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REVIEW

Eph-dependent cell-cell adhesion and segregation in development and cancer

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Abstract Numerous studies attest to essential roles for Eph receptors and their ephrin ligands in controlling cell positioning and tissue patterning during normal and oncogenic development. These studies suggest multiple, sometimes contradictory, functions of Eph-ephrin signalling, which under different conditions can promote either spreading and cell-cell adhesion or cytoskeletal collapse, cell rounding, de-adhesion and cell-cell segregation. A principle determinant of the balance between these two opposing responses is the degree of receptor/ligand clustering and activation. This equilibrium is likely altered in cancers and modulated by somatic mutations of key Eph family members that have emerged as candidate cancer markers in recent profiling studies. In addition, cross-talk amongst Ephs and with other signalling pathways significantly modulates cell-cell adhesion, both between and within Eph- and ephrin-expressing cell populations. This review summarises our current understanding of how Eph receptors control cell adhesion and morphology, and presents examples demonstrating the importance of these events in normal development and cancer.

Keywords Eph receptor · Ephrin · Receptor tyrosine kinase · Cell-cell adhesion · Cancer

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Abbreviations

ADAM10	A disintegrin and metalloprotease 10
ALL	Acute lymphoblastic leukaemia
Cas	Crk-associated substrate
CRC	Colorectal cancer
Crk	CT10 regulator of kinase
ECD	Extracellular domain
ECM	Extra-cellular matrix
EGFR	Epithelial growth factor receptor
FAK	Focal adhesion kinase
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GPI	Glycosyl phosphatidylinositol
JNK	c-Jun N-terminal kinase
MMP	Matrix metalloprotease
NMDA	N-methyl D-aspartate
PBM	PDZ-binding motif
PDZ	Postsynaptic density protein/discs large/zona occludens protein (domain)
PH	Pleckstrin homology (domain)
PI3K	Phosphatidylinositol 3′-kinase
PLC	Phospholipase C
PTB	Phospho-tyrosine binding (domain)
PTP	Protein tyrosine phosphatase
PY	Phospho-tyrosine
ROCK	Rho-associated coiled-coil-containing protein kinase
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SAM	Sterile alpha motif
SH2	Src homology 2 (domain)
TNFα	Tumour necrosis factor α

Introduction to Eph receptors and their ligands

Eph receptors represent the largest family of receptor tyrosine kinases [1–3]. They play essential roles during the development of invertebrate and vertebrate embryos where they guide cell migration and positioning during tissue modelling programs such as gastrulation and patterning of vascular, skeletal and nervous systems [2–4]. Their diverse biological functions are achieved principally by modulating the adhesion or de-adhesion/segregation between Eph-expressing cells and cells expressing their membrane-bound ligands, the ephrins. Emerging evidence suggests that Eph activation can also affect cell proliferation and apoptosis, an area recently reviewed elsewhere [5, 6].

A distinguishing feature of Eph-ephrin interactions is the phenomenon of bidirectional signalling. Ephrin binding promotes receptor clustering and phosphorylation of tyrosine residues, initiating forward signalling in Eph-bearing cells while, in parallel, clustering of the ephrins on the cell surface of ligand-expressing cells triggers a reverse signal into these cells [1]. Thus, the instructions derived from Eph/ephrin interactions are relayed into both cell populations to facilitate their mutual adhesion or segregation, which within the context of a tissue layer governs cell positioning and cell-cell contacts underlying normal and oncogenic patterning [7].

Eph receptors (and ephrins) are highly conserved in vertebrates and have been found in invertebrates including *Drosophila*, *C. elegans* and, interestingly, also in sponges [8–10]. The fact that the invertebrates express only one Eph receptor, VAB-1 in *C. elegans*, which interacts with four ephrins [11, 12], might indicate that increasing body plan complexity correlates with the growing size of the Eph/ephrin family [8, 9]. While Ephs and ephrins are predominantly expressed and active during development, their roles in normal tissue maintenance and homeostasis as well as their aberrant expression in a wide range of cancers is increasingly recognised, wherein both tumourigenic and tumour-suppressive functions have been described [4, 5]. In addition, Eph mutations are emerging as prevalent in cancers, which, as will be discussed below, likely modulate Eph-mediated cell-cell adhesion and de-adhesion events promoting tumourigenesis.

Nomenclature of Eph receptors and ephrins

There are two subtypes of Eph receptors distinguished by structural features and ephrin-binding preferences [1]—the EphA subtype, which comprises nine members in mammals (EphA1–A8, EphA10), and the EphB group with five members (EphB1–EphB4, EphB6) [13, 14]. The first isolated family member (EphA1) was found in a cDNA sample from an *E*rythropoietin-*P*roducing *H*epatocellular carcinoma cell line [15], explaining the acronym EPH-like receptors. The ligands of Eph receptors, the ephrins (*Eph r*eceptor *in*teracting proteins), are cell membrane-bound and in vivo usually present on the surface of opposing cells. Ephrins are also divided into two subclasses based on the type of their cell-membrane attachment: A-type ephrins (ephrin-A1–A6) are linked to the membrane via a GPI (glycosyl-phosphatidylinositol) anchor, while type B ephrins (ephrin-B1–B3) contain a single transmembrane domain and a short cytoplasmic tail [13, 14]. The A subclass receptors bind preferentially to type A ephrins, while EphBs favour type B ephrins [13, 14], although cross-subclass interactions have also been reported: EphA4 is able to bind A- and B-type ephrins with comparable affinities [16–18], and ephrin-A5 binds and activates EphB2 in addition to A-type Ephs [19]. In comparison, receptor-ligand interactions within the same subclass are very promiscuous, whereby within a subclass every Eph receptor can bind to multiple ephrins with very similar affinities [1].

Eph structure

The general structure of Ephs is highly conserved throughout the animal kingdom [9] (see Fig. 1a). The extracellular domain consists of an N-terminal globular domain responsible for ephrin binding, a cysteine-rich EGF-like domain and two fibronectin III repeats [20, 21]. The globular, together with the cysteine-rich domain, is additionally involved in ephrin-independent receptor dimerisation and clustering [22–25]. Analogous to other RTKs, Eph receptors contain a single transmembrane spanning domain. The intracellular domain is composed of a juxtamembrane region containing two conserved regulatory tyrosine residues that control kinase activity, a single tyrosine kinase domain, a SAM (sterile alpha motif) protein-protein interaction domain and a PDZ protein-binding motif (PBM), which can bind PDZ domain-containing proteins serving as scaffolds for the assembly of multi-protein signalling complexes [3, 26]. Amongst RTKs the presence of a SAM domain is unique to the Eph receptor family and may, along with the ligand-binding and cysteine-rich domains within the extracellular region, play a role in receptor-receptor interactions aiding homo- or heterotypic oligomerisation [27], as well as in regulating receptor endocytosis [28, 29].

Receptor clustering and activation

Unlike ligands of other RTK subfamilies, only membrane-bound or artificially clustered ephrins can trigger receptor signalling [30], while non-clustered soluble ephrins act as

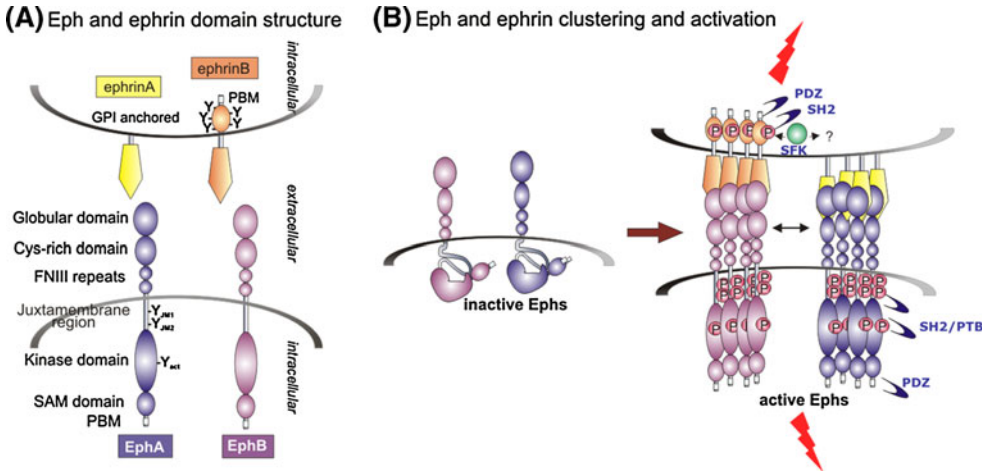

Fig. 1 Models of the structure and activation of Eph receptors. **a** Eph receptors contain an extracellular region with a globular ligand-binding domain, followed by cysteine-rich and fibronectin type III domains. Ligand binding promotes clustering and phosphorylation of the tyrosine kinase-containing intracellular region, at conserved sites within the juxtamembrane and kinase domains. **b** Inactive Ephs display an auto-inhibited conformation (*left*), in which the kinase domain interacts with the juxtamembrane domain, which is released upon activation to enable robust phosphorylation and phosphorylation-dependent signalling (*right*). Receptor-receptor interactions in the ECD further promote clustering [22], including co-clustering of EphA and EphB receptors [57]. The transmembrane ephrin-Bs also become phosphorylated on tyrosine residues by Src family kinases, which likely also promote signalling by the GPI-linked ephrin-As via clustering of lipid raft microdomains and associated signalling molecules

antagonists [23, 31–33]. More specifically, at least tetramerisation of Ephs is necessary to achieve full biological activation [34, 35]. Prior to ephrin contact, Ephs are loosely arranged within the plasma membrane displaying minimal kinase activity [1, 36], although this may be elevated by high receptor expression levels [37–40]. Eph/ephrin signalling is initiated by 1:1 high-affinity interactions between the Eph globular domain and a conserved Eph-binding domain of ephrins. Additional protein interfaces in these domains then tether dimers so that each ephrin binds two Ephs and each Eph binds two ephrins leading to the assembly of Eph/ephrin tetramers [20, 22, 23, 41, 42]. Further analysis of signalling cluster assembly demonstrated that Eph/Eph interactions resulting in expansion of these clusters progress independent of ephrin contact [24] through Eph protein interfaces located within the ligand-binding and cysteine-rich domains [22, 25]. This explains how, similar to ErbB receptors [43], Eph receptors spontaneously cluster at high cell surface densities, a phenomenon that could be further enhanced by transmembrane domain interactions [44], whereas a suspected role of cytoplasmic interactions promoting Eph clustering is yet to be confirmed.

Eph kinase domain activation relies on conformational changes that release inhibitory interactions between the kinase domain activation loop and the juxtamembrane region, and are triggered by transphosphorylation of three invariant tyrosines, one within the activation loop of the kinase domain and two within a highly conserved 10-amino acid motif in the juxtamembrane region (see Fig. 1b). While phosphorylation of the kinase domain tyrosine directly activates the enzyme, additional phosphorylation of the juxtamembrane tyrosine residues is required for full kinase activity [45–48], with recent work suggesting the second juxtamembrane tyrosine being most important for Eph activation [49]. The three invariant and potentially other tyrosines, for example in the SAM domain [50, 51], once phosphorylated, serve as docking sites for SH2 domain- and PTB-containing proteins, linking to signalling pathways that will be described below.

Co-clustering of Eph family members

Although RTK hetero-oligomerisation is a concept common, for instance, for receptors of the EGFR family [52], to date the evidence for cross-talk between Eph receptors is mostly limited to intra-subclass interactions. Given the promiscuous nature of ephrin-Eph interactions within subclasses, it is expected that ephrins will concurrently bind Ephs of the same subclass when co-expressed, and indeed EphB1 and EphB4 can form functional hetero-oligomeric complexes with kinase-dead EphB6, resulting in its transphosphorylation [53, 54]. However, there is also evidence for cross-class transphosphorylation: the EphA4 kinase domain is capable of phosphorylating the juxtamembrane tyrosines of EphB2 and EphA2 in vitro [51], and a phosphoproteomics approach of ephrin-B1-stimulated palate mesenchyme cells revealed phosphorylation of EphA2, EphA3, EphA7 and EphB4, in addition to EphB2 and EphB3, which are known to bind ephrin-B1 [55].

Similarly, phosphorylation of various EphAs was modulated in EphB2-expressing HEK293 cells when stimulated with ephrin-B1-expressing cells [56], although interestingly EphA2 phosphorylation decreased. Emerging evidence suggests that co-clustering occurs also between EphA and EphB receptors: selective activation of EphA3 with an agonistic monoclonal antibody [35] also co-clusters and activates co-expressed EphB2 in HEK293T cells, as well as other endogenous EphA and EphB receptors frequently co-expressed in tumour cells [57]. Overall, the notion of Eph co-clustering impacts profoundly on the understanding of Eph-mediated cell behaviour, since cellular responses of Eph-bearing cells in contact with ephrin-bearing cells will not be limited to those able to directly bind the activating ephrin, but by the overall abundance of EphA and EphB receptors on that cell.

Cross-talk with other receptors

It is highly likely that RTK signalling networks are necessarily interconnected as they share prominent cytoplasmic signalling cascades, such as the MAPK or PI3K pathways [58]. Thus, it is not surprising that co-clustering and interdependent signalling between Ephs and other RTKs has been suggested. For example, EphA4 and FGFR were reported to transphosphorylate each other and synergistically activate shared downstream signalling, such as MAPK activation [59–61]. By contrast, FGFR1 appears to inhibit ephrin-dependent EphB2 signalling by blocking a positive feedback loop via transcriptional down-regulation of the Ras-ERK pathway while indirectly increasing the phosphorylation of unstimulated EphB2 [62]. Interestingly, EGFR reportedly colocalises with EphA2 at cell-cell contacts [63] and adhesion-induced EphA2 expression is thought to be regulated by EGFR activation [64], while EphA3 silencing modulates EGFR downstream signalling in squamous cell carcinoma cells [65]. Accordingly, analysis of the EGFR interactome revealed amongst many other interaction partners ligand-independent association of EphA2 and EphB4 [66, 67]. Moreover, EphA2 co-operates with ErbB2 to amplify its tumourigenic effects, conferring resistance to anti-ErbB2 therapy [68]. Interactions between Ephs and VEGFR2 [69, 70] as well as the atypical RTK RYK [71–74], which contains an inactive kinase domain and shows overlapping biological functions with Eph RTKs, have also been proposed.

In addition, there is considerable interest in the crosstalk between Ephs and the receptor CXCR4 for the chemokine SDF-1 [75, 76], which plays important roles during embryonic and oncogenic development [77] and has been proposed to control endothelial cell movement in cooperation with EphB2 and EphB4 [75]. Furthermore, crosstalk of EphBs with NMDA-type [78–81] and AMPA-type [82] glutamate receptors is important for the regulation of synaptic plasticity. Further insight into the crosstalk between Ephs, other RTKs and other types of transmembrane receptors will be essential for a better understanding of Eph function and its potential application in the development of combination therapies, particularly for cancer treatment regimes.

Biological roles of Eph/ephrin-mediated adhesion and repulsion

Cell positioning as a central mechanism underlying Eph function

Eph receptors primarily function during embryogenesis to position motile cells and cell layers within surrounding tissues. They control the direction of cell movement and the choice of interacting cells or cell layers by initiating assembly or disassembly of cell-cell contacts. During embryonic development Ephs and ephrins are expressed in temporally and spatially restricted patterns in developing tissues and organs, where they participate in germ layer formation, gastrulation, organogenesis and tissue patterning [3, 83–86]. Originally identified as axon guidance molecules, their next best studied role lies within the developing vascular system. Eph and ephrin expression and function in healthy adult tissues are less prevalent, while re-emerging cell guidance functions during tumour invasion, neoangiogenesis and metastasis are now well established (see below).

Eph/ephrin-mediated cell positioning relies on cell-cell contact since both receptors and ligands are membrane-bound. Thus, migration of an Eph-expressing cell into an area of graded ephrin expression is directed by contact-dependent cell-cell segregation, instructing a change of direction to avoid the ephrin-rich area and a final destination that is determined by competition for ephrin interactions with other Eph expressing cells or axons [87, 88]. However, cell location is not only determined by graded expression and the overall abundance of interacting Ephs and ephrins defining the final position, but also by their crosstalk with other signalling systems [89–91]. Together, these multifaceted interactions facilitate accurately controlled segmentation and tissue boundary formation—processes that are vitally important during most developmental patterning programs [14, 92].

Cell sorting and boundary formation

One of the earliest studied developmental mechanisms of Eph/ephrin-mediated cell-cell segregation restricting cell movement and defining tissue compartments is boundary

formation between zebrafish rhombomeres: during this developmental stage Ephs and ephrins are expressed in complementary segments, and their bi-directional signalling maintains boundaries between even and odd rhombomeres [16, 93]. This biological concept has been replicated in vitro in models of cell-cell segregation using zebrafish blastomeres [92] and more recently in mammalian cell co-cultures of Eph- and ephrin-expressing cells [56, 62, 94]. Also during mouse embryogenesis, EphB2 and EphB3 interactions with ephrin-B2 in developing colonic crypts mediate sorting and positioning of epithelial cells along the crypt-villum axis [95]. Furthermore, Eph/ephrin interactions were shown to restrict intermingling between ephrin-positive astrocytes and Eph-expressing fibroblastic meningeal cells [96] or Schwann cells [97]. Interestingly, during nerve wound repair EphB2-mediated segregation of Schwann cells and fibroblasts relies both on repulsion between the two cell types as well as tight adhesion of Schwann cells within their collective [98].

A number of pathways are considered to be vital for cell-cell segregation and maintenance of boundaries, including inhibition of gap junction communication [92, 99], MAPK activation downstream of EphB2 signalling [62] and Wnt pathway-dependent RhoA activation [37]. Indeed, an siRNA screen for proteins required for EphB2/ephrin-B1-mediated cell sorting yielded 200 targets, roughly a quarter of which displayed altered levels of tyrosine phosphorylation [56]. Amongst these were known regulators of the cytoskeleton, endocytosis, cell adhesion and migration and cell polarity, highlighting the importance of a range of cellular processes in Eph-mediated cell segregation [56], which will be discussed below. In addition to these roles in cell positioning and sorting Eph/ephrin signalling is also implicated in mesenchymal-to-epithelial transition (MET) and endothelial-to-mesenchymal transition (EMT), which are essential cell-cell communication mechanisms involved in establishing identity and well-defined boundaries between endothelial/epithelial and mesenchymal tissues during somitogenesis [100–102] and heart development [103, 104].

Axon guidance and neural patterning

Amongst the receptor/ligand families implicated in axonal guidance, Ephs and ephrins constitute the most prevalent system orchestrating topographic mapping and neural patterning during early embryonic development [83, 105]. Eph/ephrin function is essential in many functional domains within the developing central nervous system, such as the retinotectal [106] and the corticospinal tract [107]. Thus, in the vertebrate visual system, ephrin-A ligands allow outgrowth of EphA-expressing retinal axons when present in low doses, but block extension at higher concentrations [89, 90, 108, 109], a system regarded as a paradigm for the establishment of topographic maps [88, 91, 105]. In accord, gene knock-outs confirmed ephrin-A2 and ephrin-A5 as essential for guidance cues for retinal axons in mammals [106, 110], which also regulate axon branching of retinal ganglion cells [111]. Ephrin-B3-induced EphA4 forward signalling is vital for corticospinal tract axon guidance, connecting brain and spinal cord [107, 112, 113].

Eph/ephrin controlled cell-cell segregation is essential for neural crest cell migration [114–116] and normal skeletal morphogenesis, and deletion of ephrin-B1 or mutation in its PDZ binding domain results in defects in neural crest derived tissues [117] and in the corpus callosum, a major commissural axon tract [118], leading to perinatal lethality due to skeletal defects and incomplete body closure [119]. Importantly, repulsive Eph/ephrin interactions between motor and sensory projections during neuromuscular circuit assembly prevent their intermingling, thereby ensuring the establishment of tightly associated yet functionally discrete peripheral nerve pathways [120].

Eph and ephrin function are also essential for synaptogenesis and synapse maturation [121, 122]. EphBs and ephrin-Bs are implicated in pre- and post-synaptic specification during formation of synapses affecting the stabilisation of initial axon/dendrite contacts, formation of postsynaptic specialisations and promotion of filopodia motility [82, 123, 124] and function of NMDA receptors [78, 81, 125]. For example, excitatory synapse maturation involves dendritic spine morphogenesis that requires EphB forward [126–128] and ephrin-B reverse [129] signalling in hippocampal neurons, while EphA4 activation triggers dendritic spine retraction [130]. Transsynaptic EphB/ephrin-B signalling also regulates structural plasticity of presynaptic nerve terminals [131].

Vasculogenesis and angiogenesis

Not surprisingly, axonal growth cones and capillary tip cells use common repulsive and attractive signals that determine directional guidance of their position [132]. Significant evidence suggests that B-type Ephs and ephrins, especially interactions between EphB2/B4 and ephrin-B2, play pivotal roles in the development of the embryonic cardiovascular system [133–137]. The prominent expression of ephrin-B2 on embryonic arteries and its cognate receptor EphB4 on veins, together with prominent and lethal vascular defects in knock-out mice lead to the concept that reciprocal signalling at the boundaries between these two types of vessels prevents arterial and venous endothelial cell intermingling [132, 138]. In agreement, deletion of full-length ephrin-B2, its intracellular domain or EphB4, although displaying differential effects on neural

crest cell migration, all cause similar defects in the remodelling of the primary capillary plexus, inhibit the formation of major embryonic vessels and cause embryonic lethality [133–136, 139, 140]. Recently, a different model challenged this view of vasculogenic assembly, proposing the formation of a precursor vessel that segregates to form the dorsal aorta and the cardinal vein—a process that again is critically dependent on signalling between EphB4 and ephrin-B2 [141]. There is evidence that EphB4 forward signalling directs these functions in early vascular development, while ephrin-B2 reverse signalling is required for later processes, such as cardiac valve formation [142]. While EphB4 seems to play a predominant role in vascular patterning, a combined knock-out of EphB2 and EphB3 has a similar phenotype, however, with only 30% penetrance, suggesting the integrated activity of additional Eph receptors [133]. Notably, EphB2 and EphB4 appear to co-operate with SDF-1 chemokine signalling during endothelial cell migration to form capillary-like structures [75].

In contrast there is only limited evidence for A-type Ephs and ephrins during cardiovascular development, the exception being EphA3 and ephrin-A1, expressed during embryogenesis on major blood vessels and heart valves [103, 104]. Their interaction is essential for segregation between the endothelial lining and cardiac jelly, trans-differentiation of endothelial to mesenchymal cells and their directed migration to form the endocardial cushion critical for proper heart septation [103]. However, emerging evidence suggests important roles for EphA receptors in physiological and pathological neo-angiogenesis, with EphA2 functioning in endothelial cell migration during post-natal vascular assembly in mice, and being expressed in endothelial cells in tumours [143, 144] (below).

Since Eph/ephrin- and VEGFR-signalling both control blood vessel growth and arterial differentiation, it is perhaps not surprising that functional interactions have also been reported. Two recent reports demonstrated that ephrin-B2 regulates VEGFR internalisation and thus critically affects VEGFR function during angiogenesis [145, 146]. Altered expression of ephrin-B2 causes severe vascular defects during VEGF-induced angiogenesis [146]. Moreover, ephrin-B2 PDZ signalling-deficient mice lack regulation of VEGFR2 signalling, thus displaying reduced vascularisation in brain and retina as well as within tumours [145]. Intriguingly, ephrin-B2 appears to directly activate VEGFR2, even in the absence of VEGF-A [145].

Interaction of endothelial cells with supporting cells

In addition to orchestrating the interaction between endothelial cells during vessel assembly and angiogenesis, Eph/ephrin interactions also guide the communication with vascular smooth muscle cells and pericytes that are essential to stabilise arteries, and to a lesser extent veins, and contribute to the maintenance of mature, non-angiogenic vessels once angiogenesis is completed [132]. EphrinB2 is expressed on vascular smooth muscle cells and pericytes [147, 148], and directs the interactions between endothelial and mural cells that are essential for the stability of blood-perfused vessels [140, 149]. Conditional gene targeting of ephrinB2 on pericytes and vascular smooth muscle cells leads to defective mural cell coverage and in leaky vasculature, a defect that is typical also for chronic diseases, such as diabetes and cancer [150]. EphrinB2 and EphB2 are also expressed in the surrounding mesenchyme [133], and ephrin/Eph interactions between mesenchymal and endothelial cells [151] may help differentiate mesenchymal cells into perivascular supporting cells. Finally, EphB4/ephrinB2 interactions are also thought to mediate monocyte adhesion and transmigration through the vascular endothelium [152].

Lymphangiogenesis

Lymphangiogenesis occurs predominantly during embryonic development, but also has a documented role in tumour metastasis and, similar to blood vessel morphogenesis, involves EphB4/ephrinB2 and VEGF signalling [153]. Interestingly, whilst mice lacking the PDZ motif of ephrinB2 show normal blood vasculature development, they display severe postnatal lymphangiogenesis defects due to inhibition of endothelial cell sprouting from a primitive plexus [154], which could be explained by the ephrinB2-mediated inhibition of VEGFR3 internalisation [146].

Eph receptors in tumour progression and metastasis

Increasing interest in the Eph/ephrin signalling system stems from its documented importance in a wide range of epithelial and mesenchymal tumours [2, 6], indeed most—if not all—family members were initially identified in tumour cell lines [7]. Eph cell guidance functions normally active during embryogenesis re-emerge unscheduled and often de-regulated in tumours, modulating cell-cell and cell-matrix attachment and survival during invasion, neoangiogenesis and metastasis [2, 7, 155]. Eph overexpression often correlates with more aggressive, invasive and metastatic phenotypes and poor prognosis. An example is melanoma, where expression of Ephs correlates with increased tumour progression and invasive potential [156–158], and primary melanomas express significantly less EphA2 than distant metastases [159]. Similarly in breast cancer, EphA3 reportedly is highly expressed in lymph

node metastases but not in the primary tumour [160]. Also EphA2 is highly elevated in breast cancer [144, 161] and associated with poor prognosis [68, 162]. Over-expression of EphA2 in the non-transformed mammary epithelial cell line MCF10A induces anchorage-independent growth, increased invasiveness in matrigel and tumour formation in nude mice [161]. EphB4 is also overexpressed in breast cancer [163] and suggested to be causally involved in its progression [164]. Tumour-promoting roles of EphB4 and EphA2 have been confirmed in MMTV/Neu breast cancer mouse models, where EphB4 overexpression appears to enhance tumour initiation and lung metastasis [165], while loss of EphA2 has the opposite effect [68]. Furthermore, co-clustering of EphA2 with ErbB2 was reported to enhance Ras-MAPK and RhoGTPase signalling [68]. In addition to melanoma and breast cancer, Eph expression is elevated in a wide variety of cancers, including colon, lung, kidney, ovarian and prostate cancers, sarcoma and neuroblastoma [7].

Tumour neo-angiogenesis

As in embryonic development, Ephs and ephrins play important roles in neovascularisation during tumour development. Interacting ephrin-A1 and EphA2 [144, 166], as well as ephrin-B2 and EphB4 [147, 148] are overexpressed in tumour vasculature, where the ligand commonly marks endothelial cells. Thus, while EphA2 is not expressed on embryonic vasculature [167], and EphA2$^{-/-}$ mice do not show vascular defects during embryonic development [143, 168], ephrin-A1 and EphA2 are prevalent in the vasculature of tumour xenografts [144]. Administration of soluble EphA2-Fc or EphA3-Fc inhibits tumour growth and endothelial cell migration in different in vivo models [32, 166, 169], while similarly EphA2 or ephrin-A1 deficiency reduces tumour angiogenesis [170, 171]. In addition, EphA2 activation on vascular smooth muscle cells might also induce the retraction of perivascular supporting cells via the inhibition of Rac/PAK (see below), thus allowing endothelial cells to respond to angiogenic cues [172]. Moreover, EphA2 was identified as an important mediator of melanoma vasculogenic mimicry [158].

Ephrin-B2 and EphB4 are also prominent in tumour angiogenesis. Ephrin-B2 expression, which in cultured human microvascular and arterial endothelial cells is induced by VEGF, bFGF and HGF [173], is up-regulated at sites of neovascularisation, such as tumours and wounds [150]. In mouse models of ischemia, ephrin-B2-mediated activation of EphB4 enhances the pro-angiogenic activity of endothelial cells [174, 175], and EphB4 expression on tumour cells increases angiogenesis by recruiting ephrin-B2-positive endothelial cells [176]. By contrast, in a retinal model of postnatal angiogenesis, EphB4 overexpression resulted in larger blood vessels, disorganised branching and reduced vascular permeability via inhibition of the angiopoitin-1/Tie2 pathway due to increased ephrin-B2 reverse signalling [177]. De-regulated EphB4/ephrin-B2 function in endothelial cells has also been implicated in Kaposi sarcoma [178] and hepatocarcinoma [179]. Interestingly, manipulation of ephrin-B2-signalling using the EphB4 extracellular domain fused to human serum albumin led to reduced blood vessel density, reduced pericyte recruitment and increased hypoxia, similar to effects seen with anti-VEGF treatment, whereby concurrent ephrin-B2- and VEGF-signalling blockade indeed showed a synergistic effect [180].

Role in tumour suppression

Interestingly, Ephs are not only implicated in tumour promotion but, depending on the context, are considered as tumour suppressors. Thus, in contrast to roles of EphA2 in tumour promotion, EphA2 knock-out mice show increased susceptibility to carcinogen-induced skin tumours and their invasive malignant progression [181]. Similarly, EphB4 can elicit promotion or suppression of tumour growth [164, 176, 182–184]: while it is found up-regulated in colon and endometrial cancers [185, 186], its loss is correlated with more invasive cancers of both colon [182] and breast [187]. One possible explanation for this dichotomy is that an initial increase in Eph receptor expression and function is followed by epigenetic silencing or modulation of function as the tumour progresses [6]. Thus, expression of EphA7 in colorectal and prostate cancers is frequently suppressed by promoter hypermethylation [188, 189], and re-expression in DU145 prostate cancer cells inhibits colony formation [188]. In accord, hypermethylation of EphB6 in breast cancer [190], of EphB2 in colorectal cancer [191] and of EphA3 [192] and almost all other Eph and ephrin family members in acute lymphoblastic leukaemia [193] has been reported.

Such a two-step model of Eph expression is best exemplified in colorectal carcinoma (CRC) where EphB receptor expression is elevated in intestinal adenomas [95, 185], but is lost during progression to a carcinoma and the initiation of invasive growth [182]. Similarly, EphA1 is overexpressed in earlier stages of CRC, but down-regulated in further advanced carcinomas, a trend that is also associated with poor survival [194, 195]. Gain-of-function mutations in the Wnt pathway are strongly implicated in the initiation of CRC and at the same time responsible for up-regulated EphB expression [196]. Mechanistically, EphB signalling may suppress cancer progression at this stage by regulating adherens junction formation and promoting compartmentalisation of colon carcinoma cells

[197], although in parallel contributing to adenoma growth via Abl-cyclin D1 proliferative signalling [198]. Loss of EphB receptor expression or activity during CRC progression leads to uncoupling of EphB function from cyclin D1 signalling and results in increased tumour invasion while maintaining a high proliferation rate [182, 183, 197, 198]. Recent evidence now also suggests that EphB2 marks tumour-initiating intestinal stem cells in aggressive CRCs potentially contributing to disease relapse [199].

Interestingly, EphA7 has recently been implicated as a tumour suppressor in follicular lymphoma [200]. Normal B lymphocytes express truncated, soluble EphA7, which can inhibit the activity of other expressed Eph receptors, notably EphA2, apparently by direct interaction, although competition for ligand binding may also occur. Frequent loss of EphA7 in this context due to methylation is suggested to promote EphA2 signalling and thus oncogenesis [200].

Kinase active versus inactive Eph oncogenic function

In addition to stage-dependent changes of expression it is likely that ephrin- or Eph kinase-independent functions may play a significant part in their tumour-promoting role; this notion reflects several reports suggesting that Eph activation by agonists attenuates malignant cell phenotypes and that effective bi-directional signalling may not be prevalent in a cancer setting [6, 161, 201, 202]. Consistent with this, Eph receptor overexpression can be associated with loss of cognate ligand expression, as exemplified by the expression pattern of EphA2 and ephrin-A1 in glioblastoma and breast cancer cells [190, 203]. Interestingly, in mouse xenografts, treatment with the recombinant EphB4 ligand ephrin-B2 inhibited tumour progression and breast cancer cell migration via the Abl-Crk pathway [176], suggesting a tumour promoter role of EphB4 in this model that is ligand-independent or requires low ligand levels while high EphB4 kinase activity inhibits tumour progression. Furthermore, overexpression of kinase-dead EphB4 in breast cancer cells promotes tumour vascularisation by inducing recruitment, survival and proliferation of ephrin-B2-positive endothelial cells [204]. The notion of both kinase-dependent and -independent Eph functions is of particular relevance given that somatic mutations likely to affect Eph signalling activity are emerging as prevalent in a wide variety of cancers.

Somatic mutations

Recent cDNA array screening approaches revealed that somatic mutations in Eph receptor RTKs occur frequently in a variety of cancers. For instance, frame shift mutations of EphB2 were found in 41% of colorectal and 39% of gastric tumours with microsatellite instability [205, 206], and somatic mutations were detected in a total of 11 Eph receptors in a set of breast, lung, ovarian and prostate cancer samples, where most of the mutations were in lung cancer [207]. Moreover, a genome-wide screen of diagnostic somatic mutations in breast and colon cancers emphasised Eph family members, particularly EphA3, among the most frequently mutated genes in colon and lung cancer [208, 209], and Ephs were also reported as a candidate cancer genes in glioblastoma, melanoma and pancreatic carcinoma [210, 211]. Furthermore, a recent study in breast cancer patient samples, investigating a potential link between susceptibility loci and somatic mutations of ‘driver kinases’, identified SNPs in EPHB1, EPHA3 and EPHA7 genes as cancer risk alleles [212].

Typically, the somatic Eph mutations identified in these screens do not tightly cluster in a specific region but are widely distributed in diverse domains. However, as exemplified by EphA3 mutations, many of these lie in regions regulating receptor clustering and activity, including kinase and extracellular domains, and may alter cellular responses, for example to promote adhesive, rather than repulsive, signalling. Accordingly, EphA3 and EphA5 somatic mutations in lung cancer samples were detected within the activation loop or in the transmembrane domain [213], and EphA3 was one of seven tyrosine kinases identified in CRC harbouring kinase domain mutations [214]. Interestingly for EphB6, a family member lacking a functional kinase, frequent somatic mutations [215] and a germline sequence variant [216] were also found in CRC samples. While the effects of these mutations on kinase activity/ligand binding and downstream responses remain to be elucidated, some indications exist for two EphB2 mutations: The G787R variant found in rare cases of hereditary colorectal cancer inhibits kinase activity [217] while the non-sense EphB2 Q722X mutation suggested to promote prostate cancer progression results in truncation and kinase inactivation [218, 219]. Thus Eph somatic mutations likely result in change of function, rather than loss of function mutants, as with classical tumour suppressors.

How does Eph signalling switch cell-cell adhesion to retraction?

The roles of Ephs and ephrins in the diverse developmental programs, and in cancer, described above rely primarily on their control of cell navigation and patterning, by modulating cell-cell adhesion and de-adhesion, or retraction. Whether Eph receptor signalling results in adhesion or retraction is determined by various parameters, including Eph and ephrin cell surface densities, composition of

signalling clusters, disruption and internalisation of Eph/ephrin complexes, participation of various cytoskeletal- and membrane-associated regulatory molecules, and cross-talk with other cell signalling systems.

The importance of kinase activity

While ample evidence supports the notion that Eph-ephrin contact results in cell contraction, de-adhesion and segregation [3, 220], Eph/ephrin interactions can also promote increased cell adhesion. As a prominent example, co-expression of a cytoplasmic-truncated EphA7 splice variant suppresses the phosphorylation of the full-length EphA7 and shifts the response of migrating cells from ephrin-A5-mediated cell-cell segregation to adhesion, an essential event during embryogenesis that induces fusion of the neural tube [221]. Demonstration of this developmentally regulated expression and dominant negative function of the truncated kinase-dead receptor confirms the essential role of this Eph kinase-controlled switch between adhesive and de-adhesive cell-cell interactions. Other examples include the fusion events occurring during tabularisation of the urethra, and the partition between urinary and alimentary tracts that are implemented by EphB/ephrin-B-facilitated cell adhesion [222]. Further kinase-independent roles have been described based on the ability of kinase-dead receptors to functionally compensate for loss of wild-type receptors in knockout mice. For example, kinase dead EphA4 can rescue some but not all aspects of neural development [112], and similarly kinase dead EphB receptors can compensate for defective retinal axon path-finding in EphB2/B3 null mice [223], or interact with NMDA receptors to promote synapse formation [78]. EphB2 kinase-dependent and -independent roles are also reported in the intestinal epithelium, where enhanced proliferation is communicated kinase-dependently through Abl-cyclin D1 signalling, while cell positioning is mediated kinase-independently via the phosphatidylinositol 3′ -kinase (PI3K) pathway [198]. PI3K was previously found to promote integrin-mediated adhesion downstream of EphA8 but autonomous of Eph kinase activity [224].

Consistent with the notion of this switch in function, Eph receptors can promote cell adhesion and de-adhesion within the same cell type depending on the density of the interacting ephrin. It has been demonstrated early on that a certain ephrin concentration threshold is required for HEK293 cells to respond with retraction, while in the same cells low ephrin density promotes integrin-mediated ECM attachment [225]. In agreement, ephrin-A2 stimulation enhances axon outgrowth at low concentrations but inhibits it at higher concentrations [226], while kinase-deficient EphB6 signals cell adhesion and migration at low ephrin-B2 concentrations, and retraction and inhibited migration at high concentrations, where it is phosphorylated by the associated Src family kinase Fyn [227]. Not only ligand concentration seems to be a determining factor but in addition also the total amount of displayed ligand, as demonstrated in a quantitative analysis of axon outgrowth [228].

The examples above all suggest a reoccurring theme where active kinase signalling, initiated by sufficient ligand levels, is required for Eph-controlled cell de-adhesion [229]. This concept is substantiated by the observation that high Protein Tyrosine Phosphatase (PTP) activity, controlling tyrosine kinase activation and phosphorylation, reverses retraction of an Eph-expressing cell from an ephrin neighbour and causes cell adhesion; in the case of EphA3-positive leukaemia cells PTP inhibition reverses adhesion to an ephrin-A5 surface [230]. Substantial further evidence attests to PTP-regulation of Eph receptor signalling: PTP-RO controls the sensitivity of retinal axons to ephrin stimulation by negatively regulating EphA4 receptor phosphorylation [231], while EphB2 activation is controlled by the leukocyte common antigen related receptor tyrosine phosphatase (LAR-1) [62]. Furthermore the prototypical phosphatase PTP1B regulates EphA3, and probably EphB2, activation and trafficking, and modulates EphA3-driven cell contraction and segregation [94].

Since Ephs are often co-expressed during normal development and in tumour cells, it is likely that the sum of adhesive and repulsive cues that are determined by the overall Eph receptor expression pattern on one cell relative to ephrin expression patterns on opposing cells relays the response resulting from their interaction. In prostate cancer cells, co-expression of EphA and EphB receptors can mediate either repulsion (inhibiting migration) or failure of repulsion (allowing migration), depending on the relative ligand density on the interacting fibroblasts [232]. Furthermore, co-clustering of EphA and EphB receptors results in signalling from both receptor types not requiring the presence of both ligands and with outcomes depending on the relative receptor expression, such that high expression of a kinase inactive EphA receptor can block EphB-mediated signalling, cell rounding and cell-cell segregation [57].

Disruption of Eph-ephrin complexes allows cell-cell repulsion to proceed

Eph/ephrin interactions form large clusters of high interaction avidity, which physically tether receptor- and ligand-expressing cells; this demands that cell repulsion can only proceed if this tether is disrupted. A number of studies now indicate a prominent role for the transmembrane metalloprotease ADAM (a disintegrin and a metalloprotease) 10, which sheds the extracellular domain

of a number of ligands and/or receptors, and is also involved in TNF, Notch and EGF/ErbB signalling pathways, amongst others [233]. ADAM10 cleaves A-type ephrins following Eph receptor activation [234], controlled by association of ADAM10 with the Eph receptor, and specific binding and cleavage of the Eph-bound ephrin in *trans* (i.e. from the adjacent cell) [235]. Co-clustering of ADAM10 with EphAs relies on ephrin-induced Eph clustering [236], while ephrin cleavage is regulated by a conformational change that occurs upon Eph activation, where the inhibitory interaction between the juxtamembrane and kinase domains is released (Fig. 2, [48]): this promotes a productive association with ADAM10 by overcoming steric hindrance by the raised kinase domain in the inactive conformation. Thus, a receptor with an open, activated conformation promotes ADAM-mediated ephrin cleavage irrespective of kinase activity, as does a receptor lacking the entire intracellular domain [237]. Such a mechanism explains previous conflicting evidence for an intact function of ADAMs lacking their cytoplasmic domain [237, 238], despite the well-established dependence of ADAM-mediated shedding of a number of cell surface proteins on intracellular kinase activity [233, 239], and may indicate a broadly applicable mechanism in particular for shedding of RTK ligands.

Interestingly, as in Notch and ErbB signalling, ADAMs appear to shed both receptors and ligands in regulating Eph signalling, but under different conditions. Thus, whereas cell-bound ligand induces ADAM-mediated ligand cleavage and receptor endocytosis, stimulation of calcium influx in the absence of ligand is thought to induce ADAM-mediated EphB2 extracellular domain shedding, followed by γ-secretase cleavage of the intracellular domain to release a cytosolic fragment capable of regulating NMDA receptor signalling [240]. These sequential cleavage events, first established for Notch1 and cadherin [241], can also be applied to ephrin-B1 and ephrin-B2, where shedding of the extracellular domain, which at least in some cases is mediated by ADAM13 [242], is subsequently followed by γ-secretase cleavage of the short intracellular domain [243, 244]. The released intracellular domain might function in ephrin-B reverse signalling, in particular in Src-family kinase activation [243]. Also EphA4 is cleaved by γ-secretase with the resulting released intracellular domain being important for Rac activation in dendritic spine formation [245]. Roles for other proteases in Eph/ephrin signalling have also been described, including cleavage of ligand-activated EphB2 by matrix metalloproteases MMP-2/MMP-8 [246], and cleavage within the transmembrane domain of ephrin-B ligands by the rhomboid serine protease RHBDL2 [247].

Protease-independent disruption of Eph/ephrin-mediated cell contacts has also been reported, implying 'trans-endocytosis' of the entire complex. Interactions of ephrin-B- and EphB-expressing cells lead to endocytosis of phosphorylated EphB primarily into receptor-expressing cells, but to a lesser extent also into ligand-expressing cells: detection of the ephrin or Eph cytoplasmic domain in respective opposing cells was thus interpreted as trans-endocytosis of the whole complex into either cell [248, 249]. This internalisation of either ligand or receptor into the 'parental' cell was dependent on Rac and cytoskeletal signalling and on an intact cytoplasmic domain: intracellular truncation of either ligand or receptor enhanced its uptake into the adjacent cell, while truncation of both blocked endocytosis and cell retraction, promoting

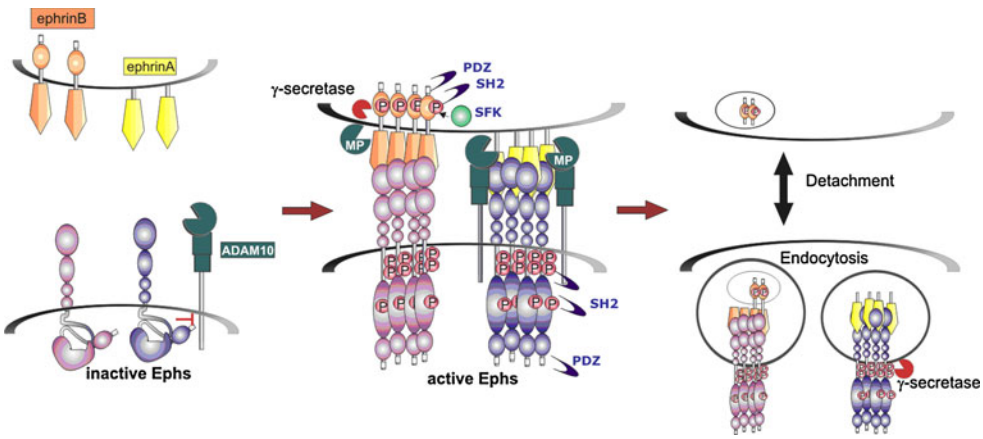

Fig. 2 Disruption of Eph/ephrin cell-cell interactions during cell-cell segregation. Engagement of Ephs and ephrins results in large, high affinity complexes that require disruption to allow cell-cell detachment. For ephrin-As this occurs through ephrin cleavage by the ADAM10 metalloprotease, which recognises the Eph/ephrin complex to release ephrin from the adjacent cell. ADAM10 association with EphAs is promoted by clustering and conformational change of the Eph receptor, to stimulate ephrin shedding. Metalloprotease (MP) cleavage of ephrin-Bs has also been described, as has γ-secretase-mediated release of the intracellular domain of both ephrin-Bs and Ephs. Alternatively, transendocytosis of the entire EphB/ephrin-B complex has also been described [248, 249]

adhesion [248, 249]. Thus, while different mechanisms may exist to achieve disruption and endocytosis of Eph-ephrin complexes at cell-cell junctions, there is a common dependence on intracellular, kinase-dependent signalling controlling endocytosis, necessary for cytoskeletal reorganisation and cell retraction.

Eph-regulated adhesion dynamics

Eph-cadherin crosstalk in cell-cell adhesion

In addition to the direct consequences of Eph/ephrin interaction, cross-talk of Ephs with adherence molecules is critically implicated in Eph-modulated cell-cell adhesion. Cadherins are responsible for homotypic and heterotypic cell-cell adhesion and also involved in cell sorting, and it is likely that the combined action of cadherin-mediated adhesion and Eph/ephrin signalling directs the final outcome during boundary formation, as was demonstrated for segmental patterning of neural-crest-derived sympathetic ganglia [250]. Likewise, spreading of EphB-expressing colorectal tumour cells into ephrin-B1-positive surrounding epithelial tissues is restricted by a compartmentalisation mechanism that relies on both EphB and E-cadherin function [197]. Moreover, EphB2-mediated collective cell migration during early nerve regeneration involves Sox2-dependent N-cadherin redistribution to cell-cell contacts [98]. Loss of E-cadherin expression leads to weakened cell-cell contacts and coincides with an altered Eph/ephrin expression pattern in embryonic stem cells [251], and alters EphA2 membrane localisation in mammary epithelial cells, embryonic stem cells and aggressive melanoma cells during vasculogenic mimicry [201, 251, 252]. Correspondingly, inhibition of β-catenin/TCF-mediated transcription downstream of E-cadherin resulted in down-regulation of EphB2 and EphB3, while ephrin-B1 was up-regulated [95, 253]. Interestingly, at EphB/ephrinB epithelial boundaries ADAM10 sheds colocalised E-cadherin, allowing segregation of the two cell populations while relative adhesiveness within the populations is increased [254]. Thus, de-regulated Eph-cadherin crosstalk might contribute to the tumorigenic potential of Eph/ephrin-positive cancer cells through alterations of cell-cell adhesion [255, 256].

Ephs and integrin-mediated cell-matrix adhesion

A variety of studies show that Eph receptors can affect extracellular-matrix (ECM) adhesion and migration by modulating integrin signalling, although both positive and negative effects have been described (summarised in Table 1). This is possibly due to distinct effects depending on the strength of ephrin-Eph interactions, since increasing ephrin density results in a biphasic pattern of cell attachment on ECM proteins, enhancing attachment at lower concentrations and inhibiting at higher ligand density [34, 225]. This is consistent with changes from adhesion to de-adhesion at higher Eph receptor activation levels, promoting cytoskeletal collapse rather than cell spreading. In accord with this (kinase-dead) EphA8-enhanced adhesion is kinase-independent [224], while EphB3 inhibition of integrin-dependent adhesion is kinase dependent [257]. Eph-mediated inhibition of integrin activity involves changes in integrin conformation and clustering [258, 259], as well as modulation of focal adhesion signalling (below). Also, EphA2 was recently reported to promote cancer cell migration through ECM in a ligand-independent manner via Akt-mediated serine phosphorylation, which was opposed by ligand-induced tyrosine phosphorylation [260]. In addition, EphB4 expression was reported to inhibit cell-substrate adhesion and migration by down-regulating β1-integrin protein levels in breast cancer cells [40]. Lastly, a direct interaction between fibronectin and EphA1 was shown to promote cell-spreading and endothelial tube formation in an Eph-kinase-independent manner [261].

Ephs and modulation of focal adhesion components

An essential event in ECM detachment and cell migration is the initiation of focal adhesion turnover and the involvement of focal adhesion modulators such as FAK, paxillin, p130Cas and Src-family kinases (SFKs) during Eph signalling is widely acknowledged (see summary in Table 2). Although evidence is conflicting as to whether activation of these proteins is up- or down-regulated upon ephrin stimulation, in general their involvement correlates with adhesive or de-adhesive cell responses. For instance, FAK and p130Cas were found phosphorylated in stimulated EphA2-expressing murine fibroblasts and are required for ephrin-A1-induced cell spreading [31]. Likewise, FAK and paxillin phosphorylation was observed during adhesion of LK63 leukemic cells to ephrin-A5 [230], while in PC3 cells FAK phosphorylation and association are lost upon EphA2 stimulation, concurrent with cell contraction [259]. However, decreased FAK phosphorylation observed in colorectal cancer cells seems to be transient and is recovered within 10 min after stimulation [95], and a recent phospho-proteomics screen confirmed increased paxillin and FAK phosphorylation in EphB2-expressing cells interacting with ephrin-B1-expressing cells [56].

Eph-directed reorganisation of the actin cytoskeleton

Eph-mediated control of cell-cell adhesion and repulsion depends not only on interactions at cell-cell contacts but

Table 1 Eph signalling regulates integrin activity

Eph	Integrin	Effect	Cell/tissue type	Via	References
EphA					
EphA2	β1	Adhesion to FN ↓ (or laminin)	PC3	SHP-2; FAK	[259]
EphA4			Hippocampal neurons	Fyn/Crk-p130Cas- Pyk2	[258]
EphA			PC3	Abl- CrkII-Rap1, RhoA-ROCK	[263]
EphA	β1	Migration on laminin↓	Schwann cells	FAK, Vav2	[97]
EphA	α5β1	Adhesion to FN ↑ (or laminin/ fibrinogen)	Rat VSMCs	Rac-PAK	[172]
EphA8			NIH3T3, HEK293	PI3K; Eph kinase independent	[224]
Eph(A2)	β1		Dendritic cells		[358]
EphA4	αIIbβ3		Platelets		[342]
EphB					
EphB1	αvβ3 α5β1	Adhesion ↑/↓	P19 terato-carcinoma; endothelial	Ligand density-dependent	[225]
EphB2	α4β1 α5β1	Adhesion to FN ↓ (or laminin)	HEK293	Ras	[302]
EphB(3)			LS174 CRC cells; HEK293	Cdc42, Rac1	[257]
EphB4	β1		MDA-MB-435; MCF7	Ligand-independent	[40]
EphB1		Adhesion to fibrinogen ↑	P19 terato-carcinoma	Nck-NIK-JNK	[359]

FN fibronectin, *VSMCs* vascular smooth muscle cells

Table 2 Change in focal adhesion (FA) modulators during ligand-stimulated Eph signalling

Eph	FA modulator	Effect	Cell/tissue type	References
EphA				
EphA2	**PY-FAK** ↓	Adhesion ↓ migration ↓	PC3	[259]
EphA4	PY-paxillin ↓		Hippocampal neurons	[258]
EphA	PY-Pyk2 ↓ PY-p130Cas ↓		Schwann cells	[97]
EphA2	**PY-FAK (Y576/577)** ↑ PY-Src ↑	Motility ↑[a]	Melanoma cells	[360]
		Adhesion ↓	PC3	[361]
EphAs	**SFKs**		Retinal neurons	[287]
EphA2	**PY-FAK** ↑ **PY-paxillin** ↑ **PY-p130Cas** ↑	Adhesion ↑	MEFs, NIH3T3	[31]
EphB				
EphB2 EphB3	**PY-FAK** ↓ **PY-paxillin** ↓	Adhesion ↓	LS174T CRC cells	[95]
EphB2	**PY-FAK** ↑ **PY-paxillin** ↑ PY-Src ↑		Hippocampal neurons	[362]
		Dendritic spine morpho-genesis/ maintenance		[272] [273]
EphB1		Migration ↑	P19 terato-carcinoma, CHO-EphB1, (endothelial)	[267]
	PY-Shc/Src ↑	Chemotaxis towards ephrin-B2 ↑		[363]

If not noted otherwise PY-FAK refers to Y397, PY-Src to Src Y418 (m)/Y416 (h) and PY-paxillin to paxillin PY31

FA modulators in bold apply to all references in groups

[a] Effect caused by receptor overexpression, ligand-independent

also on global control of their cytoskeletal function, via adaptor proteins and small GTPases that regulate actin reorganisation (for overview see Fig. 3). For example, the SH2/SH3 adaptor proteins of the Crk family, which have a well-documented function in cell migration, adhesion and actin reorganisation, are involved in the regulation of cell repulsion and membrane ruffling during Eph signalling [33, 262]. CrkII recruitment to ligand-activated EphA3 and ensuing RhoA signalling are required for cell repulsion in EphA3/HEK293T and melanoma cells [33], while Crk is recruited to nascent focal complexes by p130Cas and essential for Rap1-mediated stabilisation of these complexes and Rac1-induced membrane ruffling during Eph signalling in human aortic endothelial cells [262].

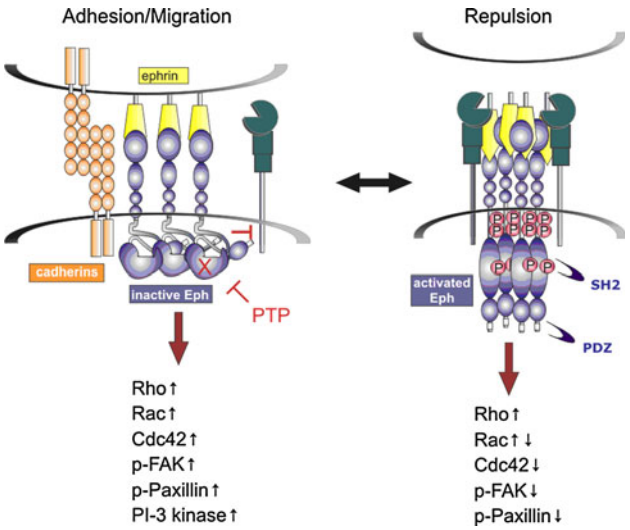

Fig. 3 Eph-mediated cell-cell adhesion versus repulsion. Cell-cell adhesion occurs under conditions of low Eph kinase activity, such as at low Eph and/or ephrin density, high protein tyrosine phosphatase (PTP) activity or inactivating Eph mutations (X). Under such conditions kinase-independent Eph signalling promotes actin-cytoskeleton-mediated cell spreading and attachment, facilitated by focal adhesions and co-operation with other adhesive molecules including cadherins. In contrast robust Eph activation at high Eph/ephrin densities promotes disruption of Eph/ephrin complexes, cell detachment, and Rho-GTPase-mediated actin-cytoskeleton collapse and cell rounding. (↑activation, ↑↓ transient activation, ↓ inactivation). In epithelial cells, ADAM10-mediated cleavage of E-cadherin also contributes to ephrin-B/EphB-mediated cell segregation [254]

Similarly, in vascular smooth muscle cells the Crk-p130Cas pathway is important for the modulation of focal adhesions and directional cell migration during bi-directional ephrin-B2/EphB signalling [140]. In ephrin-A1-stimulated PC3 prostate cancer cells, Abl-CrkII-C3G signalling inhibits Rap1 activation and cooperates with the Rho-ROCK1 pathway induced by serum factors to facilitate cell detachment [263]. Interestingly, Abl-mediated Crk phosphorylation mediates the tumour suppressor function of ephrin-B2-activated EphB4 in breast cancer cells partly by attenuating cell motility and invasion, via inhibition of Crk/p130Cas complex formation and potentially Rac1 activation [176].

Nck SH2/SH3 adaptor proteins also link RTK and other molecules like p130Cas to modulators of the cytoskeleton [264]. Nck binds to the juxtamembrane tyrosines of activated EphB and EphA receptors [265, 266] and likely recruits paxillin [267]. Selective inactivation of Nck in the developing nervous system results in similar neuronal guidance defects as seen in EphA4$^{-/-}$ mice [268], and Nck has also been shown to contribute to the inhibitory effect of EphA3 on cell migration [269]. Signalling network modelling from mass spectrometric analysis of tyrosine phosphorylation events during EphB2/ephrin-B1-mediated cell segregation also identified Nck, as well as SHC1 and SHB, another adaptor protein not previously described in Eph signalling, which likely links EphB2 with Crk, RasGAP (RASA1) and the PI3K regulatory subunit p85α (PIK3R1) [56].

Dynamic changes in Rho GTPase activity during Eph signalling

As indicated above, the Rho family GTPases—including the most commonly known members RhoA, Rac1 and Cdc42—plays a major role in Eph kinase signalling [270]. Like other GTPases, Rho GTPases act as molecular switches that cycle between an active GTP-bound form and an inactive GDP-bound form regulated by guanine nucleotide exchange factors (GEFs) elevating GTP loading and GTPase-activating proteins (GAPs) that stimulate the intrinsic GTPase activity that hydrolyses GTP to GDP [271]. Transient RhoA activation is crucial for actin contractility and disassembly to facilitate repulsion downstream of activated Eph receptors (see Table 3), and can be mediated indirectly through FAK and/or Crk [31, 33, 262, 272] or directly through GEFs (see below). RhoA commonly signals via ROCK (Rho-associated kinase) during Eph activation, and is important for EphB2/ephrin-B1-mediated cell segregation in *Xenopus* animal cap explants and dendritic spine remodelling, via LIMK-1 [37, 273, 274]. Another possible link between EphA1 and RhoA/ROCK signalling could be integrin-linked kinase (ILK), which has been suggested to interact via its ankyrin region with the SAM domain of EphA1 and modulate ephrin-induced cell contraction [275]. The ILK/PINCH/Parvin complex negatively regulates actomyosin contractility directly downstream of β-integrins [276], by which it may also affect Eph receptor signalling.

In some instances RhoA signalling alone appears insufficient for cytoskeletal contraction [172], and co-operation with Cdc42 signalling through myotonic dystrophy-related Cdc42 kinase (MRCK) may be required for full contraction [277]. In general, however, Cdc42 appears to function in opposition to RhoA, with its activity being more associated with cell adhesion and migration (Table 3): in PC3 prostate cancer cells it is thought to facilitate EphB3/4-mediated migration and lack of contact inhibition, as compared to Rho-mediated ephrin-A-stimulated retraction [232]. Previously, Cdc42 has been implicated in regulating actin polymerisation during EphB-mediated dendritic spine morphogenesis [278], while in *Xenopus* blastomere adhesion, in addition to down-regulated RhoA activity, Cdc42 function was reported to be indirectly reduced via sequestration by xPAK1 activated downstream of EphA4 [279].

In addition, Eph receptor signalling also modulates the activity of Rac1, which, after transient activation, is typically down-regulated during ephrin-stimulated cell

Table 3 Changes in activity of Rho GTPases in Eph downstream signalling

Eph	GTPase	Effect	Via	Cell/tissue type	References
EphA					
EphA4	**RhoA↑** **Rac1↓** Cdc42↓	Growth cone collapse	Ephexin-1	Retinal neurons	[286]
					[274]
EphA		CXCR4-mediated chemotaxis ↓		T-cells	[76]
		Spreading ↓ adhesion ↓		VSMCs	[172]
EphA2			LMW-PTP p190RhoGAP	PC3	[283]
EphA1			ILK	HEK293, endothelial	[275]
EphA2		Invasiveness ↑		Melanoma	[360]
		Retraction ↑ adhesion ↓ blebbing ↑		PC3	[232]
			Src/FAK		[361]
EphA3			CrkII	HEK239, melanoma	[33]
EphA4	**RhoA↑**		Ephexin-5	VSMCs	[288]
		Growth cone collapse	Ephexin-1	Retinal neurons	[285]
		Dendritic spine retraction	Cdk5-ephexin-1	Cortical/hippo-campal neurons	[130]
EphA2		Destabilisation of adherens junctions	Src-LMW-PTP-p190RhoGAP	MCF-10A	[298]
EphA3		CXCR4-mediated chemotaxis↓		Rhabdo-myosarcoma	[364]
EphA1		T-cell migration ↑	Lck-Pyk2/Vav1 PI3K	$CD8^+$ T-cells, HEK293	[365]
EphA	**Rac1↓**	HGF-induced cell protrusions ↓		MDCK	[366]
EphA4		Neurite outgrowth	α2-chimerin	Cortical neurons, COS7, HEK293, HeLa	[293] [292] [295]
		Migration ↓	β2-chimerin		[297]
EphA	**Rac1↑**	Growth cone collapse		Retinal neurons	[280]
EphA2		Neurite outgrowth	Tiam1	Cortical neurons, neuroblastoma	[291]
		Migration ↑ invasiveness ↑	PI3K	Endothelial cells	[143]
			Vav2/Vav3		[290]
			ephexin-4-RhoG ELMO2/DOCK4	MDA-MB-231, MCF7	[284]
EphA		Migration ↓	FAK, Vav2	Schwann cells	[97]
EphA	**(Rac1)**	Growth cone collapse	Vav2	Retinal neurons	[289]
	RhoA				[367]
EphA4		Adhesion ↓	Vav	Astrocytes	[368]
	RhoA↓		Grb4-PAK1	*Xenopus* blastomeres	[279]
EphA5	**Cdc42↑**	Dendritic spine maturation	Ca^{2+}-PKA-PI3K; NMDAR	Hippocampal neurons	[125]
EphB					
EphB2	**RhoA↑** **Rac1↓**	Retraction ↑ FAs ↑		Kidney medullary tubule cells	[369]
EphB2	**RhoA↑**	Sorting ↑	Dsh	*Xenopus* animal caps	[37]
EphB4		Migration ↑		Murine melanoma	[370]
EphB2		Dendritic spine morphogenesis/ maintenance	FAK	Hippocampal neurons	[272] [273]
EphB3	**Rac1↓** **Cdc42↓**	HGF-induced chemotaxis ↓ adhesion ↓		PC3	[257]
EphB4	**Rac1↑**	Adhesion ↓ ruffling ↑		Fibroblasts, endothelial cells	[248]
EphB		Dendritic spine morphogenesis	Kalirin	Hippocampal neurons	[128]
EphB2			Tiam1-(NMDAR)		[371]
	Rac1	Ruffling ↑ spreading ↑	P130Cas-Crk	Human aortic endothelial cells	[262]
EphB	**Cdc42** **RhoA**	Retraction↑	MRCK-MLC ROCK-MLC	HUVECs	[277]
EphB2	**Cdc42↑**	Dendritic spine morphogenesis	Intersectin-1	Hippocampal neurons	[278]
EphB3/4		Filopodia/migration ↑		PC3	[232]

VSMCs vascular smooth muscle endothelial cells, *HUVECs* human umbilical cord venous endothelial cells, *Dsh* dishevelled, *FA* focal adhesion
RhoGTPases in bold apply to all references in groups

retraction (Table 3), for example during RhoA activation in ephrin-A5-activated retinal ganglion cells [274]. Thus, ephrin-B1 stimulation of CRC and kidney epithelial cells resulted in Rac1/CDC42 inhibition and increased Rho signalling [257]. However, Rac is required for endocytosis and actin re-organisation during ligand-induced nerve growth cone collapse [280], suggesting that Rac1 needs to be activated, at least temporarily. Upon RTK phosphorylation, Rac1 activation stimulates NADPH-oxidase-mediated production of reactive oxygen species (ROS), which is thought to be responsible for allowing sustained phosphorylation and signalling by inactivating associated PTPs [281]. Rac-induced ROS generation is likely implicated in cross-talk between integrins and RTKs, potentiating downstream signalling and effects such as cell adhesion and spreading [282]. Thus, ephrin-A1 stimulation in PC3 cells reportedly decreases Rac activation, inhibiting integrin-induced ROS production, and indirectly leading to increased Rho activation and thus inhibited spreading [283]. On the other hand, prolonged Rac1 activation reportedly is associated with adhesion and migration, is promoting vascular assembly of endothelial cells [143] and migration and invasion of breast cancer cells [284]. The importance of Rac1 for Eph/ephrin-mediated cell segregation was confirmed by phosphoproteomics, which identified a significant number of proteins involved in regulating Rac1 activity, such as the GEF complex DOCK180/ELMO and GAPs like α- and β-chimerin [56].

GEFs and GAPs of Rho family GTPases involved in Eph signalling

As would be anticipated, GEFs and GAPs are critical in regulating Rho GTPase activities downstream of Eph receptor signalling. Ephexin-1, a member of the Dbl family of Rho family GEFs, is activated after EphA4 stimulation and leads to growth cone collapse in retinal ganglion cells [285, 286]. Tyrosine phosphorylated ephexin-1 has preferential activity towards RhoA that changes the overall balance between RhoA, Cdc42 and Rac1 activities, while in the absence of EphA4 activation, ephexin-1 activates all three Rho GTPases to promote neurite extension [285, 286]. Activation of ephexin downstream of EphA receptors might occur via SFKs during retinal axon repulsion [287] or via Cdk5 in dendritic spine retraction [130]. In addition to ephexin-1, other ephexin family members, such as the vascular smooth muscle cell-specific Vsm-RhoGEF [288] and ephexin-4 [284], have also been implicated in Eph signalling. Thus, in response to EGF stimulation, EphA2 interacts with ephexin-4 acting as a GEF for RhoG, which in turn activates Rac1 via ELMO/DOCK4 and promotes breast cancer cell migration and invasion [284]. Vav proteins, also members of the Dbl family acting downstream of many RTKs, are also implicated in ephrin-induced growth cone collapse [289] and thought to facilitate Rac1 activation during EphA2-induced angiogenesis [290]. Other GEFs connecting Eph activation and RhoGTPase/PAK1 downstream response during EphB-facilitated dendritic spine morphogenesis are the Cdc42-GEF intersectin-1 [278] and the Rac1 GEF kalirin [128], while the Rac1 GEF Tiam1 facilitates neurite outgrowth in ephrin-B1-mediated reverse and EphA2-mediated forward signalling [291].

Examples of negative Rho GTPase regulators implicated in Eph signalling include the Rac-specific RhoGAPs α- and β-chimerin: α2-chimerin binds to EphA4, is activated by ephrin-B3 stimulation and is essential for corticospinal axon guidance during the development of motor circuits [292–295]. Interestingly, loss of α-chimerin function in mice mimics the characteristic hopping gait phenotypes of EphA4 and ephrin-B3 knock-out animals [292, 293, 295]. However, α-chimerin does not seem to impact on EphA4-mediated axon guidance within the anterior commissure [296]. β2-Chimerin also binds to the kinase domains of EphA2 and EphA4, and has been proposed to act in a similar way as α2-chimerin [297]. In addition, LMW-PTP-mediated dephosphorylation of p190RhoGAP leads to RhoA activation, important in destabilisation of adherens junctions [298].

Ras family of small GTPases

Although the Ras family GTPases, including H-Ras, R-Ras and Rap amongst others, are best known as regulators of proliferation, they also have documented roles in regulating adhesion and migration, for example via integrin activation [299]. Indeed, during Eph signalling the Ras-Raf-MEK-MAPK cascade functions not only to modulate cell proliferation, but also to influence cell adhesion and axon guidance (see Table 4). For example, the down-regulation of Ras activity via p120RasGAP is required for retraction of NG108 neuronal cells [300, 301]. R-Ras phosphorylation was also suggested to be essential for EphB2 activation-induced loss of integrin-mediated adhesion [302]. Further, it was proposed that R-Ras function during EphB2 signalling is regulated through two parallel mechanisms: down-regulation of Ras activity via p120RasGAP, which is sufficient to trigger COS7 cell retraction, while both decreased Ras activation level and tyrosine phosphorylation are needed to mediate cell migration and growth cone collapse [303]. Interestingly, R-Ras was also phosphorylated and vital in promoting invasion in EphA2-positive glioma cells [304]. SHEP-1, which contains a SH2 domain interacting with activated Eph and ErbB receptors, and an inactive RasGEF domain that binds Ras and Cas proteins [305], was suggested to link Eph receptors and Ras and Rap1 [306]. Rap1 inactivation synergises with

Table 4 Eph-modulated cytoskeletal responses involve altered activity of Ras family GTPases

Eph	GTPase	Effect	Via	Cell/tissue type	References
EphA					
EphA4	**Rap1↓**	Growth cone collapse	SPAR	Hippocampal neurons	[308]
EphA		Adhesion ↓	Abl- CrkII-C3G	PC3	[263]
EphB					
EphB2	**PY-Ras↑**	Adhesion ↓ invasion ↑		NIH3T3	[302]
				Glioma	[304]
	Ras↓ PY-Ras↑	Neurite retraction	p120RasGAP	NG108	[300] [301]
		Adhesion ↓ migration ↓		COS7; hippocampal and retinal neurons	[303]
	Ras↑Rap1↓	Adhesion ↓		EphB2-overexpressing colon cells	[307]
	Rap1↑	Ruffling ↑ adhesion ↑	p130Cas-Crk	Human aortic endothelial cells	[262]

activated RhoA to signal cell repulsion of ephrinB1-stimulated EphB2-overexpressing colon carcinoma cells [307] and ephrinA-mediated growth cone collapse is facilitated by the GTPase-activating protein spine-associated Rap-GAP (SPAR) [308]. In some cases ephrin stimulation can also promote Rap1 activity, mediating adhesion rather than repulsion by stabilising nascent focal complexes in human aortic endothelial cells [262], and MAP kinase-dependent neurogenesis [309].

Ephrin-mediated reverse signalling and co-ordination with Eph forward signalling

Ephrin signalling is not as well understood as that of Eph receptors; however, recent studies identified several analogies between Eph- and ephrin-mediated cytoskeletal responses [3, 310, 311] (for a summary refer to Table 5). For example, ephrin-mediated cell retraction and adhesion have been described, and it is likely that mechanisms underlying the switch between co-ordinated cell-cell adhesion and segregation rely on intricately linked Eph forward and ephrin reverse signalling. Emerging evidence for this notion comes from quantitative mass spectrometry analysis of interacting EphB2- and ephrin-B1-expressing HEK293 cells, which revealed different networks being activated in EphB2-expressing cells, depending on the presence or absence of the ephrin-B1 intracellular signalling domain [56]. In agreement, also mutation of the C-terminal ephrin-B1 PDZ-binding motif also influences EphB2 signalling in neighbouring cells [56], implying that the response in each of the interacting Eph- and ephrin-expressing cells reflects the signalling capacity in both cells. Considering these findings it may be instructive to review the concept of 'unidirectional' Eph/ephrin signalling that has been used to describe functions of kinase-deficient Ephs, such as EphB6 lacking a functional kinase domain [53] and cytoplasmic-truncated splice variants of EphA7 [221]. Not only do these kinase-deficient Ephs continue to act as signalling hubs, typically conferring cell-cell adhesion rather than retraction, but are likely altering the signalling in interacting ephrin cells.

Further evidence for this concept of co-regulation is implicit in the need for co-ordinated endocytosis of ephrin and Eph that is facilitated by the disruption of the Eph-ephrin complex. Just as endocytosis is an important step in Eph and other RTK signalling, endocytosis of ephrins is similarly critical for cell retraction and cytoskeletal signalling [248, 249]. Ephrin-induced Eph receptor phosphorylation, which signals endocytosis of Eph clusters, at the same time signals ADAM10-mediated ephrin cleavage to facilitate Eph/ephrin internalisation, so that indeed a kinase deficient Eph blocks ephrin cleavage [237]. This would suggest that Eph kinase-dependent ephrin cleavage simultaneously may co-ordinate endocytosis of ephrin signalling complexes within the ephrin-bearing cell, to ensure coordinated induction and progression of cell-cell segregation in both cell populations.

Since ephrin signalling induces cytoskeletal changes comparable to those seen upon Eph forward signalling, involved signalling pathways are likely to be similar. Interestingly, proteomics analysis showed asymmetric tyrosine phosphorylation profiles in EphB2 and ephrin-B1 interacting cells for some but not all signalling proteins, predominantly affecting those that were shown to be essential for cell sorting [56]. Such an asymmetry is perhaps not surprising given the distinct localisation of Ephs and ephrins to different domains within the plasma membrane, as shown for EphAs and ephrin-As, that are co-expressed in spinal cord motor neurons [312]. Indeed, unlike Ephs, ephrin-Bs and ephrin-As are both harboured in detergent resistant membrane microdomains or lipid rafts, although there is some evidence that their association with these domains differs to some degree [313, 314]. Since ephrin-As are membrane-tethered via a GPI anchor and do not contain an intracellular domain like ephrin-Bs,

Table 5 Ephrin-mediated cytoskeletal dynamics

Ephrin(s)	Effect	Via	Cell/tissue type	References
Ephrin-As				
Ephrin-A5 (ephrin-A2)	**Adhesion ↑**	β1 integrin	Ephrin-A5/NIH3T3, retinal neurons, astrocytes	[334]
				[372]
		Fyn		[315]
Ephrin-A			Motor neurons	[312]
	Growth cone collapse	p75NTR; PY-Fyn↑	Retinal neurons	[336]
Ephrin-A5	**Invasion↑**	Src	NIH3T3[a]	[354]
Ephrin-Bs				
Ephrin-B1	**Adhesion ↓**	Grb4-PY-FAK↑	NG108; fibroblasts	[321]
		TAK1; MKK4/MKK7 PY-JNK↑	Ephrin-B1/HEK293	[340]
		Lck-CrkL-Rac1	Jurkat T-ALL cells	[355]
Ephrin-B3	**Growth cone collapse**	Grb4-DOCK180 Rac1↑-PAK	Hippocampal neurons	[344]
Ephrin-B1	**Adhesion ↑ migration ↑**	PY-JNK↑ αvβ3/α5β1 integrins	Endothelial	[339]
Ephrin-B2				[373]
		β1 integrins	Melanoma	[338]
Ephrin-B2 (ephrin-B3)	**Migration ↑** invasion ↑	PY-Src↑; PI3K	Endothelial	[346]
		RhoA/ROCK; JNK, actomyosin-dependentc		[325]
		Rac1↑	Glioma cells	[345,356]
Ephrin-B1	**Neurite outgrowth**	Tiam1↑- Rac1↑	Cortical neurons, neuroblastoma cells	[291]
	Adhesion ↑	Rap1; PI3K/PLCβ αIIbβ3 integrins	Platelets	[342]
	CXCR4-mediated chemotaxis ↓	PDZ-RGS3	Cerebrellar granule cells	[324]
	Migration↓	FGFR; Dsh-PCP↓	Retinal progenitors	[349] [350]
Ephrin-B1	**Boundary formation**	Dsh- RhoA↑	*Xenopus* animal caps	[37]
Ephrin-B2		α5 integrins	Zebrafish somites	[341] [102]
		Cdc42↓	Chicken embryos	[348]
Ephrin-B1	**Gap junction communication**	Connexin43	Mouse embryonic bone mesenchyme	[99]
	Tight junction integrity	Par-6	*Xenopus* blastomeres	[352]

p75NTR p75 neurotrophin receptor, *Dsh-PCP* dishevelled/planar cell polarity pathway, *MET* mesenchymal-to-epithelial transition

[a] Effect caused by ephrin overexpression, Eph stimulation-independent

their localisation to these discrete membrane microdomains appears necessary for their signalling capacity to facilitate co-localisation with SFKs [314, 315]. It is interesting in this context to note that in medial spinal cord neurons EphA3 and ephrin-A5 seem to colocalise to the same membrane patches, while in lateral spinal cord neurons they occupy distinct membrane domains [316].

Ephrin-B signalling

The transmembrane ephrin-B ligands contain a cytosolic domain with five conserved tyrosine residues, a proline-rich- and a C-terminal PDZ-domain binding motif [3, 311]. In response to Eph-ligation, ephrin-Bs are clustered and phosphorylated on the conserved tyrosines [317, 318]. Src family kinases, such as Src and Fyn, are thought to be critical for this phosphorylation and thus the biological functions of B-type ephrins [317–319]. For example, Src-dependent ephrin-B2 phosphorylation is required for in vitro assembly of endothelial cells into vascular structures [149], whereby MMP/γ-secretase-mediated cleavage of ephrin-Bs appears to be required for Src activation [243]. In addition, crosstalk with RTKs, such as Tie-2 and PDGFR, shown in vitro [133, 317] and FGFR in *Xenopus* embryos in vivo [320], leads to ephrinB phosphorylation.

One of the important adaptors recruited to activated ephrin-B1 via SH2-domain-dependent interactions is Grb4, mediating FAK activation and cell rounding [321]. Several

PDZ domain-containing proteins, including PICK1, GRIPs and syntenin, were reported to bind the C-terminal PDZ binding motif of ephrin-Bs [124, 322, 323]. Furthermore, the GAP PDZ-RGS3 associates with activated ephrin-B1 and negatively regulates SDF-1-induced G-protein coupled signalling [324]. Ephrin-B1 tyrosine phosphorylation is negatively regulated through PDZ-dependent recruitment of the phosphatase PTP-BL, which concurrently dephosphorylates and thus inactivates Src [319]. Interestingly, effects on cell contraction and (lymph) angiogenic sprouting [145, 146, 154, 325] seem to be independent of the five conserved ephrin-B tyrosines but rely on PDZ-domain interactions. Similarly, phosphotyrosine-independent ephrin-B2 signalling occurs during embryonic midline development and relies on PDZ domain function [326], and both tyrosine-dependent and -independent ephrin-B3 signalling contribute to post-synaptic neuron maturation [327].

In addition to Eph-ephrin-stimulated signalling, ephrins also bind other proteins and can therebymodulate diverse signalling pathways. Ephrin-B2 co-clustering with VEGFR2 [145] is essential for VEGFR internalisation and biological function in endothelial cells [145, 146], while in turn ephrin-B2 expression is regulated by VEGF and hypoxia [147, 328]. Ephrin-B2 and -B3 also act as receptors for the Hendra and Nipah viruses [329, 330], and similarly EphA2, together with the EGF receptor, can act an entry cofactor for the hepatitis C virus [331]. In addition, ephrin-B3, as well as ephrin-A3, binds sulphated proteoglycans [332, 333], including heparin sulphate, required for efficient ephrin-mediated cell-rounding and growth cone collapse.

Ephrin-A signalling

Despite lacking a cytoplasmic domain, there is little doubt that signalling by GPI-linked A-type ephrins modulates cell adhesion and morphology. However, the underlying signalling concepts are poorly understood. While recruitment of Fyn to clustered ephrin-A5 has been demonstrated, the mechanism leading to MAPK pathway activation and increased cell adhesion remains to be identified [315, 334]. Also in T cells, where EphA2-induced ephrin-A activation results in the phosphorylation of Lck, Fyn and Akt, and inhibits antigen receptor-induced apoptosis, signal transduction from the ephrin signalling cluster remains undefined [335]. While the aforementioned co-localisation of ephrins and SFKs to similar membrane microdomains likely facilitates signalling upon ephrin clustering, transmembrane proteins are also thought to be important. Thus, in retinal ganglion cells, Ephrin-A-mediated clustering of lipid rafts and associated SFKs likely involves co-clustering with the neurotrophin receptor p75 (p75NTR), required for EphA7 and Fyn-mediatedaxon retraction [336]. Accordingly, recruitment of the neurotrophin receptor TrkB into ephrin-A5 clusters seems to modulate BDNF-promoted retinal axon branching [337].

Ephrin-modulation of adhesion and repulsion

As with Eph receptors, ephrin clustering can modulate integrin-mediated adhesion. For example, activation of ephrin-A5 increases cell attachment and neurite outgrowth, which depends on β1-integrin and involves prolonged ERK activation [334]. In addition, ephrin-B2 is highly expressed in the invasive front of advanced malignant melanoma, and its overexpression in mouse melanoma cells was suggested to increase migration and β1 integrin-mediated adhesion [338]. In endothelial cells, ephrin-B1 signalling activates integrin $\alpha v\beta 3$- and $\alpha 5\beta 1$-mediated adhesion and migration via JNK [339], while in ephrin-B1-overexpressing HEK293 cells JNK activation results in cell rounding, an effect that is apparently independent of ephrin-B1 phosphorylation or PDZ interactions [340]. Moreover, integrin and ephrin-B2 reverse signalling act in co-operation during somitogenesis in developing zebrafish [102, 341].

Not surprisingly, small GTPases are also involved in ephrin signalling, including Rap1, which together with PI3K and PLCβ facilitate platelet aggregation [342]. In *Xenopus*, ephrin-B1 stimulation leads to downstream activation of Rho and ROCK via dishevelled, a scaffold protein implicated in the Wnt signalling pathway [37, 343]. This mechanism appears to be critical for repulsion and segregation of EphB2- and ephrin-B1-positive cell populations [37]. Recruitment of Grb4 to phosphorylated ephrin-Bs is important to relay cytoskeletal effects, including regulating focal adhesions and actin stress fibres concurrent with cell rounding [321] and dendritic spine morphogenesis via G-protein-coupled receptor kinase-interacting protein (GIT) [129]. Activation of Rac1 and its effector PAK is also communicated by Grb4-mediated recruitment of the GEF DOCK180 resulting in axon retraction and pruning [344]. Rac1 further regulates EphB2-induced invasion of ephrin-B3-positive glioblastoma cells in vitro and in vivo [345] in a similar fashion to EphB2-mediated promotion of glioma cell invasion [304]. Parallels between Eph and ephrin cytoskeletal signalling via Src and PI3K pathways were similarly reported for ephrin-B2-controlled cell migration of retinal endothelial cells [346] and EphB4 response in microvascular endothelial cells [347]. Interestingly, ephrin-B2-mediated down-regulation of Cdc42 activity was suggested to induce mesenchymal-to-epithelial transition and gap induction during chick somitogenesis [348].

More recently, a cell-autonomous function of ephrin-B2 signalling was proposed in endothelial cells [325]. Ephrin-B2 overexpression in endothelial cells caused enhanced but less directional cell migration, and Eph-independent

cell shape oscillations, which relied on ROCK and JNK activation and actomyosin contractility and seemed to be specific for B-class ephrins [325]. Similarly, ephrin-B2 was reported to control cell motility and adhesion in vSMCs independently of cell-cell contact [140]. Another cell-autonomous function reported for ephrin-B involves ephrin-B1 phosphorylation by FGFR, modulating the planar cell polarity pathway and thereby regulating the movement of retinal progenitors into the *Xenopus* eye field [349, 350].

Ephrin-B1 is additionally involved in manipulating gap junction communication by regulating connexin43 distribution [99] and the regulation of cell-cell adhesion and tight junctions, for instance via the interaction with claudins in epithelial and cancer cells [351] or through the Par polarity complex during early *Xenopus* development [352]. Interestingly, in the latter example, overexpressed ephrin-B1 acts by competing with Cdc42 for association with Par-6, a major scaffold of the Par polarity complex, causing loss of tight junctions while ephrin-B1 phosphorylation on Y310 disrupts this interaction. Par-6 is important for activation of atypical PKC indicating that maintenance of tight junction integrity during normal development requires ephrin-B1 phosphorylation [352]. The authors further propose a model where loss of Eph/ephrin interaction might contribute to increased tumour invasion [353]. However, ephrin reverse signalling has also been implicated in promoting cancer cell migration and invasion. Ephrin-A5 overexpression in NIH3T3 fibroblasts increases their invasive potential, branching morphogenesis in matrigel and anchorage-independent growth [354] while ephrin-B signalling modulates invasiveness of T-ALL [355], melanoma [338] and glioma cells [345, 356], which might additionally be fostered by ephrin-induced MMP-8 secretion, as has been described for pancreas carcinoma cell lines [357].

Concluding remarks

The Eph/ephrin signalling system has emerged as one of the principle regulators of cell-cell interactions that guide tissue patterning during development. Re-emerging in many cancer types, Ephs and ephrins are mainly implicated in tumour cell migration and invasion, neoangiogenesis and metastasis. The diametrically opposing outcomes of their activities in normal development, leading to cell-cell adhesion or de-adhesion and segregation, are matched during oncogenesis by their apparently opposing roles in promoting or suppressing tumour progression in different cancers. Clearly, Eph kinase activity is essential in determining the direction of the outcome: cell-cell repulsion depends on robust Eph kinase activation and cytoskeletal re-arrangement, while Eph kinase-independent signalling promotes adhesion and/or cell migration and invasion. Thus, somatic mutations of Ephs, which likely effect receptor clustering and activation, have been described in a range of highly metastatic cancers, suggesting that tumour progression favours change-of-function rather than loss-of-function mutations. It may be that increased levels of Eph receptors in some instances necessitate their inactivation for tumour cell survival or that the change of function actively promotes tumour progression. However, the correlation of Eph receptor expression and tumorigenicity in the absence of mutation in many cancers suggests that in some cases Eph activity is directly tumour promoting, advising caution to over-generalise. Despite the diversity of functions reported for Ephs in various cancers, their frequent overexpression and their implication in the progression of most metastatic cancers suggest Ephs as attractive prognostic markers and promising targets for cancer therapeutics. The effectiveness of such therapeutics, however, will depend on improving our emerging understanding of the distinct Eph functions in specific cancer types.

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