Published in final edited form as:

Biochim Biophys Acta. 2013 October ; 1834(10): 2160–2165. doi:10.1016/j.bbapap.2013.04.020.

Eph/ephrin recognition and the role of Eph/ephrin clusters in signaling initiation

Dimitar B. Nikolov, Kai Xu, and Juha P. Himanen

Structural Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065

Abstract

The Eph receptors and their ephrin ligands play crucial roles in a large number of cell-cell interaction events, including those associated with axon pathfinding, neuronal cell migration and vasculogenesis. They are also involved in the patterning of most tissues and overall cell positioning in the development of the vertebrate body plan. The Eph/ephrin signaling system manifests several unique features that differentiate it from other receptor tyrosine kinases, including initiation of bi-directional signaling cascades and the existence of ligand and receptor subclasses displaying promiscuous intra-subclass interactions, but very rare inter-subclass interactions. In this review we briefly discuss these features and focus on recent studies of the unique and expansive high-affinity Eph/ephrin assemblies that form at the sites of cell-cell contact and are required for Eph signaling initiation.

1. Introduction

Eph receptors, the largest family of receptor tyrosine kinases (RTKs), and their ephrin ligands are membrane-anchored molecules that regulate cell-cell communications. Especially well studied is their participation in directing axon pathfinding and neuronal cell migration, but they also play important roles in many other cellular trafficking events, and have been referred to as the global positioning system of multicellular organisms (Fig. 1) [1–4]. There are several unique features of these molecules that differentiate them from other transmembrane RTKs. For example, the binding of ephrins to the extracellular part of Eph receptors not only activates their cytoplasmic tyrosine kinase domain [5], but also leads to the transduction of a reverse signal into the ephrin-bearing cell [6]. In addition, the Ephs and the ephrins are divided in A and B subclasses based on their affinities for each other and on sequence conservation [7]. With few exceptions, the ten different human EphA's promiscuously bind to and are activated by six A-ephrins while the EphB receptors (EphB1–B6) interact with three different B-ephrins (B1–B3) [8, 9]. The interaction of Ephs and ephrins leads to the formation of unique expansive high-affinity assemblies or clusters at the cell-cell contact regions, with the most prevalent signals generated from the interaction being repulsive. Because of this, special mechanisms exist to facilitate the disassembly of the Eph/ephrin clusters, thus allowing movement of the cells in opposing directions [10]. In this review we discuss the characteristics of the unique receptor/ligand clustering at both cellular and molecular level, and suggest directions for future studies of Eph/ephrin signaling initiation.

© 2013 Elsevier B.V. All rights reserved.

Correspondence: nikolovd@mskcc.org or himanenj@mskcc.org, Tel: 212-639-7884; Fax: 212-717-3135.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

2. Eph and ephrin clustering on the cell surface

Soon after the discovery of the first Eph receptor [11], it became clear that these are not activated in a similar way as other RTK's but require pre-clustered ligands to induce efficient down-stream signaling [12]. Prior to activation, the Eph receptors are loosely distributed on the cell surface and display minimal kinase activity, unless receptor expression levels are considerably elevated [13, 14]. After activation, Eph clusters appear fairly rapidly at discrete spots on cell surface, which can be monitored with confocal microscopy [8]. More recent studies document that on cell surface, ephrins localize to and are concentrated in membrane microdomains or rafts [15]. Thus, in contrast to other RTK's where receptor dimerization is enough to trigger biological activity, Eph receptors need high local density of ligands to induce downstream signaling. Moreover, the oligomerization state of the ligands seems to control not only the architecture of the receptor/ligand signaling assemblies, but also the precise downstream cellular responses [16].

There have been observations in the past on the existence of different Eph receptor members within the same receptor cluster [17]. Moreover, proteomic and transcriptomic studies on the regulation of Eph/ephrin signaling have shown a surprising phosphorylation of several different Eph receptors (both A and B class) by a single ligand, ephrin-B1 [18, 19], suggesting co-clustering. However, it was only recently confirmed that the Eph clusters can indeed contain various types of receptors that cross-activate each other [20]. In this elegant study, the authors used transfected HEK cells and glioblastoma cell lines that express high levels of both EphB2 and EphA3. They observed that an antibody that has an exquisite specificity for EphA3 can pull down EphB2, in addition to EphA3, documenting a physical interaction between these receptors. While similar results have been reported for other cell types, e.g. B-lymphocytes [21], Janes and co-authors were able to demonstrate a remarkable cross-class activation. They used a kinase-dead EphA3 receptor in COS7 and HEK293 cells that was shown to get phosphorylated when the cells were activated by an EphA3-specific antibody, thus showing a cooperative signaling via the endogenous EphB2. The level of phosphorylation was also correlated with changes in cell morphology. Moreover, the relative expression levels of the mutant EphA3 and wild-type EphB2 determined whether the receptors get activated or inhibited. Such studies can have a great impact on our understanding of Eph signaling and potential therapeutic applications in light of the the varied expression levels of different receptors in different tumor cells.

3. Eph- and ephrin-associated proteins

To fully understand the receptor clustering as an activation mechanism, it is also crucially important to obtain a comprehensive picture of all other molecules associated with the Eph/ephrin clusters potentially influencing the downstream signaling. Recent studies have revealed interactions of Ephs with several other RTKs and non-RTK receptors. These include the EGF [22], FGF [23], and VEGF [24] receptors, the chemokine receptor CXCR4 [25], and the NMDA receptors [26]. Many of these molecules are, however, not well studied in terms of their role in the formation of cell surface Eph receptor assemblies, but rather, at the level of expression profiles. Nevertheless, these studies have revealed potentiation of FGFR-mediated downstream signaling by the EphA4/FGFR1 complex [23], correlation between the EphA2/VEGF interaction and tumor angiogenesis [24], participation of EphB2 and EphB4 in the SDF-1-induced endothelial cell movement and assembly into cord-like structures [25], and decrease of Ca-dependent desensitization of synaptic NMDA receptors by EphB2 [27]. One well-studied membrane-tethered molecule involved in Eph signaling is ADAM10, a member of the transmembrane metalloproteinases that cleave a variety of proteins off cell surfaces [28, 29]. ADAM10 specifically cleaves the A-class ephrins [30] allowing termination of EphA signaling. A tight interaction of ADAM10 with the Eph

receptor assemblies is promoted by ephrin-binding, provoking conformational changes that position the ADAM protease for efficient ligand cleavage [31], thus allowing the de-adhesion of Eph- and ephrin-containing cells (Fig. 2). Ephrin binding also changes the conformation of the cytoplasmic domain of EphA receptors, extending it away from the cell membrane and promoting association of ADAM10 with the Eph/ephrin complexes [32]. The involvement of other members of the metalloproteinase family in similar interactions with Ephs or ephrins has also been suggested [33–36].

The exact role of dephosphorylation in RTK activation or Eph receptor clustering is not yet fully understood and only few Eph- or ephrin-specific protein tyrosine phosphatases (PTPs) have been identified. After engagement of Eph receptors, ephrin-B ligands were shown to recruit cytoplasmic PTP-BL that causes ephrin dephosphorylation [37]. LMW-PTP, on the other hand, acts as a terminator of EphA2 signaling by controlling the net level of its tyrosine phosphorylation and, hence, negatively regulating the ephrin-A1-mediated cell proliferation and adhesion [38]. Tyrosine phosphatase receptors type O (Ptpro) also control the phosphorylation status of both EphA and EphB receptors, as well as the threshold response of Eph receptors and the sensitivity of retinal axons to ephrins [39]. The phosphatases Slingshot1 (SSH1) and calcineurin/protein phosphatase 2B (PP2B) are involved in EphA-dependent actin filament depolymerization and F-actin distribution in dendritic spines [40]. Taking into consideration that the kinase domain of EphA4 is partially active in solution even without ligand-induced activation [41], the cellular control mechanisms utilize PTPs to ensure that the kinases are not overly active, potentially causing cancerous and other aberrant cellular responses. In support of this, it was recently shown that changes in PTP1B expression affect the cell surface concentration of EphA3 and the level and duration of its phosphorylation and signaling [42]. In addition, images from Fluorescence Lifetime Microscopy (FLIM) have shown a direct interaction between PTP1B and EphA3 prior to ligand-stimulation [42]. These observations confirm that the PTPs regulate Eph/ephrin signaling and might be important for controlling the formation or dissolution of the receptor/ligand clusters.

4. Eph-ephrin recognition

Although the cell-biological and imaging experiments discussed above have confirmed the necessity of ordered Eph/ephrin clusters for signaling, until recently we had a relatively poor picture of the molecular level details of Eph/ephrin recognition and binding. However, since the publication of the first crystal structure of an Eph/ephrin complex [43], there has been a string of papers that have elucidated many of the specific molecular characteristics of these receptor/ligand interactions and assemblies.

Unlike other RTK, Eph receptors bind their ligands with a 2:2, stoichiometry [44]. A 'classical' RTK receptor/ligand system usually has a 2:1 stoichiometry where one ligand (or a compact ligand dimer) pulls two receptor molecules in close contact to initiate the cross-receptor phosphorylation and down-stream signaling [45–48]. The first structure of an Eph/ephrin complex [43], however, revealed a uni-molar binding mode and a circular tetrameric configuration for a signaling competent complex. In the crystals, the EphB2/ephrin-B2 complex utilized two interacting surfaces, one responsible for the initial 1:1 heterodimer formation, the second one causing the formation of a 2:2 tetrameric receptor/ligand assembly. The initial recognition and binding is centered around a hydrophobic cavity on the Eph surface where a long ephrin loop penetrates and drives complex formation. Later studies with the minimal interaction domains of other B/B, A/A, or A/B type Eph/ephrin complexes have revealed only minor differences in this initial binding pattern. However, none of these other complexes have shown similar 2:2 circular tetramers as the EphB2/ephrin-B2 complex [8, 49–52].

5. Structural features of the complete Eph receptor ectodomains

Recent structures on the entire ectodomains (ECD) of Eph receptors have revealed structural details explaining aspects of the Eph receptor clustering [53] that are essential for signaling. In essence, these studies showed that the Eph receptors use two separate interfaces for the assembly of signaling-competent clusters (Fig. 3) [54, 55]. The first one, within the ligand-binding domain, is responsible for the formation of receptor dimers (thus called 'dimerization' interface). This interface can be present even in the unbound receptors, as also seen in the earlier dimeric structure of the EphA2-LBD [49]. The other interface is within the cys-rich domain of the Eph molecules and is responsible for the formation of the higher-order assemblies. Thus, it is referred to as 'clustering interface'. The existence of these two separate and independent interacting interfaces allows the Eph receptors to utilize a so-called 'seeding' mechanism for the assembly of clusters. Once two receptors are in ligand-induced close contact, they can independently use the 'clustering' interface to bind other receptor dimers (or 2:2 Eph/ephrin complexes), thus forming a 'trimer of receptor dimers'. This process would then go on until large-sized assemblies, consisting of hundreds of receptors, are formed. The precise size or composition of these assemblies, however, is still unknown. The role of the ligand seems to be to increase the local concentration of the receptors on cell surface above the critical value for efficient clustering. It is worth mentioning that the existence of these two interaction interfaces also in the crystals of unbound Eph ectodomain could explain earlier observations that the recruitment of Eph molecules into receptor clusters does not necessarily require contact with ephrins [56]. Ligand-independent signaling can also occur if the receptor concentration is high enough, e.g. on the surface of tumor cells.

A recent X-ray structure of an EphA4-ECD reveals how unexpected intra-molecular interactions can influence receptor assemblies (Xu et al., unpublished). Thus, while the EphA4-ECD in complex with ephrin-A5 forms similar heteromeric assemblies as the EphA2-ECD/ephrin-A5 complex discussed above, when not bound to a ligand it forms head-to-tail homotypic complexes (Fig. 4). In these complexes, the ligand-binding domain of one EphA4-ECD molecule binds the FNIII-domain of a neighboring receptor molecule, thus forming Eph assemblies independent of ephrin binding. It is tempting to speculate that the head-to-tail interactions between Eph molecules represent another example of the subtle ways Eph/ephrin signaling is controlled that maybe related to earlier findings of co-expression of ephrin ligands and Eph receptors on the same cell. Although controversial conclusions have been drawn on the exact role of co-expressed ephrins and Ephs [15, 57], it is fairly well established by now that, at least on the cell surface of some axons, Ephs and ephrins are not only co-expressed but can also interact in *cis*, regulating Eph receptor phosphorylation [58] and pathway selection by spinal motor axons [59]. More structural studies are needed to confirm the exact binding mode of these in-*cis* interactions [57, 58]. These findings further emphasize the importance of Eph clustering and suggest that cells might have more delicate ways to control the size of the receptor assemblies than we thus far have appreciated [60, 61].

6. Eph/ephrin binding interfaces as drug targets

Because many Eph receptors are either upregulated or mutated in various tumors and because there is a growing evidence for their functional role in cancer proliferation and metastasis [62–64], there is a wide interest in targeting the Eph clusters for therapeutic purposes [65, 66]. Recent studies have also suggested multiple outcomes of the Eph/ephrin signaling, which can sometimes promote cell-cell adhesion and sometimes increase cytoskeletal collapse, and how these opposing responses are likely altered in cancers [67]. Several studies have shown that small organic molecules or peptides can either agonize or

antagonize Eph signaling [68–72] by selectively targeting the initial Eph/ephrin recognition interface. These findings are very intriguing in terms of Eph/ephrin-related therapeutics, since Eph receptors can act as tumor suppressors in normal tissues but are potentially converted into oncogenic proteins in tumor cells [73]. Considering the recent structural studies on the importance of higher-order receptor oligomers in Eph signaling [54, 55], we anticipate that the Eph-Eph interfaces responsible for Eph/ephrin clustering could also represent attractive targets for therapeutics. Further studies on these and other promising Eph/ephrin modulators are impeded by the fact that there are currently no crystal or EM structures or high-resolution cellular images available of the full-length transmembrane Eph receptors or their higher-order assemblies either in solution or on cell surface. It is also intriguing that the higher-order Eph assemblies can be modulated by Eph-specific antibodies [74]. A pre-clustered EphA3-specific antibody is able to trigger EphA3 conformations that promote the formation of signaling-competent receptor/ligand complexes. It would be interesting to see if such antibodies could be developed into drugs [75].

7. Producing full-length Eph receptors

Although the studies outlined above have given us a fairly comprehensive structural and biochemical understanding on the initial steps of Eph/ephrin recognition and formation of receptor/ligand assemblies, high-resolution structural and architectural information for the full length Eph receptors are needed to fully understand their signaling properties. The progress in this direction has been hampered by the lack of efficient expression systems for producing milligram amounts of full-length, transmembrane(TM)-containing RTK receptors that are needed for structural studies.

A relatively new technology, Expressed Protein Ligation (EPL), offers an alternative way to tackle the problem of receptor expression. This semi-synthesis technique has been previously used for the synthesis of proteins that are difficult to obtain using purely recombinant methods [76–78]. Though EPL has been applied successfully for many intracellular proteins [79, 80], its application to transmembrane proteins with large multi-domain extracellular regions was documented only recently. A major technical difficulty comes from the fact that oxidizing conditions are needed to maintain the disulfide-containing extracellular regions in their correctly folded state, while thiol molecules with reducing properties are required for the semi-synthesis. However, EPL technology now exists that can be used for the production of full-length Eph receptor kinases [41, 81]. Using this approach, milligram amounts of a functional Eph RTK, containing its complete ECD and cytoplasmic domain (CPD), but lacking the TM segment, has been generated [41]. According to the general principals of EPL, the authors separately produced several Eph ECD constructs containing a reactive C-terminal thioester group and Eph kinase domains containing an N-terminal cysteine residue. Upon mixing, these two preparations undergo a spontaneous, native ligation to produce a semisynthetic ECD-CPD receptor (Fig. 5).

The semisynthetic Eph receptor was used to study the ligand-induced phosphorylation of its cytoplasmic domain. A sequential and ordered auto-phosphorylation process was documented where the C-terminal juxta-membrane segment (JMS) tyrosine is always phosphorylated first, followed by the N-terminal JMS tyrosine and, finally, by the activation loop tyrosine [41]. This is in contrast with observations for most other RTKs where the kinase activation loop is phosphorylated first. The observed phosphorylation time-dependences, coupled with site-directed mutagenesis, further revealed that the Eph kinase activity directly correlates with the phosphorylation status of the JMS, and in particular with the C-terminal JMS tyrosine, and not that of the activation loop tyrosine. This is also in contrast to the accepted view for other characterized RTKs and might be related to the prerequisite of clustering associated with Eph activation. Such semisynthetic protein

production method has potential applications for reconstituting many other families of single-pass transmembrane proteins.

Although EPL was used to generate milligram amounts of a functional Eph RTK, it still contains only the complete extracellular and intracellular regions, but lacks the transmembrane segment. Thus, it would be necessary to next generate Ephs containing also the TM region. This could be achieved by further extending the EPL method [76, 82]. A similar approach was recently used for a total chemical synthesis of an *E. coli* integral membrane enzyme, diacylglycerol kinase in detergent micelles [83].

8. Future directions

The cellular and structural studies of Eph/ephrin recognition and receptor clustering discussed above have made it clear that a more interdisciplinary approach is needed to understand the precise molecular events associated with Eph signaling initiation. This could be achieved by either reconstituting the receptor clusters on controlled lipid vesicles or by obtaining high-resolution images on the cell surface. The first approach requires further developments in producing biologically active full-length receptors. EPL, for example, has shown great promise in this respect. Reconstitution of membrane proteins has earlier been done successfully for other molecules, including multidrug transporters [84], ion channels and porins [85–87], the Gag protein of HIV-1 [88], and SNARE proteins [89]. Similar approaches should be tested for Eph receptors once enough purified full-length protein is available. The reconstituted Eph-vesicles would offer great advantages since the size, density, and the composition of the clusters could be easily controlled. This would provide major insight into the Eph phosphorylation and activation mechanisms.

Thus far, cell-surface imaging studies of Eph receptors and their higher-order assemblies have been performed with methods that can produce only modest resolution. This is somewhat surprising taking into consideration that an elegant study using a higher-resolution TIRF (Total Internal Reflection Fluorescence) imaging illustrated how to measure lateral movement of EphA2 receptors and to observe receptor-ligand binding and clustering on human breast cancer cells [90]. This study showed that the physical reorganization of EphA2 on the cell surface involves changes in the cytoskeleton morphology and revealed a mechanism for spatiomechanical regulation of the Eph signaling pathways. Surely, we expect to see more studies on Eph/ephrin signaling assemblies using live cells and similar high-resolution imaging devices, such as atomic force microscopy (AFM) or simulated emission depletion (STED) microscopy. These techniques would complement the high-resolution structural studies performed with isolated protein molecules, in a way similar to how cryo electron microscopy is nowadays commonly used to supplement traditional biochemical and cell biological studies in e.g. virus research [91, 92].

Acknowledgments

We thank all members of our laboratory for their work on Eph receptors and ephrins. This research was supported by a grant from the National Institutes of Health RO1NS038486 to D.B.N.

References

1. Flanagan JG, Vanderhaeghen P. The ephrins and Eph receptors in neural development. *Annu Rev Neurosci.* 1998; 21:309–345. [PubMed: 9530499]
2. Frisen J, Holmberg J, Barbacid M. Ephrins and their Eph receptors: multitasking directors of embryonic development. *The EMBO journal.* 1999; 18:5159–5165. [PubMed: 10508149]
3. Kullander K, Klein R. Mechanisms and functions of EPH and ephrin signalling. *Nat Rev Mol Cell Bio.* 2002; 3:475–486. [PubMed: 12094214]

4. Lackmann M, Boyd AW. Eph, a protein family coming of age: more confusion, insight, or complexity? *Sci Signal*. 2008; 1:re2. [PubMed: 18413883]
5. Wilkinson DG. Multiple roles of EPH receptors and ephrins in neural development, *Nature reviews. Neuroscience*. 2001; 2:155–164. [PubMed: 11256076]
6. Holland SJ, Gale NW, Mbamalu G, Yancopoulos GD, Henkemeyer M, Pawson T. Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands. *Nature*. 1996; 383:722–725. [PubMed: 8878483]
7. Gale NW, Holland SJ, Valenzuela DM, Flenniken A, Pan L, Ryan TE, Henkemeyer M, Strebhardt K, Hirai H, Wilkinson DG, Pawson T, Davis S, Yancopoulos GD. Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. *Neuron*. 1996; 17:9–19. [PubMed: 8755474]
8. Himanen JP, Chumley MJ, Lackmann M, Li C, Barton WA, Jeffrey PD, Vearing C, Geleick D, Feldheim DA, Boyd AW, Henkemeyer M, Nikolov DB. Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling. *Nat Neurosci*. 2004; 7:501–509. [PubMed: 15107857]
9. Himanen JP. Ectodomain structures of Eph receptors. *Seminars in cell & developmental biology*. 2012; 23:35–42. [PubMed: 22044883]
10. Mancia F, Shapiro L. ADAM and Eph: how Ephrin-signaling cells become detached. *Cell*. 2005; 123:185–187. [PubMed: 16239135]
11. Hirai H, Maru Y, Hagiwara K, Nishida J, Takaku F. A novel putative tyrosine kinase receptor encoded by the eph gene. *Science*. 1987; 238:1717–1720. [PubMed: 2825356]
12. Davis S, Gale NW, Aldrich TH, Maisonpierre PC, Lhotak V, Pawson T, Goldfarb M, Yancopoulos GD. Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity. *Science*. 1994; 266:816–819. [PubMed: 7973638]
13. Noren NK, Yang NY, Silldorff M, Mutyala R, Pasquale EB. Ephrin-independent regulation of cell substrate adhesion by the EphB4 receptor. *The Biochemical journal*. 2009; 422:433–442. [PubMed: 19552627]
14. Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. *Cell*. 2008; 133:38–52. [PubMed: 18394988]
15. Marquardt T, Shirasaki R, Ghosh S, Andrews SE, Carter N, Hunter T, Pfaff SL. Coexpressed EphA receptors and ephrin-A ligands mediate opposing actions on growth cone navigation from distinct membrane domains. *Cell*. 2005; 121:127–139. [PubMed: 15820684]
16. Stein E, Lane AA, Cerretti DP, Schoecklmann HO, Schroff AD, Van Etten RL, Daniel TO. Eph receptors discriminate specific ligand oligomers to determine alternative signaling complexes, attachment, and assembly responses. *Genes Dev*. 1998; 12:667–678. [PubMed: 9499402]
17. Freywald A, Sharfe N, Roifman CM. The kinase-null EphB6 receptor undergoes transphosphorylation in a complex with EphB1. *J Biol Chem*. 2002; 277:3823–3828. [PubMed: 11713248]
18. Bush JO, Soriano P. Ephrin-B1 forward signaling regulates craniofacial morphogenesis by controlling cell proliferation across Eph-ephrin boundaries. *Genes Dev*. 2010; 24:2068–2080. [PubMed: 20844017]
19. Bush JO, Soriano P. Eph/ephrin signaling: genetic, phosphoproteomic, and transcriptomic approaches. *Seminars in cell & developmental biology*. 2012; 23:26–34. [PubMed: 22040918]
20. Janes PW, Griesshaber B, Atapattu L, Nievergall E, Hii LL, Mensinga A, Chheang C, Day BW, Boyd AW, Bastiaens PI, Jorgensen C, Pawson T, Lackmann M. Eph receptor function is modulated by heterooligomerization of A and B type Eph receptors. *J Cell Biol*. 2011; 195:1033–1045. [PubMed: 22144690]
21. Oricchio E NG, Wolfe AL, Schatz JH, Mavrakis KJ, Jiang M, Liu X, Bruno J, Heguy A, Olshen AB, Socci ND, Teruya-Feldstein J, Weis-Garcia F, Tam W, Shaknovich R, Melnick A, Himanen JP, Chaganti RSK, Wendel H-G. The Eph-Receptor A7 Is A Soluble Tumor Suppressor And Therapeutic Reagent Against Follicular Lymphoma. *Cell*. 2011
22. Komurov K, Padron D, Cheng T, Roth M, Rosenblatt KP, White MA. Comprehensive mapping of the human kinome to epidermal growth factor receptor signaling. *J Biol Chem*. 2010; 285:21134–21142. [PubMed: 20421302]

23. Fukai J, Yokote H, Yamanaka R, Arao T, Nishio K, Itakura T. EphA4 promotes cell proliferation and migration through a novel EphA4-FGFR1 signaling pathway in the human glioma U251 cell line. *Molecular cancer therapeutics*. 2008; 7:2768–2778. [PubMed: 18790757]
24. Shao Z, Zhang WF, Chen XM, Shang ZJ. Expression of EphA2 and VEGF in squamous cell carcinoma of the tongue: correlation with the angiogenesis and clinical outcome. *Oral oncology*. 2008; 44:1110–1117. [PubMed: 18485799]
25. Salvucci O, de la Luz Sierra M, Martina JA, McCormick PJ, Tosato G. EphB2 and EphB4 receptors forward signaling promotes SDF-1-induced endothelial cell chemotaxis and branching remodeling. *Blood*. 2006; 108:2914–2922. [PubMed: 16840724]
26. Dalva MB, Takasu MA, Lin MZ, Shamah SM, Hu L, Gale NW, Greenberg ME. EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. *Cell*. 2000; 103:945–956. [PubMed: 11136979]
27. Nolt MJ, Lin Y, Hruska M, Murphy J, Sheffler-Colins SI, Kayser MS, Passer J, Bennett MV, Zukin RS, Dalva MB. EphB controls NMDA receptor function and synaptic targeting in a subunit-specific manner. *J Neurosci*. 2011; 31:5353–5364. [PubMed: 21471370]
28. White JM. ADAMs: modulators of cell-cell and cell-matrix interactions. *Curr Opin Cell Biol*. 2003; 15:598–606. [PubMed: 14519395]
29. Edwards DR, Handsley MM, Pennington CJ. The ADAM metalloproteinases. *Molecular aspects of medicine*. 2008; 29:258–289. [PubMed: 18762209]
30. Hattori M, Osterfield M, Flanagan JG. Regulated cleavage of a contact-mediated axon repellent. *Science*. 2000; 289:1360–1365. [PubMed: 10958785]
31. Janes PW, Saha N, Barton WA, Kolev MV, Wimmer-Kleikamp SH, Nievergall E, Blobel CP, Himanen JP, Lackmann M, Nikolov DB. Adam meets Eph: an ADAM substrate recognition module acts as a molecular switch for ephrin cleavage in trans. *Cell*. 2005; 123:291–304. [PubMed: 16239146]
32. Janes PW, Wimmer-Kleikamp SH, Frangakis AS, Treble K, Griesshaber B, Sabet O, Grabenbauer M, Ting AY, Saftig P, Bastiaens PI, Lackmann M. Cytoplasmic relaxation of active Eph controls ephrin shedding by ADAM10. *PLoS biology*. 2009; 7:e1000215. [PubMed: 19823572]
33. Yumoto N, Wakatsuki S, Kurisaki T, Hara Y, Osumi N, Frisen J, Sehara-Fujisawa A. Meltrin beta/ADAM19 interacting with EphA4 in developing neural cells participates in formation of the neuromuscular junction. *PLoS One*. 2008; 3:e3322. [PubMed: 18830404]
34. Lin KT, Sloniowski S, Ethell DW, Ethell IM. Ephrin-B2-induced cleavage of EphB2 receptor is mediated by matrix metalloproteinases to trigger cell repulsion. *J Biol Chem*. 2008; 283:28969–28979. [PubMed: 18713744]
35. Wei S, Xu G, Bridges LC, Williams P, White JM, DeSimone DW. ADAM13 induces cranial neural crest by cleaving class B Ephrins and regulating Wnt signaling. *Dev Cell*. 2010; 19:345–352. [PubMed: 20708595]
36. Solanas G, Cortina C, Sevillano M, Battle E. Cleavage of E-cadherin by ADAM10 mediates epithelial cell sorting downstream of EphB signalling. *Nat Cell Biol*. 2011; 13:1100–1107. [PubMed: 21804545]
37. Palmer A, Zimmer M, Erdmann KS, Eulenburg V, Porthin A, Heumann R, Deutsch U, Klein R. EphrinB phosphorylation and reverse signaling: regulation by Src kinases and PTP-BL phosphatase. *Molecular cell*. 2002; 9:725–737. [PubMed: 11983165]
38. Parri M, Buricchi F, Taddei ML, Giannoni E, Raugei G, Ramponi G, Chiarugi P. EphrinA1 repulsive response is regulated by an EphA2 tyrosine phosphatase. *J Biol Chem*. 2005; 280:34008–34018. [PubMed: 16051609]
39. Shintani T, Ihara M, Sakuta H, Takahashi H, Watakabe I, Noda M. Eph receptors are negatively controlled by protein tyrosine phosphatase receptor type O. *Nat Neurosci*. 2006; 9:761–769. [PubMed: 16680165]
40. Zhou L, Jones EV, Murai KK. EphA signaling promotes actin-based dendritic spine remodeling through slingshot phosphatase. *J Biol Chem*. 2012; 287:9346–9359. [PubMed: 22282498]
41. Singla N, Erdjument-Bromage H, Himanen JP, Muir TW, Nikolov DB. A semisynthetic Eph receptor tyrosine kinase provides insight into ligand-induced kinase activation. *Chem Biol*. 2011; 18:361–371. [PubMed: 21439481]

42. Nievergall E, Janes PW, Stegmayer C, Vail ME, Haj FG, Teng SW, Neel BG, Bastiaens PI, Lackmann M. PTP1B regulates Eph receptor function and trafficking. *J Cell Biol.* 2010; 191:1189–1203. [PubMed: 21135139]
43. Himanen JP, Rajashankar KR, Lackmann M, Cowan CA, Henkemeyer M, Nikolov DB. Crystal structure of an Eph receptor-ephrin complex. *Nature.* 2001; 414:933–938. [PubMed: 11780069]
44. Himanen JP, Nikolov DB. Purification, crystallization and preliminary characterization of an Eph-B2/ephrin-B2 complex, *Acta crystallographica. Section D. Biological crystallography.* 2002; 58:533–535. [PubMed: 11856847]
45. Schlessinger J. Signal transduction. Autoinhibition control. *Science.* 2003; 300:750–752. [PubMed: 12730587]
46. Hubbard SR, Miller WT. Receptor tyrosine kinases: mechanisms of activation and signaling. *Curr Opin Cell Biol.* 2007; 19:117–123. [PubMed: 17306972]
47. Hunter T. Tyrosine phosphorylation: thirty years and counting. *Curr Opin Cell Biol.* 2009; 21:140–146. [PubMed: 19269802]
48. Hunter T. Why nature chose phosphate to modify proteins, *Philosophical transactions of the Royal Society of London. Series B. Biological sciences.* 2012; 367:2513–2516. [PubMed: 22889903]
49. Himanen JP, Goldgur Y, Miao H, Myshkin E, Guo H, Buck M, Nguyen M, Rajashankar KR, Wang B, Nikolov DB. Ligand recognition by A-class Eph receptors: crystal structures of the EphA2 ligand-binding domain and the EphA2/ephrin-A1 complex. *EMBO Rep.* 2009; 10:722–728. [PubMed: 19525919]
50. Chrencik JE, Brooun A, Kraus ML, Recht MI, Kolatkar AR, Han GW, Seifert JM, Widmer H, Auer M, Kuhn P. Structural and biophysical characterization of the EphB4*ephrinB2 protein-protein interaction and receptor specificity. *J Biol Chem.* 2006; 281:28185–28192. [PubMed: 16867992]
51. Bowden TA, Aricescu AR, Nettleship JE, Siebold C, Rahman-Huq N, Owens RJ, Stuart DI, Jones EY. Structural plasticity of eph receptor A4 facilitates cross-class ephrin signaling. *Structure.* 2009; 17:1386–1397. [PubMed: 19836338]
52. Qin H, Noberini R, Huan X, Shi J, Pasquale EB, Song J. Structural characterization of the EphA4-Ephrin-B2 complex reveals new features enabling Eph-ephrin binding promiscuity. *J Biol Chem.* 2010; 285:644–654. [PubMed: 19875447]
53. Smith FM, Vearing C, Lackmann M, Treutlein H, Himanen J, Chen K, Saul A, Nikolov D, Boyd AW. Dissecting the EphA3/Ephrin-A5 interactions using a novel functional mutagenesis screen. *J Biol Chem.* 2004; 279:9522–9531. [PubMed: 14660665]
54. Himanen JP, Yermekbayeva L, Janes PW, Walker JR, Xu K, Atapattu L, Rajashankar KR, Mensinga A, Lackmann M, Nikolov DB, Dhe-Paganon S. Architecture of Eph receptor clusters. *Proc Natl Acad Sci U S A.* 2010; 107:10860–10865. [PubMed: 20505120]
55. Seiradake E, Harlos K, Sutton G, Aricescu AR, Jones EY. An extracellular steric seeding mechanism for Eph-ephrin signaling platform assembly. *Nat Struct Mol Biol.* 2010; 17:398–402. [PubMed: 20228801]
56. Wimmer-Kleikamp SH, Janes PW, Squire A, Bastiaens PI, Lackmann M. Recruitment of Eph receptors into signaling clusters does not require ephrin contact. *J Cell Biol.* 2004; 164:661–666. [PubMed: 14993233]
57. Yin Y, Yamashita Y, Noda H, Okafuji T, Go MJ, Tanaka H. EphA receptor tyrosine kinases interact with co-expressed ephrin-A ligands in cis. *Neuroscience research.* 2004; 48:285–296. [PubMed: 15154674]
58. Carvalho RF, Beutler M, Marler KJ, Knoll B, Becker-Barroso E, Heintzmann R, Ng T, Drescher U. Silencing of EphA3 through a cis interaction with ephrinA5. *Nat Neurosci.* 2006; 9:322–330. [PubMed: 16491080]
59. Kao TJ, Kania A. Ephrin-mediated cis-attenuation of Eph receptor signaling is essential for spinal motor axon guidance. *Neuron.* 2011; 71:76–91. [PubMed: 21745639]
60. Egea J, Klein R. Bidirectional Eph-ephrin signaling during axon guidance. *Trends Cell Biol.* 2007; 17:230–238. [PubMed: 17420126]
61. Dudanova I, Klein R. The axon's balancing act: cis- and trans-interactions between Ephs and ephrins. *Neuron.* 2011; 71:1–3. [PubMed: 21745632]

62. Chen J. Regulation of tumor initiation and metastatic progression by Eph receptor tyrosine kinases. *Advances in cancer research*. 2012; 114:1–20. [PubMed: 22588054]
63. Miao H, Wang B. EphA receptor signaling--complexity and emerging themes. *Seminars in cell & developmental biology*. 2012; 23:16–25. [PubMed: 22040915]
64. Herath NI, Boyd AW. The role of Eph receptors and ephrin ligands in colorectal cancer, *International journal of cancer. Journal international du cancer*. 2010; 126:2003–2011. [PubMed: 20039322]
65. Nievergall E, Saunders T, Lackmann M. Targeting of EPH receptor tyrosine kinases for anticancer therapy. *Critical reviews in oncogenesis*. 2012; 17:211–232. [PubMed: 22471709]
66. Pasquale EB. Eph receptors and ephrins in cancer: bidirectional signalling and beyond. *Nat Rev Cancer*. 2010; 10:165–180. [PubMed: 20179713]
67. Nievergall E, Lackmann M, Janes PW. Eph-dependent cell-cell adhesion and segregation in development and cancer. *Cellular and molecular life sciences : CMLS*. 2012; 69:1813–1842. [PubMed: 22204021]
68. Noberini R, Koolpe M, Peddibhotla S, Dahl R, Su Y, Cosford ND, Roth GP, Pasquale EB. Small molecules can selectively inhibit ephrin binding to the EphA4 and EphA2 receptors. *J Biol Chem*. 2008; 283:29461–29472. [PubMed: 18728010]
69. Murai KK, Nguyen LN, Koolpe M, McLennan R, Krull CE, Pasquale EB. Targeting the EphA4 receptor in the nervous system with biologically active peptides. *Mol Cell Neurosci*. 2003; 24:1000–1011. [PubMed: 14697664]
70. Koolpe M, Burgess R, Dail M, Pasquale EB. EphB receptor-binding peptides identified by phage display enable design of an antagonist with ephrin-like affinity. *J Biol Chem*. 2005; 280:17301–17311. [PubMed: 15722342]
71. Petty A, Myshkin E, Qin H, Guo H, Miao H, Tochtrop GP, Hsieh JT, Page P, Liu L, Lindner DJ, Acharya C, MacKerell AD Jr, Ficker E, Song J, Wang B. A small molecule agonist of EphA2 receptor tyrosine kinase inhibits tumor cell migration in vitro and prostate cancer metastasis in vivo. *PLoS One*. 2012; 7:e42120. [PubMed: 22916121]
72. Noberini R, Lamberto I, Pasquale EB. Targeting Eph receptors with peptides and small molecules: progress and challenges. *Seminars in cell & developmental biology*. 2012; 23:51–57. [PubMed: 22044885]
73. Wang B. Cancer cells exploit the Eph-ephrin system to promote invasion and metastasis: tales of unwitting partners. *Sci Signal*. 2011; 4:pe28. [PubMed: 21632467]
74. Vearing C, Lee FT, Wimmer-Kleikamp S, Spirkoska V, To C, Stylianou C, Spanevello M, Brechbiel M, Boyd AW, Scott AM, Lackmann M. Concurrent binding of anti-EphA3 antibody and ephrin-A5 amplifies EphA3 signaling and downstream responses: potential as EphA3-specific tumor-targeting reagents. *Cancer Res*. 2005; 65:6745–6754. [PubMed: 16061656]
75. Keane N, Freeman C, Swords R, Giles FJ. EPHA3 as a novel therapeutic target in the hematological malignancies. *Expert review of hematology*. 2012; 5:325–340. [PubMed: 22780212]
76. Flavell RR, Muir TW. Expressed protein ligation (EPL) in the study of signal transduction, ion conduction, and chromatin biology. *Accounts of chemical research*. 2009; 42:107–116. [PubMed: 18939858]
77. Muir TW. Semisynthesis of proteins by expressed protein ligation. *Annual review of biochemistry*. 2003; 72:249–289.
78. Muir TW. Studying protein structure and function using semisynthesis. *Biopolymers*. 2008; 90:743–750. [PubMed: 18924136]
79. Hofmann RM, Muir TW. Recent advances in the application of expressed protein ligation to protein engineering. *Current opinion in biotechnology*. 2002; 13:297–303. [PubMed: 12323349]
80. McGinty RK, Kim J, Chatterjee C, Roeder RG, Muir TW. Chemically ubiquitylated histone H2B stimulates hDot1L-mediated intranucleosomal methylation. *Nature*. 2008; 453:812–816. [PubMed: 18449190]
81. Singla N, Himanen JP, Muir TW, Nikolov DB. Toward the semisynthesis of multidomain transmembrane receptors: modification of Eph tyrosine kinases. *Protein Sci*. 2008; 17:1740–1747. [PubMed: 18628240]

82. Mende F, Seitz O. 9-Fluorenylmethoxycarbonyl-based solid-phase synthesis of peptide alpha-thioesters. *Angewandte Chemie*. 2011; 50:1232–1240. [PubMed: 21290490]
83. Lahiri S, Brehs M, Olschewski D, Becker CF. Total chemical synthesis of an integral membrane enzyme: diacylglycerol kinase from *Escherichia coli*. *Angewandte Chemie*. 2011; 50:3988–3992. [PubMed: 21433227]
84. Sharom FJ. Characterization and functional reconstitution of the multidrug transporter. *Journal of bioenergetics and biomembranes*. 1995; 27:15–22. [PubMed: 7629046]
85. Morera FJ, Vargas G, Gonzalez C, Rosenmann E, Latorre R. Ion-channel reconstitution. *Methods in molecular biology*. 2007; 400:571–585. [PubMed: 17951760]
86. Yanagisawa M, Iwamoto M, Kato A, Yoshikawa K, Oiki S. Oriented reconstitution of a membrane protein in a giant unilamellar vesicle: experimental verification with the potassium channel KcsA. *Journal of the American Chemical Society*. 2011; 133:11774–11779. [PubMed: 21702488]
87. Pszon-Bartos K, Hansen JS, Stibius KB, Groth JS, Emneus J, Geschke O, Helix-Nielsen C. Assessing the efficacy of vesicle fusion with planar membrane arrays using a mitochondrial porin as reporter. *Biochem Biophys Res Commun*. 2011; 406:96–100. [PubMed: 21295545]
88. Carlson LA, Hurley JH. In vitro reconstitution of the ordered assembly of the endosomal sorting complex required for transport at membrane-bound HIV-1 Gag clusters. *Proc Natl Acad Sci U S A*. 2012; 109:16928–16933. [PubMed: 23027949]
89. Gao Y, Zorman S, Gundersen G, Xi Z, Ma L, Sirinakis G, Rothman JE, Zhang Y. Single reconstituted neuronal SNARE complexes zipper in three distinct stages. *Science*. 2012; 337:1340–1343. [PubMed: 22903523]
90. Salaita K, Nair PM, Petit RS, Neve RM, Das D, Gray JW, Groves JT. Restriction of receptor movement alters cellular response: physical force sensing by EphA2. *Science*. 2010; 327:1380–1385. [PubMed: 20223987]
91. Baker TS, Olson NH, Fuller SD. Adding the third dimension to virus life cycles: three-dimensional reconstruction of icosahedral viruses from cryo-electron micrographs. *Microbiology and molecular biology reviews : MMBR*. 1999; 63:862–922. table of contents. [PubMed: 10585969]
92. Topf M, Lasker K, Webb B, Wolfson H, Chiu W, Sali A. Protein structure fitting and refinement guided by cryo-EM density. *Structure*. 2008; 16:295–307. [PubMed: 18275820]

Highlights

- The Eph receptors are the largest family of receptor tyrosine kinases
- The Eph receptors and the ephrins mediate a variety of cell-cell interactions.
- Unique Eph/ephrin signaling assemblies form at the sites of cell-cell contact.
- Here we review the molecular mechanisms of Eph/ephrin signaling.

Fig. 1.

Schematic representation of the structural domains of ephrins and Eph receptors. Ephrins are shown in red and the Eph receptor in blue/purple. A-class ephrins are attached to the cell membrane via a glycosyl-phosphatidyl-inositol (GPI) linker, while B-class ephrins have a transmembrane helix and a small cytoplasmic domain (CPD). RBD, Receptor-Binding Domain; LBD, Ligand-Binding Domain; cys, cysteine-rich domain; FN III, fibronectin type III repeat; SAM, Sterile Alpha Motif

Fig. 2.

ADAM10 interacts with the EphA3/ephrin-A5 complex and cleaves receptor-bound ephrin in trans (from the surface of the opposing cell). The formation of a biologically active Eph/ephrin complex creates a molecular recognition motif for the cysteine-rich domain of ADAM10 that positions the proteinase domain for efficient ephrin cleavage. The structural ephrin and Eph domains are shown as in Fig. 1.

Fig. 3.

Crystal structure of an Eph/ephrin hetero-octamer. Eph receptors use two separate interfaces for the assembly of signaling-competent clusters. The first one, within the ligand-binding domain (Eph 'dimerization' interface), is responsible for the formation of receptor dimers (or 2:2 Eph/ephrin complexes). The other interface is within the cys-rich domain of the Eph molecule and is responsible for the formation of the higher-order assemblies ('clustering interface'). The existence of these two separate and independent interacting interfaces allows the Eph receptor to use a so-called 'seeding' mechanism for the assembly of receptor clusters containing hundreds of receptors. Ephrins are in pink or red and the Eph-ECDs are in purple or blue. Abbreviations are as in Fig. 1.

Fig. 4.

EphA4 engages in additional, homotypic, head-to-tail Eph-Eph interactions. While the EphA4-ECD, when in complex with ephrin-A5, forms similar heteromeric assemblies as the EphA2-ECD/ephrin-A5 complex (Fig. 3), it forms head-to-tail homotypic receptor complexes when not bound to a ligand. The ligand-binding domain of one EphA4 molecule binds the FN-domain of a neighboring EphA4, which, as illustrated, could be important for expanding the Eph clusters beyond the area of ephrin contact. Abbreviations are as in Fig. 1

Fig. 5. Expressed Protein Ligation (EPL) for generating Eph receptors. Eph extracellular domain (ECD) -thioester is generated by intein-mediated thiolysis of a purified EphECD-intein fusion protein (LBD: ligand binding domain, CR: cysteine rich region, FN III: fibronectin type III repeat). The cysteine residue at the N-terminus of the Eph kinase domain, on the other hand, is created by a protease treatment of an expressed fusion protein where a purification tag is located N-terminally to the kinase domain (Kinase: tyrosine kinase domain; SAM, Sterile Alpha Motif). The two building blocks are then ligated by a spontaneous chemical reaction to generate the semisynthetic Eph RTK.