

Eph/ephrin signalling during development

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Summary

Eph receptors and their membrane-tethered ligands have important functions in development. Trans interactions of Eph receptors with ephrins at cell-cell interfaces promote a variety of cellular responses, including repulsion, attraction and migration. Eph-ephrin signalling can be bi-directional and controls actin cytoskeleton dynamics, thereby leading to changes in cellular shape. This article provides an overview of the general structures and signalling mechanisms, and of typical developmental functions along with cell biological principles.

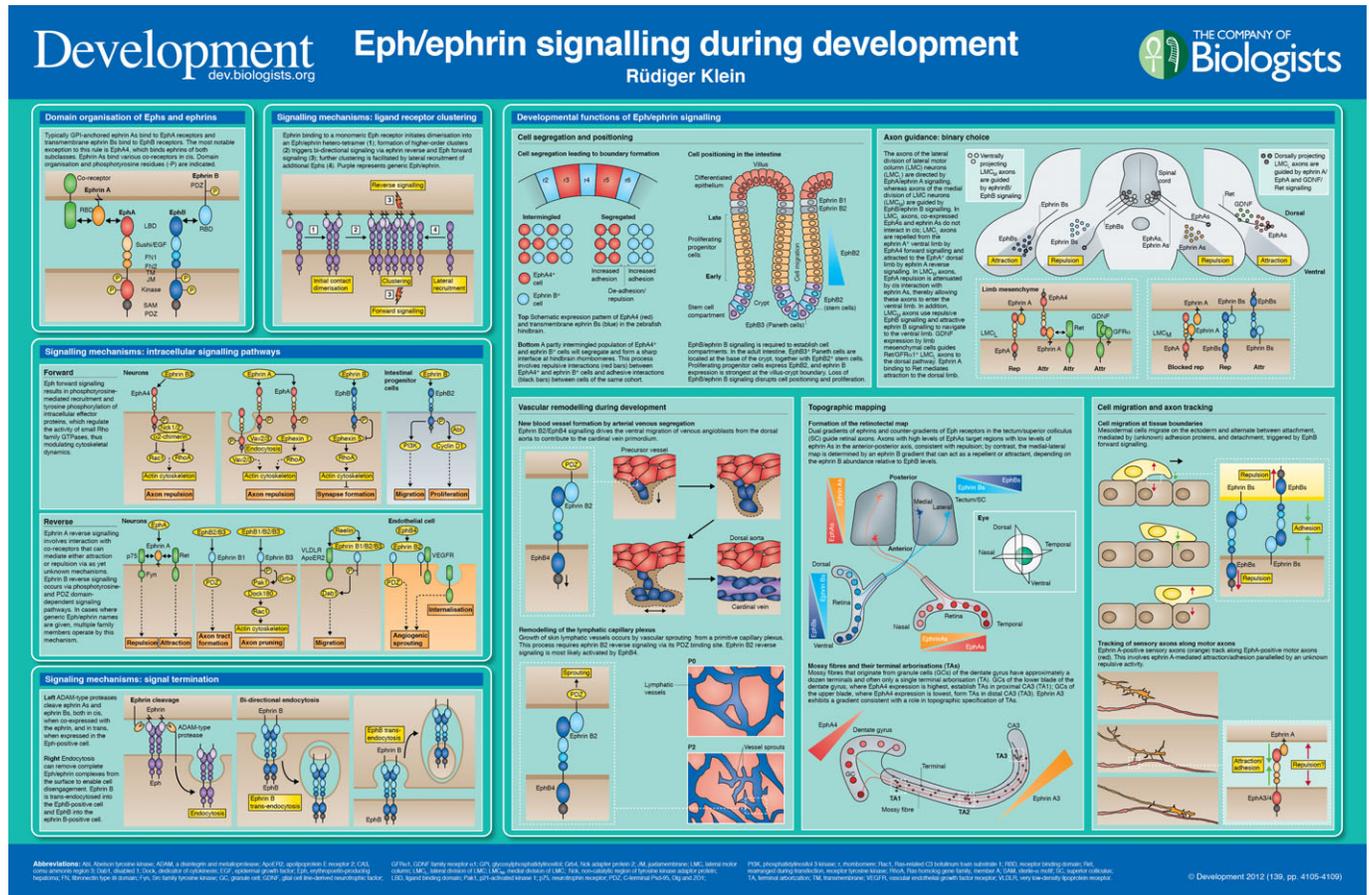
Key words: Cell migration, Eph, Ephrin-mediated vascular development, Axon guidance, Topographic mapping

Introduction

Morphogenesis in the embryo requires coordinated cell behaviour, which is stimulated by cues from the cells' environment that are generated as a result of patterning processes in early development. Eph receptors lie functionally at the interface between pattern formation and morphogenesis. They are expressed in all germ layers and are required for: cell segregation and positioning; tissue boundary formation and segmentation; cell migration, axon guidance and topographic mapping; and vascular and skeletal morphogenesis. Eph receptors constitute the largest family of receptor tyrosine kinases (RTKs). They exclusively bind membrane-tethered ligands known as ephrin proteins. Hence, Eph activation requires direct cell-cell contact. Moreover, Eph/ephrin signalling can be bi-directional, with intracellular pathways operating downstream of both the Eph receptor (forward signalling) and the ephrin ligand (reverse signalling) (Kullander and Klein, 2002). In the majority of cases, Eph forward signalling causes cell repulsion away from the ephrin-expressing cell, although adhesive responses have been described. Ephrin reverse

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signalling in neurons can also be repulsive or adhesive (Kao et al., 2012). Mechanistically, Eph/ephrin signalling controls local cytoskeletal dynamics and thereby allows cellular shape changes that underlie repulsive or migratory responses. Eph and ephrin proteins interact with a number of other ligand/receptor systems to influence how cells translate environmental signals to orchestrate morphogenetic events.

Domain organisation of Eph and ephrin proteins

Eph receptors are subdivided into two subclasses, termed EphA and EphB, based on sequence similarity and their preference for binding a particular subclass of ephrins. Typically, EphA receptors bind to glycosylphosphatidylinositol anchor (GPI)-linked ephrin A proteins and EphB receptors bind to transmembrane ephrin B proteins. Some cross-reactivities have been reported, most notably for EphA4, which binds both ligand subclasses (Pasquale, 2008). The domain organisation of Eph receptors is conserved across different animal phyla. The ectodomain contains a globular ligand binding domain (LBD), a cysteine-rich region with a Sushi and an epidermal growth factor (EGF)-like domain, and two fibronectin type III domains (FN1 and FN2). FN2 is followed by a transmembrane (TM) helix, and an intracellular part consisting of a juxtamembrane (JM) region with several conserved tyrosine (Y) residues, a tyrosine kinase domain, a sterile- α motif (SAM) protein-protein interaction domain, and a C-terminal Psd-95, Dlg and ZO1 domain (PDZ)-binding motif (Pasquale, 2008). In its inactive conformation, Eph kinase activity is auto-inhibited through interaction with its own JM region (Wybenga-Groot et al., 2001). On activation of Eph, phosphorylation of JM tyrosine residues (Y-P) relieves auto-inhibition, and allows the kinase domain to adopt an active conformation, initiating downstream signalling. Ephrins possess an extracellular receptor-binding domain (RBD). GPI-anchored ephrin A proteins can interact with other transmembrane co-receptors in cis (Bonanomi et al., 2012; Xu and Henkemeyer, 2012). Ephrin B proteins have a TM helix, an intracellular part with several conserved tyrosine residues and a C-terminal PDZ-binding motif. Tyrosine residues of the ephrin B cytoplasmic part are also phosphorylated upon engagement with Eph receptors (Kullander and Klein, 2002).

Eph/ephrin signalling mechanisms

Ligand-receptor clustering

Eph/ephrin signalling involves formation of higher order Eph/ephrin clusters (Himanen et al., 2010; Seiradake et al., 2010). Initial contact between Ephs and ephrins in 'trans' at the contact site between two cells may lead to the formation of heterotetramers (two Ephs and two ephrins) with limited signalling capacity. Functional forward and reverse signalling requires aggregation of Ephs and ephrins into larger signalling clusters. Eph clusters can incorporate additional Eph receptors in an ephrin-independent fashion by lateral recruitment.

Intracellular signalling pathways

Eph/ephrin signalling pathways have been investigated in many cellular systems and model organisms (Pasquale, 2008; Jørgensen et al., 2009; Bashaw and Klein, 2010; Bush and Soriano, 2012; Hruska and Dalva, 2012; Miao and Wang, 2012). Here, I summarise only a selection of pathways and mechanisms for which there is good *in vivo* evidence in mouse models. Eph forward signalling typically involves activation of the intrinsic tyrosine kinase activity and tyrosine phosphorylation of intracellular effector proteins. These in turn regulate the activity of small Rho family

GTPases such as Rac1, Cdc42 and RhoA, thus modulating cytoskeletal dynamics. For example, interaction between EphA4 and the RacGAP α 2-chimerin is important for axon repulsion in spinal circuits that control locomotion, such that mice lacking either EphA4 or α 2-chimerin have a rabbit-like hopping gait (Beg et al., 2007; Iwasato et al., 2007; Wegmeyer et al., 2007). The SH2-adaptor proteins Nck1 and Nck2/Grb4 have been implicated in the same pathway (Fawcett et al., 2007). The Vav family of RhoGEFs directly interacts with EphA4 and EphB2, and promotes EphA endocytosis, leading to growth cone collapse. *Vav2/Vav3* double knockouts show axon guidance defects (Cowan et al., 2005). The ephexin family of RhoGEFs also binds to Eph receptors, become activated by tyrosine phosphorylation and activate RhoA. ephexin 1-mediated RhoA activation causes growth cone collapse in pathfinding axons (Sahin et al., 2005). However, EphB signalling can also suppress ephexin activity: ephexin 5 (Arhgef15 – Mouse Genome Informatics) prevents synapse formation, and this function is suppressed by EphB-mediated tyrosine phosphorylation and subsequent degradation of ephexin 5 (Margolis et al., 2010). In the intestine, EphB2-dependent migration of progenitor cells involves PI-3 kinase activity, while proliferation is promoted by Abl kinase and cyclin D (Pitulescu and Adams, 2010).

Compared with Eph forward signalling, less is known about ephrin reverse signalling. Ephrin B reverse signalling typically involves tyrosine phosphorylation of its intracellular domain and recruitment of PDZ domain-containing proteins (Xu and Henkemeyer, 2012). Disruption of the ephrin B1 PDZ target site leads to malformation of the corpus callosum, a major forebrain axon tract (Bush and Soriano, 2009). A similar mutation in ephrin B2 disrupts sprouting of blood and lymphatic capillaries (Makinen et al., 2005; Sawamiphak et al., 2010). Endothelial ephrin B2 and its PDZ target site are also required for internalisation of vascular endothelial growth factor receptors VEGFR2 and VEGFR3 (Sawamiphak et al., 2010; Wang et al., 2010). In the absence of ephrin B proteins, stimulated VEGF receptors are retained in the plasma membrane and fail to signal properly, leading to reduced endothelial sprouting. Activation of B-class ephrin signalling in neurons induces phosphorylation of Dab1 (possibly by activation of Src family kinases), the major effector of the reelin pathway (Senturk et al., 2011). Loss of ephrin B family proteins causes neuronal migration defects similar to the reeler mouse. Ephrin B3 phosphorylation-dependent reverse signalling in neurons via the adaptor protein Nck2/Grb4, p21 activating kinase (Pak1), RacGEF Dock180 and Rac1 promotes the pruning of specific axon bundles in the postnatal hippocampus (Xu and Henkemeyer, 2009). Despite lacking intracellular sequences, ephrin A proteins are also capable of eliciting reverse signalling by coupling to co-receptors. In retinal axons, the p75 neurotrophin receptor and ephrin A proteins form a complex that is required for Fyn tyrosine kinase activation and axon repulsion (Lim et al., 2008). By contrast, ephrin A coupling to the Ret tyrosine kinase receptor in motor axons promotes axon attraction (Bonanomi et al., 2012), and ephrin A interaction with the TrkB neurotrophin receptor controls axon branching and synaptogenesis (Marler et al., 2008).

Signal termination

Eph/ephrin-mediated cell repulsion and disengagement require that the high-affinity interaction between Eph and ephrin proteins is terminated. Two mechanisms that can achieve this are ectodomain cleavage and endocytosis. A disintegrin and metalloprotease (ADAM)-type proteases cleave ephrin A and ephrin B proteins, both in cis, when co-expressed with these ephrin proteins, and in

trans, when expressed in the opposing cell (Hattori et al., 2000; Janes et al., 2005; Georgakopoulos et al., 2006). Eph receptors are also subject to cleavage by metalloproteases and γ -secretase (Bai and Pfaff, 2011). Endocytosis can remove complete Eph/ephrin complexes from the surface to enable cell disengagement. In cell culture assays, interaction of EphB-positive and ephrin B-positive cells results in the rapid formation of intracellular vesicles containing both full-length proteins in both cell populations. Hence, ephrin B is trans-endocytosed into the EphB-positive cell and EphB into the ephrin B-positive cell. The balance between uni- and bi-directional endocytosis may depend on cellular context and the intracellular signalling pathways at work in the two cell populations (Marston et al., 2003; Zimmer et al., 2003; Pitulescu and Adams, 2010).

Developmental functions of Eph/ephrin signalling

Eph/ephrin functions have been best studied in the nervous system, although multiple roles in other tissues are emerging. Here, we discuss key examples that showcase the range of activities of this pathway.

Cell segregation and positioning

Complementary expression of ephrin B and EphB is sufficient to segregate intermingled cells and is required to maintain sharp borders (Batlle and Wilkinson, 2012). Such reciprocal expression of Eph and ephrin proteins occurs in many tissues during development – most notably in hindbrain rhombomeres and somites – and underlies cell segregation. In zebrafish, two mechanisms operate in parallel: repulsive interactions between ephrin B-expressing and EphA4-expressing cells at rhombomere boundaries; and adhesive interactions between cells of the same cohort (Cooke et al., 2005). In mammalian epithelial tissues, an EphB3-ephrin B1 interaction triggers ADAM10-mediated cleavage and shedding of the cell-adhesion protein E-cadherin at the Eph/ephrin interface, thereby creating differential adhesion (Solanas et al., 2011).

Cell positioning along the crypt-villus axis of the small intestine is controlled by EphB/ephrin B interactions between epithelial cells (Batlle and Wilkinson, 2012). In newborn mice, EphB2 and EphB3 are expressed in the intervillus pockets, whereas ephrin B1 distribution is complementary to EphB proteins, being excluded from the bottom-most cells. In the adult, EphB/ephrin B expression is more complex. EphB2 is expressed by proliferating progenitor cells and its expression decreases towards the top of the crypts. At the crypt bottom, EphB2 marks stem cells and EphB3 is expressed by differentiated Paneth cells (Merlos-Suarez et al., 2011). Ephrin B expression is strongest at the villus-crypt boundary. Disrupting EphB/ephrin B signalling in embryonic and adult intestine disrupts cell positioning and proliferation. For example, Paneth cells are mispositioned in the upper crypt region (Batlle et al., 2002). The EphB/ephrin B expression pattern in the intestine is controlled by Wnt signalling: Wnt promotes expression of EphB2 and EphB3, and at the same time represses expression of ephrin B1 (Batlle et al., 2002).

Vascular remodelling during development

A functional blood vessel system develops early in embryogenesis and blood circulation has to be maintained as the system grows by generation of new and remodelling of existing vessels. EphB4/ephrin B2 signalling is required for the formation of two major axial vessels, the dorsal aorta (DA) and the cardinal vein (CV). In zebrafish embryos, angioblasts initially form a

single precursor vessel that expresses ephrin B2 and EphB4. Ventral sprouting of EphB4-positive venous-fated endothelial cells and subsequent repulsion from the ephrin B2-expressing precursor cells leads to the separation of the DA and CV (Herbert et al., 2009). Ephrin B2 and EphB4 are also co-expressed in developing lymphatic vessels. Postnatal growth of skin lymphatic vessels occurs by vascular sprouting from a primitive capillary plexus. This process requires ephrinB2 reverse signalling via its PDZ-binding motif (Makinen et al., 2005) and is most likely activated by EphB4.

Decision making at intermediate targets – binary choices

The trajectories of axons to their distant target tissues are often intricate, but they can be deconstructed into a series of sequential and often binary decisions. For example, in vertebrates, limb-innervating motor axons make an early decision at the base of the limb, such that axons select a dorsal or ventral pathway. This system has been used by many labs to extract general mechanisms of axon guidance via Eph/ephrin signalling. The axons of the lateral division of lateral motor column (LMC) neurons (LMC_L) are directed by EphA/ephrin A signalling, whereas axons of the medial division of LMC neurons (LMC_M) are guided by EphB/ephrin B signalling (Luria et al., 2008). Eph-expressing axons are repelled by ephrin-expressing limb mesenchymal cells. This mechanism drives LMC_L and LMC_M axons into dorsal and ventral limb, respectively. LMC axons also express ephrins, whereas Eph proteins are expressed in the limb. Cis interactions between EphA and ephrin A proteins can block EphA forward signalling (Carvalho et al., 2006). However in LMC_L axons, EphA and ephrin A proteins are segregated into separate plasma membrane microdomains and do not interact in cis (Kao and Kania, 2011). Hence, forward and reverse signalling can occur in parallel. Reverse signalling from limb-derived EphA4 to axonal ephrin A proteins results in attraction of LMC_L axons towards the dorsal limb (Kao and Kania, 2011; Dudanova et al., 2012). In LMC_M axons, EphA4 signalling is attenuated by cis interaction with ephrin A proteins, providing an explanation for the failure of these axons to be repelled by the ephrin A-positive ventral limb. Similar mechanisms are proposed to underlie EphB/ephrin B signalling (Kao and Kania, 2011).

Other signalling systems function in conjunction with EphA/ephrin A signalling to enhance the fidelity of axon guidance decisions. For example, glial cell line-derived neurotrophic factor (GDNF) expression by limb mesenchymal cells and signalling by the axonal Ret/GFR α 1 receptor complex also guides LMC_L axons to the dorsal pathway (Kramer et al., 2006; Dudanova et al., 2010), and reverse signalling from axonal ephrin A is thought to promote Ret-dependent pathways, and to mediate attraction of these axons towards the dorsal limb mesenchyme (Bonanomi et al., 2012).

Topographic mapping

Topographic maps are connections in the brain in which neighbour-neighbour relationships of neurons are maintained when choosing synaptic partners in the target field. The retinotectal map [tectum in lower vertebrates, superior colliculus (SC) in higher vertebrates] requires Eph and ephrin proteins to be expressed in complementary gradients in the retina and tectum, acting bi-directionally and through association with other signalling systems. Neighbouring retinal ganglion cells (RGCs) along the retinal temporal-nasal axis require differential EphA/ephrin A signalling to map onto the tectal anterior-posterior axis, whereas EphB/ephrin B signalling is required for RGCs along the retinal dorsal-ventral axis to map onto

the tectal medial-lateral axis (Feldheim and O'Leary, 2010; Suetterlin et al., 2012). Interestingly, axons with high levels of EphA proteins target regions with low levels of ephrin A proteins in the anterior-posterior axis, consistent with repulsion; by contrast, the medial-lateral map is determined by an ephrin B gradient that can act as a repellent or attractant depending on the ephrin B abundance relative to EphB levels. Topographic projections can also form postnatally through structural remodelling (Feldheim and O'Leary, 2010).

Dentate gyrus granule cells (GCs) project so-called mossy fibres to the CA3 region of the hippocampus. Initially, in mice, a single mossy fibre forms a dozen evenly spaced terminals, one of which remodels into a much larger terminal arborisation (TA) along topographic principles: GCs of the lower blade of the dentate gyrus, where EphA4 expression is highest, establish TAs in proximal CA3; GCs of the upper blade, where EphA4 expression is lowest, establish TAs in distal CA3. Interference with EphA4 expression in brain slices abolishes the topographic map (Galimberti et al., 2010). The role of ephrins has not been addressed, but ephrin A3 is expressed in a counter-gradient in pyramidal neurons along CA3, consistent with a role in topographic specifications of TAs.

Cell migration and axon tracking

Migration of cells or cellular processes on top of another cell substrate requires a fine balance of attractive/adhesive and repulsive forces to allow migration while preventing migrating cells from invading the underlying tissue. During *Xenopus* gastrulation, migration of the mesoderm over the ectoderm requires multiple EphB and ephrin B proteins on each side of the boundary. The migrating mesodermal cells alternate between attachment mediated by (unknown) adhesion proteins and detachment triggered by EphB forward signalling (Rohani et al., 2011). In the peripheral nervous system, ephrin A-positive sensory axons track along EphA-positive motor axons. This tracking event is independent of EphA forward signalling but requires that the EphA ectodomain triggers ephrin A-mediated attraction/adhesion. Loss of motor EphA3/4 or sensory ephrin A shifts the balance towards repulsion, mediated by an as yet unidentified activity (Wang et al., 2011).

Perspectives

Over the past 20 years, Eph and ephrin proteins have become established as important regulators of diverse morphogenetic processes. Because of the large numbers of receptors and ligands and their complex signalling properties, new biological functions are still being unravelled and the underlying cell biological and molecular mechanisms elucidated. Some of the interesting unanswered questions include the following. How are Eph/ephrin clustering and endocytosis linked to signalling, and how is the latter translated into highly diverse biological readouts? How do Ephs and ephrins interact with other surface proteins that control adhesion/de-adhesion and with other signalling systems that regulate cellular dynamics? Can the knowledge from developmental studies help to explain the roles of Eph and ephrin proteins in adult physiology and plasticity, and be translated to issues of regeneration and disease?

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Competing interests statement

The authors declare no competing financial interests.

Development at a Glance

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