

# Eph Receptor Signaling and Ephrins

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The Eph receptors are the largest of the RTK families. Like other RTKs, they transduce signals from the cell exterior to the interior through ligand-induced activation of their kinase domain. However, the Eph receptors also have distinctive features. Instead of binding soluble ligands, they generally mediate contact-dependent cell–cell communication by interacting with surface-associated ligands—the ephrins—on neighboring cells. Eph receptor–ephrin complexes emanate bidirectional signals that affect both receptor- and ephrin-expressing cells. Intriguingly, ephrins can also attenuate signaling by Eph receptors coexpressed in the same cell. Additionally, Eph receptors can modulate cell behavior independently of ephrin binding and kinase activity. The Eph/ephrin system regulates many developmental processes and adult tissue homeostasis. Its abnormal function has been implicated in various diseases, including cancer. Thus, Eph receptors represent promising therapeutic targets. However, more research is needed to better understand the many aspects of their complex biology that remain mysterious.

The Eph receptors have the prototypical RTK topology, with a multidomain extracellular region that includes the ephrin ligand-binding domain, a single transmembrane segment, and a cytoplasmic region that contains the kinase domain (Fig. 1). There are nine EphA receptors in the human genome, which promiscuously bind five ephrin-A ligands and five EphB receptors, which promiscuously bind three ephrin-B ligands (Pasquale 2004, 2005). Additionally, EphA4 and EphB2 can also bind ephrins of a different class. Two members of the family, EphA10 and EphB6, have modifications in conserved regions of their kinase domains that pre-

vent kinase activity. Furthermore, a variety of alternatively spliced forms identified for many Eph receptors differ from the prototypical structure and have distinctive functions (Zisch and Pasquale 1997; Pasquale 2010).

Both ephrin classes include a conserved Eph receptor-binding domain, which is connected to the plasma membrane by a linker segment whose length can be affected by alternative splicing (Fig. 1). The ephrin-As are attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor, although they can also be released to activate EphA receptors at a distance (Bartley et al. 1994; Wykosky et al. 2008), whereas the

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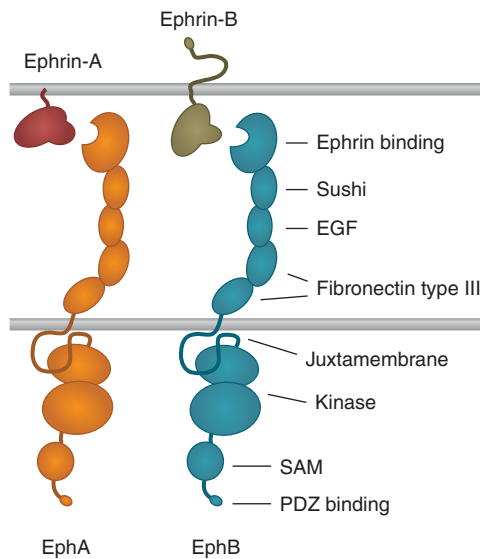
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**Figure 1.** Domain structure of Eph receptors and ephrins.

ephrin-Bs contain a transmembrane segment and a short cytoplasmic region. Ephrin-A3 and ephrin-B3 also bind heparan sulfate proteoglycans through an interaction that involves their extracellular linker region and that, at least in the case of ephrin-A3, potentiates EphA receptor activation and signaling (Irie et al. 2008; Holen et al. 2011).

The Eph receptor family has greatly expanded during evolution, and includes almost one fourth of the 58 human RTKs (Schlessinger and Lemmon 2013). A large number of Eph receptors and ephrins may be required to achieve and maintain the sophisticated tissue organization of higher organisms. Indeed, many are highly expressed in the most complex organ, the brain, particularly during the establishment of its complex architecture and intricate wiring of neuronal connections (Yamaguchi and Pasquale 2004). Besides the brain, Eph receptors and ephrins are also present in most—if not all—other tissues, often in a combinatorial manner and with dynamically changing expression patterns (Pasquale 2005). In some regions, Eph receptors and ephrins are both coexpressed in the same cells, in others they have mutually exclusive expression patterns or they

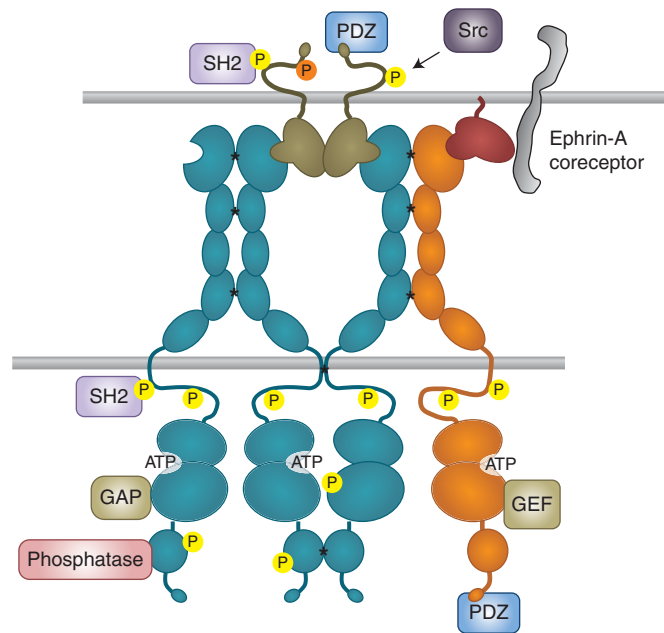
can be expressed in complementary gradients. These situations likely reflect different signaling modalities with different biological outcomes.

Eph receptors and ephrins engage in a multitude of activities. They typically mediate contact-dependent communication between cells of the same or different types to control cell morphology, adhesion, movement, proliferation, survival, and differentiation (Pasquale 2005). Through these activities, during development, the Eph/ephrin system plays a role in the spatial organization of different cell populations, axon guidance, formation of synaptic connections between neurons, and blood vessel remodeling. In the adult, the Eph/ephrin system regulates remodeling of synapses, epithelial differentiation and integrity, bone remodeling, immune function, insulin secretion, and stem cell self-renewal (Pasquale 2008; Genander and Frisen 2010). In addition, Eph receptors and ephrins are often up-regulated in injured tissues, where they inhibit some regenerative processes but promote angiogenesis, as well as in cancer cells, where they seem to be able to both promote and suppress tumorigenicity (Du et al. 2007; Pasquale 2008, 2010).

Here we provide an overview of Eph receptor and ephrin signaling mechanisms and biological effects, with an emphasis on recent findings. More detailed information on specific aspects of Eph receptor/ephrin biology and downstream signaling networks can be found in other recent reviews (Pasquale 2005, 2008, 2010; Arvanitis and Davy 2008; Lackmann and Boyd 2008; Klein 2009; Genander and Frisen 2010).

## EPH RECEPTOR FORWARD SIGNALING

“Forward” signaling corresponds to the prototypical RTK mode of signaling, which is triggered by ligand binding and involves activation of the kinase domain. However, the activation mechanisms of Eph receptors have unique features as compared to other RTK families (Barton et al. 2013). Binding between Eph receptors and ephrins on juxtaposed cell surfaces leads to oligomerization through not only Eph receptor–ephrin interfaces but also receptor–receptor *cis* interfaces located in multiple domains



**Figure 2.** Eph receptor clustering and bidirectional signaling. SH2 and PDZ indicate proteins containing these domains. All types of signaling proteins shown can associate with both EphA and EphB receptors. Asterisks indicate receptor–receptor interactions favoring clustering; yellow circles indicate tyrosine phosphorylation and the orange circle indicates serine phosphorylation.



(Fig. 2) (Himanen et al. 2010; Seiradake et al. 2010). In fact, Eph receptor clusters induced by ephrin binding can enlarge to incorporate Eph receptors that are not bound to ephrins (Wimmer-Kleikamp et al. 2004). The cellular context can also affect Eph receptor clustering ability, which depends on association with the actin cytoskeleton (Salaita and Groves 2010). Given the promiscuity of Eph receptor–ephrin interactions, and also receptor–receptor *cis* interactions, the clusters can include Eph receptors of both A and B classes (Janes et al. 2011).

The proximity of clustered Eph receptor molecules leads to *trans*-phosphorylation. Phosphorylation of two conserved tyrosines in the juxtamembrane domain relieves inhibitory intramolecular interactions with the kinase domain, enabling efficient kinase activity (Binns et al. 2000; Zisch et al. 2000; Wybenga-Groot et al. 2001). Phosphorylation of the conserved tyrosine in the activation loop appears to be less critical for Eph receptor activation than it is for many other RTKs, although it may be important

for maximal activity (Binns et al. 2000; Singla et al. 2011). There are also differences in the kinase domains within the Eph receptor family. For example, the “gatekeeper” residue in the hinge region between the kinase domain lobes, which controls access to a hydrophobic pocket adjacent to the ATP-binding site, is a threonine in most Eph receptors but a valine in EphA6 and an isoleucine in EphA7. Hence, EphA6 and EphA7 likely differ from the other Eph receptors in their sensitivity to kinase inhibitors and possibly substrate specificity (Skaggs et al. 2006; Zhang et al. 2009a).

The Eph receptors modulate many of the same networks of adaptor and effector proteins that also function downstream of other RTK families (Wagner et al. 2013). Various tyrosine autophosphorylation sites in activated Eph receptors—including the two regulatory juxtamembrane sites—enable recruitment of downstream signaling proteins that contain SH2 domains, including nonreceptor tyrosine kinases of the Src and Abl families and adaptors such

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as Nck and Crk, which are crucial for signal transduction (Fig. 2) (Jorgensen et al. 2009; Pasquale 2010). Binding of PDZ domain-containing proteins to the carboxy-terminal tails of Eph receptors also contributes to signaling. Particularly important effectors are Rho and Ras family GTPases and Akt/mTORC1. Interestingly, whereas most other RTK families use these central regulators of cellular physiology to stimulate cell proliferation, survival, and forward movement, the Eph receptors can use them to inhibit cell growth and achieve cell repulsion. In cancer cells, this can result in tumor suppression.

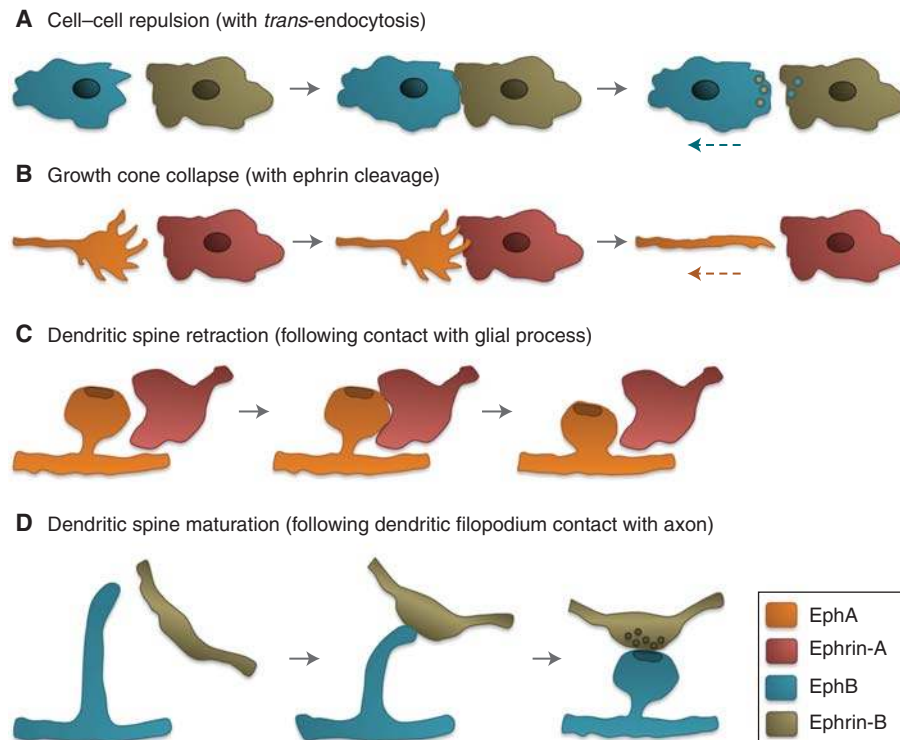
Signaling by the Eph receptors, however, is not always consistent and can lead to divergent outcomes. The kinase-inactive Eph receptors and alternatively spliced forms lacking the kinase domain can modulate signaling outcome by reducing signal strength in the clusters as well as by contributing distinctive signals. For example, the kinase inactive EphB6 can be phosphorylated by other Eph receptors and subvert the effects of EphB4 in breast cancer cells (Truitt and Freywald 2011). Moreover, a truncated membrane-anchored form of the EphA7 extracellular domain can convert repulsion to cell–cell adhesion in the developing neural tube by decreasing signaling by full-length EphA7 (Holmberg et al. 2000), and a secreted truncated form of EphA7 acts as a tumor suppressor in follicular lymphoma by binding EphA2 and blocking its oncogenic signals (Oricchio et al. 2011). There is also evidence that small and large Eph receptor clusters differ in their ability to recruit certain signaling molecules (Salaita and Groves 2010). Other aspects of the cellular context, and implementation of positive and negative feedback loops, further contribute to the diversity of Eph receptor activities.

### Rho Family GTPases

The Eph receptors are well known for their effects on the actin cytoskeleton, which impact cell shape, adhesion, and movement through regulation of the Rho GTPase family, including RhoA, Rac1, and Cdc42 (Pasquale 2008, 2010). GTPases cycle between a GDP bound (inactive)

state and a GTP bound (active) state that binds downstream effectors. The Eph receptors can influence these conversions by regulating both guanine nucleotide exchange factors (GEFs, which facilitate GDP to GTP exchange) and GTPase-activating proteins (GAPs, which promote GTP hydrolysis to GDP). Regulation of GEFs and GAPs by Eph receptors can involve constitutive or ephrin-induced association, tyrosine phosphorylation, or even ubiquitination and degradation.

RhoA is mostly involved in the formation of stress fibers and focal adhesions as well as contraction of the actomyosin cytoskeleton, whereas Rac1 and Cdc42 drive the formation of protrusive structures such as lamellipodia and filopodia, respectively (Heasman and Ridley 2008). An increased balance of RhoA versus Rac1/Cdc42 activities has been implicated in the characteristic repulsive effects of Eph receptor forward signaling, including process retraction and inhibition of cell migration/invasiveness (Fig. 3A–C). The collapse or local retraction of neuronal growth cones and dendritic spines (the small protrusions on dendrites bearing excitatory synapses) are well-known repulsive effects of EphA receptors that depend on Rho family GTPases (Fig. 3B,C) (Wahl et al. 2000; Murai et al. 2003; Fu et al. 2007). Growth cone collapse involves RhoA activation, for example, by the GEF Ephexin1 (Shamah et al. 2001; Sahin et al. 2005), and Rac1 inactivation, for example, by the GAP  $\alpha$ 2-Chimaerin (Beg et al. 2007; Iwasato et al. 2007; Shi et al. 2007; Wegmeyer et al. 2007). However Rac1 activation, which can occur downstream of Vav family GEFs, is also required for growth cone collapse and to process retraction by enabling endocytic removal of adhesive Eph receptor–ephrin complexes from sites of cell–cell contact (Cowan et al. 2005; Yoo et al. 2011). Activation and inactivation of Rho family GTPases may occur with different spatial and/or temporal resolution to achieve growth cone collapse and regulate dendritic spines. In other cell types, Eph repulsive signaling involving Rho family GTPases can lead to mesodermal–ectodermal tissue separation during gastrulation (Park et al. 2011; Rohani et al. 2011), Schwann cell-astrocyte segregation in the in-



**Figure 3.** Eph receptor–ephrin repulsive effects and dendritic spine maturation. (A) An EphB-expressing cell encounters an ephrin-B-expressing cell and retracts after the internalization of EphB–ephrin-B complexes enables cell separation. (B) An EphA-expressing growth cone at the leading edge of an axon encounters an ephrin-A-expressing cell, collapses, and begins to retract after the cleavage of ephrin-A molecules enables cell separation. (C) An EphA-expressing spine on a dendrite (bearing an excitatory postsynaptic terminal represented as a darker oval) comes in contact with an ephrin-A-expressing glial process and retracts becoming shorter. (D) An EphB-expressing filopodial protrusion on a dendrite acquires an enlarged “head” and shortens following contact with an ephrin-B-expressing axon. The presynaptic terminal also matures following contact.

jured nervous system (Afshari et al. 2010), and contact inhibition of locomotion (Astin et al. 2010).

Regulation of Rho family GTPases by Eph receptors can also control cellular processes beyond repulsion. For example, the maturation of neuronal filopodial protrusions into dendritic spines (Fig. 3D) entails ephrin-B/EphB-dependent activation of the Rac-GEFs Kalirin and Tiam1 and the Cdc42-GEF Intersectin to promote the formation of branched actin filaments that enlarge the distal portion of the filopodial protrusions (Irie and Yamaguchi 2002; Penzes et al. 2003; Tolia et al. 2007), whereas RhoA activation through focal adhesion kinase and a RhoGEF shortens the protrusions (Moeller

et al. 2006). Interestingly, EphB receptor forward signaling can also promote synapse formation through ubiquitination and degradation of the Rho-GEF Ephexin5, which decreases RhoA activity without obvious effects on spine morphology (Margolis et al. 2010). Furthermore, EphA2 forward signaling can promote endothelial angiogenic responses by activating Rac1 through Vav family GEFs (Hunter et al. 2006) and enhance epithelial characteristics by inhibiting RhoA through p190RhoGAP (Wakayama et al. 2011). In addition, EphB receptor activation by ephrin-B-expressing stromal cells promotes HGF-dependent invasiveness of metastatic PC3 prostate cancer cells through sustained Cdc42 activation (Astin et al. 2010).

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Further work is needed to understand in detail the mechanisms leading to activation versus inhibition of Rho GTPases by Eph receptors and examine the role of the many less well-characterized Rho family members in the biological activities of Eph receptors.

### Ras Family GTPases

Perhaps the most prototypical RTK signaling pathway involves activation of the H-Ras GTPase by the GEF Sos, which is recruited by the adaptors Shc and/or Grb2 bound to activated RTKs (McKay and Morrison 2007). H-Ras-GTP triggers a phosphorylation cascade that culminates in activation of the Erk1 and Erk2 serine/threonine kinases. Through phosphorylation of cytoplasmic effectors and nuclear transcription factors, the Ras-Erk pathway regulates many physiological processes—including cell proliferation, survival, differentiation, adhesion, and migration—and its deregulation can cause cancer and other diseases.

Remarkably, Eph receptor forward signaling frequently inhibits the Ras-Erk pathway and can override its activation by other RTKs (Pasquale 2008, 2010). For example, polarized Eph receptor activation in progenitor cells of the ascidian embryo attenuates Erk activation by the FGF RTK, leading to asymmetric division and fate specification (Picco et al. 2007; Shi and Levine 2008). Furthermore, ephrin-A/EphA signaling induced by contact between myoblasts suppresses Erk activation by the IGF-1 RTK, facilitating myogenic differentiation (Minami et al. 2011). In neurons, EphA-dependent Erk inhibition suppresses the effects of the TrkB RTK on growth cone motility and gene expression (Meier et al. 2011) and promotes growth cone collapse (Nie et al. 2010). In cancer cells, ephrin-A/EphA signals that suppress Erk activation by RTKs can inhibit tumorigenicity (Miao et al. 2001; Macrae et al. 2005).

A common mechanism of Eph receptor-dependent Erk inhibition is through p120RasGAP, which inactivates H-Ras (Elowe et al. 2001; Minami et al. 2011). Through p120RasGAP, the Eph receptors can also inhibit another Ras family GTPase, R-Ras, causing the reduced integrin

activity that is important for retraction of cell processes and decreased malignancy (Dail et al. 2006). Eph receptors can also negatively regulate Rap1, another member of the Ras family involved in integrin activation, by inhibiting the GEF C3G or activating the GAP SPAR (Riedl et al. 2005; Richter et al. 2007; Huang et al. 2008; Pasquale 2008).

In some cases, however, Eph receptors behave similarly to other RTKs and activate the Ras-Erk pathway. For example, in cultured mouse mesenchymal cells, ephrin-B1-EphB signaling activates Erk to promote proliferation and regulate immediate early gene transcription (Bush and Soriano 2010). In P19 embryonal carcinoma cells and microvascular endothelial cells, ephrin-stimulated EphB1 recruits the adaptors Shc and Grb2 to activate H-Ras and increase cell migration (Vindis et al. 2003). Interestingly, the activation of EphB4 by ephrin-B2 in MCF7 breast cancer cells promotes Erk1/2 activation through an unusual pathway that seems to require the PP2A serine/threonine phosphatase (Xiao et al. 2012). In stably transfected HEK293 cells, EphB2 forward signaling activates Erk to promote cell repulsion (Poliakov et al. 2008). The interplay between Eph receptors and Ras GTPases also involves feedback loops in which Ras-Erk signaling reciprocally influences Eph receptors, for example, by reinforcing ephrin-B1/EphB2 signaling or up-regulating EphA2 gene transcription (Menges and McCance 2008; Poliakov et al. 2008).

### Akt

Akt is a serine/threonine kinase that regulates cell size, proliferation, and survival through various downstream effectors such as mTOR complex 1 (mTORC1). RTKs typically activate Akt through PI3 kinase, a lipid kinase that initiates a pathway leading to Akt activation through phosphorylation on T308 and S473 (Manning and Cantley 2007). In contrast, Eph receptor forward signaling can suppress Akt activation. For instance, in a variety of cancer cells, ephrin-dependent EphA2 activation leads to rapid dephosphorylation of Akt T308 and S473, which likely depends on regulation of a phosphatase,



leading to mTORC1 inactivation and decreased cell growth and migration (Menges and McCance 2008; Miao et al. 2009; Yang et al. 2011). Remarkably, this can occur even in cancer cells where the PI3 kinase-Akt pathway is activated by oncogenic mutations. EphB3 kinase activation can also inhibit Akt, which leads to suppression of non-small-cell lung cancer migration and metastasis, by promoting the assembly of a complex involving the EphB3-binding partner RACK1 (receptor for activated C-kinase 1), the serine/threonine phosphatase PP2A and Akt itself (Li et al. 2012). However, Eph receptors can also activate Akt, for example, in pancreatic cancer cells stimulated with ephrin-A1 (Chang et al. 2008) or in malignant T lymphocytes where ephrin-B treatment suppresses apoptosis (Maddigan et al. 2011).

Akt signaling can reciprocally influence Eph receptors through feedback loops. For example, phospho-RTK arrays suggest an up-regulation of several tyrosine-phosphorylated Eph receptors in cancer cells treated with Akt inhibitors (Chandarlapaty et al. 2011). Furthermore, Akt can phosphorylate EphA2 on S897 drastically altering receptor function (see below), whereas ephrin-A1 stimulation causes loss of S897 phosphorylation (Miao et al. 2009).

### EPHRIN REVERSE SIGNALING

Besides forward signaling, the Eph receptors can also stimulate “reverse” signaling in the ephrin-expressing cells (Fig. 2) (Pasquale 2005, 2010). A central feature enabling signaling by the ephrins, which lack an enzymatic domain, is the activation of Src family kinases. Eph receptor binding causes ephrin-B phosphorylation by Src kinases, creating binding sites for the SH2 domains of signaling proteins such as the adaptor Grb4 (Cowan and Henkemeyer 2001; Palmer et al. 2002). Ephrin-B signaling through Grb4 controls axon pruning, synapse formation and dendritic spine morphogenesis in the developing mouse hippocampus (Segura et al. 2007; Xu and Henkemeyer 2009). Phosphorylation of a serine near the ephrin-B carboxyl terminus, which is also induced by EphB receptor binding, leads to stabilization of AMPA neurotransmit-

ter receptors at synapses (Essmann et al. 2008). This might regulate synaptic plasticity in concert with ephrin-B tyrosine phosphorylation (Bouzioukh et al. 2007).

Recruitment of signaling proteins containing PDZ domains to the ephrin-B carboxyl terminus is also crucial for reverse signaling. For example, the adaptor PDZ-RGS3 connects ephrin-B to G-protein-coupled receptors that control neuronal cell migration and neural progenitor self-renewal (Lu et al. 2001; Qiu et al. 2010). Ephrin-B interaction with PDZ domain proteins also promotes angiogenesis and lymphangiogenesis by enabling VEGF receptor endocytosis, and can regulate axon guidance and synaptic plasticity (Makinen et al. 2005; Bouzioukh et al. 2007; Bush and Soriano 2009; Sawamiphak et al. 2010; Wang et al. 2010). Furthermore, ephrin-B signaling controls neuronal migration in the developing mouse brain through cross talk with the secreted glycoprotein Reelin (Senturk et al. 2011), modulates epithelial cell–cell junctions through the Par polarity complex (Lee et al. 2008), disrupts gap junctional communication (Mellitzer et al. 1999; Davy et al. 2006), and enhances glioma cell invasiveness by activating Rac1 (Nakada et al. 2006).

The ephrin-As lack a cytoplasmic domain and it is not well understood how they activate intracellular signaling. Studies in neurons have implicated the p75 neurotrophin receptor and the TrkB and Ret RTKs as transmembrane-binding partners that enable ephrin-A-dependent reverse signals involved in axon guidance and branching (Fig. 2) (Lim et al. 2008; Marler et al. 2008, 2010; Bonanomi et al. 2012). Through these and likely other binding partners, the ephrin-As have diverse signaling activities. Ephrin-A2 reverse signaling can inhibit neural progenitor cell proliferation, perhaps opposing the positive effects of ephrin-B1 (Holmberg et al. 2005). In the adult hippocampus, glial ephrin-A3 functions together with neuronal EphA4 to modulate uptake of the neurotransmitter glutamate by glial cells and, thus, synaptic plasticity (Carmona et al. 2009; Filosa et al. 2009). Ephrin-A4 can inhibit apoptotic cell death in Jurkat immune cells by activating Src family kinases and Akt (Holen et al. 2008).

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Ephrin-A5 reverse signaling in pancreatic  $\beta$  cells can stimulate Rac1 activity, which is necessary for insulin secretion after glucose stimulation (Konstantinova et al. 2007). Ephrin-A5 can also increase cell-substrate adhesion in fibroblasts and astrocytes by activating the Src family kinase Fyn and integrins, and seems able to also promote invasiveness (Davy et al. 1999, 2000; Campbell et al. 2006). Furthermore, this ephrin promotes Fyn activation in glioma and HEK293 cells, leading to Cbl-dependent EGF RTK ubiquitination and degradation (Li et al. 2009). Interestingly, Fyn can in turn function in a negative feedback loop to down-regulate cell surface ephrin-A levels by modulating the metabolism of sphingomyelin (Baba et al. 2009).

## BEYOND BIDIRECTIONAL SIGNALING

### Internalization and Proteolytic Cleavage

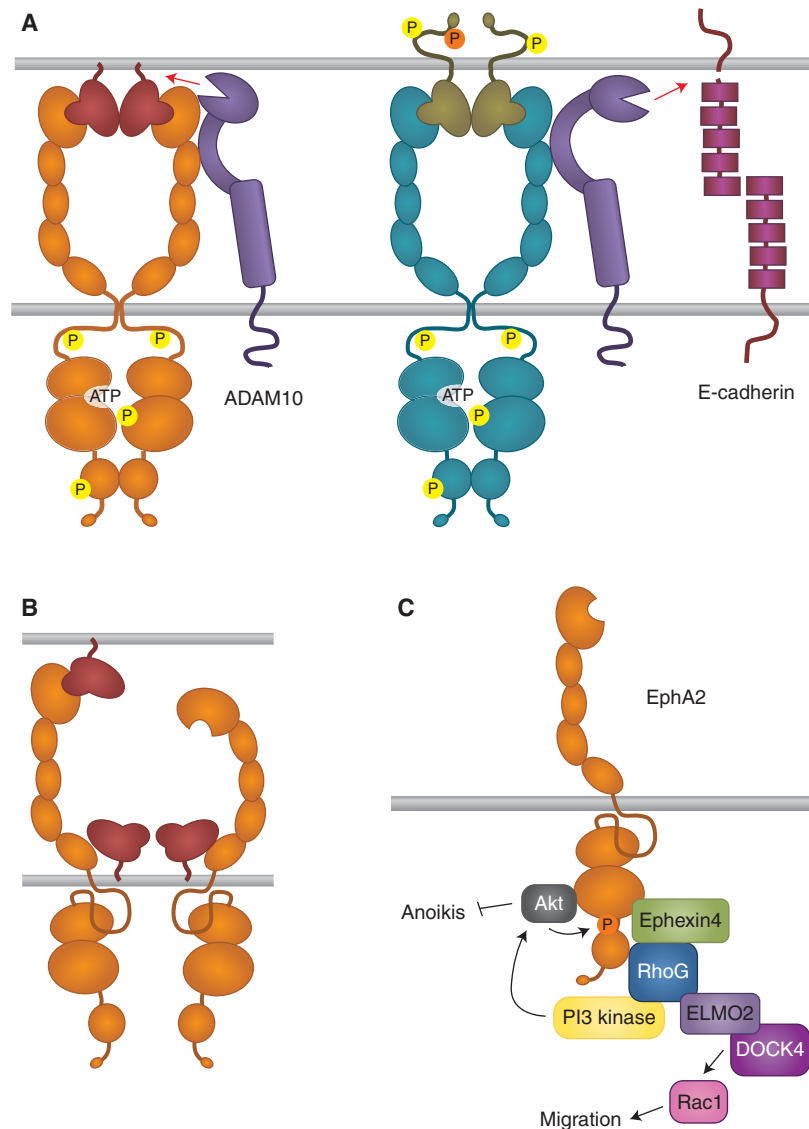
Following ligand-dependent activation, RTKs are typically internalized by endocytosis and can continue to signal from intracellular compartments until they are inactivated by dephosphorylation and degradation or traffic back to the cell surface (Goh and Sorkin 2013). For the Eph receptors, this process has unique features as a result of the plasma membrane association of the ephrins (Marston et al. 2003; Zimmer et al. 2003; Pitulescu and Adams 2010). Eph receptor–ephrin complexes can be internalized into either the Eph receptor- or the ephrin-expressing cells through the formation of vesicles containing plasma membrane fragments derived from both cells (Fig. 3A). This Rac1-dependent process, which has been defined “*trans*-endocytosis,” is critical for removal of adhesive complexes from cell–cell contact sites to allow cell separation and repulsive effects. Another protein that contributes to Eph receptor internalization and degradation is the ubiquitin ligase Cbl, which can interact with several Eph receptors promoting their ubiquitination (Walker-Daniels et al. 2002; Fasen et al. 2008).

Besides *trans*-endocytosis, Eph receptor–ephrin complexes can convert adhesive interactions into cell repulsion by activating metalloproteases, such as ADAM family members. For

example, the transmembrane ADAM10 protease can associate with ephrin-A2 on the same cell surface and cleave it following EphA receptor binding in *trans* to enable repulsive axon guidance (Hattori et al. 2000). ADAM10 can also associate with EphA3, whose active conformation promotes protease activity toward the ephrin in *trans* (Fig. 4A) (Janes et al. 2005, 2009). EphB receptors also interact with ADAM10, as well as the cell-adhesion molecule E-cadherin, and their binding to ephrin-Bs in *trans* provokes shedding of E-cadherin by ADAM10 preferentially in the ephrin-B-expressing cells (Fig. 4A) (Solanas et al. 2011). Cleavage by metalloproteases also plays a role in other Eph receptor/ephrin activities. For example, ephrin-B cleavage by ADAM13 can terminate EphB/ephrin-B signals that inhibit canonical Wnt signaling in the *Xenopus* embryo, thus enabling cranial neural crest induction (Wei et al. 2010). Furthermore, ADAM19 functions independently of its protease activity to stabilize developing neuromuscular junctions by preventing internalization of the complexes between ephrin-A5 on the muscle and EphA4 on the innervating motor neuron (Yumoto et al. 2008).

Ephrin binding and other stimuli can also induce cleavage of the Eph receptor extracellular domain, followed by further intramembrane proteolytic processing via  $\gamma$ -secretase to generate cytoplasmic fragments capable of signaling (Litterst et al. 2007; Inoue et al. 2009; Xu et al. 2009). For example, calcium influx can induce combined metalloprotease/ $\gamma$ -secretase processing of both EphA4 and EphB2 (Litterst et al. 2007; Inoue et al. 2009). The released EphA4 cytoplasmic fragment increases dendritic spine numbers through kinase-independent Rac1 activation (Inoue et al. 2009). Instead, the EphB2 cytoplasmic fragment can phosphorylate NMDA neurotransmitter receptors, which promotes their cell surface localization and may lead to a positive feedback loop by increasing NMDA receptor-mediated calcium currents (Litterst et al. 2007; Xu et al. 2009). Interestingly, stress in mice can also cause cleavage of EphB2 by the extracellular serine protease neuropsin in the amygdala (Attwood et al. 2011). This cleavage results in EphB2 dissociation from the





**Figure 4.** Eph receptor/ephrin signaling activities beyond bidirectional signaling. (A) Roles of the ADAM10 metalloprotease in Eph receptor/ephrin signaling. (B) Inhibition of Eph receptor forward signaling by *cis* interaction with ephrins. (C) Ephrin-independent Eph receptor signaling.

NMDA receptor as well as enhances NMDA receptor ion currents and the behavioral signatures of anxiety. Whether this may also be a consequence of NMDA receptor phosphorylation by a proteolytically released EphB2 cytoplasmic fragment remains to be determined.

Ephrin-B ligands can also undergo metalloprotease/ $\gamma$ -secretase processing following binding to EphB receptors. The released ephrin-B2

cytoplasmic fragment can promote Src activation and Src-dependent phosphorylation of uncleaved ephrin-B2, which is important for reverse signaling (Georgakopoulos et al. 2006). This involves regulating the interplay between Src- and the Csk-binding protein Cbp/PAG, an adaptor that controls Src activity (Georgakopoulos et al. 2011). Moreover, ephrin-B1 cytoplasmic fragments present in the developing

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mouse brain can associate with the ZHX2 transcriptional repressor and enhance its activity in the nucleus to prevent neural progenitor differentiation (Wu et al. 2009).

### Ephrin-Mediated *Cis* Attenuation of Eph Receptor Forward Signaling

Eph receptors and ephrins can be coexpressed in normal and cancer cells (Carvalho et al. 2006; Pasquale 2010; Kao and Kania 2011). In contrast to the autocrine signaling occurring when other RTKs and their soluble ligands are coexpressed (Zwick et al. 2002), a lateral *cis* interaction between Eph receptors and ephrins on the same cell surface can attenuate forward signaling (Fig. 4B) (Bohme et al. 1996; Yin et al. 2004). For example, EphA *cis* attenuation plays a role in topographic mapping of retinal axons (Hornberger et al. 1999; Carvalho et al. 2006) and ephrin-B3 inhibits signaling by EphB2 coexpressed in hippocampal synapses, decreasing tyrosine phosphorylation of NMDA neurotransmitter receptors (Antion et al. 2010). Ephrins also cause *cis* attenuation of EphA and EphB signaling in spinal cord motor neuron populations where they are highly expressed, which is important for proper axon guidance in the limb (Kao and Kania 2011). In contrast, in motor neuron populations where they are present at lower levels, ephrin-As segregate in different membrane microdomains than the coexpressed EphA receptors (Marquardt et al. 2005; Kao and Kania 2011). This segregation allows parallel activation of forward and reverse signaling in the same neurons. Biochemical and structural studies have implicated the second Eph receptor fibronectin type III domain in the *cis* interaction (Fig. 4B) (Carvalho et al. 2006; Seiradake et al. 2010). Consistent with this, an ephrin-A5 mutant that cannot engage the EphA ephrin-binding pocket was shown to still induce *cis* attenuation (Bohme et al. 1996; Carvalho et al. 2006; Kao and Kania 2011). However, how *cis* binding inhibits forward signaling remains unclear. Whereas a mechanism involving decreased Eph receptor cell surface localization seems unlikely (Yin et al. 2004; Carvalho et al. 2006), the association with coexpressed ephrins might in-

duce Eph receptor translocation to an environment rich in phosphotyrosine phosphatases or sterically inhibit Eph receptor clustering, which is necessary for activation. Through *cis* attenuation of Eph receptor forward signaling, coexpressed ephrins can fine-tune the responsiveness of cells to ephrins in *trans* beyond what is achieved by mere regulation of Eph receptor levels.

### Ephrin-Independent Activities of Eph Receptors

In addition to their ephrin-dependent activities, the Eph receptors can signal independently of ephrin ligands, for example through cross talk with other receptor systems and cytoplasmic signaling molecules. Ephrin-independent signaling can have opposite effects compared to ephrin-dependent signaling, as exemplified by EphA2. This receptor is widely up-regulated in many cancers, which often correlates with low ephrin-A expression or failure of coexpressed ephrin-As to activate forward signaling (Zelinski et al. 2001; Macrae et al. 2005; Wykosky et al. 2005; Pasquale 2010; Tandon et al. 2011). This is consistent with the ability of EphA2 forward signaling to inhibit the Ras-Erk, Akt-mTORC1 and other oncogenic pathways. However, EphA2 overexpression can induce oncogenic transformation, suggesting that this receptor also has tumor-promoting activities that may not depend on ephrin binding (Zelinski et al. 2001; Tandon et al. 2011; Udayakumar et al. 2011). Recent studies have begun to unravel the mechanism of tumor promotion by EphA2. In cancer cells where Akt is highly activated by oncogenic mutations or growth factor stimulation, EphA2 is phosphorylated at S897 by Akt, which leads to an increase in cell migration/invasion that is independent of both ephrin binding and EphA2 kinase activity (Fig. 4C) (Miao et al. 2009). Other stimuli increasing Akt activation also cause S897 phosphorylation. For example, binding of extracellular Hsp90 to the LRP1 receptor induces Akt-dependent EphA2 S897 phosphorylation and association of EphA2 with LRP1, leading to glioblastoma cell invasiveness (Gopal et al. 2011). Moreover, ephrin-B3 expression in

lung cancer cells can enhance the levels of EphA2 in its S897 phosphorylated form, concomitant with increasing resistance to  $\gamma$ -radiation (Stahl et al. 2011). Because ephrin-B3 has poor affinity for the ephrin-binding pocket of EphA2 (Gale et al. 1996), it will be interesting to investigate the connection between ephrin-B3 and EphA2 phosphorylation.

Remarkably, EphA2 seems to be at least in part responsible for the proliferative, migratory and tumorigenic activities of the EGF RTK family, as shown in several cultured cancer cell lines and in a mouse ErbB2 mammary tumor model (Larsen et al. 2007; Brantley-Sieders et al. 2008; Hiramoto-Yamaki et al. 2010; Argenzio et al. 2011). EGF stimulation can promote the association of EphA2 with the Rho-GEF Ephexin4, and it will be interesting to investigate the involvement of S897 phosphorylation in this ephrin-independent association (Hiramoto-Yamaki et al. 2010). The EphA2–Ephexin4 interaction promotes RhoG activation and recruitment of the RhoG-GTP-binding protein ELMO2 and the Rac-GEF DOCK4 to EphA2, leading to Rac1 activation and cancer cell invasiveness (Fig. 4C). The EphA2-Ephexin4-RhoG pathway also suppresses cell death due to detachment from the extracellular matrix (anoikis) in epithelial and cancer cells (Harada et al. 2011). This involves activation of PI3 kinase and Akt, which might also create a positive feedback loop further enhancing EphA2 S897 phosphorylation. Overexpression of EphB3 was also recently shown to promote lung cancer cell tumorigenicity through a kinase-independent mechanism (Ji et al. 2011). It will be important to investigate the full extent of ephrin- and kinase-independent activities of Eph receptors and how they differ from forward signaling.

### Dephosphorylation

Phosphotyrosine phosphatases can modulate the Eph receptor/ephrin system by terminating forward signaling and favoring tyrosine phosphorylation-independent activities. For instance, the cytoplasmic phosphotyrosine phosphatase LMW-PTP can dephosphorylate EphA2, thus counteracting the tumor suppressive effects of

EphA2 forward signaling and promoting cell transformation (Kikawa et al. 2002; Chiarugi et al. 2004; Parri et al. 2005). Similarly, the cytoplasmic phosphotyrosine phosphatase PTP1B can attenuate ephrin-induced EphA3 phosphorylation, endocytosis, and repulsive effects (Nievergall et al. 2010), while elevated endogenous phosphatase activity in pre-B leukemia cells can switch the EphA3-mediated response to ephrins from repulsion to adhesion (Wimmer-Kleikamp et al. 2008). In addition, PTP1B anchored to the endoplasmic reticulum has been reported to dephosphorylate EphA2 at sites of cell–cell contact where the endoplasmic reticulum comes in close proximity to the plasma membrane (Haj et al. 2012). Phosphatase activity induced by glucose in pancreatic  $\beta$  cells attenuates EphA phosphorylation and forward signaling, which are inhibitory for insulin secretion (Konstantinova et al. 2007). The protein tyrosine phosphatase receptor type O can dephosphorylate both EphA and EphB receptors, and it targets in particular the second of the two conserved phosphotyrosine residues in the juxtamembrane domain, which is the most critical for activation (Shintani et al. 2006). The LAR protein tyrosine phosphatase receptor can dephosphorylate EphB2, and LAR down-regulation by the FGF RTK results in increased ephrin-independent EphB2 tyrosine phosphorylation (Poliakov et al. 2008). LMW-PTP is also involved in EphB receptor signaling, being recruited to EphB clusters to promote cell attachment (Stein et al. 1998). However, it is not known whether this involves EphB receptor dephosphorylation by the phosphatase. Furthermore, the lipid phosphatase Ship2 can interact with EphA2 and decrease its ephrin-dependent tyrosine phosphorylation, internalization, and degradation through a mechanism likely not involving direct receptor dephosphorylation (Zhuang et al. 2007; Lee et al. 2012). Other phosphotyrosine phosphatases, such as the PDZ domain-containing PTP-BL, dephosphorylate ephrin-Bs to terminate reverse signaling (Palmer et al. 2002). Future studies will likely implicate additional phosphatases, including serine/threonine phosphatases (Yang et al. 2011), in the regulation of Eph receptor/ephrin signaling.

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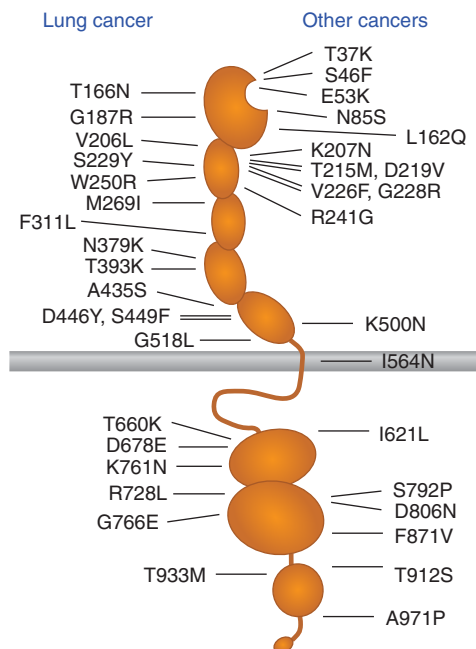
### GENE MUTATIONS

Given the importance of the Eph receptor/ephrin system in developmental processes and adult tissue homeostasis, it is not surprising that its aberrant functioning has been implicated in a variety of diseases (Pasquale 2008, 2010). In particular, somatic and germline mutations in Eph receptors and ephrin genes are beginning to be linked to cancer and other pathologies. Large-scale sequencing of tumor specimens identified somatic mutations in all the Eph receptors, with frequencies of up to 2%–6% for some Eph receptors in lung cancer and melanoma (Ding et al. 2008; Prickett et al. 2009; Peifer et al. 2012) ([www.sanger.ac.uk/genetics/CGP/cosmic](http://www.sanger.ac.uk/genetics/CGP/cosmic)). The mutations are scattered throughout the Eph receptor domains (Fig. 5), and their functional consequences are mostly unknown. However, many of the nearly 40 missense mutations identified in EphA3 (Fig. 5), the receptor found to be the most highly mutated in cancer, have been recently shown to cause various degrees of loss-of-function through multiple mechanisms

(Lisabeth et al. 2012; Zhuang et al. 2012). Most mutations in the ephrin-binding domain and the neighboring sushi domain impair ephrin binding either by directly affecting the high affinity ephrin-binding pocket or by causing overall conformational alterations. Mutations in the EphA3 kinase domain inhibit receptor tyrosine phosphorylation and kinase activity. A further consequence of many EphA3 mutations is a reduction in cell surface localization, which suggests that the mutations cause misfolding and/or alter receptor trafficking.

These findings suggest that the mutations disrupt a tumor suppressive function of EphA3 that depends on ephrin binding and kinase activity and, thus, forward signaling. The EphA3 cancer mutations indeed have different characteristics compared to mutations in other RTK families, which are typically clustered in “hot spots” and promote constitutive activation and tumorigenesis (Lee et al. 2006; Sharma et al. 2007; Greulich and Pollock 2011). Furthermore, wild-type EphA3, but not several mutants identified in tumor specimens, can suppress lung cancer cell growth in mouse xenograft models of lung cancer (Zhuang et al. 2012). Two mutations in EphA6 and EphA7 correspond to inactivating mutations in EphA3, suggesting that these Eph receptors may also suppress tumorigenesis (Lisabeth et al. 2012). EphB2 inactivating mutations identified in prostate cancer also suggested a tumor suppressor role for this receptor, consistent with the growth inhibition induced by EphB2 overexpressed in DU145 prostate cancer cells (Huusko et al. 2004). However, other Eph receptors like EphA2 or EphA4 do not seem to be frequently mutated in cancer, perhaps suggesting differences in the oncogenic activities of these receptors. A mutation in the first FNIII domain of EphA2 identified in lung cancer has indeed been proposed to promote invasiveness and survival (Faoro et al. 2010).

Although classical tumor suppressors are typically inactivated by homozygous mutations, most of the EphA3 inactivating mutations are heterozygous (Lisabeth et al. 2012). Hence, the EphA3 mutants may act as dominant negatives, disrupting the function of the wild-type receptor (Zhuang et al. 2012) and possibly other Eph



**Figure 5.** EphA3 receptor somatic mutations in cancer.

receptors that may be part of the same signaling clusters. Furthermore, concurrent mutations in multiple Eph receptors have been found in a relatively high proportion of tumor samples, suggesting that they may be advantageous for tumor cells by more severely disrupting signaling in Eph receptor clusters than a single mutation (Lisabeth et al. 2012).

Germline mutations in Eph receptors and ephrins also play a role in human disease. For instance, EphA2 mutations enhancing basal receptor activation or possibly increasing EphA2 association with the LMW-PTP phosphatase have been associated with cataract development (Shiels et al. 2008; Jun et al. 2009; Zhang et al. 2009b). Inactivating mutations in the X-linked ephrin-B1 gene cause craniofrontonasal syndrome as a result of inhibition of gap junctional communication and improper tissue boundary formation in the developing skull (Bush and Soriano 2010; Makarov et al. 2010; Zafeiriou et al. 2011). On the other hand, loss-of-function EphA4 mutations in amyotrophic lateral sclerosis patients are associated with long survival (Van Hoecke et al. 2012) and single nucleotide polymorphisms in various Eph receptors and ephrins have been implicated as modifiers in the pathogenesis of amyotrophic lateral sclerosis as well as Parkinson's disease (Lesnick et al. 2008). Furthermore, a common EphA1 polymorphism was recently associated with late-onset Alzheimer's disease (Naj et al. 2011) and an EphA6 polymorphism with responsiveness to an antischizophrenic drug (Ikeda et al. 2010). Studies on the functional effects of Eph receptor and ephrin mutations and polymorphisms will undoubtedly provide a wealth of new information on the physiological and pathological roles of this intriguingly complex signaling system.

### CONCLUDING REMARKS

Understanding signaling by the Eph RTK family has been challenging because of the many idiosyncrasies that distinguish it from the other RTK families. The peculiar characteristics of the Eph RTKs include the membrane-bound nature of the ephrins, the bidirectional mode of Eph re-

ceptor-ephrin signaling, the ability of the ephrins to stimulate but also attenuate Eph receptor signaling, and the ability of the Eph receptors to signal without ephrin involvement and even independently of kinase activity. Given the emerging view that different coexpressed Eph receptors signal cooperatively (Janes et al. 2011), to correctly interpret the results of signaling studies it will be important to profile the entire repertoire of Eph receptors present in a biological system (Noberini et al. 2012b) as well as survey their posttranslational modifications, including tyrosine and serine/threonine phosphorylation and ubiquitination. Systems biology approaches are also essential for a comprehensive understanding of the complexities of Eph receptor signaling networks and feedback loops, and the ability of these receptors to produce widely different biological outcomes (Jorgensen et al. 2009; Bush and Soriano 2012). In vivo analysis of Eph receptor/ephrin signaling as well as perturbations by designed or naturally occurring mutations and gene deletions will be critical to elucidate Eph receptor/ephrin physiological functions in the complex in vivo environment. Many fascinating activities of the Eph/ephrin system are only beginning to be appreciated, including key roles in stem cell biology (Genander and Frisen 2010) and in diseases such as Alzheimer's (Cisse et al. 2011; Hollingworth et al. 2011), or their emerging ability to regulate microRNAs (Arvanitis et al. 2010; Bhushan and Kandpal 2011; Khodayari et al. 2011) and gene transcription (Lai et al. 2004; Bong et al. 2007; Bush and Soriano 2010, 2012; Parrinello et al. 2010). Resolution of the paradoxes that plague our understanding of Eph receptor/ephrin function will enable effective exploitation of the many therapeutic opportunities that the Eph/ephrin system offers (Pasquale 2010; Noberini et al. 2012a).

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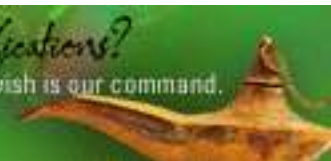


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