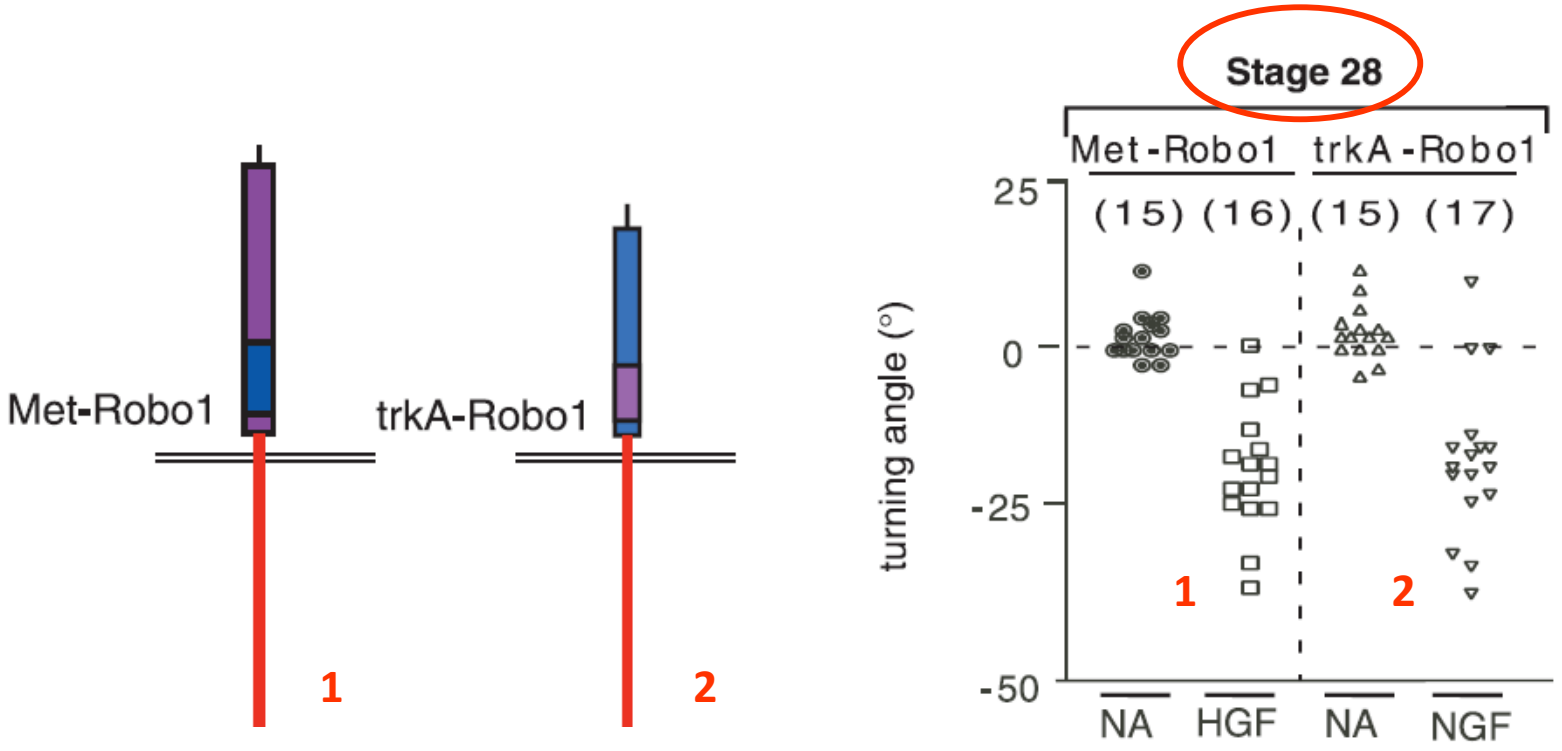


Could activation of the Robo signaling pathway by a heterologous ligand also lead to silencing of netrin attraction?





- chimeric receptors comprising the cytoplasmic domain of rRobo1 and the ectodomain of either Met or the trkA 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



• **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

• these studies strongly suggest 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)

Which technique can be used to investigate if Slit and Robo interact?

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°C with ligands (control medium, netrin-1, Slit2, HGF, NGF)

- total proteins were extracted

- proteins were subjected to immunoprecipitation, using the indicated antibodies

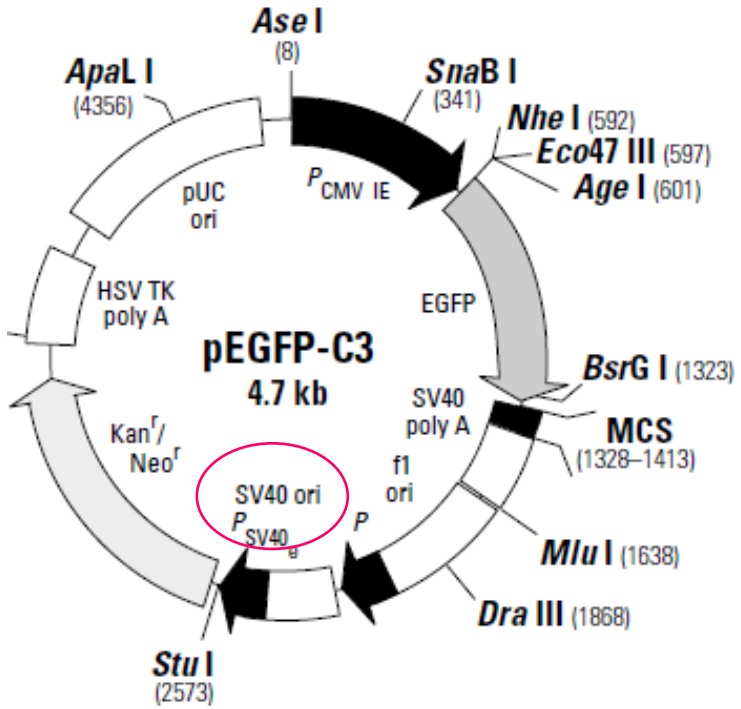
- proteins were analyzed by Western blotting

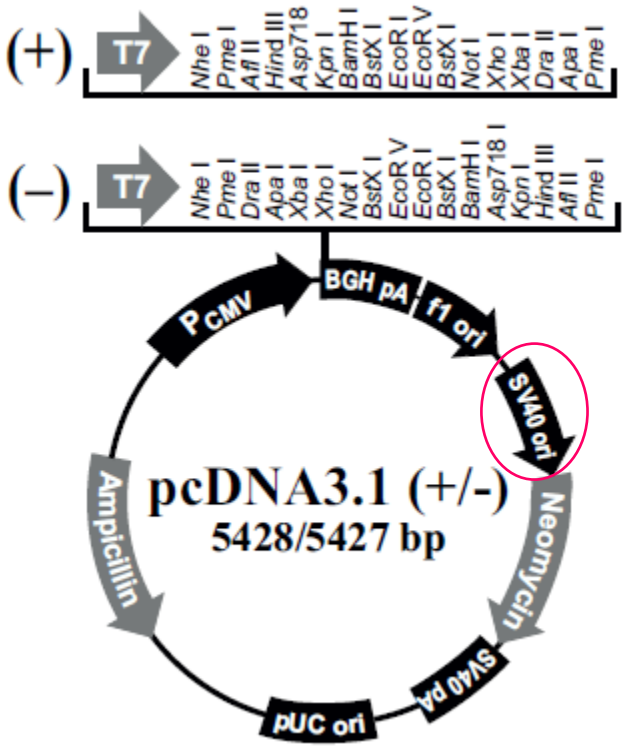
COS cells

Organism	*Cercopithecus aethiops*
Tissue	kidney
Cell Type	CV-1 cell line was derived from the kidney of the African green monkey. COS cells are obtained by immortalizing CV-1 cells with a version of the **SV40 virus that can produce large T antigen** but has a defect in genomic replication.
Morphology	fibroblast –like cells
Culture Properties	adherent
Applications	This is an African green monkey kidney fibroblast-like cell line **suitable for transfection by vectors requiring expression of SV40 T antigen**. This line contains T antigen, retains complete permissiveness for lytic growth of SV40, supports the replication of ts A209 virus at 40°C, and supports the replication of pure populations of SV40 mutants with deletions in the early region. The acronym "COS" is derived from the cells being **C**V-1 (simian) in **O**rigin, and carrying the **S**V40 genetic material. Two forms of COS cell lines commonly used are COS-1 and COS-7.

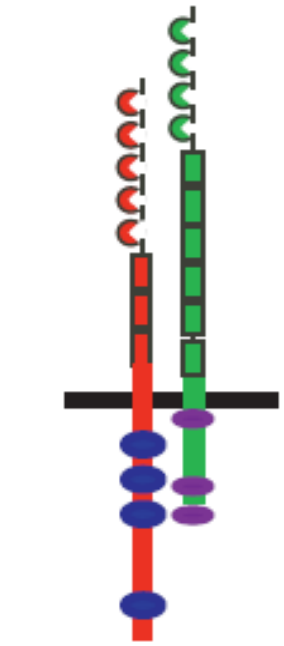
SV40 large T antigen (Simian Vacuolating Virus 40 TAg) is a hexamer protein that is a dominant-acting oncoprotein derived from the polyomavirus SV40.

SV40 large T-antigen is a product of an early gene transcribed during viral infection by SV40, and is involved in **viral genome replication** and regulation of host cell cycle.

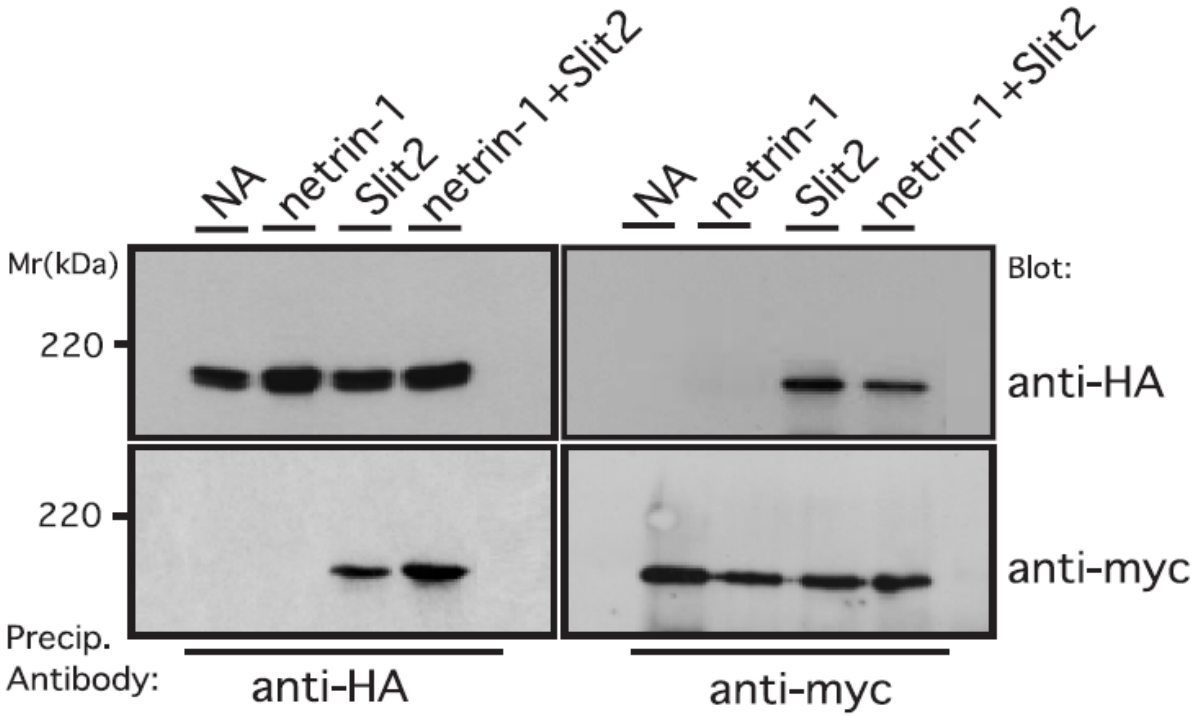




• **SV40 origin of replication** (**SV40 ori**) allows autonomous (as an **episome**) replication in mammalian cells expressing the **SV40 large T-antigen**, such as COS cells.

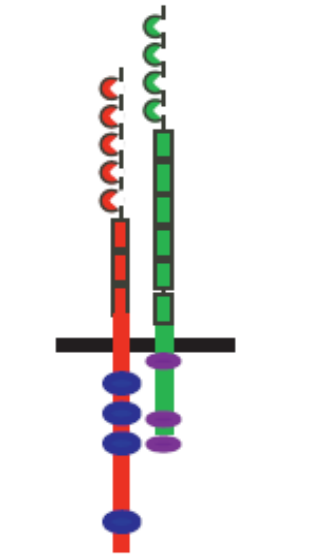


DCC(HA)
+ rRobo1(myc)

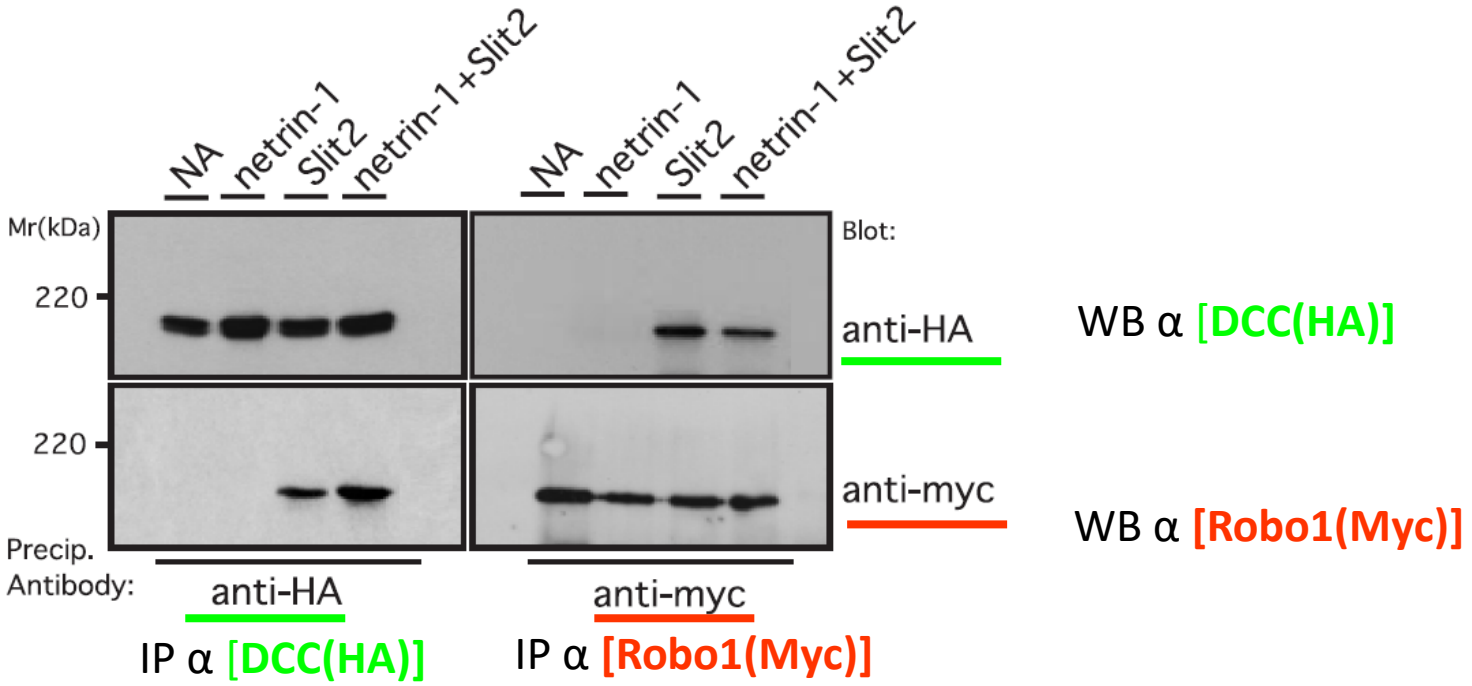


- Please, describe this result

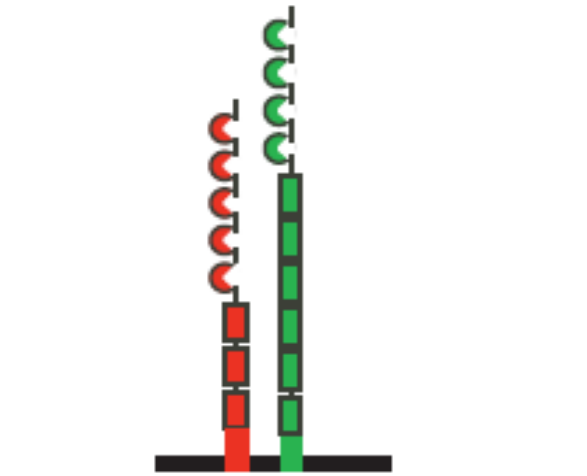
Could Robo and DCC form a receptor complex in transfected cells?



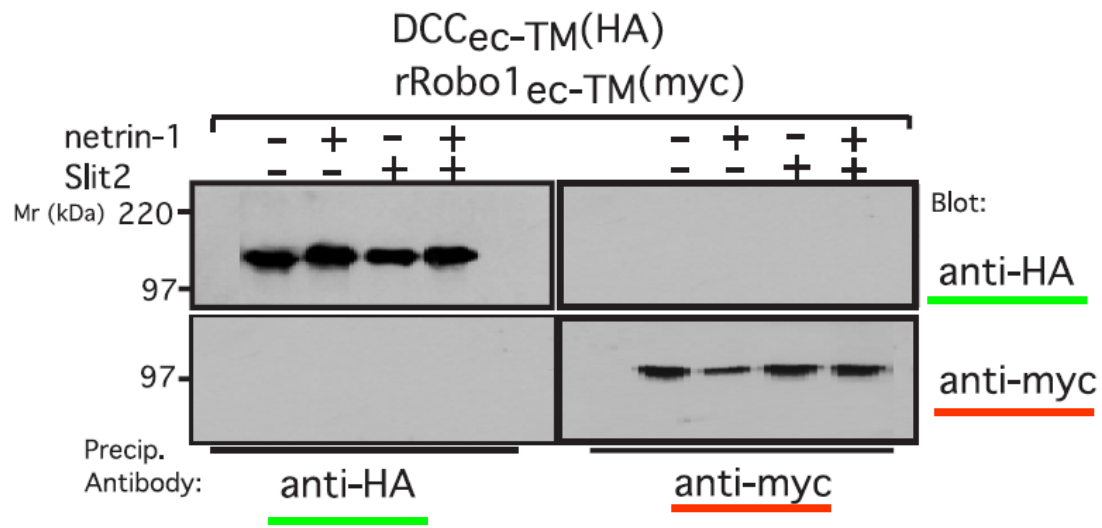
DCC(HA)
+ rRobo1(myc)

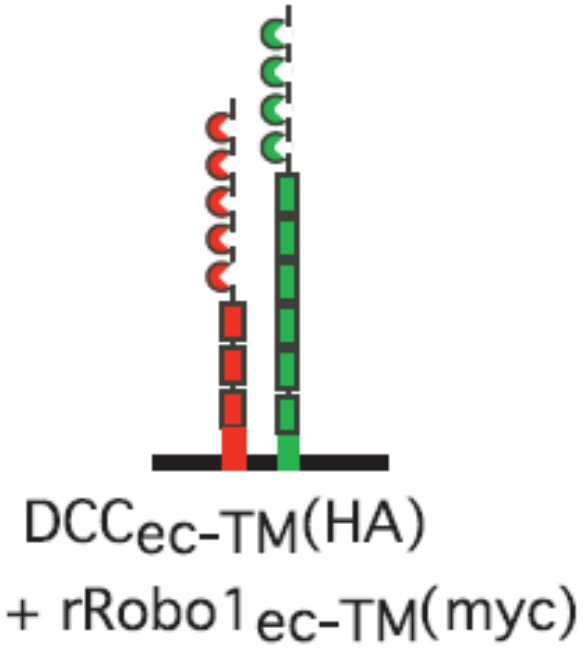


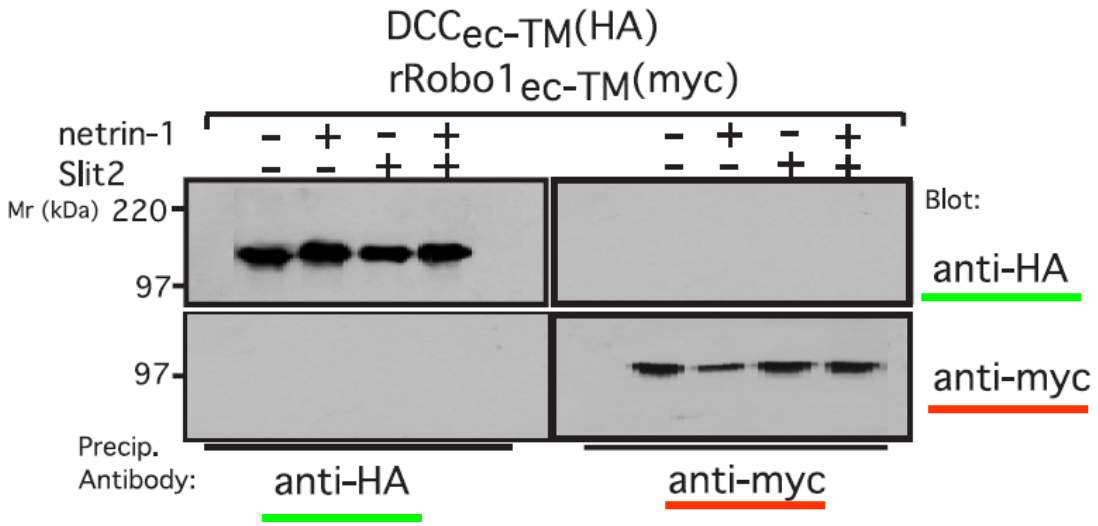
- a DCC construct tagged with an HA epitope **[DCC(HA)]** was co-expressed with a Robo1 construct tagged with a Myc epitope **[Robo1(Myc)]**
- when DCC was immunoprecipitated with an antibody to the HA tag, Robo1 did not coimmunoprecipitate under control conditions or when the cells were exposed to netrin-1, 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



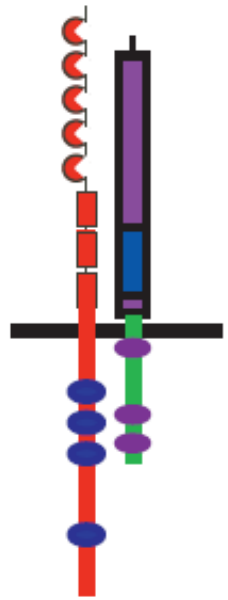
$DCC_{ec\text{-}TM}(HA)$
+ $rRobo1_{ec\text{-}TM}(myc)$



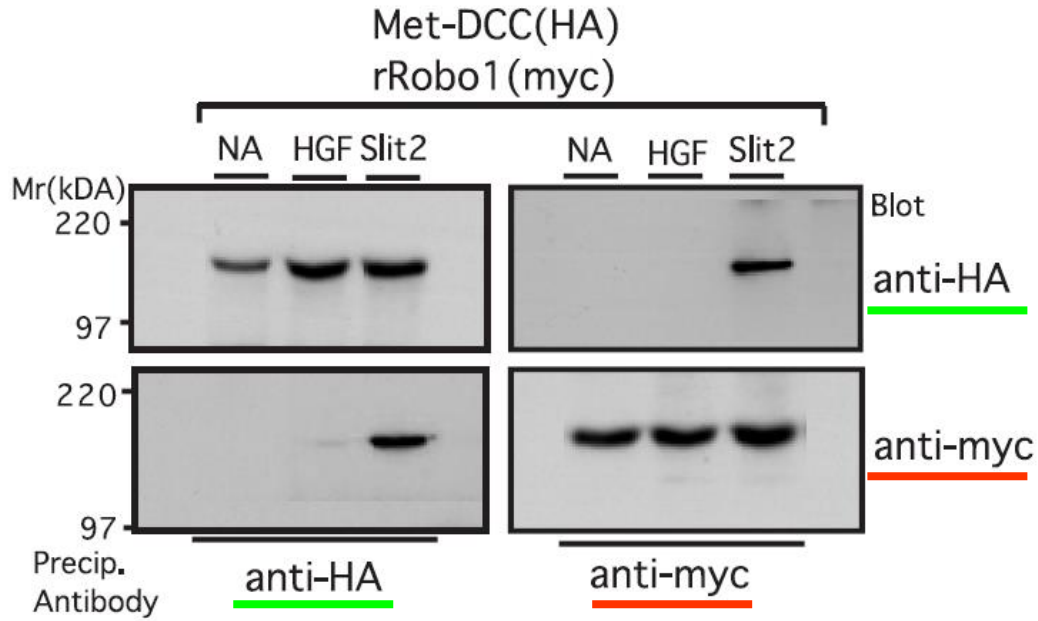


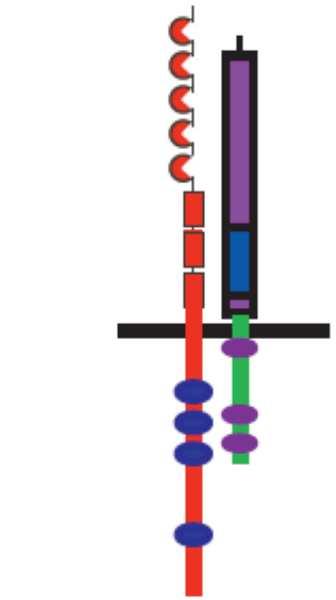


- when most of the cytoplasmic domains of the two proteins are removed, neither Slit2 nor netrin-1 induces the formation of a receptor complex

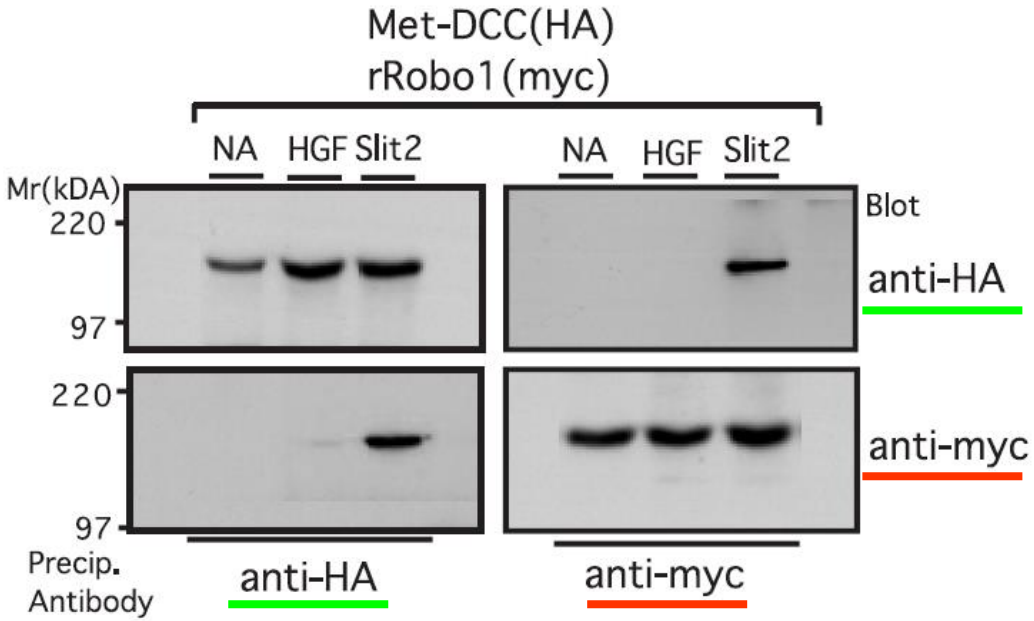


Met-DCC(HA)
+rRobo1(myc)

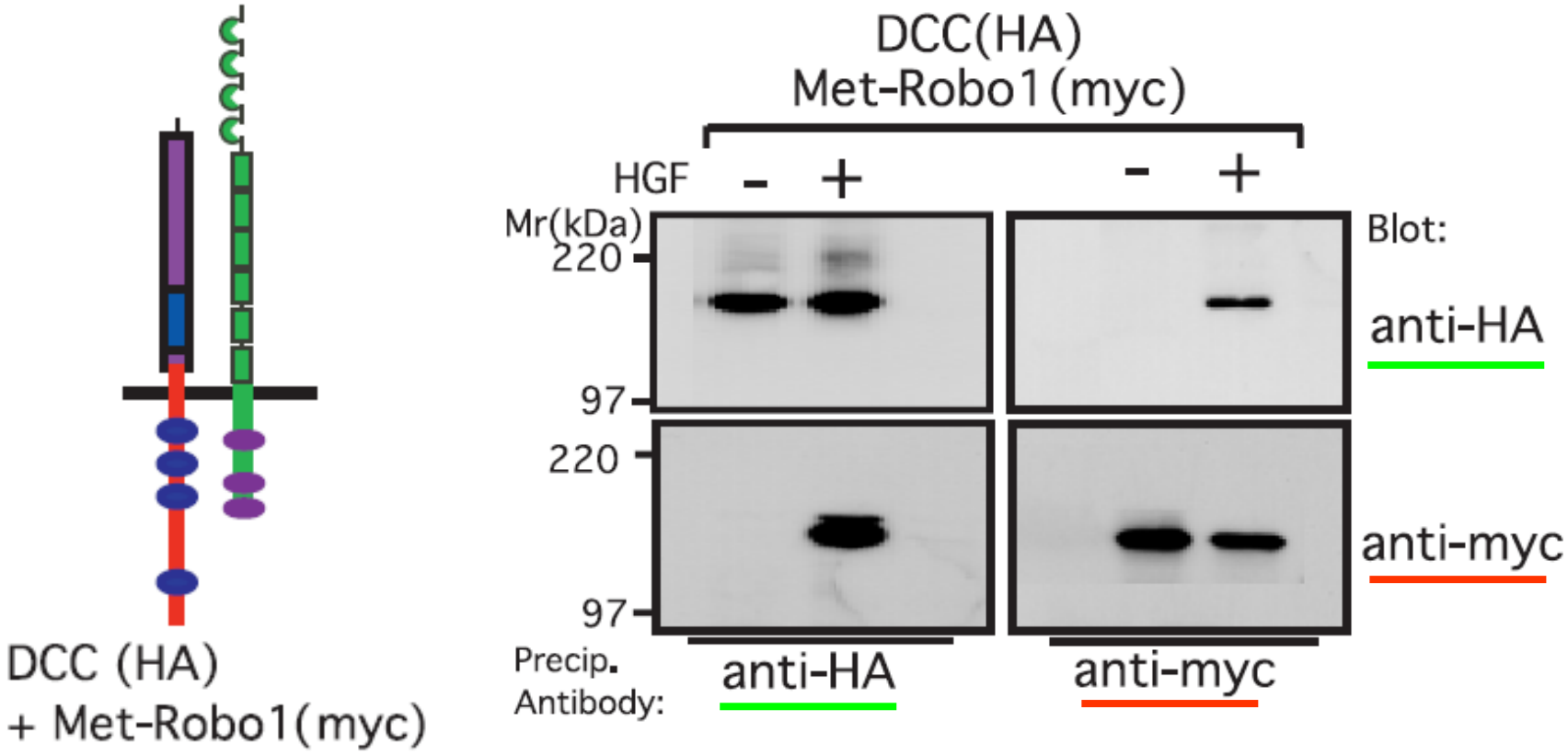




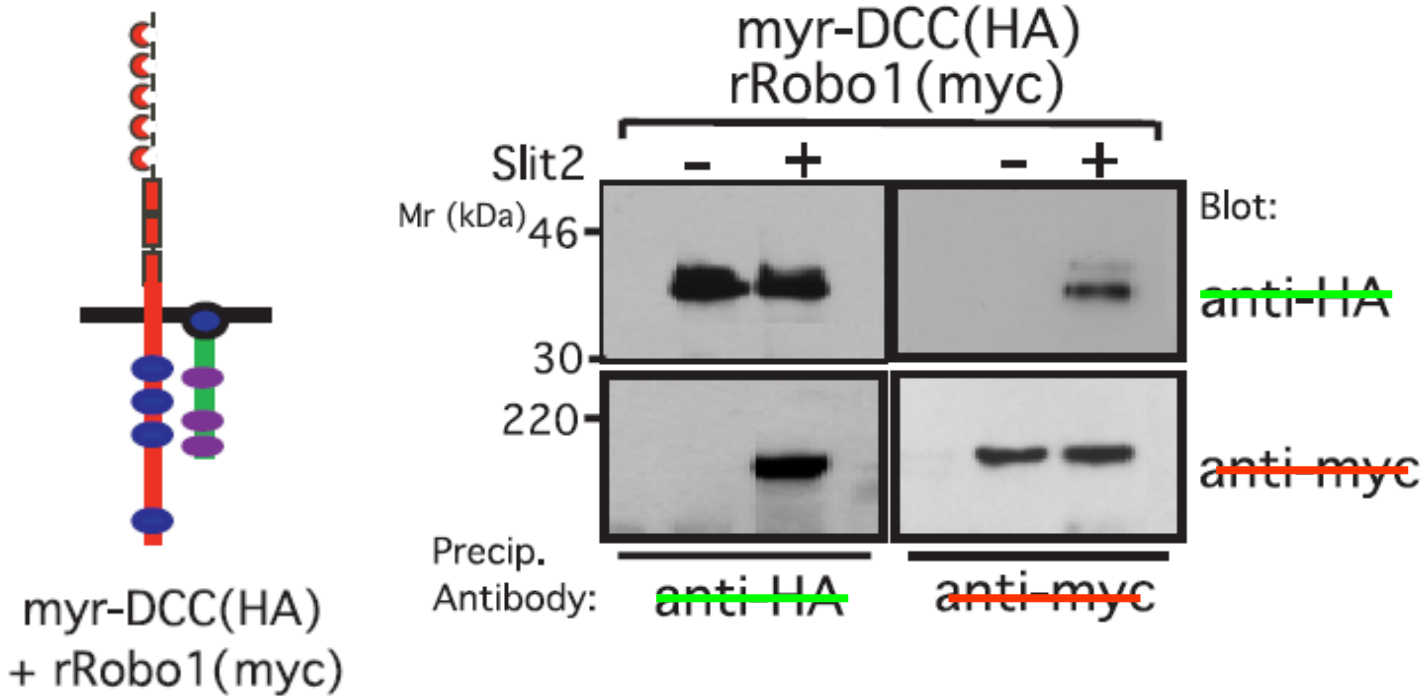
Met-DCC(HA)
+rRobo1(myc)



• 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



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

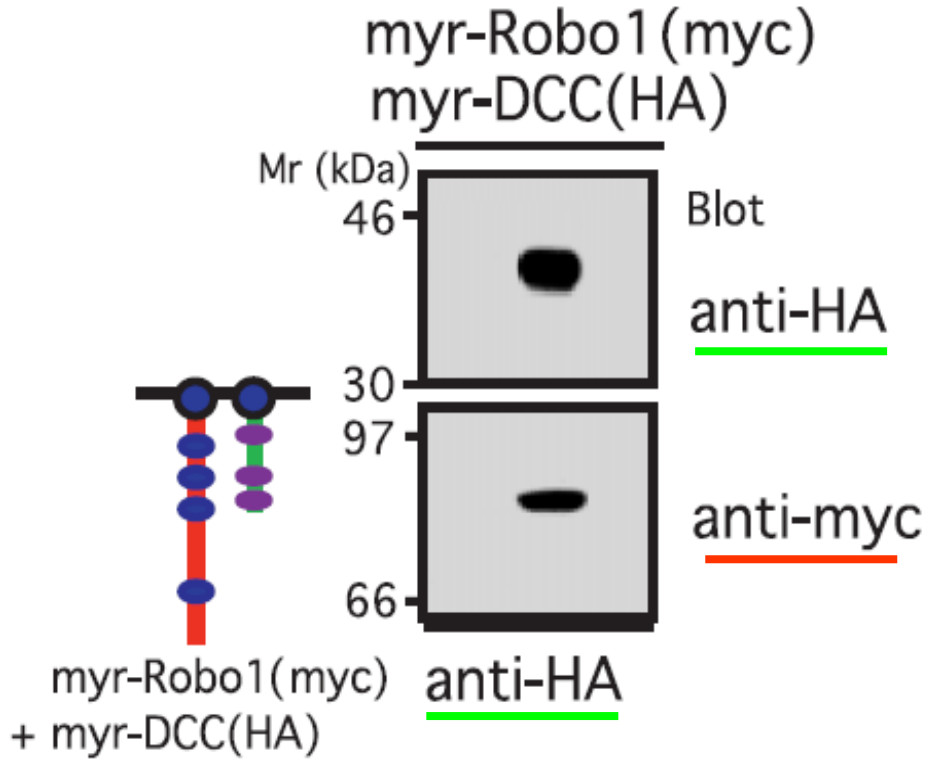


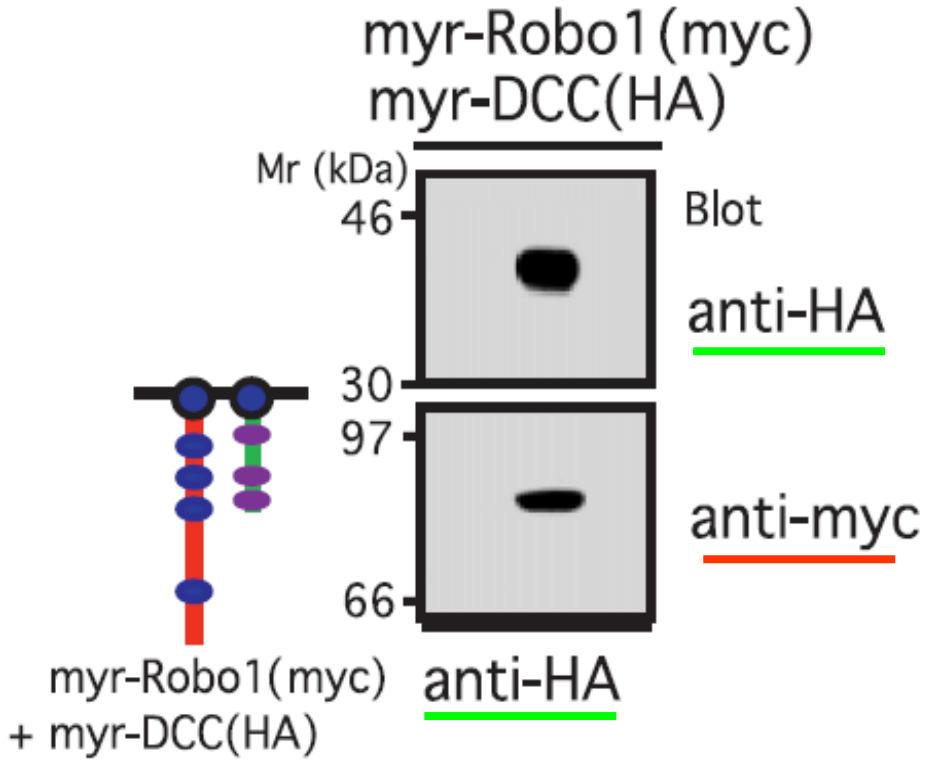
• activation of Robo1 by Slit2 even enabled it to bind the isolated cytoplasmic domain of DCC expressed as a myristoylated protein targeted to the inner leaflet of the plasma membrane

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

$\rightarrow$ 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)

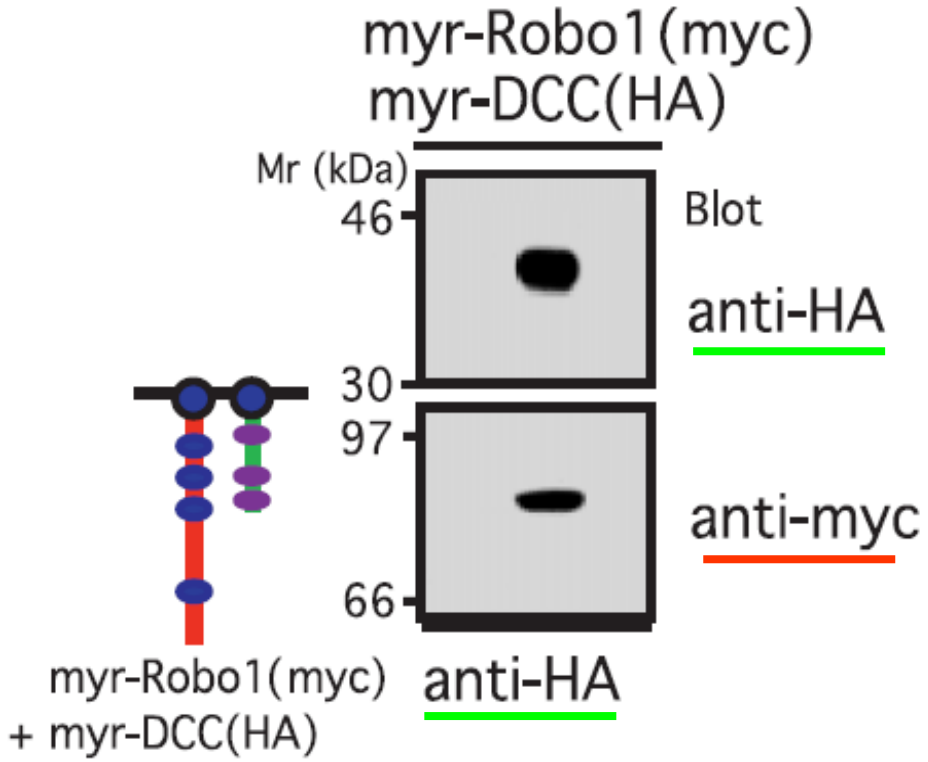
The binding relation is asymmetric: activation of Robo causes binding to DCC, but activation of DCC does not cause binding to Robo


myr-Robo1(myc)
myr-DCC(HA)
Mr (kDa)
46
30
97
66
Blot
anti-HA
anti-myc
anti-HA
myr-Robo1(myc)
+ myr-DCC(HA)



• 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

- If Robo and DCC cytoplasmic domains spontaneously interact, why stimulated full length proteins do not interact?
- What happens when full length Robo is stimulated by Slit?



• 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

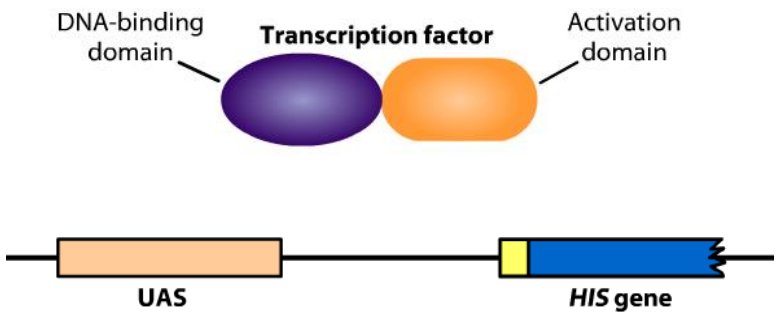
→ the cytoplasmic domains can associate but this association is repressed in the context of the full-length receptors

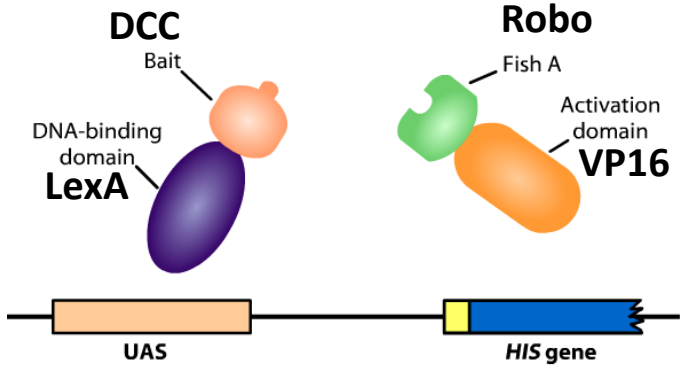
→ Slit2 functions to derepress this interaction, presumably by causing a conformational change in the cytoplasmic domain of Robo1

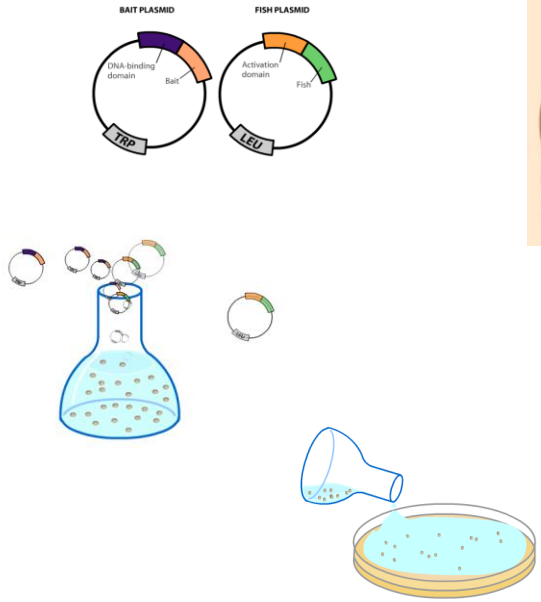
$\rightarrow$ cytoplasmic domains can associate, but this association is repressed in the context of the full-length receptors

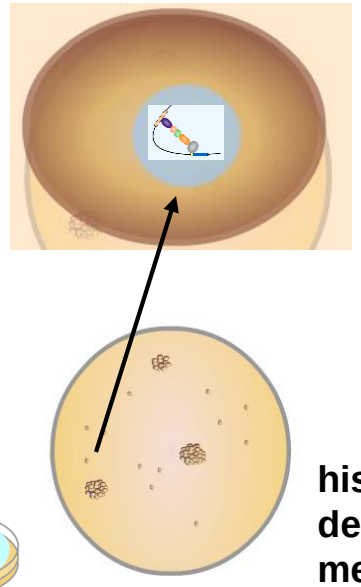
- Which technique would you use to identify domains responsible of interaction between receptors?

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**

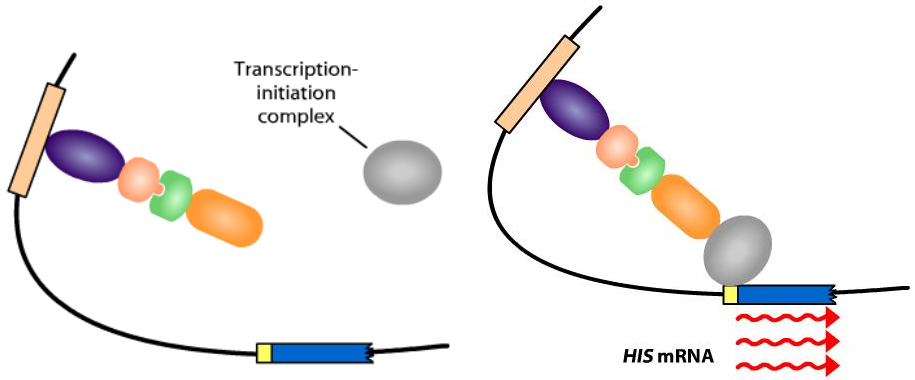




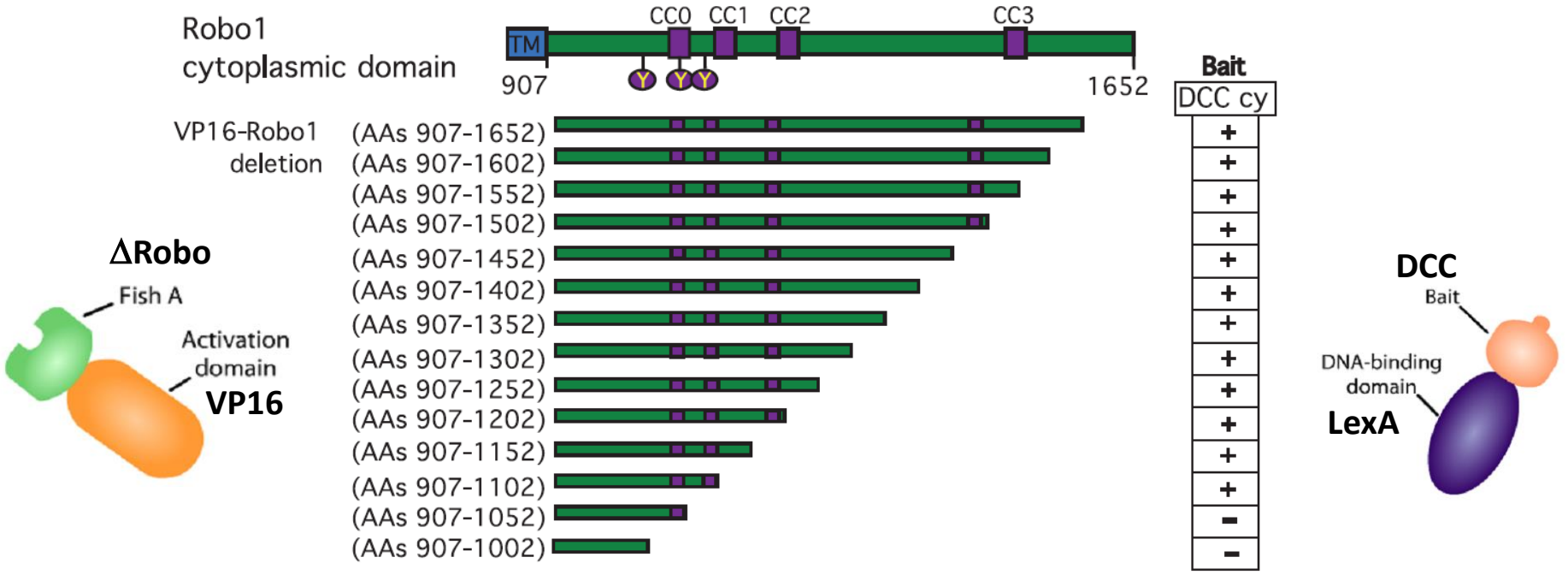




histidine-deficient media



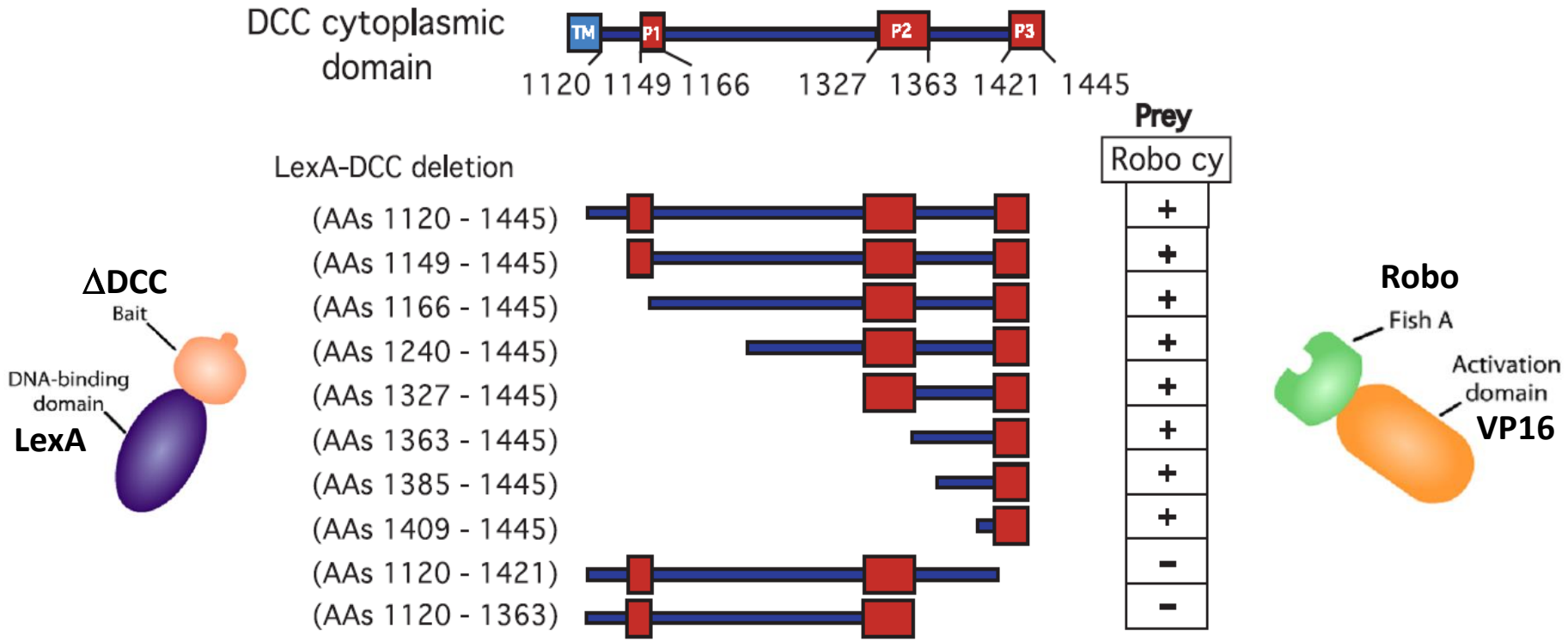
Yeast two-hybrid analysis of the interaction between the cytoplasmic domains of Robo1 (as VP16 fusion fish) and DCC (as LexA fusion bait)



- 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**

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