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# *Attenuation of Eph Receptor Kinase Activation in Cancer Cells by Coexpressed Ephrin Ligands*

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# ABSTRACT

The Eph receptor tyrosine kinases mediate juxtacrine signals by interacting "in trans" with ligands anchored to the surface of neighboring cells via a GPI-anchor (ephrin-As) or a transmembrane segment (ephrin-Bs), which leads to receptor clustering and increased kinase activity. Additionally, soluble forms of the ephrin-A ligands released from the cell surface by matrix metalloproteases can also activate EphA receptor signaling. Besides these trans interactions, recent studies have revealed that Eph receptors and ephrins coexpressed in neurons can also engage in lateral "cis" associations that attenuate receptor activation by ephrins in trans with critical functional consequences. Despite the importance of the Eph/ephrin system in tumorigenesis, Eph receptor-ephrin cis interactions have not been previously investigated in cancer cells. Here we show that in cancer cells, coexpressed ephrin-A3 can inhibit the ability of EphA2 and EphA3 to bind ephrins in trans and become activated, while ephrin-B2 can inhibit not only EphB4 but also EphA3. The cis-inhibition of EphA3 by ephrin-B2 implies that in some cases ephrins that cannot activate a particular Eph receptor in trans can nevertheless inhibit its signaling ability through cis association. We also found that an EphA3 mutation identified in lung cancer enhances cis interaction with ephrin-A3. These results suggest a novel mechanism that may contribute to cancer pathogenesis by attenuating the tumor suppressing effects of Eph receptor signaling pathways activated by ephrins in trans (Falivelli et al. 2013).

# INTRODUCTION

## 1- RECEPTOR TYROSINE KINASES

Receptor tyrosine kinases are part of the larger family of protein tyrosine kinases. Protein tyrosine kinases encompass the receptor tyrosine kinase, which are a single pass transmembrane proteins, and the non-receptor tyrosine kinases, which do not possess a transmembrane domain (Robinson et al. 2000).

A search of the human genome identified ninety tyrosine kinase genes containing a highly conserved catalytic domain, of these fifty-eight encode receptor tyrosine kinases. Nineteen of the twenty-four human chromosomes contain tyrosine kinases genes, which through a phylogenetic analysis of the amino acid sequence of the kinase domain can be grouped into twenty receptor and ten non-receptor classes. The twenty receptor tyrosine kinase classes include: RTK class I (EGF receptor family) (ErbB family), RTK class II (Insulin receptor family), RTK class III (PDGF receptor family), RTK class IV (FGF receptor family), RTK class V (VEGF receptors family), RTK class VI (HGF receptor family), RTK class VII (Trk receptor family), RTK class VIII (Eph receptor family), RTK class IX (AXL receptor family), RTK class X (LTK receptor family), RTK class XI (TIE receptor family), RTK class XII (ROR receptor family), RTK class XIII (DDR receptor family), RTK class XIV (RET receptor family), RTK class XV (KLG receptor family), RTK class XVI (RYK receptor family), and RTK class XVII (MuSK receptor family) (Robinson et al. 2000).

The non-receptor tyrosine kinases are critical components of the signaling pathways triggered by the receptor tyrosine kinases and other cell surface receptors. This group of proteins includes Src, Abl and Janus (Jaks) kinases and many others (Hubbard and Till 2000).

Receptor tyrosine kinases possess intrinsic cytoplasmic enzymatic activity, with which they promote the transfer of the ATP γ-phosphate to tyrosine residues of protein substrates. They function as receptors for cytokines, growth factors, hormones and other signaling molecules and they play critical roles in a variety of cellular processes including growth, differentiation, angiogenesis and metabolism as well as in the development and progression of many types of cancer. In fact, mutations that impair receptor functions result in developmental defects, emphasizing how important the regulated activity of these proteins is (Lemmon and Schlessinger 2010).

### 1.1- DOMAIN ORGANIZATION OF RECEPTOR TYROSINE KINASES

Generally the receptor tyrosine kinases consist of several domains: the extracellular domain, which is important for the binding of the ligand or external signal, a

transmembrane helix, which connects the extracellular domain to the intracellular domain, a small juxtamembrane region followed by the tyrosine kinase domain, which retains catalytic activity. The tyrosine kinase domain is highly conserved in the receptor tyrosine kinase family and is generally followed by a C-terminal region. The cytoplasmic domain contains multiple binding sites for adaptor proteins. Most of the receptor tyrosine kinases are monomeric in the absence of ligand and consist of a single polypeptide chain. The exceptions are the insulin-like growth factor 1 (IGF1) receptor, which is formed by two disulfide-linked dimers, and the Met HGF receptor, which contains a short α-chain disulfide-linked to a transmembrane β-chain (Hubbard and Till 2000). The global structure of the tyrosine kinase domain is comparable to that of serine/threonine kinases and is formed by an amino-terminal lobe comprising an α-helix and a five-stranded β sheets followed by a bigger carboxy-terminal lobe composed of several α-helices. Some receptor tyrosine kinases contain a kinase insert domain, which together with the C-terminal region and the juxtamembrane domain contains tyrosine residues that are autophosphorylated upon ligand binding to the receptor (Cadena et al. 1994; Hubbard 1999). In contrast, the extracellular portion of the receptor is structurally more complex and accommodates different globular domains, such as Immunoglobulin Ig-like domains, fibronectin type III-like domains, cysteine rich-domains and EGF-like domains (Fig. 1) (Hubbard and Till 2000).

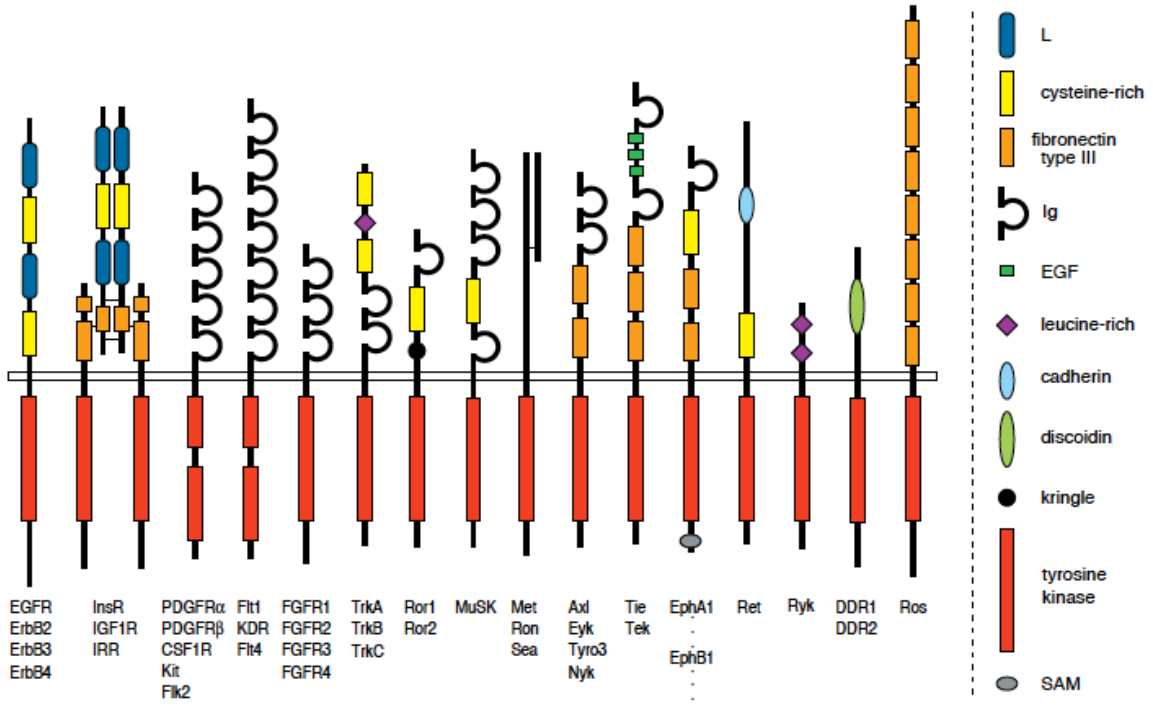


**Figure 1: Domain organization of several receptor tyrosine kinases.** Legend on the right side. The horizontal double line represents the plasma membrane, which divides the extracellular portion (top) from the intracellular portion (bottom) (Hubbard and Till 2000).

# 1.2- RECEPTOR TYROSINE KINASE SIGNALING

High affinity ligand binding is the first step in the activation of the receptor. Most receptor tyrosine kinase ligands are soluble polypeptides, except for the ligands of the Eph receptors, which are membrane-bound proteins. The ligand in its monomeric or dimeric form binds the extracellular domain of the cognate tyrosine kinase receptor and induces conformational changes that result in receptor dimerization and oligomerization. The activation of the receptor requires the binding of the ligand to stabilize the active conformation of the receptor and to destabilize cis-autoinhibitory interactions, whether the initial inactive state of the receptor is monomeric or dimeric. Receptor dimerization promotes activation of the tyrosine kinase catalytic domain, which autophosphorylates on specific tyrosine residues localized in the activation loop of the kinase domain. In addition, this process promotes the phosphorylation of tyrosine residues in the C-terminal and juxtamembrane domains, disrupting autoinhibitory interactions with the kinase domain and creating binding sites for adaptor proteins containing phosphotyrosine recognition domains of 50-100 amino acids, such as Src homology 2 (SH2) domains and phosphotyrosine-binding (PTB) domains. The adaptor proteins are recruited directly by the activated receptor or indirectly by binding to docking proteins that are associated with the phosphorylated receptor, such as FRS2, IRS1 (insulin receptor substrate-1) and Gab1 (the Grb2-associated binder) (Pawson 1995; Lemmon and Schlessinger 2010). Once the adaptor proteins are recruited by the receptor at the cell membrane, the activated receptor catalyzes the transfer of the adenosine triphosphate γ-phosphate (ATP) to the hydroxyl group of side-chain tyrosine residues in the protein substrates. Some of these proteins are the GTPase activating protein (GAP), the phospholipase C-γ (PLC-γ) and the phosphatidylinositol 3-kinase (PI 3-kinase). Finally, the activated adaptor proteins can transduce the signals downstream in the intracellular compartment (Fig. 2) (Hubbard 1999).

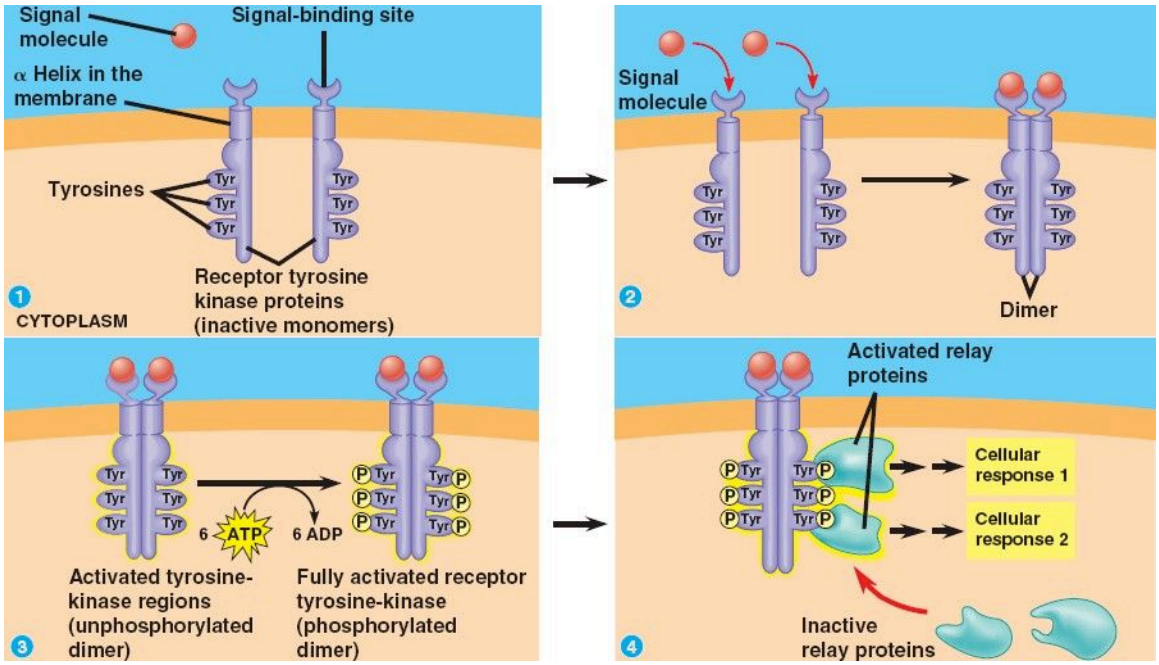


**Figure 2: Schematic representation of receptor tyrosine kinase activation and signal transduction.** 1- ligand binding to the receptor extracellular domain, 2- receptor dimerization, 3- receptor autophosphorylation and fully activation, 4- binding of adaptor proteins containing phosphotyrosine-recognition domains to the activated receptor and activation of downstream signal pathways (bio1151.nicerweb.com).

An example of a signaling pathway activated by the binding of growth factors to a tyrosine kinase receptor is the Ras-dependent Erk1/2 mitogen-activated (MAP) kinase pathway. This signaling pathway is one of the most studied and most frequently deregulated in human cancer. In this signaling pathway the binding of the growth factor to the receptor tyrosine kinase induces receptor dimerization and autophophorylation of tyrosine residues in the C-terminal region. This creates binding sites for the adaptor proteins such as Grb2, which subsequently recruits the guanine nucleotide exchange factor Sos. By catalyzing the substitution of GDP with GTP, Sos activates the membrane-bound Ras, which, in turn, phosphorylates and activates the Raf kinase (MAPKKK). Raf catalyzes the activation of MEK1 and MEK2 (MAPKKs), which subsequently activate the MAP kinases, Erk1 and Erk2. Once activated, Erk1/2 translocates in the nucleus where it regulates several cellular responses, such as cell proliferation, growth and survival (Fig. 3). CI-1040 was the first MEK1/2 inhibitor to enter clinical trials, however the insufficient antitumoral activity and poor solubility of this compound precluded further clinical development. PD0325901 and AZD6244 are second-generation inhibitors of MEK1/2 with better potency and solubility than CI-1040. AZD6244 is currently undergoing further clinical trials, while the clinical development of PD0325901 has been stopped due to its ocular toxicity in cancer patients (Fig. 3) (Fremin and Meloche 2010). Other promising MEK inhibitors are in development, such as BAY867966 and GSK1120212, which are currently undergoing clinical trials (Chang-Yew Leow et al. 2013).

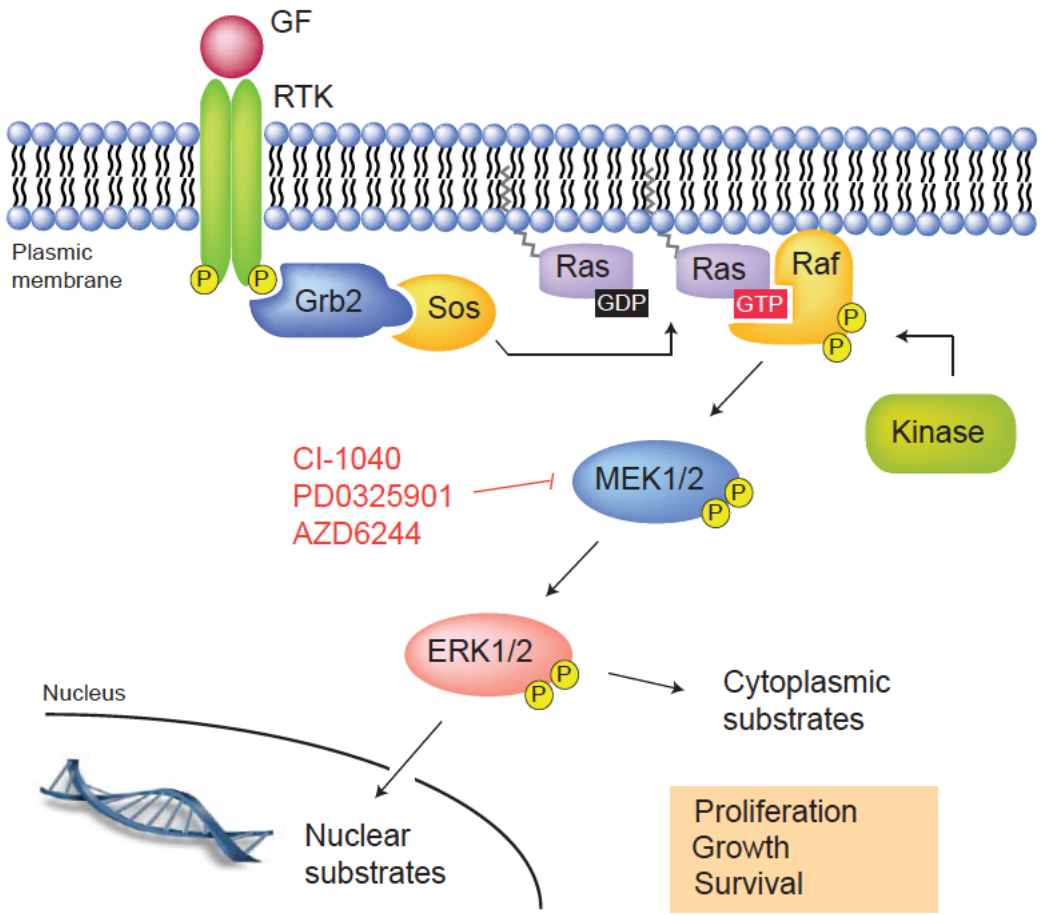


**Figure 3: Rapresentation of the Ras-dependent Erk1/2 mitogen-activated (MAP) kinase pathway** (Fremin and Meloche 2010).

Termination of receptor tyrosine kinases signaling occurs via distinctive processes: dephosphorylation by protein tyrosine phosphatases (PTPs), protein degradation following ubiquitination and receptor endocytosis (Hubbard and Till 2000). Upon ligand binding and receptor clustering, the occupied receptor can be internalized through clathrin-mediated endocytosis. In the endosomal lumen the ligand dissociates from the receptor, which becomes dephosphorylated and ubiquitylated. At this point the receptor may be degraded or recycled to the plasma membrane, which affects receptor number and signaling (Lemmon and Schlessinger 2010).

## 1.3- RECEPTOR TYROSINE KINASES AND CANCER

Receptor tyrosine kinases not only are key regulators of normal cellular processes but also have a critical role in the development and progression of many types of cancer. Deregulation of receptor tyrosine kinases has been found in a variety of human diseases. Abnormal receptor tyrosine kinases activation in human tumors may be caused by receptor overexpression, gain-of-function mutations, chromosomal translocation and

autocrine activation. Recent sequencing studies in a wide array of human cancers have identified mutations in numerous receptor tyrosine kinases (Lemmon and Schlessinger 2010).

Activating mutations in the KIT and PDGFR families of receptors have been identified in several human tumors, such as melanoma and gastrointestinal tumors. The gain-of-function-mutations of KIT are usually clustered in exons 8, 9, 11 and 17 of the KIT gene and cause constitutive receptor dimerization by removing the normal receptor cis-autoinhibition (Yuzawa et al. 2007).

Deregulation of EGFR family receptors also has a strong impact in human cancer. Different mechanisms of constitutive activation of EGFR in tumors have been identified (Gschwind et al. 2004). Amplifications of the EGFR gene and activating mutations have been found in ~35% of glioblastoma cases (Lemmon and Schlessinger 2010). Impaired receptor downregulation is another mechanism of EGFR deregulation (Peschard and Park 2003). In addition, ErbB2 protein overexpression is frequent in breast cancer patients and is associated with poor prognosis (Lemmon and Schlessinger 2010).

Furthermore, receptors of the FGFR family are mutated in several human cancers. In lymphoblastic lymphoma, multiple myeloma and myelogenous leukemia the FGFR receptor undergoes chromosomal translocation, which results in the formation of a constitutively active heterodimer containing FGFR1 and FGFR3 (Eswarakumar et al. 2005).

Small molecule inhibitors targeting the ATP-binding site, which is located in the receptor kinase domain, and monoclonal antibodies that block receptor activation or target the cell expressing the receptor tyrosine kinase for destruction by the immune system have been developed and approved by the US Food and Drug Administration (FDA). An example is the HER2-specific monoclonal antibody (mAb) Trastuzumab (Herceptin, Genentech, Inc) used to treat metastatic breast cancer. This antibody binds HER2 on the surface of tumor cells and induces receptor internalization, inhibition of cell-cycle progression and recruitment of immune cells (Gschwind et al. 2004). Although several treatments have been successfully used to fight different cancers, unfortunately the gain of drug resistance and the side effects that arise from lack of selectivity of the small molecule inhibitors towards an individual target are still a big limitation of these anticancer therapies. New strategies to solve the problem of drug resistance in the clinical therapies are focused on using simultaneously multiple receptor tyrosine kinase inhibitors that target different receptors and on developing inhibitors that target key processes engaged by all receptor tyrosine kinases (Lemmon and Schlessinger 2010).

# 2- EPH RECEPTOR SIGNALING AND EPHRINS

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 co-expressed 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 (Lisabeth et al. 2013b).

## 2.1- GENERAL FEATURES OF EPH RECEPTORS AND EPHRINS

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. 4). 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; Pasquale 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 prevent 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. 4). 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 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).

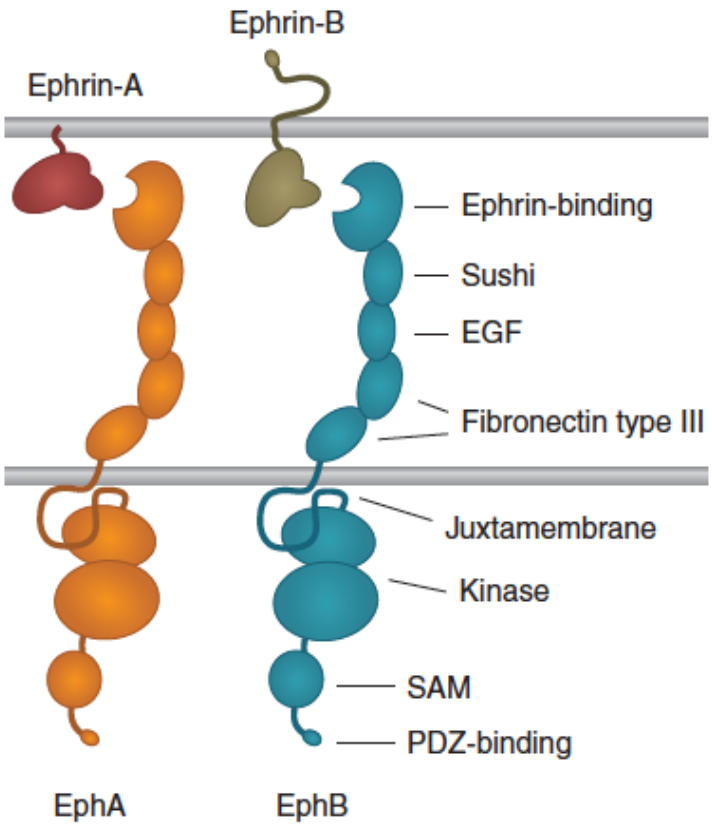


**Figure 4: Domain structure of Eph receptors and ephrins** (Lisabeth et al. 2013b).

The Eph receptor family has greatly expanded during evolution, and includes almost a fourth of the 58 human RTKs (Schlessinger and Lemmon, this issue). 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 co-expressed 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 upregulated 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; Pasquale 2010).

Here we provide an overview of Eph receptor and ephrin signaling mechanisms and biological effects, with 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; Arvanitis and Davy 2008; Lackmann and Boyd 2008; Pasquale 2008; Klein 2009; Genander and Frisen 2010; Pasquale 2010).

## 2.2- 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 (Nikolov, this issue). 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 (Himanen et al. 2010; Seiradake et al. 2010) (Fig. 5). 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 (Pawson, this issue). 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 non-receptor tyrosine kinases of the Src and Abl families and adaptors such as Nck and Crk, which are crucial for signal transduction (Jorgensen et al. 2009; Pasquale 2010) (Fig. 5). 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, while 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.

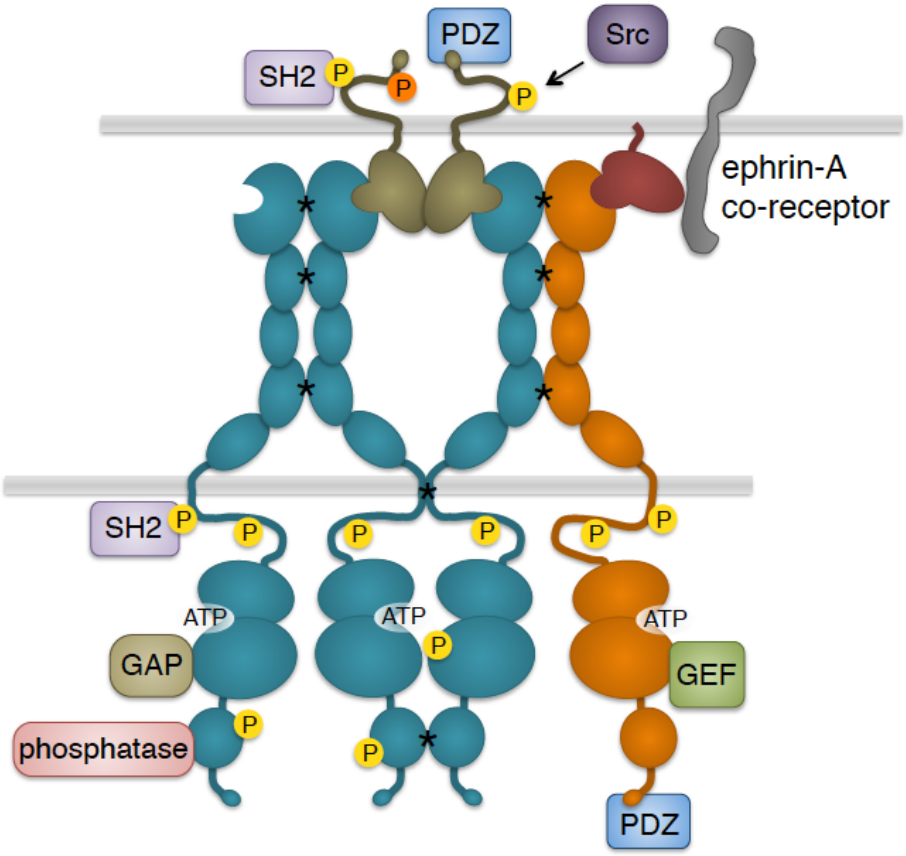


**Figure 5: Eph receptor clustering and bidirectional signaling.** SH2 and PDZ indicate proteins containing these domains. All types of signaling proteins shown can associate with both EphA and EphB receptors. Asterisks indicate receptor-receptor interactions favoring clustering; yellow circles indicate tyrosine phosphorylation and the orange circle indicates serine phosphorylation.

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.

## 2.2.1- 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; Pasquale 2010). GTPases cycle between a GDP bound (inactive) state and a GTP bound (active) state that binds dowstream 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. 6A-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 (Wahl et al. 2000; Murai et al. 2003; Fu et al. 2007) (Fig. 6B,C). 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 α2-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 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 injured 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. 6D) 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; Tolias et al. 2007) while 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).

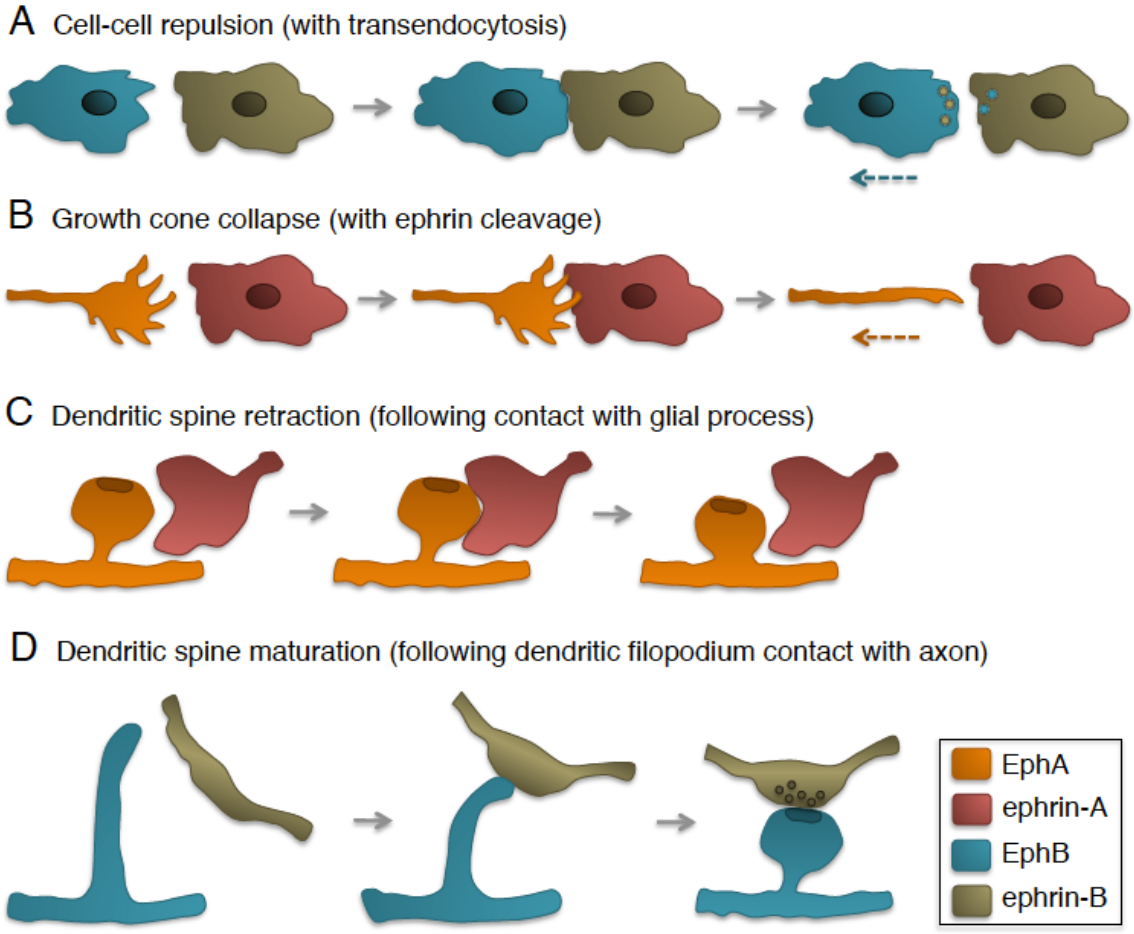


**Figure 6: Eph receptor-ephrin repulsive effects and dendritic spine maturation.** (A) An EphB-expressing cell encounters an ephrin-B-expressing cell and retracts after the internalization of EphB-ephrin-B complexes enables cell separation. (B) An EphA-expressing growth cone at the leading edge of an 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 post-synaptic 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 pre-synaptic terminal also matures following contact.

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.

## 2.2.2- 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; Pasquale 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 where Ras-Erk

signaling reciprocally influences Eph receptors, for example by reinforcing ephrin-B1/EphB2 signaling or upregulating EphA2 gene transcription (Menges and McCance 2008; Poliakov et al. 2008).

### 2.2.3- 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 stimuated 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 upregulation 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), while ephrin-A1 stimulation causes loss of S897 phosphorylation (Miao et al. 2009).

## 2.3- EPHRIN REVERSE SIGNALING

Besides forward signaling, the Eph receptors can also stimulate “reverse” signaling in the ephrin-expressing cells (Pasquale 2005; Pasquale 2010) (Fig. 5). 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 carboxy terminus, which is also induced by EphB receptor binding, leads to stabilization of AMPA neurotransmitter 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 carboxy 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 lymphangiogensis 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 crosstalk 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 (Lim et al. 2008; Marler et al. 2008; Marler et al. 2010; Bonanomi et al. 2012) (Fig. 5). 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). Ephrin-A5 reverse signaling in pancreatic β 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; Davy et al. 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 downregulate cell surface ephrin-A levels by modulating the metabolism of sphingomyelin (Baba et al. 2009).

## 2.4- BEYOND BIDIRECTIONAL SIGNALING

### 2.4.1- 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 (Sorkin, this issue). For the Eph receptors, this process has unique features due to 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. 6A). This Rac1-dependent process, which has been defined "transendocytosis", 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 transendocytosis, 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 towards the ephrin in trans (Janes et al. 2005; Janes et al. 2009) (Fig. 7A). 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 (Solanas et al. 2011) (Fig. 7A). 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 γ-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/γ-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 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/γ-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 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).

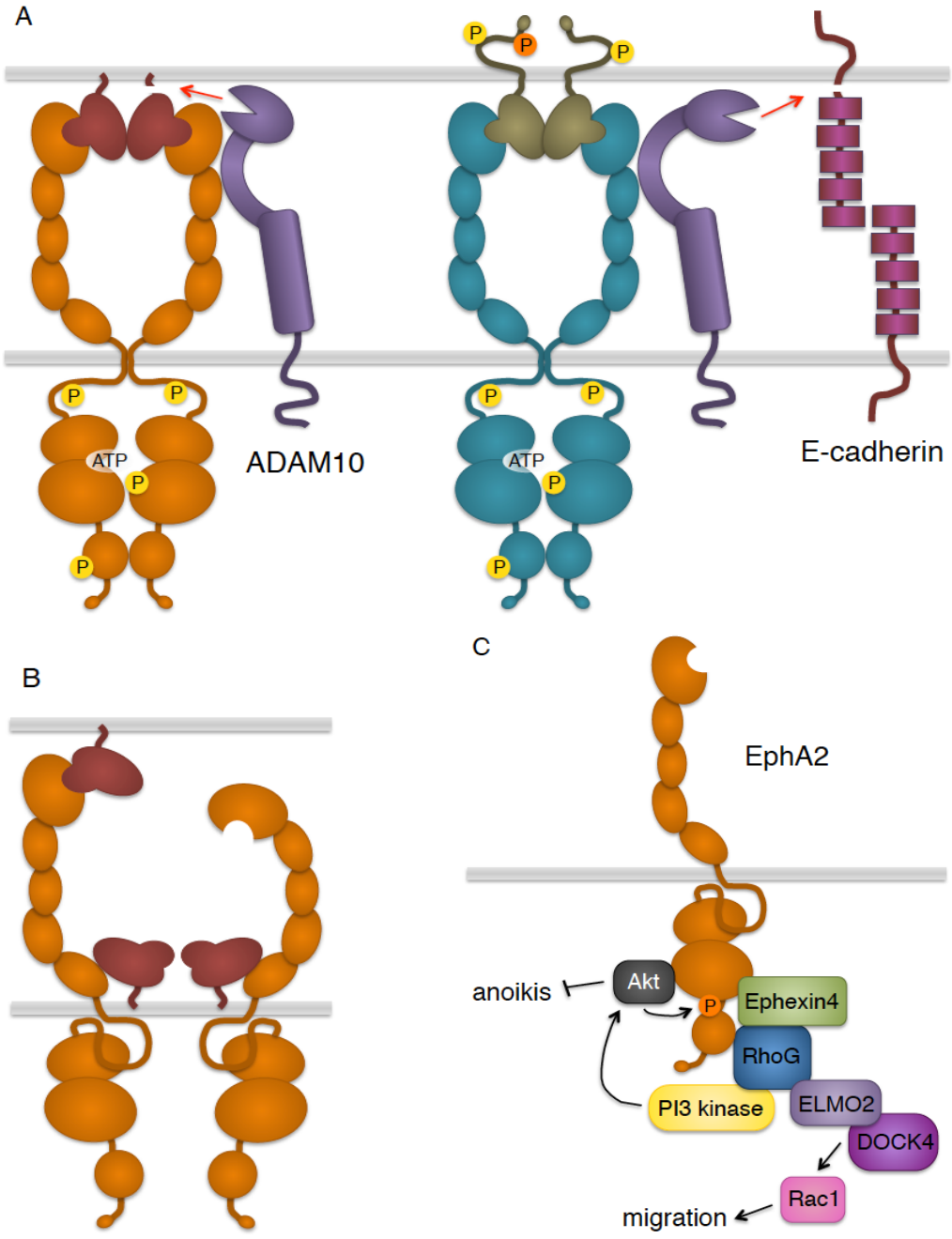


**Figure 7:** (A) Roles of the ADAM10 metalloprotease in Eph receptor/ephrin signaling. (B) Inhibition of Eph receptor forward signaling by cis interaction with ephrins. (C) Ephrin-independent Eph receptor signaling.

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

Eph receptors and ephrins can be co-expressed 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 co-expressed (Zwick et al. 2002), a lateral cis interaction between Eph receptors and ephrins on the same cell surface can attenuate forward signaling (Bohme et al. 1996; Yin et al. 2004) (Fig. 7B). 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 co-expressed 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 co-expressed 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 (Carvalho et al. 2006; Seiradake et al. 2010) (Fig. 7B). 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. While a mechanism involving decreased Eph receptor cell surface localization seems unlikely (Yin et al. 2004; Carvalho et al. 2006), the association with co-expressed ephrins might induce 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, co-expressed ephrins can fine-tune the responsiveness of cells to ephrins in trans beyond what is achieved by mere regulation of Eph receptor levels.

## 2.4.3- 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 crosstalk 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 upregulated in many cancers, which often correlates with low ephrin-A expression or failure of co-expressed 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 (Miao et al. 2009) (Fig. 7C). 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 γ-radiation (Stahl et al. 2011). Since 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. 7C). 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.

## 2.4.4- Dephosphorylation

Phosphotyrosine phosphatases can modulate the Eph receptor/ephrin system by terminating forward signaling and favoring tyrosine phosphorylation-independent activities. For instance, the cytoplasmic 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 β-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 downregulation 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.

## 2.5- 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; Pasquale 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) (http://www.sanger.ac.uk/genetics/CGP/cosmic/). The mutations are scattered throughout the Eph receptor domains (Fig. 8), and their functional consequences are mostly unknown. However, many of the nearly 40 missense mutations identified in EphA3, the receptor found to be 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 a 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).

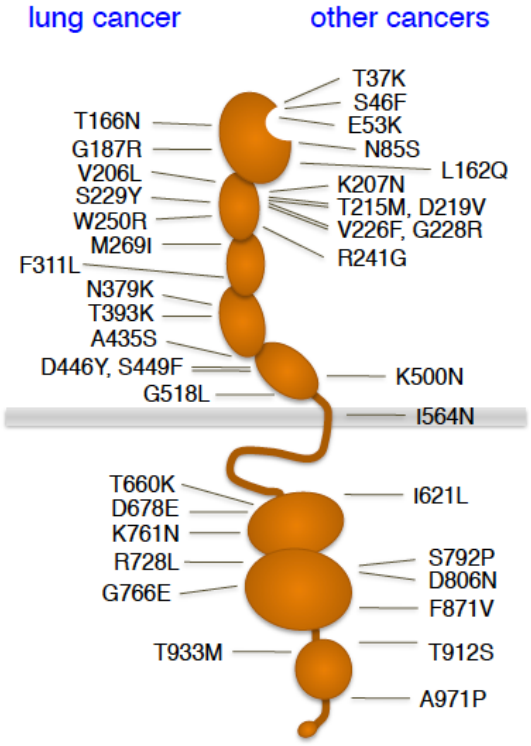


**Figure 8: EphA3 receptor somatic mutations in cancer.**

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 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 due to 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 Parkinsons'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 anti-schizophrenic 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.

## 2.6- CONCLUDING REMARKS

Understanding signaling by the Eph RTK family has been challenging due to 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 receptor-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 co-expressed 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 post-translational 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; Parrinello et al. 2010; Bush and Soriano 2012). 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).

# 3- RECEPTOR-LIGAND CIS-INTERACTIONS IN THE NERVOUS SYSTEM

Several juxtacrine signaling systems in addition to the Eph-ephrin system exhibit inhibitory cis-interactions between receptor and ligand co-expressed in the same cell. These interactions have been observed mainly in the nervous system and in the immune system and they result in the attenuation of receptor activation by the ligand in trans (Nitschke 2009; Ware and Sedy 2011; Yaron and Sprinzak 2012). The receptor-ligand cis-inhibition mechanism has been identified in three different families of proteins that play an important role in the development of the nervous system: Notch-Delta/Serrate/DSL, Eph-ephrin and plexin-semaphorins.

## 3.1- CIS-INHIBITION IN THE NOTCH SIGNALING PATHWAY

The Notch signaling pathway is important for the regulation of neuronal differentiation during neurogenesis and neuronal plasticity in the adult nervous system. It is involved in the neurogenesis process in both central and peripheral nervous system (Gaiano and Fishell 2002; Kageyama et al. 2008; Alberi et al. 2011; Kuzina et al. 2011; Lieber et al. 2011).

The Notch receptor family comprises four members, Notch1, Notch2, Notch3 and Notch4, which are conserved type I transmembrane proteins that recognize three different transmembrane ligands, Delta, Serrate and LAG-2 (DSL). Following the interaction in trans with the ligand, the Notch receptor is cleaved at two different sites near the cell membrane and the intracellular portion of the receptor translocates to the nucleus where it serves as a co-transcription factor to activate downstream genes target (Fig. 9a). Instead, the coexpression of the ligand and the Notch receptor in the same cell causes cis-inhibition of the receptor and consequently blocks Notch signaling (Fig. 9b) (Bray 2006; Artavanis-Tsakonas and Muskavitch 2010; Yaron and Sprinzak 2012).

Evidence of Notch pathway inhibition by different expression levels of ligand was initially reported in Drosophila. It was observed that during wing development, the Notch ligand Serrate has a dominant negative effect on the activity of Notch that depends on its level of expression. Excess of Serrate can attenuate Notch activation and this effect can be suppressed by Notch overexpression. Therefore, during wing development the control of Serrate expression is important for the regulation of Notch activation that leads to a normal wing phenotype (Klein et al. 1997).

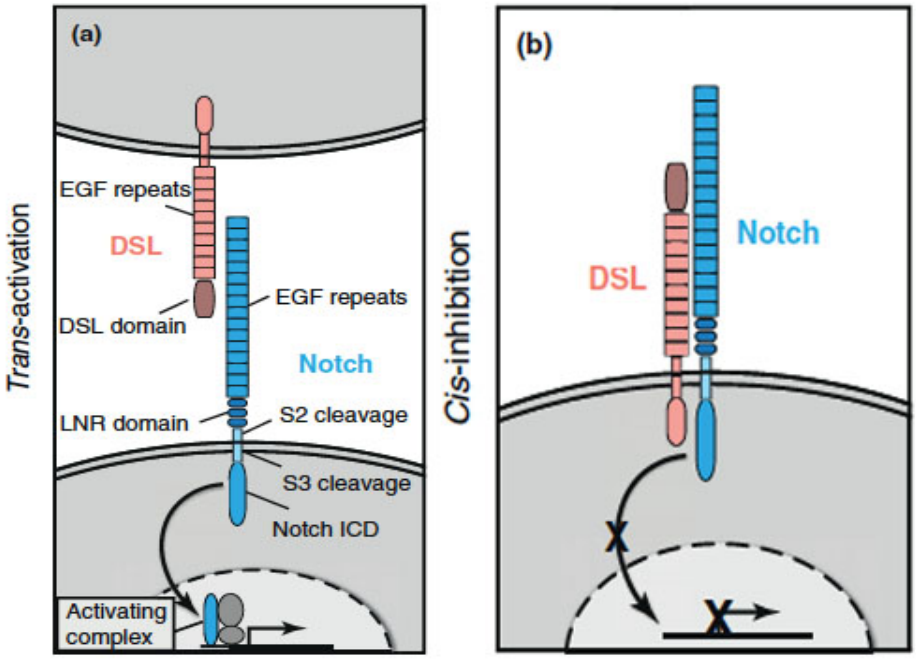


**Figure 9: Notch signaling. a:** Notch receptor activation by interaction in trans with the ligand. **b:** Notch receptor silencing by interaction in cis with the ligand co-expressed in the same cell (Becam et al. 2010; Yaron and Sprinzak 2012).

A recent study has shown that the Notch receptor cis-inhibition by Delta ligand coexpression in the same cell regulates cellular differentiation in the Drosophila ommatidia, guiding photoreceptor precursor cells through their distinctive final fate (Miller et al. 2009). During development the neuronal photoreceptor precursors (R) are organized in a group of equivalent cells. All the cells express the Notch receptor but different levels of Delta ligand. In the precursor cells with higher Delta levels the Notch receptor is silenced, and consequently the cells adopt the R1 or R6 fate. In the precursor cells with lower Delta level, the Notch receptor is activated by Delta ligand in trans and the cells adopt the R7 fate (Fig. 10). In a mutant precursor cell lacking Delta (R1 Dl-/-), the Notch receptor cannot be silenced by Delta and the cell adopts an erroneous fate instead of the R1 fate. Thus, the cis-inhibition of Notch is important for regulation of Delta-mediated unidirectional signaling (Fig. 10) (Miller et al. 2009; Yaron and Sprinzak 2012).

Sprinzak et al. developed a mathematical model using a quantitative cell culture assay in which Notch signaling response to both cis and trans Delta was mapped in order to explain the formation of the wing veins in Drosophila (Sprinzak et al. 2011). According to this model, the cells can be either sender or receiver depending on the relative level of receptor and ligand expression on their surface. A cell with high ligand expression is a sender cell, which can send signals but cannot receive them. A cell with high receptor expression is a receiver cell, which can receive signals but cannot send them. The two states are mutually exclusive and when ligand and receptor are expressed at the same level there is no signaling in the cell. When the receptor is uniformly

expressed across a tissue and the ligand is expressed in a gradient, the cells in the tissue divide in two distinct regions with a sharp boundary defined by the transition between sender and receiver cells. This model explains how Notch cis-silencing by Delta ligand co-expression regulates the formation of the Drosophila wing veins. Therefore, receptor-ligand cis-inhibition generates an additional fine level of receptor modulation, which is important during cell differentiation (Klein et al. 1997; Klein and Arias 1998; Sprinzak et al. 2010; Yaron and Sprinzak 2012).

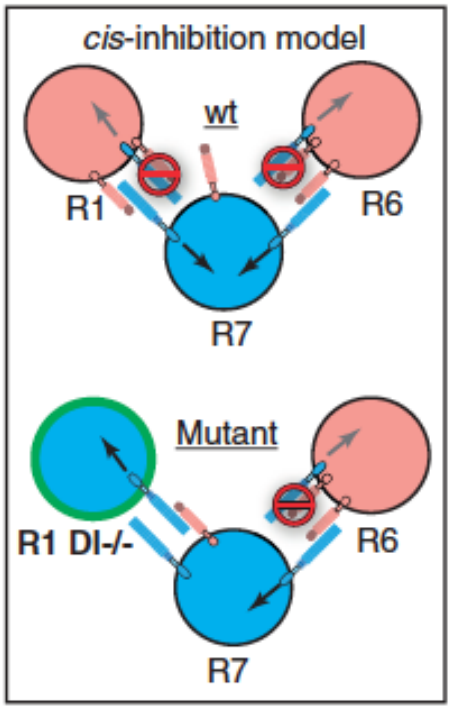


**Figure 10: Cis-inhibition model in the Drosophila ommatidia.** During the differentiation of Drosophila ommatidia, Notch signaling in the R1 and R6 cells (pink) is inhibited by endogenous Delta co-expression. The Delta mutant precursor cell R1 Dl -/- lacks Notch cis-inhibition by the ligand and therefore adopts the R7 fate (blue) (Yaron and Sprinzak 2012).

## 3.2- CIS-INHIBITION IN THE SEMAPHORIN SIGNALING PATHWAY

Receptor-ligand cis-inhibition is also involved in the regulation of the axonal guidance process. During neuronal development, two signaling systems that are responsible for directing the growing axons towards specific guidance cues are the Eph-ephrin system and the semaphorins-plexin system (Yaron and Sprinzak 2012).

The semaphorins are a large family of secreted and membrane-bound axon guidance molecules containing more than twenty genes in vertebrates, which are divided into five classes according to structural homology (Fig. 11). They are involved in diverse

developmental and biological events: repulsive axon guidance, neuronal migration, dendritic guidance, modulation of the immune system, tumor progression and angiogenesis.

The semaphorins bind to two different families of receptors: the neuropilins and the plexins. The neuropilin family consists of two genes and neuropilins were the first to be identified as semaphorins receptors. They have a short intracellular domain, which makes them dependent on additional co-receptors for intracellular signaling. The plexins are a large family of transmembrane proteins, which are divided into four classes according to sequence similarity (A-D for vertebrate plexins). They are direct signal-transducing elements for the semaphorins and co-receptors of the neuropilins (Fig. 11) (Trusolino and Comoglio 2002; Haklai-Topper et al. 2010; Yaron and Sprinzak 2012).

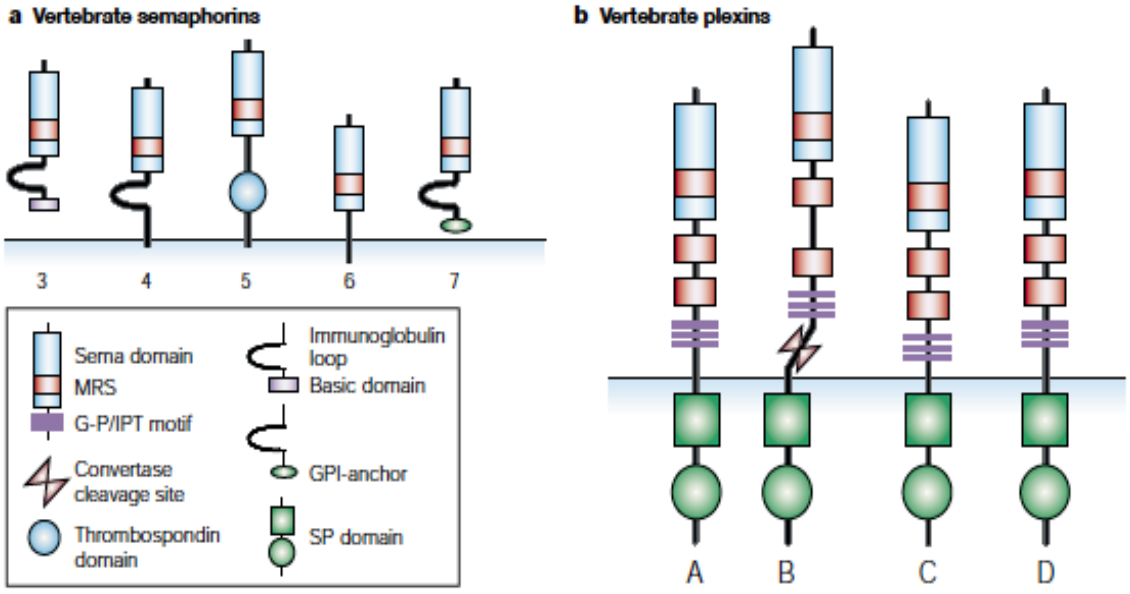


**Figure 11: Semaphorins and plexins. a:** Schematic domain representation of the five classes of vertebrate semaphorins. **b:** Schematic domain representation of the four classes of vertebrate plexins (Trusolino and Comoglio 2002).

It has been observed that sympathetic neurons, which express Plexin-A4, retract in the presence of the ligand samaphorin6A (Sema6A) (Haklai-Topper et al. 2010). In contrast, the dorsal root ganglion neurons (DRG, sensory neurons), which express not only Plexin-A4 but also Sema6A, are not responsive to Sema6A. A recent study has shown that DRG explants from cells expressing wild type Sema6A were able to grow on a layer of COS7 cells transfected with Sema6A or control plasmid. In contrast, elimination of Sema6A (Sema6A -/-) sensitized DRG neurons to respond to Sema6A and retract in a Plexin-A4 dependent manner (Fig. 12). Thus, cis-inhibition of the Plexin-A4 receptor by the co-expressed transmembrane ligand semaphorin attenuates the axonal response towards exogenous ligands and enables axonal growth.

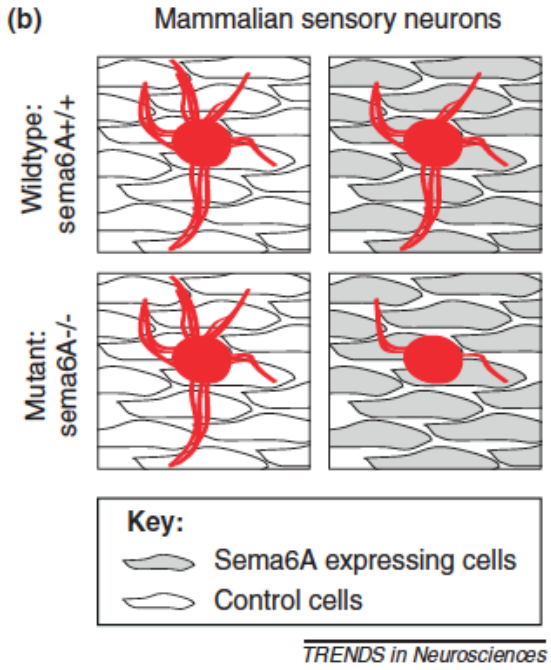


**Figure 12: Plexin-A4 cis-inhibition by Sema6A coexpression in sensory neurons.** Sensory neurons are not responsive to Sema6A in trans. In contrast, Sema6A$^{-/-}$ sensory neurons retract in response to Sema6A in trans (Yaron and Sprinzak 2012).

Sema6A and Plexin-A4 form a stable complex when co-expressed in the same cell. The cis-interaction between Sema6A and Plexin-A4 depends on the Sema domain of the Plexin-A4 receptor. Coexpression of the ligand Sema6A and the receptor Plexin-A4 inhibits the binding in trans of exogenous Sema6A ligands to Plexin-A4 through a mechanism that does not involve competition with the ligand in trans. Instead, it is possible that the cis-interaction may lock the receptor in a non-funtional conformation. Further studies need to be done in order to explain the biological function of semaphorin cis-inhibition in the guidance of sensory neurons (Haklai-Topper et al. 2010; Yaron and Sprinzak 2012).

## 3.3- COMMON FEATURES OF THE CIS-INHIBITION MECHANISM

The three distinct neuronal signaling systems, Notch-Delta, Eph-ephrin and plexin-semaphorin present common features in the receptor-ligand cis-inhibition process. In vivo and in vitro studies demonstrated that coexpression of the ligands Delta, ephrin or Sema6A together with their respective receptor on the cell membrane precludes the binding of the ligand to the receptor in trans (Sakamoto et al. 2002; Carvalho et al. 2006; Haklai-Topper et al. 2010).

The receptor later inhibition is a process occurring on the cell surface. Co-immunoprecipitation and pull down experiments showed that the cis-interaction between ligand and receptor involves the extracellular domain of the receptor, but not necessarily the ligand-binding domain. Furthermore, biochemical studies demonstrated that co-expression of the ligand does not decrease the level of receptor expression on the cell surface and cleavage of the ligand from the cell surface is able to restore the ability of the

receptor to be activated in trans (Hornberger et al. 1999; Sakamoto et al. 2002; Carvalho et al. 2006; Haklai-Topper et al. 2010).

Although cis-inhibition between ligand and receptor has been observed in several signaling systems, the underlying mechanism is still not clear. It is possible that co-expression of ligand and receptor on the same cell surface blocks the receptor clustering that is important for its activation or changes the conformation of the receptor extracellular domain disabling binding of the ligand in trans. Further experiments are required to elucidate the mechanism of receptor cis-inhibition by its ligand.

# 4- RECEPTOR-LIGAND CIS-INHIBITION IN THE IMMUNE SYSTEM

In addition to the nervous system, receptor-ligand cis-interactions have been reported in the immune system. The herpes virus entry receptor protein HVEM can engage in either cis or trans interactions with its ligands, which lead to opposite signaling outputs. HVEM is a cell surface receptor of the TNF receptor superfamily and it was discovered as first entry route for the herpes simplex virus HSV. HVEM recognizes two different families of ligands, the TNF-related cytokines LIGHT (TNFSF14) and lymphotoxin-α (LTα) and the Ig superfamily members BTLA (B and T lymphocyte attenuator) and CD160. In addition, the herpes simplex virus HSV uses the envelope glycoprotein-D (gD) as a viral ligand for HVEM to infect epithelial cells. Inflammation and adaptive immune responses are regulated by a complex network of signaling pathways initiated by HVEM, the LTβ receptor and two receptors for TNF, which cross utilize the same ligand proteins.

HVEM signaling is mainly controlled at the cell membrane. Similarly to the Eph receptors, the activation of HVEM by its ligands can generate bidirectional signaling. Moreover HVEM can also function as a ligand for BTLA. The soluble or membrane-bound forms of a specific ligand and the cis and trans ligand-receptor interactions can control the initiation of inflammatory signaling pathways mediated by HVEM in T cells (Cheung et al. 2009; Ware and Sedy 2011).

The ligand protein LIGHT can activate the receptor HVEM as a membrane-bound trimer or as a soluble form by binding in trans to the ectodomain of HVEM (Ware and Sedy 2011). In the same way, BTLA in the trans configuration can activate the receptor HVEM. The soluble form of ligand LIGHT can bind to the receptor together with BTLA, because the two ligands recognize different regions of the receptor ectodomain. In contrast, this is not possible with the membrane-bound form of LIGHT, which blocks BTLA binding to HVEM by an uncompetitive mechanism. In addition, CD160 in his trimeric form can activate HVEM in trans. Once activated, HVEM starts the TRAF2 E3 ligase pathway, which culminates with the activation of the NFkB subfamily protein RelA and cell survival (Fig. 13) (Ware and Sedy 2011).

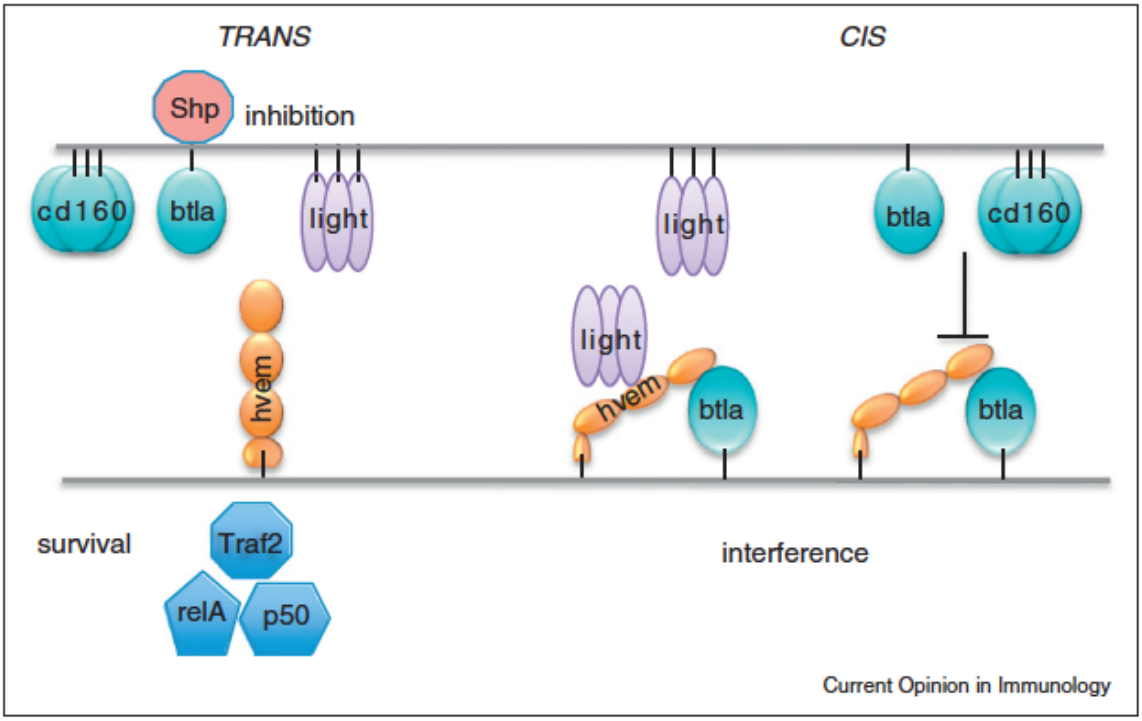


**Figure 13: HVEM receptor cis and trans binding.** On the left side of the figure: the binding in trans of the ligand proteins BTLA, CD160 and LIGHT activates HVEM signaling and the NFkB survival pathway. HVEM can also activate BTLA, generating bidirectional signals. On the right side of the figure: the cis-inhibition of HVEM by BTLA ligand coexpression blocks the binding in trans of other ligands to HVEM and therefore its signaling.

It has been reported that HVEM and BTLA are constitutively co-expressed on the cell surface of naive human and mouse T cells, where they form a stable cis-complex. Mutations that affect the binding in trans of BTLA to HVEM also interfere with the binding in cis, demonstrating the involvement of the same receptor interface. The cis-interaction between HVEM and BTLA prevents the binding in trans of the ligands BTLA and CD160 to the receptor and inhibits HVEM-dependent NFkB activation, maintaining the T cells in the naive state. The soluble form of the ligand LIGHT can bind to the receptor ectodomain within the cis-complex with BTLA, but cannot activate HVEM signaling. In contrast, the membrane-bound form of LIGHT can bind and activate the receptor, but much less compared to the level of activation observed when HVEM is expressed alone (Fig. 13). Interestingly, the viral protein gD can engage HVEM in a cis-complex, but with an opposite effect to that of BTLA, since gD activates the NFkB signaling pathway. The cis-interaction of the viral gD protein with the HVEM receptor may induce a conformational change that activates the receptor and the cell survival signaling pathway, providing a selective advantage for the virus (Cheung et al. 2009; Ware and Sedy 2011). Further studies to understand the biological role of HVEM cis and trans regulation can provide useful information for the control of inflammation and autoimmune disorders.

# 5- EPH RECEPTORS AND CANCER

The Eph receptors play an important role in the regulation of fundamental cellular processes, like axon guidance, homeostasis and cell development. A growing body of evidence has implicated Eph receptor and ephrin signaling in oncogenic processes, including cell proliferation, invasion, metastasis and tumor angiogenesis (Pasquale 2005). Beside the Eph-ephrin system, other signaling molecules involved in axon guidance have recently been linked to cancer, for example Notch and Delta, semaphorins and plexins, VEGF and the VEGF receptor (Chen 2012).

The role of the Eph-ephrin system in cancer is complex and controversial. Recent studies have shown that Eph receptor expression levels are often dysregulated in cancer (Pasquale 2010). Both increased and decreased receptor expression level can be linked to carcinogenesis. Furthermore, the Eph receptors can both promote and inhibit cancer progression depending on the presence of their ligand or cross-talk with other proteins. For example, EphA2 and EphB4 upregulation has been linked with cancer progression and tumor malignancy in several tumor types. In contrast, decreased expression levels of EphA1 and EphB6 have been observed in colorectal and lung cancer respectively. Eph receptor downregulation can be a secondary step that manifests in advanced stages of cancer after an initial cancer initiation process with higher expression level. This downregulation can be due to epigenetic silencing, chromosomal alterations and modifications of mRNA stability (Pasquale 2010).

Somatic mutations in numerous Eph receptors have also been identified in most cancers. The EphA3 receptor is one of the most mutated in human lung adenocarcinoma. The Eph receptor mutations are scattered throughout the entire receptor gene and is still not clear if some are “driver” mutations of cancer pathogenesis. Nevertheless, the participation of the Eph receptors in cancer progression makes them promising therapeutic targets (Pasquale 2010; Chen 2012).

## 5.1- PRO AND ANTI- ONCOGENIC ROLES OF EPHA2

The expression and function of the EphA2 receptor are often altered in human cancers. This protein is highly expressed in most cancer cell types, like breast, colon, prostate and lung cancer. EphA2 overexpression has also been linked to malignant progression in glioblastoma multiforme (GBM) (Ireton and Chen 2005). In human breast cancer and mouse skin cancer an unbalanced expression between the EphA2 receptor and his ligand ephrin-A1 has been observed. Indeed, the role of EphA2 as oncoprotein is independent of ephrin-ligand binding and kinase activity (Macrae et al. 2005; Miao et al. 2009).

In contrast, EphA2 stimulation by its ephrin ligands inhibits integrin signaling and the RAS-MAP kinase pathway, which is often upregulated in malignant cancers, revealing EphA2 as a tumor suppressor protein (Miao et al. 2000; Pasquale 2010).

Miao et al. (2009) analyzed the role of EphA2 in human glioma and described a possible cause for this controversial and dual role of EphA2 in cancer, linking this kinase receptor to the RTK-PI3K-Akt pathway, which regulates chemotactic cell migration

through cytoskeletal reorganization (Miao et al. 2009). In the absence of ephrin-A1 and in the presence of multiple growth factors, Akt phosphorylates the EphA2 receptor on serine S897 in the segment between the kinase and the SAM domain. This S897 phosphorylation promotes assembly of the actin cytoskeleton and extension of lamellipodia, which is required for tumor cell invasion and malignant progression.

Interestingly, the ligand-activated form of EphA2 negatively regulates Akt. Upon stimulation of EphA2 with ephrin-A1, Akt is inactivated and EphA2 is rapidly dephosphorylated at the S897 site with a resulting anti-oncogenic effect. The tumor suppressor role of EphA2 was observed even when the PTEN phosphatase is mutated and consequently the Akt-mTORC1 pathway is hyperactivated. This pathway promotes cell growth, migration and invasiveness and is regulated by the tumor suppressor lipid phosphatase PTEN. Ephrin-dependent activation of EphA2 causes dephosphorylation of Akt and inactivation of the Akt-mTORC1 oncogenic pathway, through a cross-talk mechanism with a PPI-like serine/threonine phosphatase (Yang et al. 2011). Miao et al. proposed a model for the EphA2 role in cancerogenesis whereby in the presence of the ephrin ligand the EphA2 receptor suppresses tumor development. However, in the presence of growth factors and in the absence of ephrin stimulation EphA2 is phosphorylated by Akt and promotes tumor progression and migration (Miao et al. 2009).

In summary, activation of EphA2 can be a new route for the treatment of tumors where the PI3K/Akt and Akt-mTORC1 pathways are hyperactivated (Miao et al. 2009; Yang et al. 2011).

## 5.2- THE MULTIFACETED ROLE OF EPHB4 IN CANCER

EphB4 is another Eph receptor highly expressed in epithelial cells. This receptor is present in epithelial cells of human breast tissue and notably tyrosine phosphorylated in MCF-10A cells, which are a non-transformed epithelial cell line derived from human fibrocystic mammary tissue (Berclaz et al. 2002; Noren et al. 2006; Noren and Pasquale 2007). EphB4 modulates with the Abl/Crk pathway, which regulates the maintenance of epithelial characteristics in MCF-10A cells (Noren et al. 2006). Like EphA2, EphB4 also has a dual role in cancer regulation. In several human breast cancer cell lines EphB4 expression is upregulated, but the receptor is poorly activated. This may be the consequence of low ephrin-B2 expression. In these cells Abl is not activated and Crk functions as an adaptor protein, which promotes oncogenic transformation (Lamorte et al. 2002). In contrast, EphB4 activation by a soluble form of ephrin-B2 (ephrin-B2 Fc) promotes the anti-oncogenic effect of the EphB4-Abl-Crk pathway, where Abl activated by EphB4 can inhibit cell invasion through inhibitory phosphorylation of Crk (Noren and Pasquale 2007). Furthermore, it has been shown that ephrin-B2 reverse signaling induced by EphB4 can promote angiogenesis and tumor progression (Noren et al. 2004).

In summary, the available information regarding the role of EphA2 and EphB4 in cancer suggests that ephrin-Eph signaling is important for the maintenance of homeostasis in normal tissues and its disruption may be the cause of cell transformation and cancer progression (Noren and Pasquale 2007).

Recent work has also shown that ephrin-B2-mediated activation of EphB4 has a completely opposite outcome in cell growth and regulation of the MAP kinase pathway

in other cellular contests (Xiao et al. 2012). This apparent paradox of EphB4 signaling is explained by the engagement of different downstream effector molecules in distinct cell types. In human umbilical vein endothelial cells (HuVECs), EphB4 stimulation by ephrin-B2 engages the protein p120 RasGAP and promotes inactivation of the MAP kinase signaling pathway leading to suppression of cell growth. Interestingly, upon ephrin-B2 treatment of MCF7 breast cancer cells, EphB4 promotes MAP kinase (Erk1/2) activation and cell growth through PP2A, an essential serine/threonine phosphatase that removes the inhibitory phosphorylation of c-Raf and consequently upregulates Erk1/2 (Fig. 14).

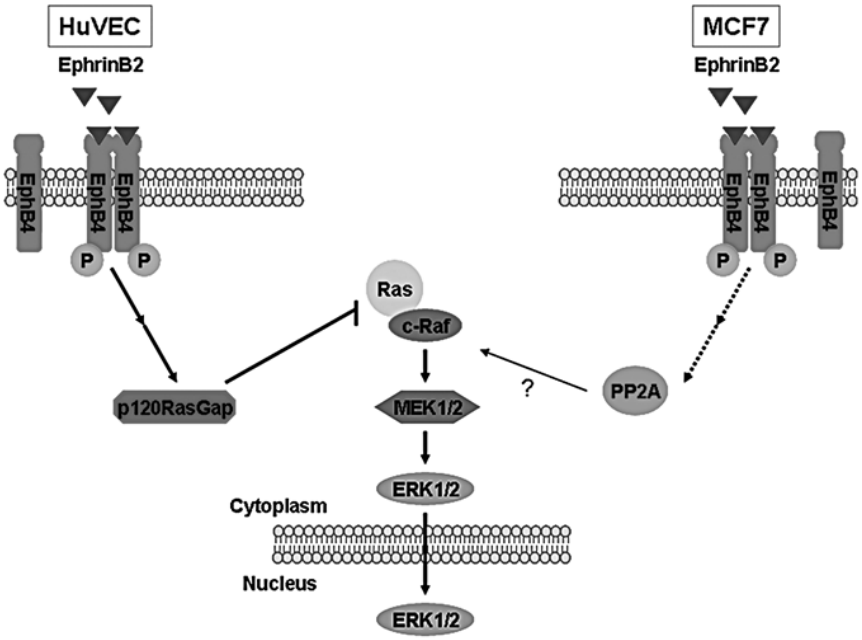


**Figure 14: Model of EphB4 signaling effects on the MAP kinase (Erk1/2) pathway upon ephrin-B2 Fc stimulation in HuVEC and MCF7 cells** (Xiao et al. 2012).

Further research is needed to better understand the controversial role of EphB4 in different types of cancer and cellular contests in order to develop appropriate EphB4-targeted cancer therapies.

## 5.3- EPHA3 AND CANCER

Among all Eph receptors, EphA3 is one of the most frequently mutated in lung cancer together with EphA5. Currently about sixty EphA3 missense mutations have been identified just in lung cancer samples, but many others have also been reported in other cancers, for example glioblastoma, melanoma, pancreatic and colorectal cancer (COSMIC http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) (Bardelli et al. 2003; Balakrishnan et al. 2007; Corbo et al. 2010). The mutations are scattered

throughout the entire EphA3 gene and the functional implications of some of the mutations have been studied (Fig. 15) (Lisabeth et al. 2012; Zhuang et al. 2012).

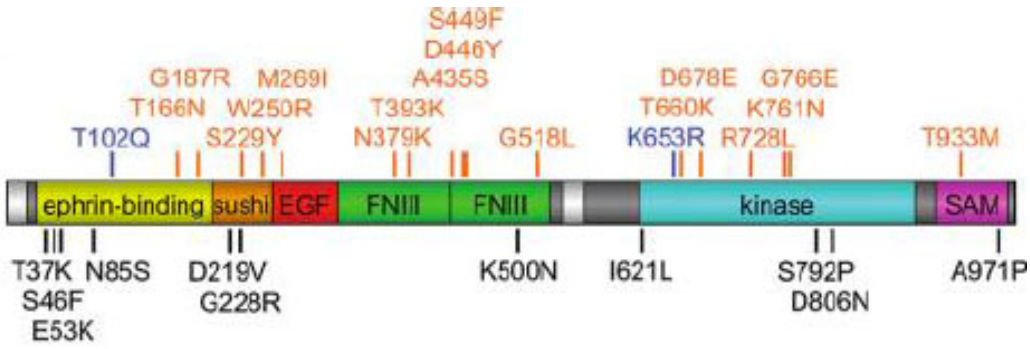


**Figure 15: EphA3 domain structure with some of the somatic cancer mutations identified.** In orange: EphA3 lung cancer mutations. In black: Different cancer mutations. In blue: control mutations known to interfere with ephrin-binding (T102Q) and kinase activity (K653R) (Lisabeth et al. 2012).

Several EphA3 mutations in the ephrin-binding domain disrupt the normal conformation of the domain and reduce the binding of the ephrins to the receptor. Some of these mutations diminish receptor surface localization, impairing the physiological EphA3-ephrin cell-cell communication system. In addition, few mutations localized within the cysteine-rich region also can affect the conformation of the receptor ligand-binding domain reducing the binding of ephrin-A5 to EphA3. Interestingly, some mutations localized in the fibronectin type III domains alter the lateral interaction between EphA3 and the ligand, and may affect receptor silencing by co-expressed ephrins (see Chapter 2, paragraph 2.4.2). Finally, mutations localized in the conserved EphA3 kinase domain completely abolish the kinase activity of the receptor (Lisabeth et al. 2012).

Although the role of EphA3 in cancer is still not well defined, these loss-of-function mutations, which impair the signaling ability of the receptor, suggest that EphA3 may be a tumor suppressor. In support of this hypothesis, it has been reported that in lung cancer cells EphA3 wild-type re-expression increases cellular apoptosis by suppressing Akt activation as well as inhibits tumor growth in vivo (Zhuang et al. 2012). In addition, in some hematopoietic tumors and in colorectal cancer, the EphA3 promoter is silenced by methylation (Dottori et al. 1999; Hinoue et al. 2009; Lisabeth et al. 2012).

However, high EphA3 expression has been reported in some tumor specimens (Guan et al. 2011; Valsesia et al. 2011; Day et al. 2013). Therefore, a tumor promoting role for EphA3 cannot be excluded. As in the case of the previously described EphA2 and EphB4 receptors, EphA3 may also have a dual role in carcinogenesis depending on ephrin ligand binding or cross-talk with other signaling proteins.

Recent work has also shown that EphA3 is highly expressed in the mesenchymal subtype of glioblastoma multiforme (GBM). This subtype is particularly aggressive and contains undifferentiated tumor-initiating cells. EphA3 plays an active role in maintaining these cells undifferentiated by reducing MAP kinase signaling, which is normally capable of promoting differentiation of neuronal progenitors. Loss of EphA3

expression in these cells shifted the balance towards differentiation and reduced cell proliferation, suggesting that the development of an EphA3-based target therapy may be effective for the treatment of glioblastoma multiforme (Day et al. 2013).

EphA3 is considered a promising target for anticancer therapies. However, it is still unclear how EphA3 expression and activity can interfere with the onset and progression of different tumors. Therefore, further studies are needed to shed light on this promising target and develop specific therapeutics approaches for different types of cancer.

# 6- AIM OF THE REASERCH

Members of the large Eph receptor tyrosine kinase family, and particularly EphA2 and EphB4, are overexpressed in a wide variety of tumor types (Pasquale 2010; Lisabeth et al. 2013a). Eph receptors signal by interacting "in trans" with ephrins expressed on neighboring cells, which promotes receptor clustering, autophosphorylation and kinase activity (Pasquale 2005). Soluble forms of the ephrin-A ligands released from the cell surface by matrix metalloproteases can also activate EphA receptors (Bartley et al. 1994; Hattori et al. 2000; Alford et al. 2007; Beauchamp et al. 2012). However, Eph receptors in cancer cells are often poorly tyrosine phosphorylated (Pasquale 2005). This suggests low activation by ephrin ligands and is consistent with the tumor suppressing effects reported for a number of Eph receptor downstream signaling pathways (Miao et al. 2001; Pasquale 2010; Yang et al. 2011).

The lack of substantial Eph receptor activation is in some cases due to low expression of ephrin ligands in cancer cells with high receptor expression (Batlle et al. 2002; Macrae et al. 2005; Noren et al. 2006; Pasquale 2010; Ji et al. 2011). In addition, several other mechanisms can keep Eph receptor activation low in cancer cells that also express ephrin ligands. For example, cancer mutations have been shown to disrupt the ephrin binding ability or kinase activity of Eph receptors (Lisabeth et al. 2012; Zhuang et al. 2012). Furthermore, lack of E-cadherin-dependent cell-cell adhesion can impair EphA2 receptor activation in breast cancer cells, suggesting inefficient EphA2 trans interaction with ephrins (Zantek et al. 1999). Another potential mechanism to attenuate Eph receptor downstream signaling in cancer cells could involve inhibitory lateral cis interactions between Eph receptors and ephrins coexpressed in the same cells (Yin et al. 2004; Carvalho et al. 2006; Lisabeth et al. 2013a). Inhibitory cis interactions with ephrins have been shown to play an important role in fine tuning Eph receptor activation in the nervous system to precisely control axon pathfinding and synaptic function (Hornberger et al. 1999; Carvalho et al. 2006; Antion et al. 2010; Pasquale 2010; Kao and Kania 2011). However, cis interactions do not occur in all neurons coexpressing Eph receptors and ephrins because in some neurons receptors and ligands occupy distinct microdomains of the plasma membrane and thus cannot intermingle (Marquardt et al. 2005; Kao and Kania 2011). Whether cis interactions between Eph receptors and ephrins can also occur in cancer cells has not been previously investigated.

Biochemical and structural studies have shown that cis interaction involves an Eph receptor-ephrin binding interface distinct from that mediating the high affinity interaction in trans (Carvalho et al. 2006; Seiradake et al. 2010). The extracellular region of both EphA and EphB receptor classes contains an N-terminal ligand-binding domain, a cysteine-rich region and two fibronectin type III domains (Pasquale 2005). The second fibronectin domain is followed by a transmembrane segment and a cytoplasmic region that includes the tyrosine kinase domain, a SAM domain and a PDZ-binding motif. The ephrins consist of an N-terminal Eph receptor-binding domain connected by a short linker region to a glycosylphosphatidylinositol (GPI) anchor for the ephrin-As and a transmembrane segment followed by a short cytoplasmic region for the ephrin-Bs. Eph

receptor-ephrin binding in trans mainly involves the interaction between the G-H loop of the ephrin and a pocket within the ligand-binding domain of the Eph receptor (Himanen et al. 2001). These interfaces predominantly support the promiscuous interactions of Eph receptors with ephrins belonging to the same A or B class. On the other hand, cis interactions have been proposed to involve the fibronectin type III domains of the Eph receptor and a region of the receptor-binding domain of the ephrin that is distinct from the G-H loop (Carvalho et al. 2006; Seiradake et al. 2010).

Here we show that Eph receptors and ephrins coexpressed in cancer cells can engage in cis interactions that inhibit Eph receptor activation by ephrins in trans. Interestingly, we detected inhibition of EphA3 activation through cis interaction with not only ephrin-A3 but also ephrin-B2, which is not an activating ligand for EphA3 (Flanagan and Vanderhaeghen 1998), suggesting that cis interactions do not exhibit the same receptor-ligand selectivity as trans interactions. We also found that a lung cancer mutation identified in the second fibronectin type III repeat of EphA3 enhances the cis association of the receptor with ephrin-A3 (Falivelli et al. 2013).

# 7- MATERIALS AND METHODS

## 7.1- Plasmids and lentiviruses

The human EphA3 cDNA was purchased from Invitrogen/Life Technologies (Carlsbad, CA; clone MGC:71556; GenBank accession number NP_005224.2), PCR amplified to include appropriate restriction sites and cloned in pcDNA3. EphA3 was also subcloned into the pLVX-IRES-ZsGreen lentiviral vector (Clontech Laboratories, Mountain View, CA). The truncated versions EphA3 ΔN, comprising a signal peptide followed by amino acids 318-984 of EphA3, was also generated by PCR amplification of full-length EphA3 and cloned in pcDNA3. The EphA3 ΔN G518L mutant was similarly generated by PCR amplification from the previously described full-length EphA3 G518L mutant (Lisabeth et al. 2012). Mouse ephrin-A3 cDNA in pcDNA3, including nucleotides 40-744 (GeneBank accession number NM_010108.1), was used as template to generate the ephrin-A3 E129K mutant using the QuickChange Site-Direct mutagenesis kit (Stratagene/Agilent Technologies, La Jolla, CA). The CS-Mm30127-Lv105-ephrin-A3 lentivirus, with mCherry inserted between the signal peptide and the mature coding sequence of mouse ephrin-A3, and the EX-mCHER-Lv105 control lentivirus encoding mCherry were purchased from GeneCopoeia. The mouse mCherry-ephrin-A3 E129K mutant was generated in pcDNA3 using the QuickChange Site-Direct mutagenesis kit and subcloned in the pLVX-IRES-Neo lentiviral vector (Clontech Laboratories). Mouse ephrin-B2 (GeneBank accession number NM_010111.5) with an N-terminal EGFP tag inserted between a signal peptide and the mature coding sequence (Makinen et al. 2005; Salvucci et al. 2009) was cloned in the pCCLsin.PPT.hPGK.GFP. pre lentiviral vector (Follenzi and Naldini 2002) replacing the EGFP insert of the vector. The pCCLsin.PPT.hPGK.GFP. pre lentiviral vector encoding EGFP was used as a control. All PCR-amplified and mutated cDNAs were verified by sequencing.

## 7.2- Cell culture, transfections and infections

The human embryonic kidney (HEK) 293T cell line (ATCC, Manassas, VA), the HEK AD-293 cell line (Cell Biolabs, Inc.), which is a derivative of the HEK 293 cell line with increased adherence, the SKBR3 and MCF7 cell lines (ATCC) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Cellgro, Manassas, VA) supplemented with 1 mM L-glutamine, 10% fetal bovine serum, 1 mM sodium pyruvate and antibiotics. The A549 human lung adenocarcinoma and NCI-H226 human squamous cell carcinoma cell lines (ATCC) were grown in Roswell Park Memorial Institute (RPMI) culture medium with the same supplements used for DMEM.

To activate EphA2 and EphA3 in lung cancer cells, the cells were stimulated for 20 min in complete medium with 2 μg/ml ephrin-A3 Fc fusion protein (R&D Systems, Minneapolis, MN) or Fc (MP Biomedical, Solon, OH) preclustered with 1/10 polyclonal

goat anti-human Fc antibody (Jackson ImmunoResearch). To activate EphA2 in breast cancer cells, the cells were stimulated for 20 min with 0.5 µg/ml ephrin-A1 Fc or Fc without preclustering. In addition, some wells were pretreated for 4 hours with 1 U/ml PI-PLC (Invitrogen/Life Technologies) and, in some experiments, some wells were pretreated for 24 hours with 100 µM GM-6001 (stock dissolved in DMSO; Enzo Life Sciences, Farmingdale, NY) or an equivalent DMSO concentration (0.4%) as a control. To activate EphB4, cells were stimulated for 20 min with 2 µg/ml ephrin-B2 Fc preclustered with 6 µg/ml anti-human Fc antibody.

To produce alkaline phosphatase fusion proteins, plasmids encoding EphA3 AP, ephrin-A3 AP, ephrin-A5 AP or ephrin-B2 AP were transiently transfected in HEK293T cells using Lipofectamine 2000 (Invitrogen/Life Technologies) according to the manufacturer's instructions. Plasmids encoding EphA3, EphA3 ΔN or EphA3 ΔN G518L were transiently transfected in HEK AD-293 cells using Lipofectamine 2000 and the cells were lysed one day after transfection. NCI-H226 and A549 cells were infected with the lentivirus encoding EphA3 and ZsGreen and FACS-sorted. The sorted cells were then infected with lentiviruses encoding mCherry-ephrin-A3 or mCherry and selected with 1 µg/ml puromycin. Alternatively, the sorted cells were infected with lentiviruses encoding EGFP-ephrin-B2 or EGFP. HEK AD-293 cells infected with lentiviruses encoding mCherry-ephrin-A3 or mCherry were selected with puromycin while cells infected with the lentivirus encoding the mCherry-ephrin-A3 E129K mutant were selected with 1.5 mg/ml G418 (Roche Applied Science, Indianapolis, IN).

## 7.3- Immunoprecipitations, pull-downs and immunoblotting

Cells were washed with cold phosphate-buffered saline (PBS) and lysed in modified RIPA buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 2 mM EDTA) or Triton-X100 buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton, 10% glycerol) with protease and phosphatase inhibitors. The cells were then briefly sonicated.

For immunoprecipitations, cells lysed in modified RIPA buffer were precleared for 15 min at 4 ºC with GammaBind Plus sepharose beads and then incubated for 90 min at 4 ºC with 2.5 µg anti-EphA2 monoclonal antibody (clone D7; Upstate Biotechnology/Millipore, Lake Placid, NY), anti-EphA3 monoclonal antibody (Invitrogen/Life Technologies), an affinity-purified rabbit polyclonal anti-EphB4 antibody to the human EphB4 C terminal region (Noren et al. 2004), or anti-dsRed polyclonal antibody (Clontech Laboratories) immobilized on GammaBind Plus sepharose beads (GE Healthcare Bio-Sciences, Piscataway, NJ). For coimmunoprecipitations, cells lysed in Triton X-100 buffer were precleared with GammaBind Plus sepharose beads and then incubated for 3 hours at 4ºC with 2.5 µg anti-EphA3 monoclonal antibody immobilized on GammaBind Plus sepharose beads.

For pull-down of ephrin-A3 from cell culture medium and cell lysates, A549 and H226 cells were grown to confluency in 60 mm plates with 1.5 ml medium for 24 hours (A549 cells) or 48 hours (H226 cells). The conditioned medium was collected and the cells were washed with cold PBS and lysed in 1.5 ml modified RIPA buffer. Culture medium and cell lysates were precleared with GammaBind Plus sepharose beads and then

incubated for 1 hour at 4 ºC with 1 µg EphA3 Fc (R&D Systems) immobilized on GammaBind Plus sepharose beads.

Immunoprecipitates, pull-downs and cell lysates were analyzed by immunoblotting with the following antibodies: anti-phosphotyrosine conjugated to horseradish peroxidase (HRP; BD Bioscience, San Jose, CA), anti-EphA3 rabbit polyclonal (sc-919, Santa Cruz Biotechnology, Dallas, TX), anti-EphA2 rabbit polyclonal (Invitrogen/Life Technologies), anti-EphB4 mouse monoclonal (Invitrogen/Life Technologies), anti-ephrin-A1 rabbit monoclonal (Abcam, Cambridge, MA), anti-ephrin-A3 rabbit polyclonal (Santa Cruz Biotechnology), anti-ephrin-A3 chicken immune serum obtained by injecting a mouse ephrin-A3 Fc fusion protein including amino acids 31-213 (Noberini et al. 2012b), rabbit anti-human Fc (Jackson ImmunoResearch Laboratories, West Grove, PA), anti-dsRed rabbit polyclonal (Clontech Laboratories, Inc), and anti-GFP rabbit polyclonal (Gentex). Incubation with primary antibodies was followed by incubation with anti-rabbit, anti-mouse or anti-chicken secondary antibodies conjugated to HRP (anti-rabbit and anti-mouse from Millipore, Billerica, MA, and anti-chicken from Sigma-Aldrich, St. Louis, MO) or fluorescently labeled anti-rabbit and anti-mouse secondary antibodies (Odyssey LI-COR, Lincoln, NE). Immunoblots were developed with ECL chemiluminescence HRP detection reagent (GE Healthcare) and the bands quantified using Photoshop. The Odyssey LI-COR system was used for detection in the immunoblots shown in Fig. 6, where the bands were quantified with Image Studio Software version 3.1.4.

## 7.4- Production of AP fusion proteins and AP cell binding assays

Culture medium containing the secreted AP fusion proteins was concentrated using Amicon Ultra Centrifugal filters (Millipore, Billerica, MA) and the concentration of the AP fusion proteins was estimated from AP activity measurements (Lisabeth et al. 2012). Assays to measure binding of EphA3 AP or ephrin AP proteins to cells were carried out as previously described (Lisabeth et al. 2012). The cells were washed once with cold Hanks' balanced salt solution (HBAH) containing 0.5 mg/ml bovine serum albumin, 0.1% $NaN_3$ and 20 mM HEPES pH 7.0 and then incubated for 90 min with 12 nM of AP fusion protein followed by 6 washes with cold HBAH. The cells were then lysed in 1% Triton X-100, 10 mM Tris-HCl pH 8.0 at room temperature, centrifuged at maximum speed in an Eppendorf benchtop microcentrifuge, and the supernatants were heated at 65ºC for 10 min to inactivate endogenous alkaline phosphatase. AP fusion proteins bound to the cells were quantified by measuring the absorbance of the cleaved p-nitrophenyl phosphate chromogenic substrate (Pierce/Thermo Scientific, Rockford, IL).

## 7.5- Cell surface biotinylation

To biotinylate cell surface proteins, A549 cells were first kept at 4 ºC for 10 min to block endocytosis. The cells were then incubated with 0.5 mg/ml of EZ-link SulfoNHS-LC-

Biotin (Pierce/Thermo Scientific) in PBS for 30 min at 4ºC, followed by two washes with cold PBS and incubation in quenching buffer (100 mM glycine in PBS) for 14 min at 4 ºC. The cells were then lysed in modified RIPA buffer. For quantification of cell surface (biotinylated) EphA3, protein A-coated 96-well plates were incubated with 100 µl anti-EphA3 polyclonal antibody recognizing an epitope in the cytoplasmic region of the receptor (Santa Cruz Biotechnology) at a final concentration of 0.5 µg/ml, washed to remove unbound antibody, then incubated for one hour with cell lysates and washed. EphA3 biotinylation was measured using a streptavidin-HRP conjugate (Pierce/Thermo Scientific) with 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid] (ABTS) chromogenic substrate followed by quantification of optical absorbance at 405 nm. For quantification of cell surface ephrin-A3, proteins on the surface of A549 cells were similarly biotinylated. The cells were then lysed in RIPA buffer, mCherry-ephrin-A3 was immunoprecipitated with dsRed antibody, and the immunoprecipitates were probed with a streptavidin-HRP conjugate.

## 7.6- Statistical analyses

All statistical analyses were performed with the Program Prism from GraphPad Software (La Jolla, CA).

# 8- RESULTS

## 8.1- Ephrin-A3 coexpression in cancer cells attenuates EphA receptor activation in trans by soluble ephrin-A3

To investigate the effect of ephrin coexpression on Eph receptor signaling in cancer cells, we examined EphA3 (an Eph receptor for which inhibitory cis interactions with ephrin-As have been extensively studied in neurons (Yin et al. 2004; Carvalho et al. 2006; Kao and Kania 2011)) and EphA2 (the EphA receptor most widely expressed in cancer cells (Wykosky and Debinski 2008; Pasquale 2010; Biao-Xue et al. 2011; Tandon et al. 2011) but for which the effects of cis interactions were not previously investigated). We infected the NCI-H226 and A549 lung cancer cell lines with lentiviruses encoding EphA3 and ZsGreen from a bicistronic transcript or only ZsGreen as a control. After selection by FACS sorting, we further infected the cells with lentiviruses encoding ephrin-A3 tagged with mCherry or only mCherry as a control, followed by selection. The two lentivirally infected cancer cell lines, which do not express detectable endogenous EphA3 or ephrin-A3 (Figure 1), were then treated with ephrin-A3 Fc (a soluble form of the ephrin-A3 ligand fused to the Fc portion of human $IgG_1$) to activate EphA3 through ephrin binding in trans. Ephrin-A3 Fc increased receptor tyrosine phosphorylation in the cells coexpressing EphA3 with control mCherry, as expected, but not in the cells coexpressing EphA3 with mCherry-ephrin-A3 (Figure 1A,B). Ephrin-A3 coexpression also attenuated ephrin-A3 Fc-induced activation of endogenous EphA2 in A549 cells (Figure 1C). Thus, in lung cancer cells, coexpressed ephrin-A3 can inhibit EphA2 and EphA3 activation by ephrin ligands.

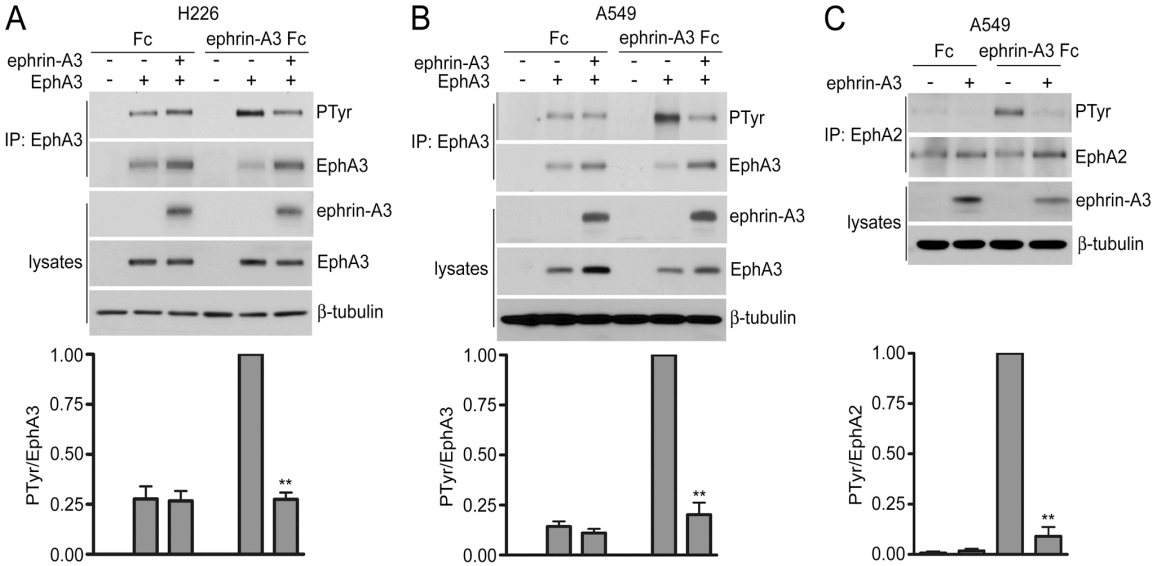

**Figure 1. Coexpressed ephrin-A3 attenuates EphA receptor activation in cancer cells.** (A,B) NCI-H226 and A549 lung cancer cells were infected with a lentivirus encoding EphA3 and ZsGreen alone or together with a lentivirus encoding mCherry-ephrin-A3; control cells were infected with lentiviruses encoding ZsGreen and mCherry. EphA3 immunoprecipitates were probed by immunoblotting for phosphotyrosine (PTyr) and reprobed for EphA3. Lysates were probed for mCherry-ephrin-A3 with an anti-dsRed antibody that also recognizes mCherry, for EphA3, and for β-tubulin as loading control. The histograms show normalized means ± SE quantified from 3 immunoblots in both A and B. In one of the A549 experiments used for quantification, the cells were stimulated with ephrin-A5 Fc. **$p<0.01$ by one sample t test for the comparison of ephrin-A3 Fc-treated cells expressing both EphA3 and ephrin-A3 with ephrin-A3 Fc-treated cells expressing only EphA3. Of note, EphA3 levels were higher in A549 cells co-expressing ephrin-A3/ephrin-B2 (see also Figs. 2A, 3B, 4A and 5C,D), suggesting that this receptor may be stabilized by the coexpressed ephrins. (C) A549 cells were infected with a lentivirus encoding mCherry-ephrin-A3 or mCherry as a control. Immunoprecipitated endogenous EphA2 was probed by immunoblotting for phosphotyrosine (PTyr) and reprobed for EphA2. Lysates were probed with an anti-dsRed antibody and β-tubulin as loading control. The histogram shows normalized means ± SE quantified from 3 immunoblots. **$p<0.01$ by one sample t test for the comparison of ephrin-A3 Fc-treated cells expressing or not expressing ephrin-A3.

## 8.2- Coexpression with ephrin-A3 in cancer cells impairs the ability of EphA3 to bind ephrin-As in trans

To examine whether in cancer cells ephrin-A3 coexpression impairs the ability of EphA3 to bind ephrin-A ligands in trans, we measured the binding of soluble forms of ephrin-A5 or ephrin-A3 fused to alkaline phosphatase (AP) to NCI-H226 and A549 cells expressing EphA3 alone or together with mCherry-ephrin-A3. We detected ephrin-A AP binding to cells only expressing EphA3 but not to cells coexpressing ephrin-A3 with EphA3 (Figure 2A). Immunoblotting verified that ephrin-A3 coexpression does not decrease overall EphA3 levels (Figure 2A). Biotinylation of cell surface proteins followed by an ELISA in which EphA3 was captured with an antibody and its level of biotinylation was detected with streptavidin conjugated to horseradish peroxidase (HRP) showed that ephrin-A3 coexpression does not affect the fraction of EphA3 present on the cell surface (Figure 2B). Thus, coexpressed ephrin-A3 in lung cancer cells inhibits ephrin binding to EphA3 in trans without reducing EphA3 expression or surface localization.

A possible explanation for these results could be that soluble ephrin-A3 released in the culture medium by matrix metalloproteases (Bartley et al. 1994; Hattori et al. 2000; Beauchamp et al. 2012) would compete with ephrin-A3 AP for binding to the EphA3 ligand-binding domain. To address this possibility, we used the extracellular domain of EphA3 fused with Fc to pull-down ephrin-A3 from the culture medium or the cells lysed in a volume equivalent to that of the culture medium. Ephrin-A3 could be detected by immunoblotting in the pull-downs from cell lysates but not from the culture medium (Figure 2C), indicating that the great majority of the ephrin-A3 remained associated with the cells during the 24-48 hour time period of our experiments. In addition, a single mCherry-ephrin-A3 band was observed in the immunoblots, making it unlikely that a

substantial portion of the ephrin was cleaved to generate a smaller form remaining associated with the cells by binding to an EphA receptor. Biotinylation of cell surface proteins followed by detection of the immunoprecipitated biotinylated ephrin-A3 with streptavidin-HRP confirmed that ephrin-A3 is similarly localized on the A549 cell surface when expressed alone or together with EphA3 (Figure 2D).

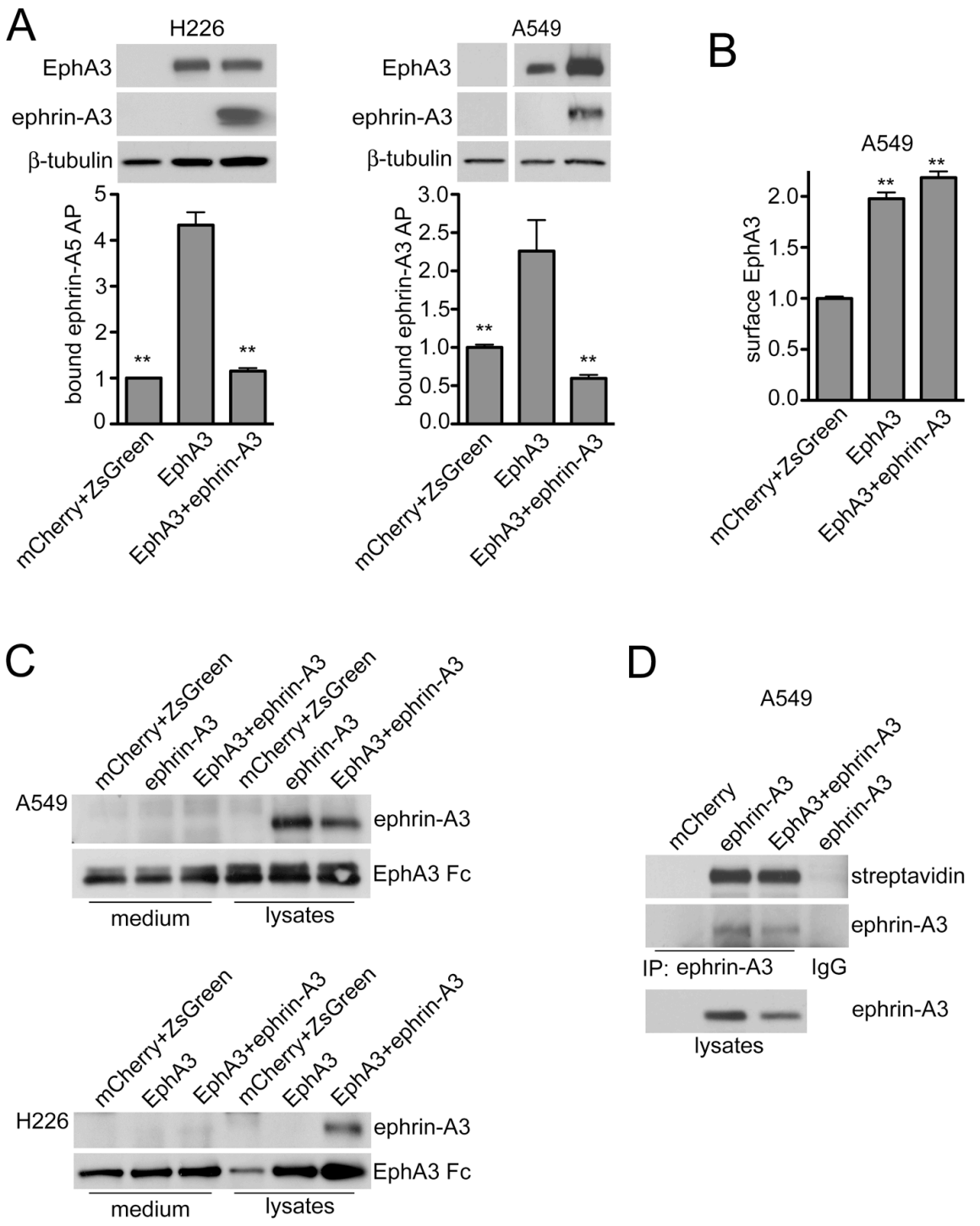


**Figure 2. Coexpressed cell surface-associated ephrin-A3 inhibits the binding in trans of soluble ephrins to EphA3 in lung cancer cells.** (A) NCI-H226 and A549 lung cancer cells were infected with a lentivirus encoding EphA3 and ZsGreen alone or together with a lentivirus encoding mCherry-ephrin-A3; control cells were infected with lentiviruses encoding ZsGreen and mCherry. The histograms show the binding of ephrin-A5 AP to NCI-H226 cells and ephrin-A3 AP to A549 cells, revealing that ephrin-A3 coexpression prevents the binding of ephrin AP proteins to EphA3. Normalized means from 2 experiments (each

with triplicate samples) ± SE are shown. **p<0.01 by one-way ANOVA and Dunnett's post-hoc test for the comparison with cells expressing only EphA3. The immunoblots show expression of EphA3, ephrin-A3, and β-tubulin as loading control in cell lysates, verifying that ephrin-A3 coexpression did not reduce EphA3 levels. In fact, EphA3 levels appeared higher in A549 cells co-expressing ephrin-A3. The white space indicates removal of an irrelevant lane. (B) Cell surface biotinylation followed by an ELISA where EphA3 was captured with an immobilized antibody and its biotinylation detected with streptavidin-HRP reveals a similar fraction of EphA3 on the surface of cells expressing EphA3 alone or together with ephrin-A3. The histogram shows means from 2 experiments (each with triplicate samples) ± SE. Incubation with twice as much lysates yielded similar results, indicating that maximal EphA3 binding to the antibody immobilized in the wells was achieved. **p<0.01 by one-way ANOVA and Tukey's post-hoc test for the comparison with cells expressing mCherry and ZsGreen; p>0.05 for the comparison of cells expressing EphA3 with and without ephrin-A3. (C) EphA3 Fc was used for pull-downs from conditioned medium and lysates of A549 or H226 cells infected with the indicated lentiviruses. By immunoblotting with an anti-dsRed antibody, ephrin-A3 was detected only in the lysates. The pull-downs were also probed for Fc to verify the levels of EphA3 Fc. (D) Surface proteins were biotinylated in cells infected with lentiviruses encoding mCherry, mCherry-ephrin-A3, or mCherry-ephrin-A3 together with EphA3 and ZsGreen. mCherry-ephrin-A3 immunoprecipitates (with anti-dsRed antibody) were probed with streptavidin-HRP, demonstrating similar cell surface levels of ephrin-A3 expressed alone or together with EphA3. IgG, control immunoprecipitate with non-immune IgGs. Lysates were probed for mCherry-ephrin-A3 with anti-dsRed antibody.

## 8.3- EphA3-ephrin-A3 cis interaction does not require the receptor ligand-binding domain

Previous studies have shown that cis interactions require membrane localization of the Eph receptor and the ephrin (Carvalho et al. 2006). Therefore, to examine whether the EphA3 ligand-binding domain is necessary for cis interaction with ephrin-A3 or whether the fibronectin type III repeats are sufficient to mediate cis binding (Carvalho et al. 2006; Seiradake et al. 2010), we transiently transfected HEK293 cells stably expressing mCherry-ephrin-A3 with plasmids encoding EphA3 ΔN (a truncated form of EphA3 that lacks the N terminal ligand-binding domain and cysteine-rich region) or full-length EphA3. In coimmunoprecipitation experiments with an anti-EphA3 antibody that recognizes the C-terminal region of the receptor, we detected association of ephrin-A3 with both full-length and truncated EphA3 (Figure 3A). This confirms that the EphA3 ligand-binding domain, which mediates high affinity binding in trans, is not necessary for EphA3-ephrin-A3 cis interaction.

To investigate the effect of mutating the ephrin G-H loop, we examined the E129K mutation in ephrin-A3. This mutation did not prevent the cis association of ephrin-A3 with EphA3 ΔN (Figure 3B), even though it abolished the trans interaction with EphA3 AP (Figure 3C). These results are consistent with those obtained with the corresponding ephrin-A5 E129K mutant, which can also still attenuate through cis interaction EphA3 phosphorylation as well as EphA-mediated growth cone collapse and

axon guidance triggered by ephrin-A ligands in trans (Carvalho et al. 2006; Kao and Kania 2011). Hence, EphA3 and ephrin-A3 can associate with each other even when lacking the regions that mediate high affinity binding in trans, supporting the general involvement in cis interactions of the Eph fibronectin type III domains and an ephrin region distinct from the G-H loop.

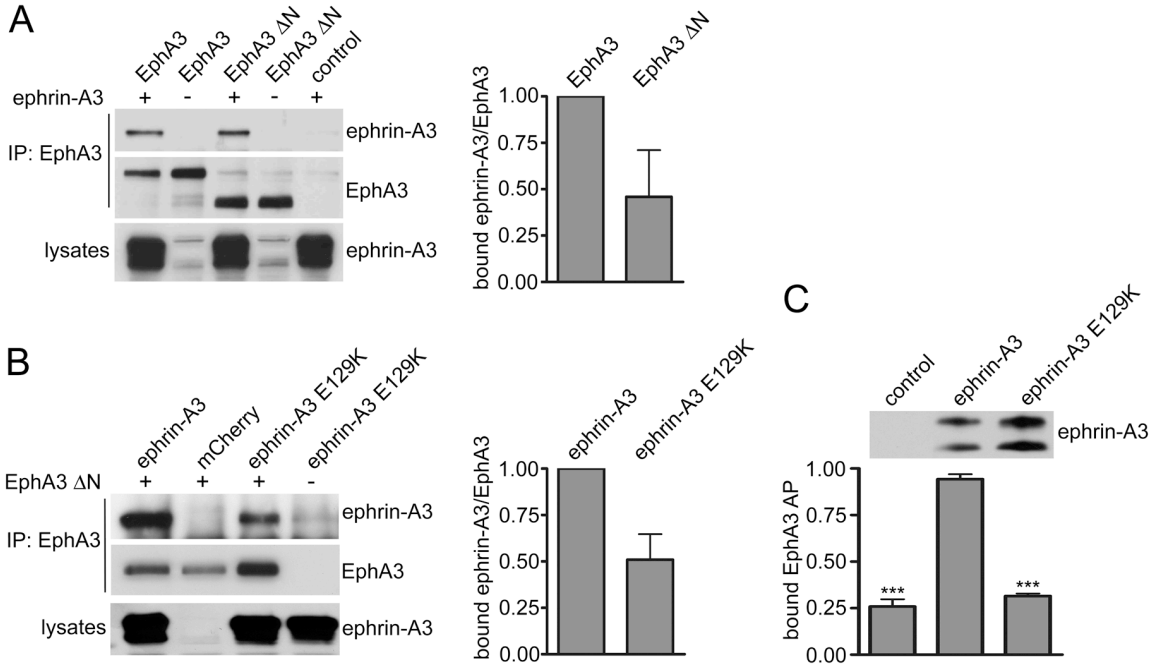


**Figure 3. Cis interaction between coexpressed EphA3 and ephrin-A3 does not require the regions involved in trans interaction.** (A) HEK AD-293 cells were infected with a lentivirus encoding mCherry-ephrin-A3 or mCherry as a control. Subsequently, the cells were transfected with plasmids encoding full-length EphA3 or a truncated form lacking the ligand-binding domain and cysteine-rich region (EphA3 ΔN). EphA3 immunoprecipitates were probed with anti-ephrin-A3 antiserum and reprobed for EphA3, revealing that ephrin-A3 association with EphA3 does not require the EphA3 ligand-binding domain. The histogram shows normalized means ± SE quantified from the immunoblots from 2 experiments. $p>0.05$ by one sample t test for the comparison of ephrin-A3 bound to EphA3 ΔN or full-length EphA3. (B) HEK AD-293 cells infected with a lentivirus encoding mCherry-ephrin-A3, the mCherry-ephrin-A3 E129K mutant, or mCherry as a control, were transfected with a plasmid encoding EphA3 ΔN. EphA3 immunoprecipitates were probed for ephrin-A3 and reprobed for EphA3, revealing that the E129K mutation does not abolish the cis interaction with EphA3. The histogram shows normalized means ± SE quantified from 3 immunoblots. $p>0.05$ by one sample t test for the comparison of ephrin-A3 E129K versus ephrin-A3 wild-type bound to EphA3 ΔN. (C) HEK AD-293 cells were transfected with control pcDNA3, pcDNA3-ephrin-A3, or pcDNA3-ephrin-A3 E129K. The histogram shows means from two experiments for the binding of EphA3 AP to ephrin-A3, confirming that ephrin-A3 E129K mutant does not bind EphA3 in trans. ***$p<0.001$ by one-way ANOVA and Dunnett's post-hoc test for the comparison with cells expressing wild-type ephrin-A3.

The immunoblot shows the expression of ephrin-A3 and ephrinA3 E129K in lanes loaded with equal amounts of total lysates. It should be noted that ephrin-A3 overexpressed in HEK cells yields two bands, with the upper band corresponding to the size of the mature full-length protein.

## 8.4- The EphA3 G518L lung cancer mutation enhances cis interaction with coexpressed ephrin-A3

Recent sequencing studies have identified EphA3 mutations in lung cancer and other cancers, and functional characterization has revealed that many are loss-of-function mutations that inhibit ephrin binding, kinase activity and/or cell surface localization, suggesting a tumor suppressor role for wild-type EphA3 (Ding et al. 2008; Lisabeth et al. 2012; Zhuang et al. 2012). One of the few mutations that were not found to impair any of the EphA3 properties examined in a previous study, but rather slightly increased EphA3 cell surface localization, is the G518L mutation in the second fibronectin type III domain (Lisabeth et al. 2012). Since G518 in EphA3 corresponds to a conserved residue that in the EphA2-ephrin-A5 crystal structure participates in the cis interface, we examined whether the G518L mutation might affect the cis association of EphA3 with coexpressed ephrin-A3. To focus on the role of the cis interaction, we used EphA3 ΔN or the EphA3 ΔN G518L mutant. Coimmunoprecipitation experiments using HEK293 cells coexpressing mCherry-ephrin-A3 with EphA3 ΔN or the ΔN G518L mutant revealed more ephrin-A3 associated with the mutant (Figure 4A). Measurement of ephrin-A5 AP binding verified that ephrin-A3 coexpression with the full-length EphA3 G518 mutant inhibited its ability to bind ephrins in trans (Figure 4B). These results suggest that the G518L mutation enhances EphA3-ephrin binding in cis and supports the involvement in the cis interface of the conserved glycine in the second fibronectin type III domain (Seiradake et al. 2010).

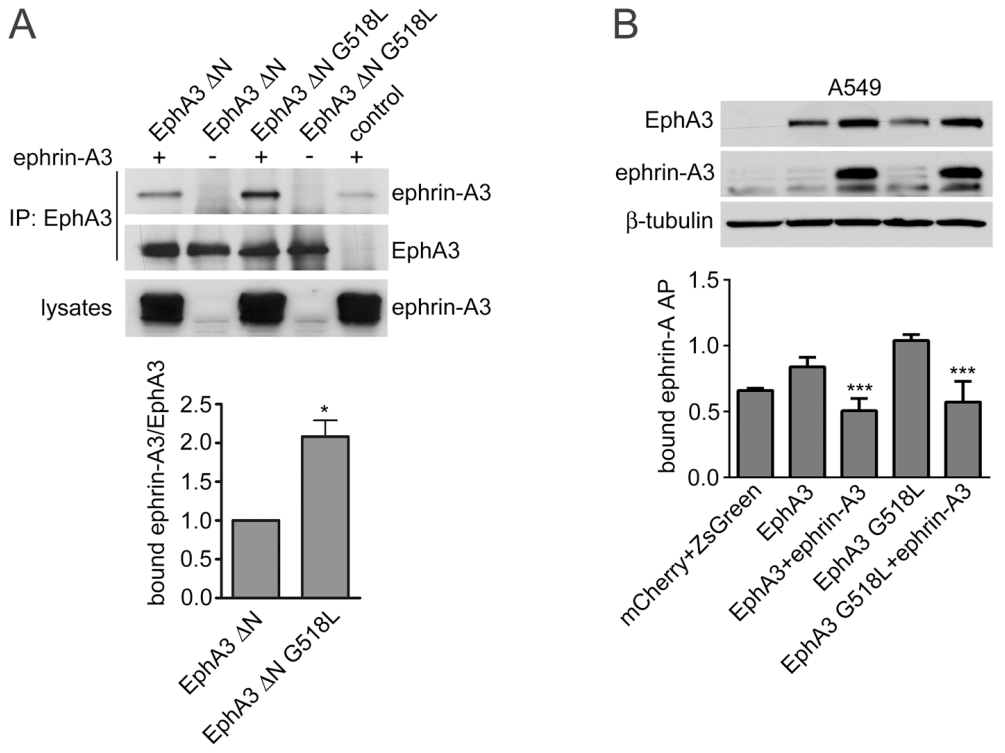


**Figure 4. The EphA3 G518L lung cancer mutation enhances cis interaction with ephrin-A3.** (A) HEK AD-293 cells were infected with a lentivirus encoding mCherry-ephrin-A3 or mCherry as a control. The cells were then transfected with EphA3 ΔN or the EphA3 ΔN G518L mutant. EphA3 immunoprecipitates were probed with an anti-ephrinA3 antiserum and reprobed for EphA3. The EphA3 G518L mutation found in lung cancer increases the affinity of the lateral interaction between EphA3 and ephrin-A3. The histogram shows normalized means ± SE quantified from the immunoblots from 3 experiments. *p<0.05 by one sample t test for the comparison of the EphA3 ΔN G518 mutant versus EphA3 ΔN. (B) A549 lung cancer cells were infected with a lentivirus encoding EphA3 wild-type or the G518L mutant and ZsGreen alone or together with a lentivirus encoding mCherry-ephrin-A3; control cells were infected with lentiviruses encoding ZsGreen and mCherry. The histogram shows cell binding of ephrin-A3 AP (one experiment) and ephrin-A5 AP (2 experiments), confirming that ephrin-A3 coexpression prevents the binding of ephrin AP proteins to the EphA3 G518L mutant. Normalized means from 3 experiments (each with duplicate samples) ± SE are shown. ***p<0.001 by one-way ANOVA and Tukey's post-hoc test for the comparison of cells coexpressing EphA3 and ephrin-A3 with cells only expressing EphA3 and for the comparison of cells coexpressing EphA3 G518L and ephrin-A3 with cells only expressing EphA3 G518L. The immunoblot of the cell lysates shows expression of EphA3, ephrin-A3, and β-tubulin as loading control.

## 8.5- Ephrin-B2 coexpression in cancer cells attenuates not only EphB4 but also EphA3 activation and ligand-binding capacity in trans

Cis interactions between the Eph fibronectin type III domains and ephrins could have distinctive selectivity compared to trans interactions involving the Eph ligand-binding domain and the ephrin G-H loop (Seiradake et al. 2010). To investigate this, we used ephrin-B2, which does not bind with high affinity to the EphA3 ligand-binding domain (Flanagan and Vanderhaeghen 1998). We infected A549 lung cancer cells and MCF7 breast cancer cells with a lentivirus encoding ephrin-B2 fused to EGFP and first examined the effects on endogenous EphB4, which binds the ephrin-B2 ligand in trans. Like EphA2, EphB4 is widely expressed in cancer cells (Noren and Pasquale 2007; Pasquale 2010) and its ability to be regulated by ephrins in cis was not previously examined. We found that ephrin-B2 expression inhibits the binding of ephrin-B2 AP to the cell surface (Figure 5A) and EphB4 tyrosine phosphorylation induced in trans by ephrin-B2 Fc (Figure 5B). Thus, cis interaction with coexpressed ephrin-B2 inhibits EphB4 ligand binding in trans and activation in cancer cells. To examine whether EphA3 can also be regulated by cis interaction with ephrin-B2, we infected A549 lung cancer cells expressing EphA3 with lentiviruses encoding EGFP-ephrin-B2 or only EGFP as a control. Interestingly, ephrin-B2 coexpression attenuated EphA3 activation by ephrin-A3 Fc (Figure 5C) and inhibited the ability of EphA3 to bind ephrin-A5 AP without decreasing overall EphA3 levels (Figure 5D). EphA3 expression only slightly increased the binding of the extracellular domain of ephrin-B2 AP to the cells (Figure 5D), confirming that ephrin-B2 does not efficiently bind to EphA3 in trans (Flanagan and Vanderhaeghen 1998). These results suggest that although ephrin-B2 is not an activating ligand for EphA3, it can affect EphA3 function through cis interaction. This implies that the binding specificities that govern cis and trans Eph receptor-ephrin interactions are not the same.

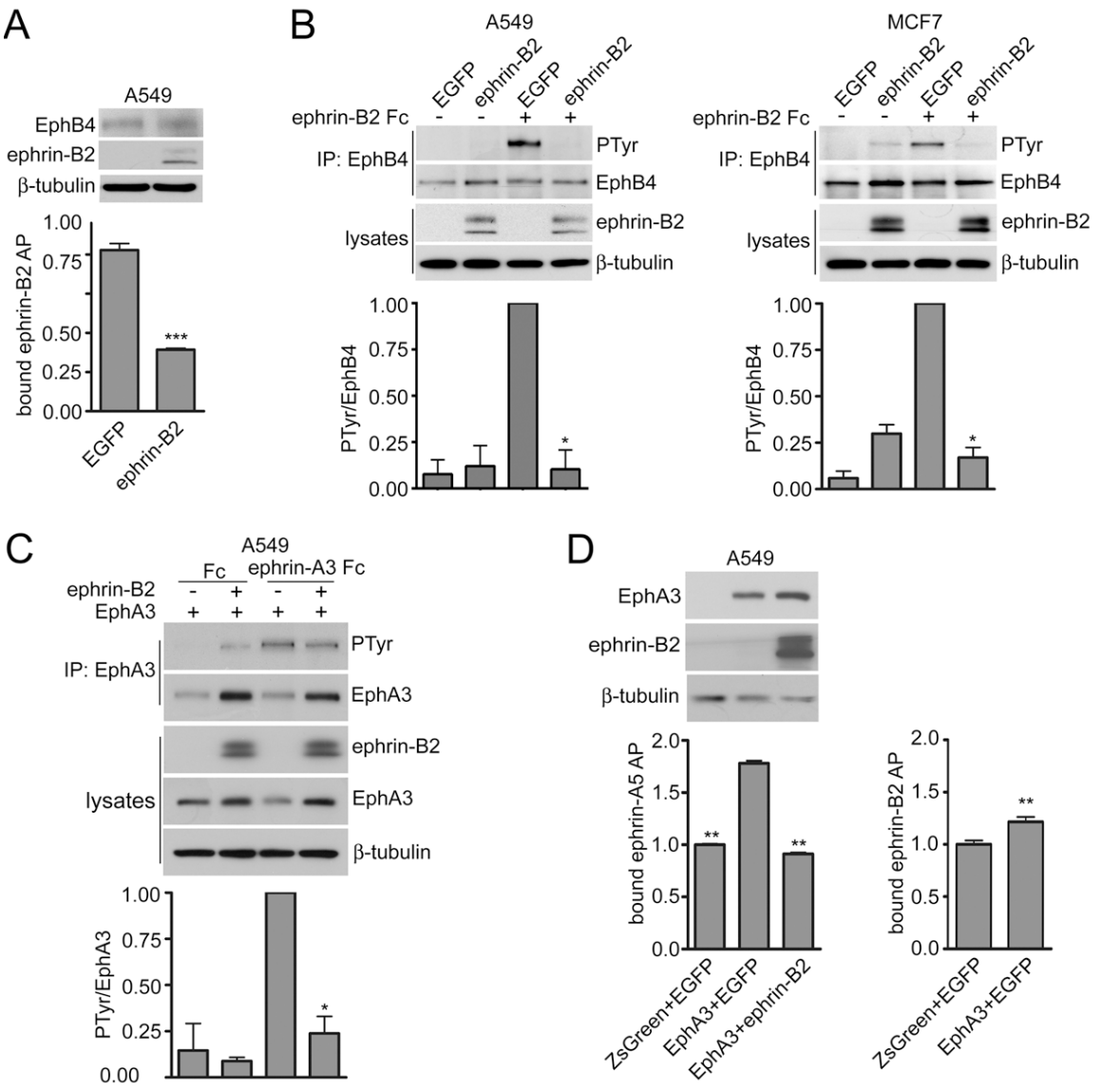


**Figure 5. Coexpressed ephrin-B2 attenuates EphB4 as well as EphA3 activation in cancer cells.** (A) The histogram shows the binding of ephrin-B2 AP to A549 cells infected with lentiviruses encoding EGFP-ephrin-B2 or EGFP, revealing that ephrin-B2 coexpression inhibits ephrin-B2 AP binding to EphB4. Normalized means from 3 experiments (each with triplicate samples) ± SE are shown. ***$p<0.001$ by unpaired t test for the comparison of cells expressing ephrin-B2 with cells not expressing ephrin-B2. The immunoblot of the cell lysates shows expression of EphB4, ephrin-B2 and β-tubulin as loading control. (B) A549 lung cancer cells and MCF7 breast cancer cells were infected with lentiviruses encoding EGFP-ephrin-B2 or EGFP. EphB4 immunoprecipitates were probed by immunoblotting for phosphotyrosine (PTyr) and reprobed for EphB4. Cell lysates were probed for ephrin-B2 with an anti-EGFP antibody and for β-tubulin as loading control. The histograms show normalized means ± SE quantified from 2 immunoblots for each cell line. *$p<0.05$ by one sample t test for the comparison of ephrin-B2 Fc-treated cells expressing ephrin-B2 with cells not expressing ephrin-B2. (C) A549 cells were infected with a lentivirus encoding

EphA3 and ZsGreen together with a lentivirus encoding EGFP-ephrin-B2 or EGFP only. Control cells were infected with lentiviruses encoding ZsGreen and EGFP. EphA3 immunoprecipitates were probed by immunoblotting for phosphotyrosine (PTyr) and reprobed for EphA3. Lysates were probed for ephrin-B2 with an anti-EGFP antibody as well as for EphA3 and for β-tubulin as loading control. The histogram shows normalized means ± SE quantified from 2 immunoblots. *p<0.05 by one sample t test for the comparison of ephrin-A3 Fc-treated cells expressing ephrin-B2 with cells not expressing ephrin-B2. (D) Ephrin-A5 AP binding to cell surface EphA3 is inhibited by ephrin-B2 coexpression. The histogram shows means ± SE from 3 experiments (each with triplicate samples) for the binding of ephrin-A5 AP or ephrin-B2 AP to the A549 cells used for the experiment in C. For ephrin-A5 binding, **p<0.01 by one-way ANOVA and Dunnett's post-hoc test for the comparison with cells expressing EphA3 and EGFP; for ephrin-B2 AP binding, **p<0.01 by unpaired t test for the comparison of cells expressing or not expressing EphA3. The immunoblot of the cell lysates shows expression of ephrin-B2, EphA3 and β-tubulin as loading control, verifying that ephrin-B2 coexpression did not reduce EphA3 levels. Of note, the doublet corresponding to overexpressed ephrin-B2 is not due to different degrees of N-linked glycosylation because removal of N-linked oligosaccharides with the PNGase-F endoglycosidase similarly increased the SDS-PAGE mobility of both bands (not shown). Whether the upper band may represent a form with O-linked oligosaccharides (Holen et al. 2011) or other posttranslational modification remains to be determined.

## 8.6- Endogenous ephrin-As attenuate activation of coexpressed EphA2 in cancer cells

To investigate whether ephrins endogenously expressed in cancer cells can also engage in cis interactions that inhibit the activation of coexpressed endogenous Eph receptors, we chose the SKBR3 and MCF7 breast cancer cell lines. These lines express high levels of ephrin-A ligands together with EphA2 (Macrae et al. 2005) (broadinstitute.org/ccle), although the receptor is expressed at relatively low levels, consistent with the complementary expression of Eph receptors and ephrins observed in many cancer cell lines (Pasquale 2010). Since both SKBR3 and MCF7 cells express multiple ephrin-A ligands, which are GPI-anchored, we used the enzyme phosphatidylinositol-specific phospholipase C (PI-PLC) to remove all ephrin-As from the cell surface. In both cell lines, removal of endogenous ephrin-As from the cell surface resulted in enhanced EphA2 activation by ephrin-A1 Fc in trans compared to untreated cells (Figure 6 A,B). In contrast, PI-PLC treatment of control Fc-treated cells decreased the low basal EphA2 activation, suggesting that endogenous ephrin-As can induce some EphA2 activation. Since ephrin-A1 has been reported to be cleaved from the surface of cancer cells by matrix metalloproteases, we also treated SKBR3 cells with the broad-spectrum matrix metalloprotease inhibitor GM-6001 (Bartley et al. 1994; Alford et al. 2010; Beauchamp et al. 2012). Treatment with the inhibitor for 24 hours further increased cell surface associated ephrin-A1. However, it did not substantially affect EphA2 tyrosine phosphorylation induced by ephrin-A1 Fc binding in trans, possibly due to already high *cis*-inhibition by the high levels of ephrin-A1 present even in the absence of GM-6001. Thus, in cancer cells cis interaction with endogenous ephrin-A ligands can attenuate

EphA2 activation by ephrin-As presented in trans, supporting the significance of cis interactions in cancer pathogenesis.

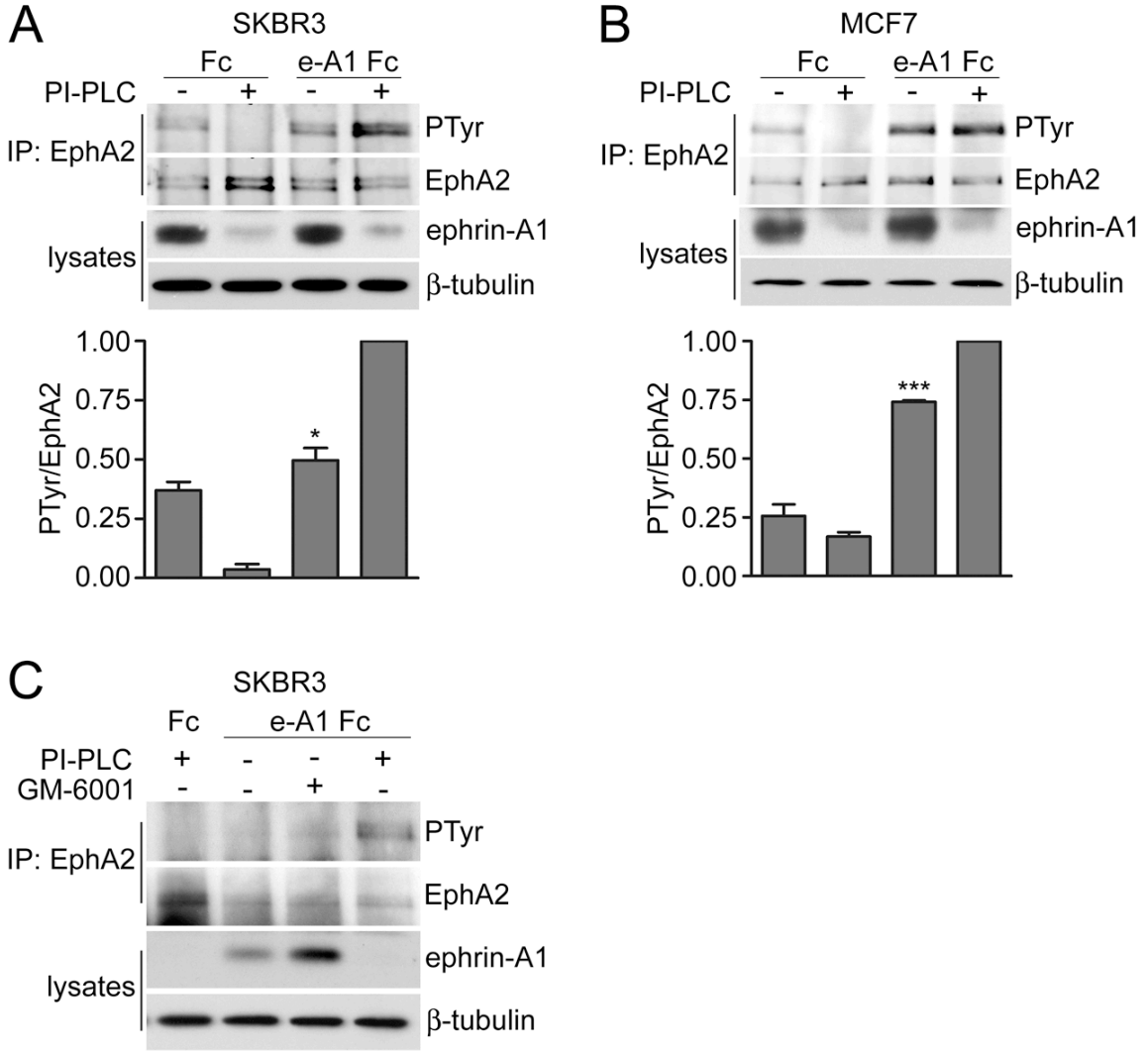


**Figure 6. Removal of endogenous ephrin-As from the cell surface potentiates EphA2 activation by soluble ephrin-A1 in trans.** (A) SKBR3 and (B) MCF7 breast cancer cells were treated with PI-PLC for 4 hours and then stimulated with ephrin-A1 Fc. EphA2 immunoprecipitates were probed by immunoblotting for phosphotyrosine (PTyr) and reprobed for EphA2. Lysates probed with anti-ephrin-A1 antibody verify removal of ephrin-As by PI-PLC; β-tubulin verifies equal loading of the lanes. The Odyssey LI-COR system was used for detection and the color images were converted to greyscale with Photoshop. The histograms show the normalized data from 3 different experiments *$p<0.05$ and ***$p<0.001$ by one sample t test for the comparison of ephrin-A1 Fc-stimulated cells treated or not with PI-PLC. (C) SKBR3 cells were treated with PI-PLC as in A or with the broad-spectrum matrix metalloprotease inhibitor GM-6001 for 24 hours. Immunoprecipitates and lysates were

probed as indicated.

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# 9- DISCUSSION

Different families of receptors and cell surface-associated ligands that together mediate juxtacrine signals by interacting in trans across cell-cell junctions can also, when coexpressed on the same cell surface, interact laterally in cis (Yaron and Sprinzak 2012). These cis interactions, which have been mostly studied in the nervous system and the immune system, typically attenuate the signals triggered by the trans interactions through mechanisms that in many cases are not well understood (Nitschke 2009; Ware and Sedy 2011; Yaron and Sprinzak 2012). Recent studies have uncovered key functional roles for inhibitory cis interactions between Eph receptors and ephrin ligands coexpressed in neurons (Hornberger et al. 1999; Yin et al. 2004; Carvalho et al. 2006; Antion et al. 2010; Kao and Kania 2011). However, despite the importance of the Eph/ephrin system in cancer pathogenesis, Eph receptor-ephrin cis interactions have not yet been investigated in cancer cells.

We have detected inhibitory cis interactions with ephrins in cancer cells not only for EphA3, which had been previously studied in neurons, but also for endogenous EphA2 and EphB4, for which the effects of cis interactions have not been previously investigated. Among the Eph receptors, EphA2 and EphB4 are the most widely expressed in epithelial and cancer cells, although most other Eph receptors including EphA3 are also aberrantly expressed in at least some cancers (Pasquale 2008; Pasquale 2010; Xi and Zhao 2011; Keane et al. 2012; Xi et al. 2012; Day et al. 2013). Cis interactions between coexpressed Eph receptors and ephrins may represent one of the strategies adopted by cancer cells to escape the tumor suppressing effects of Eph receptor signaling induced by ephrins binding in trans, including inhibition of cell growth and invasiveness (Clevers and Batlle 2006; Chen et al. 2008; Pasquale 2008; Astin et al. 2010; Pasquale 2010; Yang et al. 2011; Li et al. 2012).

We found that in cancer cells cis interactions can inhibit ephrin binding to Eph receptors in trans, consistent with previous studies in other systems (Yin et al. 2004; Kao and Kania 2011). This effect, which likely explains the observed inhibition of Eph receptor activation by ephrins *in* trans, could be due to different underlying mechanisms. We have shown that the levels of EphA3 on the cancer cell surface are not decreased by coexpression of ephrin-A3. We have also excluded occupancy of the EphA3 ligand-binding domain by ephrin-A3 that may be released into the medium by proteases (Beauchamp et al. 2012). Another possible mechanism by which cis interactions could lead to inhibition of the binding of soluble ephrins in trans could be by stabilizing the assembly of coexpressed Eph receptors and ephrins into lattice-like arrays that span cell-cell contacts and engage both cis and trans interfaces (Seiradake et al. 2010). However, we did not observe enrichment of EphA3 and ephrin-A3 in regions of cell-cell contact in A549 lung cancer cells coexpressing these proteins (not shown). Furthermore, coexpressed ephrins can block ephrin binding to Eph receptors in trans even in the absence of cell-cell contacts (Yin et al. 2004; Kao and Kania 2011). Taken together, these data suggest that ephrin binding in cis to the fibronectin type III domains of an Eph receptor may promote an additional cis interaction between the ephrin-binding pocket of the Eph receptor and the G-H loop of the ephrin. This may occur even when the second interaction is very weak, as in the case of EphA3 and ephrin-B2, since we found that

ephrin-B2 coexpression can prevent ephrin-A3 binding to EphA3 in trans. A contribution of the Eph receptor ligand-binding domain is also consistent with the trend towards a weaker cis association and the weaker attenuation of EphA receptor activation and functional effects observed when interaction between the Eph receptor ligand-binding domain and co-expressed ephrin is prevented (Fig. 6 and (Carvalho et al. 2006)). However, other possible mechanisms explaining the inhibitory effects of cis interactions on Eph receptor activation cannot be excluded, including allosteric conformational changes blocking access to the ephrin-binding pocket of the Eph receptor or intercalation of the ephrin preventing the receptor clustering needed for activation (Yin et al. 2004; Carvalho et al. 2006; Kao and Kania 2011; Yaron and Sprinzak 2012).

Previous studies have assumed that Eph receptor-ephrin cis interactions exhibit the same A or B class selectivity as trans interactions (Yin et al. 2004; Carvalho et al. 2006; Kao and Kania 2011). However, the fibronectin type III domains of an Eph receptor could conceivably bind a different subset of ephrins than the ligand-binding domain, particularly because the two Eph receptor regions also interact with distinct parts of the ephrins. Our data indeed show that coexpressed ephrin-B2 can strongly inhibit EphA3 interaction with ephrins in trans and tyrosine phosphorylation, even though this ephrin does not efficiently bind to the EphA3 ligand-binding domain (Flanagan and Vanderhaeghen 1998). However, we could not detect coimmunoprecipitation of ephrin-B2 with EphA3 (data not shown), suggesting that the cis association of EphA3 with ephrin-B2 may be weaker than with ephrin-A3 or ephrin-A5 (Figure 3) (Carvalho et al. 2006). Nevertheless, the *cis*-inhibition of EphA3 by ephrin-B2 suggests that in at least some cases ephrins that cannot activate a particular Eph receptor can instead inhibit its signaling ability through cis association. This represents a novel facet of Eph receptor-ephrin signaling and has functional implications in cancer cells, which can express multiple Eph receptors and ephrins of different classes (Stahl et al. 2011; Noberini et al. 2012b) (http://www.broadinstitute.org/ccle/). It will therefore be interesting to investigate the extent of these interclass cis interactions and whether this mechanism could explain some puzzling findings. For example, ephrin-B3 knockdown revealed that this ephrin increases EphA2 expression in the U-1810 lung cancer cell line (Stahl et al. 2011). Since ephrin-B3 is not an activating ligand for EphA2 (Flanagan and Vanderhaeghen 1998), an explanation for these findings could be that ephrin-B3 interacting in cis prevents EphA2 activation and degradation induced by ephrin-A1 in trans (Walker-Daniels et al. 2002; Stahl et al. 2011; Yang et al. 2011).

Studies in the nervous system have suggested that cis interactions are favored under conditions of high ephrin expression, which promotes colocalization of Eph receptors and ephrins in the same plasma membrane microdomains enabling their intermingling (Kao and Kania 2011). We indeed found that ephrin-A3 overexpressed in lung cancer cells can inhibit EphA2 and EphA3 activation by ephrins in trans while overexpression of ephrin-B2 can inhibit activation of EphA3 and EphB4. Importantly, ephrins endogenously expressed at high levels in cancer cells can also participate in inhibitory cis interactions, since removal of endogenous GPI-linked ephrin-As from the surface of SKBR3 and MCF7 breast cancer cells with PI-PLC allows increased activation of endogenous EphA2 by soluble ephrin-A1 in trans. In contrast, inhibiting the release of GPI-linked ephrin-As through inactivation of matrix metalloproteases in SKBR3 cells did not detectably affect EphA2 activation in trans by ephrin-A1 Fc under the conditions of

our experiments, presumably due to the already high levels of ephrin-A1 expressed in these cells. It will be interesting to determine whether in cancer cells with moderate ephrin-A levels, inhibiting matrix metalloproteases could enhance the inhibitory effect of cis interactions on EphA receptor signaling.

Some of the Eph receptor residues that are predicted to participate in cis interaction with ephrins have been reported to be mutated in cancer specimens (Seiradake et al. 2010). We found that the EphA3 G518L lung cancer mutation strengthens the cis association of EphA3 with coexpressed ephrin-A3. It will be interesting to examine whether other cancer mutations involving residues predicted to participate in the cis interface of other Eph receptors – such as EphA1 R337Q, EphB1 R327H and I332M, and EphB3 E358K in the first fibronectin type III domain as well as EphA5 G547S, EphA6 T493K and R494M, and EphA7 E482D in the second fibronectin type III domain (http://cancer.sanger.ac.uk/cosmic/) – also have functional consequences on cis associations with ephrins.

In summary, our data reveal a signaling mechanism previously uncharacterized in cancer cells whereby ephrin-mediated cis attenuation of Eph receptor signaling can inhibit responsiveness to ephrins expressed by other cancer cells or by cells of the tumor microenvironment. Further investigations of the selectivity and functional effects of Eph receptor-ephrin cis interactions will provide new information on Eph receptor signaling mechanisms in cancer pathogenesis, which may help the development of new therapeutic approaches (Falivelli et al. 2013).

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