Advanced Cell Biology and Biotechnology

ACBB 2021/22

…the lecture of December 10th is about to begin…

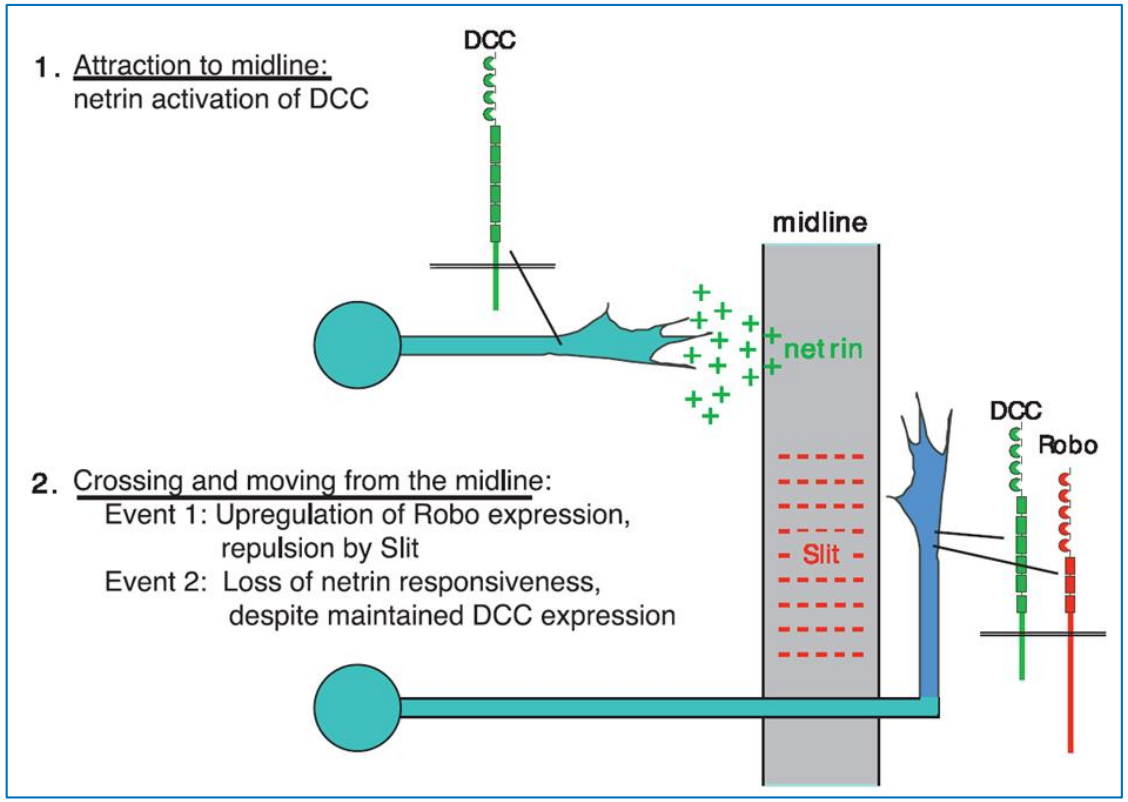
CELL-CELL COMMUNICATION

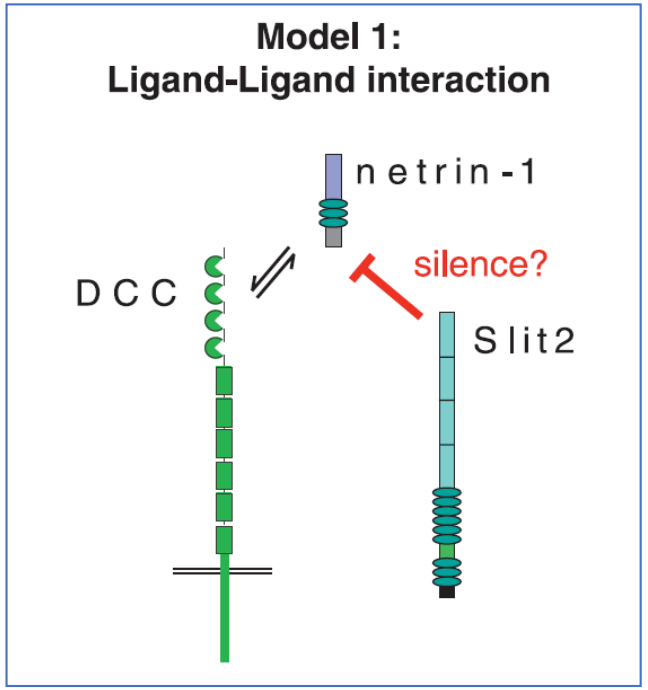
- methods to study cell-cell communication:
  - chemotaxis & chemokinesis
  - attraction & repulsion
  - substrate preference
  - bidirectional signalling

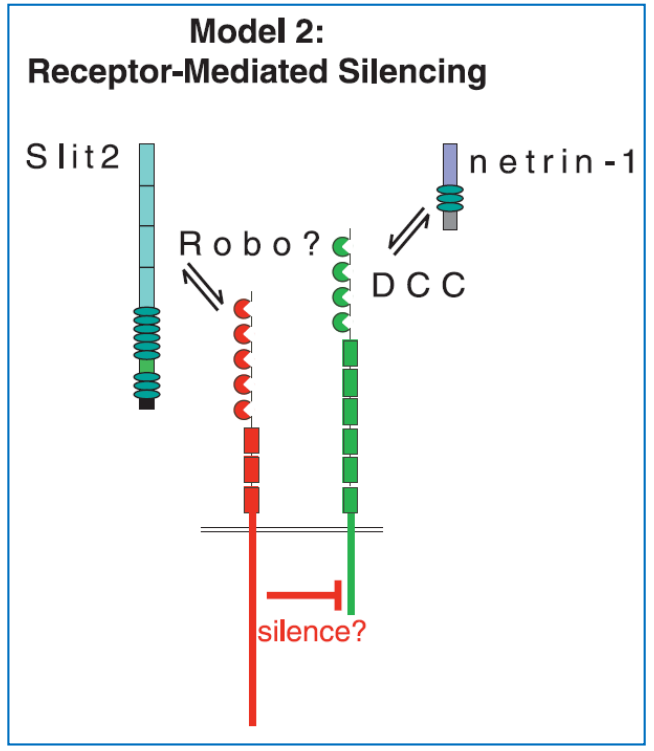
CELL-CELL COMMUNICATION

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

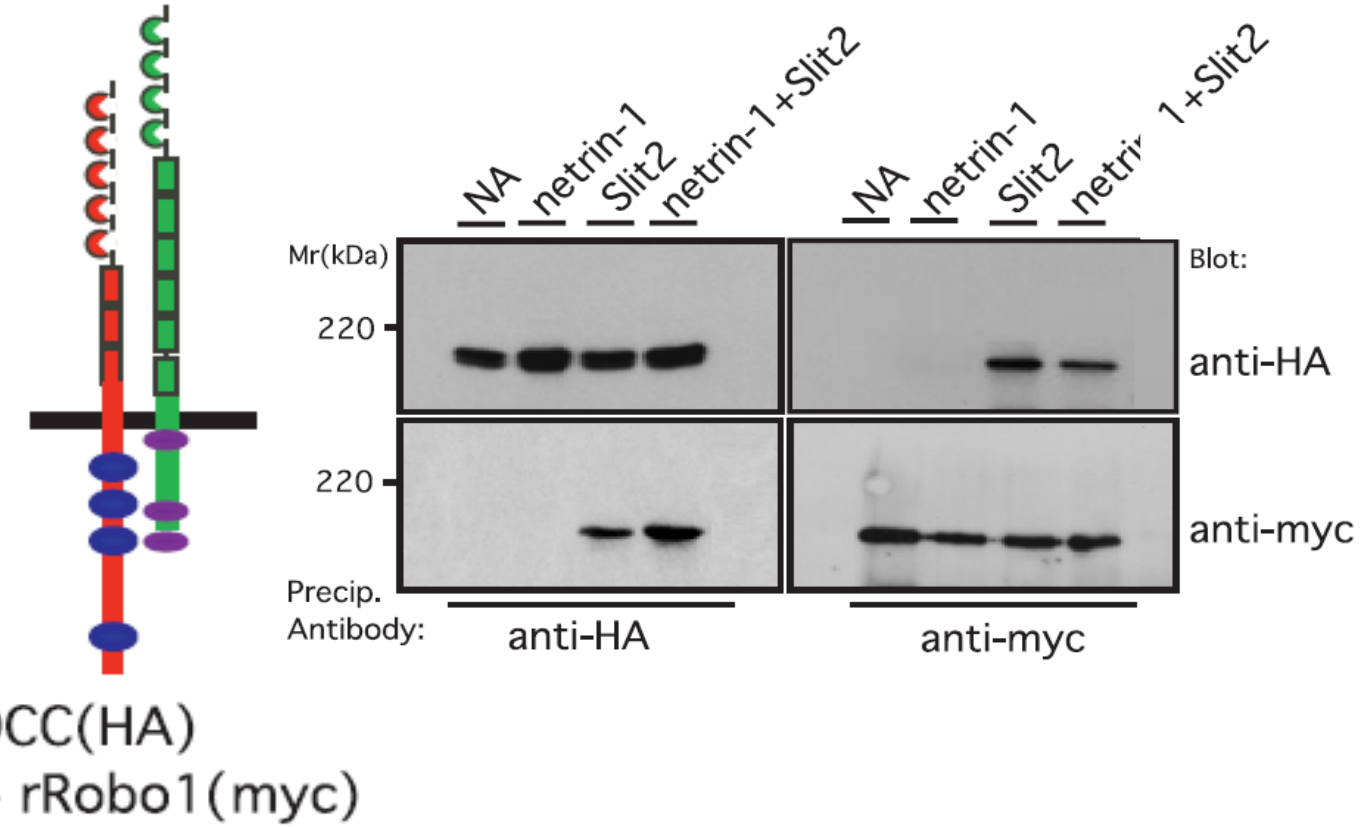
Silencing of attraction



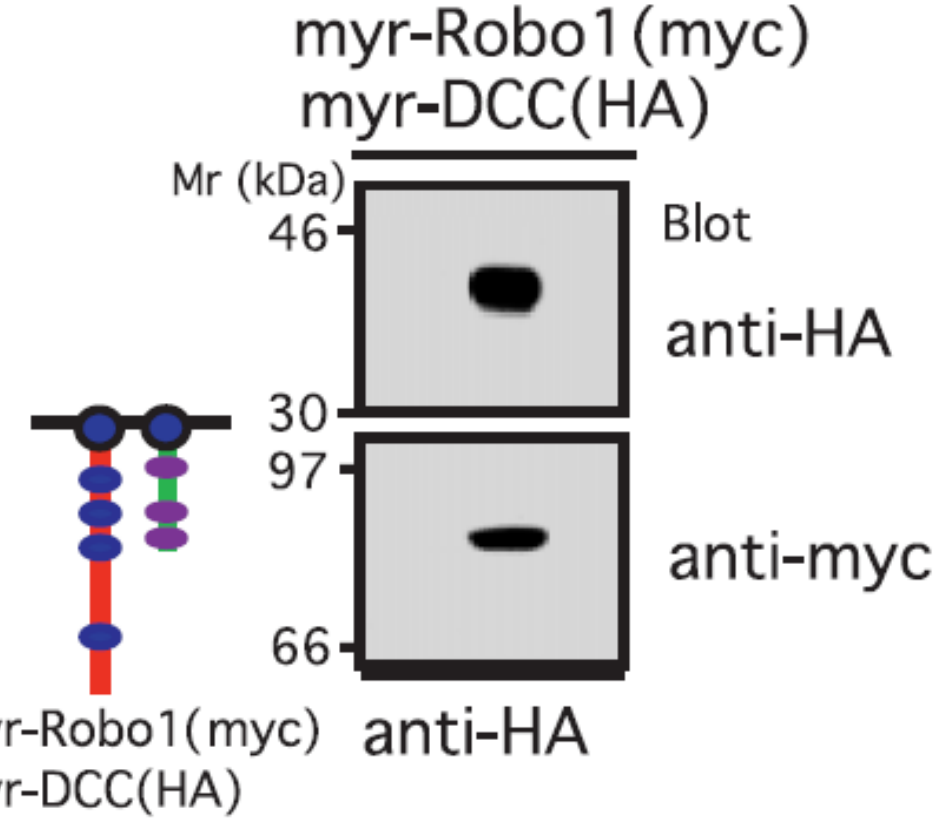




Silencing of attraction



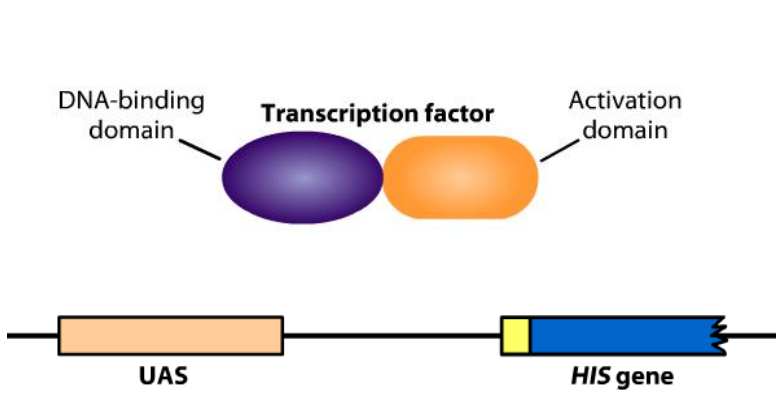
DCC(HA)
+ rRobo1(myc)

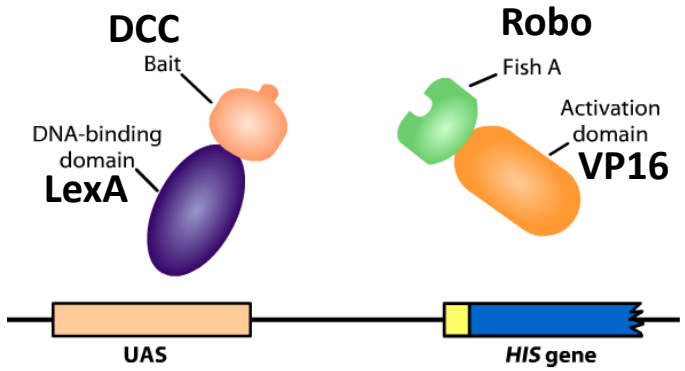


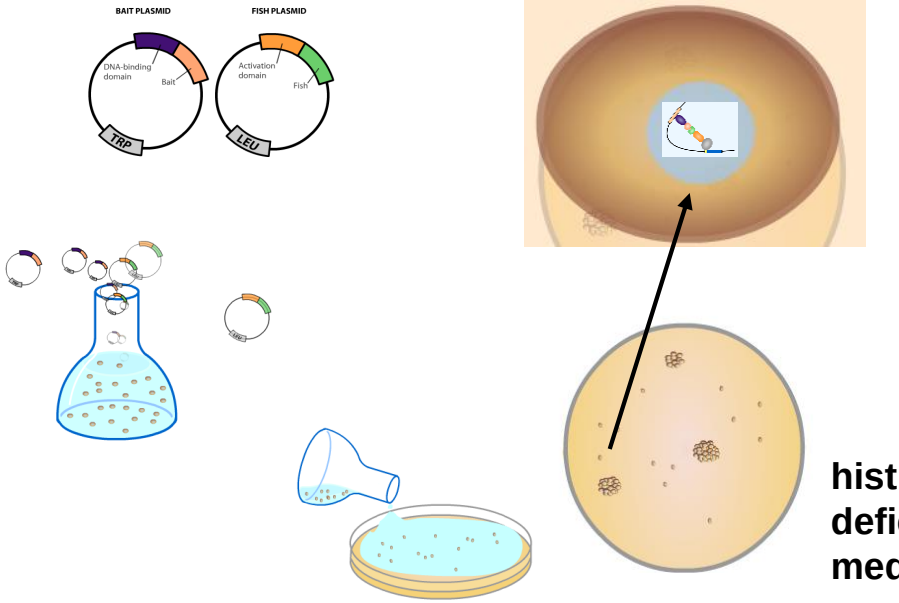
myr-Robo1(myc)
+ myr-DCC(HA)

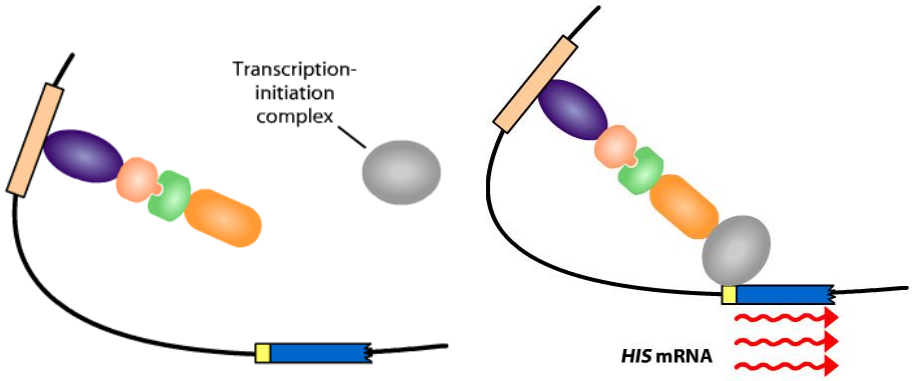
anti-HA

Yeast two-hybrid analysis



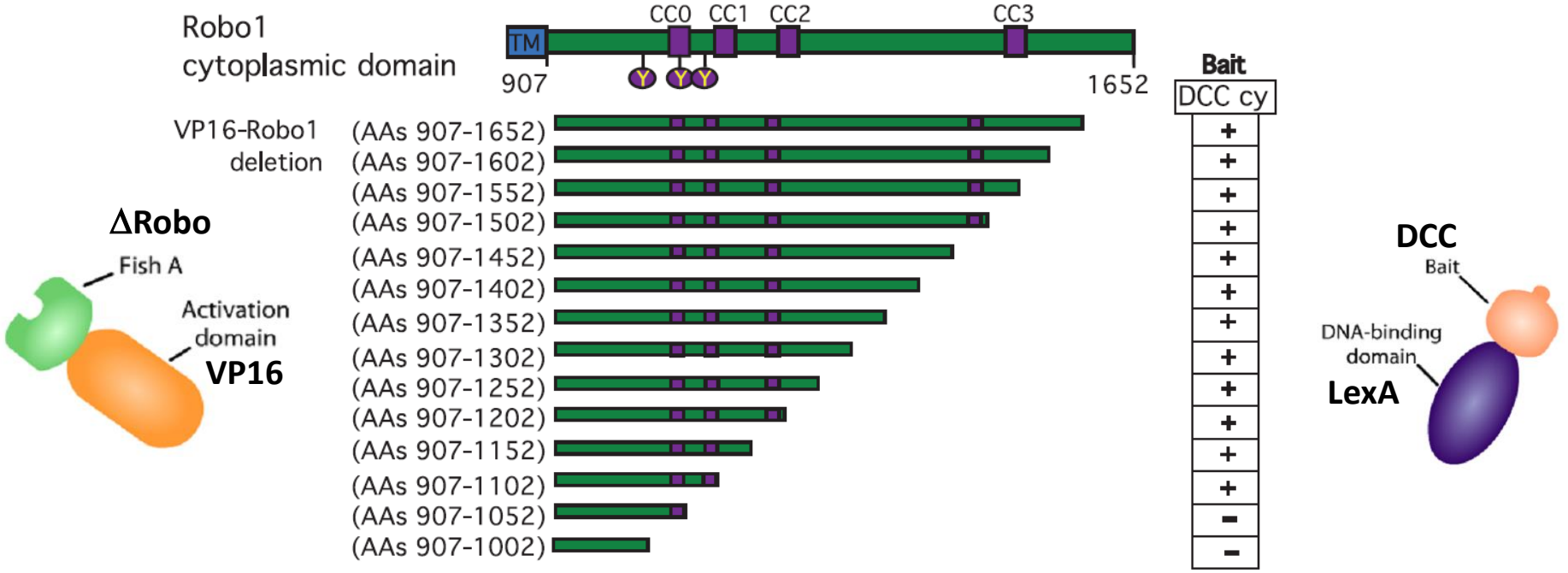






To determine whether the association of cytoplasmic domains is causally involved in silencing, regions in these domains that are required for the interaction were identified through a **yeast two-hybrid analysis**

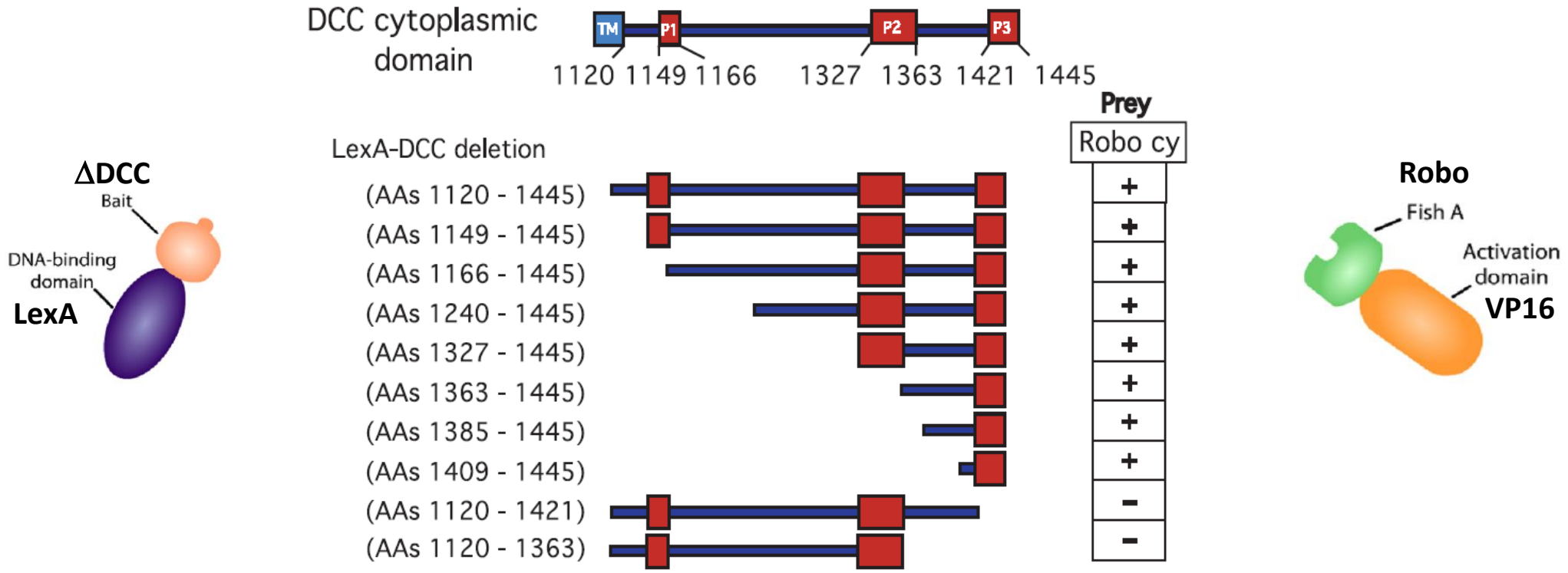
Yeast two-hybrid analysis of the interaction between the cytoplasmic domains of Robo1 (as VP16 fusion fish) and DCC (as LexA fusion bait)



- Robo 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**

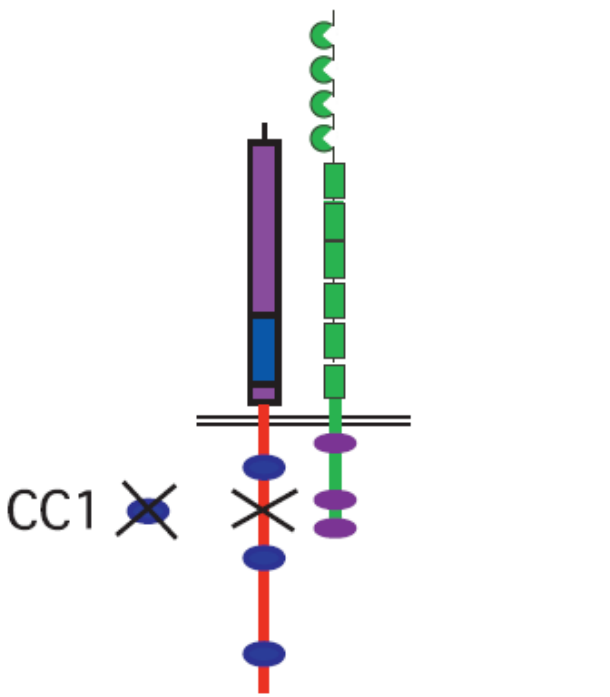
Yeast two-hybrid analysis of the interaction between the cytoplasmic domains of DCC (as LexA fusion bait) and Robo1 (as VP16 fusion prey)



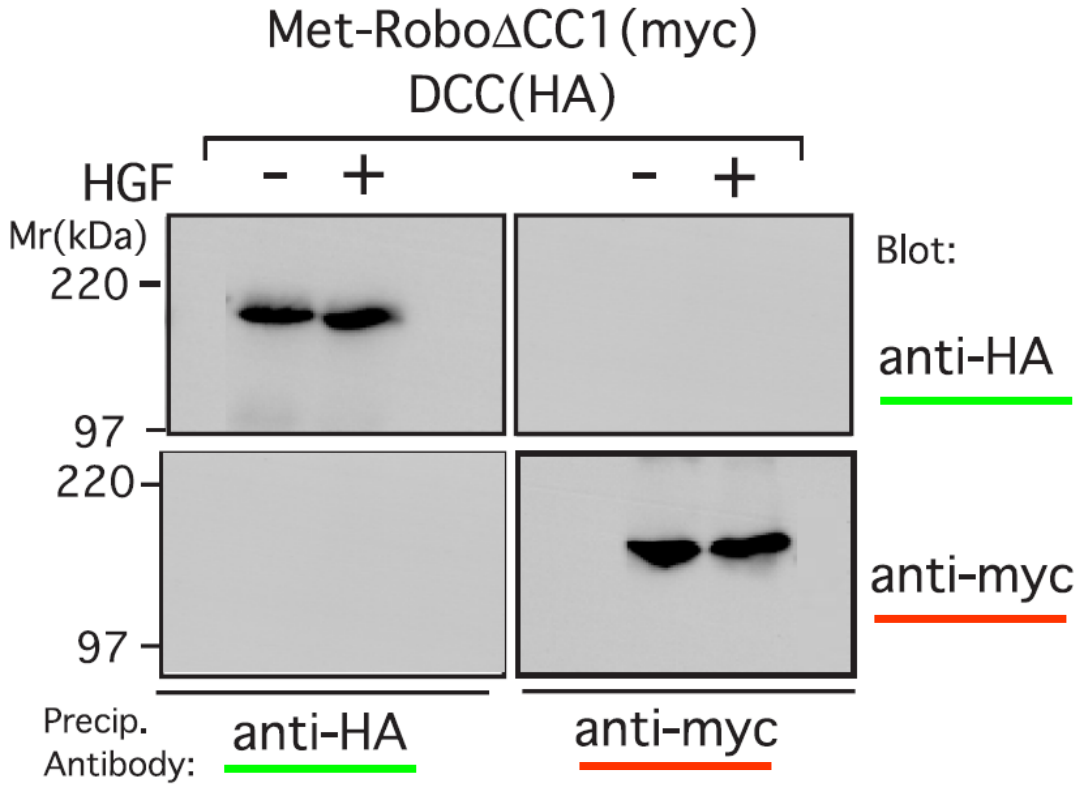
- DCC deletion constructs and their ability to interact with the Robo1 cytoplasmic domain prey
- interactions were assessed by the ability to rescue growth on histidine-deficient plates
  (+, rescue; -, no rescue)

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

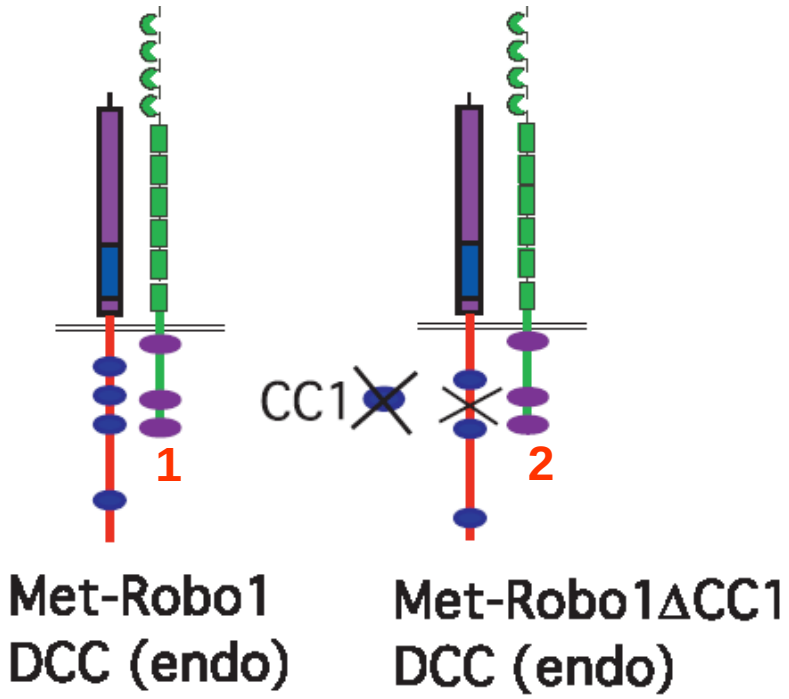
Specific deletion of CC1 abolished the association between DCC and Met-Robo1 that is induced by HGF

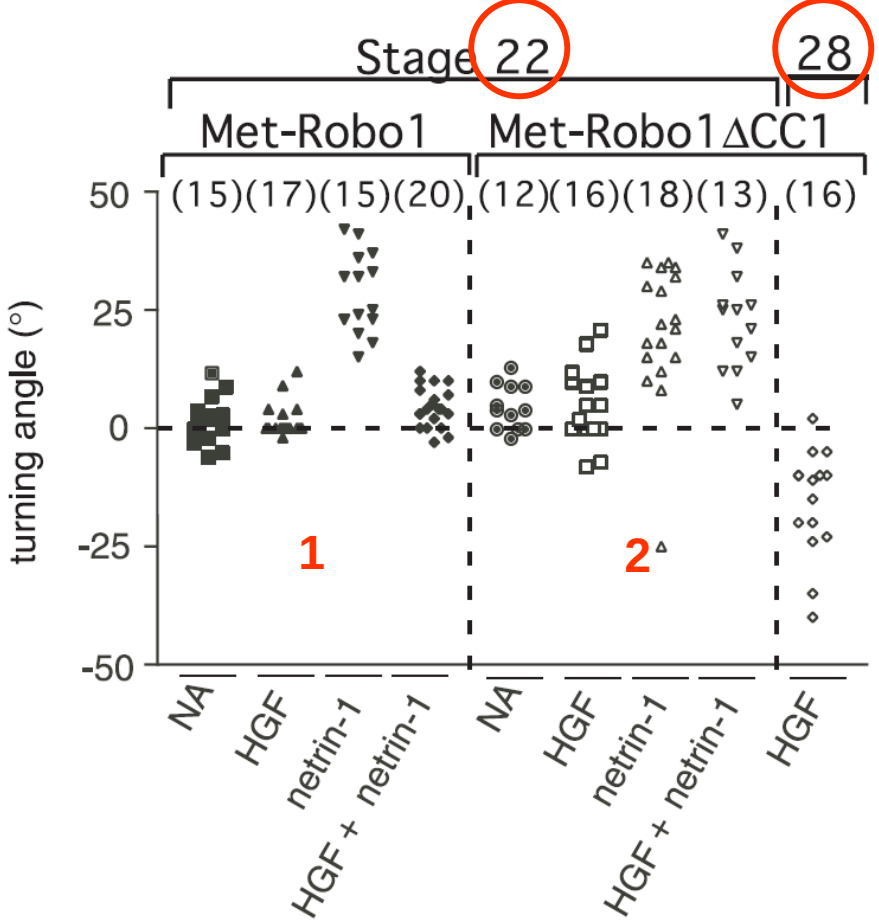


Met-Robo1ΔCC1(myc)
+ DCC (HA)



Interfering with the interaction,
does interfere with silencing?



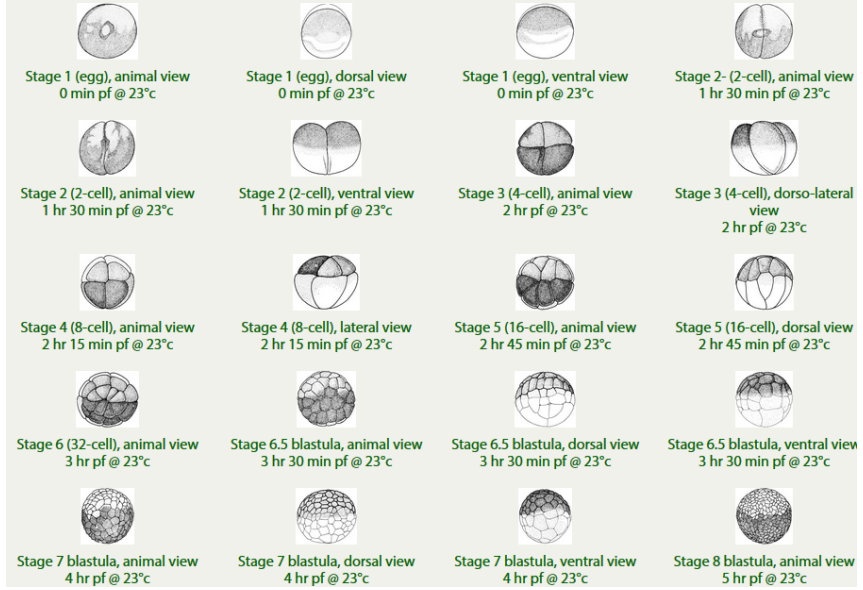


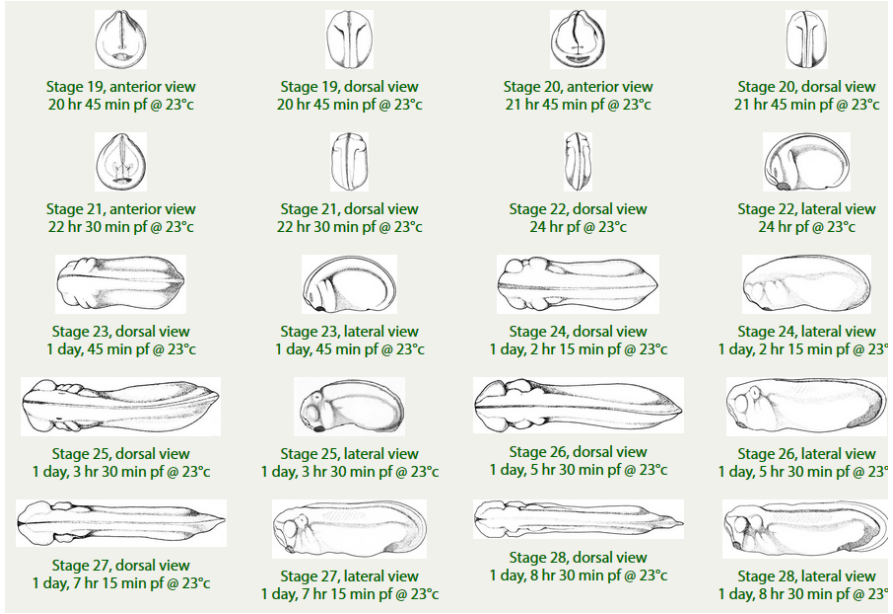
- Met-Robo1ΔCC1 receptor could not silence netrin attraction in stage 22

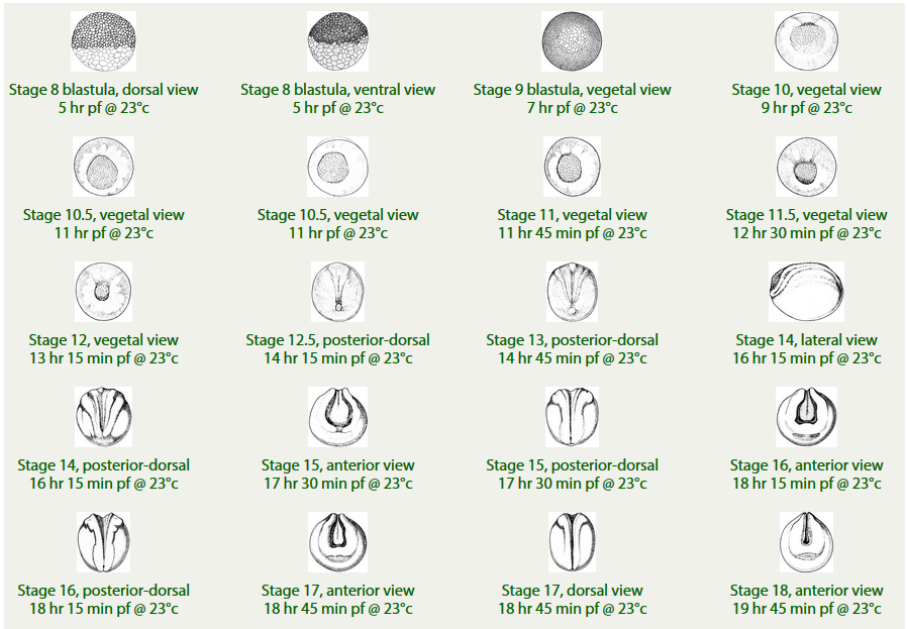
→ **this result supports the idea that silencing requires direct cytoplasmic domain binding**

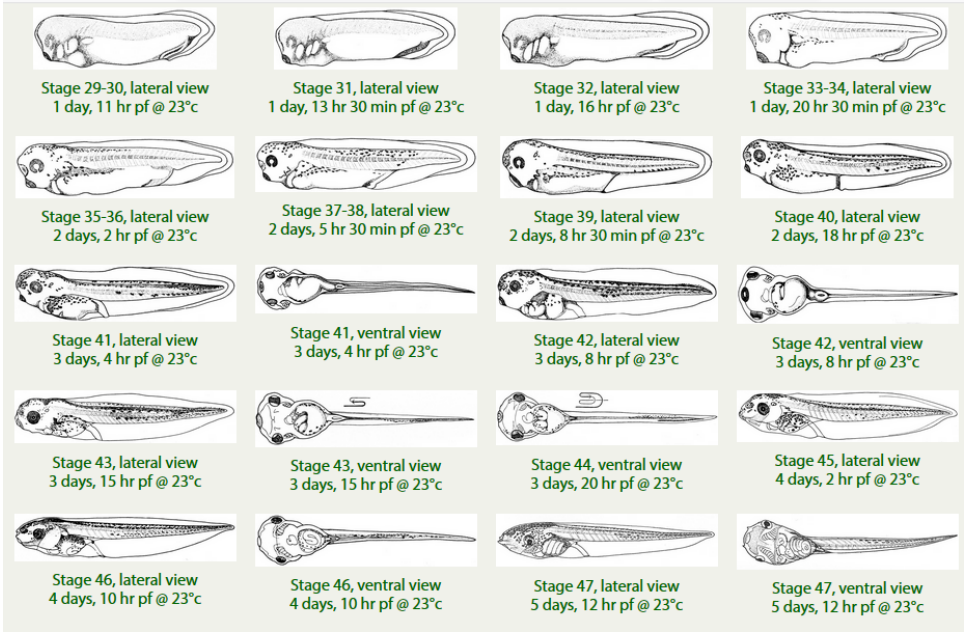
- Met-Robo1ΔCC1 induces a repulsive response to HGF in stage 28 neurons

→ **this result shows that the receptor functions in silencing and in repulsion are separated**





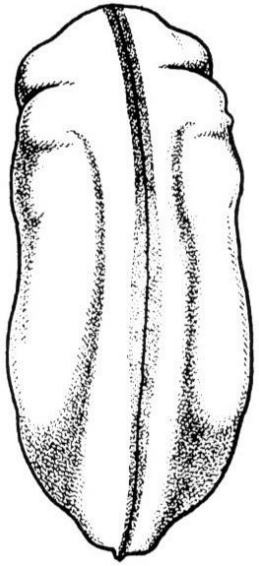


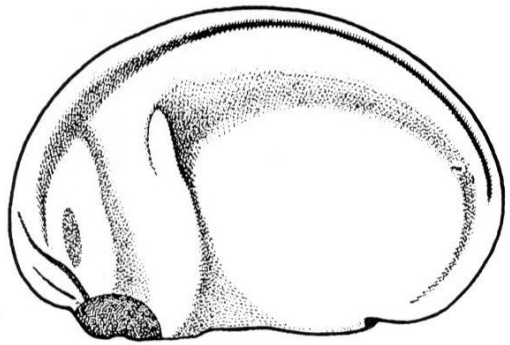


http://www.xenbase.org/anatomy/alldev.do

http://www.xenbase.org/anatomy/alldev.do

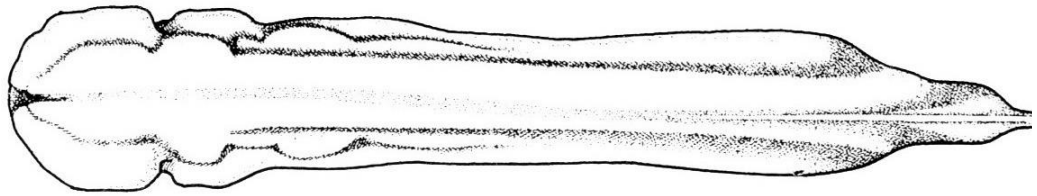
Stage 22, dorsal view 24 hr pf @ 23°c

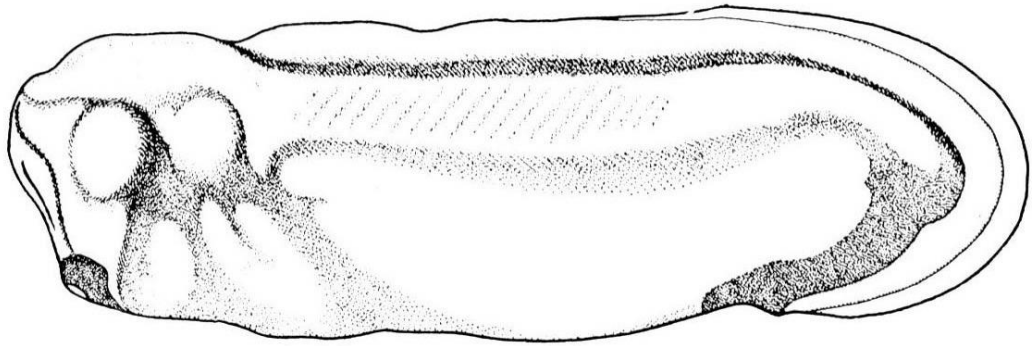




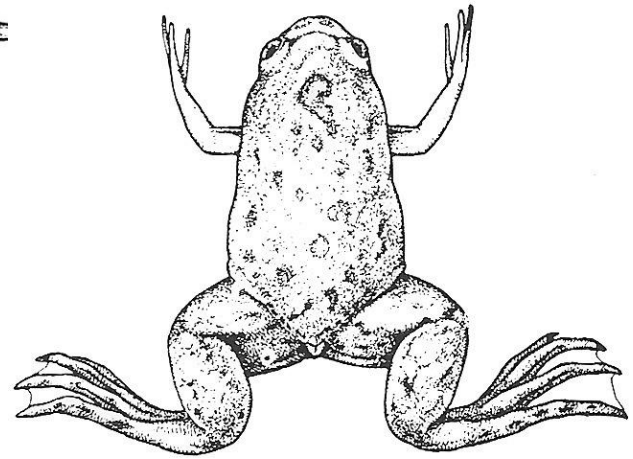
Stage 22, lateral view 24 hr pf @ 23°c

Stage 28, dorsal view 1 day, 8 hr 30 min pf @ 23°c



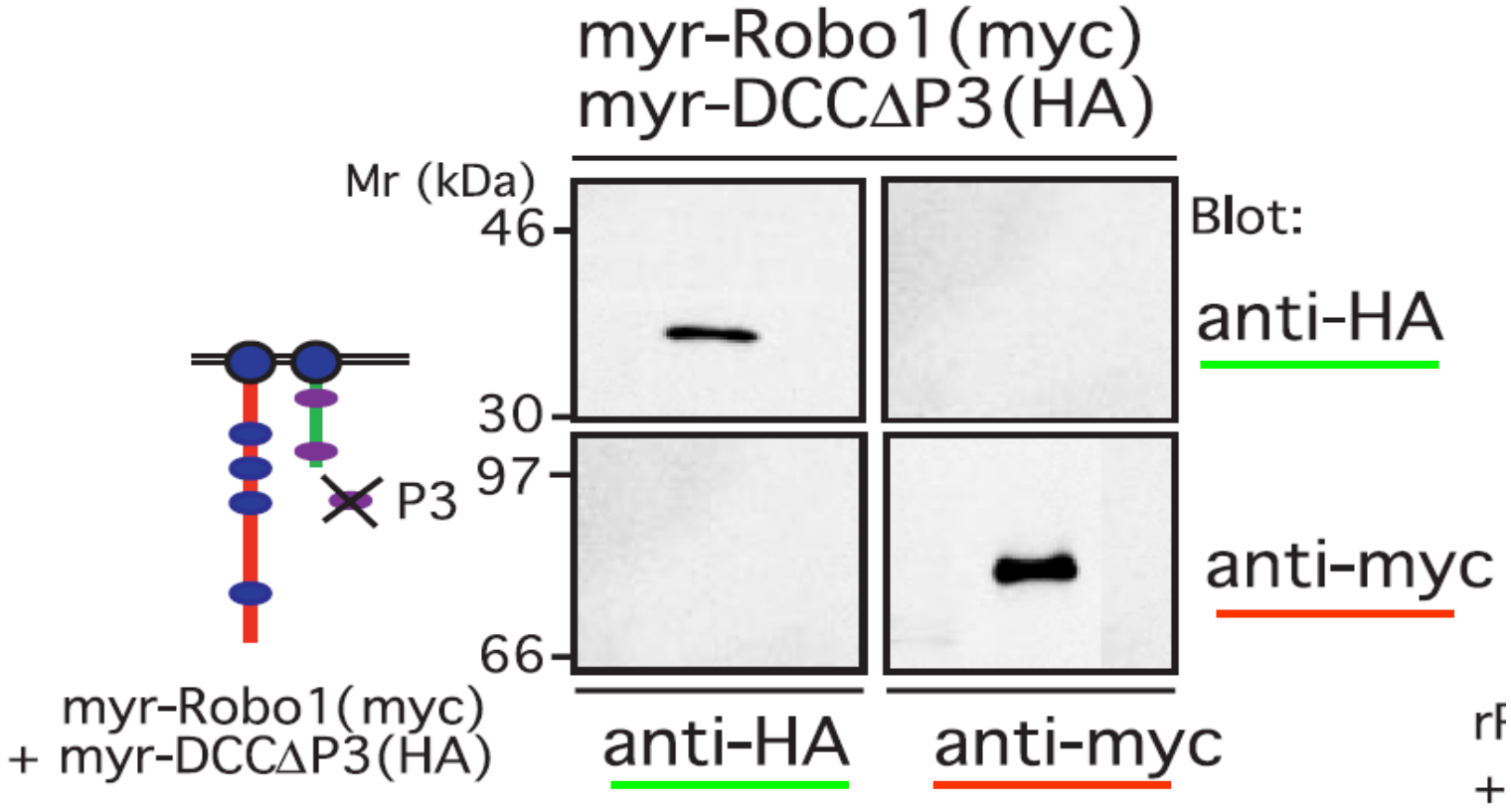


Stage 28, lateral view 1 day, 8 hr 30 min pf @ 23°c

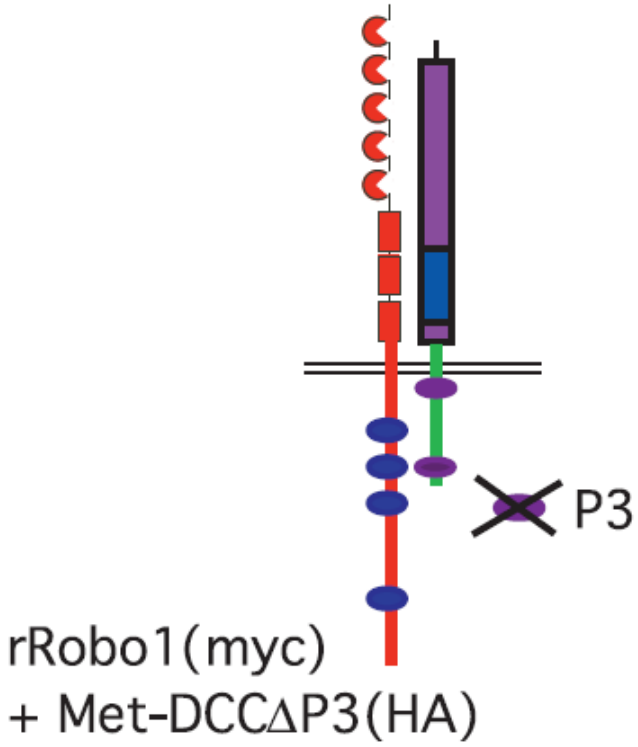


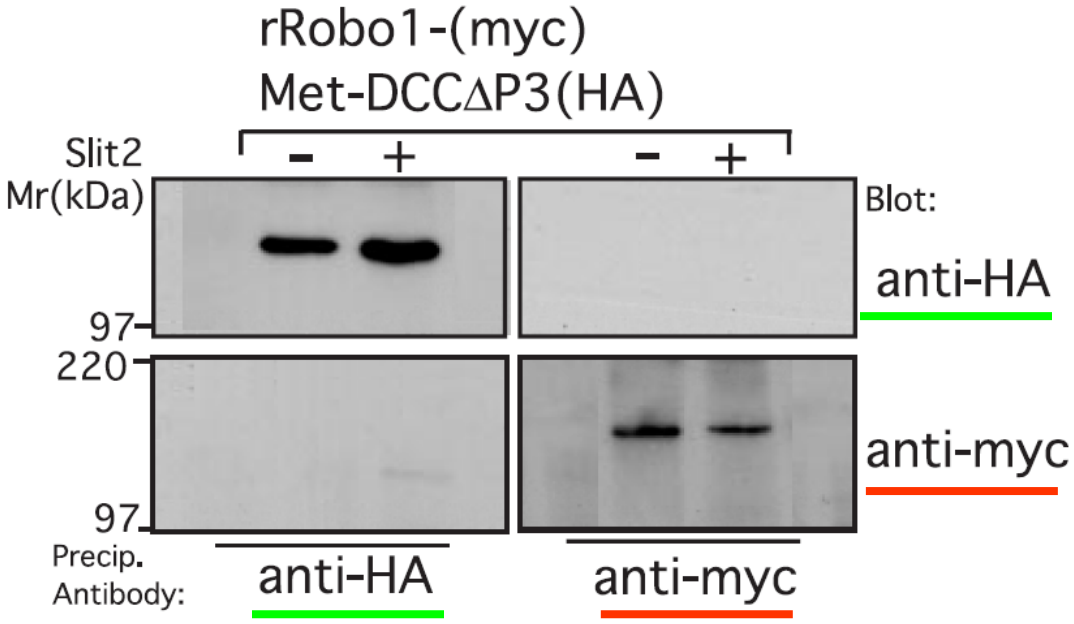
Stage 66, dorsal view 58 days pf @ 23°c

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



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



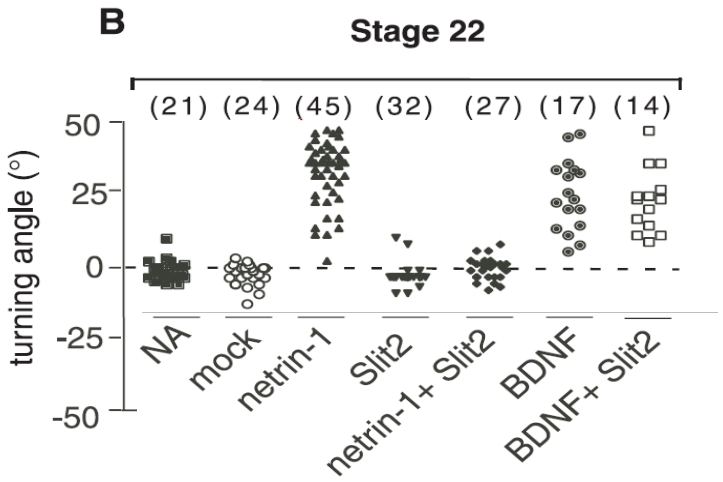


Does deletion of P3, by blocking the DCC-Robo interaction, also block silencing?

one impediment to testing this is the fact that **P3 is also required for the function of DCC in attraction**

• previous data showed 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

If I eliminate attraction, how can I study the silencing of attraction ?



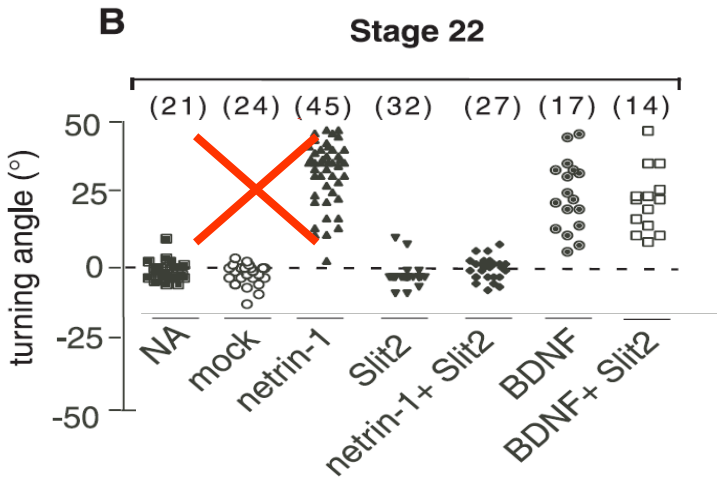
Does deletion of P3, by blocking the DCC-Robo interaction, also block silencing?

one impediment to testing this is the fact that **P3 is also required for the function of DCC in attraction**

- previous data showed 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

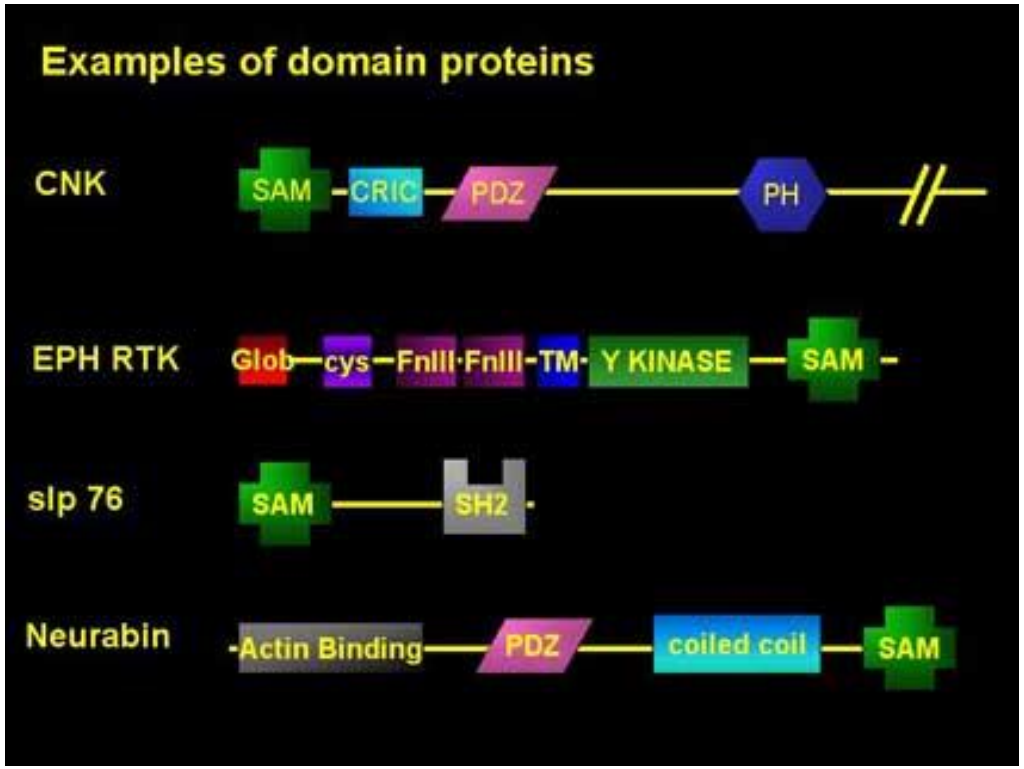
If I eliminate attraction, how can I study the silencing of attraction?

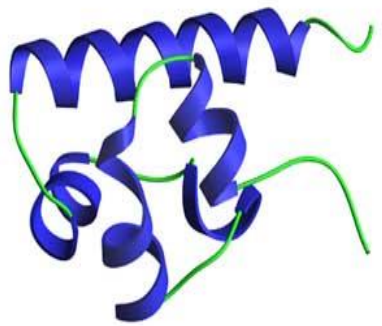
• replacing P3 with a different multimerization domain, the **SAM** domain of the EphB1 receptor, can **restore the multimerization of both DCC and Met-DCC** in response to their ligands, as well as the ability of the Met-DCC receptor to induce an attractive response in neurons in response to HGF



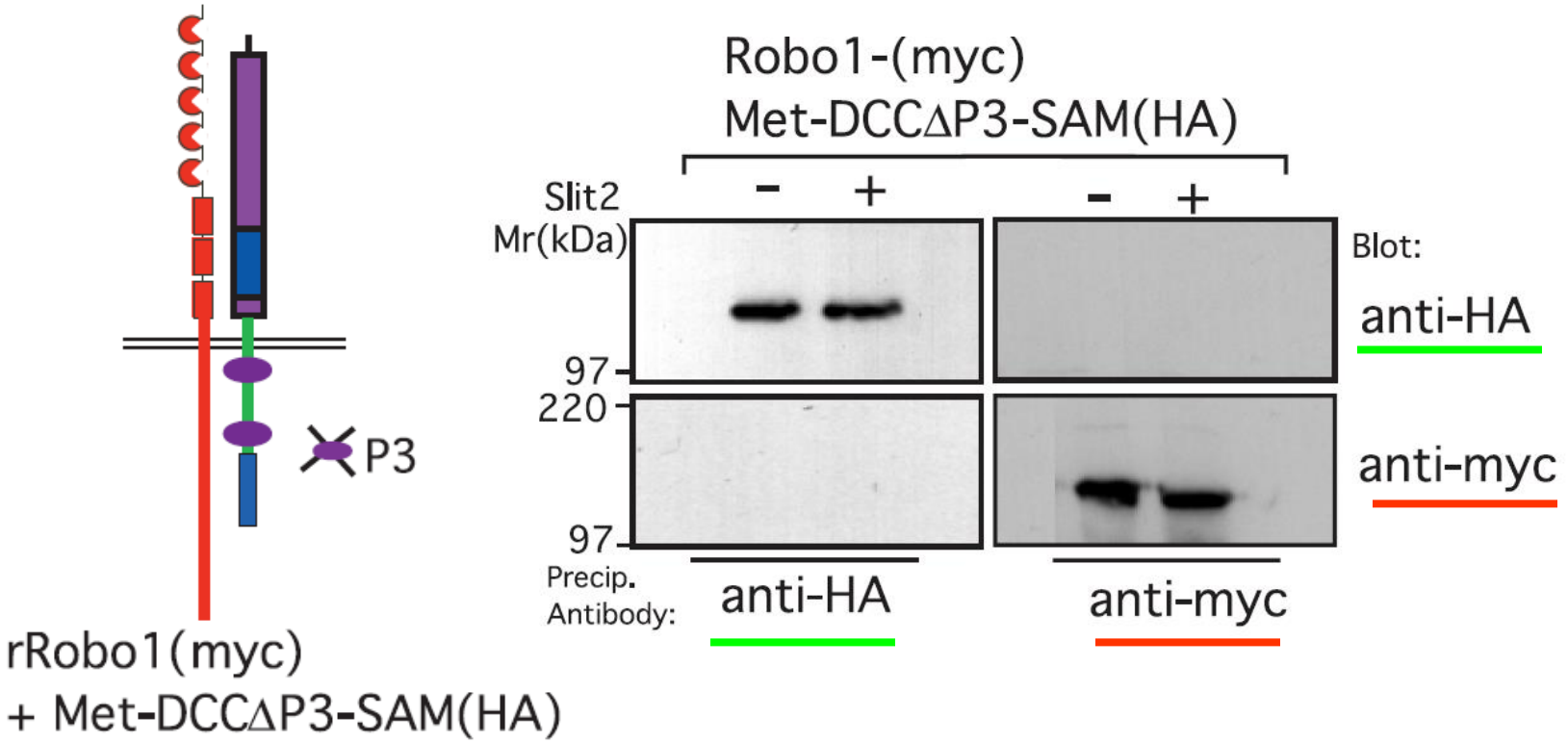
SAM Domain Binding and Function

The approximately 70 amino acid SAM (Sterile Alpha Motif) domain has been identified in over 400 different proteins with diverse cellular functions, 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.

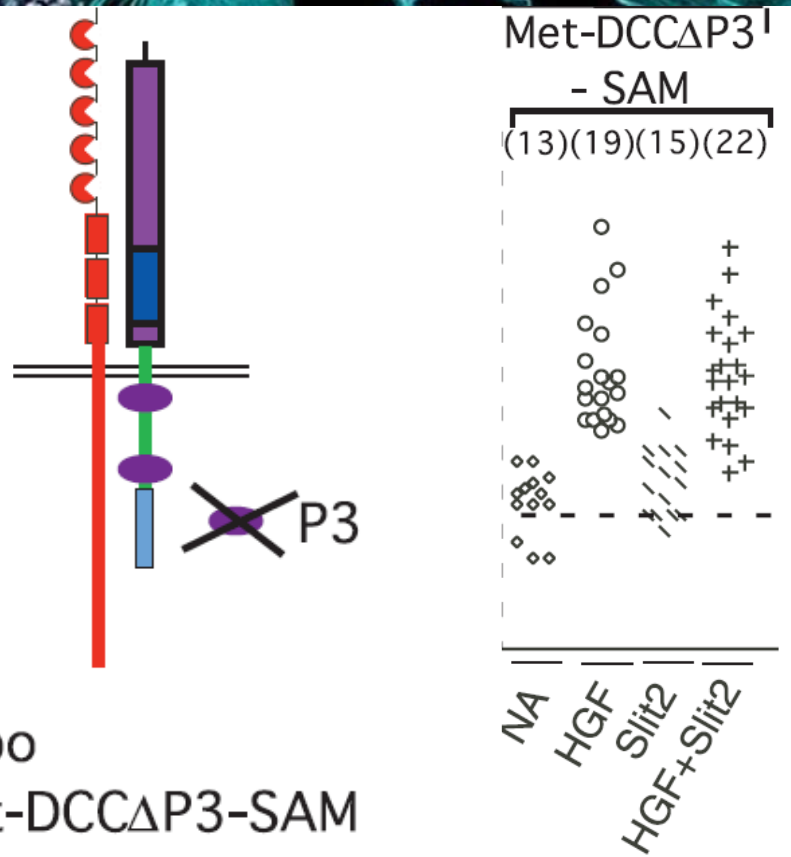




Met-DCCΔP3-SAM does not associate with Robo in response to Slit

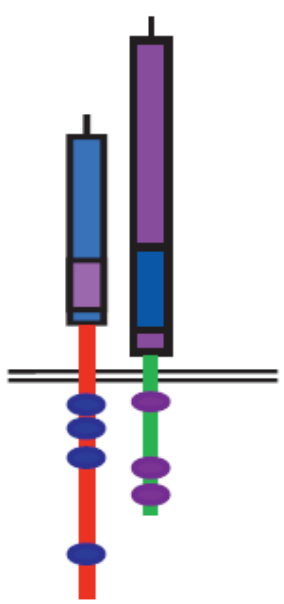


Met-DCCΔP3-SAM mediates attraction

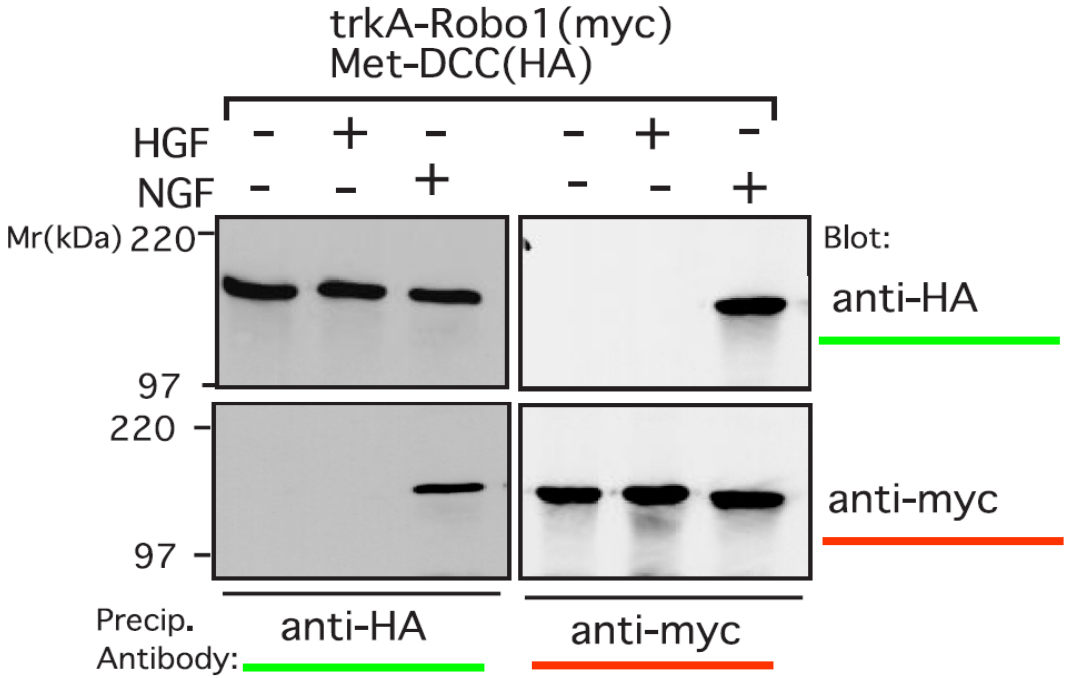


• the Met-DCC receptor in which P3 is replaced with the EphB1 SAM domain (**Met-DCCΔP3-SAM**), introduced into stage 22 neurons, can mediate attractive response to HGF, but does not bind Robo1 and is not silenced by Slit2

Restoring Robo-DCC Binding Synthetically does Restore Silencing?



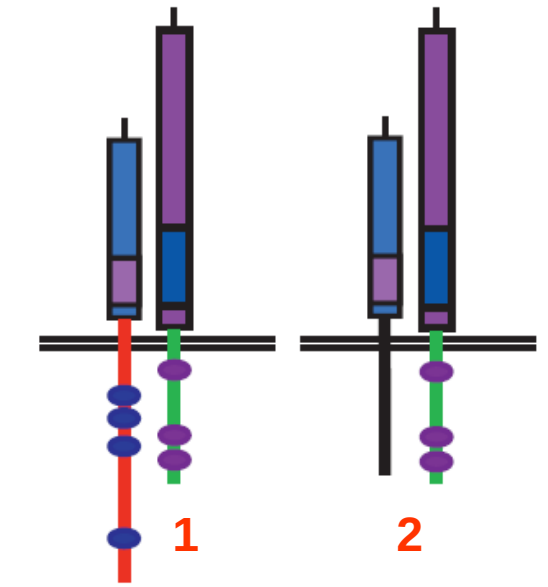
trkA-Robo1(myc)
+ Met-DCC(HA)

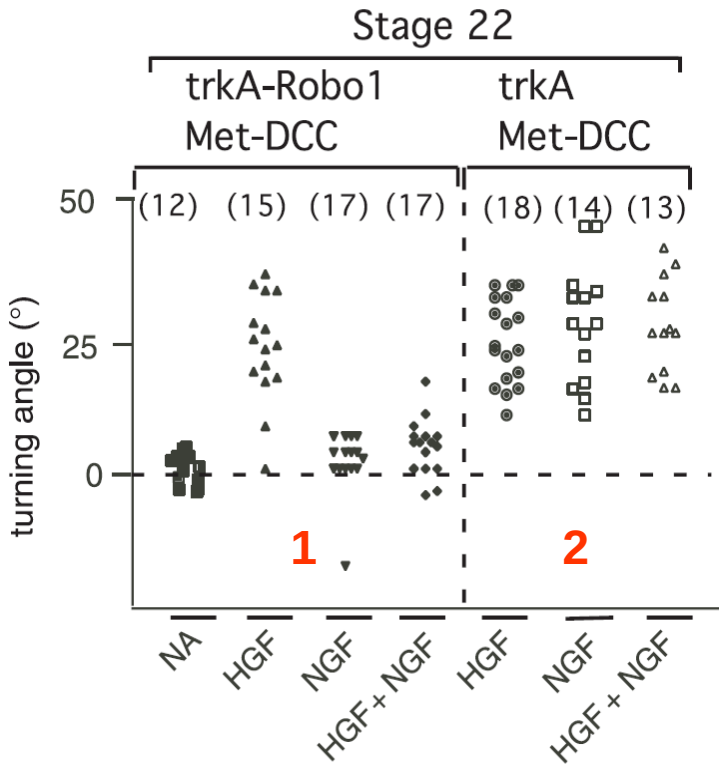


- to avoid confusion from the activities of endogenous receptors, two chimeric receptors were used: the **trkA-Robo1** chimera and the **Met-DCC** chimera

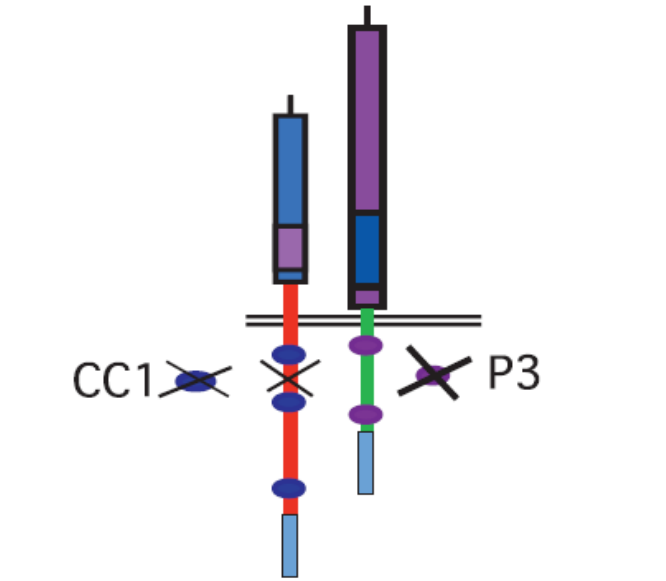
- as expected, in transfected cells, NGF but not HGF, induced formation of a receptor complex

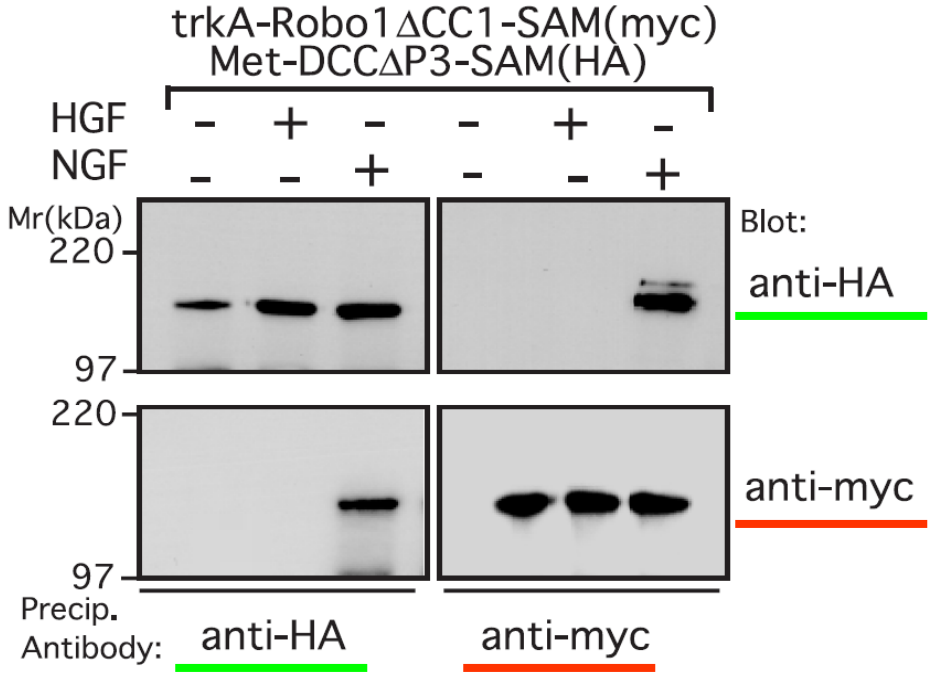
in neurons expressing the **trkA-Robo1** and the **Met-DCC** chimeras, HGF elicited an attractive response that was silenced by NGF





Restoring Robo-DCC Binding Synthetically does Restore Silencing?

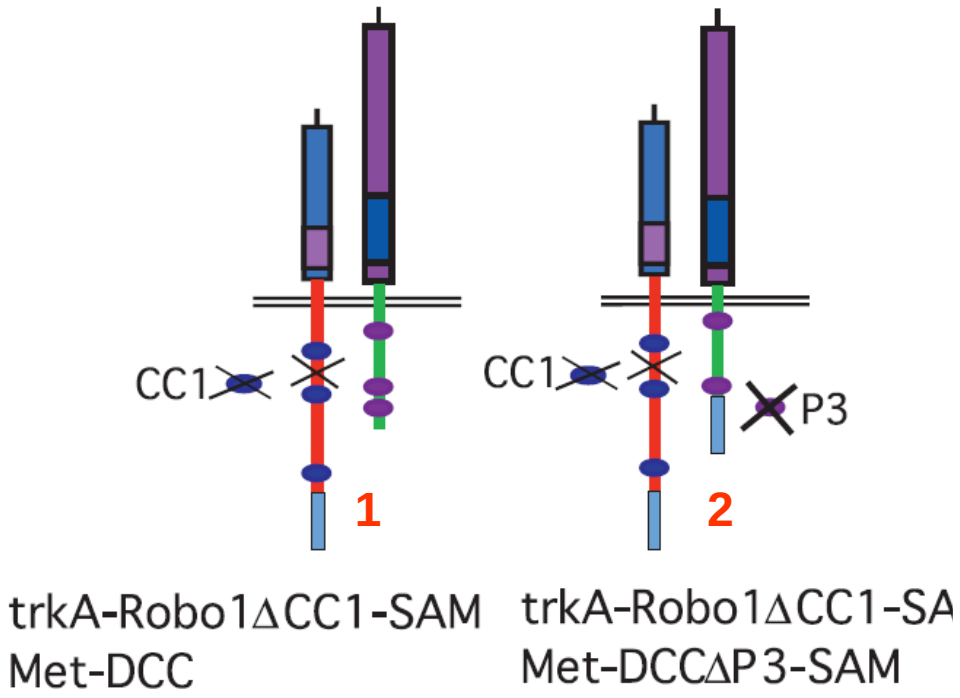


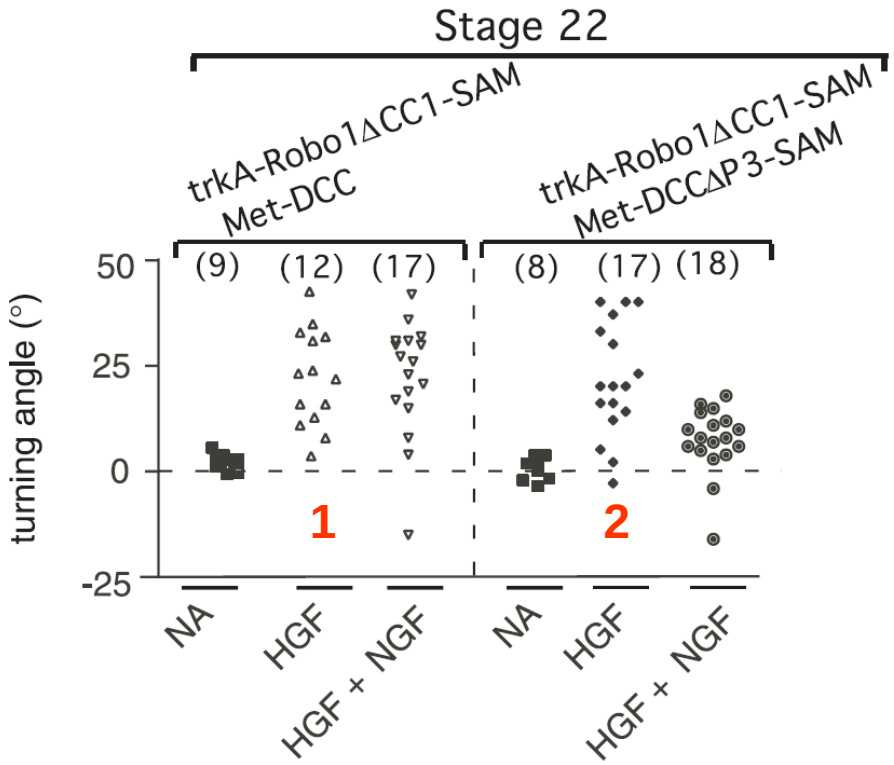


- addition of an EphB1 SAM domain to trkA-Robo1ΔCC1 might enable it to associate with Met-DCCΔP3-SAM, because of the multimerization function of the SAM domain

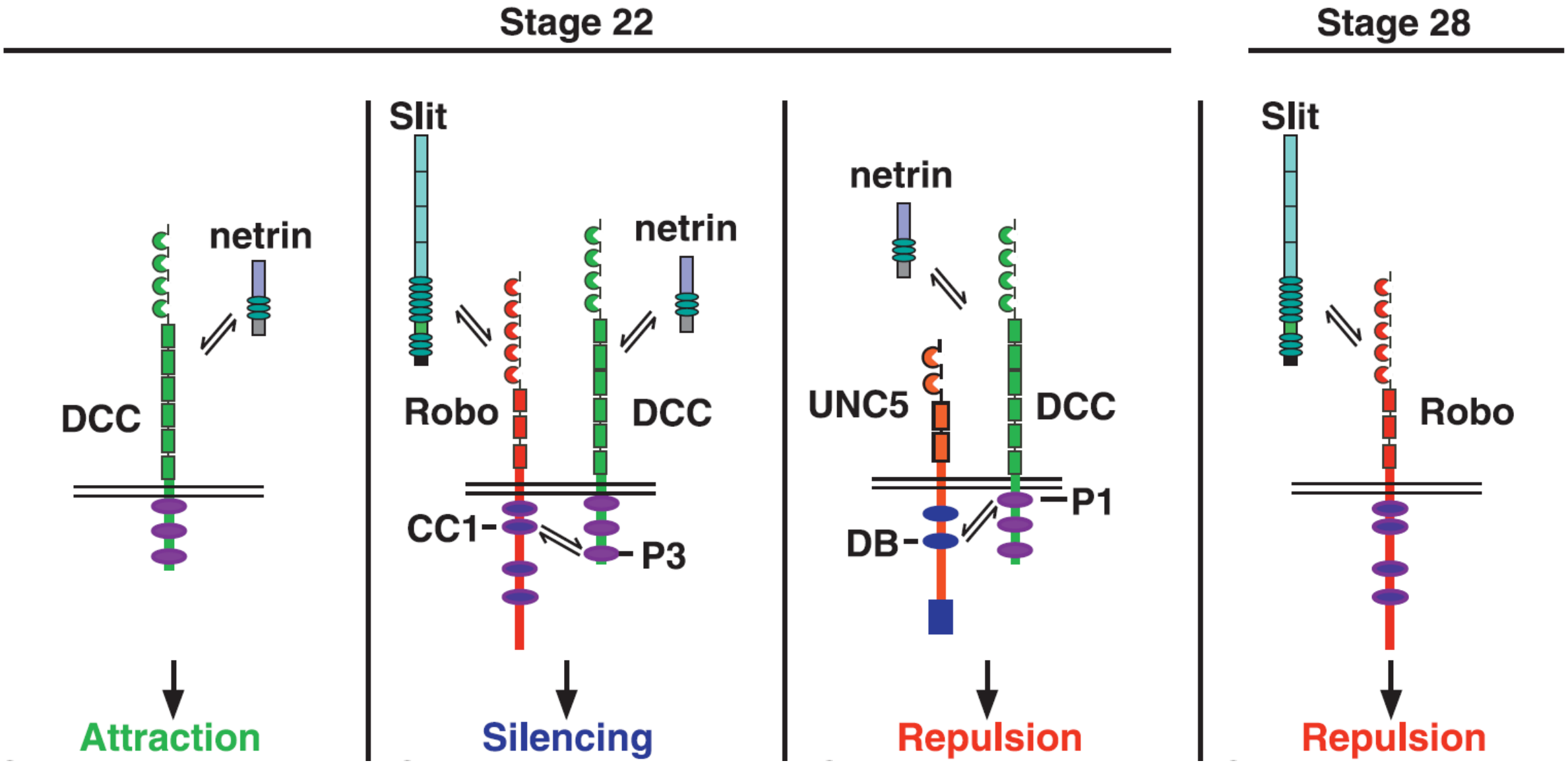
- NGF induces binding of trkA-Robo1ΔCC1-SAM to Met-DCCΔP3-SAM

Restoring Robo-DCC Binding Synthetically Restores Silencing





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

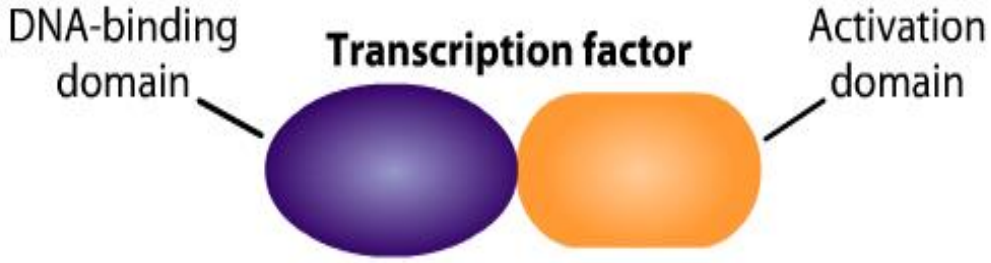

Stage 22
Stage 28
netrin
DCC
Attraction
Slit
netrin
Robo
DCC
CC1
P3
Silencing
netrin
UNC5
DCC
P1
DB
Repulsion
Slit
Robo
Repulsion

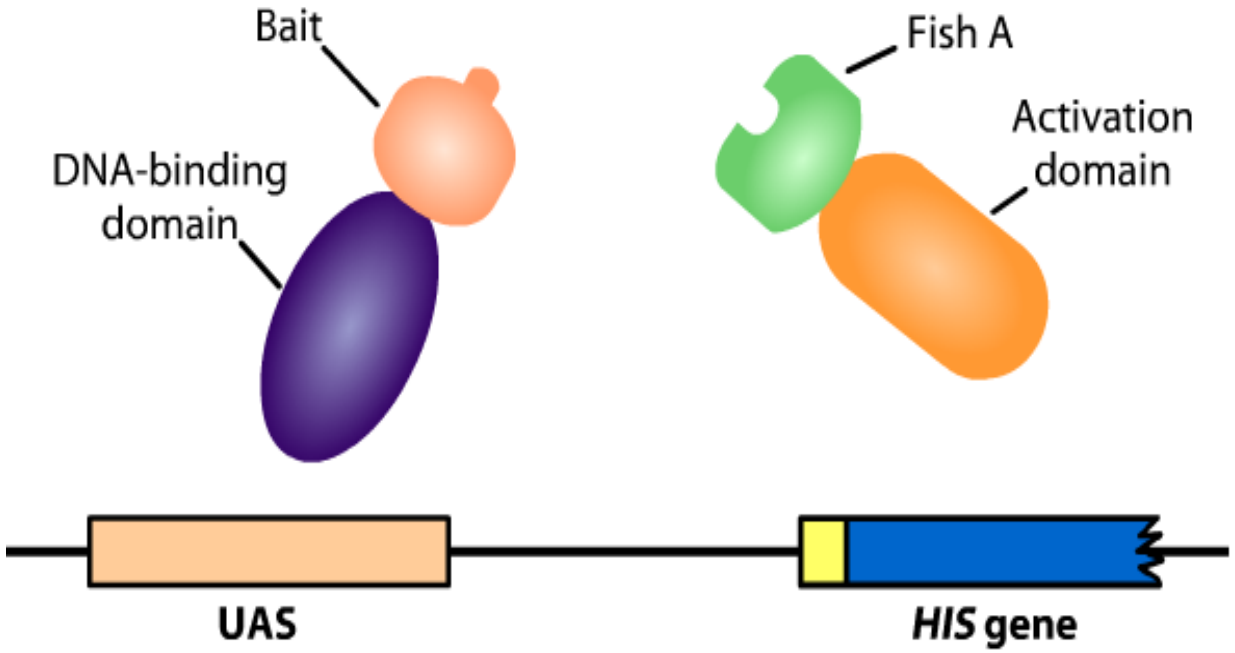
protein-protein interactions

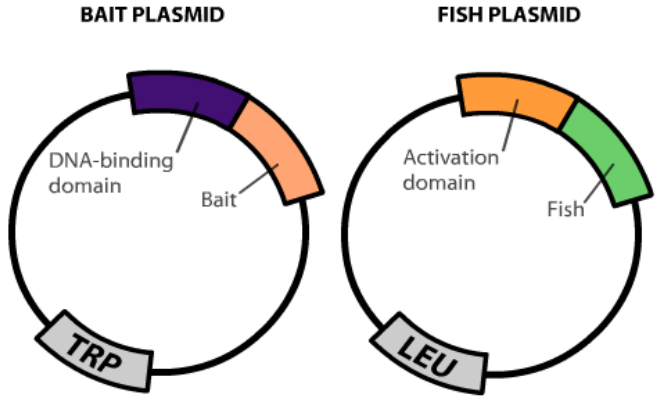
- co-IP
- Two-hybrid
- Split-TEV
- Fluorescence Resonance Energy Transfer (FRET)

Fragment complementation assays such as the yeast two-hybrid (Y2H) system and split-TEV are based on split proteins that are functionally reconstituted by fusions of interacting proteins.

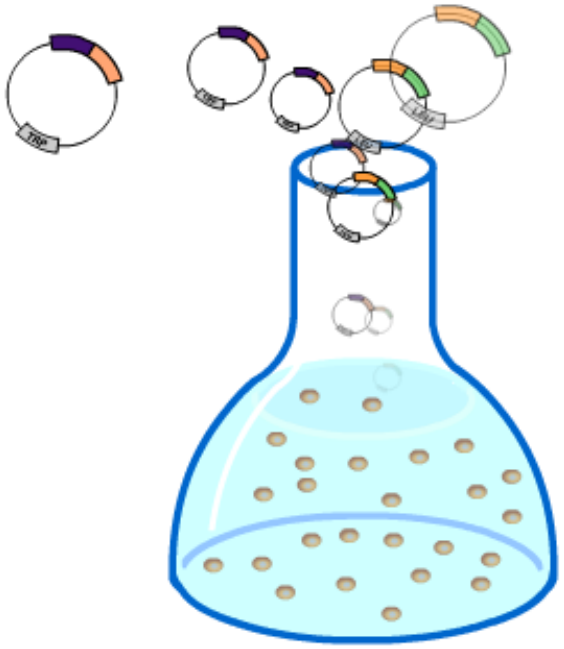
Yeast two-hybrid screening

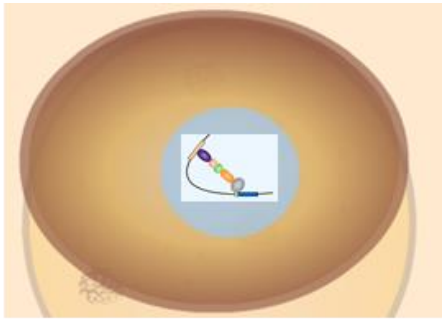


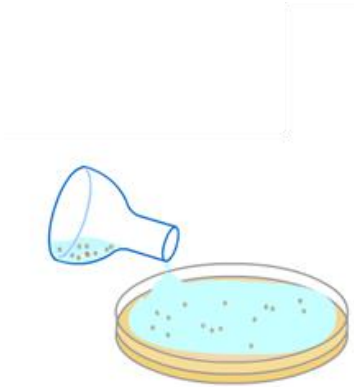


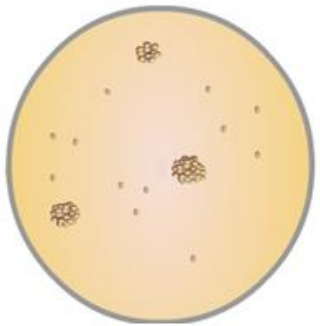


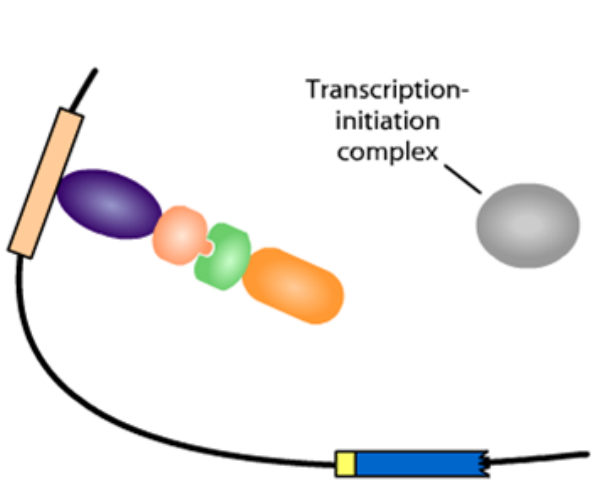
Yeast two-hybrid screening

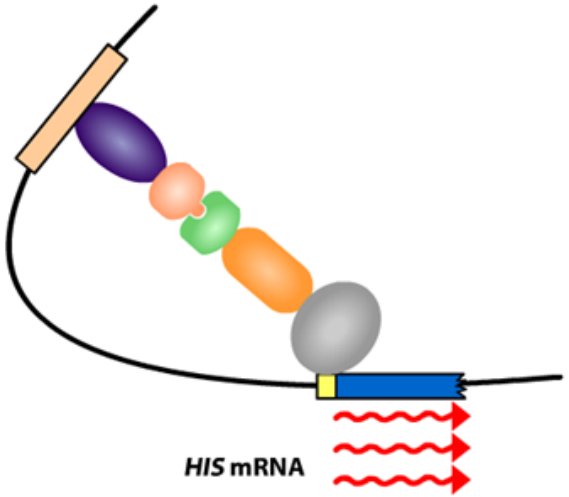




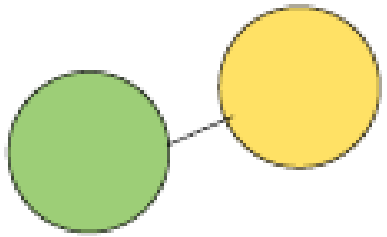








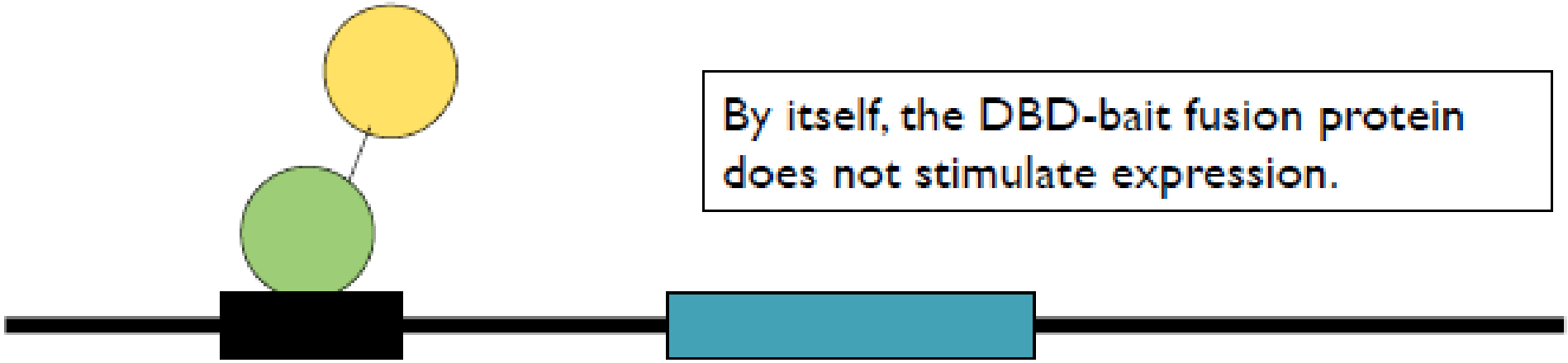
Yeast two-hybrid screening



DBD — Bait

DNA binding domain

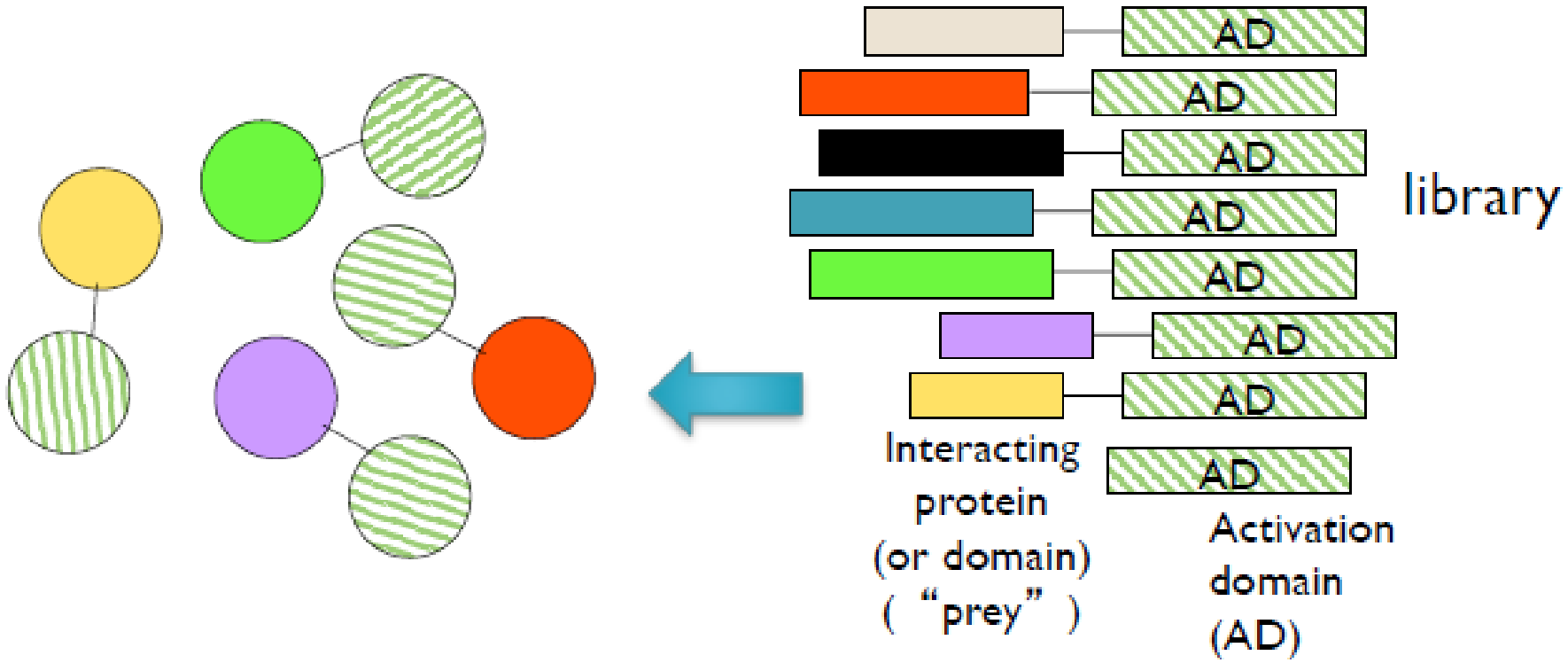
Protein (or domain) of interest ("bait")



Binding site

By itself, the DBD-bait fusion protein does not stimulate expression.

Yeast two-hybrid screening

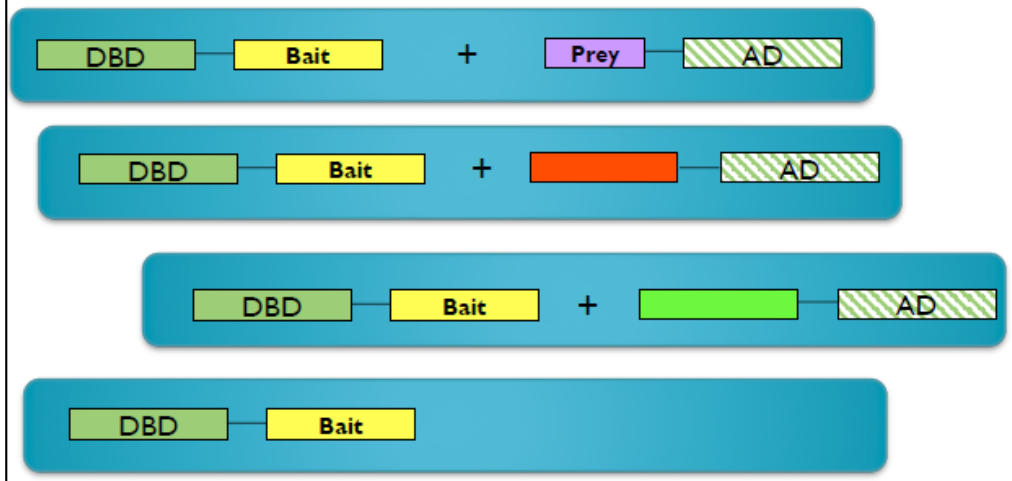


By itself, the AD-prey fusion protein does not stimulate expression.

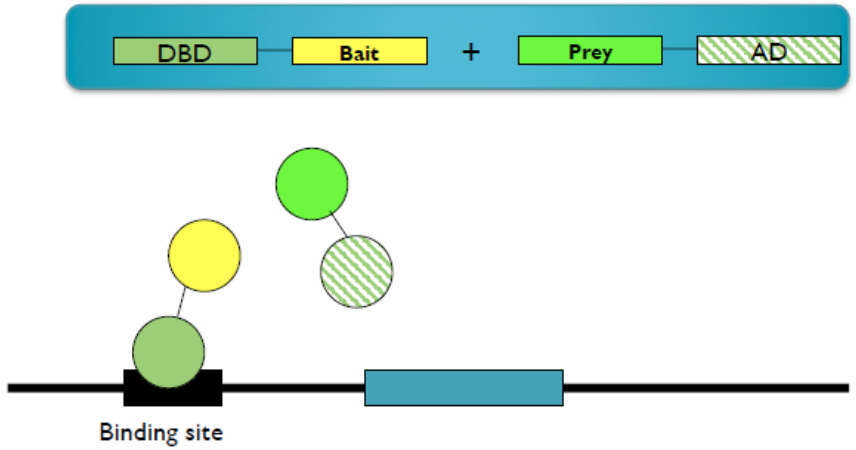
Yeast two-hybrid screening

Screening of the library

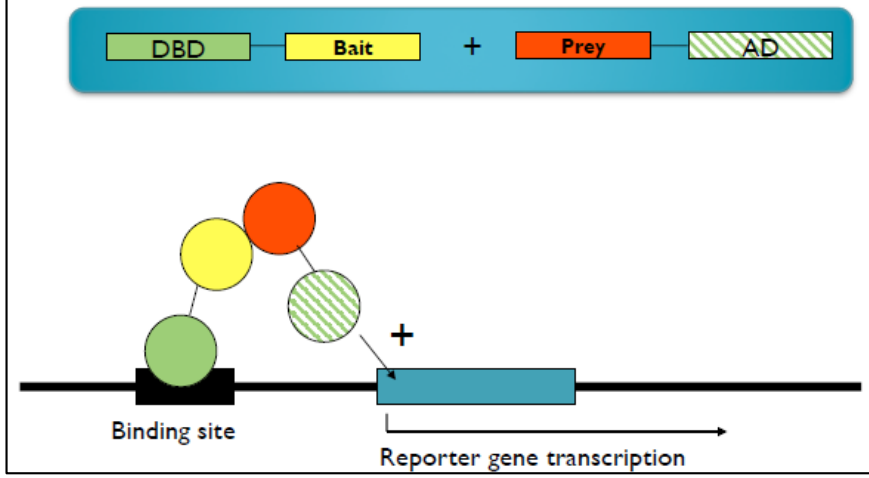
Pairs of bait- and prey-fusion proteins are coexpressed in yeast cells.



No transcription of the reporter gene: prey and bait do not interact

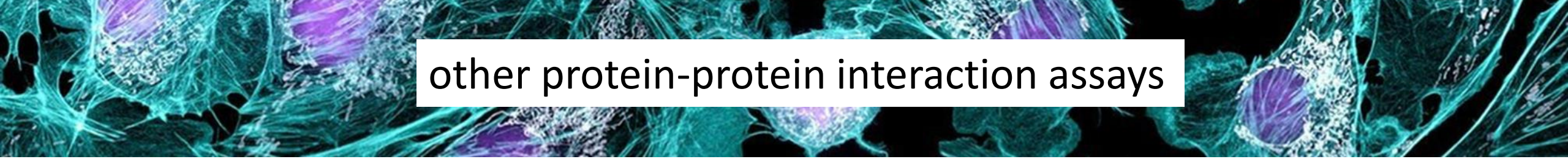


Transcription of the reporter gene: Prey and bait do interact



Limits of Y2H approach

- performing two-hybrid screens in yeast is a powerful method for identifying novel protein binding relationships that involve a particular protein of interest;
- however, the conformation of a mammalian protein expressed in yeast may be quite different from its normal conformation in a mammalian cells;
- it is important to perform a **protein-protein interaction assay** in mammalian cells to confirm that the suspected interactions also take place when the proteins are folded and modified as they would be in their native environment;
- the protein-protein interaction mammalian assay often reflects interactions between mammalian proteins with greater authenticity than can be achieved in yeast.



other protein-protein interaction assays

- co-IP
- Two-hybrid
- Split-TEV
- Fluorescence Resonance Energy Transfer (FRET)

Fragment complementation assays such as the yeast two-hybrid (Y2H) system and split-TEV are based on split proteins that are functionally reconstituted by fusions of interacting proteins.

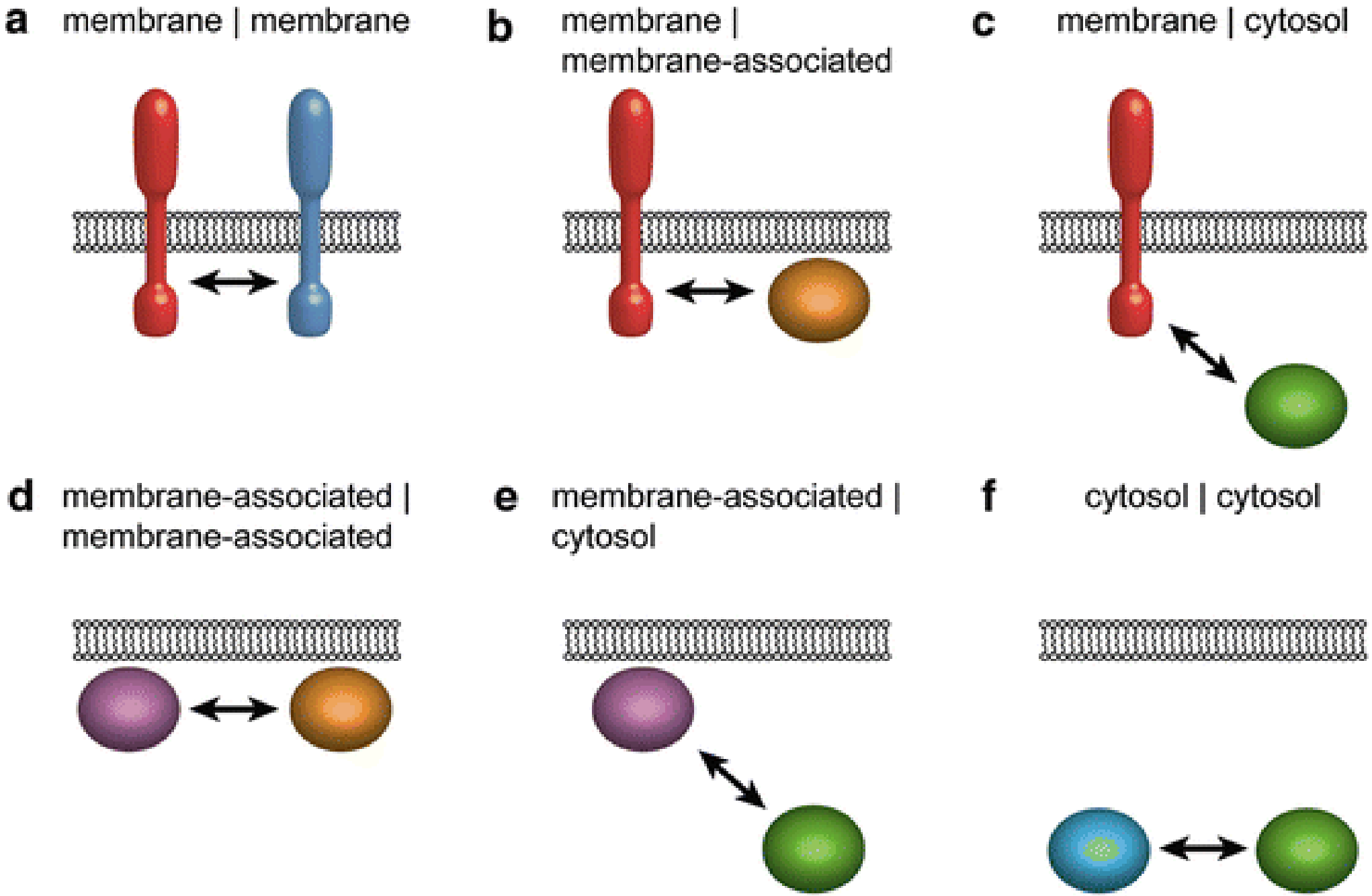
Monitoring regulated protein-protein interactions using split TEV

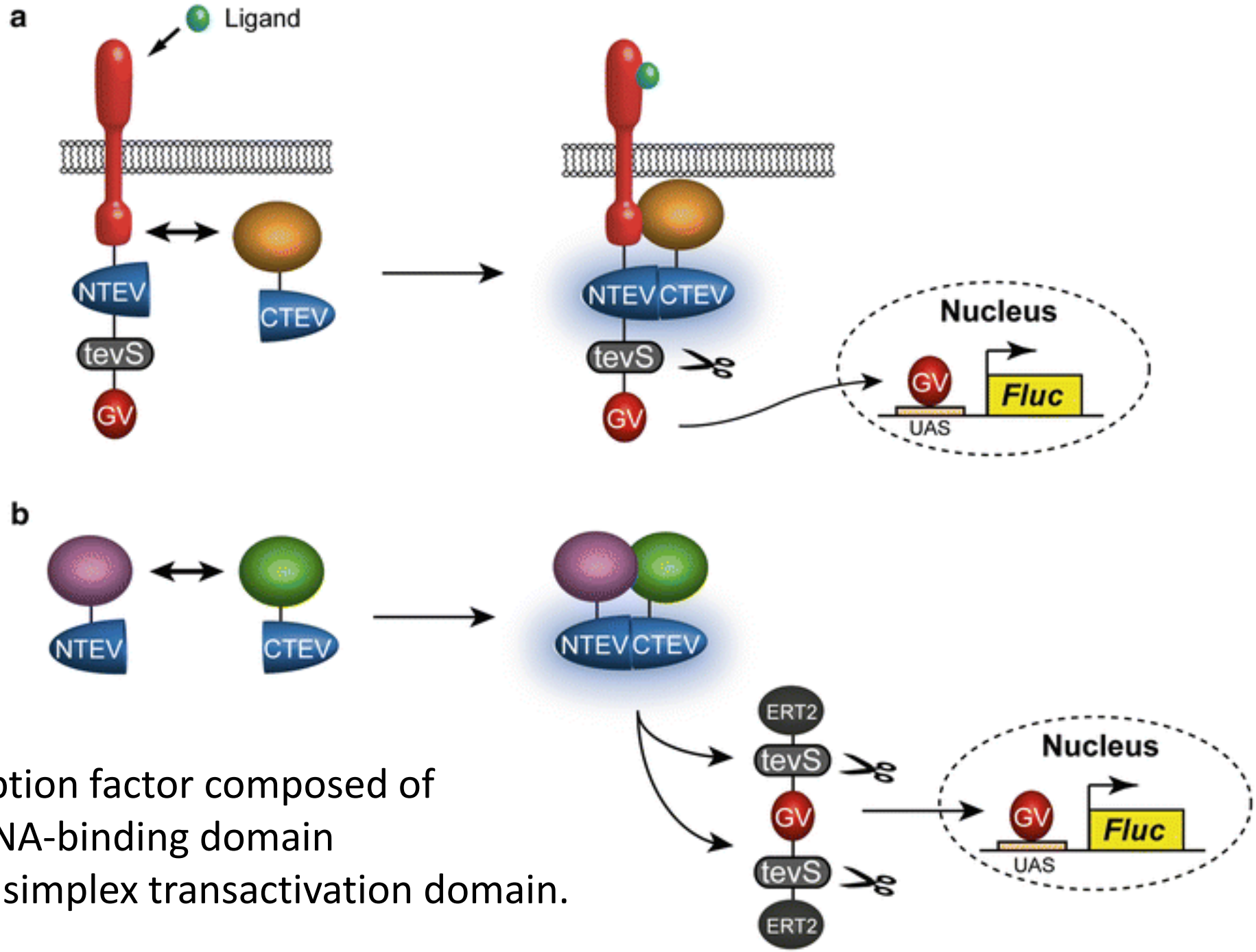
Genetically encoded split protein biosensor assays, such as the split TEV method, have proved to be highly valuable when studying regulated dynamic protein–protein interactions (PPIs) in living cells.

- based on the functional complementation of two previously inactive TEV protease fragments fused to interacting proteins and provides a robust, sensitive and flexible readout to monitor PPIs both at the membrane and in the cytosol.

- can be used to analyze interactomes of receptors, membrane-associated proteins, and cytosolic proteins.

- uses genetically encoded readouts, including standard reporters based on fluorescence and luminescence.


a
membrane | membrane
b
membrane |
membrane-associated
c
membrane | cytosol
d
membrane-associated |
membrane-associated
e
membrane-associated |
cytosol
f
cytosol | cytosol

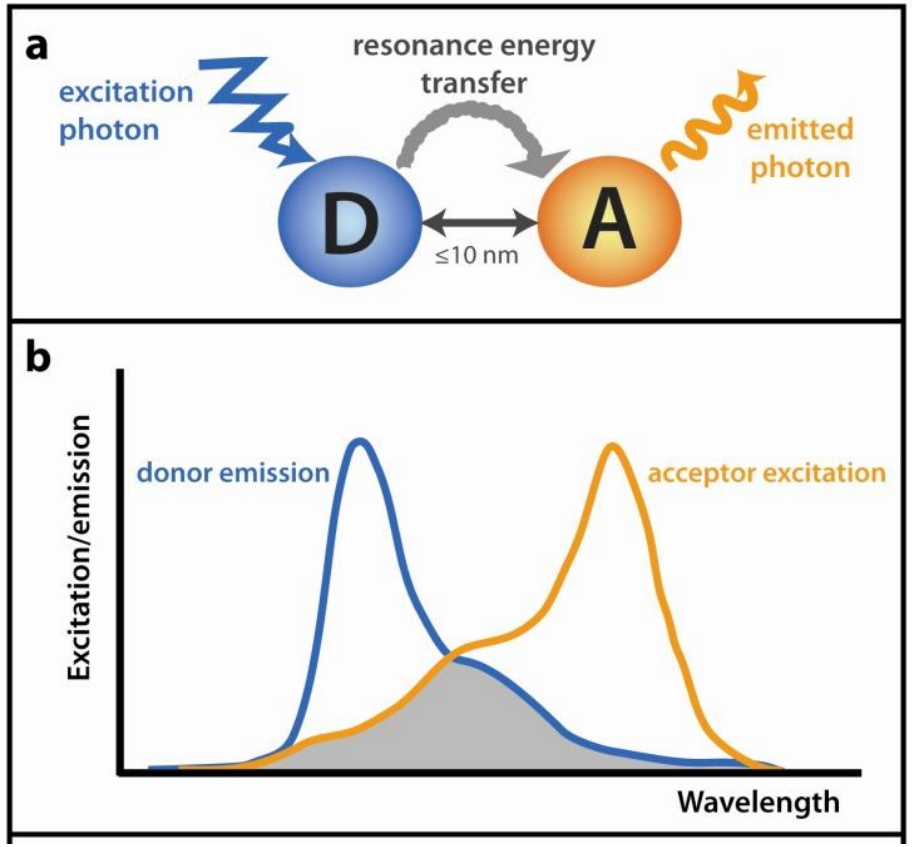


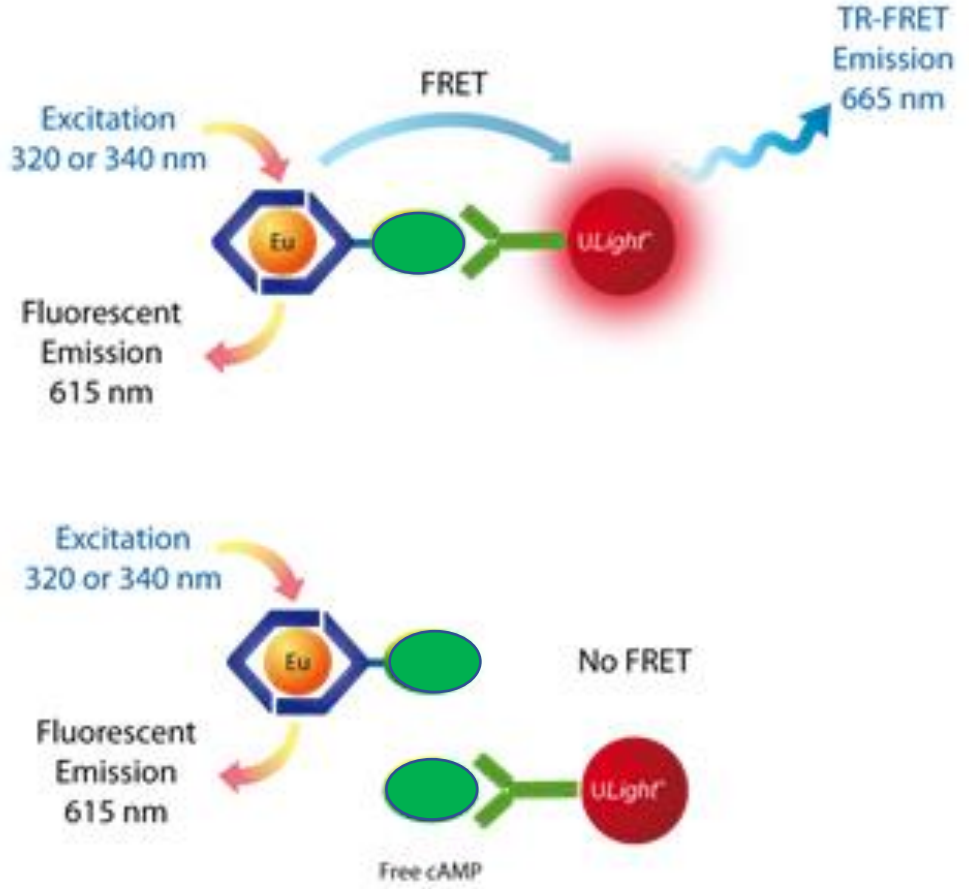
GV = a transcription factor composed of
Gal4: a yeast DNA-binding domain
VP16: a herpes simplex transactivation domain.

protein-protein interactions

- co-IP
- Two-hybrid
- Split-TEV
- Fluorescence Resonance Energy Transfer (FRET)

Fluorescence Resonance Energy Transfer (FRET)





Fluorescence resonance energy transfer (FRET) is a distance-dependent physical process by which energy is transferred from an excited molecular fluorophore (**the donor**) to another fluorophore (**the acceptor**) by means of intermolecular long-range dipole–dipole coupling.

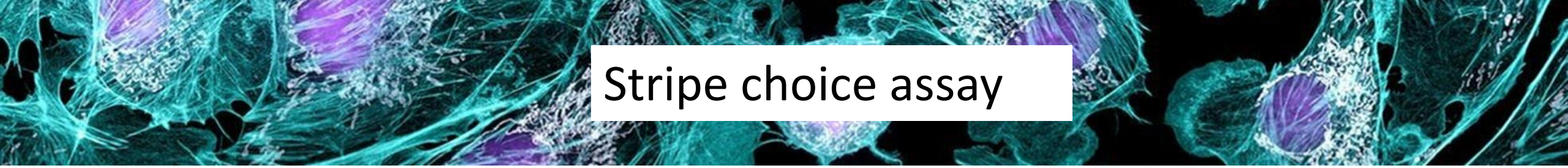
CELL-CELL COMMUNICATION

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

Stripe choice assay

Cells expressing the receptor are plated on alternating stripes expressing or not the ligand

Cells expressing the ligand are plated on alternating stripes expressing or not the receptor



Stripe choice assay

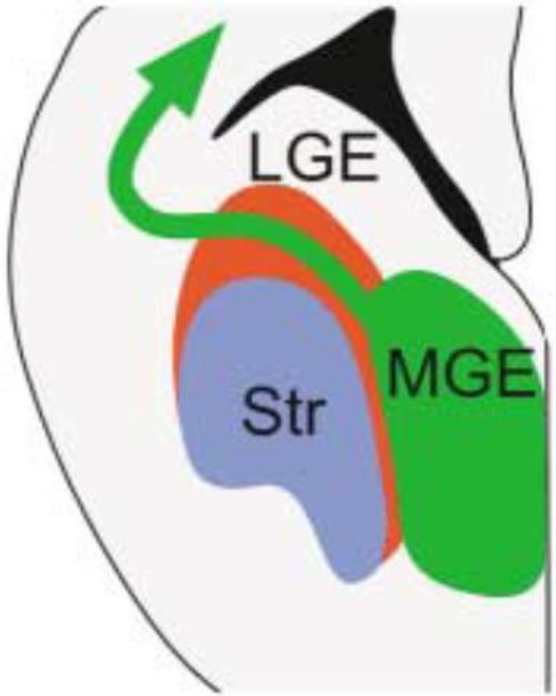
Materials and Methods

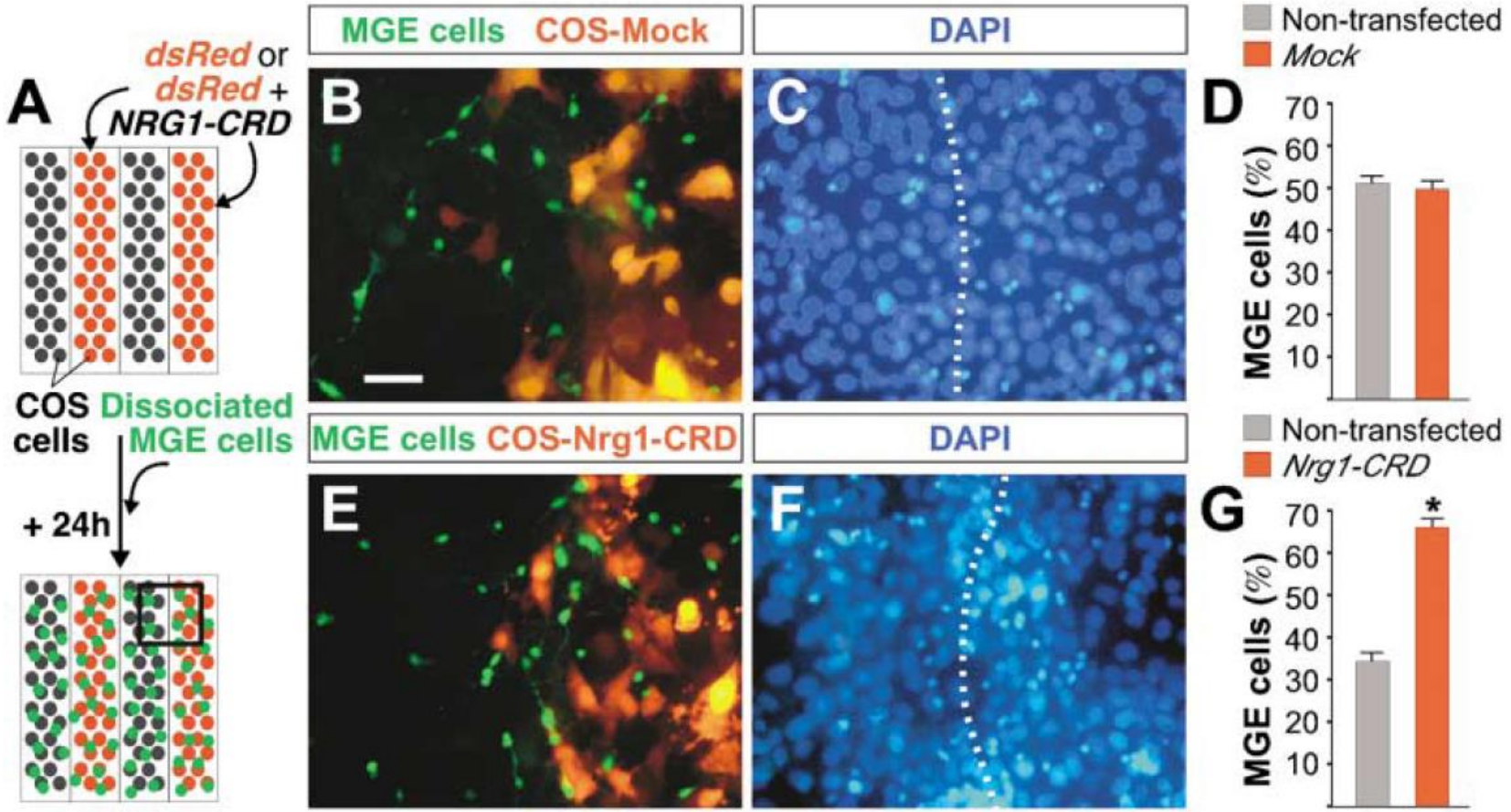
Stripe Choice Assay.
COS cells were plated in a two-well chamber slide (Lab-Tek Chamber Slide System Permanox Slide 177429), transfected at approximately 70% confluence, and incubated overnight.
To produce the stripes, transfected cells were removed with a pipette tip (one line every 2 mm), and non-transfected cells were plated on top (350.000 cells per well). Slides were subsequently incubated for 30 min to allow cell attachment to the empty stripes. The excess of cells was then washed out with three rinses of PBS, new media was added, and after 12 hr, GFP-expressing dissociated cells from the MGE were plated on top (10^5 cells per well). Analysis was performed after 24 hr.

(Flames *et al,* 2004)

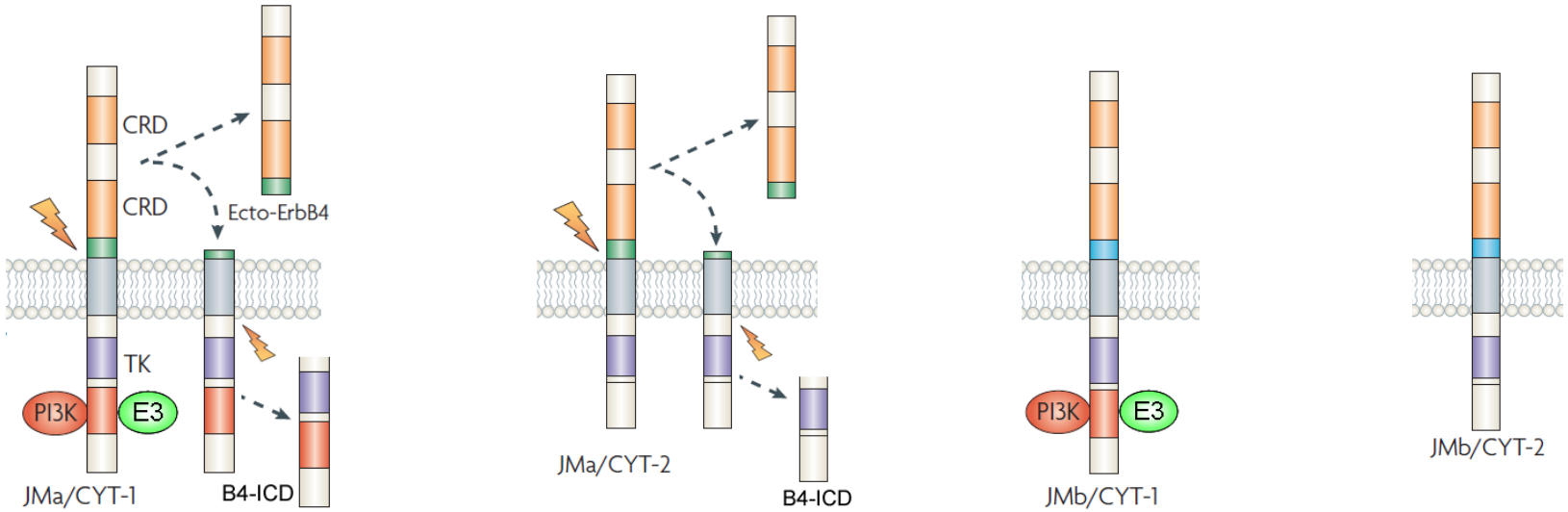
Short- and Long-Range Attraction of Cortical GABAergic Interneurons by Neuregulin-1

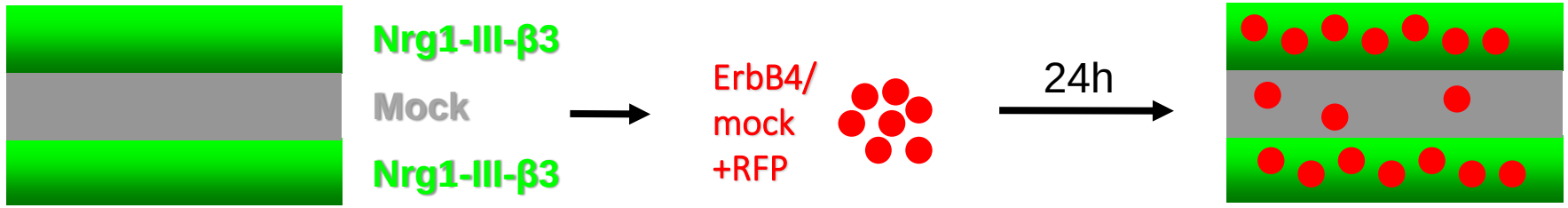
Nuria Flames,[1] Jason E. Long,[2]
Alistair N. Garratt,[3] Tobias M. Fischer,[4]
Martin Gassmann,[5] Carmen Birchmeier,[3] Cary Lai,[4]
John L.R. Rubenstein,[2] and Oscar Marín[1,*]



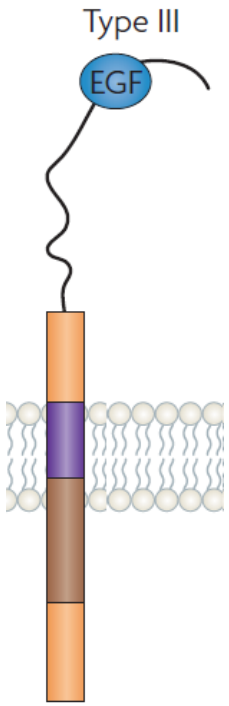


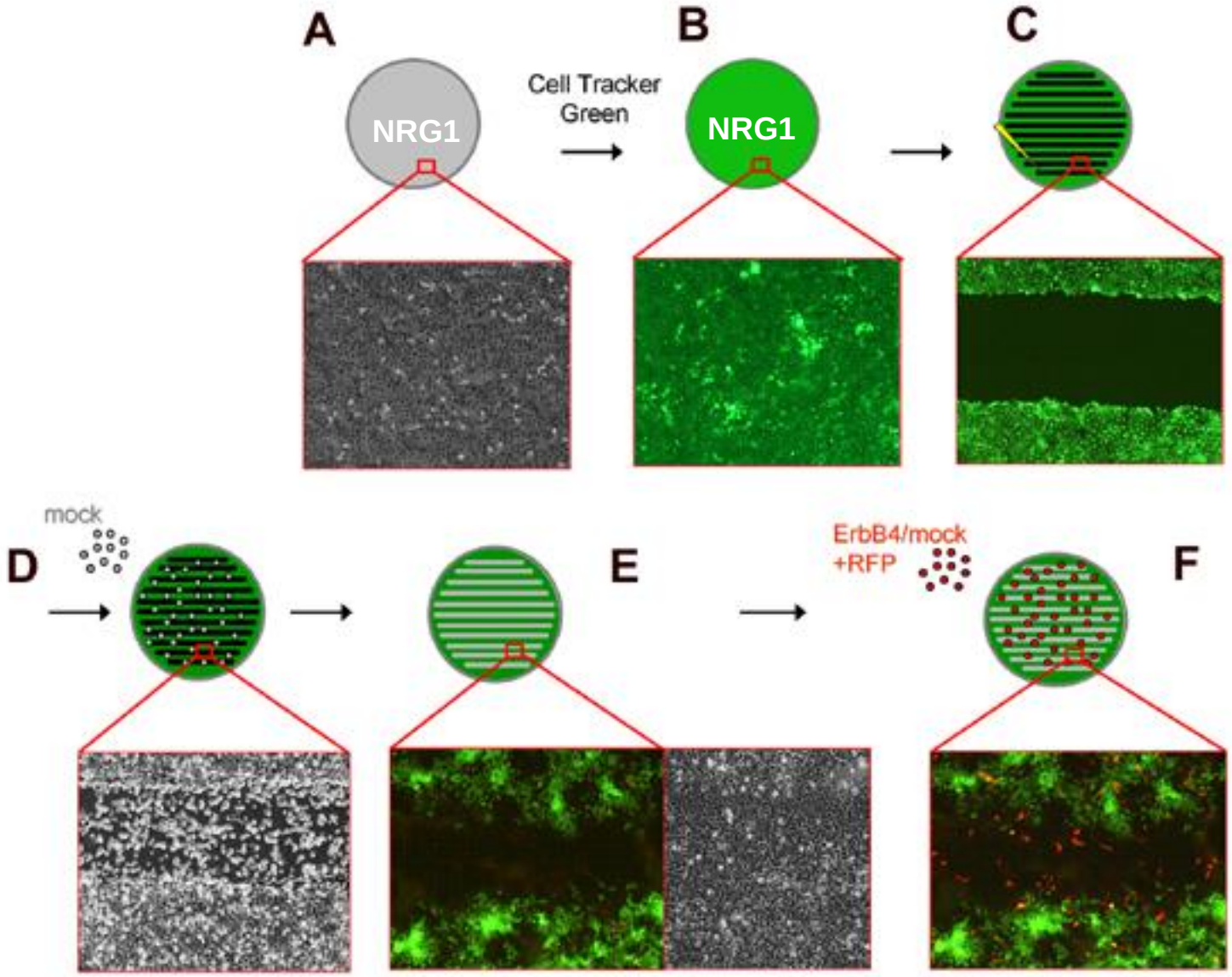
in vitro analysis of substrate preference mediated by different ErbB4 isoforms following interaction with transmembrane NRG1



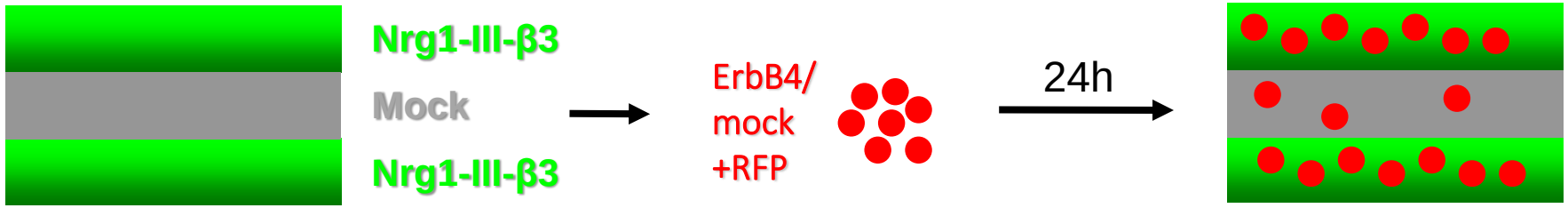


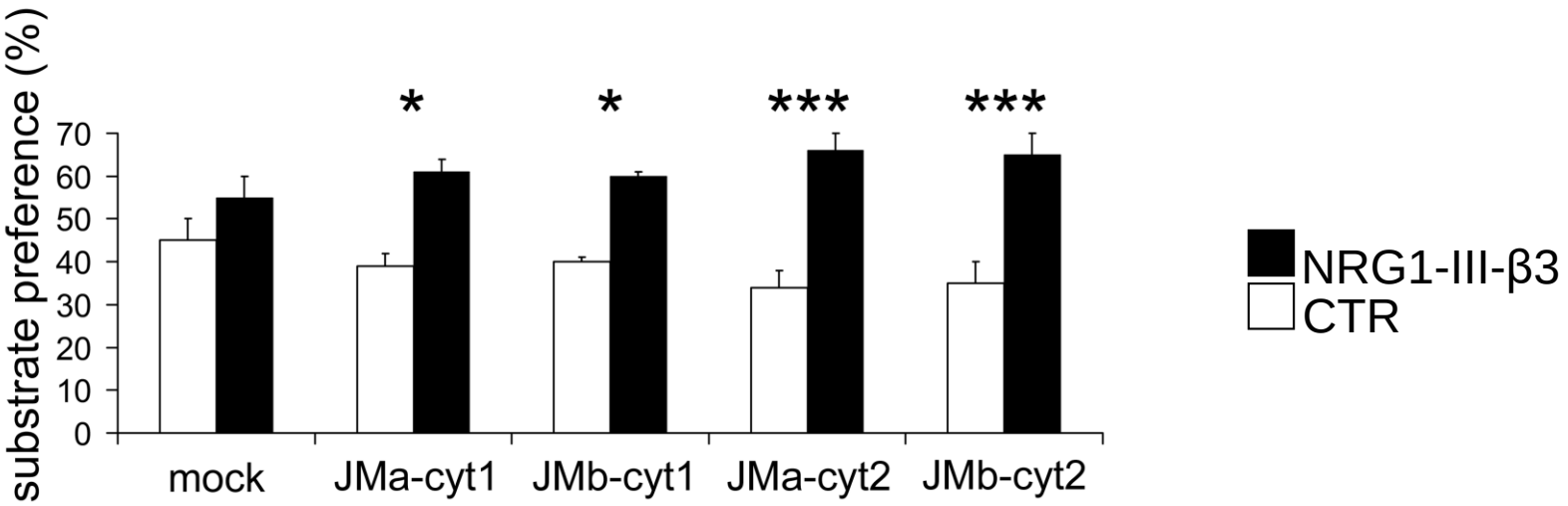
Stripe choice assay





Stripe choice assay





Stripe choice assay

Materials and Methods

The stripe choice assay was performed by partially modifying the protocol described by others. Briefly, ST14A cells stably expressing transmembrane NRG1-type III-β3 were plated in a 6 cm diameter plate, grown at ~95% confluence, and stained with 10 µM Cell Tracker Green (Life Technologies) in serum-free medium (Dulbecco's modified Eagle medium) for 40 min. To produce the empty stripes, green cells were removed with a pipette tip (one line every 2 mm, about 20 stripes/plate) and then washed twice with PBS to remove detached cells. 1.8 x 10^6 mock cells/well (stably transfected with the empty vector) were plated on top. Plates were incubated for 40 min to allow cell attachment to the empty stripes. The cell excess was washed out with three rinses of PBS, and then Dulbecco's modified Eagle medium containing 10% fetal bovine serum was added. On the same day, COS7 cells or ST14A neuronal progenitor cells were transiently cotransfected with 10 µg expression vector for one of the four ErbB4 isoforms (and mock transfected with the empty vector) and 1 µg pDsRed1-N1 (Clontech) to obtain RFP-expressing cells. Twenty-four hours later, cells in the stripes reached confluence; RFP-expressing cells (5 x 10^5 cells/plate) were plated on top of cell stripes (green=NRG1 expressing; uncolored=mock). Analysis was carried out 24 h later. Cells were fixed 40 min with 4% paraformaldehyde, and then washed with PBS and stored at 4°C in PBS with 0.02% sodium azide. For each plate, at least 15 stripes were photographed (…). In each photo, a green-labeled stripe and an uncolored stripe were included. Images were edited using Image Pro-Plus software (Media Cybernetics); for each photo, the green area and the uncolored area were measured, and the number of red cells on the green area and the number of red cells on the uncolored area were counted. For each clone expressing an ErbB4 isoform, the number of red cells/green area with the number of red cells/uncolored area was compared. At least three independent experiments (biological triplicate) were conducted.