Cellular and molecular mechanisms of EPH/EPHRIN signaling in evolution and development

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Abstract

The EPH receptor tyrosine kinases and their signaling partners, the EPHRINS, comprise a large class of cell signaling molecules that plays diverse roles in development. As cell membrane-anchored signaling molecules, they regulate cellular organization by modulating the strength of cellular contacts, usually by impacting the actin cytoskeleton or cell adhesion programs. Through these cellular functions, EPH/EPHRIN signaling often regulates tissue shape. Indeed, recent evidence indicates that this signaling family is ancient and associated with the origin of multicellularity. Though extensively studied, our understanding of the signaling mechanisms employed by this large family of signaling proteins remains patchwork, and a truly "canonical" EPH/EPHRIN signal transduction pathway is not known and may not exist. Instead, several foundational evolutionarily conserved mechanisms are overlaid by a myriad of tissue -specific functions, though common themes emerge from these as well. Here, I review recent advances and the related contexts that have provided new understanding of the conserved and varied molecular and cellular mechanisms employed by EPH/EPHRIN signaling during development.

1. Introduction

The EPH/EPHRIN signaling family is the largest receptor tyrosine kinase system in the vertebrate genome and has myriad roles in development, bone homeostasis, the immune system, and cancer (Arthur & Gronthos, 2021; Darling & Lamb, 2019; Kania & Klein, 2016; Pasquale, 2010). The most conserved and reiterated functions of EPH/EPHRIN signaling in development relate to guidance of axon or cell movements, and to the establishment of boundaries for tissue separation, but it is clear that EPH/EPHRIN signaling can also regulate cell proliferation, cell fate specification, apoptosis, cytokinesis, and mitotic spindle orientation in various contexts (Kania & Klein, 2016). It should not be surprising that such a large signaling family, highly elaborated through gene duplication in vertebrates, would have acquired widely-varied cellular functions during development. Further, the diversity of cellular function also comes into play in a number of different cancers where deregulation of EPH/EPHRIN signaling has been implicated in growth, migration and invasion of tumor cells, as well as in tumor angiogenesis and in immune response to cancer, and for these reasons have been considered as attractive therapeutic targets (Barquilla & Pasquale, 2015; Janes, Vail, Ernst, & Scott, 2021; Nievergall, Lackmann, & Janes, 2012; Noberini, Lamberto, & Pasquale, 2012; Noberini & Pasquale, 2009; Pasquale, 2010). It is clear that many of these functions reflect the developmental roles of EPH/EPHRIN signaling, but

our understanding of the basic molecular and cellular mechanisms employed remains incomplete. In this chapter, I will review and highlight recent discoveries that reveal both conserved and divergent functions for EPH/EPHRIN signaling in development across evolutionary time and across distinct tissue contexts during development. I will focus on the cellular and molecular mechanisms by which EPH/EPHRIN signaling acts in development, and particularly look for opportunities to examine *in vivo* evidence in whole organisms supporting mechanisms proposed in cell culture.

1.1 Structure and specificity

The EPH receptors are the largest family of receptor tyrosine kinases and comprise EPHA and EPHB subfamilies, which are defined based on sequence similarity and on their binding preference to the two different types of EPHRIN signaling partner thus named EPHRIN-A and EPHRIN-B (Fig. 1A). Both classes of EPHRINs are cell membrane-bound and are defined by sequence homology as well as by their mode of membrane attachment: the B-type EPHRINs have a highly conserved transmembrane domain while the A-type EPHRINs are tethered to the membrane by a GPI-anchor. The intracellular domain of B-type EPHRINs includes several conserved tyrosines and a carboxy-terminal PSD95/Dlg/ZO1 (PDZ)-binding motif. The human genome encodes five EPHB receptors and nine EPHA receptors, including two receptors, EPHB6 and EPHA10, which lack intrinsic kinase catalytic activity. The domain structure of EPH receptors is conserved between subfamilies and includes an extracellular globular ligand binding domain, a cysteine rich region that includes sushi and epidermal growth factor (EGF)-like domains, two fibronectin type III (FN) domains, the transmembrane helix, a juxtamembrane region, a tyrosine kinase domain, a sterile α motif (SAM) domain, and PDZ-binding motif (Fig. 1A) (Himanen & Nikolov, 2003).

The human genome encodes five EPHB receptors, nine EPHA receptors, three EPHRINs-B and five EPHRINs-A. For the most part, EPH/EPHRIN interactions are promiscuous within sub-class, specificity likely enabled by the presence of a "class-specificity loop" of distinct sizes in the extracellular domain of the EPHA and EPHB receptors (Himanen, Henkemeyer, & Nikolov, 1998). Exceptions to sub-class specificity include EPHB2, which can bind to EPHRIN-A5, and EPHA4, which can bind to EPHRIN-B2 and EPHRIN-B3 (Gale et al., 1996; Himanen et al., 2004). Crystal structure studies have revealed that EPHA4 has conformational

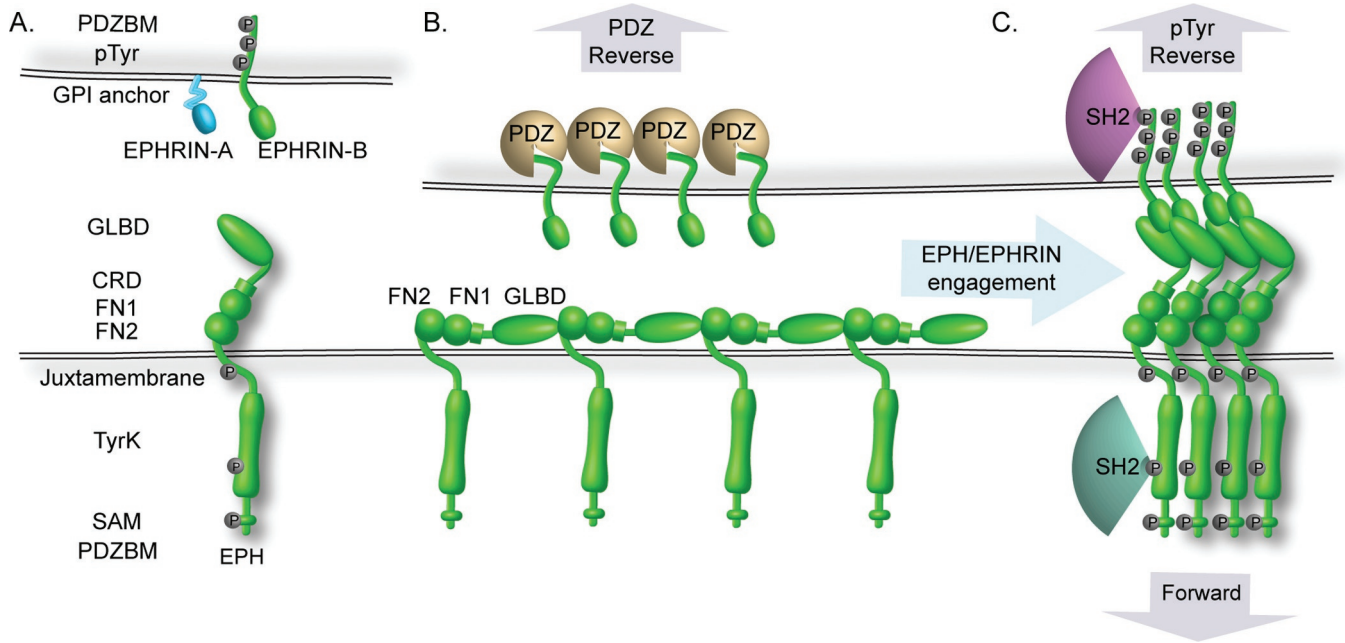

Fig. 1 Schematic representation of EPH/EPHRIN structure and activation. (A) Schematic depiction of the domain structure of EPHRIN-A, EPHRIN-B, and EPHB proteins (EPHA domain structure is similar). (B) Prior to EPH/EPHRIN signaling activation, EPH receptors make homotypic contacts between their GLBDs and FN2 domains. EPHRIN-B signaling partners can bind to PDZ-domain containing proteins independently of EPHB binding. (C) EPH/EPHRIN activation begins with heterodimerization followed by receptor autophosphorylation and recruitment of oligomeric signaling clusters which mediate maximal signaling. Binding of the EPH to EPHRIN-B signaling partners results in a conformational change that allows accessibility of the EPHRIN-B intracellular domain to a variety of tyrosine kinases that mediate its intracellular phosphorylation. SH2 domain-containing proteins bind to phosphorylated tyrosines on both the EPHRIN-B (mediating phosphorylation-dependent reverse signaling) and on the EPHB (mediating kinase-dependent forward signaling). Other modes of signaling are reviewed in (Kania & Klein, 2016). PDZBM, PSD95/Dlg/ZO1 (PDZ)-binding motif; pTyr, phospho-tyrosines; GPI, Glycosylphosphatidylinosil; GLBD, globular ligand binding domain; CRD, cysteine-rich domain; FN, Fibronectin type III domains; TyrK, tyrosine kinase; SAM, sterile α-motif.

plasticity in its ligand binding interface, such that when binding EPHRIN-A2, it resembles other A-class receptors, whereas when it binds EPHRIN-B2 or EPHRIN-B3 it takes on structural hallmarks of B-class receptors (Bowden et al., 2009; Guo & Lesk, 2014). EPHRIN-B1 binding to EPHA4 is not observed, likely because of a steric clash between a large tyrosine residue present at position 121 compared with a relatively small leucine found at the comparable position in EPHRIN-B2 and EPHRIN-B3 (Guo & Lesk, 2014). Interestingly, this amino acid difference enabling cross-class binding of these two EPHRINs with EPHA4 only exists in gnathostomes, and it is intriguing to consider the diversification of signaling functions that was enabled by this difference between otherwise well-conserved B-type EPHRINs (Arcas, Wilkinson, & Nieto, 2020).

In a signaling-off state, EPHs and EPHRINs are loosely homotypically clustered at cell membranes. Both un-liganded EPHA4 and EPHB2 can form head-to-tail interactions between the ligand binding and fibronectin domains of the extracellular domain that are autoinhibitory in nature (Fig. 1B) (Xu et al., 2021, 2013). Signaling interaction initiates through formation of a high-affinity EPH/EPHRIN heterodimer followed by the formation of higher-order oligomers and leading to full activation of kinase signaling (Fig. 1C) (Himanen et al., 2010; Ojosnegros et al., 2017; Seiradake, Harlos, Sutton, Aricescu, & Jones, 2010). A chemical genetic approach using a synthetic dimerizer with high affinity to the FK506 binding protein domain (FKBP) was used to drive the formation of EPHB2 receptor signaling complexes of varying sizes, and the strength of the resulting signal was measured by biochemical and cell biological assays, demonstrating that the degree of EPH receptor clustering determines the strength of kinase-dependent forward signaling and the strength of the cellular response (Schaupp et al., 2014). Unlike most receptor tyrosine kinases, which are activated upon dimerization, EPHB2 and EPHA4 were activated during the transition from dimers to trimers/tetramers. Smaller trimeric and tetrameric clusters were maximally effective in producing a cellular response, though increased activation was not observed upon driving formation of hexamers. Instead, graded responses in EPHB2 activation are achieved through the relative ratio between inactive clusters (monomers/dimers) and active multimers (trimers or greater). Surprisingly, this study also demonstrated that the PDZ binding motif and SAM domain negatively regulated EPHB2 oligomerization to throttle back EPHB2 signaling strength (Schaupp et al., 2014). It is worth noting, however, that crystal structures of EPHA2 in complex with EPHRIN-As indicated the ability to form large

extended signaling arrays, whereas EPHA4 in complex with EPHRIN-B3 formed smaller clusters with a closed circular arrangement (Himanen et al., 2010; Seiradake et al., 2010, 2013). This clustered structure was determined by distinct ectodomain structure, suggesting that different EPH/EPHRIN signaling partners regulate cluster formation differently to achieve varying degrees of graded signaling responses. Further, EPH receptors can hetero-oligomerize both within and across sub-class, and, in cell culture, hetero-oligomerization can modulate signaling strength and cellular response (Freywald, Sharfe, & Roifman, 2002; Janes et al., 2011; Marquardt et al., 2005; Wimmer-Kleikamp, Janes, Squire, Bastiaens, & Lackmann, 2004). Hetero-oligomerization can occur through shared EPHRIN partnership, or independently of EPHRIN specificity through EPH-EPH interactions (Janes et al., 2011; Wimmer-Kleikamp et al., 2004). As multiple EPH/EPHRINs often exhibit overlapping expression within tissues during development, this property of EPH/EPHRIN signaling drastically increases possible signaling complexity beyond the already promiscuous EPH/EPHRIN signaling partnerships. Detailed mechanistic studies that manipulate the homo- and hetero-multimerization of EPH receptors *in vivo* will be required to more fully comprehend combinatorial EPH/EPHRIN signaling codes.

1.2 EPH/EPHRIN SH2-protein mediated signaling

Upon engagement of EPH receptor signaling, autophosphorylation of the juxtamembrane domain results in full kinase activation and phosphorylation of several intracellular tyrosines which act as binding sites for Src Homology 2 (SH2)-domain containing proteins, often resulting in their own tyrosine phosphorylation. Traditional biochemical approaches (Hock et al., 1998; Holland et al., 1997), together with mass spectrometry-based phosphoproteomic approaches, have revealed an extensive network of EPH receptor phosphorylation targets (Bush & Soriano, 2010, 2012; Jorgensen et al., 2009; Zhang, Fenyo, & Neubert, 2008; Zhang, Spellman, Skolnik, & Neubert, 2006). These include proteins with catalytic activity such as p120-RasGAP and α-chimaerin (Elowe, Holland, Kulkarni, & Pawson, 2001; Iwasato et al., 2007), as well as adaptors lacking catalytic activity such as NCK, SHC, and CRK (Hock et al., 1998). Compared with other receptor tyrosine kinases, understanding of the crucial proximal signal transduction mechanisms employed by EPH receptors has remained somewhat rudimentary. Recent work has confirmed and extended these findings by

more detailed examination of the function of different SH2-domain containing proteins, mostly in cell culture, but in some cases, parallel phenotypes in genetic experiments shed light on relevant *in vivo* contexts (reviewed in Bush & Soriano, 2012).

The NCK SH2-adaptor proteins, NCK1 and NCK2, have long been known to interact with and be phosphorylated by EPH receptors, and genetic evidence supports their roles in mediating EPH receptor signaling-driven axon guidance functions in multiple contexts (Chang et al., 2018; Fawcett et al., 2007; Hock et al., 1998; Holland et al., 1997; Srivastava, Robichaux, Chenaux, Henkemeyer, & Cowan, 2013). Multiple biochemical functions for the NCK adaptor have been identified. NCK, together with p62DOK, forms a complex with p120-RasGAP, which negatively regulates RAS/mitogen activated protein kinase (MAPK) pathway activation (Elowe et al., 2001; Holland et al., 1997). NCK and p62DOK recruitment to EPHB1 and EPHB2 also mediates activation of the NCK interacting kinase (NIK) and modulates integrin-mediated attachment and activation of c-Jun Kinase (JNK), which may contribute to cytoskeletal regulation downstream of EPH/EPHRIN signaling (Becker et al., 2000; Stein, Huynh-Do, Lane, Cerretti, & Daniel, 1998). Indeed, NCK appears to facilitate EPH/EPHRIN actomyosin cytoskeletal regulation in multiple contexts. It is required for retraction of cellular protrusions and cell migration activated through EPHA3 (Hu et al., 2009). In *Xenopus,* EphA4 activation and phosphorylation provides sites for NCK2 binding and results in recruitment of p21 activated kinase (Pak1) which regulates levels of Rac, RhoA and Cdc42 GTPases and controls cell contact strength (Bisson, Poitras, Mikryukov, Tremblay, & Moss, 2007). A recent study has revealed that termination of EPH receptor tyrosine kinase downstream signaling depends on the phosphorylation of a conserved tyrosine in the SH3 domain of NCK, which prevents its association with multiple protein targets, including PAK1, and results in dismantling of the signaling network. This mechanism may therefore enable a rapid and reversible mechanism for EPH/EPHRIN transduction which is sufficiently dynamic to mediate rapid changes in the actomyosin cytoskeleton and its effects on cell shape (Dionne et al., 2018).

The SH2 domain-containing scaffold SHB was found to be highly phosphorylated by EPHB2 in HEK293 cells and in secondary palate mesenchyme cells, and appeared to have functional significance based on shRNA knockdown in an EPH/EPHRIN cell sorting assay (Bush & Soriano, 2010; Jorgensen et al., 2009). Recently, detailed examination by co-immunoprecipitation and mass spectrometry revealed that SHB scaffolds

the formation of a multi-SH2-domain protein complex including NCK1/2, p120-RasGAP, and α- and β-chimaerin RacGAP proteins that depended on EPHB2 kinase activation (Wagner et al., 2020). Phosphorylation of distinct tyrosine residues on SHB enabled binding of each of these proteins, and mutation of these tyrosines to phenylalanine reduced EPH/EPHRIN cell sorting. Interestingly, interaction of SHB with EPHB2 was relatively weak, suggesting that it may be transient or indirect. Nevertheless, formation of this complex was also observed upon stimulation of signaling through EPHA4, EPHA8, or EPHB4, suggesting that multiple EPH receptors may utilize the SHB scaffold for simultaneous multi-protein interactions. Though some *Shb*$^{-/-}$ knockout mouse embryos exhibited tantalizingly parallel vascular or neural tube defects, most *Shb*$^{-/-}$ null mice were viable and fertile, suggesting partial compensation for SHB adapter function, for example, by related scaffolds SHD, SHE, or SHF (Kriz et al., 2007; Wagner et al., 2020).

The SH2-containing phosphoinositide phosphatase, SHIP2, is a conserved target of EPH receptor phosphorylation with functional significance in the regulation of cell segregation (Ashlin, Wu, Xu, & Wilkinson, 2019; Jorgensen et al., 2009). Upon EPHRIN-A1 stimulation, SHIP2 binds to the EPHA2 SAM domain and inhibits EPHA2 endocytosis, independent of EPHA2 kinase activity (Zhuang, Hunter, Hwang, & Chen, 2007). Interestingly, SHIP2 does not bind to EPHA3, EPHA4, or EPHB1, but does interact with activated EPHB2, albeit via its SH2 domain rather than its SAM domain (Ashlin et al., 2019). Further, SHIP2 does not appear to regulate EPHB2 endocytosis, but rather regulates directional migration of EPHB2 cells away from EPHRIN-B1 cells, possibly through effects on the actin cytoskeleton. Indeed, previous studies have shown that SHIP2 has an important role in cell polarization and migration through restriction of PI(3,4,5)P_3 localization at the leading edge of migrating cells and through interaction with RhoA, which also can mediate actomyosin contractility mediated effects on EPH/EPHRIN cell-cell contacts (Kato et al., 2012; Kindberg et al., 2021).

The related CRK, CRKII and CRK-L SH2 domain-containing adapter proteins bind to several activated EPH receptors, and CRKII has been shown to mediate EPHA3 signaling effects on cell adhesion and Rho family GTPase signaling in cell culture (Lawrenson et al., 2002). In human breast cancer cells, CRK is tyrosine phosphorylated upon EPHB4 stimulation, mediated at least in part by ABL and ARG, non-receptor tyrosine kinases that can also be phosphorylated by EPH receptor signaling (Huang, Wu, Jin, Stupack, & Wang, 2008; Noren, Foos, Hauser, & Pasquale, 2006).

Curiously, whereas ABL-CRK signaling enables inhibition of cell proliferation and migration by EPHB4 in breast cancer cells, ABL also acts downstream of EPHB2 to promote tumor growth during intestinal adenoma formation (Genander et al., 2009; Noren et al., 2006). These distinct activities of ABL hint at the broader paradoxical roles of the EPH/EPHRIN family in cancer, which probably reflect the broad array of cellular behaviors that are regulated by EPH/EPHRIN signaling, many of which become dysregulated in cancer (Pasquale, 2010).

1.3 Reverse signaling mechanisms

The signaling complexity of this pathway is further multiplied by an additional unique signaling property: famously, there is a capacity for bidirectional signaling, wherein an EPHRIN-expressing cell signals to an EPH-expressing cell (forward signaling) with the simultaneous signaling of the EPH-expressing cell to an EPHRIN-expressing cell (reverse signaling). Bidirectional signaling has been examined in numerous *in vitro* and *in vivo* studies and occurs in A- and B-type subfamilies, albeit by different mechanisms. Upon hetero-tetramerization with EPHB receptors, the EPHRIN-B transmembrane and cytoplasmic domains are repositioned to allow their phosphorylation by Src-family kinases, as well as PDGF and FGF receptors (Fig. 1C) (Brückner, Pasquale, & Klein, 1997; Chong, Park, Latimer, Friesel, & Daar, 2000; Himanen & Nikolov, 2003; Palmer et al., 2002). Tyrosine phosphorylation of EPHRIN-B allows the binding of SH2 domain-containing proteins and can impact cytoskeletal remodeling in cell culture (Brückner et al., 1997; Cowan & Henkemeyer, 2001; Holland et al., 1997). The EPHRIN-B C-terminal PDZ binding motif has also been shown to bind to a handful of PDZ-proteins independent of EPH-receptor binding to transduce a reverse signal (Lin, Gish, Songyang, & Pawson, 1999; Torres et al., 1998). Both phosphorylation-dependent and PDZ-dependent modes of reverse signaling have been well-studied, but other less-studied mechanisms, including serine phosphorylation of EPHRIN-B2, also exist (Bush & Soriano, 2012; Daar, 2012; Davy & Soriano, 2005; Niethamer & Bush, 2019). We recently published an extensive review of genetic interrogation of forward and reverse signaling function of B-type EPHRINs in mice (Niethamer & Bush, 2019), which led us to speculate that some of the *in vivo* functions currently attributed to B-type reverse signaling may instead be due to impacts of EPHRIN-B intracellular mutations on forward signaling;

here, I will not dwell on this challenging question beyond noting whether forward or reverse signaling has been implicated.

A-type EPHRINs also engage in reverse signaling. For example, activation of EPHRIN-A5 reverse signaling results in β1 integrin-dependent FAK tyrosine phosphorylation and MAPK activation as well as changes in cell morphology and adhesion (Davy & Robbins, 2000). Indeed, several studies have indicated that EPHRIN-A reverse signaling may generally regulate integrin-mediated cell adhesion or cell migration in distinct cellular contexts (Daar, 2012). In addition to integrins, EPHRIN-A reverse signaling often occurs through interaction with other membrane associated signaling molecules such as TrkB, p75NTR, and the ADAM10 membrane metalloprotease, all of which impacts axon pathfinding in multiple contexts (Xu & Henkemeyer, 2012). One challenge inherent to understanding the roles of EPHRIN-A reverse signaling *in vivo* is that EPHRIN-As can also interact with EPHAs within the same cell, resulting in cis-inhibition/attenuation of EPHA forward signaling (Carvalho et al., 2006; Marquardt et al., 2005). EPHRIN-As interact in cis with the membrane proximal fibronectin III domain of EPHA receptors, a different domain than the one utilized for trans-signaling, and reduce EPHA tyrosine phosphorylation and downstream signaling response (Carvalho et al., 2006). As such, modulating reverse signaling through increasing or decreasing EPHRIN-A expression also disrupts the balance between trans-activation and cis-inhibition (Kao & Kania, 2011). Indeed, the balance between trans-activation and cis-attenuation is likely to be a key property of EPHRIN-A mechanisms in axon guidance in the retina, tectum, and spinal motor neurons, and possibly in systems beyond axon guidance (Kao & Kania, 2011). Similar cis-attenuation mechanisms have not been identified for B-type EPH/EPHRIN signaling.

2. EPH/EPHRIN function through an evolutionary lens

It has been recognized for some time that EPH/EPHRIN signaling is evolutionarily ancient and is connected to the origin of animal multicellularity and to the elaboration of morphological complexity (Drescher, 2002; Mellott & Burke, 2008a). Recent work has refined the origin and evolution of this signaling family and has revealed new insights into its likely earliest functions (Fig. 2A–F) (Arcas et al., 2020; Krishnan et al., 2019). The exponentially increasing genome sequencing data in a wide variety

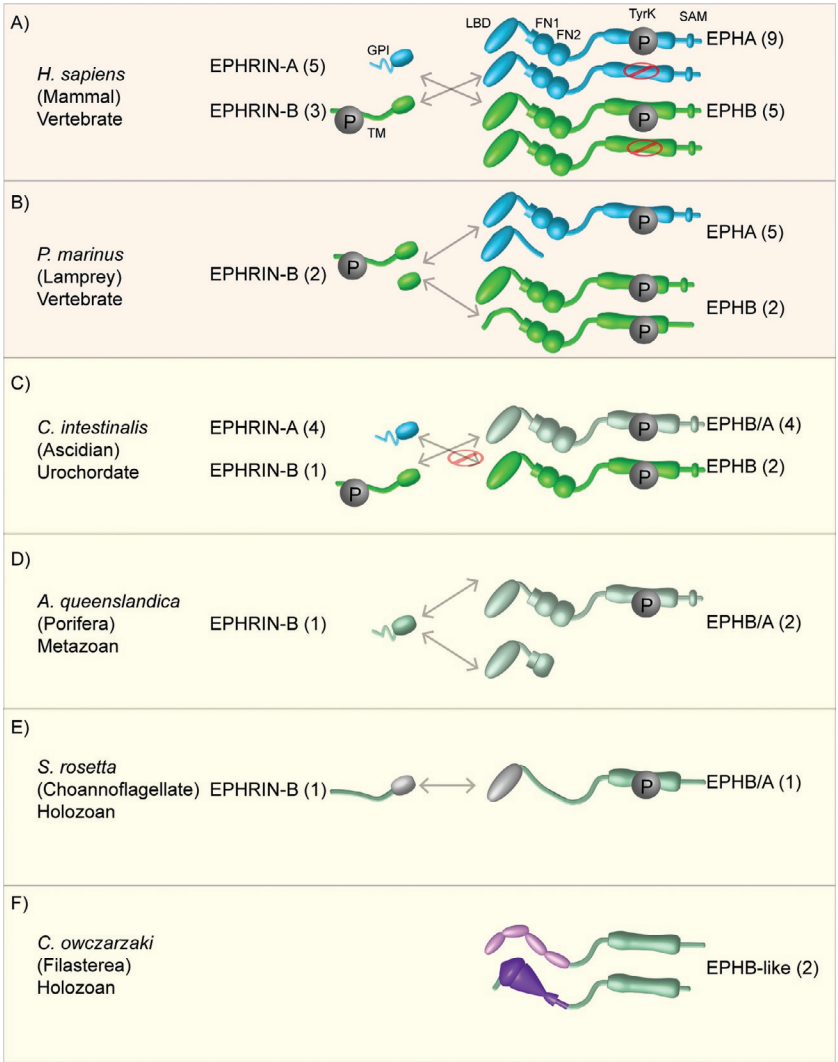

Fig. 2 Summary of evolutionary history of EPH/EPHRIN signaling. Schematic depiction of number of EPH/EPHRINs identified in different species (in parentheses) with an approximation of predicted EPH/EPHRIN binding relationships based on (Arcas et al., 2020; Krishnan, Degnan, & Degnan, 2019), which the reader is highly recommended to explore for more detailed information. (A) EPHA/EPHB subtypes in H. sapiens including kinase-active receptors which are found in EPHs from sponges to humans, and two kinase-dead EPH receptors. While EPHB6 is only kinase dead in mammals (prohibition symbol), EPHA10, which is also kinase dead, is the most ancient A-type receptor and arose in vertebrate lineages. EPHA receptors are only found in vertebrate lineages (peach boxes). (B) EPHB/EPHRIN-A Cross-subclass signaling likely arose in a common ancestor to the gnathastomes, but P. marinus lacks EPHRIN-As, EPHB/EPHRIN-A signaling does not occur in this species. (C) *C. intestinalis* have four EPHB-like receptors that also signal with EPHRIN-As while two receptors are closely related to vertebrate EPHB4,

(Continued)

of organisms has recently led to a more precise understanding of the diversification of the signaling networks that enabled the early evolution of cellular organization, proliferation, cell movement, and cell type specification. Unlike metazoan-specific modalities such as WNT signaling, tyrosine kinase signaling is ancient, arising in the earliest holozoans, and underwent extensive diversification in holozoans and metazoans (Suga, Torruella, Burger, Brown, & Ruiz-Trillo, 2014). Further, EPH receptors are amongst the most ancient receptor tyrosine kinases identified. Though it has been known for some time that bona fide EPHs and EPHRINs are found in sponges (*Porifera*) (Fig. 2D), one of the most evolutionarily basal animals, more recently, proteins with sequence homology to metazoan EPHs and EPHRINs were also identified in choanoflagellates, and structural predictions suggest they bind each other, enabling functional signaling (Arcas et al., 2020; Krishnan et al., 2019; Suga et al., 1999) (Fig. 2E). As the choanoflagellates are colonial unicellular eukaryotes and are the earliest ancestor that exhibited all metazoan EPH domain components together in one protein (Fig. 2E), EPH/EPHRIN signaling could already have regulated cellular organization in the multicellular colonies formed by these creatures.

Sequences corresponding to the domain architecture of part of metazoan EPH receptors, and with homology to the tyrosine kinase domain, have even been identified in unicellular Filasterea such as *C. owczarzaki* (Fig. 2F), a holozoan that shares a common ancestor with choanoflagellates and animals. This suggests the ancestral presence of a "proto-EPH-like" signaling molecule; as this protein lacks an EPHRIN binding domain, and an EPHRIN protein is not present in Filasterea, this "EPH" signals in an EPHRIN-independent manner (Arcas et al., 2020; Krishnan et al., 2019). An EPHRIN is first identifiable in choanoflagellates and has a B-type receptor binding domain (Fig. 2E). Sponges have EPHRIN orthologs that have either a transmembrane region, a GPI-anchor, or both in the same sequence, but are always predicted

Fig. 2—Cont'd suggesting specialized EPHRIN-B binding (prohibition symbol). (D) Bona-fide orthologs of EPHs and EPHRINs have been identified in Porifera, where all EPHRINs harbor a B-type ligand binding domain, though they may exhibit a transmembrane domain, a GPI-anchor, or both. (E) An EPH/EPHRIN-like pair was recently identified in choanoflagellates, which are closely related to those in Porifera and other ancient metazoan species. This appears to be the most ancient lineage to have the typical domain structure of metazoan EPH receptors. (F) Filasterea have sequences with distant homology to EPH receptors, which share part of the domain structure of metazoan EPH receptors. As Filasterea lack EPHRINs, these receptors may have bound other ligands, or acted as cell-cell adhesion molecules mediated by distinct extracellular domains (purple).

to be tethered to the membrane, indicating that EPH/EPHRIN signaling originated as a cell-contact communication system (Krishnan et al., 2019) (Fig. 2D). Whereas EPHRIN-A sequences originated in the common ancestor of tunicates and vertebrates (Fig. 2C), EPHA class receptors arose in the vertebrate lineage (Fig. 2A and B), indicating that EPH receptors in tunicates could bind to both EPHRIN-A and EPHRIN-B subtypes (Mellott & Burke, 2008a). As the sponge *A. queenslandica* and choanoflagellates also possess orthologs of downstream forward signaling effectors such as RhoA, Erk1/2, CDC42, it is possible that EPH/EPHRIN signals by similar core mechanisms in the earliest multicellular organisms. ROCK is present in *A. queenslandica* but not in choanoflagellates, suggesting that if EPH/EPHRIN signaling regulates actomyosin contractility in choanoflagellates, it does so through a different pathway. Interestingly, EPH and EPHRIN sequences are also found in some bacteria, suggesting that they acquired these sequences by horizontal gene transfer; whether they have a function in these prokaryotes is not yet known (Arcas et al., 2020).

2.1 Conserved functions for EPH/EPHRIN signaling in actomyosin contractility and cell positioning

Based on sequence similarities between orthologs, the ancestral EPH was a B-type receptor most similar to vertebrate EPHB4. Whereas the dramatic increase in the size of the EPH/EPHRIN signaling family in vertebrates does correlate loosely with the evolution of the nervous system, it is axiomatic, based on the existence of EPH/EPHRIN signaling in creatures lacking a nervous system, that ancestral function did not relate to neurodevelopment. Instead, it has been hypothesized that ancestral function may relate to regulation of cell movement through regulation of the actin cytoskeleton and cell adhesion, a hypothesis that accounts for the need for cell positioning information in the evolution of multicellular organisms (Drescher, 2002). A form of cell segregation in sponges achieves species-specific recognition during aggregation, and requires actomyosin contractility for cell motility (Arcas et al., 2020; Gaino, Bavestrello, & Magnino, 1999; Gaino & Burlando, 1990). Further support for an evolutionarily basal function of EPH/EPHRIN signaling in cell segregation and boundary formation is provided by the observation of complementary EPH/EPHRIN expression boundaries in the tentacles and developing buds of *Hydra vulgaris*, a simple cnidarian that evolved prior to the split of bilaterians from other metazoans. Functional experiments in an increasingly diverse array

of organisms will be extremely informative to understanding the earliest functional roles for EPH/EPHRIN signaling in evolution.

A role for EPH/EPHRIN signaling in cell position through regulation of cell migration has also been discovered in the sea urchin, *S. purpuratus,* but here EPH/EPHRIN (Eph/Efn) signaling acts as an attractive cue rather than a repulsive one as it does in most scenarios in vertebrate development (Krupke, Zysk, Mellott, & Burke, 2016). Whereas Sp-Efn is expressed in the dorsal ectoderm, Sp-Eph is expressed within pigmented cells called immunocytes that normally migrate to and insert into the ectoderm. Blocking Eph/Efn signaling in sea urchins resulted in a failure of immunocyte migration and insertion into the dorsal ectoderm, and conversely, ectopic high levels of expression of Sp-Efn within the ventral ectoderm resulted in the aberrant attraction of immunocytes into these regions, where they also inserted. Interestingly, Sp-Efn localizes to long cellular protrusions called cytonemes, which deliver it to Sp-Eph-expressing immunocytes to facilitate long-distance attraction. These cytonemes are similar to filopodia-like protrusions that mediate long-distance EphrinB1/EphB3b signaling during hepatoblast positioning in zebrafish (Cayuso et al., 2016; Kornberg, 2019; Krupke et al., 2016). More recently, it was shown that EPHRIN-A1 is also localized to signaling filopodia which deliver it to EPHA2-expressing cancer cells to mediate a repulsive response, and filopodia also can facilitate EPHB2/EPHRIN-B1 repulsive signaling in HEK293 cells (Prospéri et al., 2015; Valenzuela & Perez, 2020). Together, these results indicate that cytoneme-mediated signaling may be an evolutionarily conserved mechanism of EPH/EPHRIN delivery at a distance. Further, these results indicate a basal function for EPH/EPHRIN signaling in cell positioning which has been dramatically expanded to accommodate cellular positioning in a wide array of processes in vertebrate embryos (Krupke et al., 2016).

2.2 An ancient and conserved EPH/EPHRIN signaling module

Even prior to the expansion of the EPH/EPHRIN signaling family in vertebrates, the pathway had already acquired multiple cellular functions. EPH/EPHRIN signaling function has been extensively examined in *Ciona intestinalis* ascidians, invertebrate urochordates that are a sister group to invertebrates. These studies have identified a repeatedly utilized general mechanism for cell fate specification that seems to be conserved throughout animals. In *Ciona* embryos, during early cleavage stages, an asymmetric cell

division between two pairs of mother cells called A6.2 and A6.4 results in the generation of notochord and neural precursor cells. FGF signaling activation of Ras/MAPK drives cells to take on a notochord fate while its suppression results in neural fate. While FGF is expressed broadly and does not provide a positional cue for cell fate specification, Ci-ephrin-Ad is expressed in adjacent animal cells and signals to Eph2 and Eph3 in the mother cells prior to their asymmetric division. Following cell division, this cell-contact localized EPH/EPHRIN signal attenuates MAPK signaling and promotes neural fate within the daughter cells that arise nearer to the Ci-ephrin-Ad source. As one of only five signaling ligands expressed zygotically at the 16-cell stage, Ci-ephrin-Ad plays many parts. It restricts the neural cell fate in epidermal cells, again through repression of MAPK (Ohta & Satou, 2013). Asymmetric inhibition of MAPK by Ci-ephrin-Ad signaling from animal cells in direct contact with the A6.3 endomesodermal precursor mother cell, whose daughters give rise to mesoderm or endoderm derivatives, promotes mesoderm over endoderm fate (Shi & Levine, 2008). Later stages of cell fate specification in *Ciona* are also governed by EPH/EPHRIN repression of MAPK. For example, EPH/EPHRIN signaling is required to restrict MAPK activation during the neural plate stage to prevent the formation of too many pigment cells, as well as during the development of the *Ciona* motor ganglion (MG), which controls rhythmic swimming behavior in the tadpole stage (Haupaix et al., 2014; Stolfi, Wagner, Taliaferro, Chou, & Levine, 2011). In MG neuron specification, Ephrin-Ab is expressed in a cell lineage positioned directly posterior to the A9.30 cell lineage, which ultimately gives rise to the four anterior motor neuron cell types. Signaling by Ephrin-Ab to cells directly anterior to the A9.30 cell represses MAPK activation in this lineage, increasing expression of the Delta2 ligand, which in turn relays Notch activation to the next-most anterior cell, thereby elaborating downstream cell fate selection (Stolfi et al., 2011).

It is notable that in every scenario described above, EPH receptor signal transduction is mediated through the activation of p120RasGAP, a multidomain GTPase-activating protein that inhibits Ras by increasing the rate of GTP hydrolysis (Haupaix et al., 2014). EPH/EPHRIN signaling also acts through p120RasGAP to inhibit Ras/MAPK in vertebrate cells, resulting in cellular responses such as neurite retraction, myogenic differentiation, and reduced endothelial cell migration and proliferation, suggesting possible evolutionary conservation of this signaling module (Elowe et al., 2001; Kim et al., 2002; Minami, Koyama, Wakayama, Fukuhara, & Mochizuki, 2011).

Indeed, EPH/EPHRIN signaling can repress Ras/MAPK signaling in a wide variety of mammalian cell culture cell types and antagonize Ras/MAPK activation by a variety of growth factor pathways (Miao et al., 2001). The evolutionary conservation of this signaling module and its deployment in so many different cell types make a strong case for its utilization in vertebrate development. In fact, treatment of human microvascular endothelial cells (HDMEC) with an EPHB4 inhibitor resulted in increased pERK1/2 and increased proliferation, whereas induction by addition of pre-clustered EPHRIN-B2-Fc resulted in a decrease in pERK1/2 and decreased proliferation, supporting, at least, that EPH/EPHRIN signaling can repress Ras/MAPK in endothelial cells (Groppa et al., 2018). Intriguingly, loss of function of p120RasGAP in mice results in vascular defects and embryonic lethality by E9.5 (Henkemeyer et al., 1995), and mutations in the *RASA1* gene that encodes p120RasGAP causes capillary malformation-arteriovenous malformation (CM-AVM) in humans (Eerola et al., 2003; Gerety, Wang, Chen, & Anderson, 1999). Similar vascular phenotypes to these are also observed upon loss of EPHRIN-B2 or EPHB4 in mice, and zebrafish with loss of function of EPHB4 and p120RasGAP also exhibit highly similar abnormalities in blood vessel formation (Gerety et al., 1999; Kawasaki et al., 2014). Moreover, recent studies have shown that germline mutations in the *EPHB4* gene also result in CM-AVM and other vascular anomalies in humans (Amyere et al., 2017; Zeng et al., 2019). Broader mechanisms for EPH/EPHRIN signaling in angiogenesis have been extensively studied and recently reviewed (Du, Li, He, Li, & He, 2020; Kania & Klein, 2016).

Parallel phenotypes have also been observed upon loss of function of EPHB4/EPHRIN-B2 and p120RasGAP in lymphatic valve development in mice, where kinase-dependent forward signaling through the EPHB4 receptor is crucial (Lapinski et al., 2017; Makinen et al., 2005; Zhang et al., 2015). Kinase inactivating mutations in EPHB4 cause lymphatic abnormalities in humans, which are also observed in CM-AVM (Li et al., 2018; Martin-Almedina et al., 2016, 2021). Further studies are likely to provide definitive *in vivo* genetic evidence of a shared EPHRIN-B2/EPHB4/p120RasGAP pathway and further illuminate the cellular mechanisms by which it regulates vascular and lymphangiogenic development. Together, these studies make a compelling case for conserved signaling function of the most ancestral EPH receptor EPHB4, which seems to utilize a similar signaling mechanism beginning prior to the emergence of vertebrates, conserved in humans, and relevant to human disease.

Tantalizingly, p120RasGAP is also found in the genome of sponges and choanoflagellates, suggesting the intriguing possibility that this signaling relationship may even be conserved from the earliest origins of EPH/EPHRIN signaling (Jaber Chehayeb, Wang, Stiegler, & Boggon, 2020).

2.3 Conserved and divergent roles for EPH/EPHRIN signaling in vertebrate gastrulation

EPH/EPHRIN signaling has both evolutionarily conserved and divergent functions in the cellular movements and fate-induction signaling that occur during vertebrate gastrulation. Initial evidence for a role of EPH/EPHRIN signaling in gastrulation came from studies in zebrafish, in which exogenous expression of human EPHA3 or EPHRIN-A5 resulted in a failure of convergent extension of mesodermal cells and a severely deformed notochord (Oates et al., 1999). These results were interpreted as dominant negative effects because simultaneous overexpression of EPHA3 and EPHRIN-A5 reduced the frequency and severity of these phenotypes. Similarly, dominant negative disruption of B-type EPHRINs caused anteroposterior shortening and lateral expansion of the notochord and prechordal plate, likely through defects in convergent extension cell movements (Chan et al., 2001). During gastrulation in *Xenopus*, convergent extension of the involuting mesoderm elongates the antero-posterior axis and is regulated by signaling between Ephrin-A1 in the ectoderm and EphA4 in the involuting mesoderm (Park, Cho, Kim, Choi, & Han, 2011). Morpholino knockdown of either of these, or dominant negative disruption by expressing a kinase-dead EphA4, resulted in a failure to undergo convergent extension. Neither disruption of EphA4 signaling in *Xenopus*, nor Ephrin-B signaling in zebrafish disrupted fate specification of the mesoderm (Chan et al., 2001; Park et al., 2011). Instead, EphA4/Ephrin-A1 signaling was found to regulate tissue separation of the involuting mesoderm and non-involuting ectoderm; failure of tissue separation and disruption of convergent extension resulting from EphA4 disruption could be rescued by expressing a constitutively active form of the low molecular weight GTPase RhoA (Park et al., 2011). Interestingly, similar tissue separation phenotypes were also observed upon expression of a dominant negative ADAM10, consistent with cleavage enabling conversion of the EphA4/ephrin-A1 adhesive interaction to a repulsive outcome (Janes et al., 2005; Park et al., 2011). Numerous EPH/EPHRIN signaling partners contribute to tissue separation during *Xenopus* gastrulation. Additive antiparallel signaling by Ephrin-B1 from the ectoderm to EphB receptors in the mesoderm,

and Ephrin-B2 from the mesoderm to EphB receptors in the ectoderm are required for ectoderm/mesoderm separation (Rohani, Canty, Luu, Fagotto, & Winklbauer, 2011). As above, Ephrin-B/EphB signaling also utilizes RhoA signaling, though the low molecular weight GTPase Rac is required as well, possibly to regulate trans-endocytosis of EPH/EPHRIN signaling complexes to remove this physical tether from the interface (Gaitanos, Koerner, & Klein, 2016) (see also subsequent section on cellular mechanisms of cell sorting for more details on RhoA in tissue separation). A further role for EPH/EPHRIN signaling in *Xenopus* gastrulation is observed in the regulation of *Brachyury* expression by signaling between EphA4 and Ephrin-B2. This drives a cellular process called "peak involution" wherein dorsal mesoderm undergo bending and anteroposterior elongation to contribute to the earliest phase convergent extension (Evren et al., 2014).

A recent study showed that pan-inhibition of Eph receptor signaling in *Ciona* and in the distantly related ascidian *Phallusia mammillata* resulted in a disruption of endoderm invagination at the beginning of gastrulation without disruption of germ layer fate specification (Fiuza, Negishi, Rouan, Yasuo, & Lemaire, 2020). Following endoderm apical constriction and flattening of the embryo, Nodal signaling drives transcriptional upregulation of the Eph1 receptor in endodermal precursors, which is required for relocalization of phospho-myosin enrichment from apical to basolateral and for subsequent apicobasal shortening of the endoderm. Nodal signaling also activates expression of EphA4 in the mesoderm of *Xenopus*, and EphA4 is required for mesendoderm internalization, suggesting that this may be a shared ancestral mechanism utilized during gastrulation (Evren et al., 2014; Wills & Baker, 2015). However, whereas invagination drives internalization of endoderm in cephalochordates and tunicates such as *Ciona*, vertebrates undergo cellular ingression by migration (Wen & Winklbauer, 2017). Live imaging studies of slice explants in *Xenopus* revealed that ingression is driven by endodermal cell lengthening and rearrangement that enables their amoeboid-like migration. Ephrin-B1 exhibits highly localized enrichment at the trailing edge of migrating endoderm cells where it undergoes trans-endocytosis to permit the retraction of the trailing edge during migration, though it is intriguing to speculate that EPH/EPHRIN signaling at this interface may also enable increased actomyosin contractility to terminate this cell contact (see also below discussion on cell sorting) (Wen & Winklbauer, 2017).

Though EPH/EPHRIN signaling clearly has numerous roles in gastrulation in multiple chordate species, no known roles have been identified in

higher vertebrate species. Gastrulation defects have not been observed upon targeted disruption of *Efnb1*, *Efnb2*, or *Epha4* in mice, and indeed, it is surprising to note that despite expression of multiple EPH/EPHRIN family members during gastrulation in mice, none have yet been reported to have functional roles in this process. This difference may point to functional redundancy in mammals, or subtle defects in gastrulation may have been missed because of the relative inaccessibility of gastrulation cell behaviors in a viviparous organism; alternatively, this difference may reflect evolutionarily divergent functions amongst vertebrates.

2.4 EPH/EPHRIN signaling family as a substrate for evolutionary variation

The dramatic expansion of the EPH/EPHRIN signaling family throughout vertebrate evolution, and the considerable inherent functional redundancy that resulted, may have enabled this pathway to serve as a substrate for evolutionary variation. One example comes from crested pigeons, which display a head crest: neck feathers that grow toward the top of the head instead of down the neck as in other pigeon breeds. The head crest exhibits simple Mendelian inheritance, and the same genetic locus appears to be responsible in multiple crested pigeon breeds. This phenotype is caused by a variant in the catalytic loop of EPHB2, which is predicted to affect kinase activity resulting in disruption in the patterning of feather placode polarity and inversion of feather bud outgrowth (Shapiro et al., 2013). A similar feather phenotype in ringneck doves is also caused by an amino acid substitution that reduces EPHB2 kinase activity, indicating that evolutionary convergence impacting EPH/EPHRIN signaling drives phenotypic variation in distinct species, suggesting a possible evolutionarily conserved function of EPHB2 in feather bud patterning in these species, and possibly in other birds (Vickrey, Domyan, Horvath, & Shapiro, 2015). As compound loss of EPHB2 and EPHB3 results in skeletal and axon guidance phenotypes, function of these receptors in key developmental processes is also likely to be overlapping in avians, allowing the adoption of additional functions and contributing to phenotypic diversity.

Our recent morphometric studies of an allelic series of *Ephb1*; *Ephb2*; *Ephb3* compound mutant mouse embryos, revealed both quantitatively additive shared effects on head shape as well as qualitatively distinct effects of loss of function of each receptor (Niethamer et al., 2020; Mincer, Niethamer, Teng, Bush, & Percival, 2022). $Ephb2^{-/-}$; $Ephb3^{-/-}$ compound homozygotes display a severe cleft palate phenotype with high penetrance

in mice which is incompatible with life (Orioli, Henkemeyer, Lemke, Klein, & Pawson, 1996; Risley, Garrod, Henkemeyer, & McLean, 2009), these results suggest that partial functional redundancy between EPH receptors ensures viability allowing partial divergence of receptor function, which may contribute to face shape variation in mammals.

3. Cellular mechanisms underlying EPH/EPHRIN-mediated cell sorting and boundary formation

Active mechanisms are employed by the embryo to establish and maintain boundaries between cell populations to withstand constant cell movements that would otherwise cause cell intermingling. Indeed, EPH/EPHRIN signaling often acts to establish and/or enforce boundaries in numerous contexts in development, including gastrulation, rhombomeres and elsewhere in the nervous system, somites, craniofacial primordia, and in the development of endodermal organs, and has been reviewed extensively (Fagotto, Winklbauer, & Rohani, 2014; Kindberg & Bush, 2019; Niethamer & Bush, 2019). In addition to these roles in early morphogenesis, EPH/EPHRIN signaling contributes to boundary formation and cellular organization of germinal centers in the immune system (Laidlaw et al., 2017; Lu, Shih, & Qi, 2017).

Our understanding of the cellular mechanisms by which EPH/EPHRIN signaling regulates cell sorting and tissue separation has continually evolved together with our understanding of the general processes of cell sorting. Extensive reviews of general mechanisms governing cell sorting can be found elsewhere (Cayuso, Xu, & Wilkinson, 2015; Fagotto, 2014; Kindberg & Bush, 2019), but in brief, cell sorting has long been known as a crucial driver of cellular self-organization during development (Townes & Holtfreter, 1955). This process can be driven by qualitative or quantitative differences in cell-cell adhesion strength between two populations of cells (termed selective and differential adhesion, respectively) (Duguay, Foty, & Steinberg, 2003), or by negative effects on the strength of cell-cell contacts exerted by cortical actomyosin cytoskeletal contractility (cortical tension) (Krieg et al., 2008; Maitre et al., 2012). Together, these intercellular forces are thought to regulate the interfacial tension (the force of attraction at an interface) to achieve differences in cell-cell contact strength, a unifying summation that is termed the differential interfacial

tenson hypothesis (DITH) (Brodland, 2002; Winklbauer, 2015). These biophysical concepts constitute the founding principles upon which our understanding of EPH/EPHRIN-driven cellular organization are based.

The discovery of the importance of EPH/EPHRIN signaling in cell sorting originated with studies of hindbrain rhombomere segmentation in zebrafish (Xu, Alldus, Holder, & Wilkinson, 1995; Xu, Mellitzer, Robinson, & Wilkinson, 1999). The hindbrain rhombomeres are distinct compartments that are initially patterned by morphogen signaling and then refined, and physically separated, by cell sorting behaviors at rhombomere boundaries. EPHs and EPHRINs are expressed in a reciprocal pattern of expression in alternate rhombomeres, creating EPH/EPHRIN heterotypic interfaces at each rhombomere boundary; disruption of EPH/EPHRIN signaling results in inappropriate intermixing of cells from neighboring rhombomeres (Kindberg & Bush, 2019; Krumlauf & Wilkinson, 2021; Xu et al., 1995). It was recognized from the very earliest studies that EPH/EPHRIN signaling could drive cell sorting by repulsion (Xu et al., 1999). When ephrin-B2 expression was driven in cells of rhombomeres r3/r5, which normally express EphA4 and not ephrin-B2, ephrin-B2 expressing cells were repelled to the boundaries of these rhombomeres; conversely, cell sorting also occurred when the extracellular domain of EphA4 was overexpressed to activate ephrin-B2 reverse signaling (Xu et al., 1999). These, and other similar results suggested that bidirectional signaling drives cell sorting at rhombomere boundaries through cell repulsion (Cayuso et al., 2015).

3.1 EPH/EPHRIN signaling and actomyosin tension in cell sorting

The broad concept of a repulsive EPH/EPHRIN positional signal comprises more specific underlying mechanisms, such as an effect of EPH/EPHRIN signaling on repolarization and migration of cells upon contact, an impact on strength of cell contact in the context of random cell movement, or some combination of the two (Cayuso et al., 2015; Kindberg & Bush, 2019). As these detailed cellular mechanisms are difficult to distinguish *in vivo*, we recently took a reductionist approach in which we placed two HEK293 cells in low adhesion agarose plates and assessed their extent of contact by measuring their cell-cell contact angles as a proxy for their interfacial tension in the absence of cell-substrate contact (Kindberg et al., 2021). We found that while EPHB2-EPHB2 or EPHRIN-B1-EPHRIN-B1 homotypic cell pairs exhibited extensive cell-cell contacts reflecting low

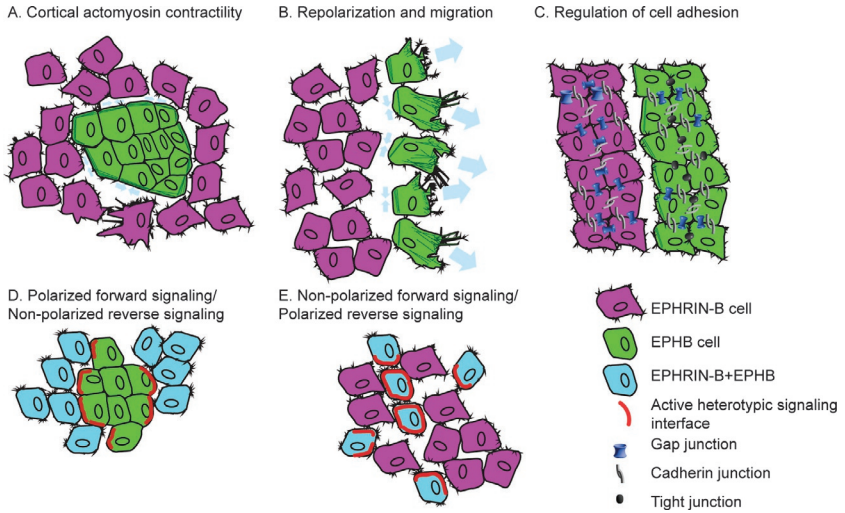

Fig. 3 Cellular behaviors driving EPH/EPHRIN-mediated cell sorting. Schematic simplification of non-exclusive cellular mechanisms that have been proposed to drive cell sorting. (A) Cortical actomyosin contractility within EPHB cells at heterotypic cell interfaces reduces the strength of heterotypic cell contacts and simultaneously drives increased homotypic cell contacts. (B) Localized collapse of EPHB cells at heterotypic cell contacts results in cell repolarization and directed migration of EPHB cells away from EPHRIN-B cells. (C) Cell-cell adhesion is regulated by EPHRIN-B through multiple mechanisms including forward signaling recruitment of ADAM10 metalloprotease and cleavage of E-cadherin at the heterotypic interface and EPHRIN-B binding to Cx43 and prevention of the formation of gap junctions at heterotypic interfaces. EPHRIN-B binding to Par-3 (not shown) prevents formation of tight junctions within EPHRIN-B cells. (D, E) Schematic representation of the concept of "Polarized" and "Non-polarized" signaling. (D) When all cells express EPHB, but are mosaic for expression of EPHRIN-B, polarized forward signaling, and non-polarized reverse signaling occur. (E) When all cells express EPHRIN-B, but are mosaic for expression of EPHB, non-polarized forward signaling, and polarized reverse signaling occur. In (E, F), productive forward signaling interfaces are highlighted in yellow to demonstrate that polarized forward signaling allows EPHB cells to differentiate between heterotypic and homotypic interfaces, whereas non-polarized forward signaling does not.

interfacial tension, EPHB2-EPHRIN-B1 heterotypic cell pairs exhibited extremely reduced cell contacts, reflecting increased interfacial tension. Inhibition of actomyosin contractility increased heterotypic cell contacts, and heterotypic cell cultures had increased stiffness when measured by atomic force microscopy, consistent with the notion that EPHB2/EPHRIN-B1 heterotypic interfacial tension is increased through effects on the cortical actomyosin cytoskeleton (Fig. 3A).

Larger scale cell mixing experiments, where EPHRIN-B1 cells are mixed with EPHB2 cells, resulting in cell sorting, also reflects the

requirement of actomyosin contractility. As for individual cell-cell contacts, inhibition of key regulators of actomyosin contractility including RhoA, Rho kinase (ROCK), Myosin light chain kinase (MLCK), or non-muscle myosin II (NMII), drastically reduced cell sorting in culture, and dominant negative disruption of ROCK, or genetic loss of function of non-muscle myosin resulted in a loss of cell sorting *in vivo*, indicating that actomyosin contractility is indeed crucial for this process (Kindberg et al., 2021; O'Neill et al., 2016; Tanaka, Kamo, Ota, & Sugimura, 2003). These results also fit with observations made during tissue separation in *Xenopus* gastrulation. In this system the ectoderm and mesoderm both express both EPHs and EPHRINs, and antiparallel EPH/EPHRIN forward signaling between them results in repeated rounds of attachment and detachment to facilitate migration of the mesoderm on the ectoderm, while maintaining tissue separation (Rohani et al., 2011). In this study, detachment required EPH/EPHRIN regulation of actomyosin contractility by RhoA at the tissue interface and was therefore one of the first studies to indicate that EPH/EPHRIN boundary formation is regulated by EPH/EPHRIN effects on cortical contractility. Interestingly, in our two-cell system, we also observed heterotypic EPHB2-EPHRIN-B1 cell pairs moving through phases of sparse or close contact over time, indicating that EPH/EPHRIN impacts on interfacial tension are dynamic in nature (Kindberg et al., 2021). This may occur through impacts of EPH/EPHRIN signaling on cadherin clustering (see also below), or through feedback regulation wherein close cell contact promoted by cadherin-based adhesion allows increased EPH/EPHRIN activation, thereby promoting actomyosin contractility-driven minimization of contact, which may in turn be terminated at the heterotypic interface through endocytosis (Fagotto, 2014; Gaitanos et al., 2016; Kindberg et al., 2021; Rohani et al., 2011; Zimmer, Palmer, Köhler, & Klein, 2003).

We proposed that EPH/EPHRIN-mediated cortical actomyosin contractility regulates cellular organization through its impact on cell-contact strength, but it is alternatively or additionally possible that disruption of actomyosin contractility might prevent cell sorting by preventing cell migration, thus preventing repulsive reorganization (see below). However, adding actomyosin contractility inhibitors Y27632 and ML7 after completion of cell sorting resulted in dramatic re-intermixing of EPHB2 and EPHRIN-B1 cells, indicating that cells still move in these conditions and that actomyosin contractility is instead required to maintain EPH/EPHRIN interfacial boundaries (Kindberg et al., 2021). These findings fit well with the previous observations of actomyosin enrichment at

EPH/EPHRIN boundaries in zebrafish rhombomeres, the *Drosophila* wing disc, the mouse secondary palate mesenchyme, and at the *Xenopus* ectoderm/mesoderm boundary during gastrulation (Calzolari, Terriente, & Pujades, 2014; Monier, Pélissier-Monier, Brand, & Sanson, 2010; O'Neill et al., 2016; Rohani et al., 2011; Umetsu, Dunst, & Dahmann, 2014).

Of course, as a predominantly cell-cell contact mediated signaling system, productive EPH/EPHRIN signaling occurs at the heterotypic interface of cells expressing an EPHRIN and EPH; as such, it is the differential between homotypic and heterotypic interfacial tensions as established by cortical actomyosin contractility that governs cell sorting through this model. This property of EPH/EPHRIN-driven cell sorting was demonstrated by Canty et al. in 2017, who expressed that a differential in homotypic interfacial tension between two cell populations need not exist to achieve heterotypic interfacial tension between the populations (Canty, Zarour, Kashkooli, François, & Fagotto, 2017). It is notable, though, that in our studies in which we reduced cadherin-based cell-cell adhesion through use of calcium-free media, we revealed a distinct cortical tension mechanism that mediates EPHB2-EPHB2 cell contact, suggesting that differential homotypic cell affinities could also contribute to cellular self-organization, at least in this cell system (Kindberg et al., 2021). Consistent with this finding, increased homotypic cell density in EPH receptor expressing cells when mixed with EPHRIN-B1 expressing cells has been observed in multiple studies (Kindberg et al., 2021; Taylor et al., 2017). Further, when we performed mixing of 3 cell types: EPHB2-expressing, EPHRIN-B1 expressing, and wtHEK293 cells, not only were the EPHB2 cells sorted out from EPHRIN-B1 cells, but wtHEK293 cells were also eliminated from EPHB2-expressing cell groups, suggesting that forward signaling into EPHB2 cells also drove non-autonomous exclusive sorting of signaling-inert cells (Kindberg et al., 2021). Further, EPHB2 kinase activity is required for the compaction of EPHB2 cell groups when mixed with EPHRIN-B1 expressing cells (Taylor et al., 2017). Together these findings suggest the possibility that a forward signaling effect on cellular cohesion might contribute to elimination of EPHRIN-expressing cells from EPH-expressing compartments, for example, as observed for ephrin-B2 expressing cells in the zebrafish hindbrain (Kemp, Cooke, & Moens, 2009; Taylor et al., 2017; Xu et al., 1999). *In vivo* experiments interrogating EPH/EPHRIN cell segregation in endoderm/mesoderm separation during *Xenopus* gastrulation, in the neural folds of mouse embryos, and in cell culture support that EPH receptor forward

signaling can be sufficient to drive cell sorting (Fuller, 2003; O'Neill et al., 2016; Rohani, Parmeggiani, Winklbauer, & Fagotto, 2014; Wu, Ashlin, Xu, & Wilkinson, 2019), though a more minor role from phosphorylation-dependent reverse signaling also contributes (Jorgensen et al., 2009; Rohani et al., 2011; Wu et al., 2019).

3.2 EPH/EPHRIN signaling and repulsive cell migration in cell sorting

In the above studies, EPH/EPHRIN may drive cell sorting through cortical actomyosin contractility or cadherin-based effects on cell-cell contact strength in the presence of random movement of cells. However, repolarization and directed migration has also been predicted by mathematical modeling to cause cell sorting (Fig. 3B) (Aharon et al., 2014). In fact, effects on cell contact strength may occur in combination with repulsive migration, defined as a cell repolarizing its protrusions and migrating away from a heterotypic cell type, akin to contact inhibition of locomotion (Kindberg & Bush, 2019; Taylor et al., 2017). This scenario fits well with the repeated demonstration of the importance of EPH/EPHRIN signaling in regulation of cell and axon migration during development (Krull, 2010). Heterotypic repulsive migration has been observed for cells cultured at low density, wherein EPHB2-expressing cells repolarize and increase their speed of migration away from an EPHRIN-B1 expressing cell (Poliakov, Cotrina, Pasini, & Wilkinson, 2008; Taylor et al., 2017). EPH/EPHRIN heterotypic boundaries can have different effects on cell polarity when cells are at high density. When human epithelial ARPE-19 cells expressing EPHRIN-B1 or EPHB3 were micropatterned into distinct regions and then allowed to intermix, they maintained their boundaries and polarized their actin cytoskeleton, microtubule organizing centers and cell shapes parallel to the EPHRIN-B1/EPHB3 interface, and cells moved along the interface, rather than away from it (Javaherian et al., 2017). These data suggest the intriguing possibility that in addition to regulating cell intermixing, EPH/EPHRIN expression boundaries may also contribute to coordinating planar cell polarity across a tissue.

Heterotypic repulsive migration, driven by phosphorylation-dependent reverse signaling, can also occur in EPHRIN-B1 expressing cells, and contributes to preventing intermixing of EPHB2 and EPHRIN-B1 expressing cells in less potent manner than forward signaling (Wu et al., 2019). In a recent study, the ability of "polarized" vs "non-polarized" forward and reverse signaling was examined. Polarized forward signaling was defined as the

situation in which EPH receptor expressing cells encounter both EPHRIN-expressing and non-expressing cells, whereas non-polarized forward signaling was defined as the situation in which EPH receptor expressing cells only encounter EPHRIN-B1-expressing cells (Fig. 3D and E). In this scenario, polarization of the reverse signal is the inverse of forward; polarized forward signaling occurs together with non-polarized reverse signaling; non-polarized forward signaling occurs with polarized reverse signaling (Fig. 3D and E). Non-polarized forward signaling and polarized reverse signaling (i.e. achieved by combining EPHB2; EPHRIN-B1-expressing cells signaling with EPHRIN-B1-expressing cells), did not drive sorting as robustly as polarized forward signaling (i.e. EPHB2; EPHRIN-B1-expressing cells signaling with EPHB2-expressing cells) (Fig. 3E) (Wu et al., 2019). Together, these results suggest that forward signaling allows EPHB2 cells to detect and respond to a heterotypic interface. It is worth pointing out that maintaining EPHB2 and EPHRIN-B1 co-expression in culture also resulted in reduced EPHB2 expression, probably through endocytosis of constantly activated receptor, a situation that has also been observed *in vivo* (Bush & Soriano, 2010; Wu et al., 2019). As such, these co-expressing cells were likely desensitized to EPHRIN-B1 at the time of mixing with EPHRIN-B1 cells. Nevertheless, based on the accumulated evidence, "non-polarized forward signaling" is not capable of driving cell sorting because a differential is not generated, as all EPHB2 cell-cell contacts are made with other EPHRIN-B1 expressing cells (Fig. 3E). This forward signaling differential may reflect the impact of cortical contractility on cell contact strength, a repulsive migratory cue, or both. Further, the above results indicate that reverse signaling is less potent than forward signaling, which drives repulsion at heterotypic relative to homotypic interfaces as a central mechanism for EPH/EPHRIN-driven cell sorting.

3.3 EPH/EPHRIN signaling and differential adhesion in cell sorting

EPH/EPHRIN signaling has also been proposed to regulate cell sorting by modulating cadherin-based intercellular adhesion, which in turn drives cell segregation through a differential adhesion mechanism (Fig. 3C) (Fagotto, Rohani, Touret, & Li, 2013; Solanas, Cortina, Sevillano, & Batlle, 2011). In colorectal cancer epithelial cells, E-cadherin accumulation was decreased at an EPHB3/EPHRIN-B1 heterotypic interface, and this effect was reversed by pan-metalloproteinase inhibition (Solanas et al., 2011). Further, EPHB3/EPHRIN-B1 cell sorting was reduced

by knockdown of ADAM10 or ADAM15 metalloproteinases, or by expressing a dominant-negative ADAM10 mutant lacking metalloproteinase activity. In the small intestine, EPHRIN-B1 expression in the villus opposes expression of EPHB2 in the crypt base and EPHB3 expression in the Paneth cells, and loss of EPHB2 and EPHB3 function result in Paneth cells becoming inappropriately positioned within the intestinal epithelium (Batlle et al., 2002). Dominant-negative loss of ADAM10 metalloprotease activity resulted in a Paneth cell intermixing phenotype that greatly mimicked the loss of EPHB3 in mice. These results formed the basis for a model in which EPHB3/EPHRIN-B1 signaling recruits ADAM10 to heterotypic interfaces to reduce cadherin-based cell adhesion (Solanas et al., 2011). A similar molecular mechanism has been recently proposed to regulate EPHA4/EPHRIN-B2-driven separation of inner pillar and outer pillar cells during the development of the cochlear sensory epithelium (Defourny, Peuckert, Kullander, & Malgrange, 2019). It is important to point out that ADAM metalloproteases can also cleave both A- and B-type EPHRINs and function to convert an adhesive molecular interaction to a repulsive cellular outcome in many contexts (Atapattu, Lackmann, & Janes, 2014; Hattori, Osterfield, & Flanagan, 2000; Janes et al., 2005; Ji et al., 2014; Wei et al., 2010). In *Xenopus*, ADAM13 cleavage of Ephrin-B1 and Ephrin-B2 is critical for Neural Crest Cell induction, and ADAM10 can cleave Ephrin-B2 upon loss of the interacting protein flotillin-1 during neural tube closure (Ji et al., 2014; Wei et al., 2010). Recently, it was shown that ADAM10 mediates cleavage of EPHRIN-B2 in human myofibroblasts to promote fibroblast chemotaxis skin and lung fibrosis (Lagares et al., 2017). Expression of dominant negative ADAM10 in a Co115 colorectal cancer cell line did not reveal dramatic differences in EPHB3 phosphorylation when mixed with EPHRIN-B1-expressing cells, however, so it was concluded that ADAM10 in that system functions specifically to mediate EPH/EPHRIN regulation of E-cadherin-based cell adhesion (Solanas et al., 2011).

EPH/EPHRIN signaling can also indirectly regulate cadherin-mediated cell adhesion through its impact on actomyosin contractility. In *Xenopus*, separation of the notochord and presomitic mesoderm is mediated by EphB4/ephrinB2 expression in the presomitic mesoderm and notochord, respectively, and morpholino knockdown of either resulted in inappropriate intermixing of the two cell types (Fagotto et al., 2013). EPH/EPHRIN signaling at this boundary mediates extensive actomyosin contractility that results in cell blebbing, but presomitic mesoderm and notochord cells

remain in contact, causing the authors to conclude that tissue separation is not directly caused by actomyosin contractility effects on cell contacts. Instead, the authors hypothesize that actomyosin contractility prevented the formation of cadherin clusters, thereby diminishing heterotypic cadherin-based adhesion relative to homotypic contacts amongst either cell type (Fagotto et al., 2013). In EPHB2/EPHRIN-B1 cell mixing experiments in HEK293 cells, knockdown of N-cadherin or depletion of cadherin-based adhesion through culture in low calcium media reduced cell-cell contacts generally, but neither disrupted cell sorting (Kindberg et al., 2021; Taylor et al., 2017). Further, N-cadherin knockdown decreased both homotypic and heterotypic repulsion as measured by cell contact time and increased protrusion collapse frequency, indicating that, at least in these cells, differential adhesion due to cadherins is not likely a major effector of EPH/EPHRIN-driven cell sorting in these cells (Taylor et al., 2017). These results suggest that EPH/EPHRIN-driven actomyosin contractility may impact interfacial tension and boundary formation through multiple mechanisms and the role of cadherin-based adhesion may be context-dependent.

EPH/EPHRIN signaling can regulate cell contact strength through multiple other mechanisms. In *Xenopus* early development, overexpression of Ephrin-B1 or its intracellular domain, leads to dissociation of blastomeres (Jones et al., 1998). This is likely because Ephrin-B1 binds to the cell polarity protein Par-6, sequestering it from the Par polarity complex (Par-3, Par-6, Cdc42, aPKC) and resulting in a loss of localized ZO-1 and a loss of epithelial tight junctions (Lee, Nishanian, Mood, Bong, & Daar, 2008). This event is regulated by tyrosine phosphorylation of the intracellular domain of Ephrin-B1, which prevents binding of Ephrin-B1 to Par-6 and restores tight junctions. In mouse craniofacial development, EPHRIN-B1 also regulates the formation of gap junctions through its interaction with Connexin 43 (Davy, Bush, & Soriano, 2006). At EPHB2/EPHRIN-B1 heterotypic interfaces, gap junction formation is prevented, effectively de-coupling EPHRIN-B1 expressing and non-expressing tissues and impacting craniofacial osteogenic differentiation. Similar regulation of gap junction formation occurs in zebrafish animal caps, where EPHB/EPHRIN-B interfaces show reduced gap junction formation and restricted communication across these boundaries (Mellitzer, Xu, & Wilkinson, 1999). As additional modes of cell contact regulation, EPH/EPHRIN regulation of tight and gap junctions, together with impacts on adherens

junctions and cortical contractility, determine the tension differential between heterotypic and homotypic interfaces, a critical part of EPH/EPHRIN boundary formation.

4. Nervous system development

EPH/EPHRIN signaling plays a vital role in the development of the vast complexity of the central nervous system architecture. In early development, EPH/EPHRIN signaling is critical for early neural patterning and neural tube morphogenesis. Its roles include EPH/EPHRIN-mediated cell sorting, as discussed above, which is crucial for processes including rhombomere boundary formation and proper distribution of Cajal-Retzius cells, which distribute throughout the cerebral cortex through EPH/EPHRIN contact-mediated repulsion (Krumlauf & Wilkinson, 2021; Villar-Cerviño et al., 2013). Later in development, through spatially nested and differing temporal levels of expression, and through use of multiple modes of signaling, EPH/EPHRIN signaling directs neuronal migration, differentiation, axon guidance and synaptic connectivity. As in other contexts, multiple signal transduction pathways, including Ras and Rho family GTPases are regulated by numerous signaling intermediaries, including guanine exchange factors GEFs such as EPHEXIN, INTERSECTIN, KALIRIN, VAV, and TIAM1 and GTPase activating proteins such as the chimaerins. These signaling pathways have been recently reviewed (Kania & Klein, 2016) and I will focus on recent studies that have further illuminated the diverse modes that EPH/EPHRIN signaling utilizes in nervous system development.

Two of the earliest known and most highly conserved vertebrate functions for EPH/EPHRIN signaling are the regulation of midline axonal crossing and axonal targeting. Several studies have revealed the importance of EPH/EPHRIN signaling in formation of the corpus callosum (CC), the largest interhemispheric axon tract in mammals (Bush & Soriano, 2009; Mendes, Henkemeyer, & Liebl, 2006; Orioli et al., 1996; Robichaux et al., 2016; Soskis et al., 2012). Whereas our previous study showed that EPHRIN-B1 expression in CC axons is required to receive a PDZ-dependent reverse signal from midline glial guidepost cells, a very recent study performing deletion of *Efnb1* within the dorsal telencephalon did not observe agenesis of the CC. Though the basis for this discrepancy is not clear, the more recent study revealed that EPHRIN-B1 is

dynamically regulated to prevent axonal midline re-crossing (Mire et al., 2018). EPHRIN-B1 increases in expression in axons following midline crossing and interacts with NRP1, a receptor for the axonal attractant SEMA3C, to silence SEMA3C/NRP1 signaling (Mire et al., 2018). This silencing allows growth of CC axons away from the midline following their crossing by effectively blinding them to the declining attractive SEMA3C gradient in the contralateral hemisphere. Whether this silencing activity requires the PDZ binding motif is not known, but N-glycosylation of the extracellular domain of EPHRIN-B1 was required, indicating this mechanism may be distinct (Mire et al., 2018).

A recent study further illuminated the role of EPHRIN-B1/EPHB signaling in axonal targeting following midline crossing of the CC. As receptors for the primary excitatory neurotransmitter in the brain, NMDA receptors (NMDAR), were presumed to play a critical role in CC formation through excitatory synaptic activity. However, in mice with cortex-specific knockout of GluN1, the essential subunit of the NMDAR, loss of NMDAR function led to loss of targeted callosal innervation of the S1/S2 border and an increase in callosal innervation in S1 during CC development. This was not due to excitatory activity and ion channel function of the receptor, but instead through physical interaction of EPHB2 with the NMDAR which resulted in stabilization of both proteins in the target neurons. EPHRIN-B1 loss specifically in projecting neurons resulted in axonal mistargeting in the contralateral cortex. It remains to be determined if the mechanism by which the callosal axons mis-target is through loss of repulsive cues from EPHB/EPHRIN signals, and if there is disruption of forward or reverse signaling or both. Though multiple studies have shown that NMDA and EPHB co-localize and physically interact (Dalva et al., 2000; Nolt et al., 2011), this is the first report demonstrating their collaborative function in neural circuit formation during development.

Neuronal target selection and determination of whether to initiate synaptogenesis must involve interpretation of repulsive and attractive cues by dendritic filopodia. Through use of a dual-color ratiometric indicator of EPHB kinase activity and manipulation of activation through use of a photoactivatable EPHB2, a recent study proposed that a dendrite's choice of whether to retract or to generate stable axo-dendritic contacts is governed by differences in the kinetics of EPHB2 activation (Mao et al., 2018). Dendritic filopodial retraction is initiated by a rapid increase in EPHB activation, while slower activation results in stabilization of contacts and synapse formation. In addition to providing the exciting insight that

EPH/EPHRIN signaling may serve both repulsive and attractive purposes in synaptogenesis, it also provides powerful new reagents for the spatio-temporal observation and manipulation of EPHB2 kinase activity that can be applied in diverse contexts.

In addition to regulating cellular position and proper neuronal connections, EPH/EPHRIN signaling also regulates cell fate specification and progenitor identity in neural development (Laussu, Khuong, Gautrais, & Davy, 2014). For example, in the vertebrate spinal cord, EPHRIN-B2 and EPHRIN-B3 are expressed in distinct domains: EPHRIN-B2 is expressed in progenitors of motor neurons (pMN) whereas EPHRIN-B3 is expressed in p3 progenitors that give rise to interneurons. Tissue-specific conditional loss of function of *Efnb2* resulted in normal initial pMN specification, but reduced maintenance of pMN neurons at later stages of development (Laussu et al., 2017). Conversely, *Efnb3* loss of function led to fewer p3 progenitor cells and increased pMN/p3 intermixing. Instead of an effect on relative cell proliferation rate, fate specification by EPHRIN-B2:EPHRIN-B3 appears to work in collaboration with SHH signaling, a key regulator of dorsoventral cell fates in the spinal cord, because compound $Efnb2^{+/-}$; $Shh^{+/-}$ mutant embryos also exhibited reduced pMN relative to p3 cell numbers while neither $Efnb2^{+/-}$ or $Shh^{+/-}$ heterozygosity had an effect on its own. This role in morphogen-dependent tissue patterning is especially interesting when considered in light of roles for EPH/EPHRINs in refining cell position following morphogenetic patterning, and suggests an additional way that these two events may be coupled.

During neural stem cell differentiation, genetic and metabolic changes are coordinated to specify distinct cellular properties that actualize distinct cell states. Activation of EPHB signaling was previously shown to promote adult neural stem cell differentiation, but the downstream signaling pathways were unclear (Ashton et al., 2012; Ottone et al., 2014). It has been recently shown that EPHRIN-B1/EPHB signaling inhibits expression of dihydrofolate reductase (DHFR), which is a key enzyme in the 1C folate metabolic pathway (Fawal et al., 2018). Lack of folate has long been linked to developmental abnormalities including neural tube defects, as well as other neurodevelopmental disorders, and 1C metabolism impacts epigenetic programs through histone and DNA methylation. Inhibition of DHFR by EPHB forward signaling leads to increased neural stem cell differentiation during development through altered 1C metabolism which modifies H2K4 methylation of key progenitor genes. These epigenetic changes lock cells into a differentiated state that is maintained and inherited in future

generations. As such, impacts of EPH/EPHRIN on 1C metabolism are propagated from cell signaling to transcriptional and epigenetic changes and may be relevant to neurological disorders involving folate deficiency and DHFR dysfunction.

5. Craniofacial and musculoskeletal development

Multiple EPH/EPHRINs regulate development of the skeleton, including roles in craniofacial morphogenesis. Numerous studies have demonstrated that EPH/EPHRIN signaling is crucial for guidance of the migratory cranial NCCs that compose most of the skeleton of the viscerocranium in vertebrates (Kindberg & Bush, 2019; Mellott & Burke, 2008b; Theveneau & Mayor, 2012). Notably, *EFNB1* is mutated in an X-linked condition called craniofrontonasal syndrome (CFNS) that affects craniofacial, axial skeletal, and neurological development (Compagni, Logan, Klein, & Adams, 2003; Twigg et al., 2004; Wieland et al., 2004). Patients with CFNS exhibit a shorter midface (frontonasal dysplasia), inappropriate fusion of calvaria of the skull (coronal synostosis), wide-set eyes (hypertelorism), and occasionally also exhibit cleft lip/palate, limb anomalies, and agenesis of the CC. CFNS is unusual in that despite being an X-linked condition, it affects females with heterozygous loss of function of *EFNB1* severely, whereas males with hemizygous loss remain mildly affected or unaffected. Targeted disruption of *Efnb1* in mice results in most of the same phenotypes as in humans, including increased severity in *Efnb1*$^{+/-}$ females, but with some differences; *Efnb1*$^{+/-}$ mutant mice do not exhibit coronal synostosis, but instead exhibit nasofrontal suture synostosis, and exhibit cleft palate only rather than cleft lip and palate (Bush & Soriano, 2010; Compagni et al., 2003; Davy, Aubin, & Soriano, 2004). It was immediately recognized that this increased heterozygous severity was likely related to cell sorting of mosaic EPHRIN-B1 expressing and non-expressing cells generated by random X chromosome inactivation, which we have since confirmed using a human induced pluripotent stem cell (hiPSC) model (Compagni et al., 2003; Davy et al., 2004; Niethamer et al., 2017).

How cell sorting occurred in this context, and how it caused dysmorphology, was more mysterious. It was initially proposed that cell sorting upon heterozygous loss of *EFNB1* caused craniofacial phenotypes by disrupting the NCC/mesoderm boundary or by disrupting NCC migration

at early stages (Davy et al., 2004; Twigg et al., 2004). Recently, we found that inducing *Efnb1* heterozygosity in the post-migratory NCC-derived craniofacial mesenchyme can drive cell sorting and craniofacial dysmorphology similar to what is observed upon germline deletion of *Efnb1*, suggesting that CFNS pathology may be caused by later effects on craniofacial shape (Niethamer et al., 2020). Using the secondary palate as a model for EPHRIN-B1 driven tissue shape change, we found that boundaries between EPHRIN-B1 expressing and non-expressing domains correlate with dysmorphology in a manner that is reminiscent of cell aggregates generated in culture. Specifically, when EPHRIN-B1 expressing and EPHB2 expressing cells are allowed to aggregate, they minimize contact with each other in accordance with a minimization of interfacial tension, causing them to be irregular in shape, just as the secondary palatal shelves are in $Efnb1^{+/-}$ mutants (Bush & Soriano, 2010; Kindberg et al., 2021; Niethamer et al., 2020). We speculate that polydactyly, which is also consistently observed in $Efnb1^{+/-}$ mutants, may also relate to tissue shape changes initiated at EPHRIN-B1 expression boundaries.

These data suggest that tissue dysmorphology in CFNS may also be caused by EPH/EPHRIN-driven heterotypic interfacial tension. We found that blocking ROCK and MLCK activity could relax tortuous tissue shape changes in aggregates, consistent with these changes also being driven by effects on cortical actomyosin contractility (Kindberg et al., 2021). We and others also demonstrated that EPHB2; EPHB3 combined loss of function recapitulated most of the $Efnb1^{Y/-}$ hemizygous craniofacial phenotype. Interestingly, using quantitative three-dimensional morphometric analysis, we found that even compound loss of function of all of the EPH receptors that bind to EPHRIN-B1 in $Ephb1^{-/-}$; $Ephb2^{-/-}$; $Ephb3^{-/-}$ mutant mice does not recapitulate $Efnb1^{+/-}$ phenotypic severity, consistent with cell sorting causing these more severe phenotypes (Niethamer et al., 2020; Risley et al., 2009). Though $Efnb1^{+/-}$; $Ephb1^{-/-}$; $Ephb2^{-/-}$; $Ephb3^{-/-}$ mutant embryos still exhibited cell sorting, it was dramatically reduced, indicating either that EPHRIN-B1 has EPH-independent functions in segregation, or that EPH receptors that lack substantial biochemical affinity may also contribute (Niethamer et al., 2020). It remains mysterious why CFNS phenotypes are so highly stereotyped. Although this may relate to constraint of dysmorphology to the domains of expression of EPHRIN-B1, we demonstrated using quantitative morphometric approaches that many $Efnb1^{+/-}$ craniofacial shape changes were present in $Efnb1^{Y/-}$ embryos as well, albeit less severe, which would not necessarily be expected if CFNS were a result

of random dysmorphology constrained to specific structures. It is therefore likely that additional morphogenetic mechanisms contribute to CFNS dysmorphology.

EPH/EPHRIN signaling also has later roles in skeletal development, and skeletal phenotypes in CFNS, the only human skeleton condition known to be caused by EPH/EPHRIN dysfunction, may relate to these functions (Arthur & Gronthos, 2021). Whereas cartilage segmentation phenotypes observed upon loss of function of *Efnb1* in the limbs and ribs in mice may relate to early patterning and progenitor cellular position, its disruption in osteoblast progenitor cells in *Osx-cre; Efnb1*$^{lox/lox}$ mice resulted in perturbed long-bone growth, reduction in osteoblast progenitor cells and osteoclasts, and disruption of growth plate structure (Compagni et al., 2003; Davy et al., 2004; Nguyen et al., 2016). Disruption of *Efnb1* function in osteoblasts using a Col1α2-Cre also resulted in skull and long bone defects and reduced bone formation and mineralization, whereas overexpression of EPHRIN-B1 within osteoblast progenitors resulted in increased bone thickness (Cheng et al., 2013; Xing, Kim, Wergedal, Chen, & Mohan, 2010). The function of EPHRIN-B1 in bone formation was found to relate to EPHRIN-B1 reverse signaling-driven dephosphorylation of the Taz transcription factor, resulting in an increase in expression of Osterix/Sp7, the master regulator of bone formation. Genetic disruption of the PDZ domain binding motif in *Efnb1*$^{\Delta V}$ mice did not result in apparent skeletal or bone defects, though the quality of bone was not examined in detail in that study (Bush & Soriano, 2009). Disruption of EPHRIN-B1 in osteoprogenitor cells also resulted in disruption of bone homeostasis with increased osteoclasts resulting in osteoporosis and delayed fracture healing (Arthur et al., 2018; Arthur, Paton, Zannettino, & Gronthos, 2020). The function of EPHRIN-B1 in regulating osteoclast numbers appears to relate to a forward signaling function, indicating that bidirectional EPHRIN-B1/EPHB2 signaling between multiple osteogenic cell populations may regulate bone homeostasis (Arthur & Gronthos, 2021).

In contrast, disruption of *Efnb2* in the same osteoprogenitor cell types in *Osx-Cre; Efnb2*$^{lox/lox}$ mutant mice resulted in impaired osteoclastogenesis at the growth plate, which in turn resulted in increased trabecular bone formation and greater cortical bone thickness (Tonna et al., 2016). This Cre drives recombination in both osteoblasts and chondrocytes, where loss of EPHRIN-B2 resulted in hypertrophic chondrocytes that failed to promote cartilage degradation. The impaired destruction of cartilage during endochondral ossification is a hallmark of the neonatal osteopetrosis phenotype that these mice exhibited, though this phenotype resolved by six weeks

of age. EPHRIN-B2/EPHB4 signaling also regulates bone homeostasis in adult mice by controlling the balance between bone resorption by osteoclasts and bone formation by osteoblasts (Zhao et al., 2006). Osteoclast progenitors primarily express EPHRIN-B2, where it is regulated by NFATC1, a master regulator of osteoclast differentiation, and signal with osteoblast progenitors that express EPHB4. PDZ-dependent reverse signaling from osteoblasts to osteoclast progenitors leads to inhibition of osteoclast differentiation through negative feedback on *Nfatc1* transcription. Forward signaling from osteoclasts to osteoblast progenitors increases osteoblast differentiation and enhances bone formation. In this way, direct cell-cell interaction between osteoblasts and osteoclasts may facilitate the dynamic transition between bone resorption and formation states. Subsequent studies refined our understanding of EPHB4/EPHRIN-B2 in bone formation and revealed that insulin-like growth factor (IGF-I) signaling is required for expression of EPHRIN-B2 in osteoclasts and expression of EPHRIN-B2 and EPHB4 in osteoblasts and chondrocytes and that EPHRIN-B2/EPHB4 mediate the effects of IGF-I signaling on endochondral bone formation (Wang et al., 2014). Furthermore, EPHRIN-B2 loss from chondrocytes resulted in reduced chondrocyte-osteoblast trans-differentiation and decreased bone formation during fracture repair suggesting a new avenue for therapeutic benefit (Wang et al., 2020).

Recent studies have revealed that EPH/EPHRIN signaling also regulates myogenic differentiation (Arnold et al., 2020). Targeted disruption of *Epha7* in mice resulted in hindlimb muscles with fewer and smaller myofibers, that exhibited slower muscle regeneration upon injury. EPHA7 is expressed by differentiated myocytes during muscle regeneration, whereas proliferating myoblasts express the signaling partner EPHRIN-A5. Treatment of satellite muscle stem cells with exogenous EPHA7 extracellular domain resulted in induction of myogenic differentiation in culture, suggesting that EPHA7 signals from differentiated myocytes to myoblasts to regulate their differentiation. Though the reverse signaling mechanisms utilized in this context are not yet known, this work suggests that contact-mediated EPH/EPHRIN signaling contributes to a "community effect" that drives coordinated myogenic differentiation to ensure a sufficient number of myocytes for fusion and formation of a muscle fiber.

6. Conclusions and perspectives

Through the continual discovery of new and surprising developmental functions of EPH/EPHRIN signaling as well as the identification

of both unique and overlapping signaling mechanisms employed, our awareness of the complexity of the EPH/EPHRIN signaling family continues to increase. Though mechanisms such as suppression of RAS/MAPK to modulate differentiation, and activation of RhoA to regulate actomyosin dynamics are shared across multiple contexts, signaling functions are just as often cell type-specific and context-dependent. As such, consideration of EPH/EPHRIN signaling from a universal perspective may have marginal utility, and continued, detailed *in vivo* study of signal transduction mechanisms is warranted. For example, while beautiful structural biology and cell culture work has provided great insight into the multimerization of EPH/EPHRIN signaling complexes and the potential for homo- and hetero-oligomers, future work that manipulates multimerization *in vivo* will be required for an understanding of how this pathway signals in the embryo, and whether context-specific regulation of these properties is at play. Approaches for the *in vivo* examination of homo- vs. hetero-oligomeric states, for example through applying bimolecular fluorescence complementation, may begin to provide insights into when and how distinct oligomeric states are utilized (Rogers & Fantauzzo, 2021). The *in vivo* context-specific utilization of proximal signaling partners also remains a major gap in knowledge. New spatial and single-cell proteomic methods applied to embryonic tissues may enlighten our understanding of overlapping functions as well as the shared and distinct context-specific downstream signal transduction pathways that allow specificity of signaling output to emerge from this complex signaling family.

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