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## Eph receptors and ephrins in cancer: bidirectional signaling and beyond

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### Abstract

The Eph receptor tyrosine kinases and their ephrin ligands have intriguing expression patterns in cancer cells and tumor blood vessels, which suggest important roles for their bidirectional signals in multiple aspects of cancer development and progression. Eph gene mutations likely also contribute to cancer pathogenesis. Eph receptors and ephrins have been shown to affect the growth and migration/invasion of cancer cells in culture as well as tumor growth, invasiveness, angiogenesis, and metastasis *in vivo*. However, Eph signaling activities in cancer appear to be complex, and are characterized by puzzling dichotomies. The Eph receptors nevertheless represent promising new therapeutic targets in cancer.

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Eph receptors and their ephrin ligands together form an important cell communication system with widespread roles in normal physiology and disease pathogenesis<sup>1</sup>. Links between Eph receptors and cancer date back to the first identified Eph family member<sup>2</sup>. EphA1 was cloned from a carcinoma cell line in a screen for novel oncogenic tyrosine kinases. The novel receptor was found to be upregulated in tumor versus normal tissues and its overexpression caused the oncogenic transformation of NIH3T3 fibroblasts<sup>2,3</sup>. The first Eph receptor-interacting (ephrin) ligand, EPHRIN-A1, was also identified from cancer cells a few years later<sup>4</sup>. The evidence implicating Eph receptors and ephrins in cancer is now extensive, and continues to grow.

The activities of the Eph system in cancer are complex, and intriguing in their paradoxical effects. For example, multiple Eph receptors and/or ephrins are present in essentially all types of cancer cells. However, not only increased but also decreased Eph expression has been linked to cancer progression. Consistent with this dichotomy, there is good evidence that Eph receptors and ephrins can both promote and inhibit tumorigenicity. The factors responsible for these divergent activities are only beginning to be uncovered.

Following a brief overview of the Eph and ephrin families and their bidirectional signaling mechanisms, the factors that regulate their expression and the remarkable multiplicity of their roles in cancer will be discussed, and the strategies under evaluation to target the Eph system for cancer therapy outlined. Other reviews provide more in depth information on Eph signaling mechanisms in development and adult physiology<sup>1,5–8</sup>.

### Eph and ephrin families

In the human genome there are 9 EphA receptors, which promiscuously bind 5 glycosylphosphatidylinositol (GPI)-linked ephrin-A ligands, and 5 EphB receptors, which

promiscuously bind 3 transmembrane ephrin-B ligands<sup>5</sup>. Exceptions are the EPHA4 and EPHB2 receptors, which can also bind ephrin-Bs and EPHRIN-A5, respectively, and EPHB4, which preferentially binds only EPHRIN-B2. Eph receptors typically interact with the cell surface-associated ephrins at sites of cell-cell contact (Fig. 1). In addition, soluble ephrin-As released from the cell surface retain the ability to activate EPHA2<sup>4,9,10</sup>.

Eph-ephrin complexes emanate bidirectional signals: forward signals that depend on Eph kinase activity propagate in the receptor-expressing cell and reverse signals that depend on Src family kinases propagate in the ephrin-expressing cell. Ephrin-dependent but kinase-independent Eph signals can also occur<sup>11–13</sup>. Eph signaling controls cell morphology, adhesion, migration and invasion by modifying the organization of the actin cytoskeleton and influencing the activities of integrins and intercellular adhesion molecules<sup>1,5</sup>. Recent work has also uncovered Eph effects on cell proliferation and survival as well as specialized cellular functions such as synaptic plasticity, insulin secretion, bone remodeling and immune function<sup>1</sup>.

Bidirectional signals can lead to removal of the adhesive Eph-ephrin complexes from cell contact sites through an unusual endocytic mechanism that involves their internalization, together with patches of the surrounding plasma membranes, into the receptor- or the ephrin-expressing cell<sup>5</sup>. This enables separation of the engaged cell surfaces to produce the characteristic Eph repulsive responses. Another mechanism allowing cell separation involves protease-mediated cleavage of the Eph or ephrin extracellular domains<sup>14–18</sup>. Internalization and cleavage result in degradation, which can profoundly downregulate Eph levels. However, in certain cellular contexts Eph-ephrin complexes persist at intercellular junctions and emanate prolonged bidirectional signals that favor adhesiveness. For example, the cell adhesion molecule E-CADHERIN promotes EPHA2/EPHRIN-A1 localization at epithelial cell junctions and the metalloprotease ADAM19 stabilizes EPHA4/EPHRIN-A5 at neuromuscular junctions independently of its proteolytic activity<sup>19–21</sup>. A combination of Eph-dependent repulsive and adhesive forces can drive the segregation of cell populations expressing different repertoires of Eph receptors and ephrins, which may include transformed and normal cells or divergent subpopulations of tumor cells<sup>5,22,23</sup>.

There is also increasing evidence that other signaling modalities beyond “conventional” bidirectional signaling contribute to the multiple activities of the Eph system in cancer. For example, an initial extracellular Eph or ephrin cleavage by metalloproteases followed by  $\gamma$ -secretase-mediated cleavage within the transmembrane segment releases intracellular domains that can generate distinctive signals<sup>14,16,24,25</sup>. Eph receptors and ephrins can also signal independently of each other, through crosstalk with other signaling systems, which produces yet more distinctive outcomes. In addition, they participate in feedback loops that may switch between different outputs depending on the state of other cellular signaling networks (Fig. 2).

## Eph and ephrin dysregulation in cancer

The Eph and ephrin families have grown in complexity during evolution, keeping pace with the increasingly more sophisticated tissue organization of higher organisms. Finely coordinated spatial and temporal regulation of Eph receptor and ephrin expression controls many processes that are critical for development and tissue homeostasis, including the formation of tissue boundaries, assembly of intricate neuronal circuits, remodeling of blood vessels, and organ size<sup>1,5</sup>. Multiple Eph receptors and/or ephrins are also expressed in both cancer cells and the tumor microenvironment, where they influence tumor properties by enabling aberrant cell-cell communication within and between tumor compartments<sup>26–32</sup>. Mutations dysregulating Eph function likely also play a role in cancer progression.

## Expression in cancer cells

Many studies have correlated Eph and ephrin expression levels with cancer progression, metastatic spread and patient survival (Table 1). *EPHA2*, for example, is upregulated in a wide variety of cancers and its expression has been linked to increased malignancy and a poor clinical prognosis<sup>27,28,31,33</sup>. Furthermore, *EPHA2* seems to be preferentially expressed in the malignant breast and prostate cancers with a basal phenotype<sup>34,35</sup>. *EPHB4* is also widely expressed in cancer cells and its increased abundance has been correlated with cancer progression<sup>29,36,37</sup>. However, decreased Eph or ephrin levels in malignant cancer cell lines and tumor specimens have been reported as well. For example, *EPHA1* is downregulated in advanced human skin and colorectal cancers<sup>38,39</sup>, EphB receptors in colorectal cancer<sup>23,40–42</sup> and *EPHRIN-A5* in glioblastomas<sup>43</sup>. Furthermore, *EPHB6* expression is lower in metastatic than non-metastatic lung cancers<sup>44</sup>. Reconciling these discrepancies, recent studies show that an initial Eph receptor upregulation (due to activated oncogenic signaling pathways and other factors) can be followed by epigenetic silencing in more advanced stages due to promoter hypermethylation, as shown for several EphB receptors and *EPHA1* in colorectal cancer<sup>23,39–41</sup>. Transcriptional repression, such as repression of *EPHB2* by C-REL (a member of the nuclear factor- $\kappa$ B family) in colorectal cancer, may also play a role in Eph silencing<sup>45</sup>. Intriguingly, differential transcriptional regulation has been reported for *EPHB2* and *EPHB4* expression during colorectal cancer progression<sup>37</sup>. This was attributed to a switch in the association of  $\beta$ -catenin from the p300 coactivator (which induces EphB2) to the CBP coactivator (which induces EphB4). An inverse expression pattern has also been observed for *EPHA2* versus ephrin-A expression in breast cancer cell lines, owing at least in part to feedback loops (Fig. 2A), and for several EphB receptors versus ephrin-Bs in early colorectal tumors and breast cancer cell lines<sup>23,46,47</sup>.

Chromosomal alterations and changes in mRNA stability also regulate Eph and ephrin expression in cancer cells (Table 1). A number of Eph receptor and ephrin genes are located in chromosomal regions frequently lost in cancer cells. For example, *EPHA2*, *EPHA8* and *EPHB2* are clustered at chromosomal region 1p36, which undergoes loss of heterozygosity in many cancers<sup>48,49</sup>. Some Eph genes, however, are in amplified regions<sup>50</sup>. Nonsense-mediated mRNA decay and interaction with mRNA-binding proteins can also regulate Eph mRNA stability in cancer cells<sup>49,51</sup>. These complex mechanisms of regulation parallel the multiplicity of Eph activities in cancer cells.

## Expression in the tumor microenvironment

Several Eph receptors and ephrins are upregulated in vascular cells by tumor-derived factors and hypoxia. For example, tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), vascular endothelial growth factor-A (VEGF-A) and the hypoxia-inducible factor HIF-2 $\alpha$  have been shown to upregulate *EPHRIN-A1* in cultured endothelial cells<sup>52–54</sup>. Endothelial *EPHRIN-B2* is upregulated by VEGF through the Notch pathway, by cyclic stretch, hypoxic stress and contact with smooth muscle cells, whereas shear stress seems to decrease *EPHRIN-B2* expression in endothelial cells but increase it in endothelial precursors by inducing their differentiation<sup>55–59</sup>. Moreover, *EPHRIN-B2* is expressed in pericytes and vascular smooth muscle cells<sup>57,60</sup>. Expression of *EPHA2/EPHRIN-A1* and *EPHB4/EPHRIN-B2* in tumor blood vessels has been most extensively characterized, but other Eph receptors and ephrins are also present in the tumor vasculature<sup>54–57,61,62</sup>. In contrast, little is known about Eph and ephrin expression in other tumor compartments, such as activated fibroblasts and infiltrating immune and inflammatory cells. Nevertheless, Eph-dependent communication between these cells and tumor cells likely plays an important role in tumor homeostasis.

## Eph mutations with cancer relevance

Screens of tumor specimens and cell lines have recently identified mutations in the genes encoding all of the Eph receptors, whereas cancer-related ephrin mutations have not been reported so far, perhaps in part because many of the screens have focused on the kinome<sup>63–67</sup> (<http://www.sanger.ac.uk/genetics/CGP/cosmic>). Mutations of at least some Eph receptors are predicted to play a role in cancer pathogenesis. For example, *EPHB2* mutations have been identified in human prostate, gastric, colorectal and melanoma tumors<sup>40,49,67–69</sup>. Some of these mutations may impair kinase function, and some are accompanied by loss of heterozygosity, suggesting a tumor suppressor role for *EPHB2* forward signaling. Furthermore, a number of Eph receptors – particularly *EPHA3* and *EPHA5* – are frequently mutated in lung cancer<sup>63,70</sup>. The mutations are typically scattered throughout the Eph domains, including the ephrin-binding domain and other extracellular regions<sup>67,70</sup>. Elucidating the effects of the mutations will provide important insight into the functional roles of the Eph system in cancer.

## Tumor Suppression

In many cancer cell lines, Eph receptors appear to be highly expressed but poorly activated by ephrins, as judged by their low level of tyrosine phosphorylation<sup>1,29,37,47,71,72</sup>. This was one of the first clues that ephrin-dependent Eph forward signaling may be detrimental to tumor progression. Furthermore, recent expression profiling of *Apc*<sup>Min/+</sup> intestinal tumors from wild-type and *Ephb4*<sup>+/-</sup> mice has revealed an extensive transcriptional reprogramming that suggests anti-proliferative and anti-invasive activities of *EPHB4* in colorectal cancer<sup>73</sup>.

## Eph forward signaling inhibits cell transformation

Forcing Eph receptor activation with soluble Fc fusion proteins of ephrin ligands can inhibit proliferation, survival, migration and invasion of many types of cancer cells in culture as well as tumor growth in several mouse models<sup>5,29,42,47,74</sup>. Conversely, a dominant negative form of *EPHB4* has been shown to promote colorectal cancer proliferation and invasion<sup>73</sup>. These studies demonstrate that Eph forward signaling pathways can lead to tumor suppression (Fig. 3). Indeed, Eph receptors activated by ephrins acquire the remarkable ability to inhibit oncogenic signaling pathways, such as the HRAS-Erk, PI3 kinase-Akt and Abl-Crk pathways. Interestingly, this may reflect a physiological function of the Eph system in epithelial homeostasis by promoting contact-dependent growth inhibition and decreasing motility and invasiveness. These changes are reminiscent of mesenchymal-to-epithelial transition (Box 1).

## Silencing of Eph forward signals in cancer cells

Cancer cells appear to use a variety of mechanisms to minimize the tumor suppressor effects of Eph forward signaling. For example, the high *EPHA2* or EphB expression and low ephrin expression observed in some cancers result in low bidirectional signaling<sup>23,46,47</sup>. Furthermore, co-expressed Eph receptors and ephrins often do not interact effectively in cancer cells<sup>20</sup>. This may be because they engage in lateral interactions that silence their signaling function, as has been shown in neurons and transfected cells<sup>5</sup>. Alternatively, loss of E- or VE-cadherin impairs endogenous *EPHA2*-*EPHRIN-A1* interaction in malignant breast cancer and melanoma cells, respectively<sup>20,75</sup>. The two cadherins appear to promote *EPHA2*-*EPHRIN-A1* interaction by stabilizing intercellular contacts and promoting the localization of *EPHA2* at cell-cell junctions. Phosphotyrosine phosphatases also negatively regulate Eph receptor forward signaling in some cancer cells<sup>76</sup>. For example, the low molecular weight phosphotyrosine protein phosphatase (LMW-PTP) has been implicated in cell transformation through its ability to dephosphorylate *EPHA2*, thus counteracting ephrin-dependent activation<sup>77</sup>. The receptor-type phosphatases PTPRO and PTPRF, and PTEN in

*C. elegans*, also dephosphorylate Eph receptors<sup>78–80</sup>. However, it is not known whether this plays a role in cancer. Eph mutations may also contribute to disrupting forward signaling by impairing ephrin binding or kinase activity. For example, the EPHA3 E53K mutation in the MeWo melanoma cell line abrogates ephrin binding<sup>66,81</sup> and the EPHB2 G787R mutation found in colorectal cancer impairs kinase activity<sup>69</sup>. It will also be interesting to investigate whether soluble Eph ectodomains generated by alternative splicing<sup>82,83</sup> or proteolysis<sup>14–18,24</sup> and proteins containing a major sperm protein (MSP) domain<sup>84</sup> (Fig. 1) may decrease Eph signaling in cancer cells by acting as naturally occurring antagonists.

### Tumor confinement by surrounding ephrins

The tumor suppressor effects of Eph forward signaling may be active at the tumor periphery if the surrounding tissues express ephrins. In mouse tumor models, ephrins present in normal tissues have been proposed to inhibit expansion and invasiveness of incipient colorectal and skin tumors expressing Eph receptors<sup>23,85,86</sup>. In addition, recent experiments in the developing zebrafish hindbrain raise the possibility that elevated Eph or ephrin levels may drive segregation of tumor cells from surrounding normal tissues, thereby decreasing invasiveness, not only through repulsive mechanisms but also by promoting adhesiveness between tumor cells<sup>22</sup>. Eph receptors may further decrease tumor invasiveness by promoting the formation of tight junctions in neighboring epithelial cells through stimulation of ephrin-B reverse signaling<sup>87</sup> (see next section). Indeed, recent systems-level studies have implicated complex, asymmetric signaling networks in the sorting of EPHRIN-B1-expressing HEK293 cells from EPHB2-expressing cells<sup>88</sup>. It is tempting to speculate that Eph receptors may contribute to tumor dormancy through these types of bidirectional signaling mechanisms that restrict tumor expansion. Accordingly, high EPHA5 levels have been detected in various dormant but not fast-growing tumor xenograft models<sup>89</sup>.

### Ephrin reverse signaling in tumor cells

Ephrin reverse signaling in cancer cells may in some cases also contribute to tumor suppression (Fig. 3). In the *Xenopus* system and HT29 colon cancer cells, EPHRIN-B1 tyrosine phosphorylation (which can be induced by interaction with EphB receptors or by activated growth factor receptors and Src) disrupts binding of the ephrin to the scaffolding protein PAR6, promoting the formation of tight junctions between cells<sup>87,90</sup>. Similar to its role in neurons, ephrin-B reverse signaling may also inhibit the migratory and invasive effects of the CXCR4 G protein-coupled chemokine receptor in cancer cells<sup>5,6</sup>. EPHRIN-A5 can downregulate epidermal growth factor receptor (EGFR) levels in glioblastoma cells<sup>43</sup>.

### Tumor promotion

Conversely, forward and/or reverse Eph-ephrin signals can enhance malignant transformation in some cases. There is also increasing evidence that the Eph receptors are capable of unconventional signaling activities that do not depend on activation by ephrin ligands and that support cancer progression. Moreover, it is well established that the Eph system promotes tumor angiogenesis.

### Eph forward signaling

In certain cellular contexts, Eph receptors activated by ephrins may have lost the ability to suppress tumorigenicity, and even acquired oncogenic ability. For example, activating mutations may render oncogenic signaling pathways resistant to inhibition by Eph forward signaling. Furthermore, EPHB2 can promote proliferation in mouse intestinal progenitor cells and *Apc*<sup>Min/+</sup> adenomas through Abl-mediated increase in CYCLIN-D1 levels even though it inhibits invasiveness through other pathways<sup>91</sup> (Fig. 4). Activation of RHOA downstream of EPHA2 and EPHB4 promotes amoeboid-type migration of cancer cells and



destabilizes epithelial adherens junctions in various cancer cell lines (Fig. 4), even though RHOA inhibits mesenchymal-type migration<sup>92–94</sup> (Fig. 3). EPHA2 forward signaling in malignant melanoma and ovarian cancer cells can also promote vasculogenic mimicry<sup>75,95</sup>.

RRAS phosphorylation downstream of EPHB2 (Fig. 4) can enhance glioma cell invasiveness, possibly by decreasing cell substrate adhesion<sup>96</sup>, even though in other cell types Eph forward signals decrease cell adhesion and migration<sup>5,47,97</sup>. Instead of inhibiting the HRAS-Erk MAP kinase pathway, depending on the circumstances, EPHB2 can sometimes activate it<sup>5,79</sup>. In turn, activation of the Erk MAP kinase pathway enhances ephrin-dependent activation of overexpressed EPHB2 in cultured cells<sup>79</sup>. This may result in different EPHB2/MAP kinase feedback loops (Fig. 2B) that could either enhance or diminish cancer cell malignancy. Indeed, activation of an engineered membrane-anchored cytoplasmic domain of fibroblast growth factor receptor 1 (FGFR1) inhibits ephrin-dependent repulsive signaling by overexpressed EPHB2 through a mechanism involving downregulation of the HRAS-Erk pathway, suggesting that FGFR1 activation could neutralize the anti-invasive effects of EPHB2 in cancer cells<sup>79</sup>. In contrast, overexpressed EPHA4 and FGFR1 associate and potentiate each other's oncogenic activities in cultured glioma and other cell types<sup>98,99</sup>. It will also be interesting to determine whether Eph receptors can downregulate PTEN levels and perhaps activity in cancer cells, as suggested by recent studies in *C. elegans*<sup>80</sup>.

### Unconventional Eph receptor activities

Downregulation of EPHA2 or EPHB4 by small interfering RNAs (siRNAs) or antisense oligonucleotides decreases cancer cell malignancy in culture and inhibits tumor growth in a number of mouse cancer models<sup>36,37,100–102</sup>. Furthermore, EPHA2 overexpression causes oncogenic transformation of mammary epithelial cells in culture as well as *in vivo*<sup>71,103</sup>. These experiments demonstrate positive effects of Eph receptors on cancer progression. Given the low levels of Eph forward signaling observed in many cancer cells, these tumor promoting activities are likely to be independent of ephrin stimulation and possibly also of kinase activity. Recent evidence indeed shows that oncogenic signaling pathways can co-opt Eph receptors to increase cancer cell malignancy.

Notable examples of how the altered signaling networks of cancer cells can subvert Eph function involve EPHA2. This receptor has been found to mediate some of the oncogenic activities of EGFR family members, including cancer cell migration in culture and tumor growth and metastasis in a transgenic mouse breast cancer model<sup>104,105</sup> (Fig. 4). EPHA2 also appears to be required for Src-dependent invasiveness of colorectal cancer cells in culture<sup>90</sup>. These effects may be ligand-independent and explained at least in part by the recently discovered crosstalk between EPHA2 and Akt, a serine/threonine kinase frequently activated in cancer cells<sup>72</sup> (Figs. 2C and 4). Phosphorylation by Akt of a single serine in EPHA2 appears to promote cancer cell migration and invasion, an effect that interestingly does not require EPHA2 kinase activity and is reversed by EPHRIN-A1 stimulation<sup>72</sup>. It will be important to investigate the details of the Akt/EPHA2 crosstalk and whether other Eph receptors may contribute to cancer progression through analogous mechanisms. EPHA2 has also been recently shown to promote epithelial proliferation and branching morphogenesis in the developing mouse mammary gland by mediating hepatocyte growth factor (HGF)-dependent inhibition of RHOA activity<sup>106</sup>, which is in contrast to the RHOA activation induced by EPHA2 overexpression, ephrin stimulation, or crosstalk with the ERBB2 receptor<sup>103,105,107</sup>. It is not yet known if an ephrin-independent EPHA2/HGF receptor crosstalk may play a role in cancer. Ephrin-independent activities of Eph receptors may also include modulation of the subcellular localization of signaling partners that are constitutively associated with an Eph receptor (Fig. 1) or become associated as a result of Eph phosphorylation by other kinases.

With regard to other receptors, the recently discovered ephrin-independent downregulation of  $\beta$ 1-integrin levels and cell substrate adhesion by endogenous EPHB4 may promote migration and invasiveness in some cancer cell types although it is inhibitory in others<sup>96,97</sup>. Additionally, distinctive signaling activities of Eph intracellular domain fragments generated by metalloprotease and  $\gamma$ -secretase cleavage may promote cancer cell malignancy. For example, the EPHA4 cytoplasmic domain released by  $\gamma$ -secretase can enhance RAC1 activity in cultured cells independently of ephrin stimulation and kinase activity<sup>24</sup>. Furthermore, EPHRIN-B3 stimulation can block apoptosis caused by caspase-dependent cleavage of overexpressed EPHA4 in cultured cells, which interestingly suggests a role for EPHA4 as a “dependence” receptor<sup>108</sup>.

### Tumor promotion by ephrin signaling in cancer cells

Little is known about the effects of ephrin-A reverse signaling in epithelial cells. One study has shown that ephrin-A1 is highly upregulated in hepatocellular carcinoma and promotes the proliferation and expression of genes associated with proliferation and invasion in human liver cancer cells<sup>109</sup>. In fibroblasts, EphA-dependent stimulation of EPHRIN-A5 activates the FYN Src family kinase, integrin-mediated adhesion and Erk MAP kinases<sup>5,6</sup> (Fig. 4). Accordingly, EPHRIN-A5 overexpression can increase fibroblast growth in soft agar, invasion and morphological transformation<sup>110</sup>. Ephrin-B reverse signaling also involves Src family kinases, which phosphorylate the ephrin-B cytoplasmic domain thus regulating its interaction with signaling molecules<sup>5,6</sup>. Src activation has been proposed to require release of the ephrin-B intracellular domain by metalloprotease and  $\gamma$ -secretase cleavage following EphB binding, which decreases Src association with its inhibitory kinase CSK<sup>14</sup>. Furthermore, homophilic engagement of claudins, which are tight junction proteins, causes Src-mediated EPHRIN-B1 phosphorylation that slows down the formation of epithelial cell junctions and may thus enhance invasiveness<sup>111</sup>. This is in contrast to the promotion of tight junction formation due to EPHRIN-B1 phosphorylation discussed above. Whether phosphorylation of different tyrosines, different levels of phosphorylation, or the cellular context may lead to positive versus negative effects of ephrin-Bs on intercellular adhesion remains to be determined.

Other recurring themes in ephrin-B reverse signaling are a localization in lipid rafts and RAC1 activation, which can occur through multiple mechanisms and increase cancer cell migration and invasion<sup>112–114</sup> (Fig. 4). For example, EPHRIN-B3 is upregulated in invading cells of glioma biopsies and promotes RAC1-dependent invasion of glioma cell lines<sup>112</sup> while EPHRIN-B2 is upregulated in invading cells of glioma and melanoma biopsies and its forced overexpression in the cultured cancer cells enhances integrin-mediated attachment, migration and invasion<sup>115,116</sup>. Furthermore, EPHRIN-B1 reverse signaling has been reported to induce secretion of matrix metalloprotease 8 (MMP8) and promote invasion of glioma, pancreatic, gastric and leukemic cancer cells *in vitro* and in mouse tumor models<sup>17,113,117</sup>.

Ephrin-B reverse signaling may also modulate gene transcription in cancer cells. Ephrin-B1 binds and activates STAT3, a transcription factor involved in cancer progression<sup>118</sup> (Fig. 4). Furthermore, in neural progenitors EPHRIN-B1 intracellular domain fragments can localize to the nucleus and bind the ZHX2 transcriptional repressor, potentiating its activity, although it is not known whether this regulation also plays a role in cancer<sup>25</sup>.

### Tumor angiogenesis

Blood vessels are critical for tumor growth and represent an important venue for metastatic dissemination. Several Eph receptors and ephrins promote angiogenesis by mediating communication of vascular cells with other vascular cells as well as tumor cells. The latter

interactions may occur especially during blood vessel growth and in tumor vessels with discontinuous endothelial lining. Furthermore, they may affect not only the endothelial cells but also, reciprocally, tumor cell behavior<sup>119</sup>.

Analysis of tumors grown in *Epha2* mutant mice or mice treated with inhibitory EphA-Fc fusion proteins suggests that EPHA2 forward signaling promotes tumor angiogenesis<sup>27,31,56</sup>. In contrast, EPHA2 does not seem to play a major role in developmental angiogenesis, and only recently abnormalities in capillary development that may be due to defective pericyte coverage have been revealed in *Epha2*-deficient mice<sup>120</sup>. *In vitro* and *in vivo* data also show that EPHA2 forward signaling can increase blood vessel permeability, perhaps in part through phosphorylation of claudins<sup>8,121</sup>. A major ligand for endothelial EPHA2 is EPHRIN-A1, whose upregulation in endothelial cells and consequent activation of EPHA2 have been reported to play an important role in the angiogenic effects of VEGF-A and TNF $\alpha$ <sup>52,53</sup>. In tumors, EPHRIN-A1 can be expressed by both endothelial and tumor cells<sup>52,122,123</sup>. Interestingly, the upregulation of EPHA2 and EPHRIN-A1 observed in pancreatic tumors of mice treated with VEGF inhibitors suggests that EPHA2-dependent angiogenesis may contribute to the development of resistance to anti-VEGF therapies, perhaps by promoting endothelial coverage by pericytes and smooth muscle cells<sup>120,124</sup>. Curiously EPHRIN-A3, another ephrin ligand for EPHA2, is downregulated in hypoxic endothelial cells in culture by the microRNA miR-210 and appears to inhibit angiogenic responses in hypoxic human umbilical vein endothelial cells<sup>62</sup>. It will be important in future studies to evaluate the combined activities of all relevant EphA receptors and ephrin-A ligands in the regulation of capillary sprouting, vessel permeability and pericyte coverage, as well as their possible redundancies and opposing functions in tumor blood vessels.

EPHB4 and EPHRIN-B2 also play a role in tumor angiogenesis. During development, they are characteristically expressed in the endothelial cells of veins and arteries, respectively, and enable arterial-venous vessel segregation and vascular remodeling<sup>55–57,125</sup>. The information available so far highlights the importance of EPHRIN-B2 reverse signaling in tumor angiogenesis, while little is known about the role of EPHB4 forward signaling<sup>56,126–128</sup>. Reverse signaling by EPHRIN-B2, and possibly other ephrin-Bs, in tumor endothelial cells, pericytes and smooth muscle cells likely depends on interaction with several EphB receptors expressed by vascular and/or tumor cells and has been shown to be important for blood vessel assembly, enlargement and decreased permeability both in cell culture and *in vivo*<sup>57,126,127</sup>. EPHRIN-B2 signaling also promotes the interaction between endothelial cells and pericytes or vascular smooth muscle cells<sup>60,128</sup>, suggesting that upregulation of this ephrin may stabilize the vessels of tumors recurring after anti-VEGF therapy<sup>129</sup>. EPHRIN-B2 in the tumor endothelium may also play additional roles. For example, it may enhance the recruitment of bone marrow-derived endothelial progenitor cells that could participate in tumor vascularization, through a mechanism involving EPHB4-dependent upregulation of selectin ligands<sup>130</sup>. It will be interesting to determine whether EPHRIN-B2 may also promote extravasation of EphB-positive metastatic tumor cells through the vascular endothelium, similar to its *in vitro* effect on monocytes<sup>58,131</sup>.

## Eph proteins as therapeutic targets

Eph receptors and ephrins represent promising new therapeutic targets in cancer. A variety of strategies are under evaluation to interfere with their tumor-promoting effects or enhance their tumor-suppressing effects, although our limited mechanistic understanding of the dichotomous Eph activities represents a challenge in the design of therapeutic agents. Other approaches that do not rely on interfering with Eph function involve using Eph receptor-



targeting molecules for the selective delivery of drugs, toxins or imaging agents to tumors, and the use Eph-derived antigenic peptides to stimulate anti-tumor immune responses.

### Interfering with Eph/ephrin function

Inhibiting the Eph system may be particularly useful for anti-angiogenic therapies, and possibly to overcome resistance to anti-VEGF therapies<sup>27,29,55,124,129</sup>. Efforts to identify small molecules that target the Eph kinase domain have begun to yield some high affinity inhibitors<sup>132–136</sup> (Table 2). Furthermore, a number of inhibitors designed to target other kinases also inhibit Eph receptors. For example dasatinib, a multi-targeted kinase inhibitor already used in the treatment of chronic myelogenous leukemia and under clinical evaluation to treat solid tumors, potently inhibits EPHA2 and other Eph receptors besides its primary targets Abl and Src<sup>34,137,138</sup> (<http://clinicaltrials.gov/ct2/results?term=epha2>). Interestingly, EPHA2 has also been identified as a biomarker for dasatinib sensitivity of cancer cells<sup>34,35</sup>. Moreover XL647, an orally bioavailable EGF and VEGF receptor inhibitor being evaluated in clinical trials for lung cancer, also targets EPHB4 (<http://clinicaltrials.gov/ct2/results?term=EphB4>).

Downregulation of EPHA2 or EPHB4 expression with siRNAs or antisense oligonucleotides has been shown to inhibit malignant cell behavior in culture and tumor growth *in vivo*<sup>36,37,100–102</sup> (Table 2). For example, delivery of *EPHA2* siRNA to tumors using neutral liposomes inhibits tumor growth and metastasis in mouse models of ovarian cancer, particularly when combined with delivery of siRNA silencing focal adhesion kinase (FAK) or with paclitaxel chemotherapy<sup>102,139</sup>. Eph receptor levels and function might also be reduced *in vivo*, as they are *in vitro*, by drugs that target the chaperone protein HSP90<sup>140,141</sup>, although other proteins will also be concomitantly downregulated.

Another strategy that shows promise for cancer anti-angiogenic therapy is to inhibit Eph-ephrin interactions. A variety of molecules can be used for this purpose (Table 2). The dimeric EPHA2 ectodomain fused to Fc (which inhibits EPHA forward signaling but promotes reverse signaling) and the monomeric soluble EPHB4 ectodomain (which inhibits both forward and reverse signaling) can both reduce tumor growth in mouse cancer models, at least in part by inhibiting tumor angiogenesis<sup>27,31,57,142</sup>. Antagonistic antibodies<sup>143,144</sup> and peptides that inhibit ephrin binding to individual Eph receptors or subsets of receptors<sup>145–147</sup> could be useful for inhibiting Eph-ephrin interactions and bidirectional signaling with greater selectivity than the promiscuous Eph ectodomains. At least two of these peptides bind to the high-affinity ephrin-binding channel of their target receptor<sup>148,149</sup>. This Eph channel also appears suitable for targeting with chemical compounds, and two isomeric small molecules that preferentially inhibit ephrin binding to EPHA2 and EPHA4, albeit with low affinity, have been identified<sup>150,151</sup>. Structural characterization of additional small molecules and peptides in complex with Eph receptors may reveal general rules enabling the rational design of chemical compounds capable of selectively targeting Eph receptors with high affinity.

Intriguingly, ephrin ligands and agonistic antibodies have also been successfully used to inhibit tumor progression in mouse cancer models despite being activators rather than inhibitors of Eph-ephrin signaling (Table 2). These agonists have been proposed to act by stimulating Eph forward signaling pathways with tumor suppressor activity and/or receptor degradation in the cancer cells<sup>47,152–154</sup>. Antibody-dependent cell-mediated cytotoxicity may also contribute to the anti-cancer effects of some of the antibodies<sup>155</sup>, perhaps explaining the discrepancies in the effectiveness of different EPHA2 antibodies with similar agonistic properties<sup>155,156</sup>. Eph agonistic antibodies may also be useful in combination with chemotherapy<sup>33,153</sup>.

Eph-targeting agents likely act through a combination of multiple effects on cancer cells and the tumor microenvironment, which may explain the efficacy of agents with opposite mechanisms of action. For example, EPHA2 agonists would be expected to enhance tumor suppressor signaling pathways and receptor degradation in the cancer cells but promote tumor angiogenesis<sup>31</sup>. On the other hand, some Eph kinase inhibitors with anti-angiogenic activity might also block possible Eph tumor suppressor activities. Such inhibitors may therefore be particularly effective for the treatment of tumors where Eph forward signaling pathways with tumor suppressor activity are not activated. EPHB4 agonists that also antagonize ephrin binding may be particularly beneficial by both enhancing EPHB4-dependent tumor suppression in cancer cells and inhibiting EPHRIN-B2-dependent angiogenesis<sup>47,127</sup>. Ultimately, how a tumor will respond to a particular Eph-targeted strategy will likely depend on the tumor type, stage and microenvironment. Selecting optimal strategies to interfere with Eph function for cancer therapy will therefore require a better understanding of Eph signaling mechanisms in the different cellular compartments of tumors. Eph-dependent oncogenic signaling networks may also represent suitable therapeutic targets. Newly developed targeting molecules, in particular those with selectivity for individual Eph receptors or ephrins, in turn represent useful research tools to further our knowledge of Eph cancer biology.

### Targeted delivery of drugs/toxins and imaging agents

Because of their elevated expression in many tumors compared to normal tissues, Eph receptors also represent attractive targets for the delivery of drugs or toxins and imaging agents to cancer tissue. Several chemotherapeutic drugs and toxins conjugated to Eph antibodies or an ephrin, which cause receptor-mediated drug internalization, appear promising in initial studies (Table 2). EPHA2- or EPHB2-targeting antibodies coupled to derivatives of the peptide drug auristatin, which disrupts microtubule dynamics, inhibit the growth of several cancers in rodent models<sup>143,157,158</sup>. Another potential application is the targeted delivery of gold-coated nanoshells conjugated to ephrins for photothermal destruction of Eph-positive cancer cells<sup>159</sup>. Importantly, systemic toxic effects have not been apparent so far and the EPHA2 antibody coupled to an auristatin derivative is under clinical evaluation (<http://clinicaltrials.gov/ct2/show/NCT00924235>). Notably, targeting Eph surfaces that are preferentially exposed on tumor cells, which may include the ephrin-binding channel, could further improve the therapeutic index<sup>152</sup>.

Antibodies, ephrins and peptides can also be used to deliver imaging agents for diagnostic purposes. EPHA2 is a particularly attractive target for this application given its widespread expression in both cancer cells and tumor vasculature and low expression in most adult tissues<sup>27,56,160</sup>. Promising results have been obtained in animal models by using an EPHA2 antibody labeled with <sup>64</sup>Cu through the chelating agent 1,4,7,10-tetraazacyclododecane N,N',N'',N'''-tetraacetic acid (DOTA) for radioimmunoPET imaging and an EPHA3 antibody coupled to <sup>111</sup>Indium for gamma camera imaging<sup>160,161</sup>.

### Immunotherapy

In addition to the immune cell-mediated cytotoxicity that can be elicited by Eph-targeted antibodies, a bispecific single-chain antibody that simultaneously binds both EPHA2 and the T-cell receptor/CD3 complex causes T-cell-mediated destruction of EPHA2-positive tumor cells *in vitro* and decreases tumor growth *in vivo*<sup>162</sup> (Table 2). Eph receptors that are preferentially expressed in tumors compared to normal tissues are also attractive targets for cancer vaccines. EPHA2, EPHA3 and an EPHB6 isoform have been identified as sources of tumor-associated peptide antigens that are recognized by cancer-specific cytotoxic T-cells<sup>163–166</sup>. Interestingly, agonists and drugs that stimulate Eph receptor degradation may inhibit tumor growth at least in part by enhancing the presentation of Eph-derived peptides

that can be recognized by effector T-cells<sup>141,154</sup>. Vaccination with Eph-derived epitopes also shows promise as a strategy to elicit tumor rejection<sup>167,168</sup>.

## Perspectives

Accumulating evidence implicates deregulation of the Eph cell communication system in cancer pathogenesis. The Eph receptors are emerging as master regulators capable of either potentiating the activities of oncogenic signaling networks or repressing them, depending on ephrin stimulation and other contextual factors. Remarkably, Eph receptors and ephrins can switch between contrasting activities by using bidirectional signaling as well as other signaling modalities to influence cancer cell behavior. We still know relatively little about how the Eph system regulates tumorigenesis at the molecular level, but clearly there is extensive cell context dependency for many Eph pathways. For example, many of the differences observed in Eph/ephrin signaling outcomes may relate to differences in spatial and temporal coordination of input signals and relays, and thus vary between cell types and *in vitro* versus *in vivo* environments<sup>169</sup>. An important step forward will be to understand in detail the Eph activities beyond bidirectional signaling and the crosstalk with oncogenic pathways. Furthermore, systems-level studies will be instrumental for: providing a comprehensive overview of the effects of Eph bidirectional and unconventional signaling mechanisms in cancer and stromal cells; comparing the signaling activities of different Eph/ephrin family members; examining the consequences of changes in Eph/ephrin expression, for example to compare the effects of Eph receptor downregulation by agonists and by transcriptional silencing; and elucidating the effects of cancer-relevant Eph/ephrin mutations. Indeed, a recent proteomic analysis combined with siRNA screening and data-driven network modeling has provided a wealth of tantalizing new information on the asymmetric bidirectional signaling networks initiated by ephrin-B1 and EphB2 at sites of cell-cell contact<sup>88</sup>. Another area of great interest is how the Eph system influences the metastatic process, including tissue invasion, dissemination through the vascular system, possible reversal of epithelial-to-mesenchymal transition at distant sites, and dormancy of Eph-expressing micrometastases seeded in ephrin-rich tissues.

To advance our understanding of Eph cancer biology, it will also be important to examine the effects of Eph or ephrin loss, increased expression, and cancer relevant mutations in genetically engineered mouse models that mimic the progression of human cancers. Such *in vivo* models are key for studying the Eph system, given its penchant for regulating communication between different cell types, which is difficult to accurately recapitulate *in vitro*. The mouse models will also be useful for preclinical evaluation of new Eph-based therapies.

Eph and ephrin expression promises to be a powerful predictor of prognosis and perhaps drug sensitivity. For example, increased EPHA2 expression can confer sensitivity to dasatinib but resistance to the ERBB2-targeting antibody trastuzumab<sup>33–35</sup>. Therefore, there is a need for a comprehensive assessment of Eph and ephrin protein expression in large cohorts of human tumors in correlation with stages of malignancy and clinical outcome. Carefully validated antibodies and quantitative proteomics approaches are needed to ensure the reliability of such studies. Understanding the complexities of the Eph system will contribute to clarify the mechanisms of cancer development, progression and metastasis as well as aid development of novel anti-cancer therapies.

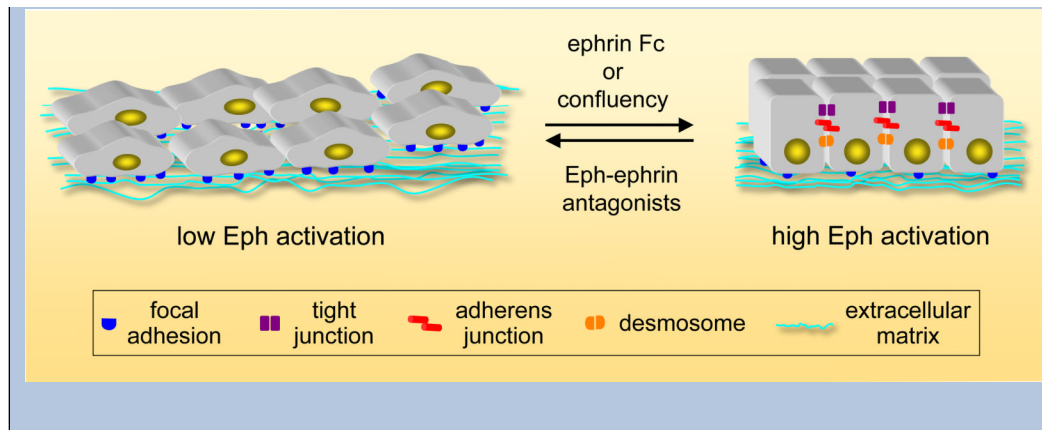
### At a glance

- The Eph receptors are the largest family of receptor tyrosine kinases. They bind GPI-linked and transmembrane ephrin ligands, generating bidirectional signals at sites of cell-cell contact.

- Eph receptors and/or ephrins are widely expressed in cancer cells and tumor stroma, but they can be downregulated at advanced cancer stages. Often Eph receptor and ephrin levels are discordantly regulated. Not only changes in expression levels, but also Eph receptor mutations likely play a role in cancer pathogenesis.
- In many cellular contexts, Eph bidirectional signaling promotes an epithelial phenotype and suppresses cancer cell-substrate adhesion, migration, invasion and growth. Consistent with this, Eph receptor signaling appears to be low in many cancer cells due to imbalance of Eph/ephrin expression or inability of receptor and ligand to interact effectively.
- Eph receptors and ephrins can also promote cancer progression through poorly understood mechanisms that do not involve reciprocal association but rather depend on crosstalk with oncogenic signaling pathways. In addition, Eph bidirectional signals promote tumor angiogenesis.
- Eph receptors and ephrins represent promising new therapeutic targets in cancer, and many Eph-based approaches show promise for prognosis and therapy.

### Box 1. The Eph system can promote an epithelial phenotype

Forward signaling by EPHA2 and several EphB receptors in epithelial and cancer cells can induce morphological changes reminiscent of mesenchymal-to-epithelial transition. For example, stimulation of EPHA2 forward signaling with EPHRIN-A1-Fc in sparse MDCK epithelial cells enhances maturation of cell-cell junctions and cell compaction<sup>170,171</sup> (see figure) In a positive feedback loop, E-CADHERIN can promote EPHA2 expression and surface localization in epithelial and cancer cells that have reached high density, thereby prolonging EPHA2 interaction with co-expressed EPHRIN-A1 and forward signaling<sup>19,20,170</sup> (Fig. 2D). Stimulation of EPHB2 forward signaling with EPHRIN-B1-Fc can also couple increased intercellular adhesion with cell contraction and apico-basal polarization in colorectal cancer cells by promoting the membrane localization of E-CADHERIN<sup>86</sup>. Interestingly, the consequences are dramatically different in colorectal cancer cells expressing EPHB2 but lacking E-CADHERIN, where EPHRIN-B1-Fc stimulation causes cell contraction and separation instead of promoting cell-cell adhesion<sup>86</sup>. Stable transfection of EPHB3 in HT29 colon cancer cells, which endogenously express ephrin-Bs and E-CADHERIN, also causes changes consistent with mesenchymal-to-epithelial transition<sup>42</sup>. Furthermore, moderate ephrin-B expression and phosphorylation can promote the integrity of adherens and tight junctions in *Xenopus* and HT29 cells<sup>87</sup>. Conversely, EPHB4-EPHRIN-B2 antagonists have been shown to disturb intercellular junctions in MCF-10A mammary epithelial cells<sup>47</sup>. Thus, interplay with E-CADHERIN can convert Eph repulsive signals into signals that promote cell-cell adhesion. It is not known whether a similar interplay may occur with N-CADHERIN, which often replaces E-CADHERIN in malignant cancer cells that have undergone epithelial-to-mesenchymal transition. Studies in normal tissues suggest that Eph receptors can promote N-CADHERIN-dependent adhesion. For example, EPHA4 forward signaling is critical for the N-CADHERIN-dependent mesenchymal-to-epithelial transition that occurs at the borders of developing zebrafish somites<sup>172</sup>. Interestingly, *EPHA2* mutations in humans and *EPHA2* or *EPHRIN-A5* loss in mice disrupt the N-CADHERIN-dependent intercellular junctions in the lens epithelium, causing cataracts<sup>173,174</sup>.



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## Glossary

<b>Basal phenotype</b>	Phenotype of highly aggressive breast and prostate cancers with gene expression profiles similar to basal cells. Basal-type breast cancers are typically negative for estrogen, progesterone and HER2 receptors. Basal-type prostate cancers have high expression of cytokeratin 5 and low expression of the androgen receptor and prostate specific antigen.
<b>Nonsense-mediated mRNA decay</b>	The process by which mRNA molecules carrying premature stop codons are degraded by a regulated pathway, thereby limiting the synthesis of abnormal proteins.
<b>Cyclic stretch</b>	Periodic stretch (or strain) to which vascular endothelial cells are subjected as a result of the rhythmic changes in vessel diameter caused by pulsatile blood flow.
<b>Shear stress</b>	The physical force exerted on endothelial cells as a result of blood flow.
<b>Pericytes</b>	Mesenchymal cell precursors to vascular smooth muscle that associate with endothelial cells during angiogenesis and provide support to small capillaries.
<b><i>Apc<sup>Min/+</sup></i></b>	Mouse that carries the Min (multiple intestinal neoplasia) point mutation in one <i>Apc</i> allele and spontaneously develops intestinal adenomas. It is a commonly used model for human familial adenomatous polyposis and for human sporadic colorectal cancer.
<b>Mesenchymal-to-epithelial transition</b>	The conversion of non-polarized and motile mesenchymal cells into polarized epithelial cells. Typically associated with increased E-CADHERIN levels and with low cancer cell invasion and metastasis. It is the reverse of the better known epithelial-to-mesenchymal transition.



<b>Ameboid-type migration</b>	Motility frequently exhibited by cancer cells and leukocytes and characterized by high speeds, lack of stable polarity and a relatively amorphous cell shape. Does not require stable integrin-dependent adhesion for traction but depends on RHOA to increase actomyosin contractility and allow invasion in the absence of extracellular proteolysis.
<b>Mesenchymal-type migration</b>	Movement of cells with elongated morphology and a front-back polarity, where traction is generated through integrin-dependent adhesion. Requires extracellular proteolysis for cell invasion and is thought to depend on RAC1.
<b>Vasculogenic mimicry</b>	The formation by the tumor cells of blood vessel-like channels that contribute to tumor blood perfusion.
<b>“Dependence” receptors</b>	Structurally unrelated receptors that can induce cell death by apoptosis when unoccupied by ligand, thus creating cellular dependence on their ligands. In the presence of ligand, these receptors mediate survival, differentiation or migration.
<b>Neutral liposomes</b>	Small vesicles made of neutral phospholipids (such as DOPC, 1,2-dioleoyl- <i>sn</i> -glycero-3-phosphatidylcholine), which can be filled with siRNA for efficient <i>in vivo</i> intracellular delivery to tumor tissue.
<b>RadioimmunoPET imaging</b>	Positron emission tomography (PET) imaging using a radioactively labeled antibody. It allows non-invasive <i>in vivo</i> visualization of a tissue of interest, such as tumor tissue, that expresses the antigen as well as quantification of antigen levels.
<b>Gamma camera imaging</b>	Imaging with a camera that detects radioisotopes emitting gamma radiation. It is also known as scintigraphy and allows non-invasive <i>in vivo</i> visualization of radioisotopes coupled, for example, to an antibody that targets tumor tissue.
<b>Epithelial-to-mesenchymal transition</b>	A complex process in which genetic and epigenetic events lead to epithelial cells acquiring a mesenchymal architecture concomitant with increased cell motility. Typically associated with the loss of E-CADHERIN expression, disruption of cell-cell junctions, and cancer cell invasion and metastasis.
<b>Apico-basal polarization</b>	Epithelial cells are polarized, with an apical membrane that faces the external environment or a lumen and is opposite the basolateral membrane, which functions in cell–cell interactions and contacts the basement membrane.

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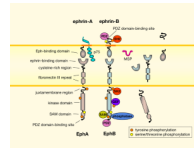
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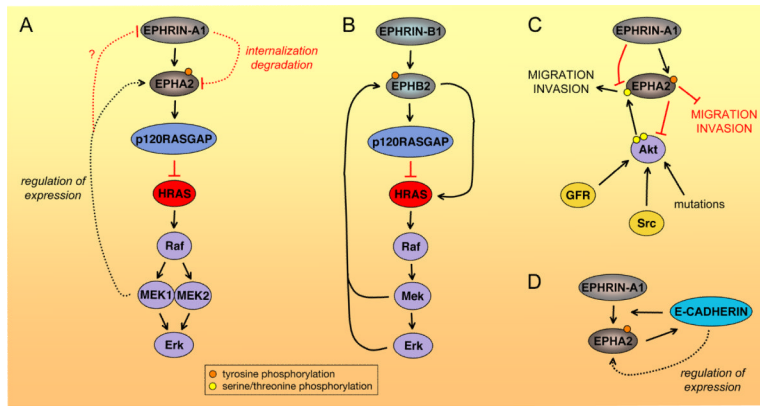
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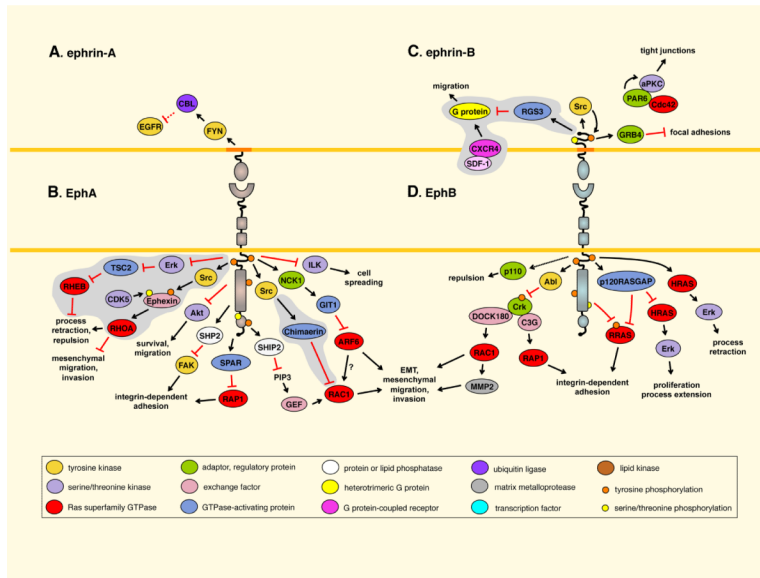
**Fig. 1. Eph receptor and ephrin domain structure and signaling interactions**

Domain structures of Eph receptors and ephrins are shown. Additionally, alternative splicing or proteolysis can generate extracellular and intracellular domain fragments of Eph receptors and ephrins of both A and B classes. The major sperm protein (MSP) domain from VAMP (vesicle-associated membrane protein)-associated protein (VAP) proteins is another Eph ligand that can compete with ephrins for binding<sup>84</sup>. Eph receptor “forward” signaling involves ephrin-induced clustering, autophosphorylation, and association with signaling effectors containing SH2, PDZ, SAM and other protein interaction domains<sup>1,5</sup>. Some signaling proteins, such as certain guanine nucleotide exchange factors (GEFs) for Rho family GTPases, can constitutively associated with Eph receptors<sup>175</sup>. The activities of some effectors are modified by activated Eph receptors, for example through phosphorylation. Phosphotyrosine phosphatases bind Eph receptors and ephrins to dampen or terminate their activity through dephosphorylation. Eph receptors are also phosphorylated on serine/threonine residues<sup>176</sup>, which can have dramatic functional consequences<sup>72</sup>. The transmembrane ephrin-Bs mediate “reverse” signals, which involve Src-dependent tyrosine phosphorylation of their cytoplasmic segment and association with SH2 and PDZ domain-containing proteins<sup>5,6</sup>. EphB binding can also affect ephrin-B function by inducing serine phosphorylation, as shown in neurons<sup>7</sup>. The glycosylphosphatidylinositol (GPI)-linked ephrin-As also mediate reverse signals, through poorly understood signaling interactions that may occur in lipid rafts (dark orange). In neurons, ephrin-As can use the p75 nerve growth factor receptor as a signaling partner to activate the FYN Src family kinase<sup>177</sup>. Most domain names are illustrated on EphA/ephrin-A and signaling interactions are illustrated on EphB/ephrin-B, but each applies to the other.



**Fig. 2. Eph feedback loops**

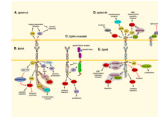
(A) EPHA2/HRAS-Erk negative feedback loop. Activation of the HRAS-Erk pathway increases EPHA2 expression through MEK1 and decreases EPHRIN-A1 expression, although it is not known if this also occurs through MEK1<sup>46,178,179</sup>. In turn, ephrin-dependent EPHA2 activation inhibits HRAS-Erk signaling and also downregulates EPHA2 levels by causing receptor internalization and degradation. (B) Positive and negative EPHB2/MAP kinase feedback loops. In a positive feedback loop, ephrin-B-dependent EPHB2 activation stimulates the HRAS-Erk pathway, and the increase in Mek and/or Erk activity in turn enables enhanced responsiveness of EPHB2 to ephrin-B stimulation through unknown mechanisms<sup>79</sup>. However, in a different cellular context EPHB2 can also inhibit the HRAS-Erk pathway<sup>5,180</sup>, which may in turn reduce EPHB2 activation by ephrin. Although not shown in the figure, EPHRIN-B1 stimulation can also downregulate EphB2 levels by causing internalization/degradation. (C) EPHA2/Akt negative feedback loop. The Akt kinase (activated by growth factor receptors (GFR), Src family kinases or mutations in upstream proteins or Akt itself) phosphorylates serine 897 in the carboxy-terminal tail of EPHA2 leading to increased EPHA2-dependent cell migration and invasion<sup>72</sup>. In turn, EPHRIN-A1-induced EPHA2 signaling inactivates Akt by causing its dephosphorylation at T308 and S473, thus decreasing EPHA2 phosphorylation at serine 897 and, consequently, cell migration and invasion. Other pathways downstream of EPHA2 can also inhibit migration and invasion. (D) EPHA2/E-CADHERIN positive feedback loop. E-CADHERIN expression increases EPHA2 expression, surface localization, interaction with EPHRIN-A1, and thus forward signaling<sup>19,20</sup> (Box 1). In turn, EPHA2 signaling enhances E-CADHERIN-mediated adhesion. Dotted lines indicate regulation of protein levels rather than activity.



**Fig. 3. Tumor suppression through bidirectional signaling**

**A.** EPHRIN-A5 reverse signaling downregulates epidermal growth factor receptor (EGFR) levels in glioma cells<sup>43</sup>. **B.** EphA receptors activate tuberous sclerosis complex 2 (TSC2) in neurons to inactivate RHEB<sup>181</sup>. EphA activation of RHOA involves Ephexin family exchange factors and other pathways (Fig. 4)<sup>5,7</sup>. EPHA2 inhibits Akt<sup>72,179</sup> and inactivates focal adhesion kinase (FAK) through the SHP2 phosphatase<sup>5</sup>. EPHA4 inhibits RAP1 through spine-associated RAPGAP (SPAR)<sup>1,7</sup>. Recruitment of the lipid phosphatase SHIP2 by EPHA2 inhibits RAC1 and EPHA2 internalization<sup>182</sup>. EPHA4 inhibits RAC1 through Chimaerins<sup>1,183</sup>. EPHA2-mediated inhibition of ADP-ribosylation factor 6 (ARF6) in epithelial cells inhibits epithelial-to-mesenchymal transition (EMT)<sup>170</sup>. EphA1 inhibits integrin-linked kinase (ILK)<sup>184</sup>. **C.** EPHRIN-B1 disrupts focal adhesions through GRB4<sup>5</sup>. Phosphorylation inhibits EPHRIN-B1 binding to PAR6, allowing PAR6 to bind GTP-bound CDC42 and activate atypical PKC (aPKC)<sup>87</sup>. Ephrin-Bs also inhibit signaling by the CXCR4 G protein-coupled chemokine receptor<sup>5</sup>. **D.** EphB signaling increases expression of the p110 subunit of PI3 kinase<sup>91</sup>. EphB receptors (and EPHA2) activate Abl, which ultimately inhibits RAP1 and RAC1<sup>42,47,107</sup>. EPHB2 inactivates RRAS through phosphorylation<sup>5</sup>. EPHB2 (and EPHA2) activates p120RASGAP to inhibit the HRAS and RRAS<sup>5,180</sup>. Finally, EPHB2 can activate Erk<sup>79</sup>. Some pathways are assembled from different sources, so the complete pathways are hypothetical. Pathways identified in neurons, and predicted to have tumor suppressing activity, are on gray background. Most other pathways were identified in cultured cells and their significance in cancer also remains to be proven. Dotted lines indicate regulation of expression levels. For more details see references 1,5–7,88,127. CDK5, cyclin-dependent kinase 5; GIT1, G protein-coupled receptor kinase-interacting ARFGAP 1; MMP2, matrix metalloproteinase 2; RAPGEF1, Rap guanine nucleotide exchange factor 1; RGS3, regulator of G-protein signaling 3; SDF-1, stromal cell-derived factor-1.





**Fig. 4. Eph tumor promoting pathways**

**A.** EPHRIN-A5 reverse signaling promotes activation of FYN,  $\beta$ 1-integrins, and Erk in fibroblasts<sup>5</sup>. **B.** Low-molecular-weight phosphotyrosine phosphatase (LMW-PTP) is activated by Src and dephosphorylates and inactivates p190RHOGAP. This increases RHOA activity to destabilize adherens junctions in EPHA2-overexpressing epithelial cells<sup>103</sup>. EPHA2 (and EPHB2) activate RHOA through focal adhesion kinase (FAK)<sup>7,94</sup>. EPHA4 activates signal transducer and activator of transcription 3 (STAT3)<sup>185</sup>. A pathway involving EPHA2, PI3 kinase and Vav family exchange factors for RAC1 operates in endothelial cells<sup>27,186</sup>. Activation of EPHA2 activates Akt in pancreatic cancer cells<sup>138</sup>. The *C. elegans* Eph receptor inhibits PTEN expression<sup>80</sup>. **C.** EPHA2-ERBB2 crosstalk activates the HRAS-Erk pathway and RHOA in a mouse mammary tumor model, enhancing tumor growth and *in vitro* cell proliferation and migration<sup>33,105</sup>. Akt, activated by ERBB2 or other pathways, phosphorylates EPHA2. **D.** Ephrin-B reverse signaling affects pathways that promote invasiveness, including matrix metalloproteinase 8 (MMP8) secretion<sup>17</sup> and activation of STAT3,<sup>118</sup> Src and RAC1<sup>113114</sup>. In contrast, non-phosphorylated EPHRIN-B1 can bind PAR6 to inhibit atypical protein kinase C (aPKC)<sup>87</sup>. **E.** EphB forward signaling activates various RAC1 and CDC42 exchange factors<sup>5,7,127</sup>, which could promote cancer cell migration and invasion. EphB4 activates RHOA<sup>93</sup>. EPHB2-mediated RRAS tyrosine phosphorylation increases glioma cell invasiveness<sup>96</sup>. EPHB2-mediated ABL activation increases CYCLIN-D1 levels<sup>91</sup>. Pathways identified in neurons, endothelial/muscle cells, or *C. elegans* that are predicted to have tumor promoting activity are on gray, light-brown or light blue background, respectively. Most other pathways were identified in cultured cells and their significance in cancer remains to be proven. For more details see references 1,5–7,127. ARF1, ADP-ribosylation factor 1; EMT, epithelial-to-mesenchymal transition; JAK2, Janus kinase 2.

**Table 1**

Examples of regulation of Eph receptor expression in cancer cells

Mechanism	Eph/ephrin	Change	Cell type/Cancer type	Ref.
<b>Frequent chromosomal abnormalities that may lead to altered Eph/ephrin expression <sup>a</sup></b>				
1p36 loss	EPHA2 EPHA8, EPHB2	down	various cancers	48, 49, 69, 187–190 <sup>b</sup>
1q21-q22 gain	EPHRIN-A1, -A3, -A4	up	various cancers	<i>b</i>
2q36.1 loss	EPHA4	down	cervical cancer	191
3p11.2 loss	EPHA3	down	lung and various other cancers	187 <i>b</i>
3q21-qter gain	EPHB3	up	early stage squamous cell lung carcinoma	50
5q21 loss	EPHRIN-A5	down	myeloid cancers, prostate cancer	187, 192 <sup>c</sup>
6q16.1 loss	EPHA7	down	various cancers	187, 192 <sup>c</sup>
7q22 loss	EPHB4	down	myeloid cancers, colon cancer	187 <i>c</i>
7q22 gain	EPHB4	up	various tumors and cancer cell lines	187, 193, 194
7q33-35 loss	EPHB6, EPHA1	down	myeloid cancers	187
7q33-35 gain	EPHB6, EPHA1	up	neuroblastoma, glioblastoma	187
13q33 loss	EPHRIN-B2	down	multiple myeloma, chronic lymphocytic leukemia, head and neck cancer	187 <sup>b,c</sup>
17p13.1-p11.2 loss	EPHRIN-B3	down	various cancers	<i>b,c</i>
19p13.3 loss	EPHRIN-A2	down	various cancers	195 <i>c</i>
<b>Promoter hypermethylation</b>				
	EPHA1	down	advanced colorectal cancer	39
	EPHA3	down	leukemias, hematopoietic tumor cells	196
	EPHA7	down	prostate, gastric, colorectal cancer	197 – 199
	soluble EPHA7 ectodomain	down	B-cell lymphomas	200
	EPHB2	down	colorectal cancer	40, 189, 201
	EPHB4	down	colorectal cancer	41
	EPHB6	down	MDA-MB-231 breast cancer cells	202
<b>mRNA stability</b>				
nonsense-mediated mRNA decay	EPHB2	down	prostate cancer	49
binding sites for RNA binding protein HuR in 3' UTR	EPHA2, EPHA4, EPHRIN-A2	down	HeLa cervical cancer and U373MG glioma cells	51
microRNA-210	EPHRIN-A3	down	endothelial cells	62, 203
<b>Transcription</b>				
Ras-MAP kinase (MEK1)	EPHA2	up	breast cancer cells, activated B-Raf-transfected fibroblasts	46, 179
p53	EPHA2, EPHB4, EPHRIN-A1	up	various p53-transfected cell lines	204 – 206

Mechanism	Eph/ephrin	Change	Cell type/Cancer type	Ref.
Twist	EPHA4, EPHRIN-A4	up	developing skull, Sézary's lymphoma?	207, 208
c-REL	EPHB2	down	SW620 colon cancer cells	45
Wnt/ $\beta$ -catenin/TCF	EPHB2, EPHB3, EPHB4	up	early colorectal cancer	23, 209
Wnt/ $\beta$ -catenin/p300/TCF	EPHB2	up	early colorectal cancer	37
Wnt/ $\beta$ -catenin/CBP/TCF	EPHB4	up	advanced colorectal cancer	37
estrogen	EPHB4, EPHRIN-B2	up	mouse mammary epithelium	210
Ras/MAP kinase	EPHRIN-A1	down	MCF10A mammary epithelial cells	46
Wnt/ $\beta$ -catenin/TCF	EPHRIN-B	down	Ls174T colon cancer cells	209

<sup>a</sup>Chromosomal locations from the NCBI Human Genome Resources ([www.ncbi.nih.gov/projects/genome/guide/human](http://www.ncbi.nih.gov/projects/genome/guide/human))

<sup>b</sup>Cancer GeneticsWeb ([www.cancer-genetics.org](http://www.cancer-genetics.org))

<sup>c</sup>Atlas of genetics and cytogenetics in oncology and haematology (<http://atlasgeneticsoncology.org/index.html>).

**Table 2**

## Eph/ephrin targeting molecules

Molecules	Targets	Activity	Ref.
<b>Kinase inhibitors</b>			
anilinopyrimidine derivatives	EPHB4 <sup>a</sup>	ATP competitors	133, 211
benzenesulfonamide derivative	EPHB4 <sup>a</sup>	ATP competitor	132
XL647 (EXEL-7647) <sup>b</sup>	EPHB4 <sup>a</sup>	ATP competitor	212
xanthine derivatives	Eph receptors	ATP competitors	135, 213
LDN-211904	Eph receptors	ATP competitor	136
pyrido[2,3-d]pyrimidine PD173955	Eph receptors	ATP competitor	214
nilotinib and analogs <sup>b</sup>	Eph receptors	ATP competitors	134, 215
dasatinib <sup>a</sup>	Eph receptors	ATP competitor	34, 35, 137, 138
<b>Inhibitors of Eph expression</b>			
siRNA	EPHA2	mRNA downregulation	101, 102, 139
oligonucleotides	EPHA2	protein downregulation	100
siRNA	EPHB4	mRNA downregulation	36, 37, 194, 216, 217
oligonucleotides	EPHB4	Protein downregulation	36, 194, 216, 217
<b>Inhibitors of Eph-ephrin interaction</b>			
EPHA2 Fc, EPHA3 Fc	EPHRIN-A	Eph competitor	53, 218–220
sEPHB4	EPHRIN-B	Eph competitor	142, 221, 222
KYL and other peptides <sup>e</sup>	EPHA4	ephrin competitor	147, 150, 223
SNEW and other peptides	EPHB2	ephrin competitor	145, 149
TNYL-RAW peptide	EPHB4	ephrin competitor	145, 148, 224
dimethyl-pyrrole derivatives	EPHA2, EPHA4	ephrin competitor	150, 151
mAb 2H9 antagonistic antibody	EPHB2	ephrin competitor	143
<b>Activators of Eph forward signaling (also downregulate Eph expression)</b>			
EA1.2 antibody	EPHA2	Eph activation/degradation, ADCC <sup>C?</sup>	100
EA2, B233, 3F2-WT (humanized B233) antibody	EPHA2	Eph activation/degradation, ADCC?	152, 155
EAS antibody	EPHA2	Eph activation/degradation, reduced Src phosphorylation & VEGF levels, ADCC?	153
Ab20, 1G9-H7 antibodies <sup>d</sup>	EPHA2	Eph activation/degradation	156
mAB208	EPHA2	Eph degradation & enhanced presentation of peptide antigens on tumor cell surface	154
YSA, SWL peptides	EPHA2	ephrin competitor, Eph activation/degradation	146
dimerized IIIA4 antibody	EPHA3	Eph activation	161

Molecules	Targets	Activity	Ref.
EPHRIN-A1 Fc	EphA receptors	Eph activation/degradation	74
EPHRIN-B2 Fc	EPHB4	Eph activation/degradation	127
<b>Cytotoxic molecules</b>			
1C1 antibody-mc-MMAF <sup>f</sup> conjugate <sup>b</sup>	EPHA2	receptor-mediated internalization & disruption of microtubule dynamics	157, 158
3F2-3M antibody (mutated 3F2-WT with enhanced effector function)	EPHA2	ADCC	155
bscEphA2xCD3 bispecific single-chain antibody	EPHA2/CD3	redirection of unstimulated cytotoxic T cells to EphA2-positive tumor cells	162
YSA-modified adenovirus <sup>e</sup>	EPHA2	adenoviral transduction of EphA2-expressing tumor cells	225
EPHRIN-A1-PE38QQR <i>Pseudomonas</i> exotoxin A conjugate	EphA receptors	EphA-mediated internalization and exotoxin-dependent cell death	226
EPHRIN-A1-gold-coated nanoshells	EphA receptors	absorption of near infrared light for photothermal ablation of tumor cells	159
2H9 antibody-vc-MMAE <sup>g</sup> conjugate	EPHB2	receptor-mediated internalization & disruption of microtubule dynamics	143
<b>Imaging agents</b>			
<sup>64</sup> Cu-DOTA-1C1 antibody	EPHA2	binding, which enables radioimmunoPET	160
YSA peptide-magnetic nanoparticles	EPHA2	binding, which enables cell capture	227, 228
<sup>111</sup> Indium-labelled IIIA4 antibody	EPHA3	binding to low affinity ephrin-binding site, which enables tumor detection	161

<sup>a</sup>Eph receptor selectivity has not been reported

<sup>b</sup>In clinical trials

<sup>c</sup>ADCC, antibody-dependent cell-mediated cytotoxicity

<sup>d</sup>not effective in vivo

<sup>e</sup>tested in vivo in a model of spinal cord injury

<sup>f</sup>mc-MMAF, stable maleimidocaproyl linker-monomethylauristatin F

<sup>g</sup>vc-MMAE, cathepsin B-cleavable valine-citrulline linker-monomethylauristatin E..