Comm Sorts Robo to Control Axon Guidance at the *Drosophila* Midline

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How are Robo levels regulated?

• Robo protein expressed from transgenes is properly regulated, even if these transgenes lack the untranslated regions of the robo mRNA
  
  → this indicates that Robo levels are regulated posttranscriptionally, probably posttranslationally

• identification of the commissureless (comm) gene. As the name indicates, commissural axons do not cross the midline in comm mutants. This is due to excess robo function, since they do cross, along with ipsilateral axons, in robo comm double mutant embryos

• ipsilateral neurons and postcrossing commissural neurons express robo but not comm

• Robo levels are high in the growth cone, whereas crossing commissural neurons express both robo and comm, and Robo levels are low

• How does coexpression of Comm prevents Robo from accumulating in the growth cone?

• to address this question, they sought to mimic these two situations by expressing comm and robo alone or together in cultured cells
Confocal micrographs of COS-7 cells expressing Robo, Fra, or chimeric Fra-Robo and Robo-Fra proteins (green), in the presence (C, D, E, F) or absence (A) of Comm (red). (B) shows a cell expressing Comm alone.
To test for a physical association between Robo and Comm, lysates from cells expressing both proteins were immunoprecipitated with antibodies against either the HA tag on Robo or the myc tag on Comm, and then probed on Western blots with anti-myc.
- coimmunoprecipitation of wild-type Comm (top) and the mislocalized L229A, P230A mutant (bottom) with Robo and Robo-Fra, but not Fra or Fra-Robo

- the association between Robo and Comm does not require their colocalization in endosomes, as Robo and the Robo-Fra chimera also associate with a mutant form of Comm (L229A,P230A) that is not sorted to endosomes but instead delivered to the plasma membrane

Costaining of cells expressing Robo (H, K, N), Comm (I, L, O), or both (J, M, P) and:

• Giantin (H, I, J) to label Golgi
• mannose-6-phosphate receptor (MPR) (K, L, M) to label Golgi and late endosome
• fluorescently labeled BSA internalized for 3.5 hr to label the entire endocytic pathway (N, O, P).
Comm could recruit Robo to endosomes either
1. by stimulating its endocytosis from the plasma membrane or
2. by sorting it directly from the trans-Golgi network

- if an appreciable fraction of Robo were trafficked via the plasma membrane in cells that coexpress Comm, then it should be possible to label Robo at the cell surface using antibodies against its extracellular HA tag and to observe the subsequent internalization of these anti-HA antibodies

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Surface labeling of cells expressing HA-Robo-V5 or HA-Fra with anti-HA, followed by 45 min chase and total protein labelling with anti-Robo or anti Fra

→ COMM DOES NOT RECRUIT ROBO TO ENDOSOMES VIA THE CELL SURFACE
(A and B) *comm* expression is the switch that controls midline crossing. In an ipsilateral neuron, *comm* is OFF: growth cone carries high levels of Robo protein and is repelled by Slit at the midline.

In a commissural neuron, *comm* is initially ON (A), keeping Robo levels low to allow crossing. Once the commissural growth cone reaches the other side, *comm* is turned OFF in order to increase Robo levels and prevent recrossing (B).

(C) Comm regulates Robo trafficking. If *comm* is OFF, Robo is packaged into vesicles delivered to the growth cone. If *comm* is ON, most (but not all) Robo is instead sorted by Comm into vesicles bound for late endosomes and lysosomes, where both Robo and Comm are degraded. Vesicles travelling to the growth cone thus contain very little Robo protein, and insertion of these vesicles at the axon tip allows it to extend across the midline.
• the secreted protein Slit repels neuronal precursors migrating from the anterior subventricular zone in the telencephalon to the olfactory bulb

• their results provide a direct demonstration of a molecular cue whose concentration gradient guides the direction of migrating neurons

• they also support a common guidance mechanism for axon projection and neuronal migration and suggest that Slit may provide a molecular tool with potential therapeutic applications in controlling and directing cell migration
Model of the quantitative and qualitative differences in the effects of attractants, repellents, inhibitors, and inducers on migration from SVZ explants.

The top colored circles represent aggregates of cells secreting putative regulators of migration. The small blue circles represent SVZ explants, and the larger light-blue circles represent the migrating cells. The size of the circle signifies the number of cells; their location relative to the inner circle shows the preferred direction of migration. The orange line shows the separation between the distal and proximal hemispheres; the dashed lines facilitate the comparison between control and experimental points. The triangle on the right depicts an expected concentration gradient of the molecules.

Repulsion of SVZa neurons by the septum and the floorplate and expression of slit genes in the postnatal septum.

a. Chains of cells migrate out of SVZa explants symmetrically when cultured alone in matrigel. In the presence of the septum (b) or floorplate (c), there are more migrating cells in the quadrant of the SVZa explant distal to the septum than in the proximal quadrant. d. the septum is repulsive to SVZa cells in collagen gel. e. A coronal section showing expression of Slit-1 in the septum and the neocortex of postnatal day 3 rats. f. A coronal section showing expression of Slit-2 in the septum, the choroid plexus and the neocortex of P3 rats. g. A sagittal section showing expression of Slit-1 in the septum and the neocortex of P3 rats. Rostral is to the left and dorsal is up.
Effect of Slit on neurons migrating from SVZa explants.

+ control HEK cells aggregates

+ HEK cells aggregates expressing xSlit

+ HEK cells aggregates expressing mSlit-1

Quantification of the effect of Slit on migrating SVZa neurons
Either Slit repels neurons, or Slit inhibits migration

To distinguish between them, two SVZa explants were placed at different distances from a single aggregate of Slit cells.

In both explants there were more cells in the distal quadrants.

→ these results are best explained by a repulsive activity of Slit, rather than inhibition of migration by Slit.

Do SVZa cells respond to the absolute concentration of Slit or to a concentration gradient of the protein?

SVZa explants were placed between two aggregates of Slit cells.

e. When an SVZa explant was placed at unequal distances from two Slit aggregates, neurons migrated away from the closer Slit aggregate.

f. When SVZa explants were placed at an equal distance from two Slit aggregates, the cells migrated out symmetrically.

→ SVZa cells respond to a concentration gradient of the Slit protein
semaphorins and their receptors are known signals for:

- axon guidance
- cell migration
- morphogenesis
- immune function
- tumor progression

- cell guidance control by semaphorins requires plexins

- the functional receptor for secreted semaphorins is a complex including neuropilins and plexins (Tamagnone, Takahashi, Rohm, 1999)

- semaphorins have multiple functions in morphogenesis and tissue remodeling by mediating cell-repelling cues through plexin receptors
Class 1 and 2 contain transmembrane and secreted semaphorins from invertebrates, respectively. Secreted semaphorins of vertebrates fall into class 3. The other vertebrate semaphorins are membrane bound, either transmembrane (class 4, 5, and 6) or GPI anchored (class 7). The Sema domain is the hallmark of this protein family, and it includes a Met-related sequence (MRS motif). Other conserved domains are immunoglobulin domains (in classes 2, 3, 4, and 7), domains rich in basic amino acids (class 3), and thrombospondin repeats (class 5).

Nine human plexins are currently known, which fall into four homology groups (A, B, C, and D subfamilies), based on sequence similarity, structural features, and tissue distribution. All plexins include a conserved cytoplasmic domain (sex-plexin domain, SP, blue) and a Sema domain (yellow). Repeated MRS motifs (orange) are found in the extracellular domain. Plexin-B subfamily members include potential cleavage sites for furin-like convertases (also found in plexin-B of *Drosophila*, here marked in red). The prototype member of the plexin-C subfamily (Vespr) is shorter, including only two MRS motifs. Plexin-D1 includes an atypical sequence in its third MRS (stippled).
Evolution of semaphorins and their receptors

Secreted (subclass 3) semaphorins are dimeric and bind to receptor complexes that include neuropilins (homo- or heterodimeric) and plexins (probably dimeric). Plexins set the semaphorin-binding specificity and mediate the intracellular signalling; neuropilin co-receptors are required for binding. Membrane-bound semaphorins bind directly to plexins and transduce intracellular signals through the sex-plexin (SP) domain. Transmembrane semaphorins might generate bidirectional signals by associating with cytoplasmic transducers (black box).
SEMAPHORINS, PLEXINS AND SF RECEPTORS

- plexins were originally identified through their homology to the extracellular domain of scatter factor receptors

- plexins function as semaphorin receptors, alone or in a complex with neuropilins (Tamagnone et al.)

- semaphorins, the plexin family (semaphorin receptors) and scatter factor receptors have in common a role in mediating cell guidance cues and share evolutionarily conserved protein modules:
  - the semaphorin domain (about 500 amino acids)
  - Met Related Sequences (MRS, about 80 amino acids)

- during development, scatter factor receptors control cell migration, epithelial tubulogenesis and neurite extension

- scatter factors and secreted semaphorins are diffusible ligands, whereas membrane-bound semaphorins signal by cell-cell interaction

In the extracellular domain, a Sema domain (yellow) and a Met-related sequence (MRS, orange) are found. The intracellular tyrosine kinase domain is autophosphorylated on several residues (conserved among the family members), two of which (Y1234 and Y1235 in Met) have an autoactivatory role on the catalytic activity, whereas phosphorylated Y1349 and Y1356 form a multifunctional docking site for signal transducers. Overall percentage homology of Ron and Sea to Met is indicated at the bottom. The ligand for Sea has not been identified as yet.
in epithelial cells (MLP29, liver progenitor cells), Semaphorin 4D (Sema 4D) triggers invasive growth, a complex programme that includes cell–cell dissociation, anchorage-independent growth and branching morphogenesis.

- the same response is also controlled by scatter factors through their tyrosine kinase receptors, which share striking structural homology with plexins in their extracellular domain.

- branching morphogenesis was considered to be a unique feature of scatter factor receptor function, as it cannot be mediated by other tyrosine kinases

they examined if Sema 4D activates the invasive growth programme by coupling Plexin B1 to the scatter factor receptor machinery.
in cells expressing the endogenous proteins, Plexin B1 (the Sema 4D Receptor) and Met (the Scatter Factor 1/ Hepatocyte Growth Factor Receptor) associate in a complex.

- binding of Sema 4D to Plexin B1 stimulates the tyrosine kinase activity of Met, resulting in tyrosine phosphorylation of both receptors.
- cells lacking Met expression do not respond to Sema 4D unless exogenous Met is expressed.

This work identifies a novel biological function of semaphorins and suggests the involvement of an unexpected signalling mechanism: the coupling of a plexin to a tyrosine kinase receptor.

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Binding of Sema4D to the Plexin B1-Met receptor complex stimulates the tyrosine kinase activity of Met.

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invasive growth