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Ephs and ephrins

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Given their multitude of functions in almost all tissues of the body, Eph receptors are one of the most underappreciated families of receptor tyrosine kinases (RTK). With fourteen receptors divided into two subfamilies, EphAs and EphBs, they are also the largest family of all RTKs. What makes Eph receptors unique is that their cognate ligands, the ephrins, are cell surface tethered, in contrast to other RTKs whose ligands are generally soluble. This phenomenon means that signalling through Eph receptors is largely cell-cell contact dependent. In this way, Eph receptors allow cells to sense their immediate surrounding cellular microenvironment and make appropriate behavioural decisions. For example, Eph receptors control whether two contacting cells are repulsed by, or attracted to, each other. As such they play an important role in normal physiological processes such as embryonic tissue boundary formation and directional guidance of developing axons, and in adult tissues they aid in wound healing and the maintenance of intestinal spatial cell populations. Aberrant expression of these receptors, however, has been implicated in many pathologies including cancer and neurodegenerative diseases. In this article we will discuss some of the key aspects of Eph and ephrin signalling that make them pivotal players in health and disease.

Eph receptors were first identified in 1987, during a screen for new oncogenic tyrosine kinases. The term Eph receptor comes from 'erythropoietin-producing hepatocellular carcinoma cell line', the cell line from which their cDNA was originally isolated. Since then a whole family of receptors and ligands have been identified and further categorised based on their sequence homology. Eph receptors are divided into two subfamilies, the type-A Eph receptors (EphA1-EphA8, Eph10), which bind to and activate type-A ephrins (ephrin-A1 - ephrin-A5), and the type-B Eph receptors (EphB1-EphB4, EphB6), which bind to and activate the type-B ephrins (ephrin-B1 – ephrin-B3). There is a degree of promiscuity between categories, for example EphB2 can bind ephrin-A5, and EphA4 is able to bind ephrin-B ligands. There is also promiscuity of binding within categories, for example EphB2 is able to bind to ephrins-B1, B2 and B3. This "redundancy" can make knockout studies difficult to interpret. EphA and EphB receptors have a similar structure; extracellularly they have a ligand-binding domain and a cysteine rich region (Cys domain) followed by

two fibronectin type III repeat domains. A single transmembrane spanning region is then followed by an intracellular juxtamembrane domain which is important for receptor activation, a kinase domain, a sterile alpha motif, which is required for receptor oligomerisation, and a PDZ domain binding site.

In contrast, the structure of the ligand classes, ephrin-As and –Bs, are very different to each other. Ephrin-B ligands contain an extracellular receptor binding domain, a short transmembrane domain and a short cytoplasmic tail, which has conserved tyrosine residues, and a PDZ domain-binding site. Ephrin-As are smaller with only a receptor binding domain anchored to the membrane via a glycosylphosphatidylinositol (GPI) anchor.

ephrin-Eph signalling

Because their ephrin ligands are membrane tethered, Eph receptors are activated by cell-cell contact; the ligand binding domain of the Eph receptor interacts with the extracellular domain of the ephrin ligand expressed on a neighbouring cell. A novel aspect of the ephrin-Eph interaction is that the resultant signalling can occur in a bi-directional manner. Signalling through the Eph receptor has been termed forward signalling, while signalling through the ephrin ligand has been termed reverse signalling.

Forward signalling

Signalling through activation of Eph receptors by ephrins expressed on another cell is considered to be the canonical signalling pathway. It can take the form of receptor auto-phosphorylation, or phosphorylation of associated effector proteins. A recently resolved X-ray crystal structure of EphB2 has demonstrated that the phosphorylation of conserved tyrosine residues in the Eph juxtamembrane region causes the receptor to adopt an open conformation, allowing further tyrosine phosphorylation of the active site of the kinase domain. Adaptor molecules containing Src homology-2 (SH2), SAM or PDZ domains can then bind and activate further downstream signalling events. As a rule, and contrary to the function of many other RTKs, Eph receptors do not directly regulate cell growth through the Ras-MAPK pathway.

Reverse signalling

Although less is known about reverse signalling, it is clear that ephrins possess a degree of signalling function. Ephrin-Bs contain five conserved intracellular tyrosine residues, which are phosphorylated by Src family kinases (SFK) upon EphB receptor engagement. The intracellular tail of ephrin-Bs also contains a PDZ binding domain, just like the Eph receptor, allowing PDZ containing proteins to bind. It has been shown that protein tyrosine phosphatase, PTP-BL, is recruited to ephrin-B upon stimulation and acts to inhibit ephrin-B phosphorylation, thereby switching signalling from phosphorylation to PDZ-dependent signalling. Ephrin-A reverse signalling is less well characterised. How a signal is transmitted from the extracellular ephrin, through a GPI anchor, to within the cell, remains to be fully elucidated. What is clear, however, is that ephrin-A reverse signalling is dependent upon other proteins, such as the Src family kinase, Fyn, being recruited to ephrin-complexes.

Signalling complexity

Eph receptor signalling is not as straightforward as one receptor binding to one ligand. Both Ephs and ephrins must be clustered in order to trigger efficient activation and, upon activation, tetramers of receptors and ligands come together to form large signalling arrays. The size of the array positively correlates with the degree of response, i.e. larger arrays have more of an effect on cell function. These arrays can be homotypic, i.e. one type of Eph receptor, or heterotypic involving the oligomerisation of different Eph receptor subtypes. This means that the activation of one Eph receptor subtype can promote the recruitment and activation of other receptor subtypes. To add further complexity, Eph receptors can also interact *in cis* with ephrins co-expressed on the same cell membrane. This interaction has been found to inhibit forward signalling through the Eph receptor. Equally, because a cell will have on its surface a number of different Eph receptor and ephrin subtypes with varying levels of expression, the behaviour of the cell upon interaction with another cell will be a function of the relative ratios of these receptor and ligand subtypes. For example, for prostate cancer cells, whether the cancer cell is attracted to, or

repulsed by, neighbouring stromal cells is dictated by the combination of Eph receptors expressed, and thus activated by, the contact.

Once activated, Eph receptors may also impact on other signalling pathways; for example, there is increasing evidence that Ephs and ephrins can also interact with other RTKs. ErbB2 (HER-2) can associate with EphA2, resulting in EphA2 phosphorylation, followed by increased activation of Rho GTPases and Ras/Erk signalling, and subsequently pro-oncogenic effects such as increased cancer cell motility and proliferation (Fig. 1).

Perhaps because of the complexities of Eph signalling, the intracellular signalling pathways downstream of specific Ephs have yet to be fully elucidated. Much of the original work in this area utilised artificial soluble ephrin ligands, an approach which has certainly provided many useful insights; however, recent advances have come from more physiologically relevant experiments using quantitative proteomic analysis. This approach involves co-culturing two labelled cell populations and using cell surface-bound ephrins on one cell type to activate the Eph receptors on the other cell type. The tyrosine-phosphorylated peptides from each cell type can then be identified and quantitated using mass spectrometry. When EphB2 and ephrin-B1 expressing cells were co-cultured, it became apparent that the bidirectional signalling was asymmetric between the two cell types, and that the two cell populations were transmitting the signals induced by cell-cell contact using very different tyrosine kinases and downstream target proteins (Fig. 1).

Eph signalling idiosyncrasies
Interestingly, Eph receptors can signal in a ligand-independent manner, particularly in situations when they are over-expressed, such as in cancer cells. For example, EphA2 is able to function in both an inhibitory and oncogenic fashion, dependent on the presence or absence of its ligand ephrin-A1. Ligand-independent signalling, triggered for example by growth factors in serum, has been shown to involve activated Akt that phosphorylates EphA2 at serine 897 leading to increased cell polarity, lamellipodia protrusion, and subsequently increased cell migration and invasion (Fig. 1). Conversely, in the presence of ephrin-A1, EphA2 is phosphorylated

at the juxtamembrane tyrosine residues and dephosphorylated at S897. This ligand-activated EphA2 then deactivates Akt, leading to disappearance of phosphorylated Akt from the leading edge of the cell. Stimulation by ephrin-A1 results in retraction of lamellipodia and a reduction in chemotactic cell migration and invasion. S897 is only conserved between EphA2 and EphA1, suggesting that ligand-independent activation via Akt is restricted to these two receptors.

A recent exciting finding is that cells can deploy Eph receptors, specifically EphB2, on extracellular vesicles called exosomes. Exosomes offer a form of communication between cells that can occur at a distance, yet that mimics cell-cell contact (Fig. 1). Importantly, the EphB2 on neuronal exosomes has been shown to be functional, triggering ephrin-B reverse signalling and growth cone collapse in target cells.

Functions of ephrin-Eph signalling

Tissue organisation

Eph receptors have been shown to play key roles in many aspects of tissue organisation, particularly during embryonic development. One such role for Eph receptors is during axon guidance. Neurones in the developing embryo must grow and migrate towards their target cell; a delicate balance between attractive and repulsive signals is required on route to ensure they do not stray off course. Eph/ephrin interactions play an essential part in this process. For example, ephrin-expressing stromal cells can repulse a neuronal growth cone (which expresses the corresponding Eph receptor) from entering into their territory. In contrast, a different Eph receptor expression profile on the growth cone can result in it being attracted to an ephrin-expressing area (Fig. 2).

Ephrin-Eph signalling also restricts cell intermingling at key tissue boundaries (Fig. 2). In the brain, Ephs and ephrins are expressed in complementary patterns, with signalling leading to cell repulsion and, as a result, preventing cell intermingling. Correct expression of Ephs and ephrins is critical in brain development; indeed, loss of EphB2 or EphB3 causes aberrant formation of the cerebral hemispheres.

Tissue Maintenance

Eph receptors not only have a vital role in many developing tissues, they are also essential in maintaining the appropriate structure of tissues in adult organisms. A good example of this is in the epithelial crypts of the small intestine (Fig. 2). At the base of each crypt intestinal stem cells proliferate and differentiate into distinct intestinal cell lineages. Progenitor cells passively move up the villus via a treadmilling effect. Newly generated absorptive entero-endocrine and goblet cells have high EphB expression, but as they move up the villus their expression profile changes to high ephrin-B expression. Repulsive events upon EphB and ephrin-B interaction, restricts the intermingling of the differentiated and proliferating cells. Another cell lineage, Paneth cells, retain high levels of EphB after differentiation and therefore are prevented from moving up the villus by EphB and ephrin-B mediated repulsion, and thus remain at the base of the crypt. The process of intestinal cell segregation is further aided by the interaction of Eph receptors with E-cadherin. This interaction occurs when cells expressing high levels of EphB such as Paneth cells, come into contact with ephrin-B expressing cells further up the crypt. The activation of the EphB receptors locally induces the activity of the metalloproteinase ADAM10, which in turn triggers cleavage of E-cadherin between cells, breaking adherens junctions and helping to retain Paneth cells to the base of the crypt.

The effect of ephrin-Eph signalling on cell-cell junctions is also evident in wound repair. Upon wounding, EphBs and ephrin-Bs are upregulated in epidermal cells back from the wound edge. Signalling between the receptor and ligand leads to a loosening of adherens junctions between cells and the dissolution of stress fibres. This results in tension release within the epidermal monolayer, aiding collective cell migration and allowing the wound to close (Fig. 2).

Adhesion, attraction, repulsion and the influences on cell migration
The full functions of Ephs and ephrins in cell-matrix adhesion are still being pieced together. Forward signalling through EphA2 down-regulates integrin function, thus reducing cell migration and integrin-mediated adhesion. In contrast, coupling of

EphB1 - ephrin-B1 *in vitro* leads to increased cell adhesion via α1β5 integrin activation, an effect that is dependent on the surface density of ephrin-B1 expression. It seems likely that cellular adhesion is dictated by the balance of expression of Eph receptors and ephrins on neighbouring cells.

As Ephs, and ephrins are activated by cell-cell contact, they are perfectly placed to control whether a cell is attracted to, or will avoid, an adjacent cell. A common consequence of Eph signalling therefore is alteration in the actin cytoskeleton to regulate cell movement, and this is most often accomplished by modulation of Rho family GTPases. This family of molecular switches modulates cell movement by promoting the formation of different actin structures required for cellular migration. The three key players in this family are RhoA, Rac1 and Cdc42, and ephrin-Eph signalling can both promote and inhibit the activation of all these GTPases. For example, in neurones, stimulation of cells with ephrin-A5 leads to growth cone collapse via activation of Rho, which causes cell contraction, and inhibition of Rac which prevents lamellipodia formation.

Eph receptors and ephrins effect activation changes in these small GTPases via GAPs and GEFs. A nice example of this is demonstrated in mice with a homozygous mutation in the gene encoding α-chimerin, a Rac-GAP. Mice with this mutation (termed *miffy*) have a distinctive 'rabbit-hopping' gait, identical to that of EphA4 and ephrin-B3 knock-out, mice. The origin of this abnormal gait is found in examination of the mouse corticospinal tract (CST), the neuronal pathway which controls voluntary limb movement. In wildtype mice the CST originates in the cerebral hemisphere, before crossing over the midline to the contralateral spinal cord. Re-crossing is then prevented through the expression of ephrin-B3 at the midline, which activates EphA4 on the axonal growth cone, inactivating Rac via α-chimerin and resulting in repulsion. In *miffy*, α-Chn$^{-/-}$, EphA4$^{-/-}$ or ephrinB3$^{-/-}$ mice however, this repulsion does not take place and the CST recrosses to the ipsilateral spinal cord, resulting in the unusual hopping gait.

Cancer

Changes in Eph receptor expression levels occur in nearly all types of cancer, with altered expression being linked to stage of disease and prognosis. EphA2 is upregulated in many different cancers including breast, melanoma, prostate, ovarian and glioblastoma, with over-expression commonly being associated with a more advanced stage of disease and a poor prognosis. The mechanisms by which Ephs and ephrins contribute to tumour progression are complex and not fully understood but, they have been implicated in cancer cell proliferation, adhesion, migration, tumour angiogenesis and invasion. Eph receptor signalling in cancer remains bidirectional; however, studies have shown that both forward or reverse signalling can result in either oncogenic or tumour suppressor effects. For example, in many cancers EphA2 appears to have a tumour suppressor function when stimulated by its ephrin ligand. However when overexpressed it activates in a ligand-independent manner and under these circumstances becomes oncogenic. For example, in breast cancer EphA2 and EphB4 receptors are upregulated and associated with a poor prognosis; however, expression of their partner ligands, ephrin-B2 and ephrin-A1, is low, as are tyrosine phosphorylation levels of the Eph receptors themselves. We now know that under these circumstances EphA2 and EphB4 signal in an ephrin-independent manner and can induce pro-oncogenic effects through crosstalk with other signalling pathways such as those downstream of the EGF receptor and erbB-2.

Another complexity of the ephrin-Eph system in cancer is that a particular Eph receptor can inhibit tumour activity in one cancer type but act in a tumour-promoting manner in a different cancer type. For example, in colon cancer low levels of EphB2 are associated with a poorer prognosis. In normal circumstances EphB2 expression is regulated by Wnt signalling in the colon and acts as a tumour suppressor. During the early stages of colorectal cancer, Wnt signalling becomes hyper-activated leading to increased expression of EphB2. As a distortion of the normal physiology mentioned earlier in this review, high ephrin-B expression in epithelial cells at the top of intestinal crypts interacts with the EphB2 on the cancer cells, repulsing them and stopping their migration out of the crypt. However, after

the initial tumour proliferation phase expression of EphB2 on the cancer cells is downregulated, allowing them to migrate unimpeded to other areas of the colon. In contrast, in breast cancer and ovarian cancer, high levels of EphB2 expression are associated with a worse prognosis.

Memory and neurodegenerative disease

In recent years Ephs and ephrins have been found to play a key role in the formation of memory, and in pathologies of memory formation such as Alzheimer's disease (AD). Our long- and short-term memories are formed through alterations in synaptic neuro-transmission. Eph receptors and ephrins are expressed both pre- and post-synaptically and are perfectly placed to effect such changes. In addition, both Ephs and ephrins are found in areas of the brain that are involved in memory formation such as the hippocampus, amygdala and cortex. Alteration of synaptic transmission can be implemented in various ways, including changes in peri-synaptic neuronal morphology, changes in pre-synaptic transmitter release, or changes in the post-synaptic response to these transmitters. Eph receptors and ephrins have been shown to play a role in all of these areas by effecting processes such as dendritic spine morphogenesis, pre-synaptic transmitter release, post-synaptic glutamate receptor trafficking, and glutamate reuptake.

In the context of Alzheimer's disease, Eph receptors potentially represent an exciting new therapeutic opportunity. Post-mortem studies in humans show a reduction in EphA4 and EphB2 expression in the hippocampal tissue of patients with incipient AD. Similar reductions in hippocampal EphA4 and EphB2 levels are seen in the AD mouse model, prior to the development of overt cognitive impairment. β-amyloid plaques are pathognomonic of Alzheimer's disease and β-amyloid oligomers have been shown to reduce Eph receptor levels by triggering Eph receptor degradation in the proteasome. If Eph receptor function is artificially restored, this can reduce β-amyloid-induced neuronal toxicity. One potential mechanism for this effect is the crosstalk between Eph receptors and the NMDA glutamate receptor. EphB has been shown to interact with NMDA at synapses, with stimulation by ephrin-B leading to NMDA clustering and calcium flux. This interaction may have important

consequences in AD since β-amyloid plaques inhibit NMDA function, leading to impaired memory function. Expressing EphB2 in a mouse model of Alzheimer's rescues NMDA function and restores memory (Fig. 3).

Future Perspectives

The list of functions attributed to ephrin-Eph signalling is growing year on year. In addition to cancer, we are now starting to better understand how dysfunction of Eph receptors contributes to pathologies such as Alzheimer's disease, viral infections, abnormal wound repair and osteoporosis. Numerous therapeutic agents that target Eph receptors are currently being investigated, including monoclonal antibodies, small molecule inhibitors, peptides, and antibodies conjugated to chemotherapy agents. Indeed, several clinical trials are already underway, looking at targeting Eph receptors in diseases such as ovarian cancer, non-small cell lung cancer and melanoma. However, more work is needed to understand the complexities of signalling redundancy and bidirectional ephrin-Eph signalling, to enable optimal therapeutic targeting of Eph receptors for the clinic.

Further Reading

Astin, W. A., Batson, J., Kadir, S., Charlet, J., Persad, R. A., Gillatt, D., Oxley, J. D., Nobes, C. D. (2010). Competition amongst Eph receptors regulates contact inhibition of locomotion and invasiveness in prostate cancer cells. Nat Cell Biol *12*, 1194-1204.

Barquilla, A., Pasquale, E.B. (2015). Eph receptors and ephrins: therapeutic opportunities. Annu Rev Pharmacol Toxicol *55*, 465–487.

Batlle, E., Henderson, J.T., Beghtel, H., van den Born, M.M., Sancho, E., Huls, G., Meeldijk, J., Robertson, J., van de Wetering, M., Pawson, T., Clevers, H. (2002). Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. Cell *111*, 251-63.

Cisse, M., Halabisky, B., Harris, J., Devidze, N., Dubal, D.B., et al. (2011). Reversing EphB2 depletion rescues cognitive functions in Alzheimer model. Nature *469*, 47–52.

Dines, M., Lamprecht, R. (2016). The role of Ephs and ephrins in memory formation. International Journal of Neuropsychopharmacology *19*, 1-14.

Gong, J., Körner, R., Gaitanos, L., Klein, R. (2016). Exosomes mediate cell contact–independent ephrin-Eph signalling during axon guidance. J Cell Biol *214*, 35-44.

Jørgensen, C., Sherman, A., Chen, G.I., Pasculescu, A., Poliakov, A., Hsiung, M., Larsen, B., Wilkinson, D.G., Linding, R., Pawson, T. (2009). Cell-specific information processing in segregating populations of Eph receptor ephrin–expressing cells. Science *326*, 1502-1509.

Kania, A., Klein, R. (2016). Mechanisms of ephrin-Eph signalling in development, physiology and disease. Nat. Rev. Mol. Cell Biol. 17, 240-256.

Miao, H., Li, D., Mukherjee, A., Guo, H., Petty, A., Cutter, J., Basilion, J. P., Sedor, J., Wu, J., Danielpour, D., Sloan, A., Cohen, M.L., Wang, B. (2009). EphA2 mediates ligand-dependent inhibition and ligand-independent promotion of cell migration and invasion via a reciprocal regulatory loop with Akt. Cancer Cell *16*, 9–20.

Nunan, R., Campbell, J., Mori, R., Pitulescu, M.E., Jiang, W.G., Harding, K.G., Adams, R.H., Nobes, C.D., Martin, P. (2015). Ephrin-Bs drive junctional downregulation and actin stress fiber disassembly to enable wound re-epithelialization. Cell Rep *13*, 1380–1395.

Pasquale, E. (2010). Eph receptors and ephrins in cancer: bidirectional signalling and beyond. Nat Rev Cancer *10*, 165-180.

Poliakov, A., Cotrina, M., Wilkinson, D. G. (2004). Diverse Roles of Eph Receptors And Ephrins in the Regulation of Cell Migration and Tissue Assembly. Dev Cell *7*, 465–480.
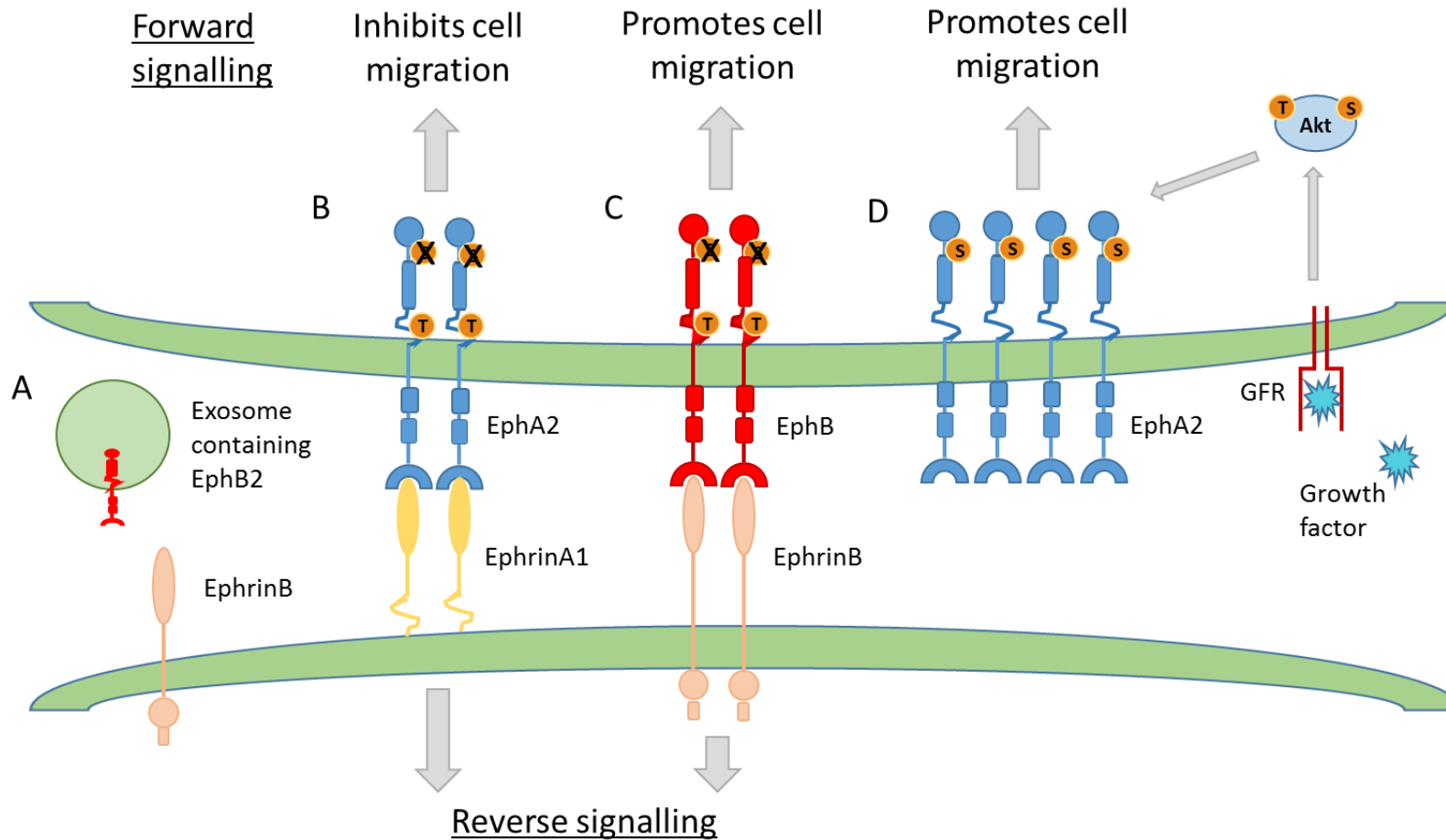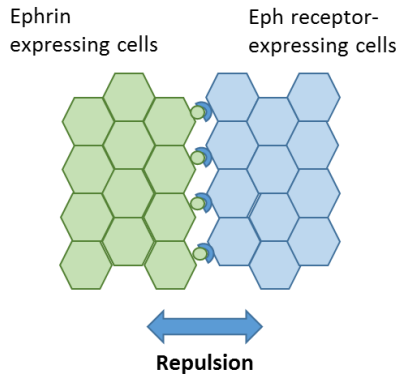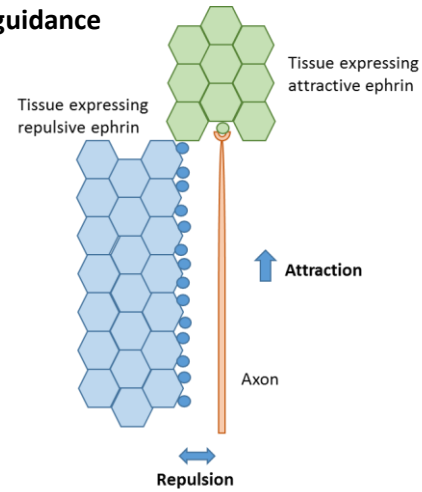
Figure 1

Figure 1 – Specific examples of Eph receptor signalling idiosyncrasies. (A) Eph receptors are able to activate ephrin receptors at a distant site via release of the full length receptor in exosomes. (B) In the presence of its ligand ephrin-A1, EphA2 is activated via tyrosine phosphorylation in the juxtamembrane domain, and dephosphorylation at S897. This leads to, amongst other effects, dephosphorylation of Akt, retraction of lamellipodia and reduced cell migration and invasion. (C) In contrast, ligand-dependent signalling through the EphB receptor promotes cell migration. (D) In the absence of its ligand, EphA2 can signal via a ligand-independent pathway. The stimulus for this could, for example, be activation of growth factor receptors, which leads to phosphorylation of Akt at T308 and S473. Akt in its activated form can then phosphorylate EphA2 at S897, resulting in increased cell polarity, lamellipodia protrusion and subsequently increased cell migration and invasion.
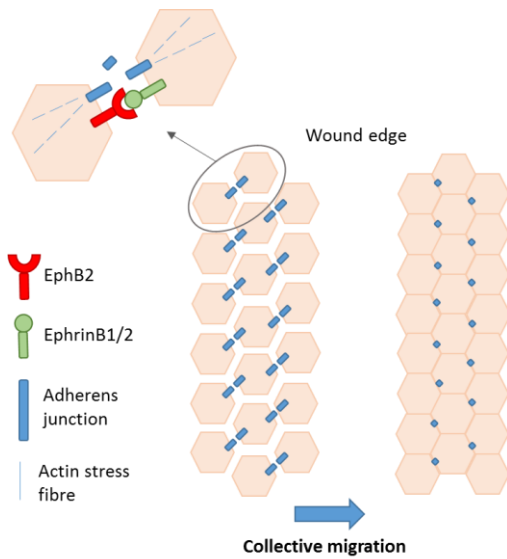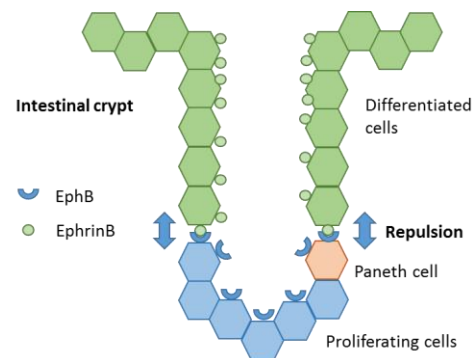
Figure 2. The varied functions of Eph receptors in physiology (A) Interaction between Eph receptors on one cell population, and ephrins on a neighbouring cell population, results in repulsion between the populations and the formation of a clear boundary. (B) Eph receptor-expressing axon is repulsed by ephrin expressing cells on one side, and attracted to a cell population expressing a different ephrin ligand, thereby guiding the direction of its growth. (C) Cells on the right side of the figure represent a normal epithelial sheet, the cells on the left show the effects of a wound on adjacent epithelial cells. The upregulation and subsequent interaction of EphB2 and ephrin-B1/2 triggers dissolution of adherens junctions between cells, and the breakdown of intracellular actin stress fibres. These changes result in a loosening of the epithelial sheet, giving cells the shuffle room needed to migrate collectively and close the wound. (D) EphB - ephrin-B repulsive interactions keep proliferating cells and Paneth cells at the base of the crypt.
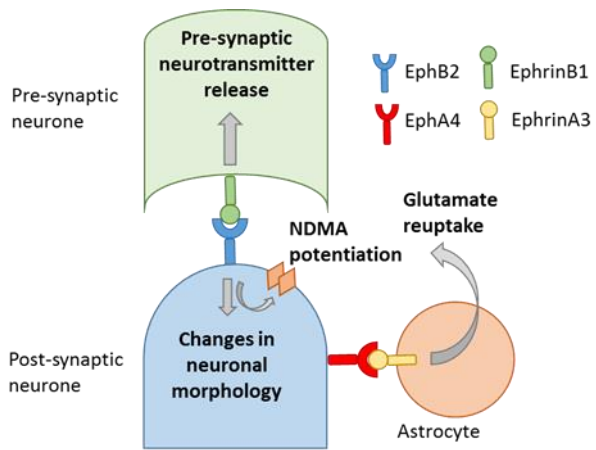
Figure 3. Memory formation. Overview of the role of Eph receptors and ephrins in memory formation.