MECHANISMS AND FUNCTIONS OF EPH AND EPHRIN SIGNALLING

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Eph receptors constitute the largest family of tyrosine kinase receptors and, together with their plasma-membrane-bound ephrin ligands, have many important functions during development and adulthood. In contrast with most receptor tyrosine kinases, unidirectional signalling can originate from the ephrin ligands as well as from the Eph receptors. Furthermore, the concept of bidirectional signalling has emerged as an important mechanism by which Ephs and ephrins control the output signal in processes of cell–cell communication.

GPI ANCHOR
The function of this post-translational modification is to attach proteins to the exoplasmic leaflet of membranes, and possibly to specific domains therein. The anchor is made of one molecule of phosphatidylinositol to which a carbohydrate chain is linked through the C-6 hydroxyl of the inositol, and is linked to the protein through an ethanolamine phosphate moiety.

FIBRONECTIN TYPE III REPEAT
A 90-amino-acid-long stretch that is repeated 15–17 times in the fibronectin molecule. It is a common motif in many cell-surface proteins.

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In numerous processes that are vital for the development and maintenance of organism function, cells must communicate crucial information to respond appropriately to the changing environment. As such, receptor tyrosine kinases (RTKs) are transmembrane proteins, which, on receiving an external stimulus, respond by transmitting a signal to the inside of the cell. Of all the RTKs that are found in the human genome, the Eph-receptor family — which has 13 members — constitutes the largest family (BOX 1). Their ligands, the ephrins, are divided into two subclasses, the A-subclass (ephrinA1–ephrinA5), which are tethered to the cell membrane by a glycosylphosphatidylinositol (GPI) ANCHOR, and the B-subclass (ephrinB1–ephrinB3), members of which have a transmembrane domain that is followed by a short cytoplasmic region (FIG. 1).

The receptors are divided on the basis of sequence similarity and ligand affinity into an A-subclass, which contains eight members (EphA1–EphA8), and a B-subclass, which in mammals contains five members (EphB1–EphB4, EphB6; BOX 1). A-type receptors typically bind to most or all A-type ligands, and B-type receptors bind to most or all B-type ligands, with the exception of EphA4, which can bind both A-type and most B-type ligands.

The Eph-receptor extracellular domain is composed of the ligand-binding globular domain, a cysteine-rich region and two FIBRONECTIN TYPE III REPEATS (FIG. 1).

The cytoplasmic part of Eph receptors can be divided into four functional units; the juxtamembrane domain that contains two conserved tyrosine residues, a classical protein tyrosine kinase domain, a STERILE α-MOTIF (SAM) and a PDZ-DOMAIN binding motif. The solved structure of the SAM domain (~70 amino acids) indicates that it could form dimers and oligomers. The PDZ-binding motif — located in the carboxy-terminal 4–5 amino-acid residues — contains a consensus binding sequence that includes a hydrophobic residue (usually valine or isoleucine) at the very carboxyl terminus.

So far, several biological functions have been attributed to Eph receptors and ephrins, including vascular development, tissue-border formation, cell migration, axon guidance and SYNAPTIC PLASTICITY. Eph receptors and ephrin ligands are both membrane bound and, therefore, binding and activation of Eph and ephrins requires cell–cell interactions. As we shall see, the influence of Eph/ephrin activation on cell behaviour differs depending on the cell type, but can generally be attributed to repulsion of neighbouring cells or of cellular processes, such as a neuronal growth cone. In a few cases Eph/ephrin activation can lead to increased adhesion/attraction. In molecular terms, many of the signalling pathways that are downstream of Eph and ephrins converge to regulate the cytoskeleton.

Functional analysis of the Eph–ephrin system is challenging for at least two reasons. First, Eph receptors can have overlapping functions in vertebrates, and loss of one receptor can be partially compensated for by another Eph receptor that has a similar expression pattern and ligand-binding specificities (BOX 2). Second, an Eph receptor can

Box 1 | **Ephs and ephrins in the genome**

With the recent release of the human and mouse genome databases, it is now possible to examine the number and localization of *Eph* and *ephrin* genes. Three clusters of receptor genes can be found on human chromosome 1 (*EphA2*, *EphA8* and *EphB2*), chromosome 3 (*EphA3*, *EphA6*, *EphB1* and *EphB3*) and on chromosome 7 (*EphA1*, *EphB4* and *EphB6*). Similar clusters, albeit on different chromosomes, are also found in the mouse genome. The gene that encodes the receptor EphB5, which has been found in chicken[86], has not been found in the available human or mouse genome databases, and thereby probably reduces the number of Eph receptors to 13 in mammals.

Similarly, *ephrinA1*, *ephrinA3* and *ephrinA4* are found in a cluster on human chromosome 1 and on mouse chromosome 3. The organization in the genome strongly implies that this family of receptors and ligands has developed through rather recent gene duplications, which might also explain the high degree of functional overlap between family members (BOX 2). This is further emphasized by the lower number of *Eph* and *ephrin* genes that are found in distant organisms such as the fruitfly, which has one *Eph* and one *ephrin* gene[87,88], and the nematode *Caenorhabditis elegans*, which has one *Eph* and four *ephrin* genes[56,62,89].

also act as a ligand in the same way that an ephrin ligand can act as a receptor[1]. As for many other RTKs, ligand binding induces so-called 'forward signalling', mostly through phosphotyrosine-mediated pathways. But ephrins can also signal into their host cell — referred to as 'reverse signalling'. Here, the ephrin cytoplasmic tail can be phosphorylated and thereby recruit signalling effectors[2,3].

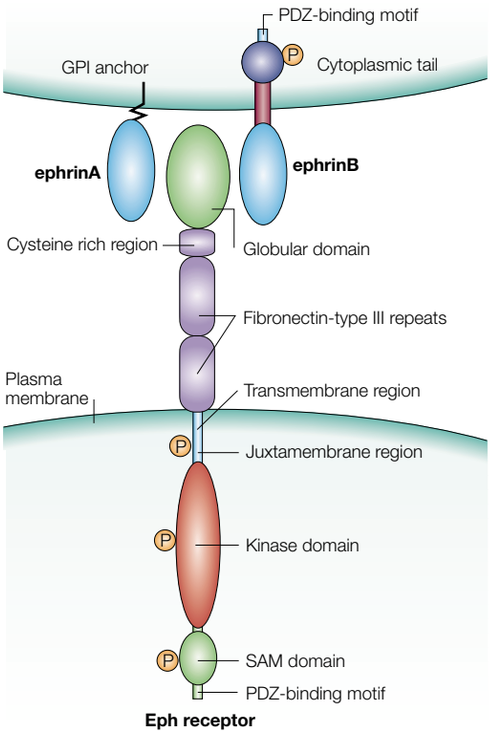

Figure 1 | **General features of Eph receptors and ephrins.** A schematic diagram, which shows an ephrin-expressing cell (top) interacting with Eph-expressing cell (bottom). Ligand–receptor interactions (green) have been mapped in detail and have uncovered dimeric and oligomeric Eph–ephrin complexes (not shown)[113–116]. GPI, glycosylphosphatidylinositol; SAM, sterile α-motif.

STERILE α-MOTIF (SAM). A domain of ~70 amino acids that is roughly conserved in many proteins and thought to participate in protein–protein interactions.

PDZ-DOMAIN (PSD-95, Dlg and ZO-1/2). A protein–protein interaction domain of around 90 amino acids that binds particularly to carboxy-terminal polypeptides.

SYNAPTIC PLASTICITY A change in the functional properties of a synapse as a result of use.

AUTOPHOSPHORYLATION The phosphorylation by a protein of one of its own residues.

SH2 DOMAIN (Src-homology-2 domain). A protein motif that recognizes and binds tyrosine-phosphorylated sequences, and thereby has a key role in relaying cascades of signal transduction.

SH3 DOMAIN (Src-homology-3 domain). A protein sequence of around 50 amino acids that recognizes and binds sequences that are rich in proline.

Guided by the concepts of reverse signalling and redundancy, we review recent data that examine mechanisms and functions of Eph and ephrin signalling.

Mechanisms of Eph-receptor forward signalling

Eph-receptor activation and downstream signalling. In contrast to most other RTKs, Eph receptors are activated when they are bound by clustered, membrane-attached ephrin ligands. This requirement for membrane clustering of the ligand indicates that contact between cells that express Eph receptors and cells that express ephrins is needed for Eph-receptor activation. Experiments have shown that membrane clustering can be mimicked by clustering tagged soluble ligands[4].

On ligand engagement, each member of the receptor dimer AUTOPHOSPHORYLATES several tyrosine residues that are located in the intracellular part of the partner receptor[5]. Autophosphorylation of juxtamembrane tyrosine residues is required for full activation of the protein tyrosine kinase domain of the receptor[6–8]. The reason for this autoregulation became apparent when the X-ray crystal structure of the EphB2 cytoplasmic region was solved[9]. The structure that was used included the entire kinase domain, together with the juxtamembrane domain, but the crucial juxtamembrane tyrosine residues had been mutated to phenylalanine residues to prevent phosphorylation of these residues. The data showed that the mutation represses catalytic activity, because interactions between the unphosphorylated juxtamembrane and kinase domains prevent proper alignment of segments in the kinase domain (FIG. 2). But when juxtamembrane tyrosine residues are phosphorylated, the juxtamembrane domain is released from the interaction with the kinase domain, which allows the kinase domain to convert into its active state. This release also allows phosphotyrosine-binding proteins to bind to the phosphorylated juxtamembrane domain.

Although it is not yet understood how the binding of ephrin ligand releases the repressed conformation of Eph receptors, these findings implicate the juxtamembrane domain as an important autoregulator of Eph-receptor tyrosine kinase activity. This regulatory mechanism has also been proposed for other RTKs, such as the platelet-derived growth factor (PDGF) receptor β[10] and the TrkB receptor[11].

Once the receptor is activated, adaptor molecules associate with it to transmit signals into the cell (FIG. 3). Adaptor proteins are a growing class of proteins that contain functional protein-interaction domains, such as SRC-HOMOLOGY-2 (SH2) and SH3 DOMAINS, and often lack intrinsic enzymatic function. They have a crucial role in the formation of protein complexes, and connect signalling molecules to upstream and downstream signalling events[12]. The past years have brought many suggestions as to which adaptors might be downstream of Eph receptors (TABLE 1); however, few of them have been shown to be of functional relevance.

Cytoskeletal regulation. In the context of cell movement, Eph receptors have long been thought to mediate effects on the cytoskeleton. The Abelson (Abl)

Box 2 | **The challenge of functional redundancy and genetic background**

The functional consequences of Eph-receptor redundancy are well described by studies in which the functions of EphB2 and EphB3 were examined by targeted deletion of these molecules in mice. Although mice that are singly deficient for EphB2 or EphB3 are viable, most *EphB2–EphB3* double mutants die at birth due to a developmental defect in the upper mouth called cleft palate, which prevents proper food intake[90]. Similarly, during retinal ganglion-cell axon guidance to the optic disc, mouse embryos that lack both EphB2 and EphB3 show increased frequency of axon guidance errors, which could not be detected in the single mutants[60]. Genetic background also has an important role for the discovery and penetrance of phenotypes in mice. By shifting mice with an *EphB2*-targeted deletion from 129 to CD1 genetic background, increased survival rates and a circling behaviour was shown in 61% of the cases. Mice that lack both EphB2 and EphB3 receptors in the CD1 background all showed a circling behaviour, which indicates that these receptors might also be redundant in the CD1 genetic background[71].

EphB2 and EphB3 bind the same set of ligands (ephrinB1–ephrinB3) and have overlapping expression patterns, which explains how these receptors can share functionality. This has methodological consequences when exploring functions of the Eph–ephrin system. As outlined in the main text, in experiments in which targeted inactivation of genes in mice are used, functions that are redundant or dependent on the genetic background are easily overlooked. Also, results from experiments that aim to disrupt receptor–ligand interactions are quite nonspecific and difficult to interpret, as several receptors and ligands can be involved.

family of non-cytoplasmic tyrosine kinases has been implicated in the organization of the actin cytoskeleton in the developing nervous system, as well as in other tissues (reviewed in REF. 13). A new signalling connection that links regions of Abl and the Abl-related gene (Arg) to the EphB2 and EphA4 receptors was found in a yeast two-hybrid screen[14]. The association involves several protein interactions. First, the SH2 domains of Abl and Arg bind to tyrosine-phosphorylated motifs in the juxtamembrane region of EphB2. Second, a phosphorylation-independent interaction between EphB2 and sequences in the carboxy-terminal tails of Abl and Arg occurs. Finally, an interaction between Abl and EphB2 is probably also mediated by an unidentified protein. This intermediary protein could turn out to be a Src family kinase (SFK), as these are known to regulate Abl kinase activity[15] and are well-characterized interactors with Eph receptors[16]. Activation of Abl kinases through stimulation with PDGF increases Abl kinase activity[15]. By contrast, treatment of a neuronal cell line with ephrinB1 to activate endogenous EphB2 decreased the kinase activity of endogenous Abl[14]. Abl and Arg have actin binding motifs in their carboxy-terminal tails, and Abl and Ephs are localized in the neuronal growth cone, which supports a role for Abl in regulating the actin cytoskeleton during Eph-mediated axon pathfinding. Mouse embryos that lack Abl and Arg show defects in NEURAL-TUBE closure[17] that are similar to those seen in *ephrinA5*-knockout mice[18], which might indicate a link between Abl/Arg-mediated signalling and ephrinA5–EphA-receptor signalling.

Small RHO FAMILY GTPASES are important regulators of the actin cytoskeleton[19] and are implicated in the regulation of neuronal morphogenesis (reviewed in REF. 20). The three best-characterized members of the Rho family are Rho, Rac and Cdc42, and these GTPases act as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state. In neuroblastoma growth cones, activated RhoA promotes GROWTH-CONE collapse, whereas activated Cdc42 and Rac1 promote the extension of FILOPODIA and LAMELLIPODIA, respectively[21].

The identification of Ephexin (Eph-interacting exchange protein), a novel guanine nucleotide exchange factor for Rho GTPases, provided a further mechanistic and functional link between EphA receptors and the cytoskeleton[22]. Like many other Eph-receptor interacting proteins, this effector molecule was found in a yeast two-hybrid screen, using the cytoplasmic region of EphA4 as bait. Mutational analysis showed that the carboxy-terminal lobe of the EphA4 kinase domain interacts directly with the tandem Dbl-homology–pleckstrin-homology (DH–PH) region of Ephexin. Transfection of a DOMINANT-NEGATIVE mutant version of Ephexin into RETINAL GANGLION CELLS inhibited ephrinA-induced growth-cone collapse, which implicates Ephexin as an important mediator of growth-cone dynamics and neurite repulsion. So how does Ephexin work? In cell-culture experiments, Ephexin has differential effects on Rho GTPases, such that RhoA is

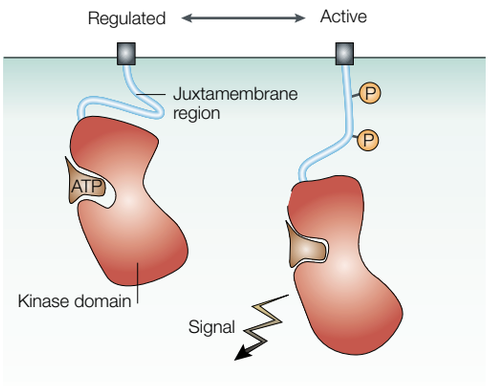

Figure 2 | **Regulation of Eph receptor catalytic activity.** EphB2-receptor kinase activity is auto-inhibited through interaction with its own juxtamembrane domain. Under regulated conditions, the conformation of the kinase domain is distorted, which causes misalignment of ATP (orange). On activation of EphB2, phosphorylation of the juxtamembrane domain tyrosine residues relieves auto-inhibition, and allows ATP to be properly aligned and the kinase domain to become activated.

NEURAL TUBE
A hollow, dorsal tube of embryonic nerve tissue that is formed by the rolling up of the neural plate. At the front, the tube expands to form the brain, whereas the posterior part narrows to form the spinal cord.

RHO FAMILY GTPASES
Ras-related GTPases that are involved in controlling the polymerization of actin.

GROWTH CONE
A motile, exploratory tip of the axon or dendrite of a growing nerve cell, which spreads out into a large cone-shaped appendage.

FILOPODIA
Long, thin protrusions at the periphery of cells and growth cones. They are composed of F-actin bundles.

LAMELLIPODIA
Flattened, sheet-like projections of crosslinked F-actin from the surface of a cell, which are often associated with cell migration.

DOMINANT-NEGATIVE
A defective protein that retains interaction capabilities and so distorts or competes with normal proteins.

RETINAL GANGLION CELLS
Cells that form the third and last layer of the retina. They are a type of interneuron that convey information from the retinal bipolar, horizontal and amacrine cells of the eye to the brain. The axons of ganglion cells form the fibers of the optic nerve.

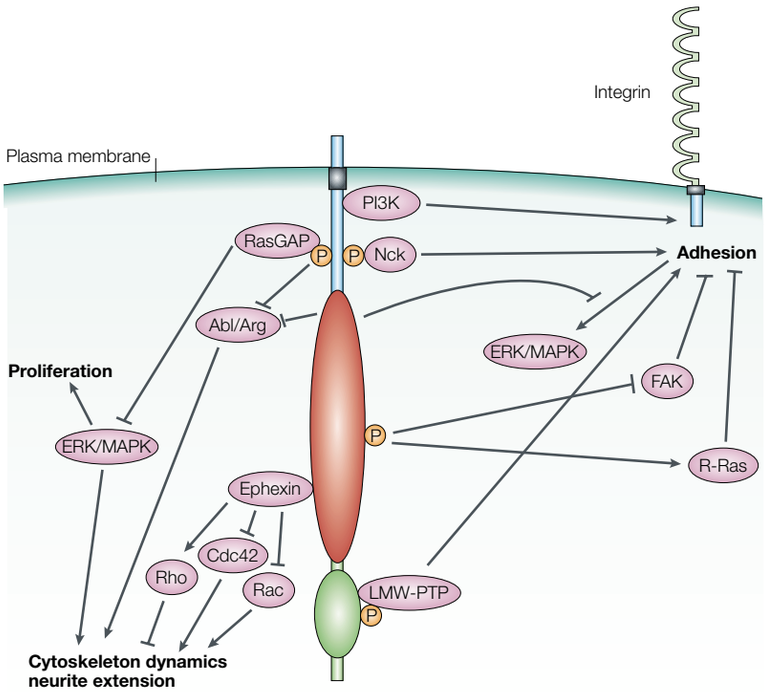

Figure 3 | **Summary of adaptor interactions described in this review.** Note that this review is not exhaustive and that further biochemical interactors of Eph receptors are listed in TABLE 1 and described in more detail elsewhere[38,117]. Abl and Arg bind the juxtamembrane region of EphB2 through a phosphorylation-independent interaction with EphB2 and through an unknown interaction. Activated Eph receptors suppress the extracellular-signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, which leads to growth-cone collapse and neurite retraction. Suppression of the ERK/MAPK pathway might be the reason that Eph receptors fail to induce a proliferative response in most cell types. Ephexin interacts with the Eph-receptor kinase domain and its activation differentially affects Rho GTPases, which are well-known cytoskeleton modulators. Several pathways link Eph receptors to regulation of adhesion or attachment (refer to text for full description); for example, suppression of integrin-mediated activation of the ERK/MAPK pathway. Abl, Abelson; Arg, Abl-related gene; GAP, GTPase-activating protein; PI3K, phosphatidylinositol 3-kinase; FAK, focal adhesion kinase. LMW-PTP, low-molecular-weight protein tyrosine phosphatase; Nck, SH2–SH3 adaptor protein.

activated, whereas Cdc42 and Rac1 are inhibited. Eph-receptor-mediated activation of Rho, and inhibition of Cdc42 and Rac, respectively, shifts actin cytoskeleton dynamics to increased contraction and reduced extension, which explains how Ephexin might mediate growth-cone collapse. In another set of experiments, ephrinA5-induced retinal growth-cone collapse was reduced using a specific inhibitor of Rho GTPases, which further supports the idea that Rho acts downstream of Eph receptors in growth-cone collapse[23]. Ephexin is not only expressed in the nervous system (for example, in the growth cones of retinal ganglion cells), but is also expressed in liver and kidney, which indicates further functions outside the nervous system that could be independent of Eph-receptor-interaction.

Effects on mitogenesis. Eph receptors were recently shown to be negative regulators of the extracellular-signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway[24,25]. This might provide an explanation for their apparent lack of mitogenic activity in cell culture[26–28], which contrasts with most other RTKs. Receptors for epidermal growth factor (EGF), PDGF or vascular endothelial growth factor (VEGF) autophosphorylate and recruit the growth-factor-receptor-bound protein 2 (Grb2)–Son-of-Sevenless (SOS) complex, which catalyses the GTP loading of Ras[29,30]. Ras then associates with Raf, which activates the dual-specificity MAPK/ERK kinase MEK, which in turn activates ERK/MAPK (for a review, see REF. 31). EphrinA1 stimulation was shown to suppress ERK/MAPK activity and proliferation of immortalized prostatic epithelial cells and primary prostatic cancer cells[24]. Furthermore, EGF- or PDGF-induced activation of the ERK/MAPK pathway was reduced after stimulation with ephrinA1. Similar conclusions were made by studies of ERK/MAPK signalling downstream of the EphB2 receptor[25]. In this study, ephrinB1-induced activation of the EphB2 receptor in neuronal cells downregulated the levels of GTP-bound Ras, which caused growth-cone collapse and neurite retraction. In addition, expression of a constitutively active variant of Ras reversed EphB2-induced neurite retraction. Therefore, suppression of the ERK/MAPK pathway by Eph receptors serves at least two roles — reduction of mitogenic signals and enhancement of cytoskeletal changes, which lead to neurite retraction and axon guidance (FIG. 3).

Cell–substrate interactions. Attachment of neuronal cells to fibronectin activates ERK/MAPK through integrin signalling[32]. Interestingly, EphB2 activation reduces ERK/MAPK activation under these conditions[25], which indicates that EphB2 might modulate integrin signalling. Eph receptors seem to regulate cell adhesion through integrin signalling in several ways, and apparently this can lead to both increased and decreased adhesion, depending on the situation. Activation of EphB2 inhibits cell adhesion through phosphorylation of R-Ras, which suppresses the ability of R-Ras to support integrin activity[33]. Activation of EphA2 in prostate carcinoma cells suppresses integrin-mediated adhesion by inducing dephosphorylation of focal adhesion kinase (FAK), which also leads to reduced adhesion[34]. By contrast, other studies have shown that EphB1 signalling enhanced α5β1 integrin-mediated cell attachment in transfected human kidney cells, a process which required EphB1 kinase activity and recruitment of low-molecular-weight protein tyrosine phosphatase (LMW-PTP) and the SH2–SH3 adaptor protein Nck[35]. Downstream activation of the Nck-interacting kinase (NIK) also couples EphB1 to activation of integrins and increased cell attachment[36]. Furthermore, in a tyrosine-kinase-independent fashion, the EphA8 receptor can localize the p110γ regulatory subunit of phosphatidylinositol 3-kinase (PI3Kγ) to the plasma membrane, which was shown to be required for integrin-mediated cell adhesion[37].

These results complicate the interpretation of Eph-regulated cell adhesion and other cell responses that involve integrins. The contrast might reflect the *in vivo* situation of the cell, such that the cellular response is determined by an intricate balance of external signals that are perceived by Eph-receptor subtypes, and the cellular context.

Table 1 | **Eph receptor and ephrin-ligand signalling adaptors**

Adaptor type	Eph receptor	References	Ephrin	References
SH2 domain	Nck	103,104	Src/Fyn/Yes	43
	RasGAP	104	Fyn	57
	Fyn	16,105,106	Grb4	48
	Src	16		
	Yes	16		
	Src-like adaptor protein (SLAP)	107		
	p85-PI3K	108		
	Grb2	109		
	Grb10	109		
	Abl/Arg	14		
	SHEP1	110		
	Crk	111		
DH–PH region	Ephexin	22		
PDZ domain	Syntenin	45	Syntenin	45,47
	Pick1	45	Pick1	45
	Grip	45	Grip	45
	AF-6	105,112	Grip2	46
			FAP-1	47
			PDZ-RGS	53
			PHIP	47
			PTP-BL	43
?	Syndecan-2	74		
	LMW-PTP	67		

Abl, Abelson; AF-6, afadin-6; Arg, Abl-related gene; FAP-1, Fas-associated phosphatase-1; GAP, GTPase-activating protein; Grb, growth-factor receptor-bound; Grip, glutamate-receptor-interacting protein; LMW-PTP, low-molecular weight protein tyrosine phosphatase; NIK, Nck-interacting kinase; PHIP, pleckstrin-homology-domain interacting protein; PI3K, phosphatidylinositol 3-kinase; Pick, protein interacting with C kinase; PTP-BL, protein tyrosine phosphatase-basophil-like; RGS, regulator of heterotrimeric G protein signalling; SH2, Src-homology 2; SHEP1, SH2-domain-containing Eph receptor-binding protein 1.

Interestingly, Eph/ephrin-mediated topographic mapping (see BOX 3) rely on repulsive forces in the visual system, whereas topographic mapping in the VOMERONASAL SYSTEM seems to depend on adhesive/attractive forces (BOX 3).

Mechanisms of ephrin reverse signalling

The intriguing concept of reverse signalling through membrane-bound ephrin ligands has emerged through biochemical and genetic studies in nematodes and mice (for reviews, see REFS 38–41).

Signalling through transmembrane ligands. The cytoplasmic domain of ephrinB ligands contains five conserved tyrosine residues, which led many researchers to propose initially that ephrinB ligands might have a signalling function. Three of these tyrosines — residues 312, 317 and 332 — have subsequently been identified as the main *in vivo* tyrosine phosphorylation sites of activated avian ephrinB1 from neural tissue[42]. These residues can be phosphorylated by several mechanisms.

Stimulation of primary neurons or ENDOTHELIAL CELLS with the soluble ectodomain of Eph receptors induces tyrosine phosphorylation of the cytoplasmic domain of endogenous ephrinB[43]. Treatment of embryonic chicken retina with fibroblast growth factor (FGF) leads to phosphorylation of endogenous ephrinB, presumably by the co-expressed FGF receptor[44]. Likewise, stimulation of the endogenous PDGF receptor in NIH 3T3 fibroblasts that ectopically express ephrinB1 induces rapid phosphorylation of ephrinB1, which indicates that a direct interaction might occur between the PDGF receptor and ephrinB1 (REF. 3). Several recent studies that are described in more detail later have significantly broadened our understanding of signalling mechanisms downstream of ephrinB ligands (FIG. 4).

Phosphorylation of the ephrinB cytoplasmic domain is thought to be an important event in ephrinB reverse signalling but, until recently, the identities of the kinase(s) and phosphatase(s) that regulate this process were unknown. Using primary endothelial cells and cortical neurons, it was shown that SFKs are positive regulators of ephrinB phosphorylation and that SFK activity is required for endothelial sprouting in a three-dimensional sprout assay[43]. It was further shown, by immunofluorescence, that EphB2-receptor stimulation induced rapid recruitment of SFKs to ephrinB-containing membrane clusters, which allowed phosphotyrosine-dependent signalling to occur (see below). Like Eph receptors, ephrinB ligands can recruit PDZ-domain proteins through a carboxy-terminal (YKV) target site (see below)[45–47]. Palmer *et al.*[43] showed that the PDZ-domain containing protein-tyrosine phosphatase PTP-BL is recruited to ephrinB membrane clusters with delayed kinetics. As PTP-BL can act as a negative regulator of ephrinB phosphorylation and Src activity, the data indicate the presence of a switch mechanism that allows a shift from phosphotyrosine-dependent signalling to PDZ-domain-dependent signalling[43] (FIG. 4).

Box 3 | **Ephs and ephrins in topographic map formation**

Recent results have indicated that topographic mapping — the mechanism by which axon terminals organize themselves onto a target area — might be regulated by either repulsive or adhesive/attractive forces. Guidance in the visual system is believed to depend on EphA receptors that are expressed in a gradient in the retina. This will project to their target in the brain, the superior colliculus, which expresses ephrinA ligands in a gradient. The progressively higher concentration of ephrinA ligand will eventually induce repulsion of EphA-bearing axons, which results in a stop–grow signal that matches the concentration of Eph receptor on the axon (reviewed in REF. 91). Uwe Drescher and co-workers[92] then studied the vomeronasal system in mice, which relays detection of pheromones between the vomeronasal organ (VNO) and the accessory olfactory bulb (AOB). Similar to the visual system, there is a topographically organized innervation, in which apical VNO neurons project to the anterior part of the AOB, and basal VNO neurons project to the posterior part of the AOB. In the vomeronasal projections the situation is different in two respects. First, the projecting axons carry ephrin ligand rather than receptor and the target area expresses Eph receptor rather than ligand. Second, the axons that carry high concentrations of ligand project onto areas expressing high concentrations of receptor. Therefore, an attractive force might guide axons in the vomeronasal system and this attraction might be mediated by ligand.

VOMERONASAL SYSTEM
A cluster of sensory neurons in the nasal arch that detects pheromones and transmits this information to higher cortical centres.

ENDOTHELIAL CELLS
Thin, flattened cells of mesoblastic origin that are arranged in a single layer that lines the blood vessels and some body cavities; for example, those of the heart.

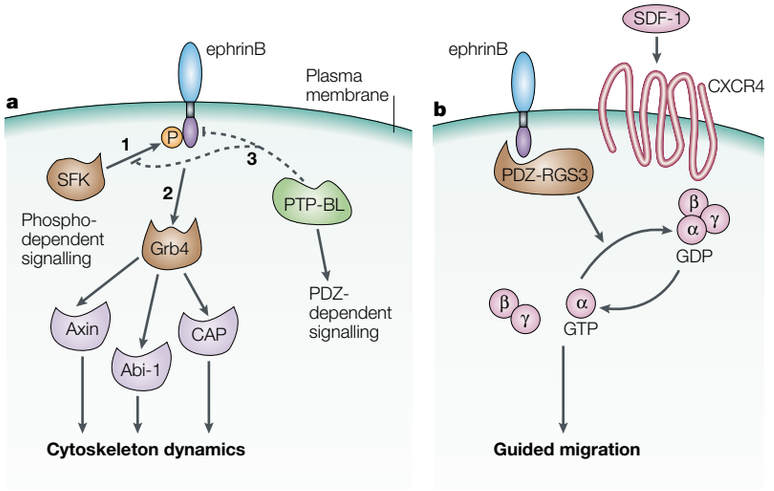

Figure 4 | **Molecular mechanisms of ephrinB reverse signalling. a** | Activation of ephrinB ligands through binding to an EphB receptor leads to the recruitment of Src-family kinases (SFKs) that phosphorylate the cytoplasmic domain of ephrinB ligands on tyrosine residues (1). Src-homology-2 (SH2)-domain-containing adaptor molecules such as Grb4 are recruited to phosphorylated ephrinB ligands, which initiates a cascade of signalling events that regulate cytoskeleton dynamics (2). With delayed kinetics, the phosphatase PTP-BL is recruited through its PDZ domain to the ephrinB carboxy-terminal tail. PTP-BL dephosphorylates ephrinB and inactivates Src, which causes a switch from phosphotyrosine-dependent to PDZ-domain-dependent signalling (3). **b** | PDZ-RGS3, among other PDZ-binding proteins, binds constitutively to ephrinBs and links regulation of G-protein-coupled signalling to ephrins. The chemoattractant stromal-cell-derived factor-1 (SDF-1) signals through its receptor CXCR4 to induce the Gα subunit to exchange GDP for GTP. This activates heterotrimeric G-protein signalling through dissociation of the heterotrimer and both the Gβγ and GTP–Gα subunits can then activate downstream effectors. PDZ-RGS3 can reverse this activation through enhancement of the intrinsic GTPase activity of the Gα subunit, which induces re-formation of the inactive heterotrimeric G protein. CAP, c-Cbl-associating protein.

Phosphorylation-dependent signalling by ephrinB ligands. A recent report[48] identified the SH2–SH3 domain adaptor protein Grb4 as a downstream effector of ephrinB ligands. Grb4 has an overall amino-acid homology of 68% to the related adaptor protein Nck, which had previously been shown to bind to EphB receptors and whose *Drosophila melanogaster* homologue dreadlocks (dock) is a crucial mediator of axon guidance and FASCICULATION in the visual system (reviewed in REF. 38). In a modified yeast two-hybrid screen that allowed for detection of protein–protein interactions that involve phosphotyrosine, Cowan and Henkemeyer[48] identified Grb4 as an interactor of ephrinB1 that was previously phosphorylated by the PDGF-receptor kinase. In pull-down experiments, the authors showed[48] that the Grb4 SH2 domain, but not the Nck SH2 domain, binds to the phosphorylated cytoplasmic tail of ephrinB1 and that this interaction is dependent on EphB2 engagement of ephrinB1. The authors went on to show that Grb4 recruitment to ephrinB1 induces loss of polymerized F-actin structures and disassembly of FOCAL ADHESIONS, which gave the cells a rounded appearance. Grb4 binds, through its SH3 domains, to several polyproline-containing proteins, including Abl-interacting protein-1 (Abi-1), c-Cbl-associated protein (CAP) and a scaffold protein, axin, in the Wnt signalling pathway. Because CAP has a regulatory role in the formation of actin STRESS FIBRES and focal adhesions[49], the downstream effects of ephrin reverse signalling on CAP were studied further. EphB2-induced stimulation of ephrinB1 led to the recruitment of Grb4 and CAP from focal adhesions to localized regions of ephrinB1 activation. This recruitment could be blocked by overexpression of the isolated Grb4 SH2 domain or by deletion of the ephrinB1 cytoplasmic tail, which indicates that SH2 binding to the ephrinB1 cytoplasmic tail is necessary for the association between ephrinB1 and CAP. Other Grb4 adaptors also function as potential links to the regulation of the actin cytoskeleton. For example, Abl is a known modifier of the actin cytoskeleton and there are at least two possible links between Abl and Grb4: through direct interaction[50] and indirectly through Abi-1 (REFS 51,52).

Phosphorylation-independent signalling by ephrinB ligands. The cytoplasmic region of ephrinB ligands also contains a PDZ-binding motif (YKV) at its carboxyl terminus (FIG. 1). The PDZ-domain proteins that interact with this motif can be subdivided into adaptor proteins that consist of only PDZ domains; for example, glutamate-receptor-interacting-protein-1 (GRIP1), GRIP2 and syntenin, or proteins with PDZ domains that are linked to a functional unit, such as the protein kinase C-interacting protein Pick1 and the tyrosine phosphatase PTP-BL. In most cases (except for PTP-BL) it is not known to what extent these proteins contribute to the localization and clustering of ephrinB1 and to downstream signalling events. A recently discovered link that connects cell migration to ephrin signalling occurs through the new cytoplasmic protein PDZ-RGS3 (REF. 53). In addition to its amino-terminal PDZ domain, this protein contains a regulator-of-heterotrimeric G-protein signalling (RGS3) domain, which can reverse the activation of the G-protein catalytic cycle downstream of G-protein-coupled receptors. The chemokine stromal-cell-derived factor-1 (SDF-1) activates the seven-transmembrane G-protein-coupled receptor CXCR4, which leads to phosphorylation of the subunit Gα–GDP and its subsequent release from the heterotrimeric Gαβγ-protein complex (FIG. 4). The released subunits further transmit the signal to eventually induce cellular

FASCICULATION
The bundling of axonal processes of neurons.

FOCAL ADHESIONS
Cellular structures that link the extracellular matrix on the outside of the cell, through integrin receptors, to the actin cytoskeleton inside the cell.

STRESS FIBRES
Axial bundles of F-actin underlying the cell bodies.

Box 4 | **Embryonic angiogenesis requires Ephs and ephrins**

Eph and ephrins carry out essential functions during formation of the vasculature. EphrinB2 is a very early marker of arterial endothelial cells, whereas one of its receptors, EphB4, reciprocally labels venous endothelial cells. Mutant animals that lack ephrinB2 (REFS 93,94) and EphB4 (REF. 95) show various cardiovascular defects. Angiogenesis in extra-embryonic tissue (yolk sac) and embryos was abolished, which resulted in a block at the primitive capillary plexus stage (see FIG. 8). Persistent and regulated expression of ephrins and Eph receptors in postnatal stages indicates further functions in therapeutic (wound healing) and pathological (tumour) angiogenesis[96–98].

Box 5 | **Cell membrane subdomains — lipid rafts**

Correct subcellular localization of proteins is a prerequisite for the functioning of signalling cascades. For the assembly of cytoplasmic and membranous signalling molecules and for polarized membrane transport, the plasma membrane is organized into lipid domains that provide subcompartments. These small (<100 nm) membrane domains, also called rafts, are enriched in certain lipids (sphingolipids and cholesterol) and specific proteins, including glycosylphosphatidylinositol-anchored proteins (such as ephrinA), transmembrane proteins (such as ephrinB), and doubly acylated proteins (such as Src family kinases)[99]. Lateral clustering of raft proteins can lead to fusion of dispersed rafts to larger patches, and thereby allow the interaction of intracellular signalling molecules.

responses, one of which includes the chemoattraction of cerebellar granule neurons. SDF-1-induced cell migration of cerebellar neurons is inhibited by the stimulation of ephrinB ligands using EphB2-receptor bodies. The inhibition is not general, as brain-derived neurotrophic factor (BDNF)-induced attraction is unaffected by ephrin stimulation. PDZ-RGS3 probably mediates this effect, as a dominant-negative version of PDZ-RGS3 prevents the ephrinB-induced inhibition. Ephrin stimulation might inhibit CXCR4-receptor-mediated chemoattraction by activating PDZ-RGS3, which induces reformation of the inactive Gαβγ-protein complex. It is not clear how ephrins stimulate the activity of PDZ-RGS3, but the interaction seems to be constitutive. Endothelial cells in culture also express a functional CXCR4 receptor and SDF-1 can induce migration of endothelial cells in culture[54,55]. It is an intriguing possibility that ephrinB2 might also regulate CXCR4-mediated signalling that occurs during angiogenesis (BOX 4).

Signalling through GPI-anchored ligands. Although most of the evidence for signalling by ephrin ligands relates to transmembrane ephrinB ligands, GPI-anchored ephrinA ligands might also have their own signalling potential. The *Caenorhabditis elegans* genome encodes four potential GPI-anchored ephrins and a single Eph receptor (VAB-1). Genetic studies indicated that the VAB-1 functions in cellular organization were, in part, kinase independent, which is consistent with reverse signalling by *C. elegans* ephrins[56]. Receptor-engaged ephrinA ligands mediate cell adhesion by activating the Src family member Fyn, which co-localizes with ephrinA in lipid-raft microdomains[57] (BOX 5).

Interestingly, in this case, integrins also seem to be involved. In contrast to Eph receptors, which in some cases inhibit integrin-mediated cell attachment, the available data consistently indicate that ephrinA ligand activation leads to enhanced integrin-dependent adhesion[57–59]. Furthermore, recent experiments that studied topographic projections in the vomeronasal system are indicative of an attractive force that is mediated by ephrinA ligands (BOX 3).

Functions of Eph-receptor signalling

In parallel with the *in vitro* biochemical studies on Eph/ephrin signalling pathways, genetic studies have attributed *in vivo* functions to the cytoplasmic domains of Eph receptors and ephrins.

A functional EphB2 receptor is necessary for proper formation of the anterior commissure, an axon bundle that connects the two cerebral hemispheres. However, mice that express a catalytically inactive EphB2 receptor, in which most of the cytoplasmic domain is replaced with a cDNA that encodes β-galactosidase, develop a normal anterior commissure (at least on certain genetic backgrounds)[41]. Likewise, in retinal-axon pathfinding, the kinase domain of EphB2 is dispensable for retinal ganglion cells to properly extend their fibres to the OPTIC DISC[60,61]. These experiments showed that certain functions of EphB2 do not require kinase activity, a finding that also prompted studies on reverse signalling. As previously mentioned, the *C. elegans* VAB-1 receptor is required for cellular movements during development. Removal of VAB-1 protein causes aberrant migration of cells, which results in severe deformations, such as VENTRAL ENCLOSURE defects, whereas inactivation of VAB-1 kinase activity causes milder phenotypes, such as tail-morphology defects that are caused by misalignment of cells[56,62].

Formation of the corticospinal tract. A clear example of signalling that is induced by Eph-receptor forward signalling has been provided by genetic studies that analysed the role of EphA4 and its ligand ephrinB3 during the formation of the corticospinal tract, a long fibre tract that connects the brain with the spinal cord and provides central control over body movements. This fibre tract begins in one lobe of the NEOCORTEX (either the left or right side), takes a complex route through the ventral part of the brain before it crosses the midline at the brain–spinal-cord junction, and then continues down in the opposite side of the spinal cord to ultimately connect with spinal-cord motor neurons. *EphA4*- and *ephrinB3*-null mutant

Receptor-kinase-dependent and ephrin-cytodomain-independent function

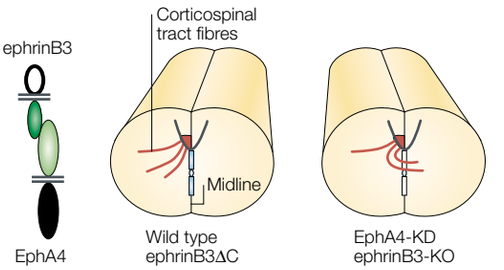

Figure 5 | **Corticospinal-tract-fibre guidance.** Forward signalling through the EphA4 receptor is necessary for correct guidance of corticospinal tract fibres (red). Drawings represent pieces of spinal cord with the left portion of the corticospinal tract fibres labelled. In wild-type mice, corticospinal tract fibres remain on one side of the spinal cord, where they contact other neurons. In mice that carry either an allele with an EphA4 catalytically inactive version (*EphA4*-KD) or a full deletion of ephrinB3 (*ephrinB3*-KO), corticospinal tract fibres will cross the ephrinB3-expressing midline (blue). By contrast, ephrinB3 does not require its cytoplasmic domain to fulfil its function as a midline repellent for corticospinal tract fibres (*ephrinB3ΔC*). A filled ellipse indicates that the cytoplasmic domain is required, a hollow circle with a thick outline indicates that it is not. KD, kinase-dead (catalytically inactive); KO, knockout.

OPTIC DISC
The exit point of retinal axons from the eye into the optic nerve (also called the optic nerve head).

VENTRAL ENCLOSURE
The ability of epithelial sheets with free edges to join together and fuse at the ventral midline.

NEOCORTEX
The 'newer cortex', which refers to the telencephalic cortex as opposed to the evolutionarily older primitive cortex (piriform cortex). It is found in higher vertebrates and is the site of higher mental processes.

SEMICIRCULAR CANAL
Liquid-filled archformed canal that is part of the inner-ear balance organ.

VESTIBULAR SYSTEM
The inner-ear balance organ that keeps track of the position and motion of the head in space. It consists of three perpendicularly oriented semicircular canals, which detect angular acceleration, and the utricle and saccule, which detect linear acceleration.

mice phenocopy each other in an unusual rabbit-like parallel hopping gait, and both mice have corticospinal tract fibres that aberrantly recross the midline in the spinal cord[63,64]. Neither *EphA4*- nor *ephrinB3*-null mice could shed light on the direction of signalling — reverse through ligand, or forward through receptor — during outgrowth of the corticospinal tract fibres. Evidence that determined the direction of signalling during corticospinal tract guidance was provided by two studies that approached the problem from opposite sides. EphA4 forward signalling was studied by producing a knock-in mouse that carried a catalytically inactive allele but, as its extracellular ligand-binding domain was still intact, it was still capable of signalling into the ephrin-expressing cell. This mouse showed the same striking rabbit-like gait and errors in corticospinal-tract-fibre pathfinding, which indicated a requirement for EphA4 kinase signalling for normal corticospinal-tract-fibre development[8].

In a complementary set of experiments, a mouse carrying an allele of *ephrinB3* that lacks its cytoplasmic tail did not show the gait abnormalities or the corticospinal-tract-fibre defects, which indicates that reverse signalling by ephrinB3 is not required for this function[65]. Expression data supported the conclusions that ephrinB3 functions in the spinal cord as a cell-attached midline repellent for EphA4-expressing neurons. EphA4 is endogenously expressed in the corticospinal tract fibre[66] and in cortical neurons, which respond to ephrinB3 stimulation with growth-cone collapse[8,64]. These studies provide an *in vivo* example in which EphA4 receptor forward signalling is necessary and sufficient for normal development of one of the main neural-fibre tracts in the mammalian nervous system (FIG. 5). However, mice that do not express *EphA4* have a defective anterior commissure — similar to the phenotype of *EphB2*$^{-/-}$ mice — and this defect is rescued in mice that carry a catalytically inactive version of EphA4. This indicates that EphA4 also has kinase-independent functions in axon-tract formation, possibly as a non-signalling partner for signalling-competent ephrinB proteins.

Eph receptors are known to require clustered membrane-attached ligand for full activation, and in controlled experiments in which multimeric ligand stimulation was compared with dimeric ligand stimulation, multimeric stimulation could induce further responses[67]. How the enhanced signal is transduced through the receptor is not clear. SAM domains have been proposed to form dimers or even oligomers on the basis of the solved structure[68–70], but the carboxy-terminal SAM domain of Eph receptors has no obvious function at present. Perhaps higher-order clustering through the SAM domain or the PDZ recognition motif regulates further signalling events. However, mice that carry an *EphA4* allele in which the SAM domain is deleted do not show any defects in corticospinal-tract-fibre axon guidance and walk normally, which indicates that the SAM domain is not an absolute requirement for EphA4-mediated axon guidance[8]. Future studies will tell us whether the SAM domain is important in other Eph-mediated functions, such as cell migration or plasticity (see below), perhaps in conjunction with the PDZ-binding motif or through mechanisms that have yet to be identified.

Receptor-kinase-independent function

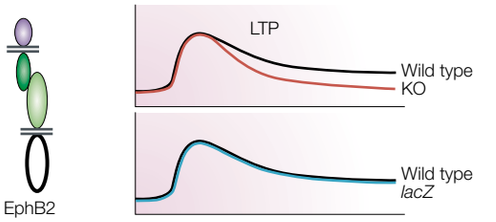

Figure 6 | **Long-term potentiation.** The cytoplasmic domain of EphB2 is dispensable for certain modifications of synaptic plasticity. In animals that lack EphB2 (knockout; KO), long-term potentiation (LTP) is reduced (red, upper panel) but is rescued in animals that carry an allele (*lacZ*) that lacks the cytoplasmic domain of EphB2 (blue, lower panel). A hollow ellipse with a thick outline indicates that the cytoplasmic domain of EphB2 is not required.

Regulating fluid homeostasis. Another example of EphB-receptor forward signalling has been shown to occur in the inner ear, where EphB2 and EphB3 have partially redundant roles in regulating fluid homeostasis in the SEMICIRCULAR CANALS. The authors of the study showed that the cytoplasmic domain of EphB2, together with EphB3, is required for normal functioning of the VESTIBULAR SYSTEM. They also showed the existence of a protein complex that contains EphB2 and Aquaporin-1, a known fluid regulator that, just like Eph receptors, can be bound by PDZ-domain containing proteins. These results showed that EphB2 forward signalling can regulate fluid homeostasis, but it is presently unclear whether this function requires EphB2 kinase activity or PDZ-domain interactions only[71].

Long-term potentiation. Recent research has shown that Eph receptors and ephrins are important in the adult nervous system; for example, during the processes of synapse formation and synaptic plasticity (BOX 6). EphrinB stimulation induces *N*-methyl-D-aspartate

Box 6 | **Synaptic plasticity**

Synapses are highly specialized structures through which neurons communicate and thereby regulate behaviour. Every synapse is made of a part of a neuron that conducts an impulse to the presynaptic side and a part of another neuron that receives the stimulus at the postsynaptic side. On electrical excitation of the presynaptic neuron, neurotransmitters are released from synaptic vesicles on the presynaptic side, cross the synaptic cleft and activate their receptors on the postsynaptic side. Dendritic spines are the principal postsynaptic targets for excitatory synapses[100]. In recent years, these small protrusions on the surface of dendrites have attracted significant interest because changes in their morphology are implicated in synaptic plasticity and long-term memory[101]. Synaptic plasticity is a term used to describe how synapses change in response to stimuli. The perforant path, an important pathway in the hippocampus, shows activity-dependent plasticity, a change now called LONG-TERM POTENTIATION (LTP)[102]. LTP is induced by a strong presynaptic stimulus and results in an increase in synaptic efficacy that can last up to hours *in vitro* and days or weeks *in vivo*. Long-term depression (LTD) is induced by a repeated low-frequency stimulus and results in the long-lasting reduction in synaptic efficacy below baseline potentiation.

(NMDA) receptors to cluster with EphB receptors through a direct interaction between the extracellular regions of the two receptors. Although the interaction is independent of EphB kinase activity, the authors showed that subsequent steps of synapse formation in neuronal cell cultures require EphB kinase activity[72]. In a follow-up study, members of the Greenberg laboratory showed that ephrinB2-stimulation of EphB receptors modulates NMDA-receptor-dependent calcium influx — an event that, in transfected neurons, also requires the presence of the EphB2 cytoplasmic region. EphB activation led to phosphorylation of NMDA receptors through Src tyrosine kinases and to a large increase in the ability of the NMDA receptor to flux calcium in response to glutamate[73]. These data present several possibilities for how Eph receptors are able to influence and regulate the formation and maturation of synapses.

Dendritic spines are the postsynaptic targets for excitatory synapses, and their formation in hippocampal neurons can be induced by the cell-surface PROTEOGLYCAN syndecan-2. EphB2 can phosphorylate and bind to syndecan-2, which leads to syndecan-2 clustering and spine formation. The association between EphB2 and syndecan-2 is dependent on the kinase activity of EphB2 and therefore represents another example of EphB forward signalling[74]. Mice that lack EphB2 protein (*EphB2*$^{-/-}$ mice) are viable and have normal hippocampal synapse morphology, which indicates that EphB2 is not critically required for synapse formation and is perhaps functionally redundant with other Eph receptors. *EphB2*$^{-/-}$ mice, however, do show impaired hippocampal long-term potentiation, the best studied cellular correlate for learning and memory processes. Moreover, in hippocampal slices that were derived from *EphB2*$^{-/-}$ mice, two forms of synaptic depression were completely absent. Expression of a truncated form of EphB2 that lacks kinase activity rescued the *EphB2*$^{-/-}$ phenotype, which indicates that EphB2 kinase signalling is not required for these EphB2-mediated functions[75,76] (FIG. 6).

It is evident from these studies that the ephrinB–EphB–NMDA-receptor interaction somehow influences signalling at the synapse and modulates synapse functions, but some of these events are dependent on Eph-receptor kinase signalling, whereas others are not.

Receptor-kinase-dependent and ephrin-cytodomain-dependent function

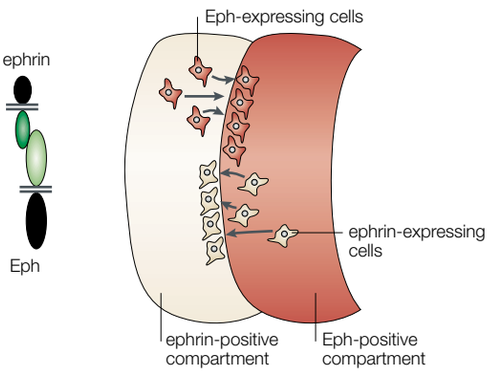

Figure 7 | **Boundary sorting.** Both Eph receptor and ephrin-ligand cytoplasmic domains are necessary for cell sorting at compartment boundaries. The schematic drawing shows red cells that express an Eph receptor sorting from a white (ephrin-positive) compartment into a red (Eph-positive) compartment. Likewise, white cells that express ephrin ligand sort to a white (ephrin-positive) compartment. A filled ellipse indicates that the cytoplasmic domain is required.

Ephrin-cytodomain-dependent function

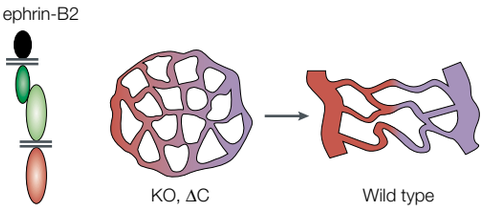

Figure 8 | **Angiogenesis and vasculogenesis.** The ephrinB2 cytoplasmic domain is required during development of the vasculature. The vascular bed (centre) remodels into a hierarchically organized vascular system that consists of large vessel and capillaries (right). Mice that carry an allele lacking the cytodomain of ephrinB2 (ΔC), or that lack ephrinB2 altogether (knockout; KO) cannot undergo this remodelling step. A filled ellipse indicates that the cytoplasmic domain is required.

Ephrin-cytoplasmic-domain-dependent functions

Hindbrain segmentation. EphB–ephrinB signalling is important for the establishing of boundaries between segments of the vertebrate hindbrain (rhombomeres; reviewed in REF. 77). Because of the repulsive action of ephrins, cells that express the ligand will sort away from cells that express the receptor, and *vice versa*. This restriction of cell intermingling will help to establish a sharp boundary between the two cell populations. Ectopic-expression studies in zebrafish embryos have shown that signalling by the cytoplasmic domain of ephrins is required to mediate cell sorting at boundaries[78] (FIG. 7). In another set of experiments that used explant cultures, it was shown that bidirectional signalling is required for the restriction of cell intermingling[79].

Vasculogenesis and angiogenesis. The functions of the cytoplasmic tail of ephrinB ligands have been genetically examined in mice by replacing wild-type alleles that encode full-length ephrinB protein with modified alleles that encode ephrinB protein lacking their cytoplasmic tail. Removal of the entire cytoplasmic tail of ephrinB3 did not result in a midline re-crossing phenotype (see above), which indicates that ephrinB3 reverse signalling is not required for midline cells to repel EphA4-expressing axons[65]. By contrast, deletion of the cytoplasmic tail of ephrinB2 caused a severe phenotype with defects in angiogenic remodelling and vasculogenesis, which strongly resembles the phenotype of mice that lack the entire protein[80] (FIG. 8). Although the exact cellular function of ephrinB2 in endothelial cells is not understood, these results indicate that ephrinB2 reverse signalling

PROTEOGLYCANS
A class of acidic glycoproteins that are found in the extracellular matrix, especially in connective tissues. They contain more carbohydrate than protein.

LONG-TERM POTENTIATION
A long-lasting increase in the efficacy of synaptic transmission, which is commonly elicited by high-frequency neuron stimulation.

NEURAL-CREST CELL
An embryonic cell that separates from the embryonic neural plate and migrates, giving rise to the spinal and autonomic ganglia, peripheral glia, chromaffin cells, melanocytes and some haematopoietic cells.

BRANCHIAL ARCH
In the higher vertebrate embryo, this is one of a series of arches — populated by neural-crest cells — that develop into structures of the ear, neck and face. The corresponding structures in fish and amphibians, sometimes referred to as gill arches, are made of bone or cartilage and are located on either side of the pharynx.

AORTIC ARCHES
Paired arches in vertebrate embryos that connect the ventral aorta with dorsal aorta(e) by running up between gill slits or gill pouches on each side.

PROTEOME
The complete set of (predicted) proteins in an organism. The term is analogous to the genome (the complete genetic material).

Ephrin-cytodomain-independent function

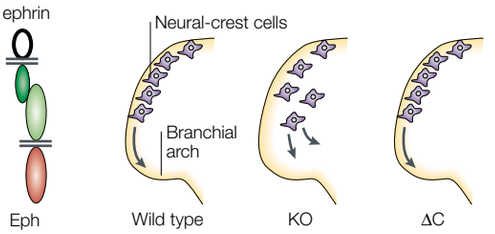

Figure 9 | **Migration of neural-crest cells.** The ephrinB2 cytodomain is not required for correct migration of neural-crest cells into branchial arches. In *ephrinB2*-null mutant mice, migration of neural-crest cells into branchial arches partially fails, because cells do not follow their normal route along the edge of the branchial arch. This defect is rescued in mice that carry an allele lacking the cytodomain of ephrinB2. A hollow ellipse with a thick outline indicates that the cytoplasmic domain is not required. KO, knockout.

might be required for remodelling of the embryonic vasculature (see BOX 4). It is possible that, in order for cells to generate new vascular sprouts, cell–cell and cell–matrix adhesions have to be suppressed and that this is mediated by repulsive actions of ephrins.

Ephrin-cytoplasmic-domain-independent functions

Neural-crest-cell migration. Interestingly, the truncated ephrinB2 ligand was able to rescue a cell migration defect that was present in *ephrinB2*-null mutants. In *ephrinB2*$^{-/-}$ embryos, NEURAL-CREST CELLS that express EphA4, EphB1 and EphB3 (which are all receptors for ephrinB2), seem to scatter and migrate in a wider and more diffuse pattern compared with wild-type mice and with mice in which the carboxyl terminus of ephrinB2 was deleted (FIG. 9). Migration of neural-crest cells has previously been reported to depend on Ephs and ephrins in various vertebrates. In *Xenopus* embryos, overexpression of ephrinB2, or truncated EphA4 or EphB2, led to scattering of neural-crest cells into adjacent regions[81], and in stripe assay experiments it was shown that ephrinB1 and ephrinB2 restrict migration of EphB-receptor-expressing crest cells[82,83]. However, these earlier studies could not pinpoint the direction of signalling (through ligand or receptor). The studies based on deletion of the ephrinB2 cytoplasmic domain indicate that migration of neural-crest-cells — and the subsequent formation of BRANCHIAL and AORTIC ARCHES — does not require reverse signalling through ephrinB2, and is instead mediated by forward signalling of Eph receptors in migrating cells. Whether or not forward signalling through Eph receptors is also necessary for angiogenic remodelling is not known. The cognate receptor for ephrinB2 on endothelial cells is EphB4, and *EphB4*$^{-/-}$mutants suffer from the same defects as *ephrinB2*$^{-/-}$embryos. It would be interesting to examine whether a catalytically inactive EphB4 receptor can rescue the angiogenic defects.

Conclusions and perspectives

The list of functions that involve Ephs and ephrins is growing and will continue to do so. Ephs and ephrins are recognized to regulate several important processes during development, and have now also extended their roles to the adult nervous system. Recent data have started to unravel the significance of forward-, reverse- or bidirectional signalling in different biological functions. In parallel, new signalling effectors are continually being discovered, and this tells us more about both the versatility and the complexity with which these receptors and ligands can carry out their functions. Clearly, one important challenge is to link signalling effectors with vital functions. Many other questions regarding Eph-receptor and ephrin signalling remain to be answered. Does ephrinB-mediated reverse signalling require phosphotyrosine-dependent signalling or do PDZ-domain interactions represent the important readout? How are ephrins recruited to rafts and is raft recruitment necessary for ephrin signalling? *In vitro*, ephrins can be artificially clustered, which circumvents the need for rafts, but ephrin phosphorylation will be deregulated[43]. Are ephrinA and ephrinB ligands co-localized in the same type of rafts? How similar are ephrin reverse and Eph-receptor forward signalling and could they be functionally exchanged? Both proteins recruit and activate SFKs and cluster PDZ-domain proteins. It will be interesting to find common and specific signalling effectors between Ephs and ephrins and to subject them to functional assays. Do different functions of Ephs (axon guidance, cell migration, angiogenesis and synaptic plasticity) require different forward and reverse signalling mechanisms? Perhaps by using emerging new technologies to analyse the PROTEOME[84,85], it will be possible to identify the networks that are crucial for differential Eph and ephrin signalling and function.

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