NOVEL NON-REPULSIVE OUTCOMES FROM EPHRIN-B

REVERSE SIGNALING

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For my parents, Elizabeth, and Mary

NOVEL NON-REPULSIVE OUTCOMES FROM EPHRIN-B REVERSE SIGNALING

by

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“Focus”

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NOVEL NON-REPULSIVE OUTCOMES FROM EPHRIN-B REVERSE SIGNALING

Publication No. _____

Christopher Dravis, Ph.D.

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Supervising Professor: Mark Henkemeyer, Ph.D.

In this dissertation, I present a detailed characterization of the physiological roles for reverse signaling through B-subclass ephrins during embryonic development. The stereotypical outcome of Eph-ephrin signaling has long been established as one of cell-cell repulsion, elicited through a localized breakdown of the actin cytoskeleton. In contrast to this dogma, I have found that ephrin-B reverse signaling is instead necessary for mediating cell-cell adhesion events during several critical midline closure events. I demonstrate that mice with germline mutations specifically disrupting the ability of

ephrin-B2 to conduct cell autonomous signals present with defects in urorectal septation of the hindgut, tubularization of the urethra, tracheoesophageal septation of the foregut, closure of the palatal shelves, and closure of the embryonic eyelid, defining roles for ephrin-B2 reverse signaling in each of these developmental events. Further, I show that mice with germline mutations either deleting *EphB2* or similarly impairing the ability of EphB2 to conduct cell autonomous forward signals indicate that EphB2 acts non-cell autonomously to activate ephrin-B1 reverse signaling in closure of the ventral body wall.

The developmental malformations in these mutant animals are each hallmarked by the failure of lateral mesenchymal folds to properly adhere, typically at the midline, which is difficult to reconcile with the canonical outcome of cell-repulsion from Eph-ephrin signaling. Consistent with a role in eliciting cell-cell adhesion at these septation events, I show that EphB and ephrin-B molecules are expressed in the epithelia where adhesion will take place. Moreover, my data specifically localizes ephrin-B reverse signaling to these adherent epithelia. Finally, an *in vitro* palatal shelf fusion assay used to determine the role of Eph-ephrin signaling in these developmental events indicates clear roles for ephrin-B2 reverse signaling in cell-cell adhesion. Taken together, my data leads me to propose that ephrin-B reverse signaling is not only capable of cell repulsion, but is also able to elicit cell-cell adhesion responses, which are employed in a bevy of adhesion-based septation events during embryonic development.

Finally, my analysis of reverse signaling-deficient *ephrin-B2* mice also indicates an additional novel, non-repulsive role for ephrin-B reverse signaling in mediating ionic homeostasis within the inner ear. I show that these *ephrin-B2* mutant mice present with a circling or “waltzing” phenotype due to severe defects within the vestibular apparatus.

My data shows that the disruption of bidirectional signaling between EphB2 and ephrin-B2 result in malformed structures within the vestibular apparatus and abnormal endolymph fluid. Further, I show that EphB2 and ephrin-B2 are expressed on non-motile, secretory epithelia within the inner ear, suggesting that these molecules play important roles in maintaining the proper volume and ionic makeup of the endolymph fluid running through the vestibular apparatus. My data leads me to propose that ephrin-B2 reverse signaling is therefore not only capable of regulating the cytoskeleton to produce either cell repulsion or cell adhesion outcomes, but that these reverse signals can mediate cellular responses independent of cytoskeletal dynamics, such as ionic homeostasis.

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Prior publications that appear in this work

Dravis, C., Yokoyama, N., Chumley, MJ., Cowan, CA., Silvany, RE., Shay, J., Baker, LA., Henkemeyer, M. Bidirectional signaling mediated by ephrin-B2 and EphB2 controls urorectal development**. Dev Biol**. 271(2), 272-290 (2004).

Dravis, C., Wu, T., Chumley, M.J., Yokoyama, N., Wei, S., Wu, D.K., Marcus, D.C. and Henkemeyer, M. EphB2 and ephrin-B2 regulate the ionic homeostasis of vestibular endolymph. **Hearing Research** 223, 93-104 (2007).

Dravis, C. and Henkemeyer, M. Ephrin-B Reverse Signaling Mediates Tracheoesophageal Septation, Palatal Shelf Fusion, and Abdominal Body Wall Closure at the Embryonic Midline. *Submitted*.

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Abi	Abl interacting
ADAM	A-Disintegrin-And-Metalloprotease
AOB	Accessory Olfactory Bulb
aPKC	Atypical Protein Kinase C
Arg	Abl-Related Gene
AVC	Anterior Vertical Canal
β-gal	Beta-Galactosidase
CAP	Cbl Associated Protein
cDNA	Complementary Deoxyribonucleic acid
CST	Corticospinal Tract
DC	Dark Cells
DG	Dentate Gyrus
Dox	Doxycycline
EMT	Epithelial to Mesenchymal Transition
EOB	Eyelid Open at Birth
EPH	Erythropoietin-Producing Hepatocellular
Ephexin	Eph-interacting exchange protein
ephrin	Eph family Receptor Interacting Protein
GAP	GTPase Activating Protein
GDP	Guanosine-5'-diphosphate
GEF	Guanine nucleotide Exchange Factor
GIT	G Protein-Coupled Receptor Kinase-interacting Protein

GPI	Glycosylphosphatydylinostitol
Grb4	Growth Factor Receptor Bound 4
GRIP	Glutamate Receptor Interacting Protein
GT	Genital Tubercle
GTP	Guanosine-5'-triphosphate
HC	Horizontal Canal
HnRNPK	Heterogeneous Nuclear Ribonucleoprotein Kinase
HSV-TK	Herpes Simplex Virus Thymidine Kinase
IF	Immunofluorescence
JAM	Junctional Adhesion Molecule
JMS	Juxtamembrane Segment
Kan	Kanamycin
kb	Kilo-Base Pair
KD	Kinase Domain
kDA	kiloDalton
KUZ	Kuzbanian
LGN	Lateral Geniculate Nucleus
LMW-PP	Low Molecular Weight Phosphatase
Lnx	Ligand of Numb X
LTEC	Laryngotracheoesophageal Cleft
MOE	Main Olfactory Epithelium
NCC	Neural Crest Cells
Neo	Neomycin

PAR-3	Partitioning-defective Protein 3
Pick1	Protein Interacting C Kinase 1
PDZ	Post-synaptic Density-95/Discs Large/Zonula Occludens-1
PTP-BL	Protein Tyrosine Phosphatase BAS-Like
Ptpro	Protein Tyrosine Phosphatase Receptor Type O
PVC	Posterior Vertical Canal
RGC	Retinal Ganglion Cells
RGS	Regulator of G-protein Signaling
RTK	Receptor Tyrosine Kinase
SAM	Sterile-alpha Motif
SDF-1	Stromal-Cell-Derived Factor 1
SFK	Src Family Kinase
SGZ	Subgranular Zone
SH2	Src Homology 2
SH3	Src Homology 3
TC	Transitional Cells
URS	Urorectal Septum
VNO	Vomeronasal Organ

Chapter 1

Introduction

The development of a mature organism from a single fertilized egg requires an innumerable series of cell migration and cell proliferation events orchestrated through a symphony of detailed molecular instruction. Most of this instruction comes from cell surface receptors sensing extracellular cues and feeding that information into the cell via signal transduction pathways. Often these signaling events occur intercellularly, at sites of cell-cell contact, and in these cases one of the more significant means of signal transduction occurs through events mediated by the large family of Eph receptor tyrosine kinases, and their membrane-bound ligands, the ephrins. In this chapter, I introduce the Ephs and ephrins. I discuss the structure of these molecules and the means by which both Eph and ephrin are capable of cell autonomous signal transduction. I next highlight a number of key molecular interactions that the Ephs and ephrins use to convert external stimuli into cellular response. I then discuss means by which this signal is terminated. Finally I delve into what effects signaling through these molecules elicit within the cell,

and how these effects have been incorporated into numerous physiological roles during development.

Eph receptors and their ephrin ligands

The Eph receptor field was born in 1987, when efforts to uncover tyrosine kinases involved in cancer identified erythropoietin-producing hepatocellular (EPH) receptor, a novel tyrosine kinase named after the hepatic carcinoma cell line from which its cDNA was isolated (Hirai et al., 1987). From this initial discovery, the Eph receptors have grown into the largest known family of receptor tyrosine kinases (RTKs), with 16 receptors in all covering two subclasses (EphA1-10 and EphB1-6), 14 of which are found in mammals (Figure 1.1) (Drescher, 2002). In general, the Eph receptors share a number of structural features. Starting from the plasma membrane and working outwards, the extracellular domain of the Eph receptor is composed of two fibronectin type-III repeats, a cysteine-rich region, and most importantly, an N-terminal globular domain of approximately 180 amino acids through which the Eph receptors interact with their ephrin ligands (Figure 1.2) (Himanen et al., 1998; Labrador et al., 1997). In the cytoplasm, following the single-pass hydrophobic transmembrane domain, the Eph receptors in order possess a juxtamembrane segment (JMS) containing two conserved tyrosine residues that regulate the kinase activity of the receptor, a canonical tyrosine kinase catalytic domain (KD), a sterile alpha motif (SAM), and a post-synaptic density-95/discs large/zonula occludens-1 (PDZ)-binding motif at the extreme C-terminus. Despite the similarities in structure, the Eph receptors have been separated into A- or B-subclass based on sequence similarity and ligand preference (Figure 1.1 dentogram). EphA receptors are more similar to each other

than they are to EphB receptors, and they preferentially bind to the five glycosylphosphatydylinostitol (GPI)-linked ephrin ligands, while EphB receptors are more similar to each other than EphA receptors, and they preferentially bind to and are activated by transmembrane ephrin ligands (Orioli and Klein, 1997). Exceptions to this latter rule include the cross-activation of the receptors EphB2 and EphA4, which can be activated by both GPI-linked and transmembrane ephrin ligands (Gale et al., 1996; Himanen et al., 2004).

The ligands for the Eph receptors are the ephrins (Eph family receptor-interacting proteins), which consist of a class of nine molecules (ephrin-A1 to -A6 and ephrin-B1 to -B3), all of which are found in mammalian species. The extracellular organization of all nine ephrins is similar, consisting of a well-conserved 20-kDa receptor-binding domain that mediates interactions with the globular extracellular domain of the Eph receptors (Nikolov et al., 2005). Beyond these similarities in the mechanism of Eph receptor interaction, the structures of A-subclass and B-subclass ephrins diverge radically. The six members of the ephrin-A subclass are all tethered to the outer leaflet of the plasma membrane by a GPI-linkage, while the three B-subclass ephrins instead have a single-pass, hydrophobic transmembrane domain, followed by a short, highly-conserved cytoplasmic tail of approximately 80 amino acids (Figure 1.2). The cytoplasmic tail of B-subclass ephrins possesses no known catalytic activity, although this region can mediate protein-protein interactions through five conserved tyrosine residues that become phosphorylated and a C-terminal PDZ-binding motif (Holland et al., 1996; Lin et al., 1999; Torres et al., 1998).

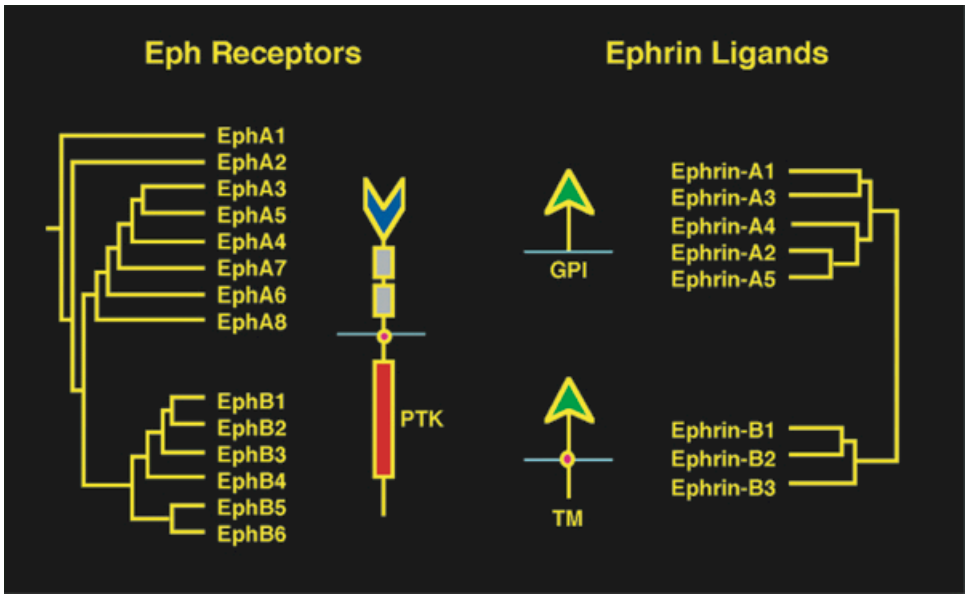


Figure 1.1

Dentogram depicting the large family of Eph receptor tyrosine kinases and their membrane-bound ligands, the ephrins. Not shown are EphA9, EphA10, and ephrin-A6.

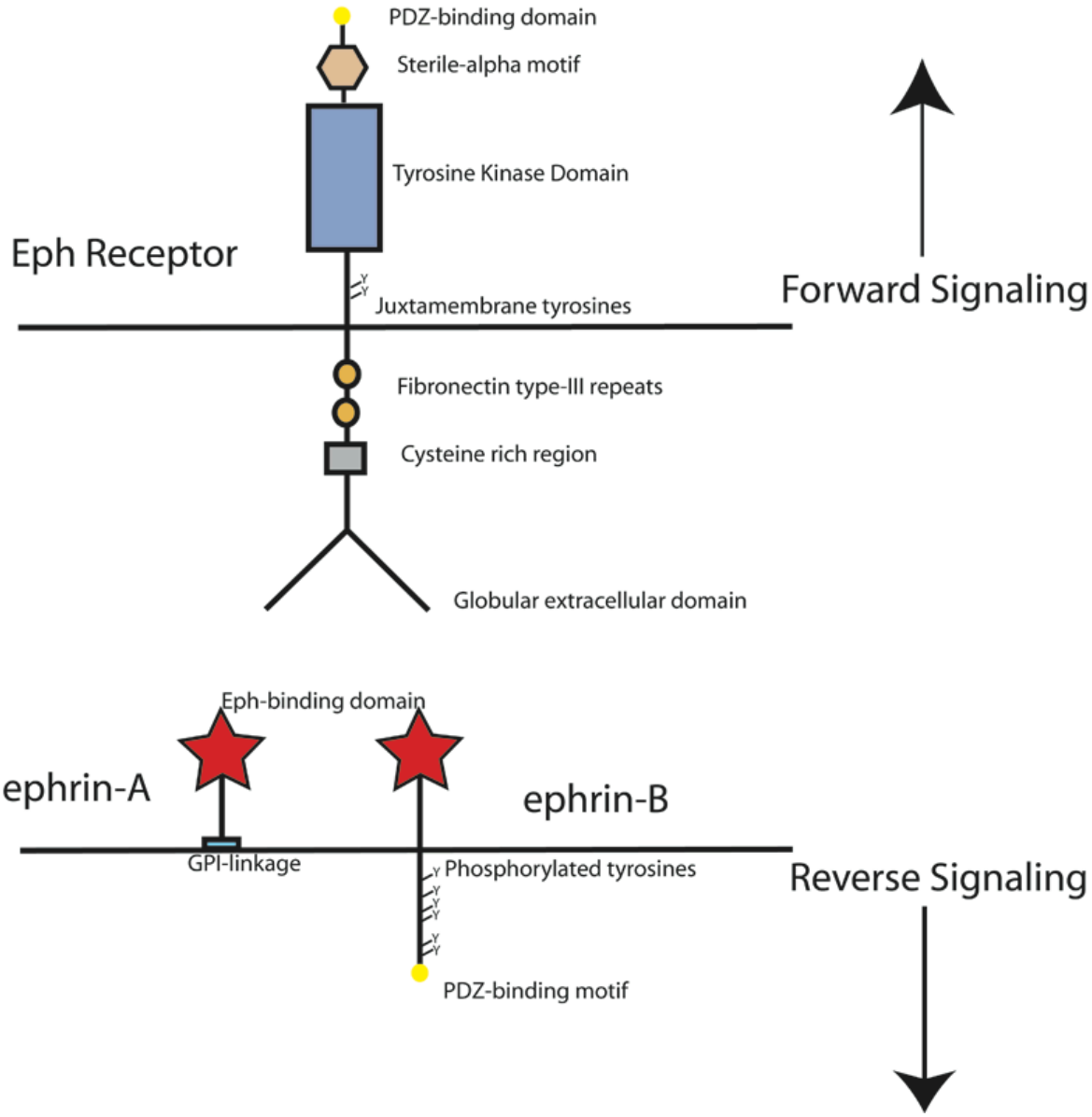


Figure 1.2

Bidirectional signaling through the Ephs and ephrins.

Bidirectional signaling between Eph and ephrin

One of the more interesting features of the Ephs and the ephrins is that because both receptor and ligand are expressed at the cell-surface, and because both Eph and ephrin possess multiple protein-protein interaction sites, the potential exists for bidirectional signaling between these molecules following receptor-ligand activation (Figure 1.2). The pioneering study demonstrating this phenomenon occurred in 1996, when it was shown that *EphB2* null mice, but not mice in possession of a germline mutation that specifically replaced the intracellular tyrosine kinase and PDZ-binding domains of EphB2 with the enzyme β-galactosidase, presented with axon-pathfinding errors in the anterior commissure, a major bundle of forebrain axons responsible for connecting the right and left hemispheres of the brain (Henkemeyer et al., 1996). This stunning discovery indicated that EphB2 could signal in a non-cell autonomous fashion through its extracellular domain interactions with the ephrins, suggesting a ligand-like role for the Eph receptors and thus by extension, a receptor-like role for the ephrins. Consistent with this hypothesis, EphB2 protein was not expressed in the axons of the anterior commissure, but rather in the surrounding regions of the brain (where it acts as a guidance cue) and the B-subclass ephrins were localized to the migrating axons of the anterior commissure (Henkemeyer et al., 1996). The potential therefore of Ephs and ephrins to function as receptors or ligands to one another adds a second layer of complexity to the study of these molecules, and forces us to distinguish between the cell autonomous and non-cell autonomous roles of these molecules in their different biological functions. Accordingly, we refer to signal transduction through the cell autonomous

functions of the Eph receptor as the *forward signal*, and distinguish signaling that takes place via the cell autonomous functions of the ephrins as the *reverse signal* (Figure 1.2).

The mechanistic details behind the activation of these bidirectional signals has been well defined through crystal structure analyses. Before cell-cell contact, Eph and ephrin molecules remain either unclustered on the cell surface, or loosely pre-clustered in cholesterol-rich lipid rafts (Bruckner et al., 1999). Upon cell-cell contact, the ephrin extracellular domain binds within the cleft of the globular domain of the Eph receptor with nanomolar affinity and at a 1:1 stoichiometry to form heterodimers (Himanen et al., 2001). In most cases, these heterodimers quickly form a very stable, ring-like tetrameric structure through an interaction of lower affinity between Eph and ephrin molecules in adjacent heterodimers, allowing for rapid multimerization into signaling centers on the cell surface (Davis et al., 1994; Himanen et al., 2001; Stein et al., 1998). The assembly of the two Eph receptors and two ephrin ligands into these tetrameric rings is thought to induce conformational changes in each set of molecules that orient them into either permissive or active signaling structures (Himanen et al., 2001; Wybenga-Groot et al., 2001). Interestingly this tetrameric assembly does not appear to be necessary, as an exception to this phenomenon occurs in ephrin-A5/EphB2 interactions, in which the oligimerization never progresses past the stage of the heterodimers, apparently without any consequence on the abilities of these molecules to transduce their respective signals (Himanen et al., 2004). Of further interest, these tetrameric ring structures can be composed of hetero-receptor complexes containing different Eph receptors, the best example of which is EphB6, which possesses a non-functional kinase domain but can be transphosphorylated through complex formation with EphB1 (Freywald et al., 2002).

Following cell-cell contact and high-affinity binding to ephrin molecules, the activation of the Eph receptors is thought to proceed in a similar fashion to other RTKs (Hubbard and Till, 2000; Schlessinger, 2000). The binding of ephrin and the subsequent assembly into higher order structures brings adjacent Eph receptors into close proximity to one another. The Eph receptors then become phosphorylated on JMS tyrosine residues, either by phosphorylating each other in *trans* or through the activation of co-localized Src-family kinases (Kalo and Pasquale, 1999; Knoll and Drescher, 2004). This event represents a critical step in upregulating the kinase activity of the molecule, as in the absence of ligand interaction, the unphosphorylated JMS of the Eph receptor interacts tightly with its own kinase domain, distorting the kinase domain and severely impinging tyrosine kinase activity (Wybenga-Groot et al., 2001). Phosphorylation of these JMS tyrosine residues sterically disrupts the interactions between the JMS and KD, thus relieving the distortion of the kinase domain and permitting the Eph receptor to phosphorylate its respective substrates. Interestingly, recent work has indicated that the activation of kinase activity in Eph receptors can also occur without major structural changes to the segment of the kinase domain that is sterically inhibited by the JMS, so further refinement of our understanding in this process is likely necessary (Wiesner et al., 2006). In addition to playing an important role in mediating the activity of the KD, the phosphorylation of the JMS tyrosines further serves two important functions: 1) the phosphorylated JMS tyrosine residues are important substrates for Src-homology 2 (SH2) domain interactions, and 2) the same conformational change in the KD that increases kinase activity might also make the SAM and PDZ-binding domains of the Eph receptors more accessible to potential protein interactions as well (Himanen et al., 2007).

On the other side of the equation, activation of ephrin reverse signaling is also initiated following the multimerization of Eph-ephrin heterodimers. On the cell surface, both A- and B-subclass ephrins in the unstimulated state are found in membrane rafts enriched in glycosphingolipids, where they co-localize with Src-family kinases (SFKs) (Cowan and Henkemeyer, 2002; Palmer et al., 2002). When the ephrins bind to the Eph receptors and undergo conformational changes associated with their assembly into heterodimers and then tetrameric rings, these SFKs become activated in a currently unidentified mechanism. In the case of the A-subclass ephrins, the SFK Fyn becomes activated and phosphorylates other molecules present at the membrane to initiate signaling events and subsequent physiological responses (Davy et al., 1999). Alternatively, with the B-subclass ephrins, the SFK Src is activated upon the conformational changes in ephrin-B, but instead of (or in addition to) phosphorylating other membranous proteins as with A-subclass ephrins, Src actually phosphorylates the conserved tyrosine residues on the cytoplasmic tail of the B-subclass ephrins (Palmer et al., 2002). The phosphorylation of these tyrosines converts the ephrin-B cytoplasmic tail into a substrate for subsequent signal transduction by creating SH2 domain-binding sites that can facilitate protein-protein interactions between the ephrins and other molecules. It has also recently been shown that the cytoplasmic tail of B-subclass ephrins is also phosphorylated on serine residues, although the kinase(s) responsible for this modification, and any potential signaling relevance from this event, have not yet been elucidated (Essmann et al., 2008).

Eph/ephrin signals target cytoskeletal regulators

Unlike the majority of tyrosine kinase signaling in the cell, signal transduction through the Ephs and ephrins does not appear to target the nucleus to regulate transcription, but has instead been consistently shown to interact with molecules and signaling pathways linked to the regulation of cytoskeletal dynamics. One of the first elements of forward signaling identified in the field was the regulation of Ras family GTPases by the Eph receptors. The source of this regulation appears to principally stem from the interaction of the phosphorylated Eph receptor JMS tyrosines with p120RasGAP, a GTPase-activating protein (GAP) for H-ras and R-ras that promotes the hydrolysis of GTP in these molecules to attenuate their activity (Holland et al., 1997). The downregulation of H-ras and R-ras activity by RasGAP has been shown to induce neurite retraction in either established cells lines or in primary neuronal cultures, while mice in which *RasGAP* has been knocked out show clear defects in cell movement (Dail et al., 2006; Elowe et al., 2001; Henkemeyer et al., 1995). Further, RasGAP has been shown to interact with activated Eph receptors through a complex including p190RhoGAP, $p62^{Dok}$, and Nck (Ellis et al., 1990; Holland et al., 1997; Jones and Dumont, 1999). RhoGAP and Nck in particular have been demonstrated to possess extensive ties to regulating actin cytoskeletal assembly (Holland et al., 1997; McGlade et al., 1993; Pawson and Nash, 2000).

The dominant measure by which Eph receptors regulate actin dynamics appears to be through the coordinated activities of small GTPases of the Rho family (Rho, Rac, and Cdc42). As with the Ras family GTPases, these Rho family members cycle between an active conformation in which GTP is bound and the molecule is capable of interacting

with effector molecules, and an inactive conformation in which GTP has been hydrolyzed to GDP. The activation state of these Rho family GTPases is controlled by the differential activity of guanine exchange nucleotide factors (GEFs), which catalyze the exchange of GDP for GTP to activate these molecules, or GAPs, which stimulate the hydrolysis of GTP into GDP (Bernards, 2003). The Rho family GTPases have been shown to directly mediate cell structure and movement. Classically, it has been understood that Rho promotes the formation of stress fibers, while Rac and Cdc42 are responsible for extending lamellipodia and filopodia, respectively, although indications that Rho can also promote actin polymerization and membrane extension, along with findings of crossregulation between the Rho family members, suggests this understanding may be overly simplistic (Higashida et al., 2004; Kurokawa and Matsuda, 2005; Nimnual et al., 2003; Nobes et al., 1995; Yuan et al., 2003). In growth cone dynamics, the activation of Rho has been shown to inhibit the outgrowth of neurites and promoted the retraction of growth cones, while Rac and Cdc42 appear to play antagonistic roles to Rho by stimulating neurite outgrowth, although this too has come into question by recent work showing Rac activation was necessary for neurite retraction (Lundquist, 2003; Xu and Henkemeyer, 2009; Yuan et al., 2003).

In addition to the recruitment of p190RhoGAP to activated Eph receptors mentioned above in relation to Ras family involvement in forward signaling, it has been shown that Eph receptors can regulate Rho family activity by interacting with a number of Rho GEF molecules. The first Rho GEF identified to bind to Eph receptors was ephexin (Eph-interacting exchange protein), which is constitutively bound to EphA receptors, where it activates Rho, Rac, and Cdc42 molecules in a balanced manner that permits

growth cone stability and axon outgrowth (Shamah et al., 2001). Following ephrin-induced activation, ephexin is tyrosine phosphorylated, which tilts the balance of Rho family activation towards RhoA, thus inducing stereotypical growth cone collapse (Sahin et al., 2005). A similar interaction between EphA4 and the exchange factor Vsm-RhoGEF produces altered cytoskeletal behavior in vascular smooth muscle cells through Rho activation (Ogita et al., 2003). Neither ephexin nor Vsm-RhoGEF bind to EphB receptors, but the exchange factors Vav2, intersectin-1, Tiam1, and kalirin have all been shown to interact with B-subclass Eph receptors (Cowan et al., 2005; Irie and Yamaguchi, 2002; Penzes et al., 2003; Tolias et al., 2007). Like ephexin, Vav2 activity is altered in response to ephrin-induced tyrosine phosphorylation to promote growth cone collapse and cell-axon repulsion, whereas intersectin, Tiam1, and kalirin regulate the morphogenesis of dendritic spines through Cdc42 (intersectin) or Rac (Tiam1 and kalirin), and as of yet have not been shown to promote growth cone collapse (Cowan et al., 2005). Recent work also suggests that Rho GEFs might not possess a monopoly on Eph receptor signaling. The Rac GAP α-chimerin has also been shown to interact with Eph receptors through SH2 domain interactions with phosphorylated JMS tyrosines to promote growth cone collapse (Shi et al., 2007). It is also worth pointing out that the Ras activity initially shown to be mediated by Eph receptor forward signaling also appears to funnel into these same Rho family effectors (Burridge and Wennerberg, 2004; Zondag et al., 2000).

Another manner in which the Eph receptors mediate cytoskeletal dynamics through the differential activation of Rho family molecules is through their interactions with non-receptor tyrosine kinases. The best example of this has been documented in the interactions that take place between EphB2, Abl, and Arg. Abl was one of the first protein

kinases to be identified and contains multiple functional regions including an SH2 domain, a Src Homology 3 (SH3) domain, a tyrosine kinase domain, and a C-terminal tail that can bind SH3 domains and actin (Sefton et al., 1981; Witte et al., 1980). Arg (Abl-related gene) shares these same functional features (Kruh et al., 1986; Kruh et al., 1990). Abl and Arg interact with the Eph receptors through at least three different mechanisms, including the recruitment of Abl and Arg to the juxtamembrane tyrosines of Eph receptors, a phosphorylation-independent recruitment of Abl and Arg to EphB2 through their C-terminal tails, and the reciprocal tyrosine phosphorylation of Eph and Abl/Arg (Yu et al., 2001). Abl and Arg then target Rho family GTPases to mediate the regulation of cytoskeletal behavior in the growth cone or at sites of adhesion (Yu et al., 2001; Zandy et al., 2007). In addition to Abl/Arg, as mentioned above, Src has also been shown to be recruited following EphB receptor activation; while JMS tyrosines on the Eph receptor represent one target of kinase, Src can also be recruited to tyrosine phosphorylate other membrane associated proteins as discussed below, providing another link to a non-receptor tyrosine kinase with proven roles in mediating cytoskeletal dynamics (Chang et al., 1995; Vindis et al., 2003).

Finally, the Eph receptors interact with an entirely different set of proteins linked to the cytoskeleton through their C-terminal PDZ binding motif. Of the molecules that interact with Eph receptors through PDZ-binding interactions, the most notable appear to be Pick-1, GRIP (Glutamate receptor interacting protein), syntenin, and AF-6/afadin (Hock et al., 1998; Torres et al., 1998). The striking commonality between these molecules appears to be their involvement in cell adhesion. AF-6/afadin is a critical element of the nectin-afadin cell-cell adhesion system, where afadin links nectin to the

actin cytoskeleton to strengthen sites of adhesion (Takahashi et al., 1999; Zhadanov et al., 1999). Syntenin contains two tandem PDZ domains, and has been shown to localize to sites of cell adhesion and actin stress fibers in cultured epithelial cells (Grootjans et al., 1997; Zimmermann et al., 2001); and Pick-1 has been shown to bind and perhaps cluster cell adhesion molecules such as nectin and a set of junctional adhesion molecules (Xu and Xia, 2006). It remains unclear exactly how the Eph receptors utilize these molecular interactions; different contexts could see the recruitment of these PDZ-domain proteins to Eph receptors used to either initiate a localized adhesion response, or to potentially disrupt these molecules from perhaps promoting adhesion elsewhere in the cell, to permit a cell retraction event. Intriguingly, there is also evidence to suggest AF-6/afadin interacts with Ras-family GTPases as well, potentially providing a secondary avenue through which the Eph receptors can tap into the same signaling pathways referenced above (Boettner et al., 2000).

As with the Eph receptors, reverse signals emanating from the ephrins invariably appear to target the regulation of cytoskeletal dynamics. Reverse signaling through the A-subclass ephrins is fairly simplistic, given the absence of a cytoplasmic domain in ephrin-A molecules. The principle means of reverse signaling through A-ephrins occurs via the activation of co-localized Src family kinases to target substrates that regulate cytoskeletal dynamics, many of which ultimately link to the same Rho family members (Brown et al., 2005; Chang et al., 1995; Davy et al., 1999; Davy and Robbins, 2000; Holen et al., 2008; Konstantinova et al., 2007). Reverse signaling through A-subclass ephrins might also require the clustering and/or activation of co-receptors into signaling centers; recently the p75 neurotrophin receptor ($p75^{NTR}$) has been identified as signaling partner for A-subclass

ephrins, where the association between $p75^{NTR}$ and ephrin-A is necessary for Fyn activation and the subsequent inducement of an axon repulsion outcome (Lim et al., 2008b).

Signaling through the B-subclass ephrins has been more successfully characterized to date. Following Eph receptor activation and phosphorylation of the ephrin-B cytoplasmic tyrosines by SFKs, the adaptor protein Grb4 is recruited to the ephrin cytoplasmic tail (Cowan and Henkemeyer, 2001). Grb4 is an adaptor protein in that it possesses no catalytic activity, but instead plays only a structural function to recruit other molecules that can activate signaling pathways (Chen et al., 1998). Recruitment of Grb4 to the phosphorylated tail of ephrin-B molecules occurs through interaction between the SH2 domain of Grb4 with phosphorylated ephrin tyrosines; recruitment of effector molecules then occurs through the three adjacent SH3 domains Grb4 possesses (Cowan and Henkemeyer, 2002; Su et al., 2004). Of interest, nearly all of the Grb4-interacting proteins appear to have significant roles in regulating cytoskeletal dynamics. These include: 1) Abl, a tyrosine kinase that serves as a key regulator of axon pathfinding through control of actin polymerization and cell adhesion in the growth cone (Coutinho et al., 2000; Lanier and Gertler, 2000); 2) Abi-1, an Abl-interacting protein (Abi) that is localized to sites of actin polymerization in lamellipodia and filopodia where it associates with GEFs for Rac (Scita et al., 1999; Stradal et al., 2001); 3) Axin, a scaffolding protein in the Wnt signaling pathway that could mediate the involvement of β-catenin in cadherin-based adhesion events; 4) CAP/ponsin, a Cbl-associated protein (CAP) that localizes to sites of cell adhesion where it binds to cytoskeletal molecules such as vinculin, paxillin, and afadin, and appears to have some regulatory function in the formation of actin stress

fibers and focal adhesions (Asakura et al., 1999; Ribon et al., 1998; Zhang et al., 2006); 5) DOCK180, a Rac GEF shown to interact with the second and third SH3 domains of Grb4, with established roles in cell migration and membrane ruffling, and recently shown to mediate the retraction of neurites following ephrin-B3 reverse signaling activation (Cheresh et al., 1999; Tu et al., 2001; Xu and Henkemeyer, 2009); 6) Dynamin, a GTPase with well-defined roles in endocytosis that is also proposed to act as an actin regulatory protein and has been previously shown to affect neurite outgrowth (Lee and De Camilli, 2002; Schafer, 2004; Torre et al., 1994); 7) Git1, the G protein-coupled receptor kinase-interacting protein (GIT), which forms a complex with β-PIX, an exchange factor for Rac, through which it regulates actin dynamics (Zhang et al., 2003); 8) hnRNPK, a ribonucleoprotein involved in transcription that can also interact with Vav, the Rho family GEF described above, which has been linked to actin cytoskeleton remodeling (Bustelo et al., 1995); and 9) Pak1, a serine-threonine kinase that is targeted by GTP-bound Rac and Cdc42 and mediates cytoskeletal changes such as actin depolymerization through the phosphorylation of substrates like LIM-kinase, or through complex formation with exchange factors such as β-PIX (Edwards et al., 1999; Manser et al., 1998). Amazingly, the pertinence of these Grb4-interacting molecules in regulating the physiological roles of ephrin-B reverse signaling *in vivo* (discussed later) still remains largely speculative.

The other major interaction site through which the B-subclass ephrins interact with effector proteins is through their C-terminal PDZ-binding motif. The most interesting and perhaps best-characterized protein to interact with the ephrin-B cytoplasmic tail through PDZ domain interactions is the molecule PDZ-RGS3. PDZ-RGS3 contains an N-terminal PDZ domain responsible for constitutively interacting with B-subclass ephrins, and a C-

terminal RGS domain that acts as a GAP for the α-subunits of trimeric G proteins (Lu et al., 2001). Following Eph-receptor activation, PDZ-RGS3 is translocated such that it catalyzes the hydrolysis of GTP to GDP in the G-protein-coupled chemokine receptor CXCR4. In an activated, GTP-bound stated, CXCR4 acts as a chemoattractant in response to its soluble ligand, stromal-cell-derived factor 1 (SDF-1), to direct cell migration (Lu et al., 2001). The silencing of CXCR4 by PDZ-RGS3 activation therefore silences this cell attraction response. Two other potentially relevant PDZ-domain interactions with B-subclass ephrins occur through PAR-3 and Syntenin. Syntenin has already been described above, while Partitioning-defective protein 3 (PAR-3) binds to B-ephrins through either its second or third PDZ domain, where it forms a complex involving PAR-6 and atypical protein kinase C (aPKC) that has been shown to target Rho family molecules Rac1 and Cdc42 or Rho family GEFs such as Tiam1 or Ect-2 to modulate cytoskeletal behavior (Johansson et al., 2000; Liu et al., 2004; Nishimura et al., 2005). PAR-3 also directly associates with junctional adhesion molecule (JAM), which may play a role in recruiting this PAR-6 complex and its Rho family regulation to sites of adhesion (Ebnet et al., 2001). The overexpression of PAR-3 in epithelial cell lines suppresses contact-mediated inhibition of cell migration and promotes membrane protrusions through Rac1 (Mishima et al., 2002).

Two other molecules heavily involved in regulating cytoskeletal dynamics have been shown to interact with the cytoplasmic tail of B-subclass ephrins as well. The Rac-specific GEF, Tiam1, referenced above as a mediator of Eph receptor forward signaling, has also been shown to bind B-subclass ephrins, and presumably makes available the same avenues involved in regulating cytoskeletal dynamics with forward signaling in reverse

signal transduction, as well (Tanaka et al., 2004). Additionally, it has been shown that Dishevelled (Dsh), which like Axin is a scaffolding protein involved in Wnt signaling, binds constitutively to the cytoplasmic tail of B-subclass ephrins. Following Eph receptor mediated activation of ephrin-B reverse signaling, Dsh dissociates from the ephrin cytoplasmic tail potentially through the phosphorylation of two C-terminal tyrosines, which has been shown to mediate RhoA activation and regulate cell repulsion in a manner that still needs to be detailed (Lee et al., 2009; Tanaka et al., 2003). As with the molecules interacting with ephrin-B through the SH2 domain docking of Grb4, the functional significance of these protein-protein interactions involving either the PDZ-binding motif or undetermined segments on the cytoplasmic tail of B-subclass ephrins remains thoroughly unestablished.

Finally, an unexpected protein interaction partner for B-subclass ephrins has emerged in claudin family molecules. The claudins are a very large family of tetraspanic molecules that are expressed in a variety of tissue-specific combinations. Classically, the claudins have been localized to tight junctions, where they interact with molecules such as ZO-1 through PDZ domain interactions to regulate the tightness of these junctions and thus the paracellular flow through epithelial sheets. Interestingly, the claudins have also been localized to sites outside of the tight junction on epithelial cells, most notably at adherens junctions as well. Recently, claudins have been shown to interact with B-subclass ephrins, and surprisingly this interaction appears to occur independently of the intracellular domain of the ephrin (Tanaka et al., 2005b). The association between ephrin and claudin directly mediates cell-cell adhesion of epithelial cells in contact, and is associated with the induction of ephrin tyrosine phosphorylation, suggesting claudin might

mediate Eph receptor activation of the ephrin, or perhaps even promote ephrin functions independent of Eph receptor activation (Tanaka et al., 2005b). It has been further suggested claudin is a target of Eph receptor phosphorylation, suggesting claudin might represent a convergent point of both forward and reverse signaling (Tanaka et al., 2005a).

Silencing Eph-ephrin bidirectional signal transduction

While the means by which Eph-ephrin signal transduction is initiated and then propagated within the cell have received most of the attention of the Eph-ephrin field, it is equally important to consider how an activated signaling complex is silenced. Given the physiological roles of these molecules in regulating the actin cytoskeleton in response to ephemeral extracellular stimuli and in a context as dynamic as cell migration, it is imperative that these signals possess some element of transience so that the cell does not continue to react to environmental cues that are no longer relevant. The most obvious manner in which this might occur is through simple dissociation of the receptor/ligand binding domains of the Ephs and ephrins, but given the nanomolar affinity of this complex, that does not appear to be nearly sufficient enough. The cell appears to principally resolve this problem through proteolytic cleavage of the ectodomains of the Ephs and ephrins, followed by endocytosis. The first involvement of proteolytic cleavage of these molecules was identified in 2000 when it was shown that ephrin-A2 forms a stable complex with A-Disintegrin-And-Metalloprotease (ADAM) 10, a mammalian homologue of Kuzbanian (KUZ) from *Drosophila*. Following the interaction of ephrin-A2 with EphA receptors, ephrin-A2 is cleaved by ADAM10 on specific residues to terminate the signal transduction (Hattori et al., 2000). Amazingly, it was since shown

that ADAM10 can also interact with EphA receptors, but rather than cleave the Eph receptor, the metalloprotease is somehow directed to cleave ephrin-A molecules *in trans* (Janes et al., 2005). B-subclass ephrins have not been shown to be targeted for cleavage by ADAM10; instead, ephrin-B molecules appear to be similarly cleaved by the aspartic protease γ-secretase (Tomita et al., 2006). In both cases, the cleavage of ephrin appears to disrupt the conduction of bidirectional signaling. As of yet, there is no evidence to suggest the Eph receptor ectodomain is similarly shed.

The Eph-ephrin complexes can be similarly removed from signaling centers at the cell surface in a cleavage-independent fashion through endocytosis, a process in which these molecules become rapidly internalized within the cell and away from the plasma membrane. Data for this phenomenon comes from two parallel studies using cell culture systems to demonstrate that when cells expressing EphB receptors come into contact with cells expressing ephrin-B molecules, intracellular vesicles containing complexes of both molecules can be found within either cell (Marston et al., 2003; Zimmer et al., 2003). The endocytosis is therefore fully *in trans*, and involves full-length protein that show no signs of proteolytic cleavage. Signaling through the Ephs and ephrins might play some role in regulating the endocytosis of these Eph-ephrin complexes. Endocytosis of the Eph-ephrin complex was disfavored from proceeding into the cytoplasm of Eph-expressing cells in which the Eph receptor was C-terminally truncated, or into the cytoplasm of ephrin-expressing cells in which the ephrin-B ligand possessed a similar deletion of its cytoplasmic tail, suggesting a requirement for the signaling domains of these molecules (Zimmer et al., 2003). In support of this notion, it was similarly shown that the endocytosis of the Eph-ephrin complex required Rac activation and the regulation of actin

polymerization, a known target and a known consequence of bidirectional signals through the Ephs and ephrins, respectively (Marston et al., 2003). Accordingly, Vav, a key exchange factor involved in Rho family activity that is targeted by Eph receptor forward signaling, along with CAP and Dynamin, two molecules linked to Grb4 and ephrin-B reverse signaling, have all been linked to endocytosis (Cowan et al., 2005; Tosoni and Cestra, 2009). One last potential connection between the Eph receptors and endocytosis comes from the ubiquitylation of these receptor tyrosine kinases, which has been shown to target these cell surface molecules for endocytosis and subsequent degradation (Mosesson et al., 2003). Both EphB1 and EphB2 have been shown to target themselves for degradation, either through the induction of the ubiquitin ligase Cbl through EphB1 kinase activity, or the recruitment of numb-protein X (Lnx) 1, which is an E3 ubiquitin protein ligase targeted to the PDZ binding motif of EphB2 (Fasen et al., 2008; Halford and Henkemeyer, unpublished).

Finally, given that Eph-ephrin signaling operates through the activation of tyrosine kinases, it makes sense that the last manner in which these signals are negatively regulated is through the recruitment of tyrosine phophatases. The most thoroughly characterized example of phosphatase involvement in mediating Eph-ephrin signaling involves the protein tyrosine phosphatase BAS-like (PTP-BL), which possesses five PDZ domains, through which it interacts with B-subclass ephrins. PTP-BL is believed to function as a downregulator of ephrin-B tyrosine phosphorylation, as when PTP-BL was overexpressed in cells containing ephrin-B1, it was recruited to sites of ephrin localization with delayed kinetics (compared to the more rapid recruitment and activation of SFKs), after which the tyrosines on the ephrin cytoplasmic tail were found to be dephosphorylated (Palmer et al.,

2002). Dephosphorylation of the Eph receptors has been shown to occur through activation of Ptpro (protein tyrosine phosphatase receptor type O), which dephosphorylates JMS tyrosines on both A- and B-subclass Eph receptors and appears to play functional roles in attenuating their signal transduction (Shintani et al., 2006). It has also been shown that EphB1 interacts with the low molecular weight phosphatase (LMW-PP), which could potentially attenuate the activity of these molecules as well (Stein et al., 1998). In general, the dephosphorylation of tyrosine residues on either Eph or ephrin appears to play a minor role in the downregulation of bidirectional signaling through these molecules, compared to the involvement of ectodomain shedding and endocytosis, although the emergence of more players like Ptpro and PTP-BL may quickly shift that balance.

Physiological roles for bidirectional Eph-ephrin signaling

Development of a mature multicellular organism from its humble origin as a fertilized egg is a supremely complex event that must be precisely orchestrated. Rapidly dividing cells within the developing organism are constantly on the move, and to prevent these processes from devolving into chaos and undermining the viability of the organism, it is essential these cells possess a navigational system instructing them on when to move, how to get there, and what to do upon arrival. The Ephs and ephrins are a logical choice to play instrumental roles in these guidance events, given their function as cell-surface receptors, their ability to directly mediate cytoskeletal behavior in response to external stimuli, and their widespread expression in developmental tissues. It comes as little

surprise then that these molecules have now been defined to play a widespread assortment of roles in development, which I will now summarize.

Axon guidance

Nowhere has Eph-ephrin signaling been better characterized than in the developing nervous system, and nowhere has that characterization within neural development been more extensive than in axon guidance. The exaggerated cytoskeletal structures of neural cells, featuring extensive dendritic or axonal protrusions that extend many times the length of the soma and terminate in easily-defined growth cone structures and the developmental requirement that these protrusions migrate great distances within the organism, along with the ease in working with neuronal cells in culture conditions, have made axon guidance an ideal system in which to study Eph-ephrin signaling.

The visual system

During development of the eye, retinal ganglion cells (RGC) within the eye will funnel into the optic disc to form the optic nerve. These RGC axons then navigate towards the midline at the optic chiasm, where the axons either cross the midline and innervate well-defined positions within the contralateral superior colliculus (known as the tectum in birds, frogs, etc.) and lateral geniculate nucleus (LGN), or bounce back to innervate similarly well-defined positions within the ipsilateral superior colliculus and lateral geniculate nucleus, where the visual input is further processed. The proper migration of RGC axons thus contains three critical decision points: 1) within the retina, projecting RGC axons must find and navigate through the optic disc, 2) at the optic

chiasm, RGC axons must know whether to continue migrating across the chiasm to contralateral termination zones, or bounce back ipsilaterally, and 3) the axons must project towards specific regions of the superior colliculus or LGN. This process is extremely well orchestrated, with RGC axons originating from specific fields within the retina terminating in specific zones within the superior colliculus and LGN in a highly reproducible fashion. As it turns out, Eph-ephrin signaling is involved in all three of these steps.

Within the retina, expression studies have shown that EphB receptors are expressed in a low dorsal to high ventral gradient, while their ephrin-B ligands are expressed in an opposing low ventral to high dorsal gradient (Holash and Pasquale, 1995; Kenny et al., 1995; Marcus et al., 1996). Disruption of EphB-ephrin-B signaling in mice with null mutations for both *EphB2* and *EphB3* revealed a striking number of RGC axons originating from the dorsal retina (which express ephrin-B) that did not properly funnel into the optic disk, but instead erroneously wandered into the ventral retina (Birgbauer et al., 2000). The expression data suggest then that migrating dorsal RGCs express ephrin and rely on the gradient of EphB receptors to provide a repulsive cue to prevent these axons from overshooting the optic disc. Consistent with this, dorsal, ephrin-expressing RGC explants showed clear growth cone collapse and repulsive outcomes in response to treatment with solubilzed, pre-clustered EphB-Fc fragments used to activate reverse signaling (Birgbauer et al., 2001). Further, mice that were null for *EphB3* and also contained a truncated allele of *EphB2* compromising its ability to forward signal only, did not show defects in optic disk targeting, suggesting a ligand-like role for the Eph receptors and cell-autonomous function for reverse signaling through ephrin-B molecules

(Birgbauer et al., 2000). These results therefore indicate that dorsal RGCs expressing ephrin-B are normally repulsed by an incremental gradient of ventral EphB expression, to guide optic disc targeting in the retina.

The optic chiasm is the next critical checkpoint for eye development. The majority of organisms with eyes located in the front of the head have binocular vision, such that the central portion of the visual field is perceived by both eyes (Williams et al., 2004). RGCs covering this binocular field within the retinas from both eyes should therefore terminate together within the superior colliculus and LGN, to permit higher order visual processing. To ensure this occurs, a subset of RGC axons extending from the ventrotemporal region of the optic disc are therefore segregated from the rest of the RGCs at the optic chiasm and instead project with RGCs from the contralateral retina. The first suggestion of involvement of Eph-ephrin signaling in directing divergence of RGCs at the optic chiasm came from expression studies, where it was shown that ephrin-B2 is strikingly expressed at the optic chiasm of mammals (which have ipsilateral projections), but not fish or birds (which do not) (Nakagawa et al., 2000). The role of ephrin-B2 in this process was then clarified in 2003, when it was shown that EphB1 is expressed exclusively in the ventrotemporal region of the retina and that *EphB1* null mice do not show ipsilateral projections, indicating that ephrin-B2 serves as a repellent at the optic chiasm to push ventrotemporal RGCs expressing EphB1 into ipsilateral projections (Williams et al., 2003). Consistent with this, recently generated forward signaling-deficient *EphB1* mice also do not show ipsilateral projections of ventrotemporal RGCs (Chenaux and Henkemeyer, unpublished).

The final element of eye development is the targeting of RGCs into the superior colliculus and LGN. Whereas the migration of RGCs at the optic disc and optic chiasm appear principally mediated by B-subclass Ephs and ephrins, the targeting of RGCs into their final termination sites appears to require both A- and B-subclasses. EphA receptors and A-type ephrins exhibit polarized gradient expressions in both the retina and the superior colliculus, and repulsive signaling through the receptor like role of both the Ephs and ephrins expressed within the extending RGC axons is responsible for their targeting into the superior colliculus along the anterior-posterior axis, as mice null for either *EphA* or *ephrin-A* present with axons terminating at topographically incorrect positions (Cheng et al., 1995; Drescher et al., 1995; Feldheim et al., 2000; Feldheim et al., 2004; Frisen et al., 1998; Park et al., 1997; Wilkinson, 2000). B-subclass Ephs and ephrins appear to play similar roles in mediating the targeting of RGC axons to the superior colliculus along the dorsal-ventral and lateral-medial axes. Interestingly, the ventral RGCs, which as mentioned above express the highest levels of EphB receptors, target to areas of the superior colliculus that express the highest levels of ephrin-B, suggesting that axon repulsion might not be the only outcome of Eph-ephrin signaling at play here to guide precise termination (Hindges et al., 2002). As with *EphA* and *ephrin-A* null animals, *EphB* receptor null animals similarly present with topographically incorrect targets within the superior colliculus (Hindges et al., 2002). Forward signaling through B-subclass Eph receptors appears particularly important in targeting RGCs into the superior colliculus, as mice with germline mutations compromising the ability of EphB2 to forward signal present with ectopic termination zones within the superior colliculus (Hindges et al., 2002; Thakar and Henkemeyer, unpublished). It is also of note that mice null for *Vav*, a key Rho

GEF implicated in transducing Eph receptor forward signaling, also present with defective axon pathfinding in RGCs, and that these axons appear insensitive to ephrin stimulation in culture (Cowan et al., 2005). This suggests that EphB forward signaling mediated by Vav molecules plays necessary roles in guiding RGC axons to their proper targets in the visual system.

Olfactory and Vomeronasal Systems

Mammals use two distinct olfactory systems to process "smells" within their environment. The main olfactory system is used to process most odorants through detection along the main olfactory epithelium (MOE) and triggers behaviors that can be controlled through learning and experience. On the other hand, the vomeronasal organ (VNO) is a distinct structure within the nasal septum of vertebrates that is responsible for the detection of pheromones, and which triggers innate, stereotypical responses that are not easily manipulated by experience (Dulac, 2000). Just as in the visual system, the targeting of neurons from the site of stimulus to higher order processing centers in both of these systems appears to be dependent upon topographic mapping involving Eph-ephrin signaling.

The VNO contains separate apical and basal domains, which send axons to either the anterior or posterior part of the accessory olfactory bulb (AOB), respectively, which then relays that information to the amygdala or hypothalamus, bypassing the cortex (Rodriguez et al., 1999). Expression studies show that apical VNO axons that express a high level of ephrin-A5 target into the anterior part of the AOB, which expresses high levels of EphA6. Similarly, basal VNO axons expressing low levels of ephrin-A5 target

into the anterior part of the AOB, which expresses low levels of EphA6 (Knoll and Drescher, 2002). The expression data thus suggests an attractive role for ephrin reverse signaling activated by EphA6, which was further supported by *in vitro* work showing that VNO axons in cell culture preferred to target to lanes expressing EphA-Fc in stripe assays (Knoll et al., 2001). Additional work is necessary to detail this further, given that the expression of EphB/ephrin-B molecules in this system has not been explored, nor is it clear why molecules such as EphA3 and ephrin-A3 are expressed without gradients in the AOB and VNO.

Topographic mapping of the main olfactory system is quite complex, given that primary olfactory neurons express one of roughly 1000 types of olfactory receptors in a mosaic pattern, and yet the axons of these dispersed neurons must properly target specific regions within the olfactory bulb (Mombaerts, 1999). Accordingly, the expression of Ephs and ephrins in the main olfactory system does not occur in well-ordered gradients as in the visual system or VNO, but instead is markedly dynamic and mosaic, reflecting the complexity of the olfactory system (St John et al., 2002). Remarkably though, the same instructive roles in axon guidance between Eph- and ephrin-expressing cells appear to be in place. The targeted deletion of both *ephrin-A3* and *ephrin-A5*, which are expressed in the primary olfactory neurons, results in abnormal axon pathfinding, indicated by posterior displacement of their axons within the olfactory bulb (Cutforth et al., 2003). Further, overexpression of *ephrin-A5* in these primary olfactory neurons pushes axon displacement towards more anterior targets (Cutforth et al., 2003). Eph receptors have been found to be expressed in mitral cells in the olfactory bulb where these primary olfactory neurons target, and the complementary expression of ephrin-A and EphA suggests the Eph

receptors might be providing repulsive cues to properly guide ephrin-expressing olfactory neurons (Serizawa et al., 2006).

Corticospinal Tract

Stroke victims whose hemorrhages occur on one side of the brain typically suffer the effects of weakness or paralysis on the opposite side of the body. The cause for this is that axons from the motor cortex of one hemisphere of the brain typically innervate targets on the contralateral side of the corticospinal tract (CST), such that the left side of the brain controls movement on the right side of the animal, and the right side of the brain controls movement on the left side of the animal. Developmentally, this occurs when the axons of corticospinal neurons from the motor cortex cross the midline at the caudal medulla, extend down the contralateral column of the spinal cord, and branch off to target contralateral pools of spinal cord motor neurons (Stanfield, 1992). The prevention then of these neural pathways from recrossing the midline and innervating motor neuron pools on both sides of the midline is a key element in permitting asymmetric body movement. Signaling between EphA4 and ephrin-B3 appears to be necessary to prevent errant recrossing of the midline by these CST axons, as both *EphA4* and *ephrin-B3* null animals present with a symmetrical, hopping locomotion and appear to have CST axons that aberrantly innervate bilateral motor neuron populations (Dottori et al., 1998; Kullander et al., 2001a; Yokoyama et al., 2001). Expression data puts EphA4 in the migrating CST axons and ephrin-B3 at the spinal cord midline, suggesting that ephrin-B3 serves as a ligand to activate EphA4 forward signaling in CST axons, and that this signal functions to promote axon repulsion back away from the midline. Consistent with this notion, a

mutation in *ephrin-B3* that specifically blocks its reverse signal while leaving the ability of the protein to activate forward signaling does not present with any defects in CST pathfinding and exhibits normal movement, while mice with germline mutations targeting *EphA4* forward signaling exhibited a hopping locomotion and presented with CST axons that had recrossed the spinal cord midline, as in the original knockout animals (Kullander et al., 2001b; Yokoyama et al., 2001).

Recently the downstream effector involved in mediating this EphA4-ephrin-B3 role in midline CST axon pathfinding has been identified. Incredibly, a spontaneous mouse mutation, *miffy*, yielded mice with a similar hopping locomotion found in either *EphA4* or *ephrin-B3* knockout animals. Histological examination revealed the hopping locomotion was the result of abnormal re-crossing of CST axon at the midline of the spinal cord. To determine what gene was disrupted in *miffy* mice, the investigators used positional cloning to identify the Rac GAP α-chimerin as the molecule in question. This was further verified by the creation of BAC transgenic mice overexpressing α-chimerin which rescued the defect in *miffy* mice, the generation of mice with targeted deletions of α-chimerin which phenocopied *miffy* mice, and biochemistry demonstrating clear association of α-chimerin with EphA4 (Iwasato et al., 2007). Further, consistent with the apparent role in repulsion of EphA4-expressing axons at the midline, the suppression of α-chimerin activity in cultured cells stymied the ability of ephrin-B3 to elicit collapse of the growth cone (Iwasato et al., 2007). That the effector of EphA4 forward signaling is a GAP instead of a GEF, and for Rac instead of Rho, unlike most of the Rho family effectors previously characterized, was unexpected; however, the conclusions from this

elegant study have been verified by follow-up work on α-chimerin by other groups (Beg et al., 2007; Shi et al., 2007).

Motor neuron limb innervation

Spinal cord motor neuron axons must properly exit the spinal cord and innervate into the limbs to form projections on specific muscles (Lance-Jones and Landmesser, 1981). Guidance of these migrating axons is well regulated and appears to require repulsive cues mediated by Eph-ephrin signal transduction. The involvement of these molecules in limb innervation was first suggested in 2000 by the discovery that *EphA4* null animals present with a defect in which lateral motor axons destined to enter the dorsal hindlimb instead bundle into the ventral nerve of the limb (Helmbacher et al., 2000). Expression data has again localized EphA4 to the migrating motor neurons, suggesting a cell-autonomous function for forward signaling. The ligands for EphA4 appear to be ephrin-A2 and ephrin-A5, which are specifically expressed in the ventral portion of the limb. Given the expression of the A-subclass ephrins, and the phenotype wherein EphA4-expressing motor neurons aberrantly project into the ventral portion of the limb, the emerging model is that A-subclass ephrins serve as repulsive cues to repel EphA4-expressing motor neurons away from the ventral portion of the limb, so that they can properly reach their dorsal muscle targets (Eberhart et al., 2004; Eberhart et al., 2002). A similar system appears to be in place for B-subclass Ephs and ephrins in guidance of medial motor axons. EphB receptors are highly expressed on medial motor axons, while the ligand ephrin-B2 is preferentially expressed in the dorsal mesenchyme of the limb, suggesting a repulsive function from ephrin-B2 activation of EphB forward signaling to

keep medial motor axons from extending dorsally. This appears to have been confirmed by analyses of *EphB1;EphB2;EphB3* compound null animals in which medial motor axons were found to extend aberrantly into dorsal regions of the limb (Luria et al., 2008).

Synaptic functions

Once axons are guided to their proper targets, they must then properly form synapses through regulated interactions between the axon and its target cell, which manipulate the function and structure of each respective cell. One focal cytoskeletal feature at the synapse is the dendritic spine, a small dendritic protrusion that is triggered through axon-dendrite contact (Bourne and Harris, 2008). Given the maturation of the dendritic spine requires cytoskeletal changes and depends upon cell-cell contact, the Ephs and ephrins seem logical candidates to regulate the formation of these structures, and indeed cell-autonomous signaling through both Eph and ephrin are now known to be important in their development. Starting with the Eph receptors, several Ephs have been localized to the dendrites and to spines, and compound triple null animals lacking *EphB1, EphB2, and EphB3* present with abnormal dendritic spines both *in vivo* and in *in vitro* culture systems (Henkemeyer et al., 2003). The localization of the Eph receptors to the spine indicates cell-autonomous signaling roles, which has been supported by the finding that EphB2 forward signaling deficient animals also present with dendritic spine defects in an *EphB1;EphB3* null background (Henkemeyer et al., 2003). The role of EphB forward signaling in dendritic spine formation appears to involve the initiation of contact with axons through the stimulation of dendritic filopodia-like protrusions (Kayser et al., 2008). Unsurprisingly, the cytoskeletal changes induced in dendritic spine formation via the Eph

receptors is mediated through Rho family GTPase activity, as Tiam1 and kalirin are recruited to Eph receptor complexes in the spine and appear necessary for spine formation (Penzes et al., 2003; Tolias et al., 2007).

Reverse signaling through B-subclass ephrins also appears to function in dendritic spine formation. When investigators look at either the hippocampus or cultured cells taken from the hippocampus, ephrin-B is localized to dendrites and their spines (Grunwald et al., 2004). Further, manipulation of ephrin-B reverse signaling by either expressing dominant-negative ephrin-B1 in cultured hippocampal neurons, or expressing a dominant-negative isoform of Grb4, a key mediator of ephrin-B reverse signaling, resulted in severe disruption of dendritic spine formation, in which the normally globular, mushroom-shaped structure of the spine was converted into less stable filopodia-like structures (Segura et al., 2007). The role of ephrin-B reverse signaling in spine formation appears to operate through Grb4 interaction with GIT1, which as previously described, interacts with the Rac exchange factor, β-PIX.

Once the synapse has been rudimentarily established, Eph-ephrin signaling has been further implicated in the activity-dependent modification of synapse strength. It was initially observed that EphB2 can directly interact with and tyrosine phosphorylate the NMDA glutamate receptor, and can activate the AMPA glutamate receptor through PDZ domain interactions involving the molecule GRIP (Dalva et al., 2000). The clustering of these glutamate receptors results in Ca^{2+} flux that can potentiate the excitability at the synapse and perhaps mediate events such as long term potentiation (LTP) (Takasu et al., 2002). EphB receptors can also tyrosine phosphorylate synaptojanin 1 to regulate AMPA glutamate receptor endocytosis at the synapse as well (Irie et al., 2005). Interestingly

though, the principal function of Eph-ephrin signaling in synaptic plasticity appears to require reverse signaling. *EphB2* null mice, but not forward signaling deficient *EphB2*-truncated mice present with defects in LTP in culture slices from the hippocampus, suggesting a role for EphB2 activation of ephrin-B reverse signaling (Henderson et al., 2001). This has been further verified by the finding that tyrosine phosphorylation of ephrin-B2 is necessary for LTP of the hippocampus *in vivo* (Bouzioukh et al., 2007). Recently, elegant work from Byung Kook Lim, a graduate student in the laboratory of Mu-Ming Poo (and a former rotation student in the Henkemeyer laboratory) has demonstrated that ephrin-B reverse signaling promotes the stabilization of the retinotectal synapse in the developing *Xenopus*, and that this morphological change correlates to increased basal synaptic activity and activity-induced LTP (Lim et al., 2008a). The emerging picture therefore seems to be that Eph-ephrin signaling is necessary for both the establishment of the synapse by mediating axon-dendrite contact and for the mediation of the strength of that synapse.

Vascular morphogenesis

Blood vessel formation is an essential developmental event in which primitive blood vessels are generated through the fusion of endothelial precursor cells, and then remodeled to form new capillaries through extensive structural overhaul, or "pruning" events that mimic the refinement processes in axon guidance (Risau, 1997). Eph-ephrin signaling has now been shown to play essential roles in each of these processes. *EphB4* and *ephrin-B2* null mice both present with an early embryonic lethal phenotype around E10.5 due to failed vascular morphogenesis in which the development of vasculature

never progresses past the establishment of primitive vascular networks (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). Expression data from these studies showed that EphB4 is expressed on embryonic veins, while ephrin-B2 shows a complementary expression pattern in preferentially localizing to embryonic arteries, suggesting the phenotype in these mice was the result of failed signaling between EphB4 and ephrin-B2. The question then became which element of this bidirectional signal was driving vascular morphogenesis: reverse signaling through ephrin-B2, forward signaling through EphB4, or both?

Attempts to answer this question spiced the field with more than its fair share of drama, as competing laboratories came to opposing conclusions on the subject. Research performed under the guidance of Rudiger Klein initially proposed in *Cell* that it was reverse signaling through ephrin-B2 that was necessary for vascular morphogenesis, as mice created in his laboratory with a germline mutation specifically deleting the cytoplasmic tail of ephrin-B2, and thus presumably eliminating only ephrin-B2 reverse signaling, phenocopied *EphB4* and *ephrin-B2* null animals in embryonic lethality and defects in the vasculature development (Adams et al., 2001). This claim was subsequently challenged by research from the Henkemeyer laboratory, which demonstrated that a mouse model in which the cytoplasmic tail of ephrin-B2 was replaced with the enzyme β-galactosidase, which also should specifically ablate ephrin-B2 reverse signaling while leaving forward signaling intact, did not present with the vascular defects found in the *EphB4* and *ephrin-B2* null mice, indicating that it was EphB4 forward signaling activated by ephrin-B2 that was responsible vascular remodeling (Cowan et al., 2004). It was shown by Chad Cowan, a graduate student in the Henkemeyer laboratory,

that the confusion came from the fact that the ephrin-B2 truncation allele utilized by Klein was flawed; this truncation allele is not properly trafficked to the plasma membrane, but instead gets stuck in the *trans*-Golgi network, meaning this allele functions as a null mutation because the truncated protein can not get to the plasma membrane to activate forward signaling in adjacent cells, nor can it obviously transduce a reverse signal of its own (Cowan et al., 2004). Given this, it should come as no surprise that the ephrin-B2 cytoplasmic tail deletion phenocopies the *ephrin-B2* null mutant animal, since both alleles constitute functionally null forms of ephrin-B2. Importantly, cell assays performed by others have further confirmed that EphB4 forward signaling is the driving force behind embryonic vascular morphogenesis (Fuller et al., 2003; Maekawa et al., 2003; Salvucci et al., 2006; Steinle et al., 2002).

Outside of embryonic development, vascular morphogenesis is also of keen interest in relation to tumor angiogenesis. An essential element for tumor growth is neovascularization of blood vessels to supply the cancerous mass with oxygen, and disruption of this event represents a major aim of cancer therapy. As in embryonic vasculature, the Ephs and ephrins are also highly present in the tumor vasculature (Brantley-Sieders and Chen, 2004). The principal Eph receptor involved in tumor angiogenesis so far has proven to be EphA2, which is surprising given that it is not expressed normally on embryonic vasculature. Stimulation of EphA2 forward signaling by ephrin-A1 in cell culture assays has been shown to induce corneal angiogenesis and stimulate endothelial cell migration and assembly, while tumors do not grow as well in *EphA2* null animals (Brantley-Sieders et al., 2005; Ogawa et al., 2000; Pandey et al., 1995). As to be expected, the targets of EphA2 forward signaling in tumor angiogenesis

appear to be Rho family GTPases and their exchange factors, as disrupting the activation of Vav family molecules attenuates the angiogenic properties of EphA2 forward signaling (Hunter et al., 2006). The role of EphB4 in tumor angiogenesis is less well-described, although recent work suggests a novel mechanism by which EphB4 might contribute to tumor neo-vascularization through the regulation of endothelial precursor cell migration, mediated by interaction with ephrin-B2 (Foubert et al., 2007).

In addition to blood vessels, the circulatory system is also comprised of lymphatic vessels, which stem from primitive venous structures and mature through similar remodeling techniques seen in blood vessel development (Alitalo and Carmeliet, 2002). As in blood vessel development, Eph-ephrin signaling similarly appears to be necessary for lymphatic maturation as well. Once again, ephrin-B2 appears to be the critical ephrin involved in this development process, but interestingly, where in blood vessel development it was the non-cell autonomous role of activating EphB4 forward signaling that ephrin-B2 was instrumental in, with the lymphatic structures it appears to be cell autonomous reverse signals that appear to be most functionally relevant. Data for this comes from the creation of mutant mice with germline mutations specifically deleting the PDZ-binding motif of ephrin-B2 (Makinen et al., 2005). These mutant mice die post-natally due to severe lymphatic defects owed to their failure to remodel primitive lymphatic structures into more hierarchical vasculature, indicating an essential role for ephrin-B2 reverse signal transduction through PDZ-domain interactions in this process. The identity of the proteins interacting with ephrin-B2 through these PDZ domain interactions in this developmental event remain unknown, but this still represents a key breakthrough in trying to define the identity of the ephrin-B reverse signal.

Neural crest cell migration

Neural crest cells (NCC) are a key population of multipotent cells that delaminate from the neural tube following closure and migrate great distances along distinct pathways to constitute a number of different cell types (Crane and Trainor, 2006). Migration of NCC occurs during early embryonic development, a time of rapid growth and morphogenesis, and thus must be very precisely regulated. The migratory process thus seems ripe for Eph-ephrin involvement, and indeed, some of the earliest studies of Eph-ephrin signaling have found these molecules play important guidance roles in NCC migration. One of the first studies implicating Eph-ephrin signaling in NCC migration came from 1997, when it was shown in *Xenopus* that EphA4/EphB1 and ephrin-B2 were expressed on adjacent streams of NCC, and that either the overexpression of ephrin-B2 or the inhibition of forward signaling using truncated alleles of the Eph receptors produced errant migration of the NCC (Smith et al., 1997). This data indicated ephrin activation of EphA4/EphB1 forward signaling in NCC in the frog was necessary for their proper guidance, presumably through cell repulsive stimuli. A similar involvement of NCC migration was seen in the chick, where ephrin-B1 and EphB3 are expressed in adjacent populations of trunk NCC, and also appear to mediate NCC migration through repulsive forward signaling activated by the ephrins (Krull et al., 1997). Finally, NCC migration in the mouse also appears to require non-cell autonomous roles for ephrin-B2 in activating forward signals, as *ephrin-B2* null mice have been reported to present with defects in branchial NCC migration, whereas ephrin-B2 reverse signaling deficient animals do not (Adams et al., 2001; Davy and Soriano, 2007).

While these early studies implicated forward signaling mediated by ephrin-B activation in NCC migration, recent work has suggested that reverse signaling may also play in this developmental event as well. Analysis of *ephrin-B1* null mice has also suggested a requirement for the ephrin in proper migration of branchial NCCs. *Ephrin-B1* null mice present with a cleft palate phenotype that is consistent with defects in NCC migration, NCC in *ephrin-B1* null animals were found to migrate aberrantly into incorrect termination sites, and these problems were present not just in *ephrin-B1* null mice, but also when *ephrin-B1* was conditionally deleted only in NCC (Davy et al., 2004). Further, these same defects were also found in mice with a specific mutation in *ephrin-B1* ablating the ability of the ephrin to interact with PDZ-domain containing proteins, suggesting that reverse signaling through ephrin-B1 is responsible for mediating NCC migration (Davy et al., 2004).

Cell sorting and boundary formation

Repulsive signaling through the Ephs and ephrins also plays fundamental roles in the segregation of mixed populations of cells into distinct structures, and in maintaining the proper organization of cells within a defined apparatus. One of the first examples of Eph-ephrin signaling in cell sorting was documented in the segmentation of the hindbrain in zebrafish and *Xenopus*. In the hindbrain, EphA4 is expressed on odd-numbered rhombomeres, while B-subclass ephrins are expressed in the even-numbered ones, suggesting repulsive signaling between these molecules either establishes or maintains rhombomere segregation (Becker et al., 1994; Flenniken et al., 1996). The microinjection of mRNA encoding dominant-negative *EphA4* in either animal model resulted in disrupted

rhombomere segregation, suggesting cell-autonomous roles for forward signaling through the receptor in this process (Xu et al., 1995). Follow-up studies suggested there might be potential roles for ephrin-B2 reverse signaling in the segregation of ephrin-expressing rhombomeres, as well, although the design of the experiment (overexpressing truncated alleles presents a mix of dominant-negative inhibition of cell autonomous signaling along with hyperactivation of non-cell autonomous signaling that makes it hard to distinguish what is causing any phenotypic perturbation) leaves that conclusion as fairly tenuous (Xu et al., 1999).

Another role for Eph-ephrin signaling in cell sorting has been characterized in formation of the somites, which are primitive masses of mesoderm flanking the neural tube that will mature into skeletal muscle and the vertebrae. As in the hindbrain, EphA4 and ephrin-B2 are expressed in alternating stripes in the somites and presomitic mesoderm, and the microinjection of dominant-negative isoforms of Eph and ephrin in zebrafish again results in abnormal segmentation (Durbin et al., 1998). Further, in zebrafish mutants in which deletion of a transcription factor results in abnormally fused somites, the ectopic expression of EphA4 is sufficient to induce somite boundary formation (Barrios et al., 2003). EphA4 and ephrin-B2 are similarly expressed in the mouse, however *EphA4* null mice have not been reported to present with defects in somite development and there have been divergent claims about somite development in *ephrin-B2* null animals, with one group reporting normal somite development, and another laboratory reporting disrupted somatic polarity and differentiation (Davy and Soriano, 2007; Wang et al., 1998). Further research is necessary to determine the precise contributions of forward and reverse signaling involving ephrin-B2 and EphA4 in these

processes, as well as to discover what other molecules might be involved in segmentation of the somites in the mouse.

Stem cells

One of the more unexpected physiological roles recently defined for Eph-ephrin signaling involves the regulation of adult stem cells, which is surprising given that the Ephs and ephrins have classically been shown to affect the cytoskeleton instead of targeting the cell cycle to regulate cell proliferation events. Closer examination of stem cell biology does suggest several potential areas in which cytoskeletal remodeling could be of fundamental importance. These include early cell migration events to bring stem cells into their proper target zone for the renewal of certain cell populations, barrier formation to ensure stem cells stay within their niche to receive appropriate molecular cues for their regulation, the establishment of cell polarity to ensure the stem cell properly divides to produce one daughter cell that will differentiate and one daughter cell that will retain similar stem cell potentiality, and the directed cell migration of differentiated daughter cells to their proper effector target areas.

The involvement of Eph-ephrin signaling has now been established in these aspects of stem cell biology. The best example of this is in hippocampal development and maintenance. The hippocampus is a unique structure of the brain involved in memory and spatial navigation that is one of just a few areas in the brain where adult neurogenesis continually takes place (Gross, 2000; Ming and Song, 2005). New neurons are constantly being generated from nestin-positive neuronal stem cells localized along the subgranular zone (SGZ) of the dentate gyrus (DG), which then extend into the molecular zone of the

dentate gyrus and presumably become established in memory networks (Kee et al., 2007). Analysis of *EphB1;EphB2* compound null animals has indicated that these Eph receptors are necessary for regulating the activity of these neuronal stem cells in adult neurogenesis. *EphB1* null animals present with fewer nestin-positive stem cells in the developing hippocampus compared to their wild-type littermates, and this disparity extends back into embryonic development, suggesting defects in the early migration events bringing these stem cells into their niche along the SGZ (Chumley et al., 2007). As well, even stem cells that did make it to the DG were ectopically located away from the SGZ, suggesting defects in barrier formation to keep the stem cells at their niche. Further, when stem cells at the SGZ did properly divide to form differentiating daughter cells, these cells presented with abnormal cytoskeletal structures; rather than initiating the branching of dendrites after extending them out through the granular layer of the DG, differentiated cells in *EphB1* null animals instead featured significant dendrite branching within the granular layer (Chumley et al., 2007). *Ephrin-B3* null animals partially phenocopy these defects seen in the Eph receptor null animals, while an ephrin-B3 reverse signaling deficient animal does not, suggesting ephrin-B3 acts as a ligand to activate forward signaling through the EphB receptors in these stem cell functions (Chumley et al., 2007). Signaling through A-subclass Ephs and ephrins has also been implicated in regulating stem cell activity at the lateral ventricle wall, another site of adult neurogenesis, although the roles of these molecules at this site have not been as well detailed as in the hippocampus (Holmberg et al., 2005).

A second area in which Eph-ephrin signaling is thought to regulate stem cell activity is in the intestine, where massive amounts of daily turnover within the intestinal

crypt require ongoing stem cell-mediated renewal. Within the intestinal crypt, progenitor stem cells are present at the base of the crypt and express EphB2 and EphB3, while differentiating cells express B-subclass ephrins and are located apically within the crypt (Batlle et al., 2002). The expression gradient of EphB and ephrin-B suggests some sort of barrier function, and indeed analysis of *EphB2;EphB3* compound null animals shows that ephrin-B1 expressing differentiated cells are no longer localized apically in the crypt, but are instead distributed throughout the crypt (Batlle et al., 2002; Holmberg et al., 2006). Similarly, the EphB-expressing progenitor cells are also mislocalized within the crypt, accompanied by reduced proliferation within the progenitor niche (Holmberg et al., 2006). Interestingly, it was suggested that Eph-ephrin signaling controls stem cell proliferation independent of these positioning effects within the crypt, as a mutant allele of *EphB2* that hyperactivates EphB2 kinase activity presented without any defects in the localization of either proliferating or differentiated cells, but still had statistically significant increases in cell proliferation (Holmberg et al., 2006). That is a bold finding within the field that will hopefully be followed up in the coming years with further verification and detail.

Cell adhesion

In addition to all of the roles in Eph-ephrin signaling mediating morphogenetic events through cell repulsion, there is an example wherein these molecules appear to mediate cell adhesion. A fraction of both *EphA7* and *ephrin-A5* null mice die shortly after birth with anencephaly, a condition arising from the failure of the neural tube to properly adhere and close during early embryonic development (Holmberg et al., 2000). The apparent defect in adhesion is difficult to make sense of, given well-characterized roles for

EphA7 and ephrin-A5 in repulsive outcomes (Frisen et al., 1998). Both EphA7 and ephrin-A5 are co-expressed at the edges of the neural tube where adhesion takes place, but importantly also expressed in these leading epithelia is a splice variant of EphA7 in which the cytoplasmic domain of the receptor is not translated. This splice variant acts in a dominant-negative fashion to interfere with the repulsion typically mediated by full-length EphA7, as verified in cell culture assays in which EphA7 expressing cells avoid ephrin-A5 expressing cells, but cells expressing both full length EphA7 and the truncated isoform of EphA7 are not repelled from ephrin-A5 positive cells (Holmberg et al., 2000). Amazingly then, the suppression of Eph-ephrin repulsive signaling is utilized to permit cell adhesion outcomes to occur, or could even potentially convert these cell-surface receptors into adhesion molecules. The role of alternative splice forms in regulating Eph-ephrin repulsion has not been since replicated with other Eph/ephrin molecules or in other physiological process, raising questions as to how relevant this system will be, although a similar role for alternative splice forms in regulating cytoskeletal behavior has been shown in axon guidance involving Robo3 and Slit (Chen et al., 2008).

Summary

I have introduced the Ephs and the ephrins by detailing how bidirectional signal transduction between these molecules occurs, through what signaling pathways and molecular interactions these signals are proposed to convert external stimuli into cellular response, and the assortment of pertinent biological processes that appear to require these bidirectional signals, including axon guidance, formation of the synapse, vascular development, segmentation, cell migration, and stem cell activity. I would like to

highlight two important messages from this introduction. First, the dominant outcome of Eph-ephrin signaling in these physiological roles appears to be cell-cell or axon-cell repulsion; even when the Ephs and ephrins are implicated in a cell adhesion event, it is the quelling of Eph-ephrin repulsive activity that is thought to mediate the adhesive outcome. Second, at the time this study was initiated, there had not been any success in linking the physiological outcomes of bidirectional signaling with the assortment of molecules thought to interact with the Ephs and ephrins. That has improved a little bit in the last few years, highlighted by EphA4 and α-chimerin, but remains a key target of Eph-ephrin research. In an effort to fill this chasm, we have generated mice with germline mutations that specifically ablate the ability of ephrin-B2 to conduct distinct components of its reverse signal. Here I report a number of experiments in which I detail novel non-repulsive outcomes from ephrin-B reverse signaling that challenge the dogma that the Ephs and ephrins are principally repulsive signaling molecules and suggest novel signaling pathways for ephrin reverse signaling transduction.

CHAPTER 2

Bidirectional signaling between ephrin-B2 and EphB2 controls urorectal development

Dravis, C., Yokoyama, N., Chumley, MJ., Cowan, CA., Silvany, RE., Shay, J., Baker, LA., Henkemeyer, M. Bidirectional signaling mediated by ephrin-B2 and EphB2 controls urorectal development**. Dev Biol**. 271(2), 272-290 (2004).

Summary

Here I report that mice lacking either reverse signaling through ephrin-B2, or forward signaling through EphB2/B3, present with malformations in urorectal development. The specific defects in these mice, failure to properly tubularize the urethra and failure to properly septate the primitive cloaca, appear to be the result of failed cell-cell adhesion at the midline. Consistent with a role in mediating these adhesion events, both EphB2 and ephrin-B2 are specifically co-localized to the epithelia and flanking mesenchyme at the sites of midline adhesion.

Urorectal development

The partitioning of common cloaca endoderm into separate urogenital and anorectal compartments is a critical developmental process during embryogenesis. The cloaca (from the Latin word for "sewer") is an endoderm-derived opening located at the caudal end of the embryo between the primitive external genitalia (the genital tubercle) and the ventral base of the tail (Rathke, 1832; Retter, 1890; Stephens, 1963; Tourneux, 1888). The cloaca is initially tucked behind a thin membrane (the cloacal membrane) and represents a convergent point for both the urinary and intestinal systems. During embryonic development, septation of the cloaca is initiated when mesenchymal wedges (termed the Rathke and Tourneux folds) invaginate into the cloaca and extend caudally around E10.5 in the mouse. These folds then meet and adhere at the midline, where they subsequently fuse together to create the urorectal septum (URS), which will separate the cloaca into a dorsal anorectal canal and a ventral urogenital sinus that extends into the genital tubercle (GT), in a process that is complete around E14.5 in the mouse.

From this point, urorectal development proceeds in a sex-specific manner. In the female the GT does not expand in size and becomes the primitive clitoris, while the urogenital sinus is incorporated into the urethra. In males on the other hand, the GT rapidly elongates to form the penis, with the urogenital sinus extending the length of the GT along the ventral base as the urethral plate. The urethral plate will then tubularize in a proximal to distal fashion as urethral folds at the ventral base of the penis adhere at the midline to enclose the urethral endoderm, forming a functional urethra in which urine can be evacuated through the distal tip of the penis. Defects in the tubularization of the urethra in males represent a common human birth defect of poorly understood etiology

called hypospadias. Hypospadias affects one in 125 infant boys, and is hallmarked by an abnormal urethral opening along the ventral base of the penis, rather than at the distal tip (Baskin et al., 1998; Paulozzi, 1999; Paulozzi et al., 1997). Defects in cloacal septation are less common than hypospadias, affecting roughly one in 500-5,000 live births (Smith, 1998). Urorectal malformations present with a spectrum of severity, ranging from mild defects in the size of the anorectal opening to complete anorectal agenesis, in which the rectum forms an abnormal fistula on the bladder. In these most severe cases the patient presents with persistent cloaca, in which one exterior opening connects the urethra, rectum, and with females, the vagina.

The molecular etiology of urorectal development remains poorly defined. The past decade has seen successful use of knockout mice and case studies to identify a number of high-order signaling and/or transcription factors in urorectal development, including Shh and its Gli transcription factors, Homeobox transcription factors, and Wnt signaling; and it has been shown that androgen signaling and perhaps even environmental exposure are mediators of this process as well (Goodman et al., 2000; Gray et al., 2001; Haraguchi et al., 2001; Morgan et al., 2003; Perriton et al., 2002; Wilson et al., 1993; Yamaguchi et al., 1999). Noticeably absent from this list is the identification of cell adhesion molecules or cell surface receptors that directly target the actin cytoskeleton, which could potentially directly mediate the midline adhesion events responsible for urorectal septation and urethral tubularization.

Gene targeting of the ephrin-B2 cytoplasmic domain

In order to identify novel physiological roles for reverse signaling through ephrin-B2, Nobuhiko Yokoyama, a postdoctoral fellow in the Henkemeyer laboratory, used homologous recombination in murine embryonic stem cells to target the *ephrin-B2* gene (Figure 2.1). The *ephrin-B2*^{lacZ} allele replaces the sequence encoding most of the cytoplasmic domain (amino acids 264–336) with an in-frame *lacZ* cassette, leading to expression of a membrane-bound ephrin-B2-beta-galactosidase (ephrin-B2-βgal) fusion protein. By specifically deleting only the cytoplasmic domain, the ephrin-B2-βgal protein traffics to the membrane and interacts with Eph receptors on adjacent cells to activate forward signaling, but it is not able to interact with intracellular SH2 and PDZ domain-containing proteins, and is therefore unable to participate in reverse signaling (Figure 2.2). Similar strategies have been used with *EphB1, EphB2* and *ephrin-B3*, to great success in elucidating the ability of these molecules to function both cell and non-cell autonomously (Chenaux and Henkemeyer, unpublished; Henkemeyer et al., 1996; Yokoyama et al., 2001). Cre-mediated deletion of the *lacZ* cassette results in creation of the *ephrin-B2*^{T} allele, in which the cytoplasmic domain of ephrin-B2 is deleted, but not conjugated to β-gal. As mentioned in Chapter 1, the *ephrin-B2*^{T} allele functions as a protein null because the *ephrin-B2*^{T} truncated protein fails to reach the cell surface (Cowan et al., 2004).

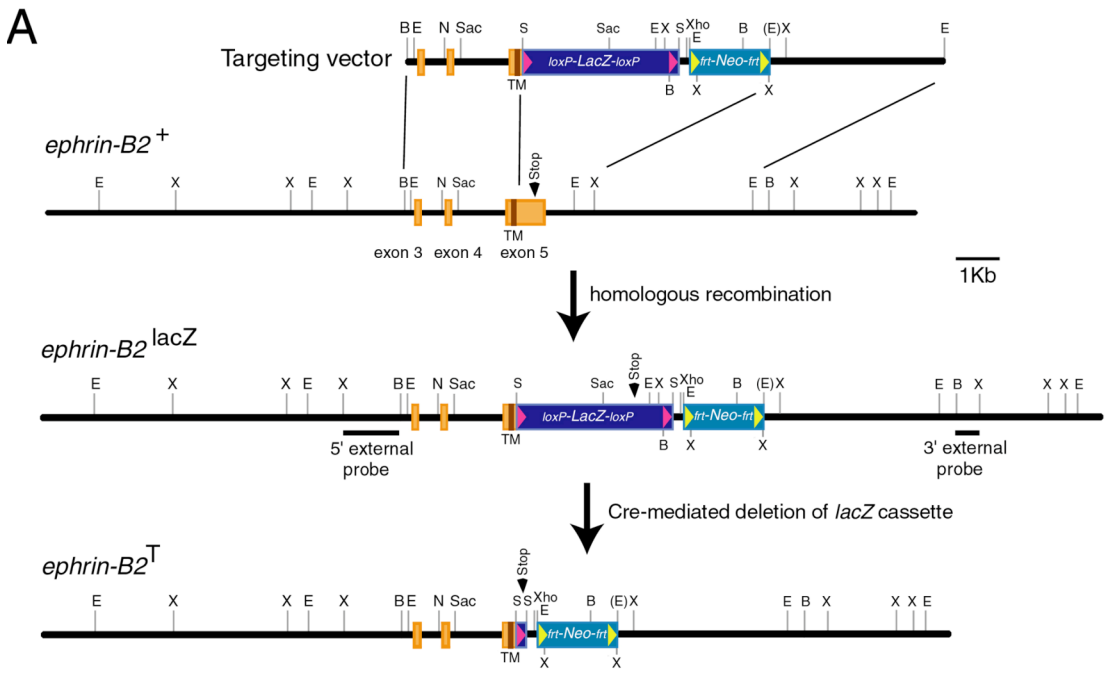


Figure 2.1

Strategy for targeting the *ephrin-B2* locus to produce the *ephrin-B2*^{lacZ} and *ephrin-B2*^{T} alleles. The *ephrin-B2*^{lacZ} allele produces an in-frame fusion of the ephrin-B2 transmembrane and extracellular domains with beta-galactosidase in place of the cytoplasmic tail of ephrin-B2, while the *ephrin-B2*^{T} allele results from Cre-mediated excision of the lacZ cassette to produce a truncation mutant of *ephrin-B2* in which the cytoplasmic tail is deleted. Image provided by Nobuhiko Yokoyama.

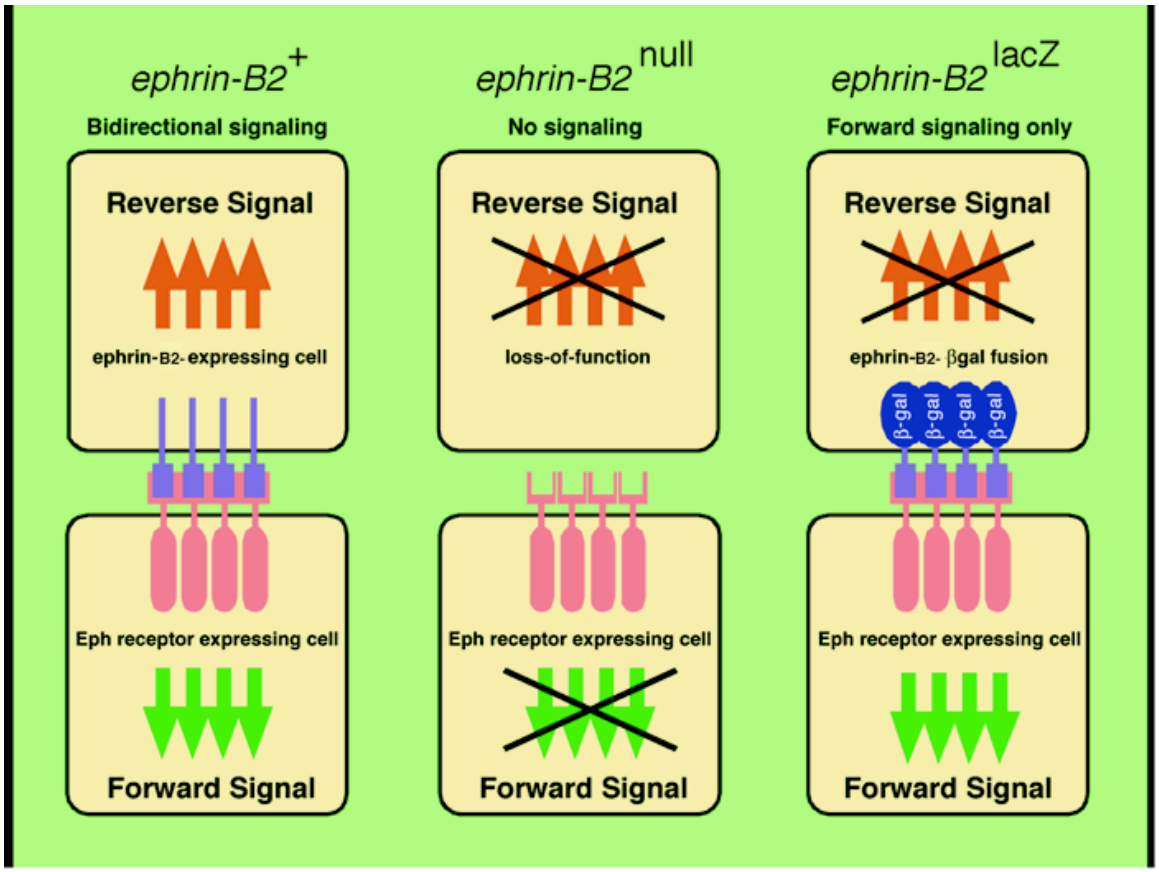


Figure 2.2

Strategy utilized to identify physiological roles for ephrin-B2 reverse signaling. In a wild-type animal, the potential exists for bidirectional signaling between Eph- and ephrin-expressing cells. In an *ephrin-B2* knockout, all signaling is lost; there is no ephrin-B2 present to transduce a reverse signal, nor is there any ephrin present to activate a forward signal in adjacent cells. To selectively remove ephrin-B2 reverse signaling, *ephrin-B2*^{lacZ} mice express an ephrin-B2-βgal fusion protein that can activate forward signaling in adjacent cells, but cannot transduce a reverse signal of its own. Comparison of these mice then allows for determination of the functional relevance of forward and reverse signaling involving ephrin-B2 in any physiological process.

Hypospadias in ephrin-B2$^{lacZ/+}$ heterozygous adult males

It was observed that 28% of *ephrin-B2*$^{lacZ/+}$ heterozygous adult male mice exhibited a normal sized, but ventrally flattened hypospadic penis resulting in sterility (Figure 2.3 and Table 2.1). Histological analysis revealed severe hypospadias as evidenced by a failure of the urethral endoderm to fuse at the ventral midline. Intimately associated with the hypospadias, *ephrin-B2*$^{lacZ/+}$ heterozygous mice present with a marked reduction in the perineal distance separating the anus from the genital tubercle, indicating defective cloacal septation (Figure 2.3). *Ephrin-B2*$^{lacZ/+}$ heterozygous mice presented with hypospadias in all of the backgrounds present in our colony, including 129, C57B6, CD1, and FVB, with an apparently complete 100% penetrance in the FVB background (data not shown). The hypospadic phenotype has never been noted in wild-type mice, or in any of the mice null singly for *EphB1, EphB2, EphB3, EphB4, EphB6, ephrin-B1*, or *ephrin-B3* presently in our colony.

Given the role of androgen signaling in urorectal development, the kidneys, ureters, bladder, and internal reproductive organs of the *ephrin-B2*$^{lacZ/+}$ heterozygous animals were examined, and nothing abnormal in their development was identified, so the defects observed are not likely a secondary product of affected sex hormones.

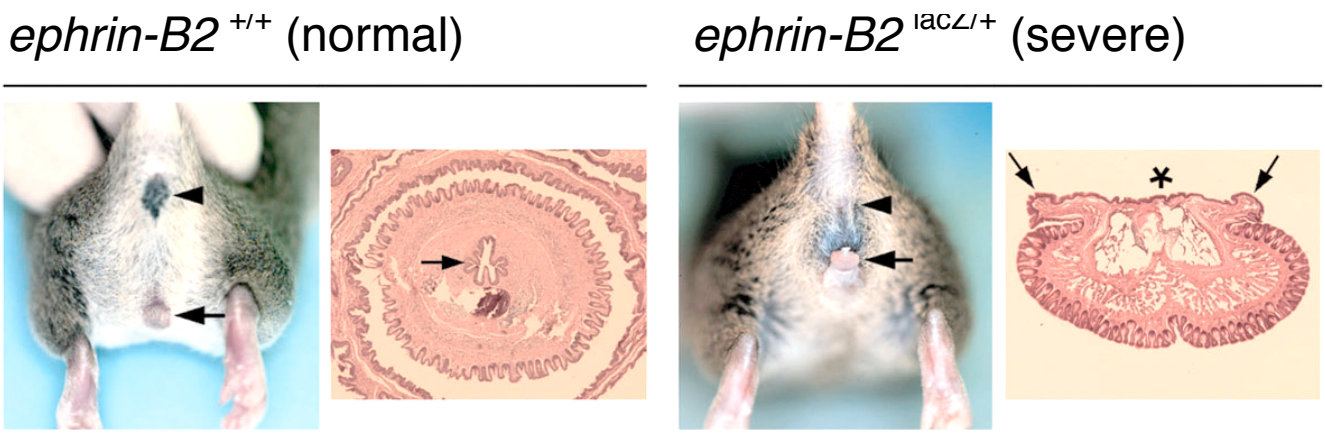


Figure 2.3

Hypospadias in adult *ephrin-B2*$^{lacZ/+}$ heterozygote males. The left panels show a wild-type male mouse with normal development of the perineum separating the penis (arrow) and anorectum (arrowhead), and proper tubularization of the urethra (arrow) in an H&E stained cross section of the penis. The right panels show an affected *ephrin-B2*$^{lacZ/+}$ heterozygote male in which the perineal distance is markedly reduced, and the penis presents with hypospadias, as evidenced by an abnormal urethral opening at the ventral base of the penis (asterisk) and the failure of the urethral folds to meet and adhere at the midline (arrows).

Table 2.1 Incidence of hypospadias in *ephrin-B2*$^{lacZ/+}$ heterozygotes.

genotype	% males with hypospadias
ephrin-B2$^{+/+}$	0 (0/482)
ephrin-B2$^{lacZ/+}$	28 (89/316)
ephrin-B2$^{T/+}$	0 (0/104)

Fertile, normal appearing *ephrin-B2*$^{lacZ/+}$ heterozygous males were mated to *ephrin-B2*$^{+/+}$ wild-type females and the resulting adult male offspring were scored for the presence of hypospadias. The total number of males that exhibited hypospadias over the total number of males scored is shown in parentheses. Results from similar matings to generate *ephrin-B2*$^{T/+}$ heterozygous males are also provided. Note reduced viability of *ephrin-B2*$^{lacZ/+}$ heterozygotes compared to the *ephrin-B2*$^{+/+}$ littermates (65.6%, 316 versus 482 expected). The *ephrin-B2*$^{T/+}$ heterozygotes do not show reduced viability (*ephrin-B2*$^{+/+}$ progeny class not shown).

Dominant-negative perturbation of reverse signaling by ephrin-B2-βgal

The hypospadias phenotype in the *ephrin-B2*$^{lacZ/+}$ heterozygous animals has not been found in *ephrin-B2* null heterozygous animals, suggesting that the ephrin-B2-βgal fusion protein possesses a dominant-negative function, perhaps in inhibiting the ability of co-expressed ephrin molecules to conduct a reverse signal (Figure 2.4B). To experimentally verify this, a postdoctoral fellow in the Henkemeyer laboratory, Michael Chumley, took COS-7 cells, transfected them with either wild-type *ephrin-B2* alone or with increasing amounts of the *ephrin-B2*^{lacZ} allele, and then stimulated reverse signaling in these cells by treating them with EphB2-Fc (the pre-clustered extracellular domain of EphB2). After stimulation, cells were lysed and probed with an antibody recognizing the tyrosine phosphorylated tail of B-ephrin, to get a readout for the intensity of the reverse signal. Strikingly, when wild-type *ephrin-B2* is expressed alone, a robust reverse signal is present, as indicated by the anti-phospho-ephrin-B immunoblot; however, as increasing amounts of the ephrin-B2-βgal fusion protein were introduced, the phospho-ephrin-B signal dramatically tapered off in intensity (Figure 2.4C). The biochemical data therefore confirms that the ephrin-B2-βgal fusion protein can inhibit the ability of wild-type ephrin-B2 to conduct a reverse signal.

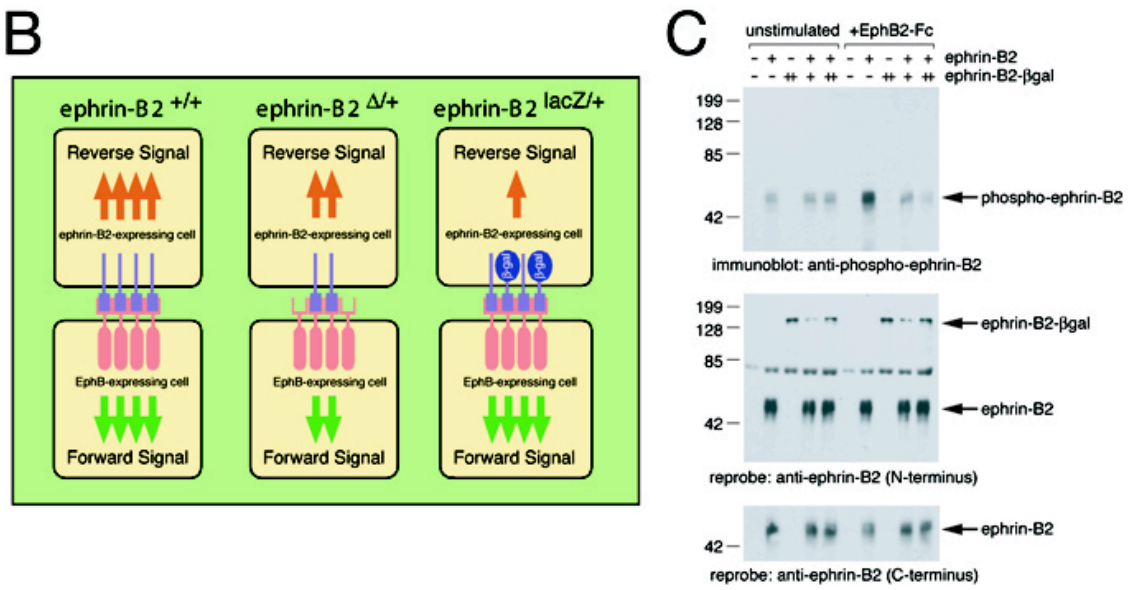


Figure 2.4

Ephrin-B2-βgal fusion protein acts in a dominant-negative fashion to inhibit its own reverse signal. (B) Schematic demonstrating how the ephrin-B2-βgal fusion protein might act in a dominant-negative fashion. In a wild-type situation, ephrin-B2 is capable of transducing a robust reverse signal (four arrows, left panel). The loss of one copy of ephrin-B2 in *ephrin-B2*$^{\Delta/+}$ animals theoretically results in a decrease in the intensity of the ephrin-B2 reverse signal (two arrows, middle panel). Expression of the ephrin-B2-βgal fusion protein in *ephrin-B2*$^{lacZ/+}$ heterozygotes may disrupt the ability of co-expressed ephrin molecules to reverse signal, resulting in less intense reverse signaling compared to the *ephrin-B2*$^{\Delta/+}$ heterozygote (one arrow, right panel). (C) Biochemistry confirming the dominant-negative behavior of the ephrin-B2-βgal fusion protein. COS-7 cells were co-transfected with wild-type ephrin-B2 cDNA and increasing amounts of the ephrin-B2-βgal fusion protein. These cells were then either left unstimulated (left) or stimulated for reverse signaling with preclustered EphB2 extracellular domain (right). Cells were then lysed and probed for phosphorylated ephrin-B to get a readout for the intensity of the reverse signal (top). Note the robust ephrin-B reverse signal in the absence of the ephrin-B2-βgal fusion protein, while the intensity of the reverse signal dramatically tapers off as more of the ephrin-B2-βgal fusion protein is co-transfected into the cell. Strip and reprobes with antibodies against N-terminal or C-terminal ephrin-B confirm the appropriate expression levels of wild-type ephrin-B2 and the ephrin-B2-βgal fusion protein (middle and bottom panels). Image provided by Michael Chumley.

Hypospadias in EphB2;EphB3 compound homozygous male mice

Ephrins do not act alone, but in concert with the Eph receptors. To identify which Eph receptors were involved in urorectal development, compound null animals for Eph receptors known to interact with ephrin-B2 were generated, given that none of the Eph receptor single null animals presented with hypospadias. It was observed that 25% of animals that were compound nulls for *EphB2* (Henkemeyer et al., 1996) and *EphB3* (Orioli et al., 1996) also exhibited hypospadias and defective cloacal septation, indicating EphB2 and EphB3 are the relevant Eph receptors in urorectal development (Figure 2.5 and Table 2.2). To determine whether EphB2 was acting cell autonomously (forward signaling) or non-cell autonomously (reverse signaling), mice encoding a truncated version of EphB2 that can activate reverse signals but can not transduce forward signals, $EphB2^{lacZ}$ (Henkemeyer et al., 1996), was crossed into an *EphB3* null background to generate $EphB2^{lacZ}$;$EphB3^{\Delta}$ compound mutants. 36% of $EphB2^{lacZ/lacZ}$;$EphB3^{\Delta/\Delta}$ compound mutant males similarly possessed hypospadias and compromised cloacal septation, indicating that in addition to reverse signaling through ephrin-B2, forward signaling through EphB2 is also important for urethral tubularization and cloacal septation.

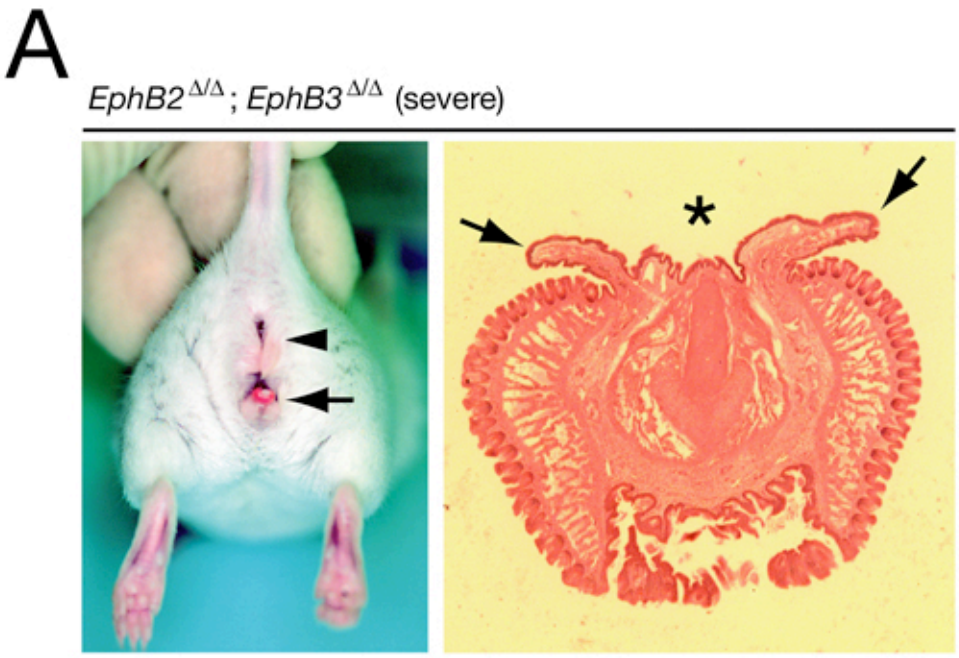


Figure 2.5

EphB2;EphB3 compound null animals present with hypospadias and reduced perineal distance. An adult *EphB2*$^{\Delta/\Delta}$;*EphB3*$^{\Delta/\Delta}$ male shows a marked reduction in the perineal distance separating the anorectum (arrowhead) and urogenital (arrow) compartments (left panel). H&E stained cross-section of the penis from the male shows an abnormal urethral opening at the ventral base of the penis (asterisk) due to a failure of the urethral folds to adhere at the midline (arrows) (right panel).

Table 2.2 Incidence of hypospadias in *EphB2*;*EphB3* compound homozygotes.

genotype	% males with hypospadias
EphB2$^{\Delta/\Delta}$;*EphB3*$^{+/+}$	0 (0/>200)
EphB2$^{lacZ/lacZ}$;*EphB3*$^{+/+}$	0 (0/>200)
EphB2$^{+/+}$;*EphB3*$^{\Delta/\Delta}$	0 (0/>200)
EphB2$^{\Delta/+}$;*EphB3*$^{\Delta/\Delta}$	0 (0/233)
EphB2$^{\Delta/\Delta}$;*EphB3*$^{\Delta/\Delta}$	25 (19/76)
EphB2$^{lacZ/+}$;*EphB3*$^{\Delta/\Delta}$	0 (0/338)
EphB2$^{lacZ/lacZ}$;*EphB3*$^{\Delta/\Delta}$	36 (26/72)

The total number of adult males that exhibited hypospadias over the total number of males scored for a given genotype class is shown in parentheses.

Ephrin-B2-βgal does not overactivate EphB forward signaling

While the biochemical data from the COS-7 cells indicates the ephrin-B2-βgal fusion protein has a dominant-negative activity that impairs the ability of co-expressed ephrin molecules to reverse signal, it is also possible that the fusion protein might function to hyperactivate forward signaling through Eph receptors as well. This is a concern because ephrin-B2-βgal may be expressed at higher levels at the cell surface than wild-type ephrin-B2, and because the tendency for the β-galactosidase to oligerimerize into tetramers on its own might confer a hyperactivated ligand state for the ephrin (Cowan et al., 2004). If the defects in urorectal development were the consequence of ephrin-B2-βgal overstimulating forward signals, a reduction of wild-type *EphB2* and *EphB3* gene copies should reduce receptor protein expression and forward signaling, thus decreasing the incidence of hypospadias. To explore this possibility, the *ephrin-B2*^{lacZ} mutation was combined with the *EphB2* and *EphB3* protein-null mutations. Strikingly, analysis of these mice revealed that 55% of the *ephrin-B2*$^{lacZ/+}$ animals that carried mutant alleles of *EphB2* and *EphB3* exhibited hypospadias (27 of 49 males), compared to the 28% of *ephrin-B2*$^{lacZ/+}$ animals that present with hypospadias in a wild-type background. This indicates more *ephrin-B2*$^{lacZ/+}$ mice present with hypospadias when combined with null mutations in *EphB2* or *EphB3* (Chi^2 statistical analysis provides a P value of <0.001). Consistent with a greater penetrance in hypospadias, mice containing both *ephrin-B2*^{lacZ} and *EphB* null mutations also presented with much more severe defects in urorectal development, and this was the case even when the *EphB2*^{lacZ} allele was included (Figure 2.6). Based on this data, it seems that the ephrin-B2-βgal fusion protein does not function to overactivate

EphB receptor forward signaling, and instead possesses dominant-negative potential only through its ability to inhibit B-ephrin reverse signaling.

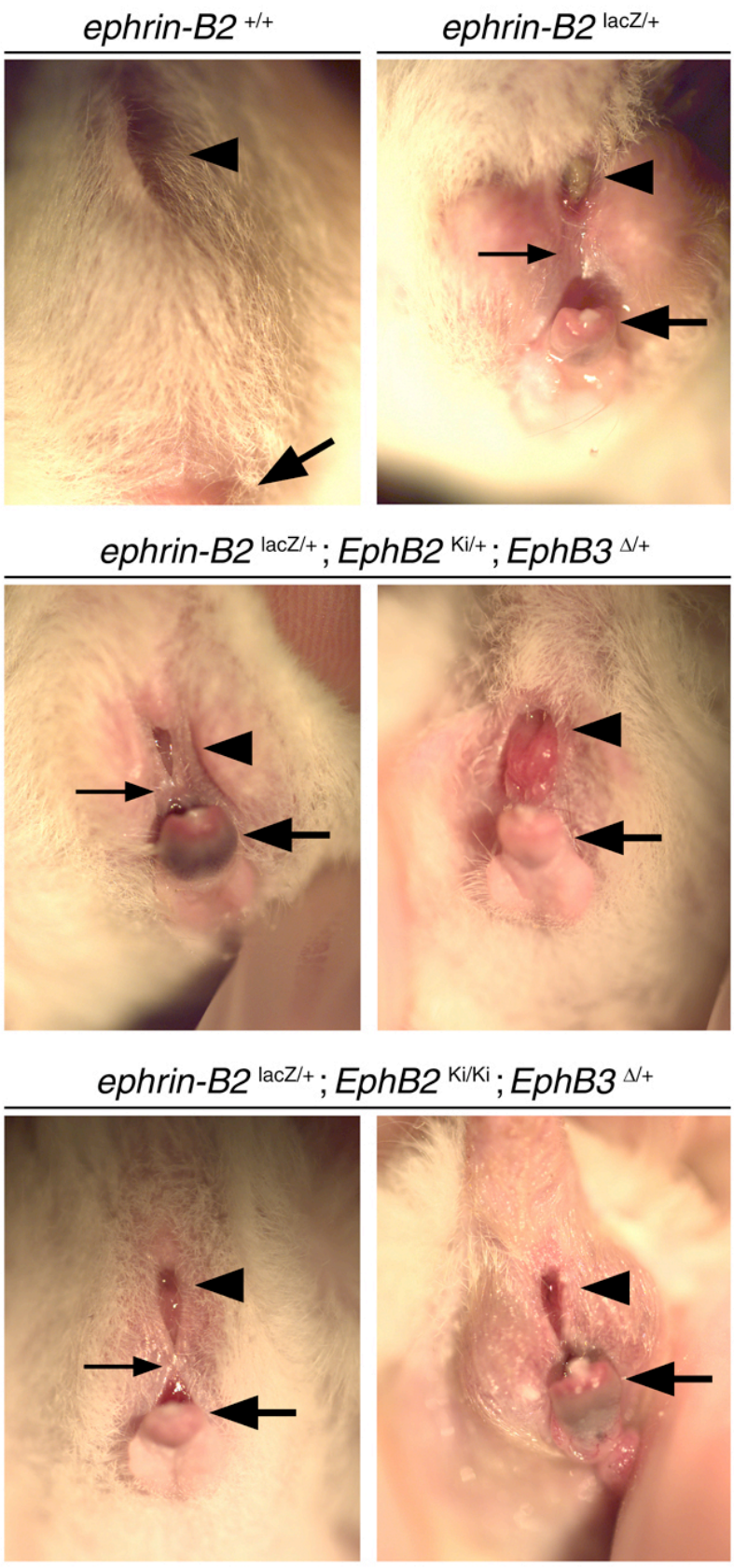

ephrin-B2 +/+
ephrin-B2 lacZ/+
ephrin-B2 lacZ/+ ; EphB2 Ki/+ ; EphB3 Δ/+
ephrin-B2 lacZ/+ ; EphB2 Ki/Ki ; EphB3 Δ/+

Figure 2.6

Ephrin-B2^{lacZ}*;EphB2;EphB3* compound mutant animals present with more severe urorectal malformations, genetically suggesting that the ephrin-B2-βgal fusion protein does not hyperactivate EphB forward signaling. The wild-type male shows a normal penis (arrow) and proper perineal distance separating the penis from the anorectum (arrowhead) (top left panel). The *ephrin-B2*$^{lacZ/+}$ heterozygote shows a more typical hypospadic penis and reduced perineum (top right panel). Four different *ephrin-B2*^{lacZ}*;EphB2;EphB3* compound mutant animals present with more severe defects than either *ephrin-B2*$^{lacZ/+}$ heterozygotes or *EphB2;EphB3* compound mutants, typified by reduced perineal distance based on the smaller amount of tissue separating the penis and anorectum (small arrow) (middle and bottom panels). *EphB2*^{Ki} here is the *EphB2*^{lacZ} allele.

Detailed phenotypic and expression analysis of *ephrin-B2*$^{lacZ/+}$ heterozygotes and *EphB2;EphB3* compound homozygotes—incomplete septation of the cloaca

In addition to the hypospadias found in both *ephrin-B2*$^{lacZ/+}$ and *EphB2;EphB3* mutant animals, these mice also present with a marked reduction in the perineal distance separating the anorectal and urogenital systems. Whole-mounts taken from late stage embryos show that whereas in the wild-type lateral folds have clearly come together and adhered at the midline to separate the cloaca into distinct anorectal and urogenital compartments, in both *ephrin-B2*$^{lacZ/+}$ and *EphB2;EphB3* animals this process has clearly failed, either moderately so where the lateral wedges have come into contact but have not properly fused together, or very severely so where no signs of adhesion are present and the animal is left with a persistent cloacal cavity (Figures 2.7 and 2.9).

To determine where these molecules are expressed during cloacal septation, X-gal stains were performed for ephrin-B2 and EphB2, using the *ephrin-B2*^{lacZ} and *EphB2*^{lacZ} alleles, respectively, on both whole-mounts and sections through the site of cloacal septation. Ephrin-B2 is noticeably expressed in whole-mounts along midline endoderm at the site of septation at E17 and along the urethral plate at E13 (Figure 2.7 and 2.8). Sections through the caudal end of an *ephrin-B2*$^{lacZ/+}$ embryo at E17 reveal a hypospadic penis with untubularized urethra compared to a wild-type littermate. Deeper sections reveal a marked reduction in the size of the URS separating the developing rectum and urethra compared to the wild-type (Figure 2.8). Utilizing the X-gal stain, ephrin-B2 is noticeably expressed on adhering epithelia at all sites of adhesion in urorectal development. This includes ephrin-B2 expression at the two sites of adhesion for urethral tubularization, at both the distal glans and the proximal ventral base, and in the midline

epithelia where the lateral folds are coming together to septate the cloaca (Figure 2.8). Once septation has taken place, ephrin-B2 becomes restricted to the urethral endoderm and not the hindgut.

Similar expression studies were performed using the *EphB2*^{lacZ} allele. Serial sections of the caudal end of an E17 *EphB2*$^{lacZ/lacZ}$;*EphB3*$^{\Delta/\Delta}$ compound mutant embryo were X-gal stained to determine EphB2 expression (Figure 2.9). Histological analysis shows, as in the affected *ephrin-B2*$^{lacZ/+}$ embryo, failed proximal closure of the urethra and incomplete midline septation of the cloaca. Strikingly, the X-gal stain localizes EphB2 to the apical edge of epithelial cells lining the lateral folds that are adhering at the midline to partition the cloaca. EphB2 and ephrin-B2 therefore both appear to be expressed in adherent epithelia at the sites of urorectal adhesion.

Because cloacal septation and urethral tubularization are typically complete before E17 in the wild-type embryo, I next examined the expression of EphB2 and ephrin-B2 at earlier stages in hindgut development. Serial sections of the cloaca from E13 *EphB2*$^{lacZ/lacZ}$ embryos were X-gal stained to detect EphB2; noticeably the Eph receptor is expressed in the epithelia at the site of adhesion, as well as in the flanking mesenchyme (Figure 2.10). After septation, EphB2 shows a reciprocal expression pattern to ephrin-B2 in that it preferentially localizes to the hindgut, instead of urethral endoderm. A similar expression profile was obtained from indirect immunofluorescence (IF) on sections of the cloaca from a wild-type embryo using an antibody directed against EphB2 (Figure 2.10). Once again EphB2 is expressed in both the epithelia at the site of adhesion and in the flanking mesoderm, compared to parallel stains in an *EphB2* null embryo, which served as a negative control. X-gal stains of E13 *ephrin-B2*$^{lacZ/+}$ embryos revealed that ephrin-B2 is

also noticeably expressed in the epithelia at the site of adhesion and in the flanking mesenchyme, and, as in X-gal stains from E17, ephrin-B2 again preferentially localizes to the urethral endoderm instead of the hindgut after septation (Figure 2.10).

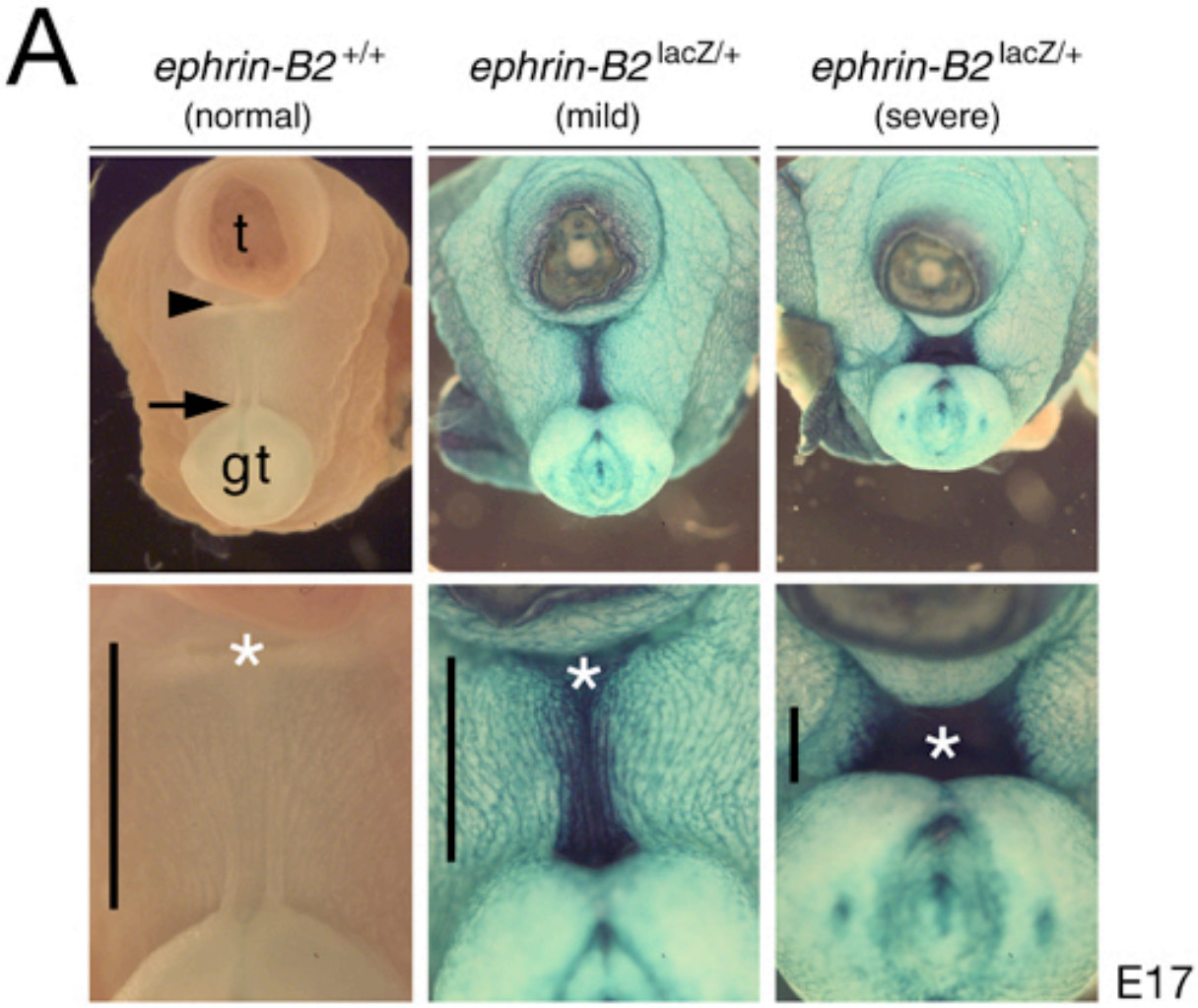


Figure 2.7

Hypospadias and incomplete cloacal septation in *ephrin-B2*$^{lacZ/+}$ heterozygotes. Whole-mount images of the caudal end of X-gal stained E17 embryos. Note the proper fusion at the midline of lateral folds to separate the anorectum (*) at the base of the tail (t) (arrowhead) and the genital tubercle (gt) (arrow) in the wild-type (left). In the two affected heterozygotes, midline fusion of these folds is either mildly (middle) or severely (right) incomplete, resulting in reduced perineal distance (vertical bars) and a persistent cloacal opening. The bottom panels represent higher magnification images. X-gal stain localizes ephrin-B2 to the point of adhesion at the midline (blue).

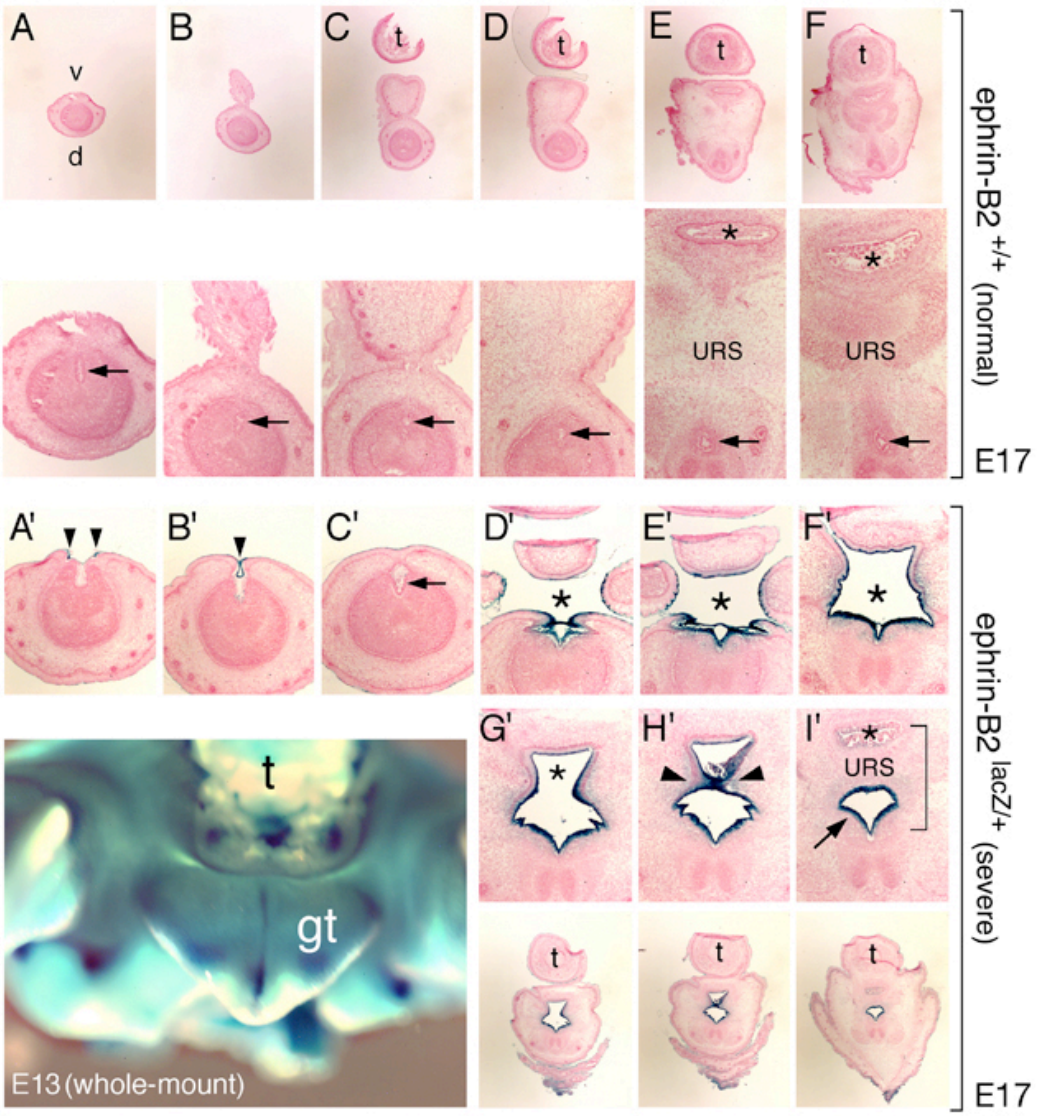


Figure 2.8

Hypospadias and incomplete cloacal septation in *ephrin-B2*$^{lacZ/+}$ heterozygotes. Serial sections from a wild-type E17 embryo show normal tubularization of the urethra (arrows) and formation of the urorectal septum (URS) to partition the urethra from the anorectum (asterisk) (A-F). In comparison, serial sections from an *ephrin-B2*$^{lacZ/+}$ heterozygote show hypospadias due to failed urethral fold adhesion (A-D′) and incomplete septation of the cloaca (D-I′). Where septation has occurred, the URS is markedly reduced in size compared to the wild-type (I′). X-gal stains show no signal in the wild-type (top), while ephrin-B2 is localized to sites of adhesion in the *ephrin-B2*$^{lacZ/+}$ heterozygote, including at urethral fold adhesion in the penis (A-D′) and cloacal septation (H′). A whole-mount X-gal stained E13 *ephrin-B2*$^{lacZ/+}$ heterozygote similarly shows ephrin-B2 is expressed at the midline where urethral fold adhesion will take place on the genital tubercle (inset bottom left).

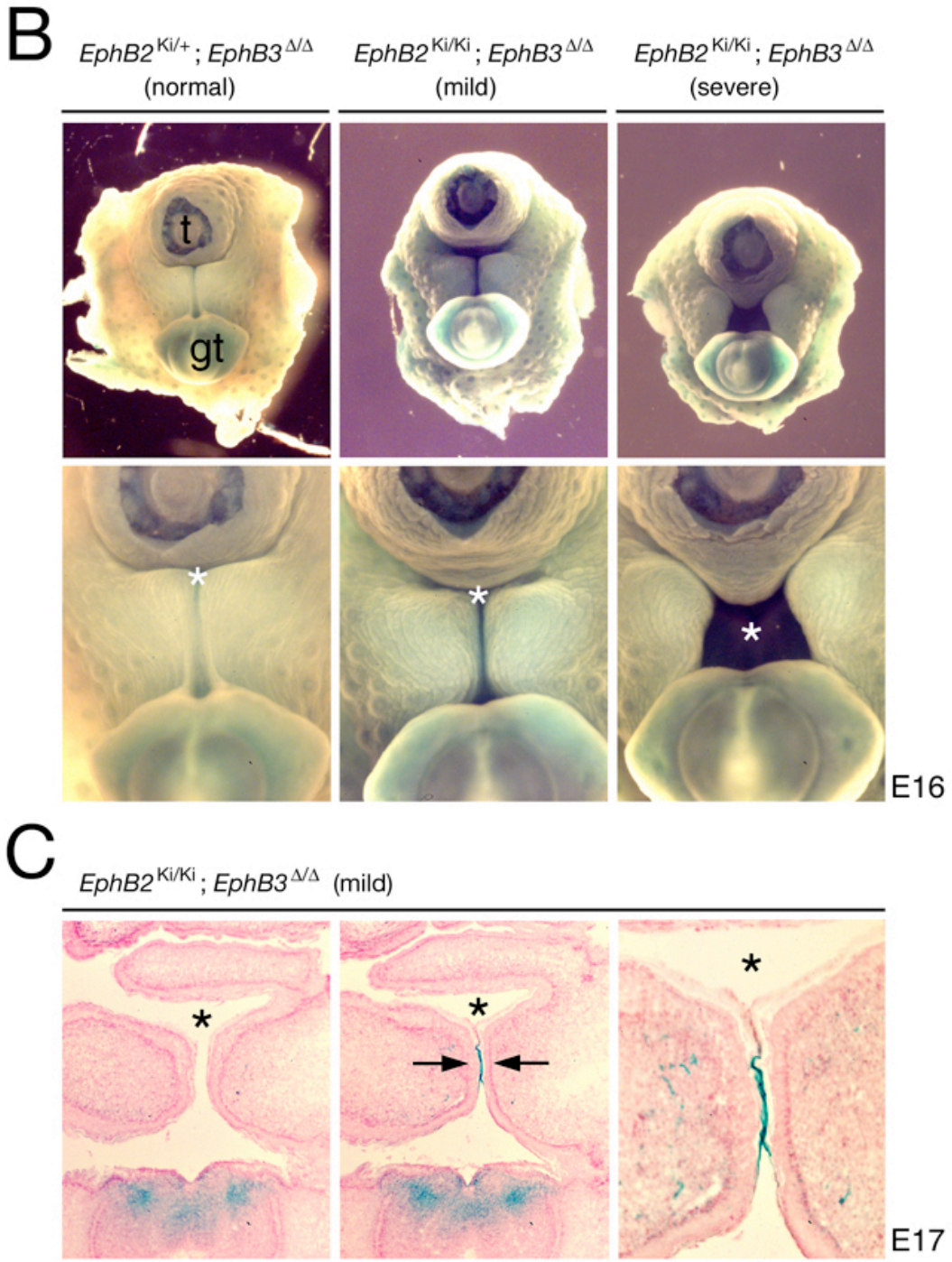


Figure 2.9 *EphB2*^{lacZ};*EphB3*$^{\Delta}$ compound mutants present with hypospadias and incomplete cloacal septation. (B) Caudal end whole-mount view of E16 embryos demonstrate that while lateral folds have properly adhered at the midline to separate the genital tubercle (gt) from the anorectum (asterisk) in the *EphB2*$^{lacZ/+}$;*EphB3*$^{\Delta/\Delta}$ embryo, in two *EphB2*$^{lacZ/lacZ}$;*EphB3*$^{\Delta/\Delta}$ mutants this process has failed, leaving behind an open cloaca cavity (right panels). (C) Serial sections of the cloaca from an *EphB2*$^{lacZ/lacZ}$;*EphB3*$^{\Delta/\Delta}$ animal were X-gal stained to detect EphB2 expression. EphB2 is expressed apically in epithelia adhering at the midline (arrows). *EphB2*^{Ki} here is the *EphB2*^{lacZ} allele.

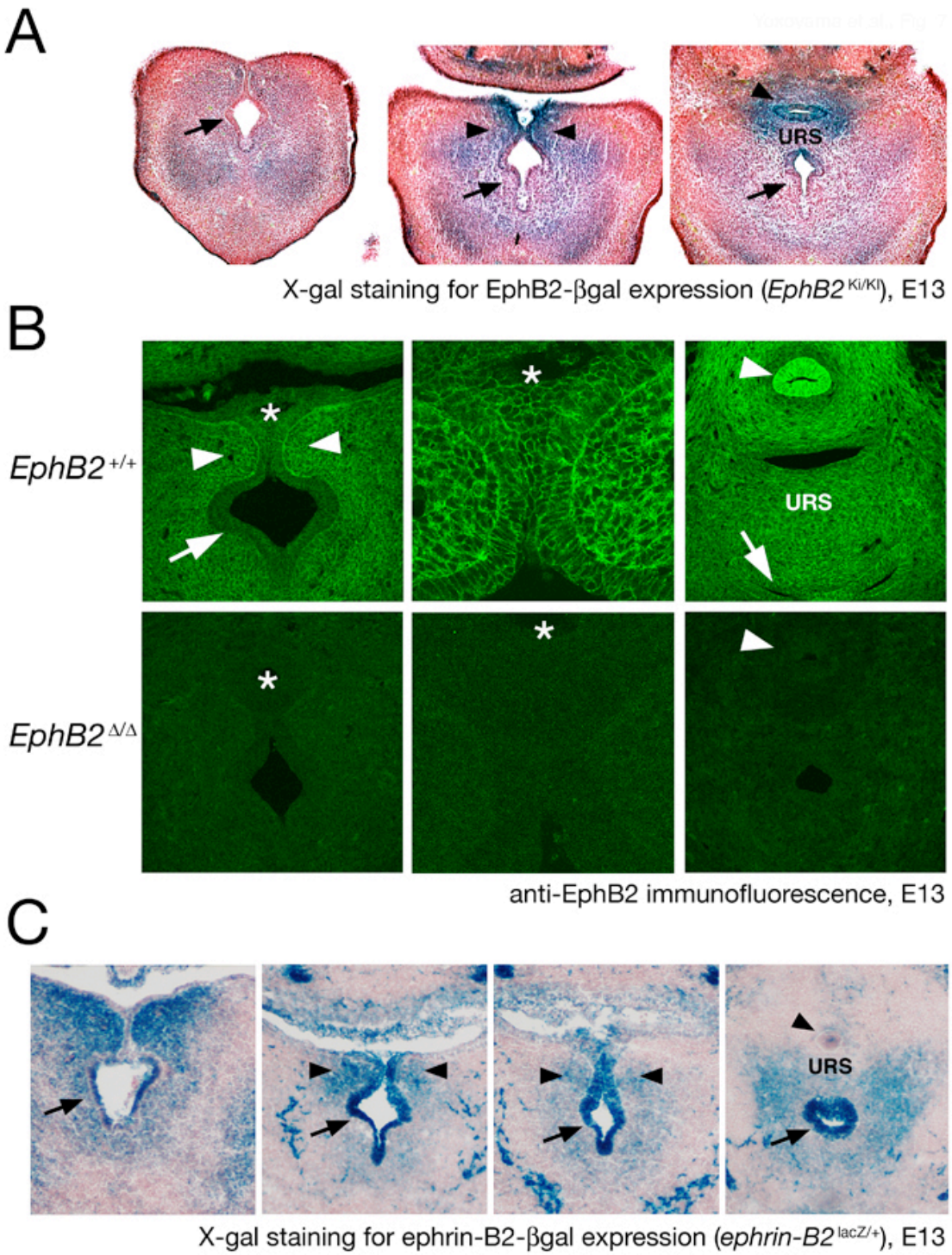


Figure 2.10

EphB2 and ephrin-B2 are expressed at the point of adhesion in urorectal development. (A) Serial sections through cloaca septation from E13 *EphB2*$^{lacZ/lacZ}$ embryos were X-gal stained. EphB2 is noticeably expressed in the epithelia and flanking mesenchyme at the point of adhesion (arrowheads). After septation, EphB2 preferentially localizes to the anorectum (arrowhead, right panel) instead of the urethra (arrow). *EphB2*^{Ki} here is the

EphB2^{lacZ} allele. (B) Immunofluorescence was performed on wild-type (top) or *EphB2*$^{\Delta/\Delta}$ tissue (bottom) for EphB2. EphB2 shows a similar expression as above, showing heavy expression along the epithelia and mesenchyme at the site of adhesion. No signal was obtained in the *EphB2*$^{\Delta/\Delta}$ negative control. (C) Serial sections of cloacal septation from E13 *ephrin-B2*$^{lacZ/+}$ heterozygotes were X-gal stained to determine ephrin-B2 expression. As with EphB2, ephrin-B2 is noticeably expressed in the epithelia and flanking mesenchyme at the point of adhesion (arrowheads). Unlike EphB2, ephrin-B2 is preferentially localized to the urethra after septation occurs (arrow).

EphB2 and ephrin-B2 are co-expressed in cells that meet at the midline

The individual expression analyses of ephrin-B2 and EphB2 at both early and late stages of urorectal development suggested that these molecules might be co-expressed in the epithelia and flanking mesenchyme at sites of adhesion. To examine this, IF was performed on E13 *ephrin-B2*$^{lacZ/+}$ embryos using antibodies against β-galactosidase to recognize ephrin-B2 and against EphB2 to recognize the Eph receptor (Figures 2.11). As was suggested by the previous expression work, EphB2 and ephrin-B2 are clearly co-expressed at the site of cloacal septation, both in the epithelia at the site of adhesion, and most noticeably in the mesenchyme flanking the site of septation.

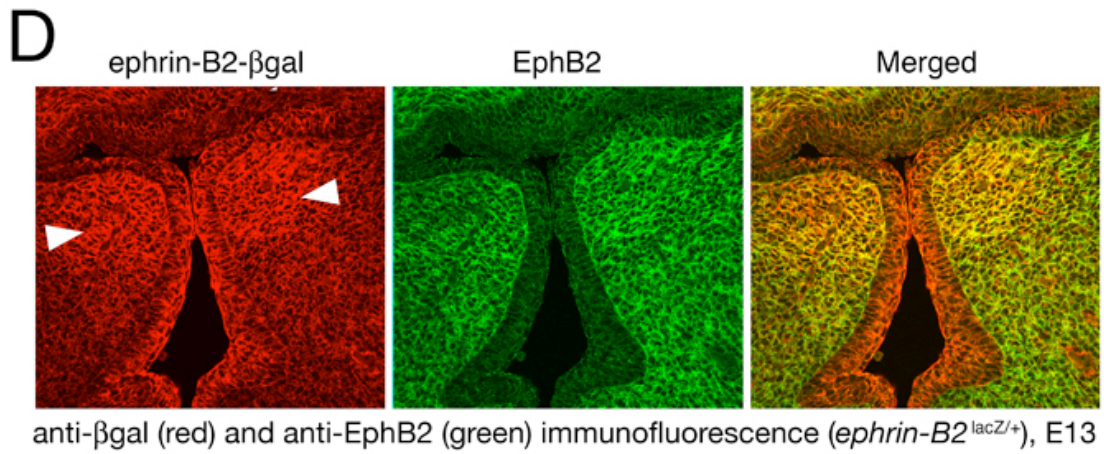


Figure 2.11

EphB2 and ephrin-B2 are co-expressed at the midline during cloacal septation. Double IF was performed on a section of cloacal septation from an E13 *ephrin-B2*$^{lacZ/+}$ heterozygote using antibodies against β-gal (red) and EphB2 (green). The individual expression profiles for EphB2 and ephrin-B2 match previous data, localizing both molecules to adherent epithelia and the mesenchyme adjacent to the adhesion point. Co-expression of EphB2 and ephrin-B2 in the epithelia and mesenchyme is apparent in the merged image (yellow).

Ephrin-B2$^{lacZ/lacZ}$ homozygotes exhibit a complete failure in cloacal septation

Given the severity of the phenotypes in *ephrin-B2*$^{lacZ/+}$heterozygote embryos, we speculated that more severe defects in cloacal septation might occur in *ephrin-B2*$^{lacZ/lacZ}$ homozygote embryos. Because *ephrin-B2*$^{lacZ/lacZ}$ homozygotes survive until P0, it is feasible to assess urorectal development in the complete absence of ephrin-B2 reverse signaling, which had previously been impossible because *ephrin-B2* null embryos die by E11 with severe vascular defects (Cowan et al., 2004). Analysis of *ephrin-B2*$^{lacZ/lacZ}$ homozygotes revealed that these animals present with complete failure in cloacal septation with 100% penetrance. As shown in sagittal sections from late stage embryos, the defect presents itself as complete anal atresia, characterized by the absence of a rectal cavity or anal opening, in which the intestine instead forms a fistula to the urethra at the base of the bladder, distal to which the embryo only has common, unseptated cloacal endoderm (Figure 2.12). This unseptated cloacal endoderm takes on the appearance of urethral endoderm, based on morphology and the fact that it expresses high levels of ephrin-B2 (Figure 2.13B).

Histological sections of *ephrin-B2*$^{lacZ/lacZ}$ homozygotes were taken from E11 and E13, when cloacal septation is just being started. While wild-type and and unaffected *ephrin-B2*$^{lacZ/+}$ heterozygote embryos showed normal septation, as in the late stage embryos, cloacal septation in the *ephrin-B2*$^{lacZ/lacZ}$ homozygotes was completely absent. This was even the case at E11, where the overall pattern of cloacal endoderm in the *ephrin-B2*$^{lacZ/lacZ}$ homozygous mutant is similar to that seen in the wild-type and heterozygote, but whereas the cloaca ultimately adheres and fuses to produce the URS in

the wild-type *ephrin-B2*$^{lacZ/+}$ control animals, the open cloacal cavity just persists in the *ephrin-B2*$^{lacZ/lacZ}$ homozygotes (Figure 2.13C). IF for ephrin-B2 and EphB2 in *ephrin-B2*$^{lacZ/lacZ}$ homozygotes at E13 shows that even though cloacal septation has failed in these mutants, EphB2 and ephrin-B2 are co-expressed where adhesion should be taking place (Figure 2.13B).

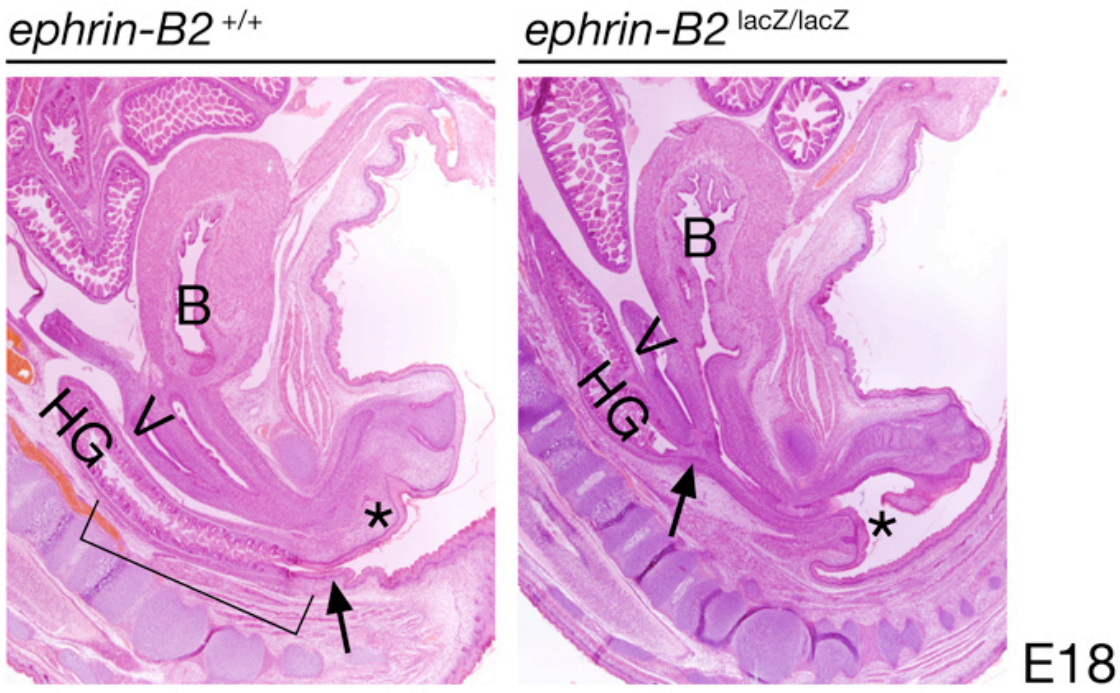
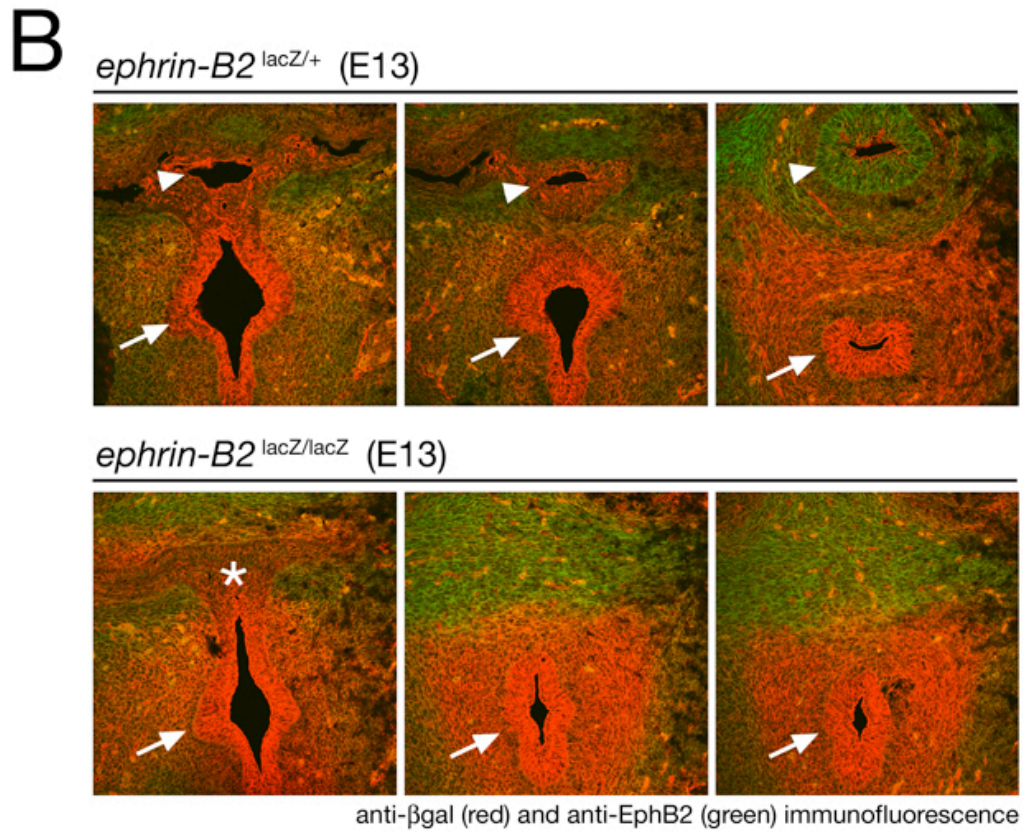


Figure 2.12

Failed midline septation of the cloaca in *ephrin-B2*$^{lacZ/lacZ}$ homozygotes. H&E stained sagittal sections from wild-type (left) and *ephrin-B2*$^{lacZ/lacZ}$ homozygous (right) E18 female embryos. The wild-type shows clear separation between the anorectum (HG) and the vagina (V) and urethra, and the hindgut extends to the anal pit (arrow). In contrast, in the homozygote, the hindgut forms a fistula with the urethra at the base of the bladder (B), distal to which there is only common endoderm that does not reach the anal pit (arrow, left panel), but instead empties into a cloaca cavity at the base of the genital tubercle (asterisk).



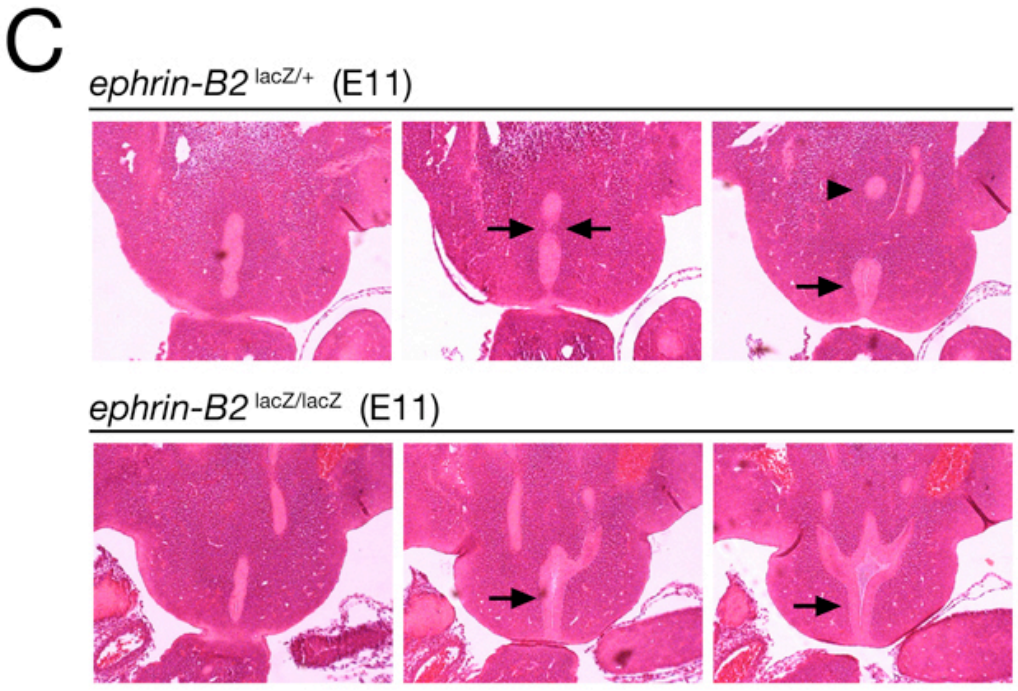


Figure 2.13
Failed midline septation of the cloaca in *ephrin-B2*$^{lacZ/lacZ}$ homozygotes. (B) Serial sections of cloacal septation from E13 *ephrin-B2*$^{lacZ/+}$ heterozygotes and *ephrin-B2*$^{lacZ/lacZ}$

homozygotes were double immunostained for β-gal (red) and EphB2 (green). *ephrin-B2*$^{lacZ/+}$ heterozygote shows cloaca septating to produce distinct anorectal (arrowhead) and urethral (arrow) endoderm. In contrast, the cloaca has failed to septate in the *ephrin-B2*$^{lacZ/lacZ}$ homozygote, leaving only common endoderm that has taken on a urethral appearance (arrow). Note that ephrin-B2 and EphB2 still segregate in the homozygote, even though septation has not taken place. Also note that ephrin-B2 and EphB2 expression overlaps where septation should be taking place. (C) H&E stained serial sections through early hindgut septation in E11 *ephrin-B2*$^{lacZ/+}$ heterozygotes (top) and *ephrin-B2*$^{lacZ/lacZ}$ homozygotes (bottom). While the overall patterning of the hindgut looks similar in both the heterozygote and the homozygote (left panels), septation into distinct primitive anorectum and urethra occurs in the heterozygote, while in the *ephrin-B2*$^{lacZ/lacZ}$ homozygote, a common cloacal cavity persists.

Signaling through ephrin-B2 SH2 and PDZ domain interaction sites play important but non-essential roles in urorectal development

To determine what signaling pathways and molecular interactions might be important for the ephrin-B2 reverse signal at the caudal midline, I developed *ephrin-B2*$^{\Delta V}$ mice that possessed a germline point mutation in *ephrin-B2* in which the C-terminal valine residue on the ephrin-B2 cytoplasmic tail was deleted, to eliminate PDZ domain interactions (Figure 2.14). In a parallel effort, another graduate student in the lab, George Chenaux, developed *ephrin-B2*$^{6YF\Delta V}$ mice, in which not only is the C-terminal valine deleted, but six tyrosines on the ephrin-B2 cytoplasmic tail are also converted to phenylalanines, in order to eliminate both PDZ and SH2 domain interactions, respectively. *Ephrin-B2*$^{\Delta V}$ and *ephrin-B2*$^{6YF\Delta V}$ heterozygotes were crossed to generate homozygote mutants, and strikingly neither *ephrin-B2*$^{\Delta V/\Delta V}$ nor *ephrin-B2*$^{6YF\Delta V/6YF\Delta V}$ mice present with any apparent defects in either cloacal septation or urethral tubularization, indicating that signals through SH2 and PDZ domain interactions are not playing essential roles in ephrin-B2 reverse signaling at the caudal midline.

However, when *ephrin-B2*$^{6YF\Delta V/+}$ heterozygotes were crossed with *ephrin-B2*$^{lacZ/+}$ heterozygotes to generate *ephrin-B2*$^{lacZ/6YF\Delta V}$ mutant mice, I saw a much greater incidence of hypospadias and cloacal malformation in these *ephrin-B2*$^{lacZ/6YF\Delta V}$ mutants than with *ephrin-B2*$^{lacZ/+}$ alone. 86.7% of *ephrin-B2*$^{lacZ/6YF\Delta V}$ mice (n=15) presented with hypospadias and defects in cloacal septation, compared with 14.2% of *ephrin-B2*$^{lacZ/+}$ mice (n=21) from the same crosses (Table 2.3). Therefore, while signaling through the SH2 and PDZ domains of ephrin-B2 is not essential for proper urorectal development, the

molecular interactions through these signaling avenues do appear to be playing some role in properly mediating these midline adhesion events.

Table 2.3 Incidence of hypospadias in *ephrin-B2*$^{lacZ/6YF\Delta V}$ animals.

genotype	% males with hypospadias
ephrin-B2$^{+/+}$	0 (0/>20)
ephrin-B2$^{lacZ//+}$	14.3 (3/21)
ephrin-B2$^{6YF\Delta V/+}$	0 (0/24)
ephrin-B2$^{6YF\Delta V/6YF\Delta V}$	0 (0/>20)
ephrin-B2$^{6YF\Delta V/lacZ}$	86.7 (13/15)

The total number of adult males that exhibited hypospadias over the total number of males scored for a given genotype class is shown in parentheses.

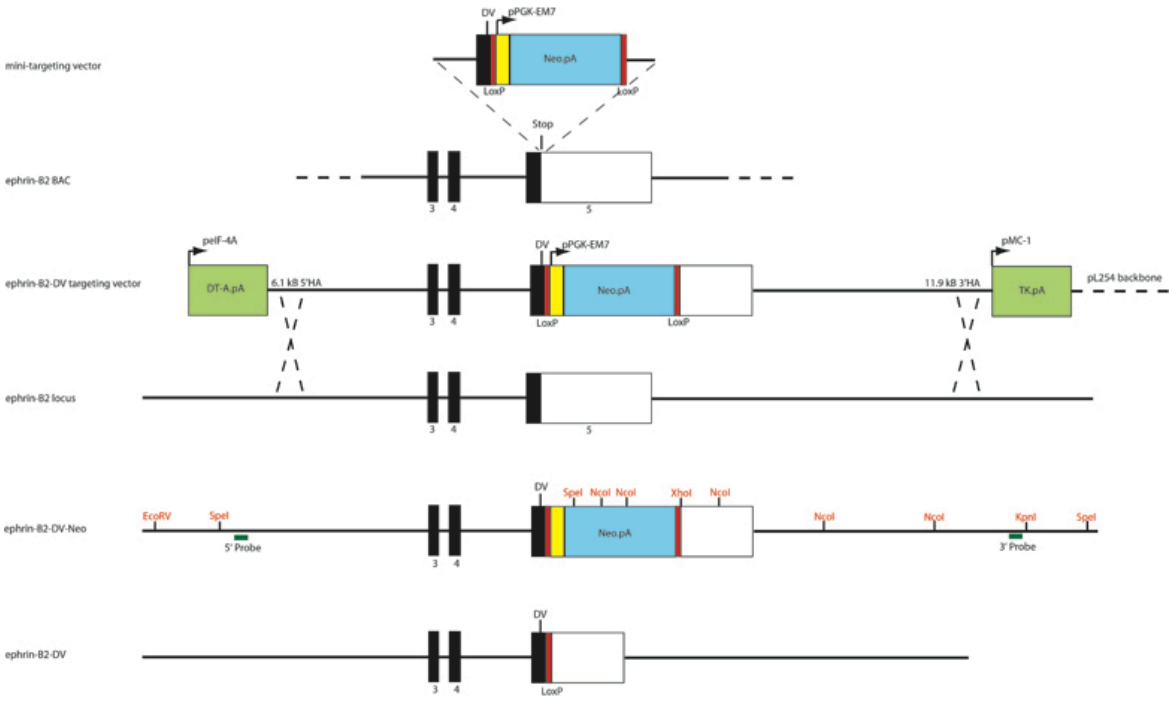


Figure 2.14

Strategy detailing the generation of *ephrin-B2*$^{\Delta V}$ mice. DV refers to the deletion of the C-terminal valine residue.

Discussion

I have described a requirement for bidirectional signaling between ephrin-B2 and the Eph receptors B2 and B3 in urorectal development. Homozygote *ephrin-B2*$^{lacZ/lacZ}$ mice lacking all ephrin-B2 reverse signaling have a fully-penetrant defect in cloacal septation, characterized by the absence of the terminal-most hindgut and a fistula of the intestine onto the urethra at the base of the bladder. Heterozygote *ephrin-B2*$^{lacZ/+}$ mice, in which ephrin-B2 reverse signaling is knocked down through dominant-negative activity of the ephrin-B2-βgal fusion protein present with a mild defect in cloacal septation, often presenting as a reduction in the perineal distance separating the anorectal and urogenital systems of adult male mice; further, these mice present with a hypospadias phenotype, in which the urethral opening is abnormally localized along the ventral base of the penis, instead of at the distal tip. *EphB2;EphB3* compound mutant animals, including a mutation in which EphB2 is rendered inactive for forward signaling only, also present with these same urorectal defects, indicating EphB2 and EphB3 are the EphB receptors activating ephrin-B2 reverse signaling in these midline adhesion events, and that forward signaling through these EphB receptors is also necessary for proper urorectal development. Expression data localizes both EphB2 and ephrin-B2 to the sites of midline adhesion in cloacal septation, and in fact these molecules appear to be co-expressed in the epithelia and flanking mesenchyme at the point of adhesion. Finally, analysis of *ephrin-B2*$^{6YF\Delta V}$ mice in which the SH2 and PDZ domain interactions with ephrin-B2 have been abolished indicate that reverse signaling through these avenues play important, but non-essential roles in caudal midline adhesion. The data therefore suggests novel roles for ephrin-B

reverse signaling in mediating cell-cell adhesion, which challenges the stereotypical outcome of cell repulsion for these molecules.

CHAPTER 3

Ephrin-B2 reverse signaling is necessary for tracheoesophageal septation of the foregut

Summary

Having demonstrated a requirement for ephrin-B2 reverse signaling in urorectal septation of the hindgut, I show that ephrin-B2 reverse signals are also necessary for midline septation of the foregut as well. As in hindgut septation, ephrin-B2 is localized to adherent epithelia at the site of septation, suggesting a similar role in mediating cell-cell adhesion.

Tracheoesophageal septation

Septation of the embryonic foregut is an essential process during early embryonic development in which common foregut endoderm becomes partitioned to distinguish the primitive esophagus from the trachea. The process by which this event occurs is of poorly

known molecular etiology and controversial mechanics (Kluth and Fiegel, 2003; Orford et al., 2001). Histological analysis shows that around E10 in murine embryonic development, the common embryonic foregut begins to septate into distinct endodermal compartments that will constitute the ventral trachea and dorsal esophagus. This process begins at the caudal end of the foregut, and extends cranially until the process is complete around E11.5. The process by which this septation occurs is speculative, although the prevailing hypothesis is that septation of the foregut proceeds much as septation of the hindgut occurs; that is, lateral wedges invaginate into the endoderm, creating adhesion sites for adjacent epithelia at the midline, which then adhere together and fuse to create a septum that divides the foregut into separate compartments that become the primitive esophagus and trachea. In short, like the cloaca, foregut septation also appears to be a midline cell-cell adhesion event. The only molecules presently linked to foregut septation through mouse genetics include, as with hindgut septation, high order signaling molecules and transcription factors such as Shh, Gli2/3, Foxf1 and Nkx2.1 (Felix et al., 2004; Mahlapuu et al., 2001; Minoo et al., 1999; Spilde et al., 2003). Case studies in humans have also linked tracheoesophageal development to various transcription factors, other DNA-binding proteins, and chromosomal deletions (Marsh et al., 2000; Ondrey et al., 2000; van Bokhoven et al., 2005; Vissers et al., 2004; Williamson et al., 2006).

Defects in septation of the foregut mimic common human birth defects, with an incidence of 1 in 3-4,000, and a range of phenotypes from a fistula in which the esophagus and trachea remain connected, to the most severe defect of laryngotracheoesophageal cleft (LTEC), in which the foregut fails to septate and a common endodermal tube serves to intake both air into the lungs and food/water into the gut (Depaepe et al., 1993; Sparey et

al., 2000). Birth defects affecting foregut septation carry significantly more severe prognoses for the affected than those associated with hindgut septation; whereas hindgut septation defects require non-emergent surgical attention and generally only affect the quality of life, defects in foregut septation can be life-threatening due to the reflux of gastrointestinal matter into the breathing apparatus and require immediate surgical intervention (Shehab and Bailey, 2001).

Laryngotracheoesophageal cleft in *ephrin-B2*^{lacZ} animals

The initial studies of *ephrin-B2*$^{lacZ/lacZ}$ homozygotes indicated a loss of ephrin-B2 reverse signaling leads to neonatal lethality, with defects in cardiac valve development, axon pathfinding, and midline adhesion/fusion of the urethra and anorectum (Cowan et al., 2004; Dravis et al., 2004). Because of the urorectal/hindgut defects, I examined *ephrin-B2*$^{lacZ/lacZ}$ homozygote embryos to determine if septation of the foregut was similarly affected. I found that approximately 50% (n=15) of late stage *ephrin-B2*$^{lacZ/lacZ}$ embryos exhibited defects in tracheoesophageal septation as evidenced by common, unseptated foregut. In normal foregut development a septum forms to separate the esophagus from the trachea (Figure 3.1A, left panel). The defects observed in the *ephrin-B2*$^{lacZ/lacZ}$ mutants appeared with a phenotypic range of moderate, in which a septum is visible but had failed to extend to the rostral apex, to severe, in which no septum was present, and only unseptated foregut was present rostral to the bronchi (Figure 3.1A, middle and right panel). Sections taken of E14.5 embryos showed the same defect in foregut septation; distinct trachea and esophagus are visible in the wild-type embryo, while an *ephrin-B2*$^{lacZ/lacZ}$ littermate showed only unseptated foregut (Figure 3.1B). The failure of foregut

septation in these *ephrin-B2*$^{lacZ/lacZ}$ homozygotes results in morphological abnormalities in the unseptated trachea. This is visualized by the abnormal appearance of disorganized cartilage rings in *ephrin-B2*$^{lacZ/lacZ}$ embryos (Figure 3.1C).

I next examined *ephrin-B2*$^{lacZ/lacZ}$ embryos at earlier stages of development to determine when foregut septation was going awry, and perhaps gain insight as to why the process was failing in these mutants. While E10.5 *ephrin-B2*$^{lacZ/+}$ embryos appeared normal and showed a common cranial foregut that septated into separate tracheal and esophageal endoderm caudally, *ephrin-B2*$^{lacZ/lacZ}$ littermates showed no signs of septation, even at the terminal caudal point of the foregut (Figure 3.2D). The overall patterning of the foregut therefore appears similar between the *ephrin-B2*$^{lacZ/lacZ}$ animals and their control littermates, the only difference being that the initiation of adhesion and septation occurs in the controls, but not in the *ephrin-B2*$^{lacZ/lacZ}$ embryos.

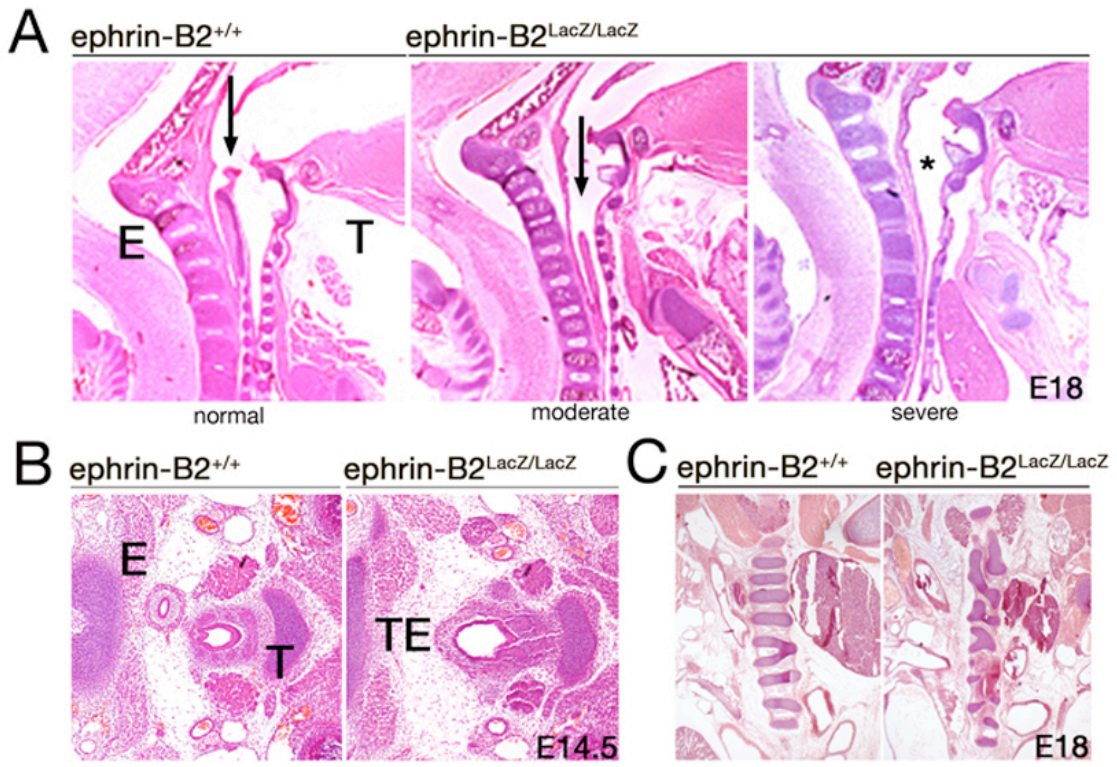


Figure 3.1

Failed midline septation of the foregut in *ephrin-B2*$^{lacZ/lacZ}$ mutants. (A) H&E stained sagittal sections from E18 *ephrin-B2*$^{+/+}$ or *ephrin-B2*$^{lacZ/lacZ}$ littermates. In the WT (left panel), a septum is discernable (arrow), separating the esophagus (E) from the trachea (T). Defects in septum formation in the mutants were classified as: moderate, where a septum is visible but fails to fully extend rostrally (arrow, middle panel); or severe, where no septum has formed (asterisk, right panel). (B) H&E stained transverse sections from E14.5 *ephrin-B2*$^{+/+}$ and *ephrin-B2*$^{lacZ/lacZ}$ littermates. In the WT (left panel), septation has occurred to produce distinct esophageal (E) and tracheal (T) endoderm. Equivalent section of a mutant (right panel) reveals unseptated foregut (TE). (C) H&E stained coronal sections of E18 *ephrin-B2*$^{+/+}$ and *ephrin-B2*$^{lacZ/lacZ}$ littermate embryos. While a normal banding of the cartilage rings surrounding the trachea is observed in the WT (left panel), mutants with failed tracheoesophageal septation present with visibly disorganized and improperly banded cartilage (right panel).

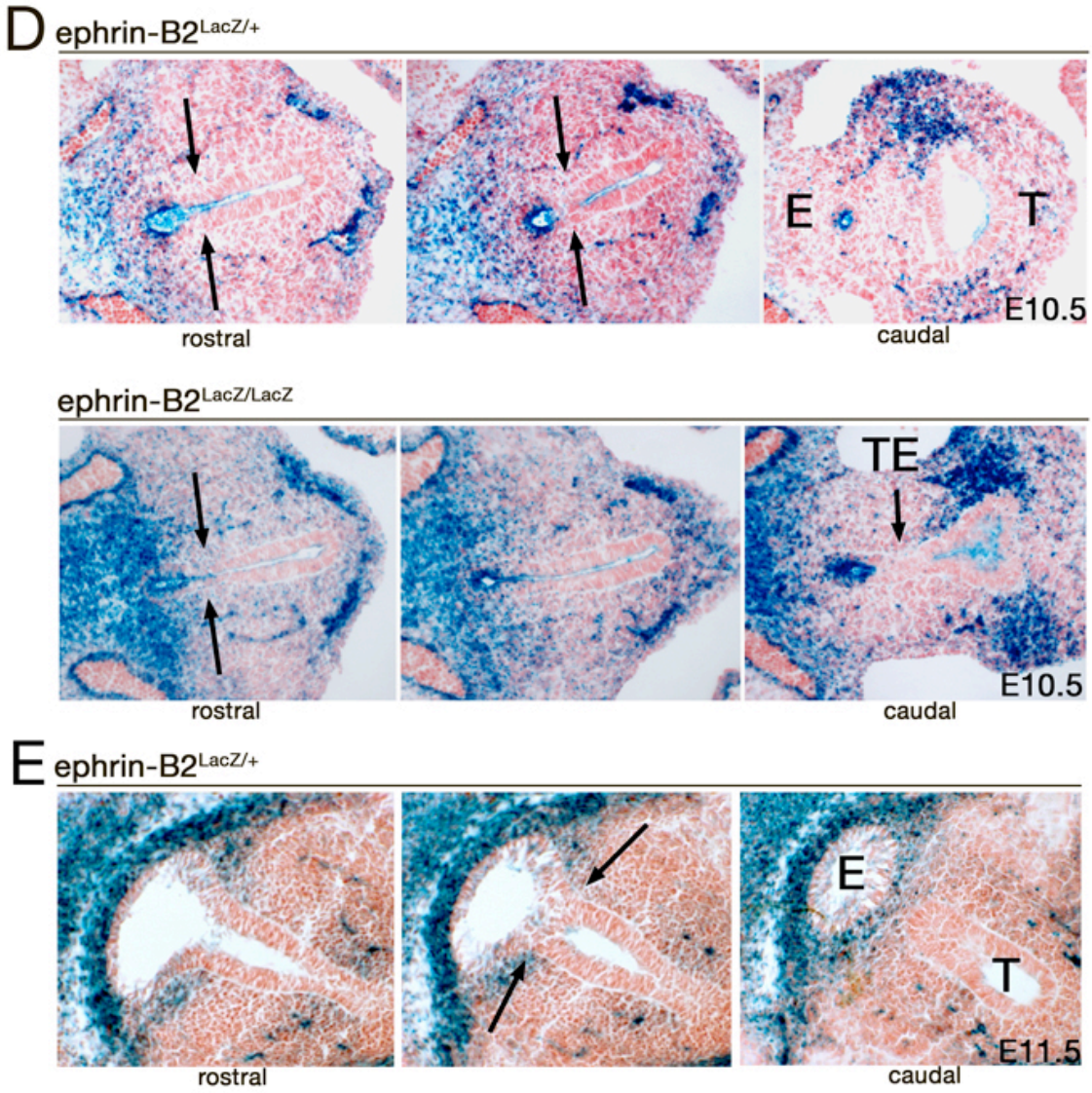


Figure 3.2 (D) Serial X-gal stained transverse sections from E10.5 *ephrin-B2*$^{lacZ/+}$ and *ephrin-B2*$^{lacZ/lacZ}$ littermates to detect expression of the ephrin-B2-βgal fusion protein (blue). Septation in the *ephrin-B2*$^{lacZ/+}$ embryo proceeds from common foregut (top panel, left) to septating foregut (arrows, middle panel), to septated foregut showing separated esophagus (E) and trachea (T) (right panel). Septation of the foregut in the *ephrin-B2*$^{lacZ/lacZ}$ mutant failed to occur (arrows, bottom panel, left), caudal to which only unseptated foregut remains (middle panel). A fistula (TE) between esophagus and the emerging bronchi is present at the very caudal end of the foregut (right panel). X-gal stain shows ephrin-B2 expression (blue) in the endoderm of the foregut at septation, with higher levels of expression detected at the future esophageal pole of the foregut. Ephrin-B2 is also found in the mesenchyme surrounding the future esophagus. (E) Rostral to caudal serial X-gal stained sections from an E11.5 *ephrin-B2*$^{lacZ/+}$ embryo. Similar to E10.5, ephrin-B2 is highly expressed in the mesenchyme and epithelia associated with future esophageal endoderm. Ephrin-B2 is also present on the endoderm and flanking mesenchyme at the point of septation (arrows, middle panel).

Genetic analysis of Eph receptor involvement

My discovery of tracheoesophageal fistula in *ephrin-B2*$^{lacZ/lacZ}$ mutants suggests that reverse signaling through the cytoplasmic domain of ephrin-B2 is necessary for proper septation of the foregut. However, the *ephrin-B2*^{lacZ} allele is not able to answer which Eph receptors are involved in activating these reverse signals. To address this, I generated a number of Eph receptor compound null animals, as these genes can show redundant functions. However, analysis of *EphB2*;*EphB3*, *EphB2*;*EphB3*;*EphA4*, and *EphB1*;*EphB2*;*EphB3*;*EphA4* compound knockouts did not reveal any defects in tracheoesophageal septation. I therefore suspect at least one additional Eph receptor (perhaps EphB4 and/or EphB6) may be paired with ephrin-B2 in foregut septation.

Ephrin-B2 is noticeably expressed at the adhesion point of foregut septation

I next sought to build an expression profile for ephrin-B2 in foregut septation by performing X-gal stains on sections from *ephrin-B2*$^{lacZ/+}$ and *ephrin-B2*$^{lacZ/lacZ}$ embryos collected at E10.5. These X-gal stains determined ephrin-B2 is expressed along the length of the foregut endoderm, and appears to be most highly expressed in the epithelia of the foregut destined to become esophagus, as well as in the mesenchyme surrounding the esophagus (Figure 3.2D). Ephrin-B2 is notably present at the site of septation in the *ephrin-B2*$^{lacZ/+}$ heterozygote or where septation should be occurring in the *ephrin-B2*$^{lacZ/lacZ}$ homozygous mutant. A similar expression profile for ephrin-B2 is also seen at E11.5 (Figure 3.2E). Indirect Immunofluorescence (IF) using a pan-ephrin-B antibody on wild-type embryos shows a similar expression profile as the X-gal stains, with ephrin-B

expressed in the mesenchyme surrounding the future esophageal component of the foregut and along septating endoderm (Figure 3.3).

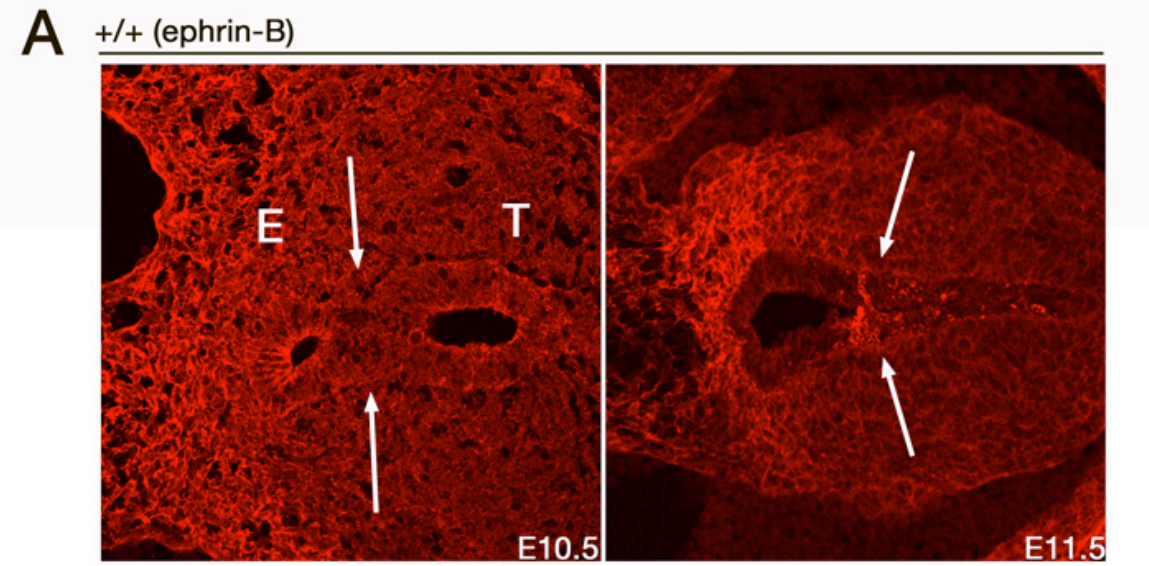


Figure 3.3

Detection of ephrin-B molecule during tracheoesophageal septation. Coronal sections capturing foregut septation from wild-type embryos immunostained at E10.5 (left panel) or E11.5 (right panel) with a pan-ephrin-B antibody. Ephrin-B is present in the mesenchyme associated with the future esophageal endoderm and in the endoderm of the foregut.

EphB2 and EphB3 expression is localized to foregut septation

While the Eph receptors involved in foregut septation are unknown, I reasoned that EphB3 was likely involved based on its role in cell-cell adhesion events during hindgut septation. Unfortunately, conventional methods to examine EphB3 expression by mRNA *in situs* or immunohistochemistry have not provided satisfactory results. To circumvent this, I generated a BAC transgenic animal that expresses the reverse tetracycline transactivator, rtTA2S-M2, under control of *EphB3* promoter sequences (Urlinger et al., 2000). I crossed this BAC-Tg-*EphB3*^{rtTA} transgene to a TRE-lacZ reporter line and collected doxycycline-induced embryos hemizygous for both transgenes (Ludwig et al., 2004). BluO-gal stains revealed EphB3 expression in the mesenchymal cells coming into contact with epithelial cells at the site of septation (Figure 3.4). To determine the expression pattern of EphB2, BluO-gal stains were also performed on foregut tissue from *EphB2*$^{lacZ/+}$ animals expressing the EphB2-βgal fusion protein (Figure 3.5). Interestingly, unlike EphB3, which is principally expressed in the mesenchyme flanking adhesion, EphB2 is more preferentially expressed on the epithelia at the point of septation. The expression data indicates EphB2 and EphB3 likely do participate in foregut septation, albeit with non-essential roles given the lack of a foregut septation defect in *EphB2;EphB3* compound null animals.

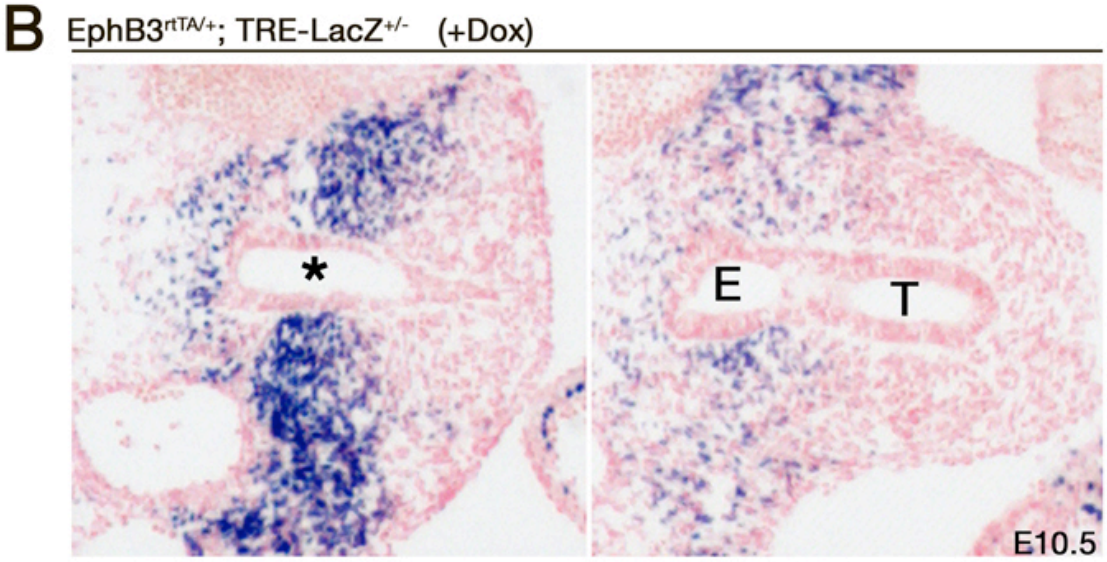


Figure 3.4

EphB3 expression during septation of the embryonic foregut. BluO-gal stained coronal sections from a BAC-Tg-*EphB3*^{rtTA};TRE-lacZ$^{+/-}$ embryo treated with doxycycline to visualize EphB3 expression (blue). EphB3 is highly expressed in the mesenchyme flanking the future point of septation (asterisk, left panel), where lateral folds will invaginate into the foregut and meet at the midline as seen in an adjacent caudal section (right panel).

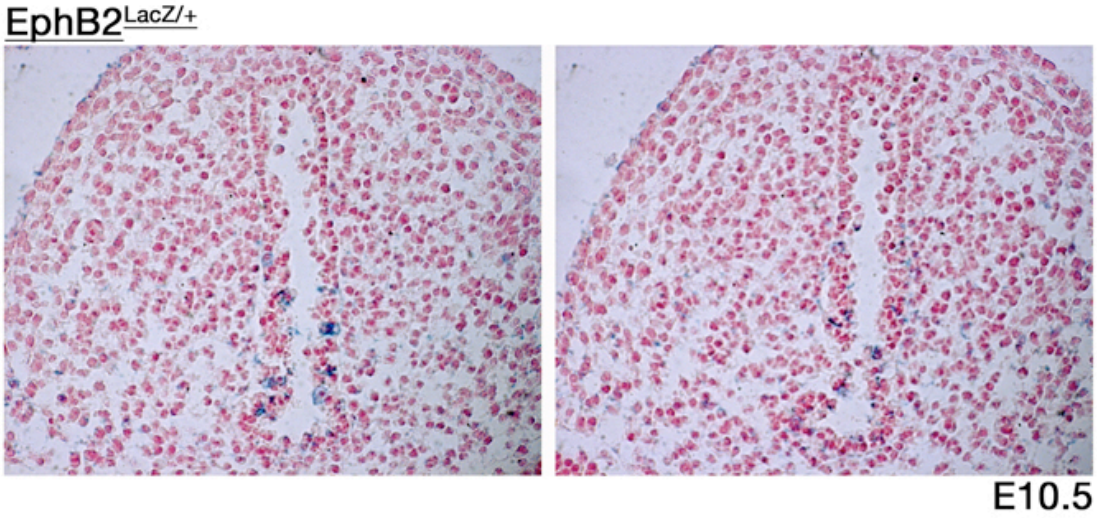


Figure 3.5

EphB2 expression labels epithelia at the site of foregut septation. BluO-gal stain (dark blue) of E10.5 *EphB2*$^{lacZ/+}$ foregut before (left panel) and at septation (right panel).

Reverse signals are being activated at the site of foregut septation

Having defined a role for ephrin-B2 reverse signaling in foregut septation, I next examined where this reverse signaling was taking place. I carried out confocal IF with two different antibodies that specifically recognize phosphorylated tyrosine residues within the conserved ephrin-B cytoplasmic domain. This analysis showed that while some ephrin-B reverse signaling occurs in the mesenchyme flanking the point of septation, the most striking phosphorylation of ephrin-B is detected along the septating endoderm (Figure 3.6). Adjacent sections treated with lambda phosphatase confirmed the phospho-specific activity of the antibodies. This data localizes active ephrin-B reverse signaling to septating foregut endoderm.

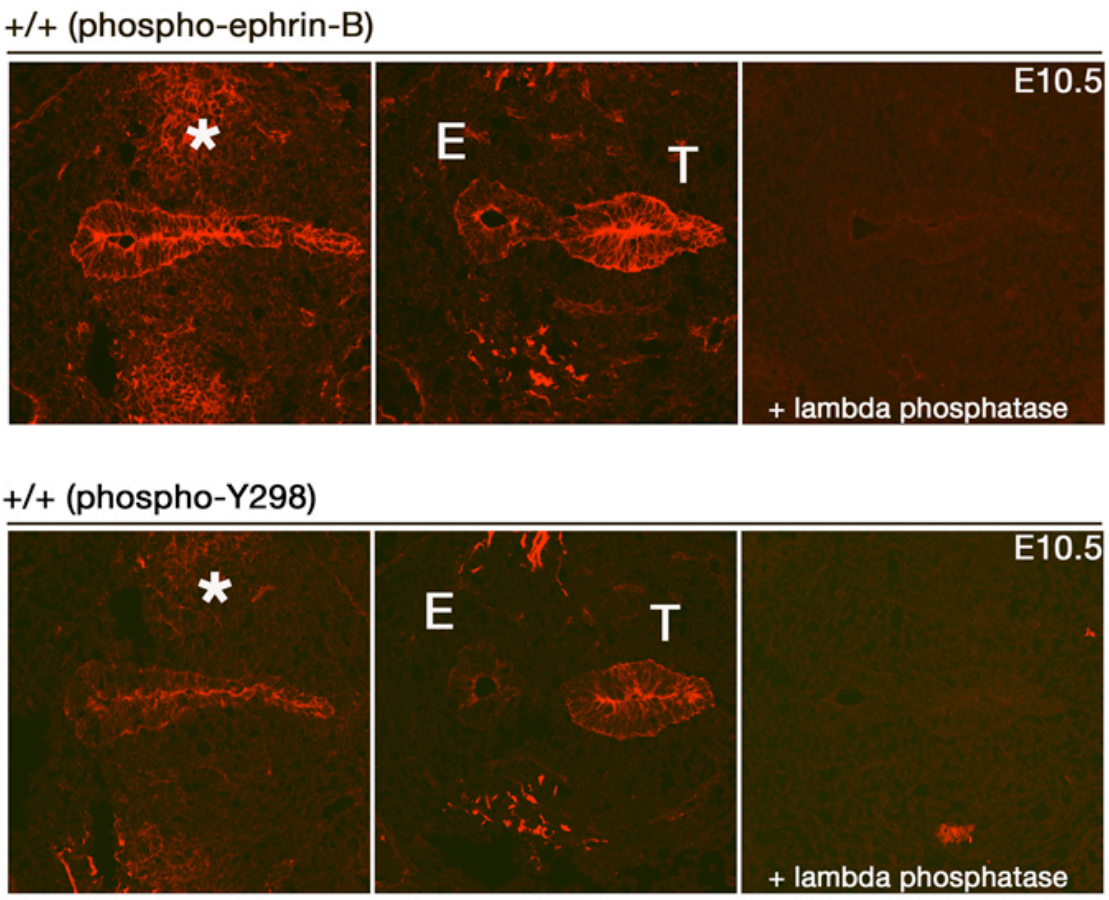


Figure 3.6

Ephrin is tyrosine phosphorylated at the site of foregut septation. Coronal sections from wild-type embryos immunostained with an antibody recognizing specific phosphorylation of tyrosines 324 and 329 (top panel) or of tyrosine 317 (bottom panel) on the conserved cytoplasmic tail of ephrin-B molecules, to detect reverse signaling activity *in vivo*. Activated ephrin-B molecules are found on the endoderm before septation (top and bottom panels, left) and after septation (top and bottom panels, middle). Activated ephrin-B also appears in the mesenchyme flanking the site of septation (asterisks). Adjacent sections pre-treated with lambda phosphatase confirm the specificity of the antibodies (top and bottom panel, right).

Discussion

I have described a requirement for ephrin-B2 reverse signaling in proper septation of the embryonic foregut. Mice lacking ephrin-B2 reverse signals present with LTEC, a severe defect in which the foregut does not partition into separate tracheal and esophageal tracts, but instead persists as one common compartment. Examination of *ephrin-B2*$^{lacZ/lacZ}$ mice at early timepoints indicates a normal overall patterning of their foregut structures, with the only defect apparently in the initiation of midline adhesion and fusion to produce septation. EphB2 and EphB3 are likely two Eph receptors involved in activating this ephrin-B2 reverse signal and mediating midline septation based on their expression at the site of septation, although the roles for these Eph receptors appear non-essential. Ephrin-B2 is localized in the epithelia at the site of adhesion, as well as in the flanking mesenchyme, and importantly appears to be actively conducting reverse signals in these cells. The phenotype and the expression data therefore suggest a role for ephrin-B2 reverse signaling in mediating cell-cell adhesion at the embryonic midline in foregut septation.

CHAPTER 4

Bidirectional signaling between ephrin-B2 and EphB2/B3 is necessary for closure of the embryonic palate

Summary

Here I present data that both forward signaling through EphB2/B3 and reverse signaling through ephrin-B2 are necessary for proper development of the palate. Expression data again co-localizes Eph and ephrin to the site of palatal shelf adhesion, suggesting a role for these molecules in mediating another midline cell-cell adhesion event.

Palate Formation

The formation of the palate represents another important developmental septation event in which the oral and nasal cavities become partitioned. Unlike the septations of the

hindgut and foregut, the closure of the embryonic palate has been characterized to a far greater extent. Mechanistically, it is accepted that the fusion of the palate involves the initial outgrowth and elevation of palatal shelves from the maxillary processes, followed by their midline adhesion to leave uniform mesenchyme that will constitute the secondary palate (Fitchett and Hay, 1989). The molecular basis of palatal closure is also much more advanced, as defects in this process have been characterized in a number of gene targeted mice (Schutte and Murray, 1999; Thyagarajan et al., 2003). Included among these are mice null for *ephrin-B1* and compound null for *EphB2* and *EphB3*, although it has not been established what contribution forward and reverse signaling plays in palatal shelf closure, or what physiological roles these signals were eliciting in these midline events (Compagni et al., 2003; Davy et al., 2004; Orioli et al., 1996). The degree to which palatal development has been so well characterized to date likely reflects a combination of the ease of detecting the defect in animal models and the commonality of the birth defect in the human population (1 in 1,000 live births) (Murray, 2002).

EphB2 forward signaling mediates palatal shelf formation

EphB2$^{\Delta/\Delta}$;*EphB3*$^{\Delta/\Delta}$ compound null animals exhibit a cleft palate phenotype that was not observed in *EphB2* or *EphB3* single mutants (Orioli et al., 1996). To determine if EphB2 was acting as a receptor to transduce forward signals important in palate development, I once again utilized the *EphB2*^{lacZ} allele in which an EphB2-βgal fusion protein is expressed that can activate reverse signaling but can not transduce a forward signal. Histological analysis of E18.5 embryos revealed that 45% of the *EphB2*$^{lacZ/lacZ}$;*EphB3*$^{\Delta/\Delta}$ and 15% of the *EphB2*$^{\Delta/\Delta}$;*EphB3*$^{\Delta/\Delta}$ compound mutants exhibited

severe cleft palates (Table 4.1). The cleft palate phenotype found when using the *EphB2*^{lacZ} allele indicates that forward signaling through EphB receptors is important in development of this midline structure. The increase in penetrance to 45% further suggests involvement of more than EphB2 and EphB3 in palate development, as the dominant negative effect seen here with the *EphB2*^{lacZ} allele is best explained by the ability of the truncated EphB2-βgal fusion protein to disrupt forward signaling of other co-expressed Eph receptors, in a manner similar to what was shown through biochemical data with the ephrin-B2-βgal fusion protein.

Table 4.1 Incidence of cleft palate associated with *EphB2*;*EphB3* compound mutants.

EphB2 mutation	background	*B2/B2;B3/B3*	*B2/+;B3/B3*	*+/+;B3/B3*
EphB2$^{\Delta}$	CD1	27 (4)	32 (1)	NA
EphB2^{lacz}	CD1	29 (12)	17 (2)	NA

EphB2$^{\Delta/\Delta}$;*EphB3*$^{\Delta/\Delta}$ (or *EphB2*$^{lacZ/lacZ}$;*EphB3*$^{\Delta/\Delta}$) males were intercrossed with *EphB2*$^{\Delta/+}$;*EphB3*$^{\Delta/\Delta}$ females (or *EphB2*$^{lacZ/+}$;*EphB3*$^{\Delta/\Delta}$) and offspring collected at E18.5 just before birth. The number of animals that had a cleft palate is in parentheses.

Ephrin-B2 reverse signaling is also necessary for palatal shelf closure

To determine if ephrin-B2 is involved in palate formation, I examined E18.5 embryos carrying the reverse signaling-deficient *ephrin-B2*^{lacZ} allele. Histological analysis of these embryos revealed that approximately 25% of *ephrin-B2*$^{lacZ/lacZ}$ homozygotes and 6% of *ephrin-B2*$^{lacZ/+}$ heterozygotes, showed a severe cleft palate (Figure 4.1 and Table 4.2). This data indicates that *ephrin-B2* is involved in palate fusion and that reverse signaling through this molecule is important for this process. Coupled with the aforementioned *EphB2*;*EphB3* genetic data, it thus appears both forward signaling through EphB receptors and reverse signaling through ephrin-B2 are important for midline adhesion of the palate. This involvement of bidirectional signaling through both EphB and ephrin-B mirrors a similar requirement for both forward and reverse signaling in urorectal/hindgut development.

Table 4.2. Incidence of cleft palate associated with the *ephrin-B2*^{lacZ} mutation.

background	*ephrin-B2*$^{lacZ/lacZ}$	*ephrin-B2*$^{lacZ/+}$	*ephrin-B2*$^{+/+}$
129/B6/CD1	35 (9)	59 (4)	>40 (0)

Ephrin-B2$^{lacZ/+}$ heterozygotes were intercrossed and offspring collected at E18.5 just before birth. The number of animals that had a cleft palate is in parentheses.

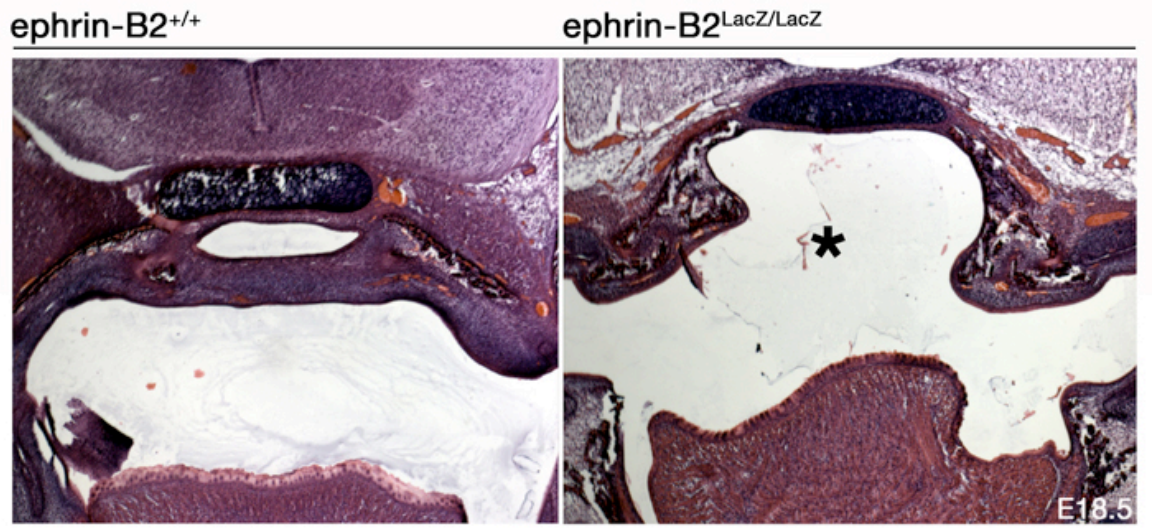


Figure 4.1

Cleft palate in *ephrin-B2*$^{lacZ/lacZ}$ mutants. H&E stained coronal sections of *ephrin-B2*$^{+/+}$ (left) and *ephrin-B2*$^{lacZ/lacZ}$ (right) E18 littermates. The *ephrin-B2*$^{lacZ/lacZ}$ homozygote presents with a cleft palate (asterisk).

EphB2 and ephrin-B2 are co-expressed in adherent epithelia at the site of palatal shelf fusion

Having determined the relevant Eph-ephrin molecules in palatal development, I next examined the expression profiles of these molecules during the process. X-gal stains of *ephrin-B2*$^{lacZ/+}$ heterozygote embryos revealed expression of ephrin-B2 throughout the mesenchyme of the palatal shelf, as well as in the leading epithelia before adhesion (Figure 4.2B). Later, when adhesion has taken place, ephrin-B2 is clearly visible on the multilayer epithelial seam, as well as in the mesenchyme flanking the midline site of adhesion.

To examine the expression of the EphB receptors, I carried out IF of palatal shelves with an antibody against EphB2. These assays showed EphB2 expression in the mesenchyme and leading epithelia of the palatal shelf (Figure 4.2C). No signal was detected in IF performed on control *EphB2*$^{\Delta/\Delta}$ null tissue. X-gal stains of E14.5 *EphB2*$^{lacZ/+}$ animals showed that as palatal shelf adhesion nears and as it occurs, EphB2 expression becomes more restricted to the leading epithelia where adhesion takes place (Figure 4.3 top panel).

To investigate EphB3 expression, I created another BAC transgenic line that expresses eYFP under *EphB3* promoter control, termed BAC-Tg-*EphB3*^{YFP}. IF for GFP to detect expression of the eYFP reporter showed that EphB3 is expressed at the leading mesenchyme of the palatal shelves (Figure 4.3 bottom left). EphB3 expression was further examined in X-gal stains of palatal shelves from doxycycline-treated embryos double hemizygous for the BAC-Tg-*EphB3*^{rtTA} and TRE-lacZ transgenes. These X-gal stains also showed EphB3 expression in the mesenchyme of the palatal shelves at E13.5,

and that this expression shifts from the mesenchyme to adhering epithelia as palatal shelf adhesion nears at E14.5 (Figure 4.3 bottom). Remarkably, the expression profiles for EphB2, EphB3, and ephrin-B2 all appear nearly identical to each other during formation of the palate.

Based on the individual expression studies taken for ephrin-B2 and the two EphB receptors, I speculated that these molecules were likely co-expressed at the site of cell-cell adhesion as the palatal shelves meet and fuse at the midline. To address this, I performed double IF analysis on *ephrin-B2*$^{lacZ/+}$ embryos using an antibody against EphB2 and an antibody against β-gal to detect ephrin-B2. This revealed that EphB2 and ephrin-B2 are co-expressed on the midline epithelia where the palatal shelves adhere (Figure 4.4). The demonstration of co-expression in palatal cells adhering at the midline mimics the previous finding of co-expression in adhering cells at the site of urorectal/hindgut septation.

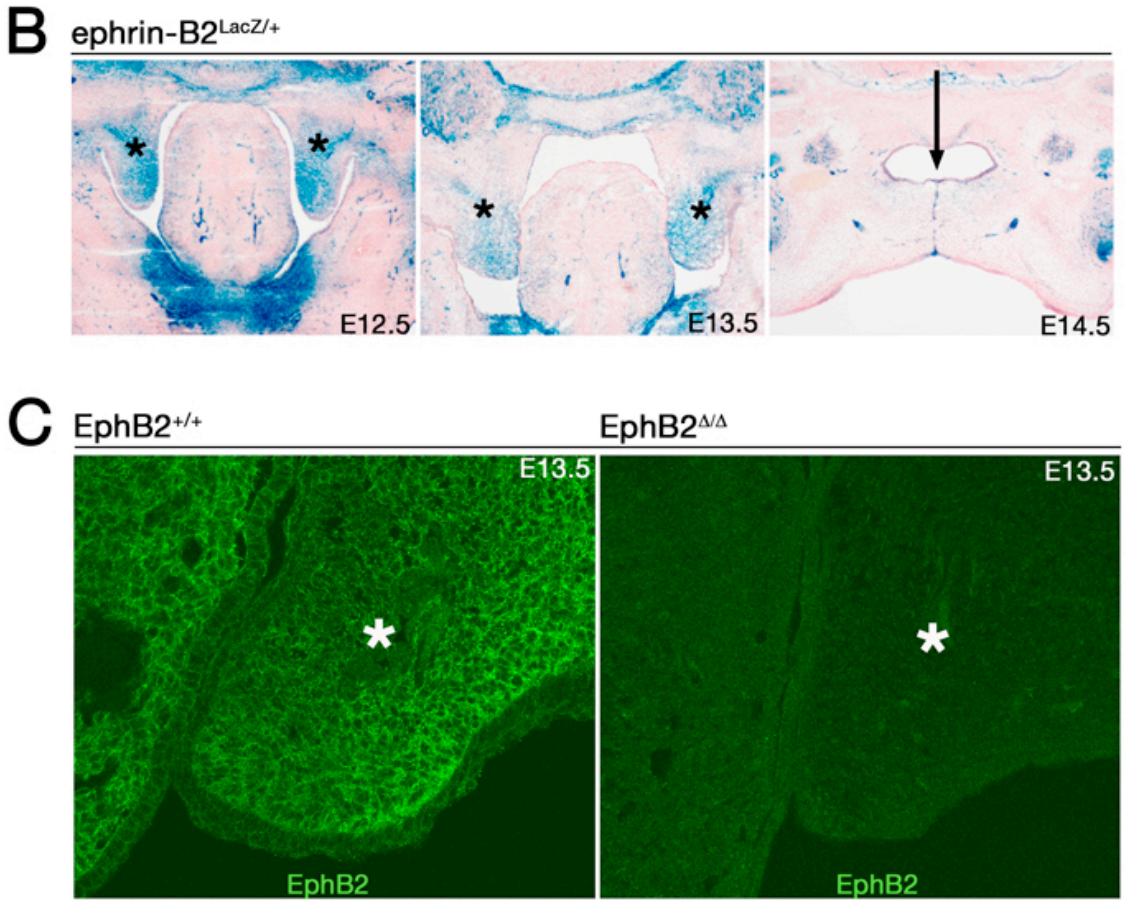


Figure 4.2

Expression of ephrin-B2 and EphB2 during palatal development. (B) X-gal stained coronal sections from *ephrin-B2*$^{lacZ/+}$ embryos at E12.5, E13.5, and E14.5 to detect expression of the ephrin-B2-β-gal fusion protein (blue). Ephrin-B2 is expressed in the mesenchyme and leading epithelia of the palatal shelves at E12.5 and E13.5 (asterisks, left and middle panel) before palatal shelf adhesion. After adhesion, ephrin-B2 expression is strongest in the midline epithelial seam (arrow, right panel). (C) Coronal sections at E13.5 from *EphB2*$^{+/+}$ and *EphB2*$^{\Delta/\Delta}$ embryos treated with anti-EphB2 antibodies. EphB2 is expressed in the mesenchyme of the palatal shelf in the WT (asterisk, left panel), while no signal is detected in the mutant.

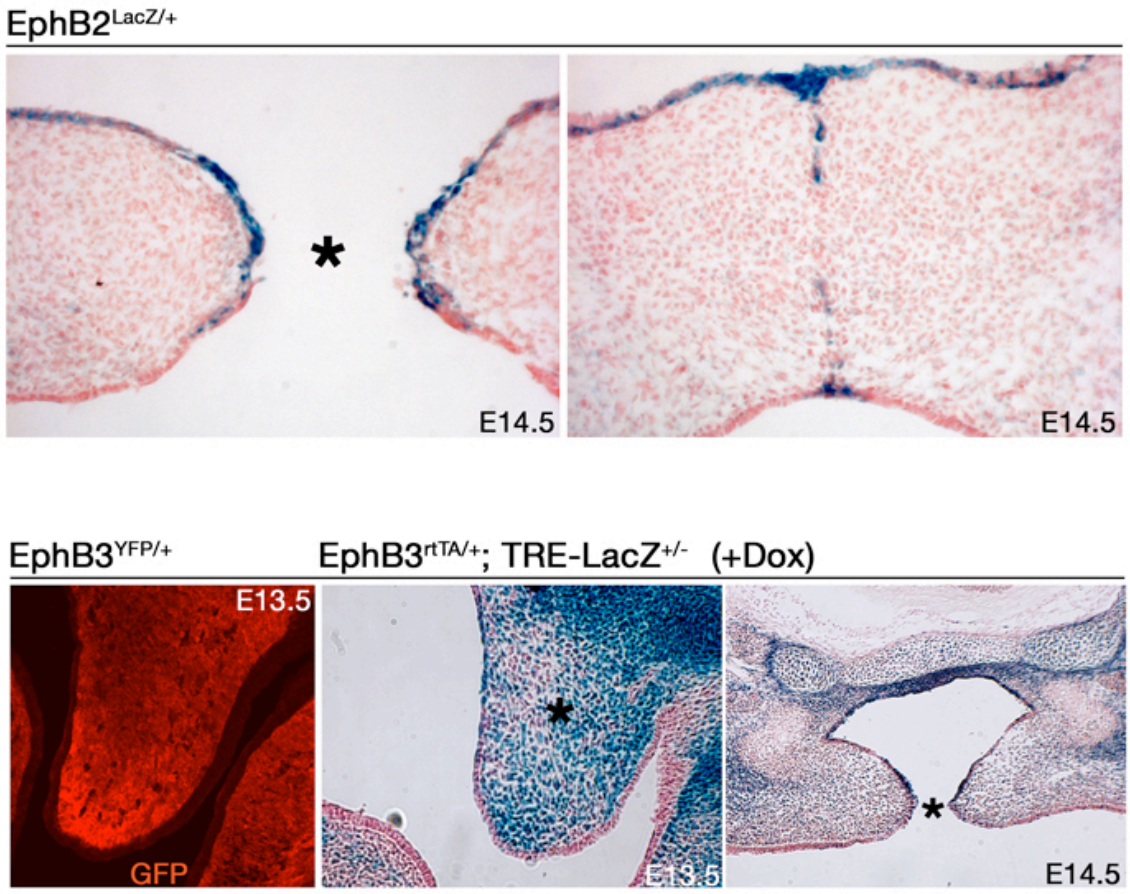


Figure 4.3

EphB2 and EphB3 expression during palatal development. Coronal sections from an E14.5 *EphB2*$^{lacZ/+}$ embryo stained with X-gal. EphB2 becomes specifically expressed in the epithelia of the palatal shelves, both immediately preceding adhesion (left panel) and at the site of adhesion (right panel). (E) Coronal section from an E13.5 BAC-Tg-*EphB3*$^{YFP/+}$ embryo treated with anti-GFP antibodies shows specific EphB3 expression in the mesenchyme of the palatal shelf (asterisk, left panel). X-gal stained coronal sections from E13.5 and E14.5 BAC-Tg-*EphB3*^{rtTA};TRE-lacZ double hemizygous embryos treated with doxycycline show EphB3 is initially highly expressed in the mesenchyme of the palatal shelf at E13.5 (asterisk, middle panel) but then becomes more preferentially expressed in the leading epithelia as midline adhesion becomes imminent at E14.5 (asterisk, right panel).

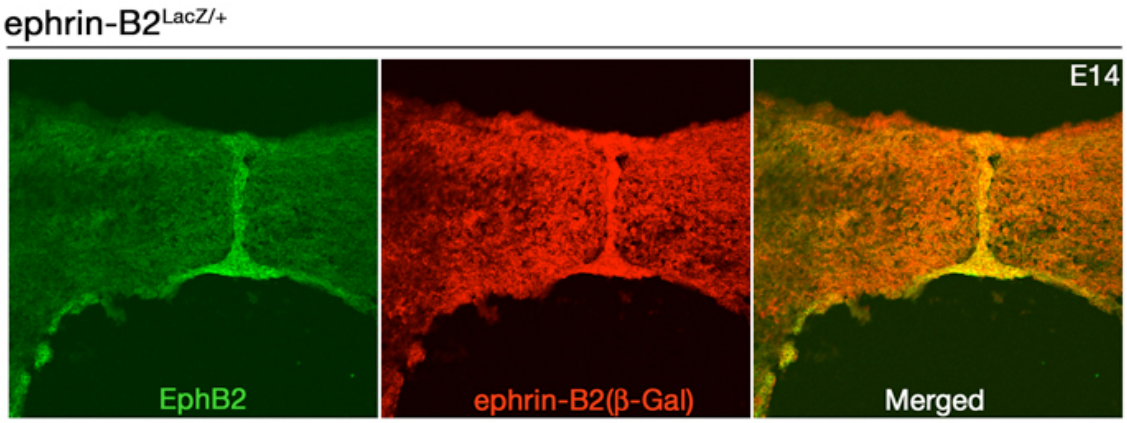


Figure 4.4

EphB2 and ephrin-B2 are expressed at the point of palatal shelf adhesion. Coronal section from an E14 *ephrin-B2*$^{lacZ/+}$ embryo treated with anti-EphB2 (left panel) and anti-β-gal (middle panel) antibodies. The merged image (right panel) shows EphB2 and ephrin-B2-β-gal are co-expressed on the midline epithelial seam where adhesion has occurred.

B-ephrin reverse signaling is activated at the site of cell-cell adhesion in the palatal shelf

Having established that reverse signaling through ephrin-B was important in palatal development, I next sought to determine where this reverse signaling was occurring. To address this, I performed IF on adjacent sections of *ephrin-B2*$^{lacZ/+}$ embryos with a β-gal antibody to detect ephrin-B2 and with the phospho-ephrin$^{Y324/Y329}$ antibody to detect activated ephrin. While the β-gal antibody labeled the leading epithelia of the palatal shelf as well as the flanking mesoderm, interestingly, the phospho-ephrin antibody indicated that only ephrin-B2 in the leading epithelia of the palatal shelf was tyrosine phosphorylated (Figure 4.5). Double IF performed on palatal shelves from E14 wild-type embryos for EphB2 and the phospho-ephrin$^{Y324/Y329}$ antibody show EphB2 is co-expressed with phosphorylated ephrin-B in these leading epithelia where adhesion will take place. This data indicates that ephrin-B2 reverse signaling in palate formation involves signal transduction into the leading adherent epithelia.

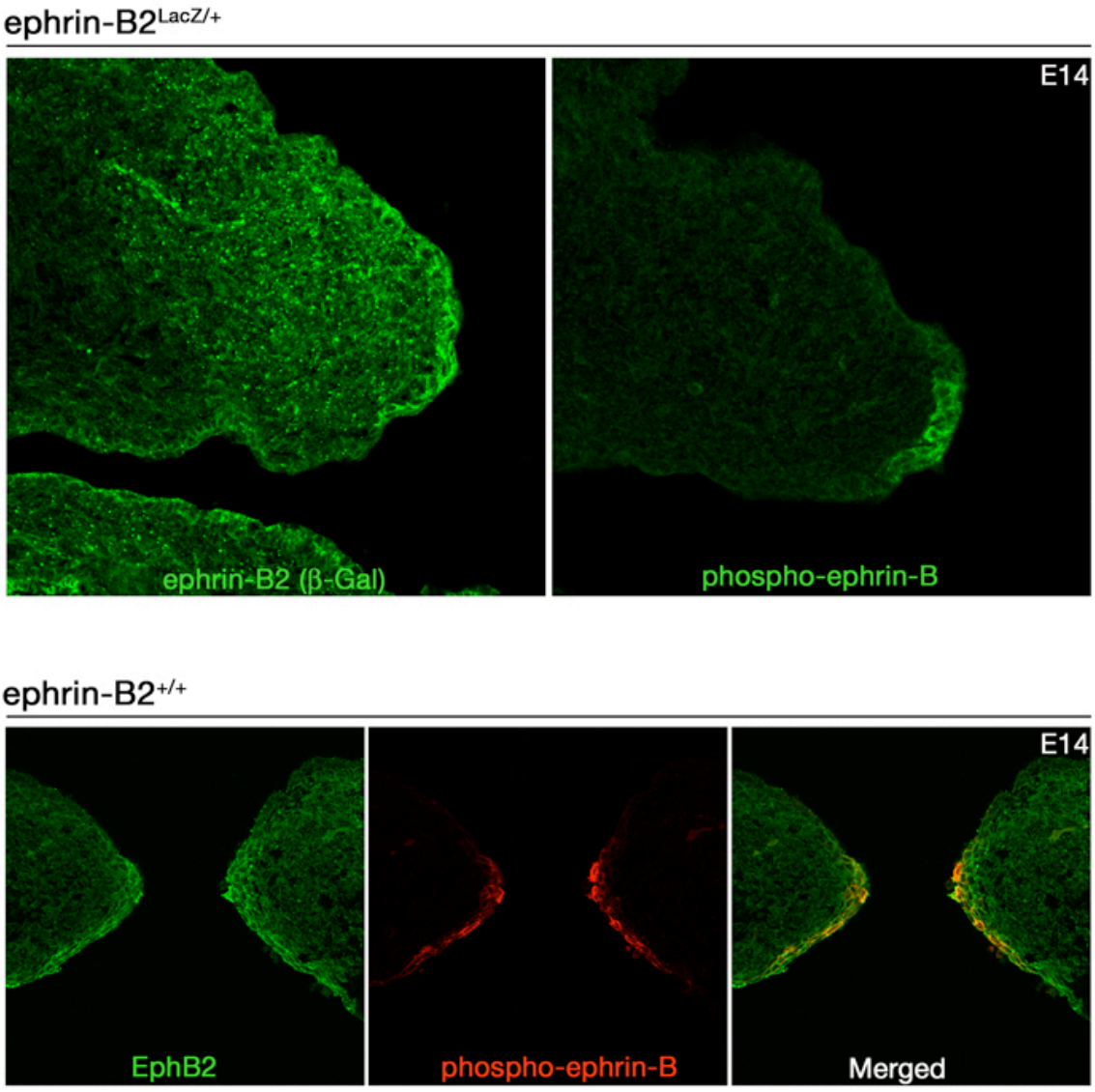


Figure 4.5

Adherent epithelia in the palatal shelf actively transduce reverse signals. (Top) Adjacent coronal sections from an E14 *ephrin-B2*$^{lacZ/+}$ embryo treated with anti-β-gal (left panel) or anti-phospho-ephrin-B$^{Y234/Y329}$ (right panel) antibodies. While ephrin-B2 is expressed in both the mesenchyme and leading epithelia of the palatal shelf, it is only the ephrin-B2 in the leading epithelia that is tyrosine phosphorylated. (Bottom) Coronal section from a wild-type E14 embryo treated with anti-EphB2 (left panel) and anti-phospho-ephrin-B$^{Y324/329}$ (middle panel) antibodies. EphB2 is co-expressed with tyrosine-phosphorylated ephrin-B in leading epithelia of the palatal shelves.

Palatal shelves from *ephrin-B2*$^{lacZ/lacZ}$ mice fail to fuse *in vitro*

In an effort to determine the function role of ephrin-B2 reverse signaling during palate formation, a collaborator, Michael Risley, a graduate student in the laboratory of William McLean, performed *in vitro* palatal shelf fusion assays with *ephrin-B2*$^{lacZ/lacZ}$ mice. The basic experiment here is that the palatal shelves from E13.5 embryos are dissected out and then cultured in direct opposition to one another for 72 hours, after which you section through them to see whether they have properly adhered together or not. The system is thus a great way to determine whether the cleft palate phenotype is the result of a failure of these palatal shelves to *meet* at the midline, or is it a failure of these shelves to properly *adhere* at the midline. *In vitro* palatal shelf fusion assays were performed on both wild-type and *ephrin-B2*$^{lacZ/lacZ}$ littermates. Strikingly, while greater than 80% of the palatal shelves from wild-type embryos fused together properly in culture (n=33), none of the five *ephrin-B2*$^{lacZ/lacZ}$ embryos from which palatal shelves were taken showed proper fusion. The data therefore strongly indicates the cleft palate phenotype seen in *ephrin-B2*^{lacZ} mice is the result of failed midline adhesion.

Discussion

I have shown that both *EphB2*$^{lacZ/lacZ}$;*EphB3*$^{\Delta/\Delta}$ and *ephrin-B2*$^{lacZ/lacZ}$ mice present with a cleft palate phenotype, indicating that both forward signaling through EphB2 and reverse signaling through ephrin-B2 are playing important roles in mediating midline closure of the palate. Expression data again shows both EphB and ephrin-B2 expressed at the site of midline adhesion, both in the epithelia that are adhering in the midline, as well

as in the mesenchyme flanking the site of adhesion. The requirement for both forward and reverse signals and the apparent co-expression of Eph and ephrin at sites of adhesion mirror similar findings in studies of EphB2, EphB3, and ephrin-B2 during midline septation of the cloaca. Similarly, as in the case of foregut septation, ephrin-B reverse signals were specifically localized to the site of adhesion at the leading edge of the palatal shelf, which when coupled to the cleft palate phenotype, suggests that these reverse signals play an integral role in mediating cell-cell adhesion. Finally, the failure of palatal shelves to adhere properly even when cultured in direct opposition to one another strongly suggests defective cell adhesion as the cause for cleft palate.

CHAPTER 5

EphB2/B3 activation of reverse signaling through ephrin-B1 is necessary for closure of the ventral body wall

Summary

Previous reports have indicated that EphB2 and EphB3, along with the ligand ephrin-B1, play important roles in mediating the closure of the ventral body wall. Here I characterize the penetrance of this phenotype and document the expression of EphB at the midline. Further, genetic analysis of forward signaling deficient *EphB2*^{lacZ} mice reveals that closure of the ventral body wall is principally driven through reverse signaling via ephrin-B1.

Ventral closure of the abdominal body wall

Closure of the ventral body wall to properly encapsulate the visceral organs is an essential part of mammalian development. Mechanistically, this process proceeds through a series of steps that appears more complex than the comparatively simple closure of the dorsal neural tube. Around E8, the embryo carries a U-shape, in which the ectoderm and neuroectoderm are located in the concave region of this "U", while the endoderm is along the convex portion of this "U" and is exposed, as there is no body wall at this point (Brewer and Williams, 2004). At this point, the embryo undergoes a poorly defined turning event in which the caudal end of the embryo seemingly flips the direction of the "U", in which the gut becomes localized within the concavity of the embryo, in a configuration that will become permanent. This turning event also produces the primary ventral body wall, which is nothing more than a very thin epithelial membrane. This primary ventral body wall will persist until E12, when the secondary ventral body wall starts to form. This occurs when lateral folds migrating across the ventral surface meet and adhere at the midline, followed by a fusion event that leaves a continuous surface outside of the umbilical ring (Brewer and Williams, 2004). The use of knockout mice has led to the identification of many genes that appear to mediate this process, many of which are higher order signaling molecules or transcription factors (Brewer and Williams, 2004).

Defects in closure of the ventral body wall occur in one in 2-4,000 live births, and can manifest themselves as either gastroschisis, omphalocele, or thoracoabdominoschisis (Baird and MacDonald, 1981; Chitayat et al., 1997). These are similar defects in which the fetus is born with the visceral organs exposed to some extent; gastroschisis and omphalocele differ in whether the visceral organs are exposed to the amniotic fluid or

remain enclosed in visceral peritoneum, respectively. Incidents of omphalocele can be detected through ultrasounds and prenatal care, and warrant emergent surgery to repair the defect; even with early detection, the mortality rate for omphalocele is around 10%.

EphB2/B3 activation of ephrin-B1 reverse signaling mediates ventral body wall closure

Having characterized cell-adhesion defects in the urethra, hindgut, foregut, and palate, I next re-visited the involvement of the Ephs and ephrins in closure of the ventral body wall. In one of the earliest studies of Eph receptor knockout mice, it was reported that *EphB2;EphB3* compound null animals present with a failure in midline closure of the ventral body wall, although this defect was not characterized in any detail (Orioli et al., 1996). The generation of $EphB2^{\Delta/\Delta}$;$EphB3^{\Delta/\Delta}$ compound nulls revealed that ~40% of these mice exhibited a failure in midline closure of the abdominal wall that resembled the human birth defect omphalocele (Figure 5.1 and Table 5.1). Interestingly, this defect appears to be dependent on the genetic background of the mouse, as omphalocele was detected in the inbred 129 strain, but not in mice from a CD1 background. To determine if forward signaling through EphB2 played a role in ventral body wall closure, I investigated the penetrance of this phenotype using the $EphB2^{lacZ}$ forward signaling deficient allele and found that only 9% of the $EphB2^{lacZ/lacZ}$;$EphB3^{\Delta/\Delta}$ compound mutant animals exhibited omphalocele. This leads me to conclude that EphB2 and EphB3 are acting principally as ligands, and that their main function in ventral body wall closure is to bind B-subclass ephrins and activate reverse signaling. Further, since it has been reported that *ephrin-B1* null mice also present with a partially penetrant omphalocele defect (Compagni et al.,

2003; Davy et al., 2004), while none of our *ephrin-B2* mutant mice have shown any defects in ventral body wall closure, it appears that it is principally the reverse signaling of ephrin-B1 that EphB2 and EphB3 are functioning as ligands for.

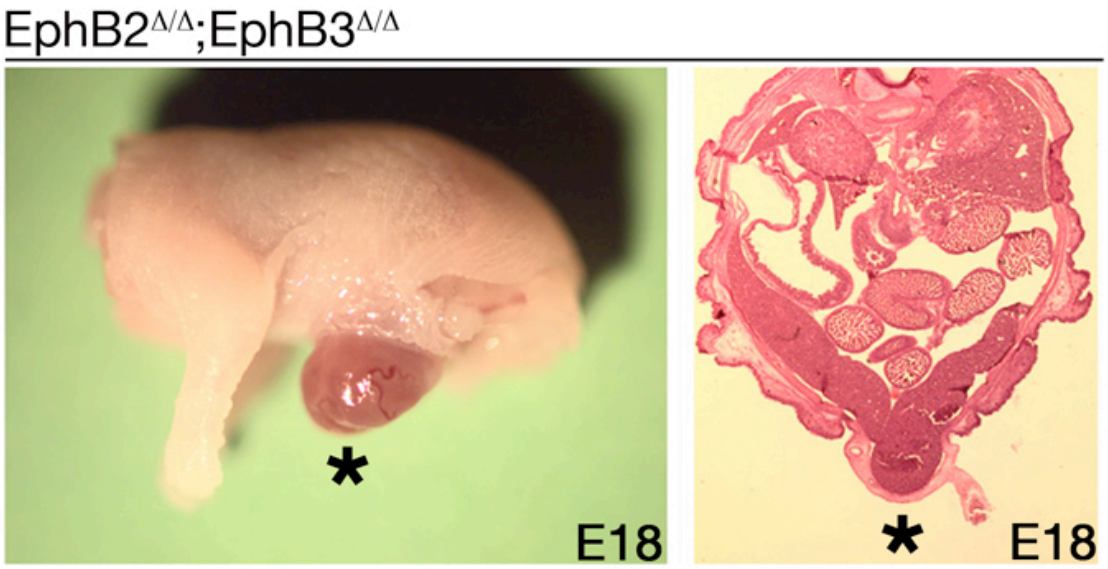


Figure 5.1

Failed closure of the ventral abdominal wall in *EphB2*;*EphB3* compound null embryos. (Left) Whole-mount image of an E18 *EphB2*$^{\Delta/\Delta}$;*EphB3*$^{\Delta/\Delta}$ compound null shows herniated visceral organs (asterisk) due to a failure in ventral abdominal body wall closure. (Right) Transverse H&E stained section of an *EphB2*$^{\Delta/\Delta}$;*EphB3*$^{\Delta/\Delta}$ E18 embryo similarly shows failed midline closure of the ventral body wall and visibly herniated visceral organs (asterisk).

Table 5.1 Incidence of omphalocele in *EphB2*;*EphB3* compound mutants.

EphB2 mutation	background	*B2/B2;B3/B3*	*B2/+;B3/B3*	*+/+;B3/B3*
$EphB2^{\Delta}$	129	38 (15)	63 (2)	42 (0)
$EphB2^{lacz}$	129	11 (1)	21 (0)	14 (0)

$EphB2^{\Delta/+}$;$EphB3^{\Delta/\Delta}$ (or $EphB2^{lacZ/+}$;$EphB3^{\Delta/\Delta}$) males were intercrossed with $EphB2^{\Delta/+}$;$EphB3^{\Delta/\Delta}$ females (or $EphB2^{lacZ/+}$;$EphB3^{\Delta/\Delta}$) and offspring collected at E18.5 just before birth. The number of animals that had defective ventral body wall closure is in parentheses.

EphB2 is expressed at the ventral midline

To determine where EphB2 is expressed during ventral midline closure of the abdominal wall, I performed whole-mount BluO-gal stains on $EphB2^{lacZ/+}$ embryos. At E13.5 expression of EphB2 was detected in cells at the ventral midline where abdominal closure takes place and at E15.5 was localized to the terminal closure spot of the umbilical ring (Figure 5.2). Once again EphB2 is localized to the site of adhesion, just as was the case in urethra, cloaca, foregut, and palate. Methods to determine the expression pattern of ephrin-B1 have so far proven unsatisfactory, however I have recently generated a novel transgenic line of mice that express the same rtTA2S-M2, used with *EphB3* previously, under promoter control of *ephrin-B1*, which will hopefully circumvent this problem.

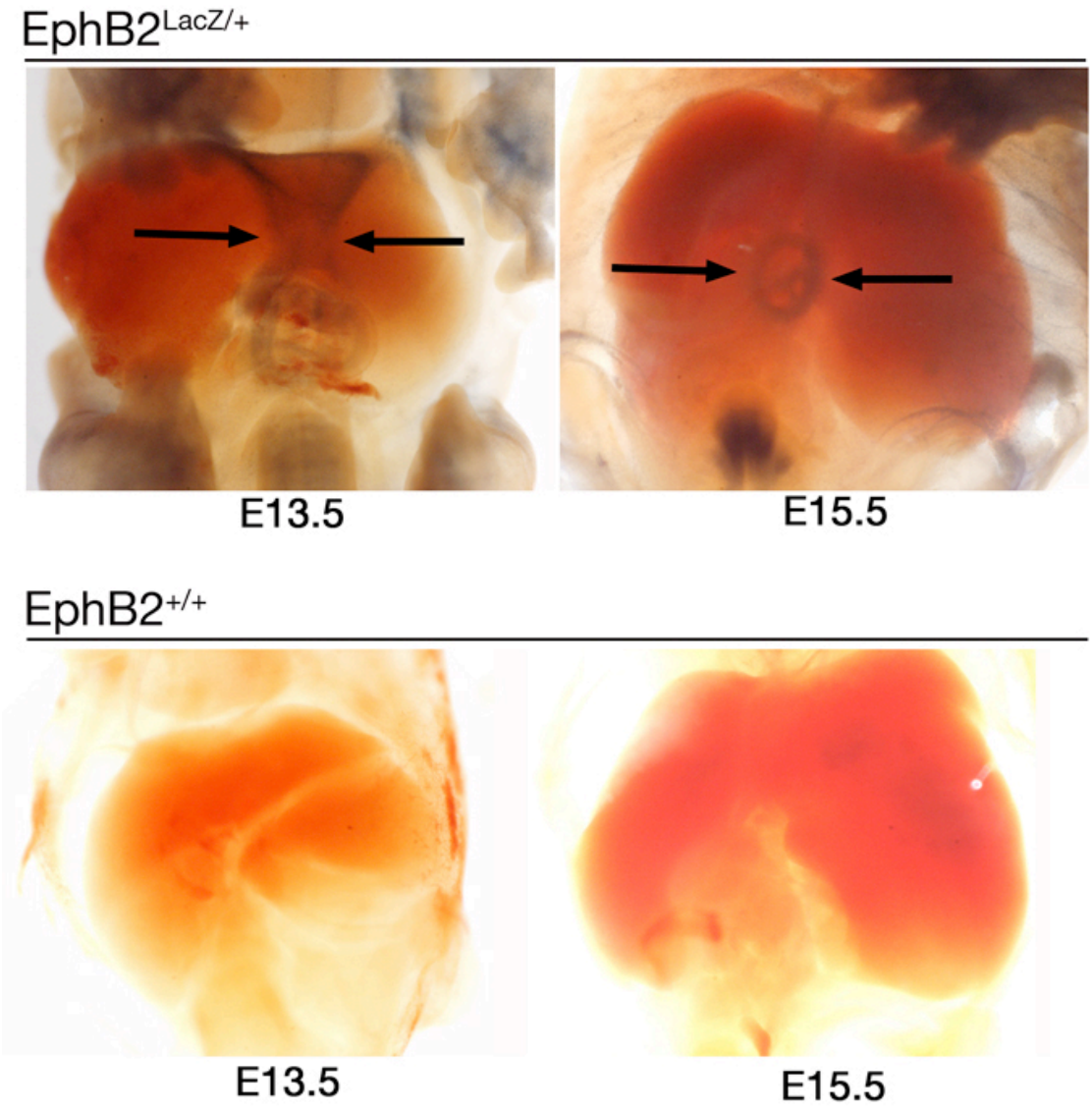


Figure 5.2

Whole-mount BluO-gal stained *EphB2*$^{+/+}$ and *EphB2*$^{lacZ/+}$ embryos at E13.5 and E15.5 to detect expression of the EphB2-β-gal fusion protein. EphB2 is expressed at the ventral midline at E13.5 (arrows, top left panel) and at leading edges of the closing umbilical ring at E15.5 (arrows, top right panel). No BluO-gal activity is detected in the WT embryos (bottom panels).

Discussion

The involvement of Eph-ephrin signaling in the closure of the ventral body wall is not new. Previous reports have indicated that both *EphB2/B3* and *ephrin-B1* knockout mice present with omphalocele, a severe embryonic malformation in which the fetus is delivered with the visceral organs exposed to the environment, instead of confined behind a ventral body wall. Absent from these initial studies however, were a quantification of the penetrance of the phenotype in *EphB2;EphB3* compound null animals, an expression profile for any of these molecules during ventral body wall closure, and most importantly, an idea for whether these relevant Eph/ephrin molecules were playing cell or non-cell autonomous signaling roles in mediating this closure event. Given my ongoing focus in detailing the roles of B-subclass Ephs and ephrins in other adhesion events in the urethra, cloaca, palate, and foregut, I naturally expanded my studies to fill in the gaps in our understanding of Eph/ephrin involvement in ventral body wall development. Here I have shown that the penetrance of omphalocele is roughly 40% in *EphB2;EphB3* compound null animals. Further, taking advantage of the EphB2-βgal fusion protein, I localize EphB2 to midline structures where the ventral lateral folds are meeting and adhering at the midline. Finally, I demonstrate that forward signaling through EphB2 is playing a minimal role in mediating midline closure here. Only 9% of $EphB2^{lacZ/lacZ}$;$EphB3^{\Delta/\Delta}$ mice presented with omphalocele, which is significantly less than the ~40% penetrance seen in the *EphB2;EphB3* compound null mice. Moreover, this 9% becomes even further minimized when you consider that the EphB2-βgal fusion protein appears to possess dominant-negative behaviors to inhibit forward signaling (where forward signaling appears important, $EphB2^{lacZ/lacZ}$;$EphB3^{\Delta/\Delta}$ mice have more penetrant phenotypes than

EphB2$^{\Delta/\Delta}$;*EphB3*$^{\Delta/\Delta}$ mice). The dominant signal in mediating midline closure of the ventral body wall therefore appears to be ephrin-B1 reverse signaling.

CHAPTER 6

EphB2/B3 activation of reverse signaling through ephrin-B2 mediates closure of the embryonic eyelid

Summary

Here I show that EphB2, EphB3, and ephrin-B2 appear to be mediating another cell adhesion event, this time in closure of the embryonic eyelid. As in the previously characterized adhesion events, reverse signaling appears to be necessary, the molecules are co-expressed at sites of adhesion, and ephrin-B reverse signaling is activated at the site of adhesion.

Embryonic eyelid closure

Closure of the embryonic eyelid is a development event common to a number of vertebrates, including mice and humans. In humans, the eyelids will adhere shut around

the 60d stage, and re-open *in utero* two months before birth (Zieske, 2004). In mice, the eyelids typically adhere around E16 and do not re-open until 12 days after birth. The process has been well characterized mechanistically, as rather simply the leading epithelia from each eyelid begin streaming towards each other across the surface of the cornea. The eyelids will eventually meet and adhere tightly to one another. While this may resemble the cell adhesion events previously described in the hindgut, foregut, palate, and ventral body wall, an important difference is that whereas in those systems the adhesion of lateral epithelial cells produces an epithelial seam that is resolved through fusion mediated by epithelial to mesenchymal transition (EMT) or apoptosis, in eyelid closure there is no fusion (Dudas et al., 2007; Martinez-Alvarez et al., 2004). Instead, the epithelial seam that forms at E16 following the adhesion of the eyelids is not immediately resolved, but instead persists until this contact is disrupted and the eyelids are re-opened.

Defective closure of the embryonic eyelid presents as an eyelid open at birth (EOB) phenotype in mice. This phenotype has been identified in a number of mutant mice now, and has implicated the involvement of molecules such as c-Jun, FGFR2, TGFα, and Smad7, which presumably play roles in mediating the cell proliferation necessary for the eyelids to grow and meet, along with integrin subunits and Rock-I, which likely mediate the adhesion and cytoskeletal changes directly impacting the adhesion of the eyelids (Carroll et al., 1995; He et al., 2002; Li et al., 2001; Luetteke et al., 1993; Thumkeo et al., 2005; Zenz et al., 2003). The involvement of Rock-I is particularly interesting, given that this serine/threonine kinase is an effector of Rho signaling, which is a major target of Eph-ephrin signaling; further, in addition to the EOB phenotype, *Rock-I*

null mice also present with omphalocele, providing a potentially significant overlap in phenotype with *EphB/ephrin-B* mutant mice (Shimizu et al., 2005).

The failure to close the eyelids has not been extensively characterized in human fetal development, presumably because the eyelids reopen before birth. However the finding that the EOB phenotype can result in corneal damage or inflammation, and is often accompanied by other embryonic malformations, suggest more attention should perhaps be placed toward monitoring prenatal eyelid development (Mann et al., 1993).

B-ephrin reverse signaling mediates embryonic eyelid closure

While characterizing palatal shelf defects in $EphB2^{\Delta/\Delta};EphB3^{\Delta/\Delta}$ compound null mice and $ephrin\text{-}B2^{lacZ}$ single mutant mice, I discovered that 38% of $ephrin\text{-}B2^{lacZ/lacZ}$ embryos at E18.5 (n=24) exhibited an eyelid closure defect as scored by any failure of the eyelid to fully close in one or both eyes, indicating a role for ephrin-B2 reverse signaling in the closure of the embryonic eyelid (Figure 6.1). Additionally, I noted that 43% of $EphB2^{\Delta/\Delta};EphB3^{\Delta/\Delta}$ compound null E18.5 embryos (n=30) also exhibited defects in embryonic eyelid closure, indicating that EphB2 and EphB3 were the relevant EphB receptors in this process. However, none of the 11 $EphB2^{lacZ/lacZ};EphB3^{\Delta/\Delta}$ compound mutant E18.5 embryos I looked at showed any defects in eyelid closure, indicating that forward signaling through these EphB receptors is not important, and that the closure of the eyelid is specifically driven by EphB2/B3 activation of reverse signaling through ephrin-B2.

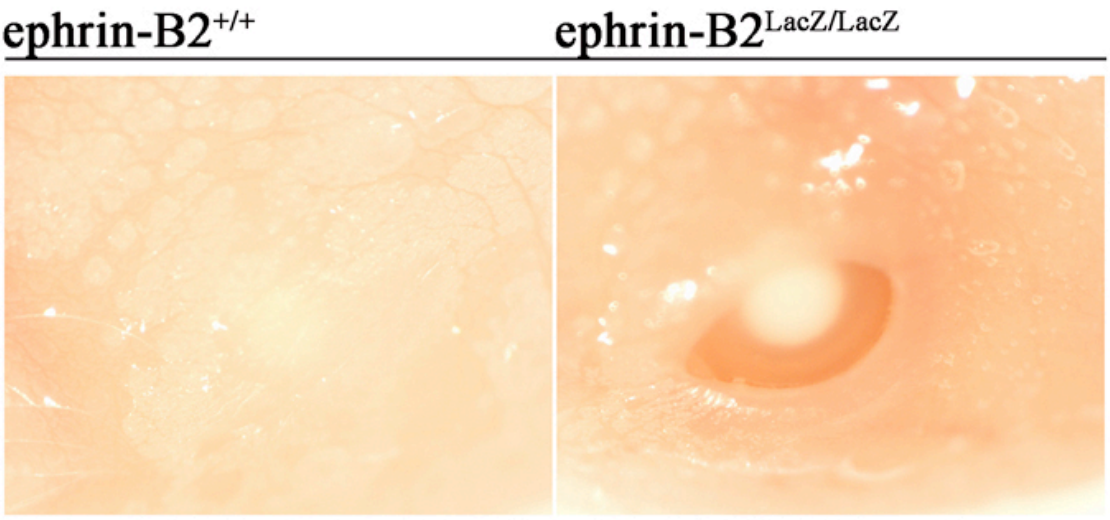


Figure 6.1

EOB in *ephrin-B2*$^{lacZ/lacZ}$ mutants. High magnification shots of whole-mount wild-type (left) and *ephrin-B2*$^{lacZ/lacZ}$ (right) embryos at E18.5. Whereas the eyelids have adhered shut in wild-type embryo, the eyelids have not adhered shut in the *ephrin-B2*$^{lacZ/lacZ}$ mutant, and the eyes remain open.

EphB and ephrin-B2 are expressed on adherent eyelid epithelia

To determine the potential roles of EphB2, EphB3, and ephrin-B2 in eyelid closure, I next examined the expression profiles of these molecules at E15.5 when the eyelids begin adhering. Eyelids from E15.*5 ephrin-B2*$^{lacZ//+}$ embryos were immunostained with antibodies specific for pan-ephrin-B or β-gal to visualize ephrin-B. These immunostains indicate that B-ephrin is highly expressed in the epithelia of the eyelids, and particularly at the site of adhesion, where ephrin-B2 is found all over the newly formed epithelial seam (Figure 6.2 top panels). EphB3 was visualized by staining E15.5 eyelids from BAC-Tg-*EphB3*^{YFP} embryos with an antibody against GFP. While no signal was detected in wild-type controls (data not shown), EphB3 expression was clearly visible in the leading epithelia of the eyelid before adhesion and at the site of adhesion, in addition to high levels of expression in the mesenchyme of the eyelid (Figure 6.2 bottom panels). To visualize EphB2 expression, eyelids collected at E15.5 from either *EphB2*$^{+/+}$ or *EphB2*$^{\Delta/\Delta}$ embryos were immunostained with an antibody recognizing EphB2. While no expression was detected in the *EphB2*$^{\Delta/\Delta}$ embryo as expected, EphB2 was clearly visible in the mesenchyme and leading epithelia of the eyelid from the wild-type embryo (Figure 6.3). This expression pattern was confirmed by X-Gal stains of *EphB2*$^{lacZ/+}$ E15.5 eyelids, which also place EphB2 in the mesenchyme and leading epithelia of the eyelid (Figure 6.3 right panel).

Given the individual expression profiles of ephrin-B2, EphB2, and EphB3, I reasoned that Eph and ephrin might be co-expressed in adhering eyelid epithelial cells. To address this, E15.5 *ephrin-B2*$^{lacZ/+}$ eyelids were immunostained for EphB2 and ephrin-B2; these co-immunostains showed similar individual expression profiles for EphB2 and

ephrin-B2, and when viewed in the merged image, provided clear evidence that both Eph and ephrin are co-expressed in adhering eyelid epithelia, both in the leading epithelia before adhesion and in the newly formed epithelial seam after adhesion (Figure 6.4).

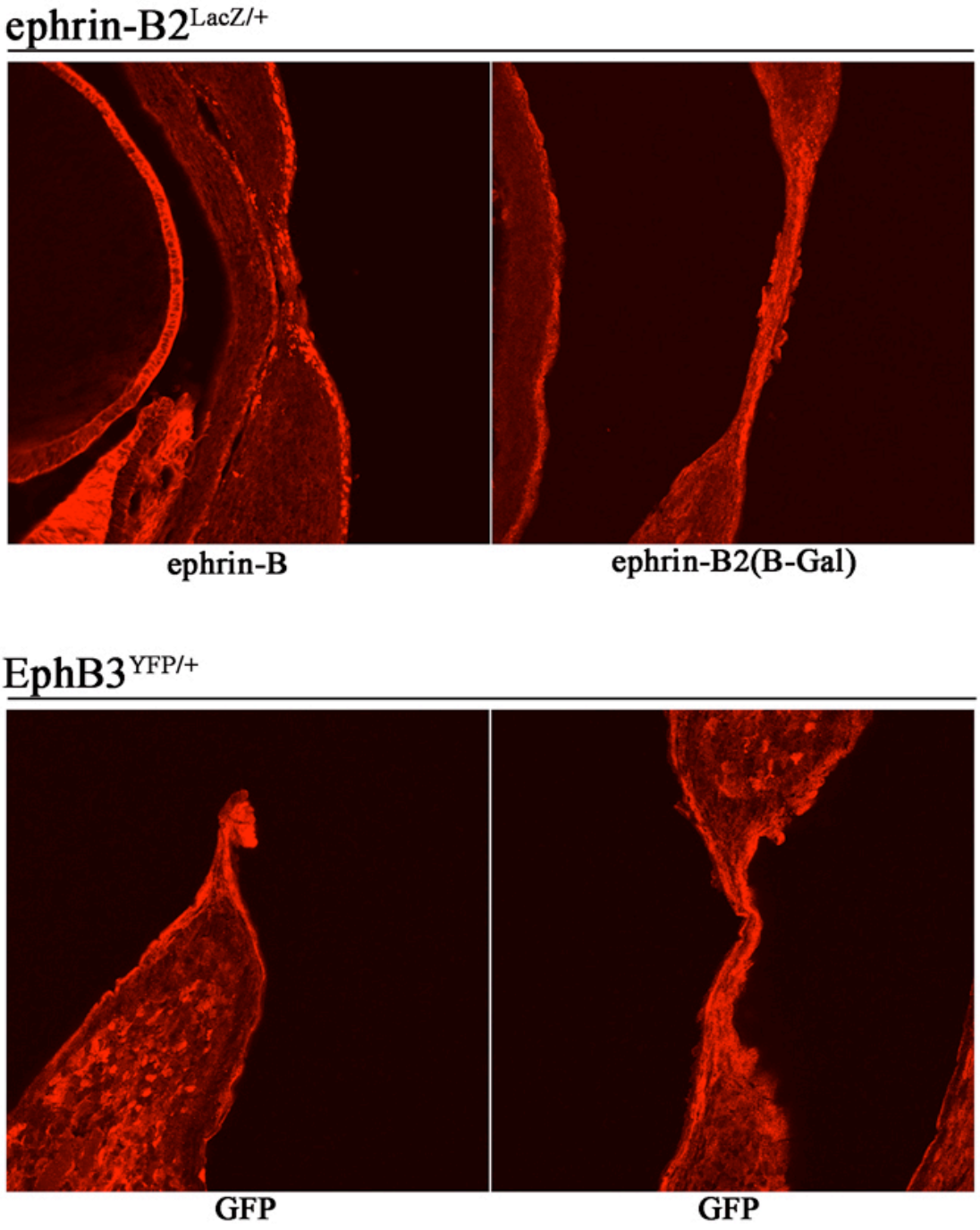


Figure 6.2 Ephrin-B2 and EphB3 are expressed at the site of eyelid adhesion. (Top) Serial sections through the eyelid of an E15.5 *ephrin-B2*$^{lacZ/+}$ embryo were stained with antibodies against either pan-ephrin (left) or β-gal (right) to detect ephrin-B2 expression. Ephrin-B2 is localized to the adherent epithelia in the eyelid. (Bottom) Serial sections through the eyelid of an E15.5 BAC-Tg-*EphB3*$^{YFP/+}$ embryo were stained with an antibody recognizing GFP to detect EphB3 expression. EphB3 is present on the adherent epithelia before and at adhesion, as well as in the flanking mesenchyme.

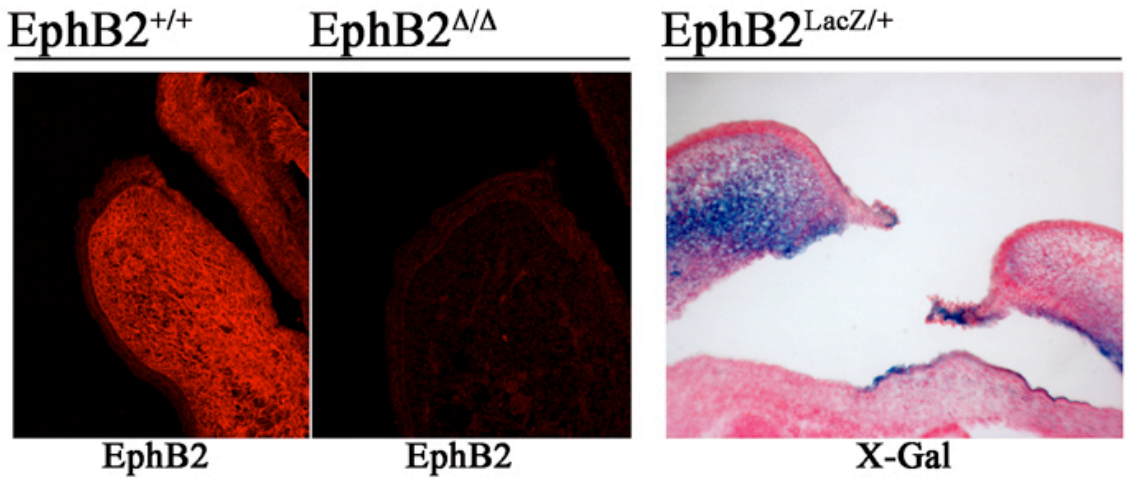


Figure 6.3

EphB2 is highly expressed in the epithelia and mesenchyme of the eyelid at adhesion. (Left and middle) Sections from E15.5 wild-type and *EphB2*$^{\Delta/\Delta}$ embryos were immunostained for EphB2. EphB2 is expressed highly throughout the mesenchyme of the eyelid, and is also present in the leading epithelia where adhesion will occur. No signal is found in the negative control. (Right) X-gal stain of pre-adhesion eyelids from E15.5 *EphB2*$^{lacZ/+}$ embryos shows a similar expression pattern, localizing EphB2 to the mesenchyme of the eyelid and the epithelia where adhesion will occur.

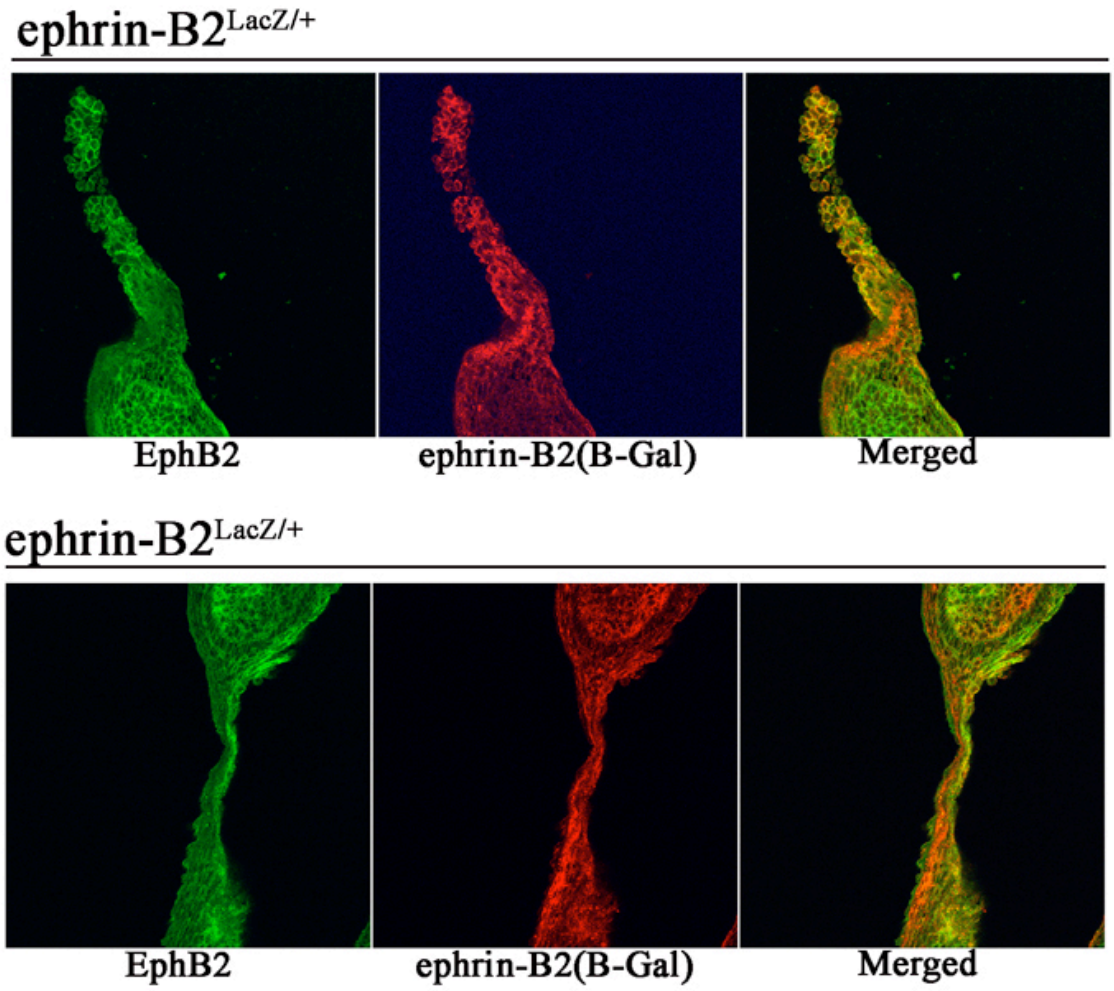


Figure 6.4

Ephrin-B2 and EphB2 are co-expressed in the adhering eyelid. Serial sections of the eyelids taken from E15.5 *ephrin-B2*$^{lacZ/+}$ embryos were stained with EphB2 and β-gal to detect EphB2 and ephrin-B2 expression, respectively. EphB2 and ephrin-B2 are co-expressed in the epithelia and flanking mesenchyme within the eyelid before (top) and after (bottom) adhesion occurs.

Ephrin-B is tyrosine phosphorylated and activated in adherent epithelia

Having determined that B-ephrin reverse signaling was involved in adhesion of the embryonic eyelid, I last sought to determine where this B-ephrin reverse signaling was taking place. To answer this, I performed IF on eyelids from wild-type E15.5 embryos with an antibody recognizing the phosphorylation of one of the key, conserved tyrosine residues on the B-ephrin cytoplasmic tail. Immunostains with this antibody did not fully overlap with serial stains using a pan-ephrin antibody, indicating the antibody does not just recognize ephrin-B, but is instead specific for tyrosine phosphorylated ephrin. Instead, signal was found only in the leading epithelia of the eyelid and in epithelia lining the inner portion of the eyelid, indicating the activation of B-ephrin reverse signaling in these cells (Figure 6.5 top panel). In a similar experiment, I used a different phospho-ephrin-B antibody to examine the activation of reverse signaling at sites where eyelid adhesion had already occurred, and found robust reverse signaling activation all over the nascent epithelial seam, much of which co-localized with EphB2 (Figure 6.5 bottom panel).

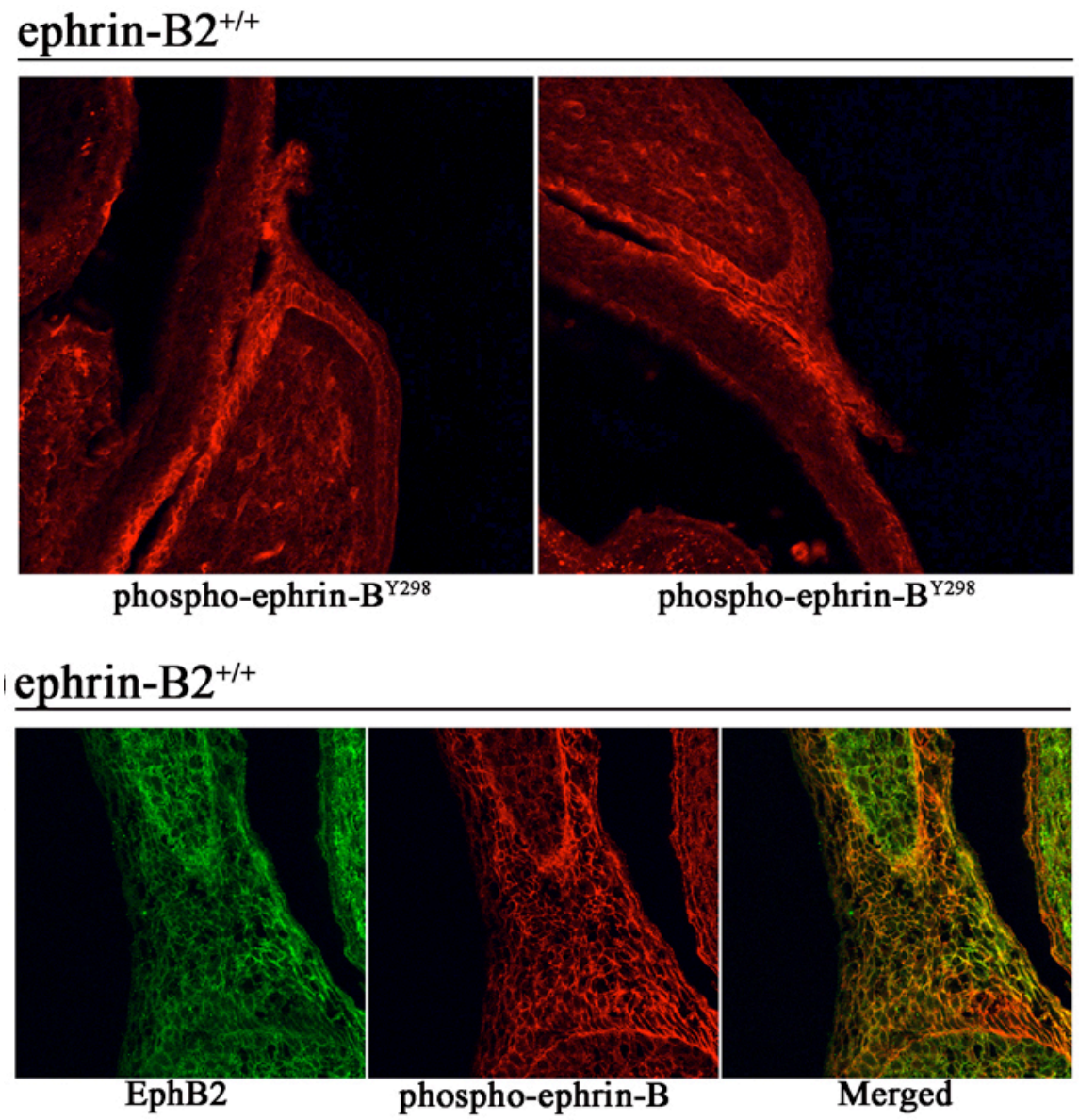


Figure 6.5

Ephrin-B is activated in adherent epithelia. (Top) IF was performed on eyelids taken from wild-type embryos with an antibody recognizing phosphorylation of tyrosine 317 on the ephrin cytoplasmic tail (Y298 in zebrafish). Ephrin-B is tyrosine phosphorylated in the epithelia lining the inner portion of the eyelid and at the site of adhesion. (Bottom) Double IF using antibodies against EphB2 and the phospho-ephrin-B$^{Y324/Y329}$ antibody in the eyelid of a wild-type embryo further revealed tyrosine phosphorylation of ephrin-B at the site of eyelid adhesion, where EphB2 is also expressed.

Discussion

I have shown that ephrin-B2 reverse signaling plays an important role in mediating closure of the embryonic eyelid, as *ephrin-B2*$^{lacZ/lacZ}$ mice present with a partially penetrant EOB phenotype. I have further defined that EphB2 and EphB3 are the relevant Eph receptors involved in this development event, and that they act non-cell autonomously, as none of the *EphB2*$^{lacZ/lacZ}$*;EphB3*$^{\Delta/\Delta}$ forward signaling deficient animals I examined presented with EOB. As in the other cell adhesion events at the caudal end of the animal and in the palate and foregut, Eph and ephrin appear to be co-expressed at the point of adhesion, and ephrin-B is actively conducting reverse signals in these adherent cells, consistent with a role for reverse signaling in adhesion. Eph/ephrin behavior in closure of the eyelid is a little different from the other adhesion events in that it does not occur at the midline, does not seem to have a role for forward signaling too, and does not follow the adhesion with fusion, which might provide some clues into how these molecules are behaving in these different morphogenetic processes.

CHAPTER 7

EphB2 and ephrin-B2 regulate the ionic homeostasis of vestibular endolymph

Dravis, C., Wu, T., Chumley, M.J., Yokoyama, N., Wei, S., Wu, D.K., Marcus, D.C. and Henkemeyer, M. EphB2 and ephrin-B2 regulate the ionic homeostasis of vestibular endolymph. **Hearing Research** 223, 93-104 (2007).

Summary

Here I describe a novel role for ephrin-B2 reverse signaling in regulating ionic homeostasis within the vestibular apparatus. Mice deficient for ephrin-B2 reverse signaling present with a circling phenotype due to defective structures and fluid homeostasis within the vestibular apparatus. Both ephrin-B2 and ephrin-B reverse signaling are localized to specialized epithelia within the inner ear, suggesting that these reverse signals play a direct role in regulating ionic homeostasis.

The vestibular apparatus

The inner ear is a bony structure that consists of two functional parts: the cochlea, which is the organ used to detect sound, and the vestibular apparatus, which is the organ used to detect elements of balance, such as gravity, acceleration, and the angle of the head. The vestibular apparatus consists of three connecting tubes, called semicircular canals, through which fluid flows in response to movement; the movement of this fluid stimulates hair cells located at the apex of specialized sensory structures called the cristae, which is then processed through higher order neural circuitry to form a sense of balance. This specialized fluid is termed endolymph, and is enclosed within the labyrinth of the vestibular apparatus. Endolymph fluid is unique in that ionically it resembles intracellular fluid, in that it is rich in K^+ but poor in Na^+. The ionic composition of endolymph fluid is established by a continuous sheet of epithelial cells that lines the bony labyrinth and encloses the endolymph. The interaction of two forms of specialized epithelial cells, the transitional and dark cells, found in the semicircular canals and utricle are responsible for maintaining the endolymph by regulating the flow of water and charged molecules into the semicircular canals (Wangemann, 1995; Wangemann, 2002). The epithelia must also form very tight junctions to serve as a stringent barrier to keep the endolymph fluid distinct from the fluid outside the epithelial sheet, the perilymph, which maintains a high Na^+, low K+ composition. The regulation of the K^+-rich nature of endolymph fluid is absolutely essential for proper vestibular function, and defects in this maintenance have been linked to human pathologies associated with chronic imbalance.

Defects in vestibular function in mice present as a circling phenotype that resembles the “waltzing” behavior of mice that was prized by mouse collectors towards

the end of the 19th century and helped lead to the establishment of inbred murine lines that now dominate biomedical research today. Previous work by the Henkemeyer laboratory uncovered that *EphB2;EphB3* compound null animals presented with this circling phenotype, indicating a role for Eph-ephrin signaling in the development of the vestibular apparatus (Cowan et al., 2000). Coincidental with this circling phenotype, the EphB receptor null animals presented with severely reduced semicircular canals. Expression analysis localized EphB2 to endolymph-producing dark cells, suggesting cell autonomous roles in regulating ionic homeostasis, which was further supported by the finding that forward signaling deficient $EphB2^{lacZ/lacZ};EphB3^{\Delta/\Delta}$ compound mutant mice also presented with the waltzing phenotype. This study did not indicate what ephrin-B molecules were playing a role in vestibular development, whether reverse signaling was also important in vestibular function, or how the endolymph fluid was altered in these circling mice.

EphB2 and ephrin-B2 are expressed on the inner ear epithelia

To determine the identity of ephrin-B molecules involved in vestibular function, I performed double IF on sections through the semicircular canals of adult $ephrin\text{-}B2^{lacZ/+}$ mice, with antibodies recognizing β-gal (to recognize ephrin-B2) and EphB2. These assays determined that ephrin-B2 is highly expressed on the transitional cells, a distinct set of epithelial cells that separates the secretory dark cells from the sensory hair cells. EphB2 expression was localized to the dark cells, as previously demonstrated (Figure 7.1 top panel) (Cowan et al., 2000). A similar expression pattern was obtained in wild-type adult mice when using a pan-ephrin-B antibody in place of β-gal (Figure 7.1 middle panel). Expression of EphB2 was confirmed by repeating the IF in $EphB2^{\Delta/\Delta};EphB3^{\Delta/\Delta}$

animals serving as a negative control (Figure 7.1 bottom panel). X-gal stains of BAC-Tg-*EphB3*^{rtTA};TRE-lacZ animals also appear to localize EphB3 to the dark cells within the inner ear as well (data not shown).

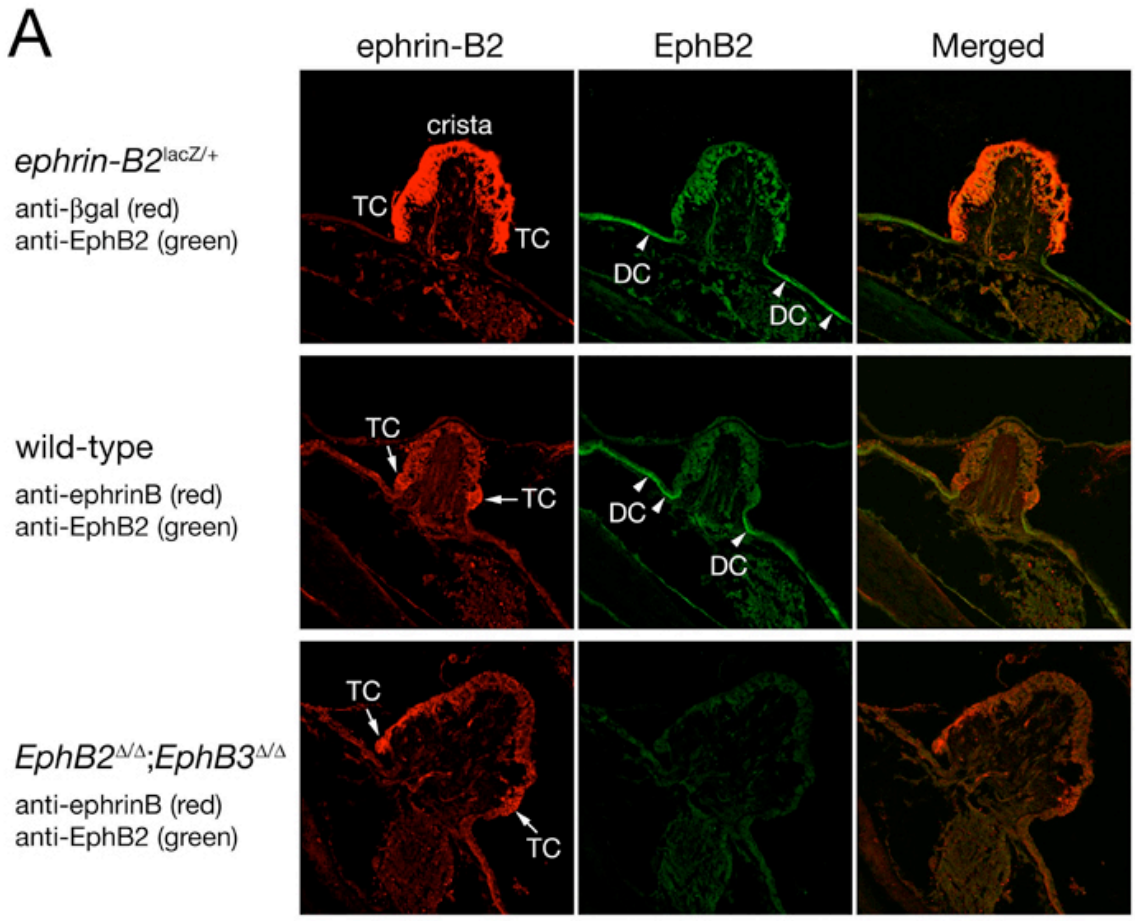


Figure 7.1

Expression of ephrin-B2 and EphB2 in the vestibular apparatus. Sections through either *ephrin-B2*$^{lacZ/+}$ or wild-type adult sensory cristae were double labeled with an anti-EphB2 antibody and either an anti-βgal antibody or a pan anti-ephrin-B antibody. Ephrin-B2 is localized to the transitional cells (TC) and supporting cells, while EphB2 is found in the vestibular dark cells (DC, arrowheads). No EphB2 expression is found in the negative control (bottom panel).

Ephrin-B reverse signaling is activated in inner ear epithelia

Given the complementary expression profile of EphB2 on the dark cells, and ephrin-B2 on the transitional cells, I looked to explore in greater detail the interface where these molecules come into contact. High magnification images of this interface show that there is an overlap of expression between EphB2 and ephrin-B2 expressing cells at this junction point, providing for a small window for Eph-ephrin signal activation (Figure 7.2). To determine if reverse signaling was occurring at this interface, I repeated my IF experiments on the inner ear from late stage *ephrin-B2*$^{lacZ/+}$ embryos. As in the adult, ephrin-B2 was preferentially expressed on the transitional cells, and EphB2 was found on dark cells (Figure 7.3 top panel). Serial sections from these same late stages embryos were then immunostained for phosphorylated ephrin-B, to get a readout for where ephrin reverse signals are being activated. Strikingly, this signal localizes specifically to the junction point between the dark and transitional cells, where EphB2 and ephrin-B2 are coming into contact (Figure 7.3).

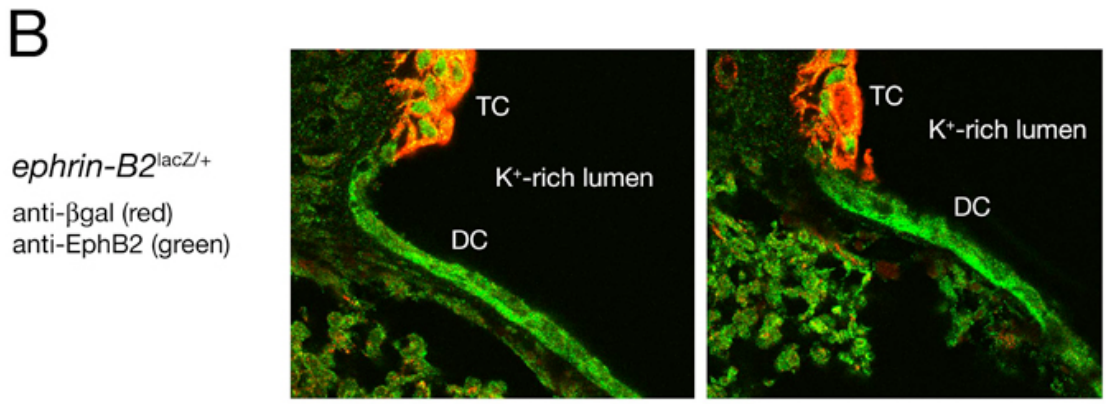


Figure 7.2

High magnification image of EphB2 and ephrin-B2 expression at the crista. Sections from the crista structure of an *ephrin-B2*$^{lacZ/+}$ adult were immunostained for EphB2 and β-gal to detect ephrin-B2. EphB2 is noticeably expressed at the basolateral membrane of the dark cells (DC), while ephrin-B2 is expressed in the transitional and support cells of the cristae. EphB2 and ephrin-B2 expression overlaps at the junction point between the dark and transitional cells (TC).

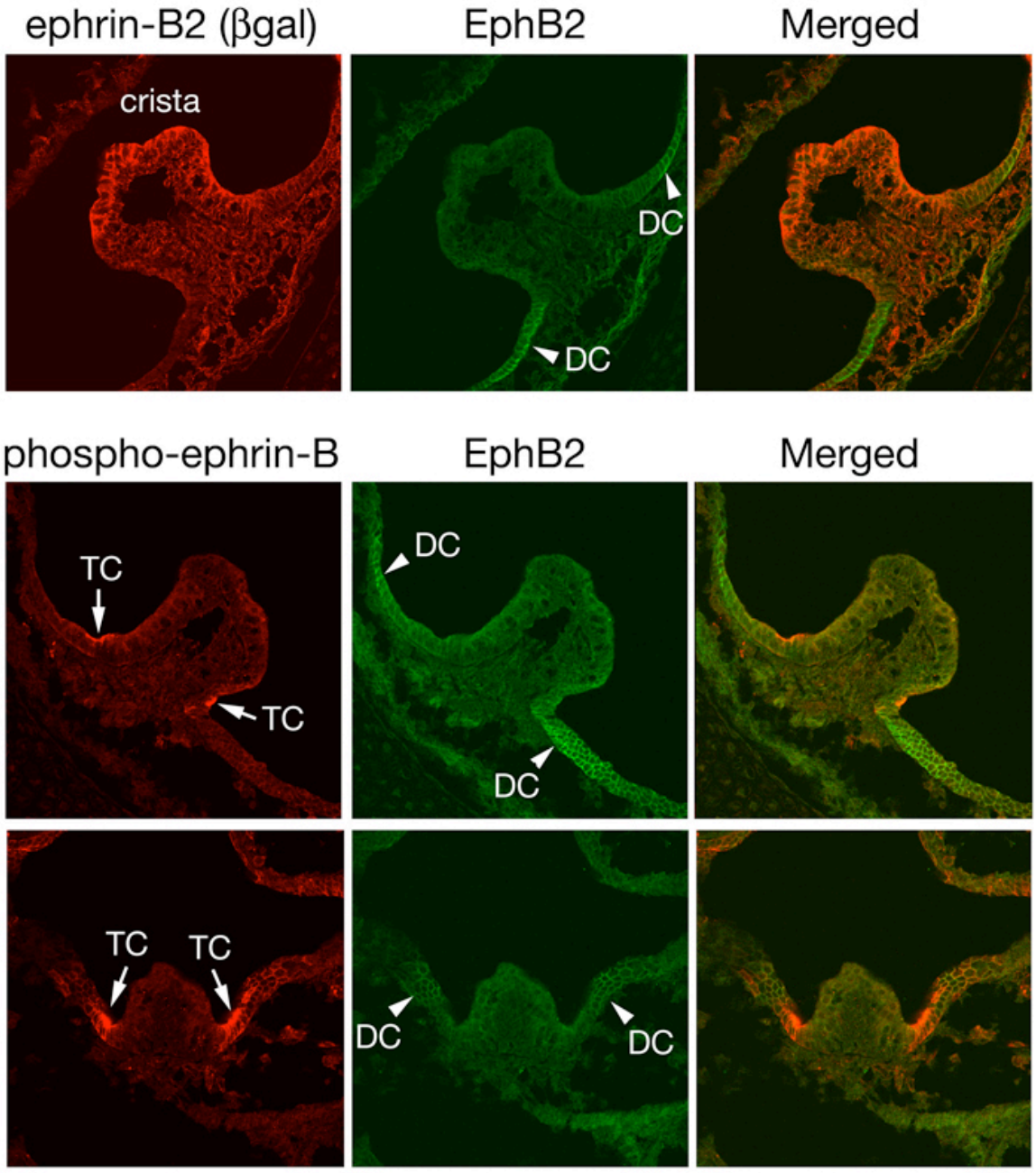


Figure 7.3

Ephrin-B2 is activated at the junction between transitional cells and dark cells. (Top) Immunostains of the crista from an E18 *ephrin-B2*$^{lacZ/+}$ embryo for ephrin-B2 and EphB2 demonstrate similar expression patterns for these molecules as in the adult, where ephrin-B2 is on the transitional cells of the crista, and EphB2 is in the flanking dark cells. (Bottom) Serial sections using an EphB2 antibody and phospho-ephrin-B antibody show that ephrin-B is specifically localized at the junction point between the transitional and dark cells, where EphB2- and ephrin-B2- expressing cells come into contact and overlap.

Mice heterozygous for a null mutation in *ephrin-B2* show vestibular dysfunction

The expression data clearly indicates a role for ephrin-B2 reverse signaling in vestibular function, so *ephrin-B2* mutant mice were scored for any circling behavior. In the 129 or C57BL/6 (B6) background strains *ephrin-B2*$^{T/+}$ heterozygote mice (as mentioned previously the *ephrin-B2*^{T} allele is a functional null for ephrin-B2) appear normal with no vestibular dysfunction. However, the circling phenotype in *EphB2;EphB3* compound mutant animals is only apparent in a CD1 background, so the *ephrin-B2*^{T} allele was backcrossed three times into a CD1 background (87.5% CD1). Surprisingly, 13.5% of the resulting *ephrin-B2*$^{T/+}$ heterozygote adult mice exhibited rapid head bobbing and a hyperactive circling locomotion (n=170) while none of the wild-type littermates showed any circling, indicating that a 50% reduction in ephrin-B2 expression is enough to elicit vestibular dysfunction. *Ephrin-B2*$^{T/T}$ embryos die very early on in embryogenesis due to severe vascularization defects, precluding an analysis of homozygotes.

Ephrin-B2 reverse signaling is important for normal vestibular function

To determine if ephrin-B2 was playing cell or non-cell autonomous roles in the transitional cells, similar crosses were utilized to drive the reverse signaling deficient *ephrin-B2*^{lacZ} allele into a CD1 background. When backcrossed into a CD1 background, 20% of *ephrin-B2*$^{lacZ/+}$ mice present with the same circling phenotype (n=190), indicating that reverse signaling through ephrin-B2 is important for vestibular function (Table 7.1 and Figure 7.4). Less than 1% of *ephrin-B2*$^{lacZ/+}$ animals in a 129/B6 background presented with the circling phenotype, demonstrating again the importance of the CD1 background in relation to vestibular function.

Table 7.1 Circling in e*phrin-B2*$^{lacZ/+}$ heterozygotes.

background	e*phrin-B2*$^{+/+}$	e*phrin-B2*$^{lacZ/+}$
129/B6	381 (0)	240 (2)
CD1	419 (0)	194 (40)

ephrin-B2$^{lacZ/+}$ heterozygote males were mated to *ephrin-B2*$^{+/+}$ wild-type females and resulting adult offspring were scored for circling. The total number of mice scored for each genotype and background is shown with the number that circled in parentheses. A mouse was scored to circle if it exhibited a rapid hyperactive circling locomotion at three inspections on three different days.

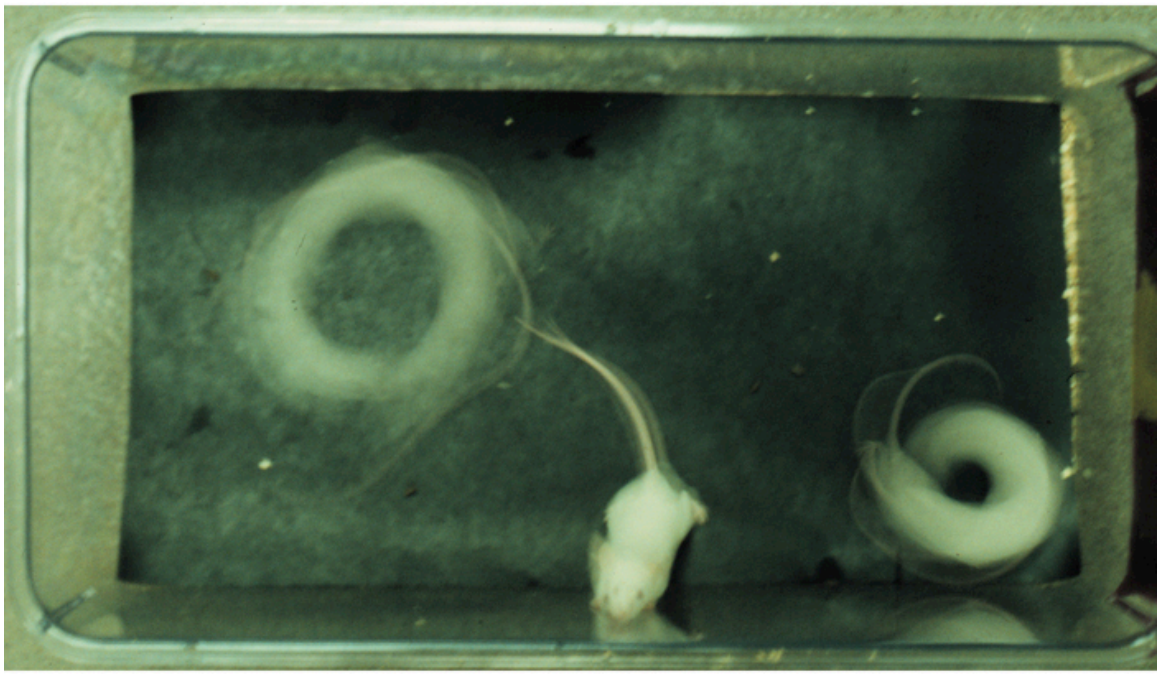


Figure 7.4

The circling or “waltzing” behavior seen in *EphB2* and *ephrin-B2* mutant animals.

ephrin-B2-βgal fusion protein does not cause vestibular dysfunction by hyperstimulating EphB forward signaling

As I have already dealt with previously in cloacal development, it is important to rule out the possibility that the defect seen in *ephrin-B2*$^{lacZ/+}$mice is the consequence of the ephrin-B2-βgal fusion protein hyperactivating forward signaling in adjacent cells. To explore this possibility genetically, the *ephrin-B2*^{lacZ} allele was crossed with *EphB2* mutant mice to generate compound mutants carrying single alleles of both mutations. As in the hindgut, the thought process here is that if the ephrin-B2-βgal fusion protein is overactivating EphB forward signaling, downregulating this forward signal by removing a copy of EphB2 should reduce the severity and/or penetrance of the vestibular phenotype; if on the other hand, it is truly the loss of ephrin-B2 reverse signaling that is responsible for the vestibular defect, the loss of a copy of EphB2 should make the severity/penetrance even worse. The generation of *ephrin-B2*$^{lacZ/+}$;*EphB2*$^{\Delta/+}$ compound heterozygotes revealed that 69% of these mice presented with the circling phenotype, compared to 13% of *ephrin-B2*$^{lacZ/+}$;*EphB2*$^{+/+}$ controls (Table 7.2). The genetic analysis therefore seems to further conclude that ephrin-B2 reverse signaling is necessary for proper vestibular function, and the defect found in the *ephrin-B2*^{lacZ} animals is not due to the hyperactivation of EphB2 forward signals.

Table 7.2 Enhanced circling in e*phrin-B2*$^{lacZ/+}$;*EphB2*$^{\Delta/+}$ compound heterozygotes.

ephrin-B2$^{+/+}$;*EphB2*$^{+/+}$	*ephrin-B2*$^{+/+}$;*EphB2*$^{\Delta/+}$	*ephrin-B2*$^{lacZ/+}$;*EphB2*$^{+/+}$	*ephrinB2*$^{lacZ/+}$;*EphB2*$^{\Delta/+}$
42 (0)	58 (0)	16 (2)	13 (9)

ephrin-B2$^{lacZ/+}$;*EphB2*$^{\Delta/+}$ compound heterozygote males were mated to *ephrin-B2*$^{+/+}$;*EphB2*$^{\Delta/+}$ females and resulting adult offspring of the indicated genotype were scored for circling. The total number of adult mice scored for each genotype class is shown with the number that circled in parentheses.

Loss of ephrin-B2 reverse signaling leads to reduced endolymph-filled lumens in the inner ear

The vestibular defects seen in the *EphB2;EphB3* compound mutant mice were the result of decreased semicircular canal volume. To determine if the circling phenotype in the *ephrin-B2*^{lacZ} mice was of a similar cause, a former graduate student in the Henkemeyer laboratory, Shiniu Wei, performed histological analysis on the vestibular structures of both *ephrin-B2*$^{lacZ/+}$ and *ephrin-B2*$^{T/+}$ adult circling mice. Unsurprisingly, the semicircular canals from mice heterozygous for both *ephrin-B2* alleles were markedly reduced in size, compared to a non-circling wild-type littermate (Figure 7.5). Additionally, because *ephrin-B2*^{lacZ} homozygotes survive until just after birth, Shiniu was able to look at vestibular development in the complete absence of ephrin-B2 reverse signaling in *ephrin-B2*$^{lacZ/lacZ}$ embryos. Sections taken from late stage *ephrin-B2*$^{lacZ/lacZ}$ embryos show an even greater reduction in the volume of the semicircular canal compared to wild-type embryos, and importantly, this defect even occurs outside of the CD1 background, highlighting the severity of a complete loss of ephrin-B2 reverse signaling (Figure 7.6). Latex paint casts of the vestibular apparatus performed by Doris Wu provide an even more striking visualization of the defects in semicircular canal size in the *ephrin-B2*$^{lacZ/lacZ}$ mutants (Figure 7.7).

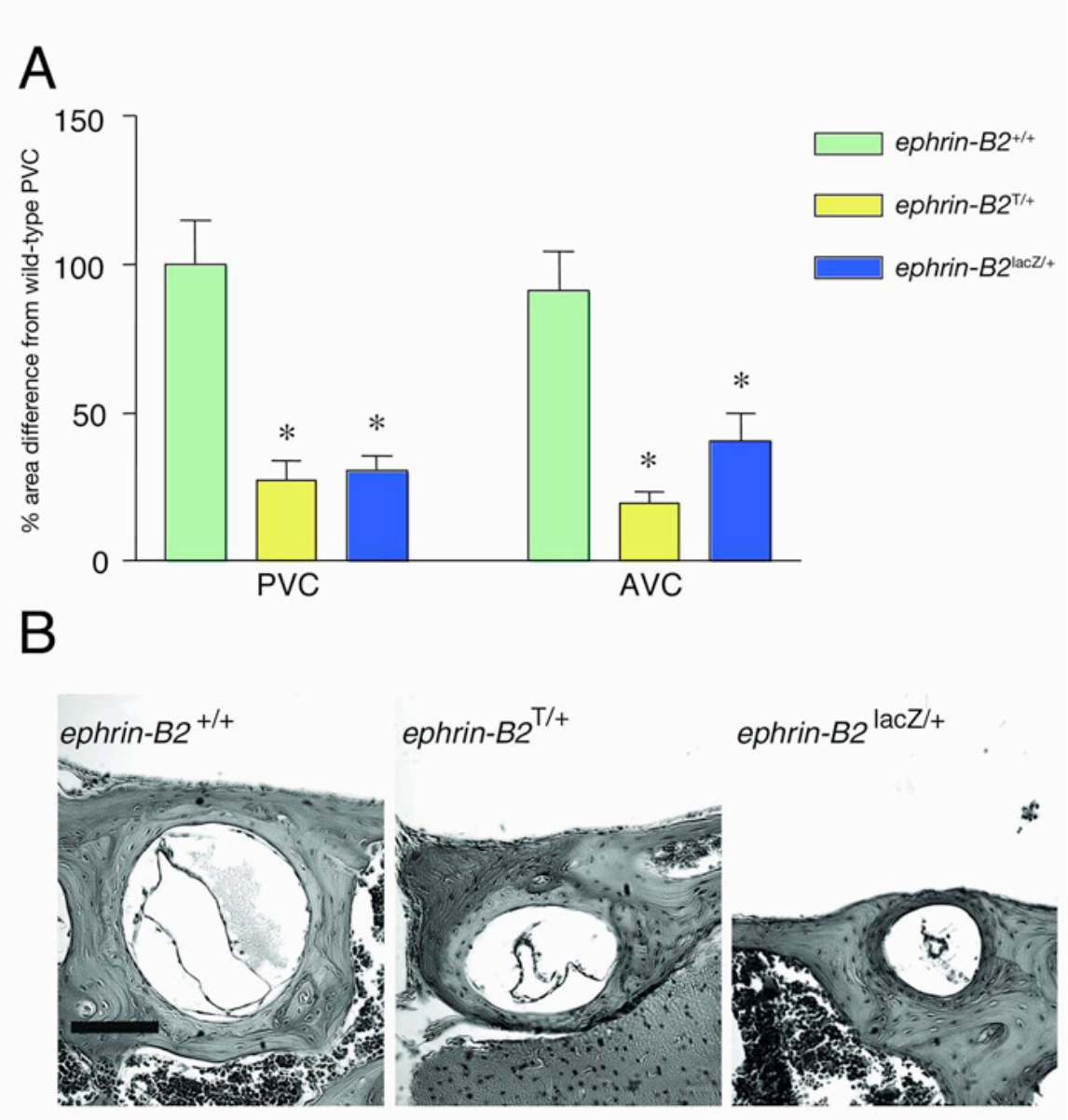


Figure 7.5

Reduced semicircular canals in *ephrin-B2*$^{T/+}$ and *ephrin-B2*$^{lacZ/+}$ heterozygous adult mice. (A) Measurements of the cross sectional area of the bony canals from the posterior vertical canal (PVC) and anterior vertical canal (AVC). Results are from seven *ephrin-B2*$^{+/+}$ non-circling, seven *ephrin-B2*$^{T/+}$ circling, and five *ephrin-B2*$^{lacZ/+}$ circling adult mice all in the CD1 background. Results are presented as percentage difference from the wild-type PVC. A one-way ANOVA and Dunnett's post-hoc test ($P < 0.01$) performed by Michael Chumley was used to determine that the heterozygotes show a significant reduction in the cross sectional area of both the PVC and AVC bony canals. (b) H&E stained cross sections bisecting the PVC in a non-circling *ephrin-B2*$^{+/+}$ wild-type and circling *ephrin-B2*$^{T/+}$ and *ephrin-B2*$^{lacZ/+}$ heterozygote adult mice all in the CD1 background. Image provided by Michael Chumley and Shiniu Wei.

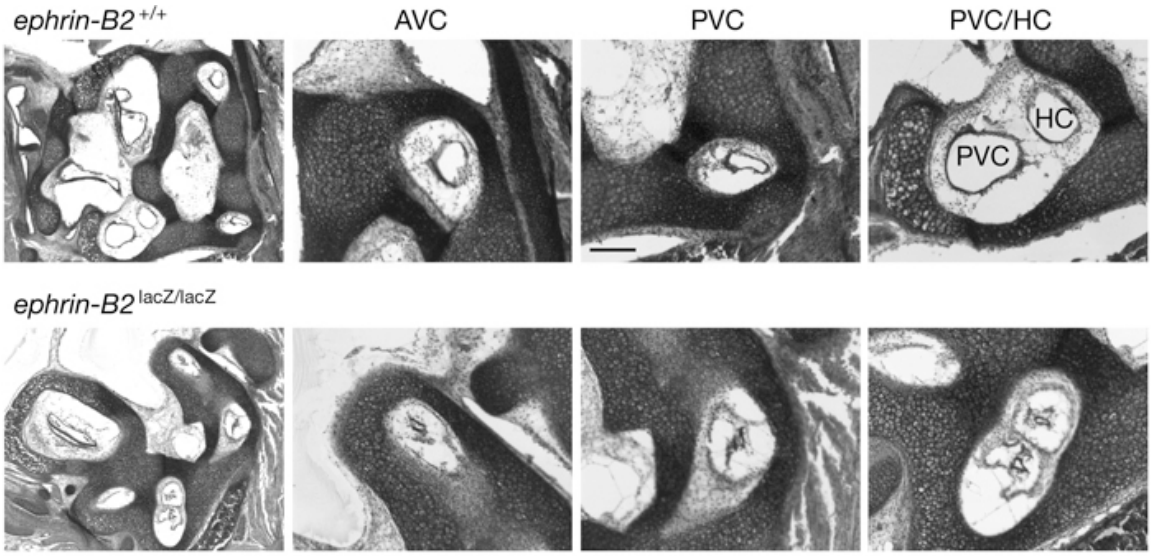


Figure 7.6

Reduced semicircular canals in *ephrin-B2*$^{lacZ/lacZ}$ homozygotes. H&E stained cross sections of the inner ear from E18.5 embryos bisecting the AVC, PVC, and horizontal canal (HC) in an *ephrin-B2*$^{+/+}$ wild-type and an *ephrin-B2*$^{lacZ/lacZ}$ homozygous littermate in a mixed 129/CD1 background. Image provided by Shiniu Wei.

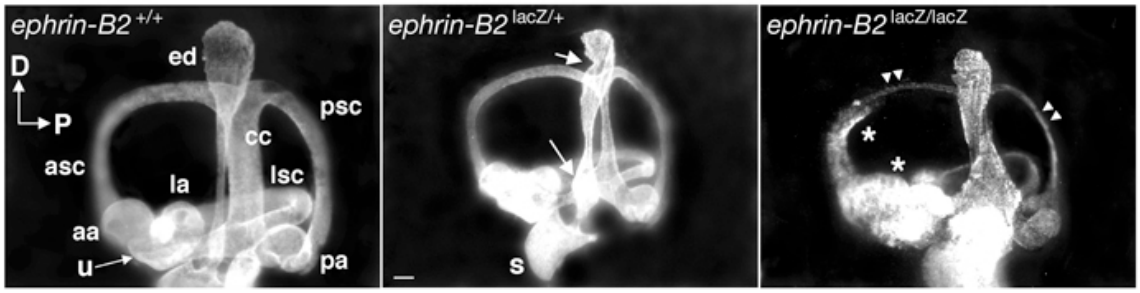


Figure 7.7

Reduced semicircular canals in *ephrin-B2*$^{lacZ/lacZ}$ homozygotes. Latex paint casts of the vestibular apparatus from wild-type, *ephrin-B2*$^{lacZ/+}$, and *ephrin-B2*$^{lacZ/lacZ}$ E18.5 littermates. Arrowheads indicate where the semicircular canals are much reduced in the *ephrin-B2*$^{lacZ/lacZ}$ animal. Image provided by Doris Wu.

EphB2 and ephrin-B2 regulate the ionic homeostasis of endolymph

The volume of endolymph in both *EphB* and *ephrin-B2* mutant mice is obviously reduced compared to the wild-type. However, given the localization of these molecules to secretory cells, it is possible the ionic makeup of this endolymph fluid is also imbalanced. To address this, a collaborator in the laboratory of Daniel Marcus, Tao Wu, isolated endolymph from wild-type, *ephrin-B2*$^{lacZ/+}$, and *EphB2*$^{lacZ/lacZ}$*;EphB3*$^{\Delta/\Delta}$ compound mutant mice, and measured the concentration of potassium within this fluid. Statistically significant decreases in endolymphatic potassium levels were found in both the reverse signaling and forward signaling deficient mice (Figure 7.8 top). Coincidental with this loss of potassium, the endolymphatic potential, measured by inserting an electrode into the inner ear endolymph, was similarly significantly reduced in both *EphB* and *ephrin-B2* mutant animals (Figure 7.8 bottom). It therefore appears that the inability to properly regulate the ionic homeostasis of endolymph fluid through the coordinated activities of Eph and ephrin in the dark and transitional cells is responsible for the vestibular dysfunction seen in these mutant mice.

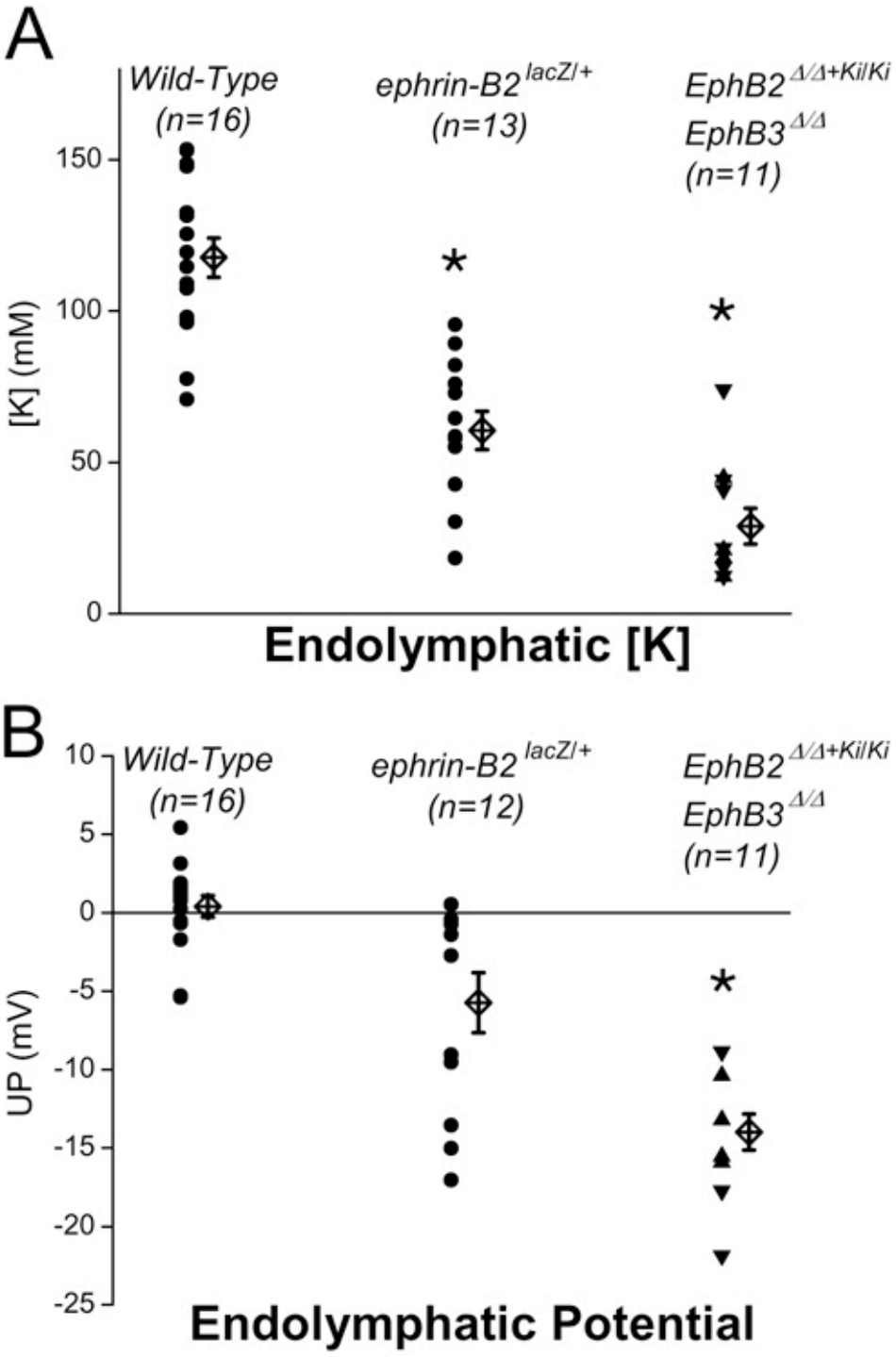

A
Wild-Type
(n=16)
ephrin-B2 lacZ/+
(n=13)
EphB2 Δ/Δ+Kil/Ki
EphB3 Δ/Δ
(n=11)
150
100
50
0
[K] (mM)
*
*
Endolymphatic [K]
B
Wild-Type
(n=16)
ephrin-B2 lacZ/+
(n=12)
EphB2 Δ/Δ+Kil/Ki
EphB3 Δ/Δ
(n=11)
10
5
0
-5
-10
-15
-20
-25
UP (mV)
*
Endolymphatic Potential

Figure 7.8

Reduced [K^+] and endolymphatic potential in circling *ephrin-B2*$^{lacZ/+}$ heterozygous and *EphB2;EphB3* compound mutant adult homozygotes in a CD1 background. The [K^+] (A) and endolymphatic potential (B) were measured by inserting a double-barrel electrode tip into the endolymph of anesthetized adult mice. *EphB2*^{Ki} here is the *EphB2*^{lacZ} allele. Image provided by Dan Marcus and Tao Wu.

Discussion

I have described abnormal vestibular function in mice with mutations that ablate the reverse signaling of ephrin-B2. Expression studies done by myself have shown that ephrin-B2 is expressed principally along the transitional cells within the inner ear, which serve as a bridge between secretory dark cells and sensory hair cells. EphB2 also appears necessary for vestibular function, and is specifically expressed within these secretory dark cells. Expression between ephrin-B2 and EphB2 overlaps at the site where dark cells and transitional cells come into contact, and consistent with a role for reverse signaling in vestibular function, ephrin-B reverse signals can be localized to this interface with an antibody recognizing the phosphorylated tail of B-ephrin. Co-workers have then shown that the endolymph fluid is misregulated in either *ephrin-B2* or *EphB2;EphB3* mutant mice, which presumably results in the abnormally reduced semicircular canals found in these animals. The combination of malformed semicircular canals and endolymph fluid not at its proper ionic composition is believed to result in abnormal vestibular sensory stimulation, which causes the head-bobbing and circling found in these affected mice.

Chapter 8

Conclusions

Summary

In my thesis I have attempted to uncover novel physiological roles for B-subclass ephrin reverse signaling through the molecule ephrin-B2. The strategy utilized in this pursuit involved the analysis of a newly generated animal model, in which the cytoplasmic tail of ephrin-B2 had been replaced with the enzyme β-galactosidase. The ensuing ephrin-B2-βgal fusion protein, coded by the *ephrin-B2*^{lacZ} allele, traffics to the plasma membrane and is expressed on the cell surface, where it can activate forward signaling in adjacent cells, but lacking any intracellular signaling domains, is unable to transduce a reverse signal through the ephrin-B2 cytoplasmic tail. This mouse model therefore represents an excellent tool by which I can readily distinguish between the "ligand-like" non-cell

autonomous and "receptor-like" cell autonomous signaling roles for ephrin-B2 in any given physiological process.

In examining these *ephrin-B2*^{lacZ} mice, I have discovered an assembly of embryonic malformations that appears to require ephrin-B2 reverse signaling. These include novel roles for ephrin-B2 reverse signaling in tubularization of the urethra and septation of the cloaca in hindgut development, as *ephrin-B2*^{lacZ} mice present with hypospadias and persistent cloaca phenotypes; septation of the primitive embryonic foregut, as *ephrin-B2*^{lacZ} mice present with laryngotracheoesophageal cleft; closure of the palate, as *ephrin-B2*^{lacZ} mice present with cleft palate; and finally, closure of the embryonic eyelid, as *ephrin-B2*^{lacZ} mice present with their eyes open at birth. Further, my analysis indicates that ephrin-B1 reverse signaling is important in ventral body wall closure, as *EphB2* null animals, but not forward signaling deficient *EphB2*^{lacZ} animals, present with omphalocele in an *EphB3* null background.

The identification that Eph-ephrin signaling is required in these different developmental events is important in advancing our understanding of a number of important developmental processes, defects in which closely resemble common human birth defects. But linking the Ephs and ephrins to embryonic development by itself is not a significant advance in the field. These molecules have been shown time and again to regulate cytoskeletal dynamics in cell migration events during development. Instead what makes this work noteworthy is what this reverse signal is doing during these developmental events. Whereas the stereotypical outcome of Eph-ephrin signaling is one of cell-cell or cell-axon repulsion, what I find here is that the reverse signal appears to be promoting a cell-cell adhesion response.

Finally, I show that *ephrin-B2*^{lacZ} animals also present with a defect in vestibular function, as these mice possess a circling phenotype. Within the vestibular apparatus, *ephrin-B2*^{lacZ} animals have disrupted inner ear structures and have lost ionic homeostasis within the critical endolymph fluid that flows through the sensory structures. If the discovery that Eph-ephrin signaling has the flexibility to modulate its control of cytoskeletal dynamics to elicit either repulsive or adhesive responses is of considerable interest to the field, it is all the more noteworthy that these same signaling molecules are apparently not limited to regulating cytoskeletal structure, but can play important roles in ionic homeostasis and the regulation of extracellular fluids as well.

Ephs and ephrins: where we were

The Eph-ephrin field was really starting to hit its stride when I began to study these molecules in 2002. Nearly all of the Eph receptors and ephrin ligands had been identified and cloned, and knockout mice for all but *EphB6* and *ephrin-B1* had been reported within the B-subclass. Roles for the Ephs and ephrins in axon guidance, cell migration, and boundary formation were well described, leading to a consensus outcome for Eph-ephrin signaling in promoting a cell repulsion outcome (Egea and Klein, 2007; Murai and Pasquale, 2003; Pasquale, 2005; Poliakov et al., 2004). Distinguishing the contributions of forward and reverse signaling in different physiological events was just starting to become a point of detail, as a series of studies started to bring into focus the likelihood that non-cell autonomous roles for the Eph receptors were more than just a triviality. The first of these studies was of course the ground-breaking finding by Mark Henkemeyer that *EphB2*^{lacZ} mice, in which a truncation abolishes only forward signaling, do not phenocopy

EphB2 null animals, indicating clear ligand-like roles for Eph receptors to transduce a signal through the ephrins (Henkemeyer et al., 1996). This was subsequently followed up by the demonstration that the cytoplasmic tail of B-subclass ephrins is phosphorylated, and that the isolated extracellular domain of Eph receptors was clustered on ephrin-expressing cells into cell-surface signaling centers. Finally, a number of protein-protein interactions with B-subclass ephrins were being reported, starting with the identification of PDZ domain containing proteins that could interact with the ephrin-B C-terminus (Bruckner et al., 1999; Lu et al., 2001), and culminating in the groundbreaking discovery by Chad Cowan that the adaptor protein Grb4 bound B-subclass ephrins (Cowan and Henkemeyer, 2001), thus linking the ephrins to a world of new effector molecules.

Despite all of this work, there was still no hard evidence that signal transduction through B-ephrin reverse signaling had any biological relevance. It was strongly suggested by the *EphB2*^{lacZ} mice of Mark Henkemeyer and their roles in the anterior commissure, and by the axon pathfinding defects of RGCs into the optic disc by Eric Birgbauer (Birgbauer et al., 2001), but there was no data clearly indicating that when you ablated the ability of an ephrin-B molecule to reverse signal, any developmental processes were clearly affected. Which is not to say that attempts had not been made, they just had not proved substantive. A β-gal truncation of ephrin-B3 did not reveal any cell autonomous functions for the ephrin, as instead the molecule was only shown to function as a ligand at the midline of the spinal cord (Yokoyama et al., 2001). A deletion of the ephrin-B2 cytoplasmic tail produced faulty data and erroneous claims for ephrin-B2 reverse signaling, that was in the process of being debunked by the Henkemeyer laboratory (Adams et al., 2001; Cowan et al., 2004). The opportunity was therefore ripe to

step in and characterize reverse signaling roles for both ephrins-B1 and B2. And with Nobuhiko Yokoyama having recently generated the *ephrin-B2*^{lacZ} animal, in which ephrin-B2 reverse signaling is specifically knocked out, the timing was right as well.

Results

I have identified a number of physiological processes that require reverse signaling through ephrin-B2. The initial role for ephrin-B2 reverse signaling was defined in caudal development, where a fraction of *ephrin-B2*$^{lacZ/+}$ heterozygote breeding studs were infertile due a condition called hypospadias, in which the penis does not properly tubularize and encapsulate the urethra, resulting in an abnormally splayed penis with a mislocalized urethral exit point along the ventral base. Coincidental with the hypospadias, affected *ephrin-B2*$^{lacZ/+}$ mice also presented with a reduction in the perineal distance separating the anorectum and genital tubercle, indicating a defect in the septation of the primitive cloaca. My analysis of *ephrin-B2*$^{lacZ/lacZ}$ homozygous mutants confirmed a requirement for ephrin-B2 in hindgut septation, as these mice have a complete failure in cloacal septation, which presents itself as the most severe anorectal malformation wherein the anorectum is absent and instead the intestine forms a fistula at the base of the bladder, leaving one common endodermal compartment to exit all waste matter. Analysis of *EphB2;EphB3* compound null animals revealed that these mice also presented with hypospadias and defective cloacal septation, indicating that EphB2 and EphB3 were likely functional ligands to activate ephrin-B2 reverse signaling. Strikingly, forward signaling deficient *EphB2*$^{lacZ/lacZ}$*;EphB3*$^{\Delta/\Delta}$ mice also presented with these same urorectal defects, suggesting that both forward and reverse signaling played important roles in urethral tubularization

and cloacal septation. Consistent with the requirement for both forward and reverse signaling components of bidirectional signaling, both EphB and ephrin-B2 are expressed on functionally relevant cells in these midline events; the two molecules are co-expressed on both adherent epithelia and in the flanking mesenchyme where lateral wedges come into contact and adhere to either tubularize the urethra or septate the cloaca.

Having defined a role for ephrin-B2 reverse signaling in hindgut septation, I next examined whether these reverse signals had a similar role in mediating tracheoesophageal septation of the foregut, as well— this despite the assurances of friend and co-worker, Chad Cowan, that they did not! Fortunately for me, they did. My histological analysis of *ephrin-B2*$^{lacZ/lacZ}$ mutant embryos indicated a clear failure in either the formation of a septum to separate the trachea and esophagus, or in the ability of that septum to properly extend to the rostral apex to separate these two compartments. Consistent with a role for ephrin-B2 in mediating septation of the foregut, ephrin-B2 is highly expressed on the epithelia at the site of adhesion, as well in the mesenchyme flanking this adhesion site and encircling the esophagus. Ephrin-B reverse signals are also actively being transduced at the site of foregut septation, which further supports a role for ephrin-B2 reverse signaling in mediating this process. Unfortunately, despite repeated attempts to analyze several different combinations of *Eph* receptor compound null animals, I could not reproduce this phenotype on the Eph receptor side of the equation, so it remains unclear 1) what combination of Eph receptors are functioning non-cell autonomously to activate ephrin-B2 reverse signaling in the foregut, and 2) if forward signaling through these molecules also has some contribution in the process, as occurs in hindgut septation. That said, the precise localization of both EphB2 and EphB3 to the site of foregut adhesion suggests that these

two receptors are likely a part of whatever combination of Eph receptors are involved. Moreover, given that the cell adhesion events these molecules are responsible for are likely going to be functionally very similar in both hindgut and foregut septation, it seems likely that if forward signaling was necessary for hindgut septation, it will be important in foregut septation as well.

While characterizing the defects in *ephrin-B2*$^{lacZ/lacZ}$ animals with regards to foregut septation, I encountered two defects in these reverse signaling deficient animals at the cranial end of the embryo. The first of these was in palatal shelf fusion, in which a fraction of *ephrin-B2*$^{lacZ/lacZ}$ homozygous mutant mice presents with a cleft palate phenotype in which the oral and nasal cavities fail to properly separate. The second of these was in embryonic closure of the eyelid, in which a fraction of *ephrin-B2*$^{lacZ/lacZ}$ homozygous mutant mice presents with EOB. As in the septation of the hindgut, EphB2 and EphB3 appear to be the relevant EphB receptors involved in these two developmental events, as *EphB2;EphB3* compound null animals present with both cleft palate and EOB. Interestingly, however, *EphB2*$^{lacZ/lacZ}$*;EphB3*$^{\Delta/\Delta}$ mice only present with cleft palate and not EOB, suggesting the forward signaling is very important in palatal shelf fusion but is not relevant to closure of the embryonic eyelid. Another conserved feature with the hindgut is that both EphB receptor and ephrin-B2 appear to be co-expressed at the sites of adhesion; once again both molecules can be found in adherent epithelia right before the palatal shelves or eyelids come into contact, as well as in the flanking mesenchyme. Moreover, as in septation of the foregut, IF performed with an antibody detecting tyrosine phosphorylated ephrin-B indicates that ephrin-B is specifically being activated in these

adherent epithelia, further confirming a role for reverse signaling in these processes and suggesting that role is in mediating cell-cell adhesion.

Ephrin-B2$^{lacZ/lacZ}$ homozygous mice present with no apparent defects in closure of the ventral body wall, but while characterizing *EphB2;EphB3* compound mutant animals I detected the same omphalocele defect described in brief over a decade ago. Given that the other defects I characterized obviously suggested roles in cell adhesion, it made sense to pay attention to this malformation, given that it too is the apparent result of failed midline adhesion. The previous description of ventral body wall defects in *EphB2;EphB3* compound mutant animals made no mention of whether or not forward signaling was involved or where these molecules were expressed. I addressed both of these by showing that forward signaling has a very minimal role in mediating ventral body wall closure, as even with the dominant-negative ability of the *EphB2*^{lacZ} allele to interfere with forward signaling, only a small fraction of *EphB2*$^{lacZ/lacZ}$*;EphB3*$^{\Delta/\Delta}$ animals present with omphalocele. I further localize EphB2 to the ventral midline where adhesion of the lateral body walls takes place. The significantly reduced penetrance of omphalocele in *EphB2*$^{lacZ/lacZ}$*;EphB3*$^{\Delta/\Delta}$ mice suggests that, as in the other adhesion defects I have characterized above, the adhesion event is driven principally by B-ephrin reverse signaling, this time through the ligand ephrin-B1.

Finally, I have helped characterize a novel role for ephrin-B2 reverse signaling in regulating ionic homeostasis within the inner ear. Both *ephrin-B2*^{lacZ} and *EphB2;EphB3* compound mutant mice present with a circling phenotype due to dysfunction within the vestibular apparatus, the part of the inner ear necessary for balance and motor coordination. Excellent work done by collaborators and co-workers have demonstrated

that the vestibular apparatus in these mice is abnormal, wherein the semicircular canals through which endolymph fluid flows to activate sensory cells is severely restricted in size. Here I have shown that the EphB receptors and ephrin-B2 have reciprocal expression patterns on specialized epithelia within the inner ear. EphB2 is expressed specifically on epithelial dark cells, while ephrin-B2 can be found on adjacent epithelial transitional cells. Both dark cells and transitional cells play critical roles in maintaining the specific ionic concentration of endolymph fluid, by regulating the exchange of water and charged molecules necessary for maintaining this homeostasis. I further show that reverse signaling is actively being conducted at this dark cell-transitional cell junctional interface where Eph/ephrin expression overlaps, which is consistent with the role for ephrin-B2 reverse signaling in vestibular function indicated by the *ephrin-B2*^{lacZ} mice. Finally, a collaborator shows that the ionic concentration of endolymph in both *ephrin-B2*^{lacZ} and *EphB2;EphB3* compound mutant mice is severely altered from the norm, further indicating a role for reverse signaling in mediating ionic homeostasis.

Discussion

My studies here indicate the involvement of the Ephs and ephrins in novel physiological roles that are difficult to reconcile with the stereotypical outcome of Eph-ephrin repulsive signaling. Here I discuss how I interpret these results, highlight important themes from the data, address criticisms of this model, and discuss what these results do to further our understanding of Eph-ephrin signaling and of the developmental events they now appear to mediate.

B-ephrin reverse signaling mediates cell-cell adhesion

The first question arising from the data is what is this ephrin-B reverse signal doing in these different septation events. If you look at development of the palate as an example, there are a number of distinct processes that need to take place. Early on in embryonic development the palatal shelves emerge from the maxillary processes through directed cell migration at E11.5; these palatal shelves then dramatically expand in size through cell proliferation and more cell migration at E12.5 and E13.5; these palatal shelves then meet and adhere at the midline at E14.5, shortly after which they fuse together through apoptosis and EMT to form a continuous septum between the oral and nasal cavities. The same basic process hallmarks closure of the urethra, eyelid, ventral body wall, and septation of hindgut and foregut. Given our understanding of Eph-ephrin signaling, as regulating cytoskeletal dynamics and utilizing tyrosine kinase signaling, it is easy to see the potential involvement of bidirectional signaling in any of these distinct processes. However, given the paradigm of Eph-ephrin signaling and cell repulsion, it is really just these early directed cell migration events that the field first points to in relation to these embryonic malformations. And accordingly there are a number of papers focused on non-cell autonomous roles for ephrin-B molecules in mediating early cell migration events through the activation of forward signaling (Adams et al., 2001; Davy and Soriano, 2007; Risley et al., 2008).

The problem with this idea is that the expression data of the relevant molecules during these processes suggests this is a limited understanding of what the Ephs and ephrins are doing in these developmental events. When the expression of ephrin-B2 and the EphB receptors are documented during any of these septation/closure events, these

molecules are strikingly localized to the epithelia at the site of adhesion. Now, these molecules are also expressed in the flanking mesenchyme as well, which is consistent with perhaps a role in cell migration as well. But most importantly, when an antibody recognizing the phosphorylated tail of ephrin-B is utilized, the reverse signal is localized specifically to the adherent epithelia. So at least for the case of cell autonomous signaling functions involving B-subclass ephrins, reverse signals appear to only function in these adherent epithelia. These adherent epithelia are not especially proliferative and cell proliferation in the epithelia would not intuitively seem to play a role in these septation/closure events, so this seems to eliminate a role for ephrin-B reverse signaling in cell proliferation. Further, these adherent epithelia are not particularly motile; they just ride along at the leading edge of the underlying mesenchyme, until they meet their proper target at the midline and initiate adhesion. This seems to further eliminate a role for ephrin-B reverse signaling in mediating directed cell migration of these lateral mesenchymal folds.

By elimination that leaves roles for B-ephrin reverse signaling in adhesion, the process by which these lateral folds become linked by a single epithelial seam, and fusion, the process by which this epithelial seam breaks down to leave continuous mesenchyme. Fusion is a tempting fate for ephrin reverse signaling. One of the dominant means of fusion is EMT, a process that requires extensive cytoskeletal remodeling, which is what the Ephs and ephrins are well known for. The other widely accepted means of fusion is apoptosis, which is not a widely accepted response from Eph-ephrin signaling, but has been proposed to be an outcome of EphA forward signaling (Depaepe et al., 2005). Activation of apoptotic pathways does seem a logical target for Eph-ephrin signaling, as in

theory, when a migrating cell is given a repulsive cue that it cannot escape, that cell is likely not where it should be and, for the sake of the overall health of the metazoan, should be eliminated. However, a key clue here in eliminating fusion from the equation is the discovery of the EOB phenotype in reverse signaling deficient mice. In the closure of the embryonic eyelid, there is no fusion event, as the epithelial seam just persists until the eyes re-open. If we assume then that the same Eph-ephrin molecules are doing similar things in these different septation/closure events, based on the similarity in the morphology of these events and the expression pattern of these molecules during them, we can rule out failure in fusion as the cause for these embryonic malformations.

That leaves cell-cell adhesion as the remaining potential role for B-ephrin reverse signaling in these different developmental processes. It is entirely consistent with the apparent adhesion defects in these mutant animals and it is consistent with the expression data localizing Eph, ephrin, and activated ephrin to adherent epithelia. Further, it is the conclusion yielded by the *in vitro* palatal shelf fusion assay, the one experiment yet utilized to determine whether the defect in palate development was the result of a failure in fusion or palatal shelf outgrowth. This simple experiment showed that even when you bring these lateral folds into direct contact to one another, and thus bypass the roles for cell proliferation and cell migration in these septation/closure events, midline fusion still fails to occur. Putting these findings together, the conclusion at this point is that ephrin-B reverse signaling is responsible for mediating cell-cell adhesion in these different development processes.

How ephrin-B reverse signaling is not eliciting a cell-cell adhesion response

Having hypothesized that ephrin-B reverse signaling is mediating cell-cell adhesion in these different events, the question then becomes how do these reverse signals produce this outcome. The only previous report of Eph-ephrin involvement in an apparent cell adhesion defect was the finding that EphA7 and ephrin-A5 are necessary for proper closure of the neural tube. In this study the authors found that inhibitory splice variants of *EphA7* were responsible for silencing the normal cell-repulsion outcome from EphA7-ephrin-A5 signaling, permitting cell adhesion to occur (Holmberg et al., 2000). Alternatively, Eph-ephrin signaling can be silenced through *cis* interactions, as ephrin can interact *in cis* with co-expressed Eph receptors through the fibronectin type-III repeats on Eph receptors to form non-functional heterodimers (Carvalho et al., 2006). Could one of these mechanisms explain the defective cell adhesion in the *ephrin-B2*^{lacZ} mice? Is normal ephrin-B2 repulsive signaling typically silenced by some means in the wild-type, which is not occurring in *ephrin-B2*^{lacZ} mutants? The answer appears no. The presence of tyrosine-phosphorylated ephrin-B at the sites of adhesion suggests that Eph-ephrin signaling is fully functional. I therefore think that the cell adhesion mediated by B-subclass ephrins is the result of an active modification of the cytoskeleton, and is not the consequence of inhibiting cell repulsion through EphB molecules.

Another proposed mechanism by which the Ephs and ephrins mediate cell adhesion is that the Ephs and ephrins are themselves adhesion molecules, and the cell repulsion events that they are well characterized in eliciting only occur after the Eph-ephrin heterodimer is either endocytosed or cleaved by a protease. This concept was put forth by two groups that found when endocytosis of Eph-ephrin complexes were inhibited,

cell repulsion events were similarly inhibited and cells remained in contact with one another (Marston et al., 2003; Zimmer et al., 2003). Could the Ephs and ephrins mediate these cell adhesion events by serving as cell adhesion molecules? Again, the answer seems no. Foremost, these molecules do not fit the classic profile of a cell adhesion molecule in that while they regulate actin dynamics, they have not been shown to directly anchor to the actin cytoskeleton. Moreover, if the adhesion response is the result of ectodomain interaction between Eph and ephrin, this interaction has not been disturbed in either *ephrin-B2*^{lacZ} or *EphB2*^{lacZ} mice, so it would not make sense for these mice to present with any of the apparent adhesion defects I have now characterized. Finally, in these initial studies on Eph-ephrin and endocytosis, it was found that expressing truncated forms of Eph or ephrin blocked endocytosis and resulted in failed repulsion in cultured cells, which they generously termed adhesion. That is very clearly quite different from what is occurring with the *ephrin-B2*^{lacZ} and *EphB2*^{lacZ} mice, in which truncated forms of either EphB2 or ephrin-B2 result in failed adhesion, not repulsion. I therefore do not believe the Ephs and the ephrins are themselves functioning as adhesion molecules, nor does it appear endocytosis has a functional consequence in mediating these cell-cell adhesion events.

How B-ephrin reverse signaling could mediate cell-cell adhesion

How then might Eph-ephrin signaling elicit a cell-cell adhesion response? The emerging answer appears to be the same way these molecules elicit cell-cell repulsion, through the differential activation of Rho family GTPases. In cell repulsion, the Ephs and ephrins target these molecules to elicit a localized breakdown of the actin cytoskeleton.

However, it is well known that these same molecules can produce the opposite outcome in creating actin-rich pockets or cytoskeletal extensions. Rac and Cdc42 in particular have long been known to promote the extension of lamellipodia and filopodia when activated, respectively, and recent work suggests that the activation of Rho family members can do the same (Aspenstrom et al., 2004; Ellis and Mellor, 2000; Murphy et al., 1999). The formation of these extensions is not only intuitively antithetical to cell repulsion, but it plays important roles in mediating cell-cell adhesion. The protrusion of filopodia-like structures are found at the leading edge of epithelia in dorsal closure in *Drosophila*, a paradigm for epithelial fusion, and are believed to be important in forming initial cell contacts between adjacent epithelia to form weak adhesions that will mature into more stable adherens junctions (Jacinto et al., 2000). Unsurprisingly, this process is compromised when Rho activity is altered (Jacinto et al., 2002). It is possible then that reverse signaling through ephrin-B2 is necessary for producing these cellular extensions to facilitate adhesion through Rho family regulation, and that defects in this process result in the apparent adhesion defects found in *ephrin-B2*^{lacZ} or *EphB2;EphB3* mutant mice.

The control over whether Rho family activity produces changes in cytoskeletal behavior consistent with either adhesion or repulsion is very delicate, with subtle perturbations in the balance of Rho, Rac, and Cdc42 eliciting outcomes that either favor the formation of filopodia-like protrusions, or call for their retraction. Interestingly, recent work has shown that similarly subtle changes in the activation of Eph-ephrin signaling might achieve the same type of control. In this study, the authors found that retinal explants expressing EphA receptors showed cell-repulsion behavior when exposed to a high level of ephrin-A (and thus presumably receive a strong activation of forward

signals), but showed cell-attraction or adhesion behavior when exposed to lower levels of ephrin-A (and thus presumably receive a weaker activation of forward signaling) (Hansen et al., 2004). The same signaling pathway through the Ephs and ephrins can therefore potentially mediate either adhesion or repulsion based on the intensity of that signal transduction.

This potentially raises some functional relevance to my observation that EphB and ephrin-B are co-expressed at the sites of adhesion in these different developmental processes. The co-expression of Eph and ephrin is not typical; in the majority of systems in which these molecules are used, one encounters distinct populations of cells that express either the Eph or the ephrin molecule, not both molecules (for example, the barrier ligand-like functions of ephrin-B3 in the spinal cord midline and ephrin-B2 at the optic chiasm), and the process appears clearly driven by cell autonomous signaling through only the Eph or through only the ephrin molecule. Most of these processes are also clearly utilizing the repulsive potential of Eph-ephrin signaling as well. In stark contrast, in these midline events, I see an apparent utilization of cell adhesion roles, I see the co-expression of Eph and ephrin in the same cell at sites of adhesion, and in several cases I see that both Eph forward and ephrin reverse signals are at play. This suggests that the co-expression of Eph and ephrin into the same cell may play some functional role in converting a repulsive signal into an adhesive outcome. The aforementioned study by Hansen and co-workers suggests that this might occur through an attenuation of signal strength for these molecules (Hansen et al., 2004). That is, the targeting of Rho family molecules by both forward signaling through the Eph receptors and reverse signaling through the ephrins might yield a balance of Rho, Rac, and Cdc42 activity that yields an adhesive response,

whereas forward signaling or reverse signaling alone would be expected to produce a cell repulsion outcome (Figure 8.1).

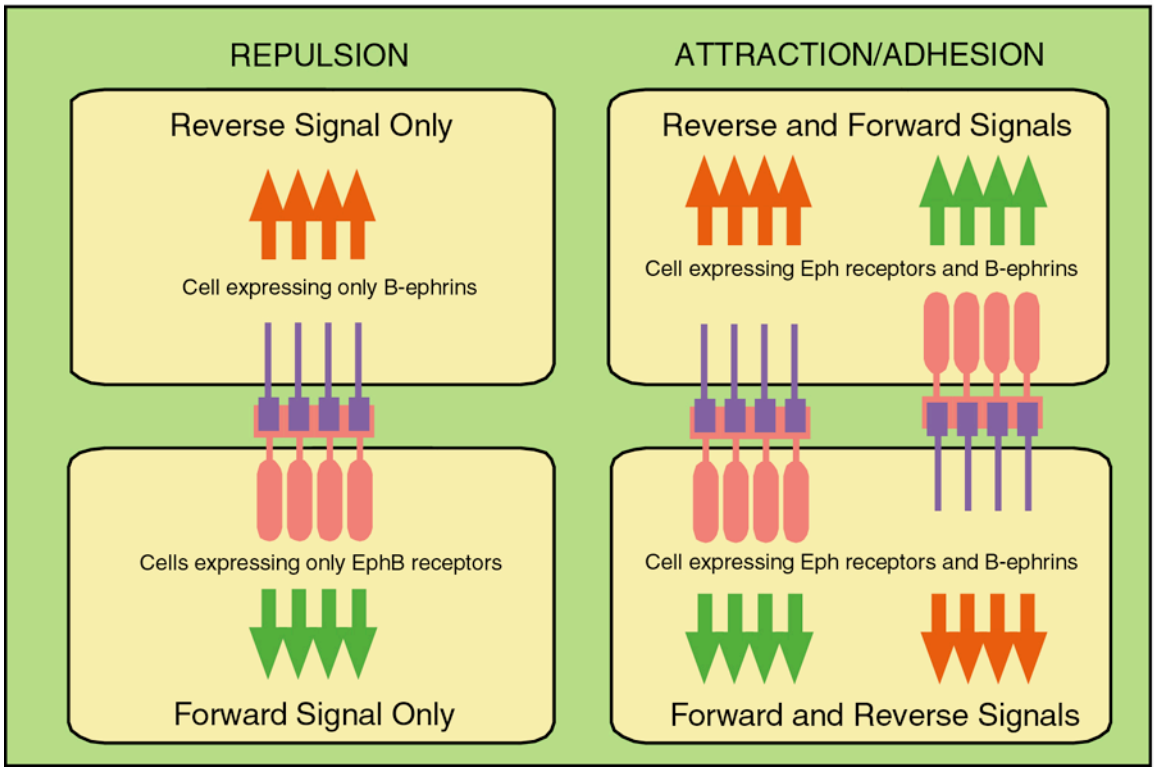


Figure 8.1

Schematic proposing how Eph-ephrin signaling may mediate both repulsion and adhesion. Cells receiving only forward or only reverse signals traditionally produce cell repulsion outcomes, whereas cells receiving both forward and reverse signals may produce a cell-cell adhesion response.

Another possibility is that ephrin-B reverse signaling is playing a more specific structural role at the adherens junction. Adherens junctions (AJ) are rich in actin and actin-binding proteins, and Rho family signaling effectors have been shown to regulate actin polymerization at the AJ, which is key for properly sealing the epithelial seam (Kobielak et al., 2004; Perez-Moreno et al., 2003). Another interesting molecule at the AJ is claudin. The claudins represent a very large family of tetraspan molecules. Classically the claudins have been defined as mediating cell-cell adhesion at tight junctions, where the claudins interact with molecules such as zonula occludens 1 (ZO-1) through PDZ binding interactions (Itoh et al., 1999; Krause et al., 2008). Contrary to this initial characterization however, claudins are not limited to tight junctions alone, but can be found at other sites of adhesion (Angelow et al., 2008). These include expression at the basolateral membrane, where claudin interacts with EpCAM, a panepithelial cell-cell adhesion molecule, and at adherens junctions (Kuhn et al., 2007; Nunes et al., 2006). At these sites claudin serves as a cell adhesion molecule, interacting tightly with other claudins through either hetero- or homophilic interactions in both *cis* and *trans* (Krause et al., 2008). Of note, B-subclass ephrins have been shown to directly interact with claudin-1 and claudin-4. This raises the intriguing possibility that ephrin-B reverse signaling could possess multiple roles in regulating adhesion at the adherens junction, either in working through Rho family molecules to promote localized polymerization of actin at the AJ, or by directly mediating the ability of cell adhesion molecules such as claudin to localize to and function at the AJ.

The Ephs and ephrins are essential midline guidance molecules

One of the emerging themes from my data is the prevalent role for Eph-ephrin signaling in mediating midline development. Previous studies have identified roles for Eph-ephrin signaling in a multitude of axon guidance roles at the midline, including the anterior commissure and corpus callosum (Cowan et al., 2004; Henkemeyer et al., 1996; Mendes et al., 2006; Orioli et al., 1996), the corticospinal tract (Kullander et al., 2001a; Kullander et al., 2001b; Yokoyama et al., 2001), and in RGC at the optic chiasm (Chenaux and Henkemeyer, unpublished; Williams et al., 2003). Further work has defined developmental roles at the midline for the Ephs and the ephrins outside of axon guidance in rib pairing and neural tube closure (Compagni et al., 2003; Holmberg et al., 2000). Here I expand upon this concept by finding several additional roles for Eph-ephrin signaling at the midline in tubularization of the urethra, septation of the cloaca, tracheoesophageal septation of the foregut, fusion of the palate, and ventral body wall closure. It has thus become increasingly clear that these molecules and the signals they transduce are critical mediators of midline development.

Eph-ephrin signaling: a molecular basis for VACTERL association

The VACTERL acronym is used to denote a non-random association of human embryonic malformations. The defining features of VACTERL include vertebral defects (V), anorectal malformations (A), cardiac anomalies (C), tracheoesophageal septation defects (TE), renal dysplasia (R), and limb abnormalities (L) (Temtamy and Miller, 1974). Cases of VACTERL are defined as presenting with two or more of these anomalies, and most of the 1 in 5,000 live births that are associated with VACTERL have only diads or

triads (Rittler et al., 1996). A number of additional congenital anomalies are also observed in VACTERL patients outside of the defining features in the acronym, including hypospadias, cleft palate, neural tube defects, and omphalocele (Botto et al., 1997; Rittler et al., 1996). Evidence has built for a genetic basis behind VACTERL, however a molecular understanding of the association remains rudimentary (Aynaci et al., 1996; Brown et al., 1999; McNeal et al., 1977; Nezarati and McLeod, 1999; Reardon et al., 2001). At present, the only animal models that mimic VACTERL association are mice impaired in Shh signaling (*Shh, Gli2, Gli3* deficient animals) or animals treated *in utero* with the anthracycline antibiotic, adriamycin (Arsic et al., 2002; Kim et al., 2001; Liu and Hutson, 2000).

Here I have presented data that animals with EphB/ephrin-B signaling ablations present with anorectal malformations, LTEC, cleft palate, hypospadias, and omphalocele, which represent two of the defining features of VACTERL as well as three associated anomalies. This data extends upon previous studies demonstrating that Eph/ephrin signaling disruptions also cause cardiac defects (Cowan et al., 2004), neural tube defects (Holmberg et al., 2000), and limb/skeletal abnormalities (Compagni et al., 2003; Davy et al., 2004). I have also noticed a distended kidney phenotype in *ephrin-B2*$^{lacZ/lacZ}$ mice, due to a defect in ureter-bladder integration (unpublished data), and a presumptive role for EphB2/ephrin-B1 in regulating kidney cytoarchitecture has also been reported, so renal development also appears linked to Eph-ephrin signaling (Ogawa et al., 2006). When I couple my data with these previous reports, I find near complete overlap between EphB/ephrin-B malformations and VACTERL association (Table 8.1). This leads me to propose Eph/ephrin signaling as a molecular basis for the VACTERL association.

VACTERL Association Defects	Eph/ephrin involved	Defect	References
Vertebral	*ephrin-B1*	Asymmetric rib attachment	(Compagni et al., 2003)
	ephrin-B2	Abnormal somite patterning	(Davy and Soriano, 2007)
Anorectal	*EphB2, EphB3* *ephrin-B2*	Failed cloacal septation leading to persistent cloaca with GI fistula	(thesis)
Cardiac	*ephrin-B2*	Enlarged cardiac valves	(Cowan et al., 2004)
TracheoEsophageal	*ephrin-B2*	Tracheoesophageal fistula with esophageal atresia	(thesis)
Renal	*ephrin-B2*	*ephrin-B2*$^{lacZ/lacZ}$ embryos present with hydronephrosis presumably due to failed ureter-bladder integration	(unpublished data)
	EphB2, ephrin-B1	Presumptive roles in regulating cytoarchitecture of medullary tubule cells	(Ogawa et al., 2006)
Limb	*ephrin-B1*	polydactyly	(Compagni et al., 2003) (Davy et al., 2004)

Peripheral VACTERL Defects	Eph/ephrin involved	References
Hypospadias	*EphB2, EphB3* *ephrin-B2*	(thesis)
Cleft Palate	*EphB2, EphB3* *ephrin-B1, ephrin-B2*	(Orioli et al., 1996) (Compagni et al., 2003) (Davy et al., 2004) (thesis)
Omphalocele	*EphB2, EphB3* *ephrin-B1*	(Compagni et al., 2003) (Orioli et al., 1996) (thesis)
Neural Tube Closure	*ephrin-A5, EphA7*	(Holmberg at al., 2000)

Table 8.1
Complete overlap between the defining and peripheral defects of VACTERL association with developmental malformations linked to Eph-ephrin signaling.

Alternate signaling avenues for B-subclass ephrin reverse signaling

One of the more unexpected findings from my work is that *ephrin-B2*$^{6YF\Delta V/6YF\Delta V}$ mutants do not phenocopy *ephrin-B2*$^{lacZ/lacZ}$ animals, as *ephrin-B2*$^{6YF\Delta V/6YF\Delta V}$ mice do not present with any apparent defects in urethral tubularization, cloacal septation, tracheoesophageal septation, palate fusion, eyelid closure, or vestibular function. Only when the *ephrin-B2*$^{6YF\Delta V}$ allele is combined with the *ephrin-B2*^{lacZ} allele do defects in these systems become apparent. In the *ephrin-B2*$^{6YF\Delta V}$ mutation, signaling through only the SH2 and PDZ domain interactions of ephrin-B2 are abolished, while the *ephrin-B2*^{lacZ} mutation represents a complete ablation of all potential molecular interactions with the cytoplasmic tail of ephrin-B2 (Figure 8.2). Based on the increased penetrance of defects in the *ephrin-B2*$^{lacZ/6YF\Delta V}$ mutants compared to their *ephrin-B2*$^{lacZ/+}$ littermates, it appears ephrin-B2 reverse signaling through these SH2 and PDZ domain interactions are involved in these midline adhesion events; however, it is a non-essential role, given the results from analysis of *ephrin-B2*$^{6YF\Delta V/6YF\Delta V}$ homozygotes, which do not present with any apparent midline adhesion defects. Given that ephrin-B2 reverse signaling is clearly essential in these midline adhesion events (provided the phenotypes in *ephrin-B2*$^{lacZ/lacZ}$ homozygotes), there must be some other avenue of reverse signaling taking place here that is disrupted in the *ephrin-B2*^{lacZ} mutation but not with the *ephrin-B2*$^{6YF\Delta V}$ mutation (Figure 8.2).

Interestingly, recent work by other groups have identified some novel modifications and protein interaction sites on the ephrin-B cytoplasmic tail that might represent additional signaling avenues for ephrin-B reverse signaling, which could perhaps explain the disconnect between the *ephrin-B2*$^{6YF\Delta V}$ and *ephrin-B2*^{lacZ} alleles. The first of

these to occur was the identification of an interaction between B-subclass ephrins and the claudins (Tanaka et al., 2005b). I have mentioned above the potential intrigue of ephrin-claudin interactions in mediating cell-cell adhesion. The interaction between these molecules has not been well characterized, but is not believed to occur through either SH2 or PDZ domain binding. The interaction of ephrin-B and claudin might therefore occur normally in *ephrin-B2*$^{6YF\Delta V}$ mice, but become disrupted in *ephrin-B2*^{lacZ} mice in an unexplained manner, leading to their spectrum of adhesion defects (Figure 8.2). Ongoing work will be needed to pursue this further (discussed below).

The next candidate for alternate means of ephrin-B signal transduction comes from the focus given to a proline-rich region in the ephrin-B cytoplasmic tail, which could potentially mediate SH3 domain interactions (Figure 8.2). Classically, SH3 domains recognize the consensus sequence of x-P-p-x-P, in which the second and fifth residues are always prolines, and the third residue is sometimes a proline. The ephrin-B2 cytoplasmic tail does contain a M-P-P-Q-S-P sequence that could represent a SH3 domain binding site. Recent work has suggested that Grb4, the adaptor protein that binds to B-ephrin through SH2 domain interactions, can also interact with this proline-rich sequence through its SH3 domain (Segura et al., 2007). Biochemistry from this study demonstrates interaction between the ephrin-B cytoplasmic tail and the SH3 domain of Grb4; further, the authors find that a mutant form of ephrin-B1, in which the conserved tyrosine residues are replaced with phenylalanine, as in the case of the *ephrin-B2*$^{6YF\Delta V}$ allele, can still interact with Grb4 (Segura et al., 2007). In data not shown, the authors also ascribe some functional relevance of this proline rich region, by claiming that the mutation of these prolines to alanines results in impaired dendritic spine formation (Segura et al., 2007).

The final novelty that might impact ephrin-B reverse signaling is the discovery that B-ephrin is not only tyrosine phosphorylated, but becomes serine phosphorylated as well (Figure 8.2). It remains to be seen to what extent this phosphorylation event can mediate signal transduction, but this is certainly another prime candidate to explain the difference between the *ephrin-B2*$^{6YF\Delta V}$ and *ephrin-B2*^{lacZ} phenotypes (Essmann et al., 2008).

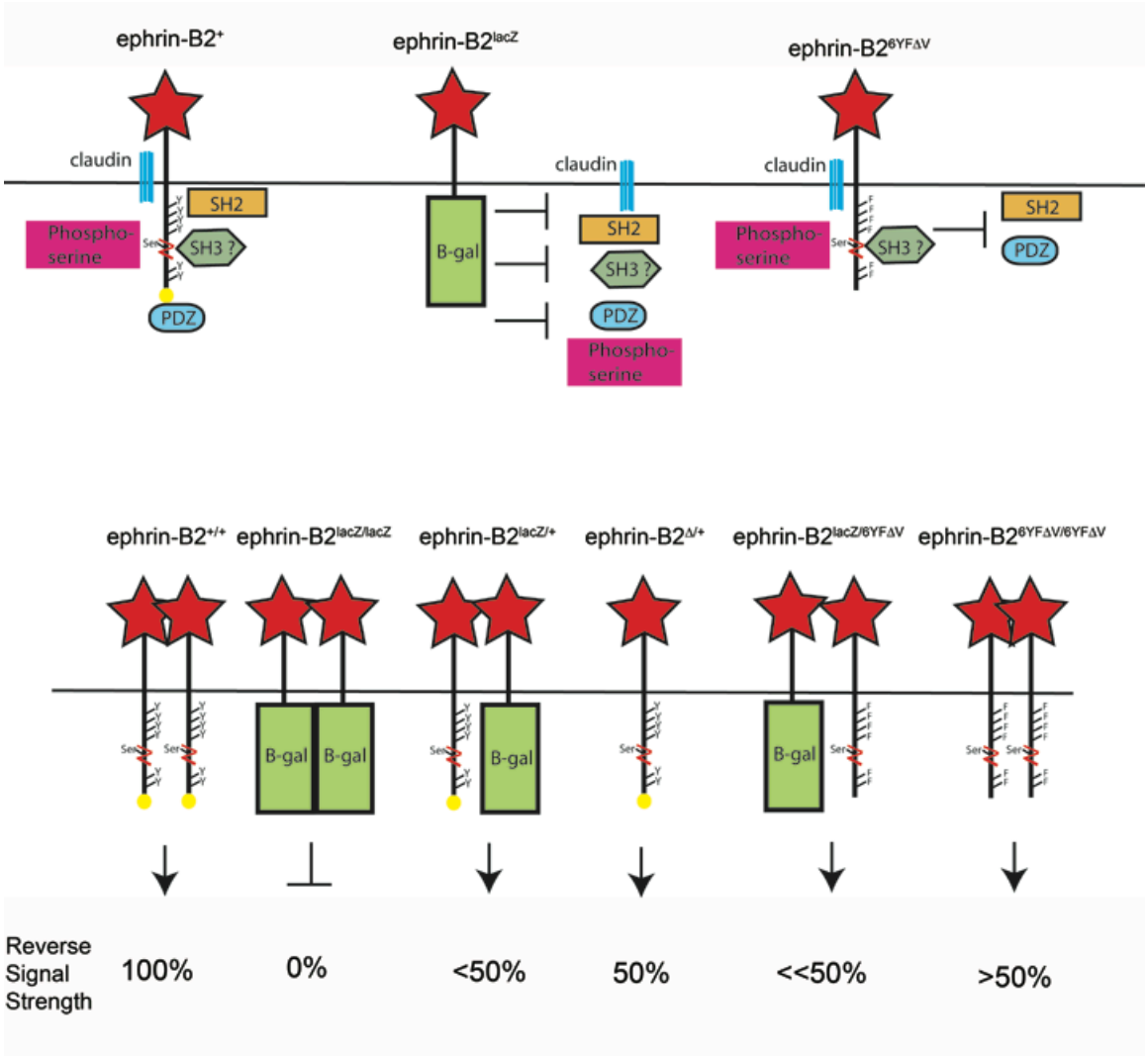


Figure 8.2

Schematic explaining the disparity between *ephrin-B2*^{lacZ} and *ephrin-B2*$^{6YF\Delta V}$ mutant animals. (Top panel) Ephrin-B2^{+} can interact with SH2 and PDZ domain-containing proteins, and can putatively interact with SH3 domain-containing proteins, claudin, and phospho-serine sensitive proteins (left). The ephrin-B2-βgal fusion protein is presumably unable to interact via any of these mechanisms (middle), while the *ephrin-B2*$^{6YF\Delta V}$ allele can not interact with SH2 and PDZ domain-containing proteins**,** but could potentially still interact with SH3 domain-containing proteins, claudin, and phospho-serine sensitive proteins (right). (Bottom panel) The relative strength of ephrin-B2 reverse signaling in mice containing different alleles of ephrin-B2 is estimated, given the presumed differential abilities of *ephrin-B2*$^{+}$, *ephrin-B2*^{lacZ}, and *ephrin-B2*$^{6YF\Delta V}$ to interact with effector molecules shown in the top panel, and given the dominant-negative activity of the ephrin-B2-βgal fusion protein. The estimated reverse signal strength correlates to the penetrance and severity of midline adhesion defects found in the different genotypes.

Mechanistic and molecular insight into hindgut and foregut septation

My studies have helped provide insight into how septation of the foregut into the trachea and esophagus and of the hindgut into the anorectum and urogenital sinus occurs within the developing embryo. The septation of these cavities have been poorly defined developmental events, with controversy extending over how they occur and what goes wrong in their respective malformations (Haraguchi et al., 2001; Nievelstein et al., 1998; Penington and Hutson, 2002a; Penington and Hutson, 2002b; Staack et al., 2003). In particular, it remains a point of debate as to whether the septation occurs through the adhesion of lateral wedges to form a septum, or whether a septum extends distally through directed cell growth and migration. Here my studies suggest that these septation events are driven through adhesion. Histological analysis shows clear points of adhesion in both the foregut and hindgut, where an epithelial seam forms to produce the septum that will partition these cavities. Further, these defects in hindgut and foregut septation present with other embryonic malformations that are clearly based on cell-adhesion, such as the fusion of the palate, ventral body wall closure, and closure of the embryonic eyelid, which further suggests a similar role in cell adhesion for the foregut and hindgut.

As well, it has been gratifying to identify a role for the Ephs and ephrins in these developmental events. The molecular understanding of hindgut and foregut septation has been very primitive to date, with only the identification of more abstract, high order signaling molecules and transcription factors as playing necessary roles in these septation events. In the Ephs and ephrins I have identified molecules that are expressed at the cell surface and play direct roles in mediating the cytoskeletal events that are necessary for these midline fusion events to properly take place. As we now work to expand signaling

pathways further downstream of the Ephs and ephrins, we can continue to build a better understanding of molecularly what needs to happen in these midline events, and hopefully apply that knowledge to a clinical setting to better deal with defects in these processes affecting the human population.

Eph-ephrin signaling controls gut development and maintenance

Another emerging theme from my data is the widespread involvement of signaling between the B-subclass Ephs and ephrins in development of the gut. The maturation of the gut during embryonic development is a remarkable process by which a common endodermal tube is extensively remodeled to produce specialized compartments and organs necessary for a variety of functions that are critical to vertebrate survival. Here my data has indicated that signaling through ephrin-B2 and the Eph receptors EphB2 and EphB3 play important roles in mediating multiple aspects of this development. At the cranial end of the embryo, I show that ephrin-B2 reverse signaling is necessary for proper closure of the palate and septation of the foregut into separate tracheal and esophageal compartments, whereas at the caudal end of the embryo, my data suggests essential roles for ephrin-B2 reverse signaling in closure of the hindgut to septate the cloaca into separate urogenital and anorectal compartments and in tubularization of the urethra to properly exit urine. Further, my data suggests roles for these molecules in gut development even in systems that are not derived from endoderm, as it appears ephrin-B1 reverse signaling is necessary for proper closure of the ventral body wall, which is essential for properly enclosing endodermal visceral organs of the midgut within the body cavity.

The Ephs and ephrins also play important roles in regulating the maintenance of the gut as well. As mentioned earlier, signaling between B-subclass Ephs and ephrins play important roles in mediating intestinal homeostasis, where these molecules are necessary for properly guiding the constant regeneration of intestinal epithelium (Holmberg et al., 2006). Additionally, the Ephs and ephrins have been suggested to play important roles in pancreatic development and maintenance. A-subclass Ephs and ephrins have been suggested to play important roles in regulating insulin secretion in pancreatic β cells, while unpublished work done in collaboration with the laboratory of Ondine Cleaver has suggested that B-subclass Eph-ephrin signaling is necessary for proper development of pancreatic structures, as well (Konstantinova et al., 2007; Villasenor and Cleaver, unpublished). Coupling my data with these other reports, Eph-ephrin signaling appears to possess an extensive involvement in development and maintenance of the gut. This is obviously of interest in furthering our understanding of the maturation and homeostasis of the gut, but is perhaps more important with respect towards understanding the bevy of disease states that are found in endodermally-derived structures. The paradigm that diseases such as cancer arise from the ectopic activation of molecules important in early development suggest that the Ephs and ephrins, molecules now shown to be important in early gut development, should be viewed as candidate molecules involved in different disease states of the gut.

Fidelity of the *ephrin-B2*^{lacZ} allele

One question I would like to address in considering my data is the reliability of the *lacZ* alleles in *EphB2* and *ephrin-B2*, which result in the synthesis of C-terminally

truncated proteins that lack their respective cytoplasmic domains and covalently attach β-gal. This has become a controversial subject given that similar animal models have produced divergent claims about the physiological roles of forward and reverse signaling events. Therefore, I would like to point out that: 1) the ephrin-B2-βgal fusion protein encoded by the *ephrin-B2*^{lacZ} allele localizes properly to the cell surface and clusters into higher order oligomers, unlike other ephrin-B2 cytoplasmic truncations which become trapped in the *trans*-Golgi network and do not traffic to the plasma membrane (Cowan et al., 2004); 2) the success of the *lacZ* alleles to properly signal non-cell autonomously has been demonstrated *in vivo* now several times over, as an *ephrin-B3*^{lacZ} allele rescues *ephrin-B3*$^{\Delta}$ defects in the corticospinal tract, an *ephrin-B2*^{lacZ} allele rescues an *ephrin-B2*$^{\Delta}$ lethality due to vascular morphogenetic failure, and the *EphB2*^{lacZ} allele rescues EphB2$^{\Delta}$ defects found in axon pathfinding, ventral body wall closure, and embryonic eyelid closure (Cowan et al., 2004; Henkemeyer et al., 1996; Mendes et al., 2006; Yokoyama et al., 2001); and 3) the *lacZ* alleles do not hyperactivate adjacent forward or reverse signals, as genetic assays have shown in my data above, and as is suggested by the fact that mice with hyperactive EphB2 forward signals do not present with any of the defects found in either *ephrin-B2*^{lacZ} or *EphB2*^{lacZ} animals (Holmberg et al., 2006). It is also important to emphasize that *EphB2;EphB3* null animals present with the same defects, and that I have now linked specific signaling avenues of ephrin-B2 reverse signaling to these midline adhesion events through analysis of *ephrin-B2*$^{6YF\Delta V}$ animals. I therefore believe the phenotypes seen in either *ephrin-B2*^{lacZ} or *EphB2*^{lacZ} animals represent the loss of cell autonomous signaling via ephrin-B2 or EphB2, respectively.

Chapter 9

Future Directions

Summary

My data has suggested novel physiological roles for B-subclass ephrin reverse signaling in mediating cell-cell adhesion and in regulating ionic homeostasis. In the last chapter I discuss where we go from here by addressing relevant questions stemming from my data and highlighting future experimental directions that I believe would expound upon my data.

In vivo system to screen candidate molecules for Eph-ephrin signal transduction

One of the more disappointing aspects of Eph-ephrin studies is that while many physiological roles for Eph-ephrin signaling have been identified, in most cases our understanding of the signal transduction events in these processes extends no further than

the Ephs and ephrins. That is, when we say, for example, the activation of EphB4 forward signaling by ephrin-B2 mediates vascular morphogenesis, we do not know which molecules interact with EphB4 to produce this effect. The exception to this rule, the identification of α-chimerin as the key mediator of EphA4 forward signaling in CST axon pathfinding (Iwasato et al., 2007), occurred through the fortune of a spontaneous mutation, which can not be realistically utilized as an experimental approach to uncover similar downstream effectors in other physiological processes.

To address this, I have taken the initiative of creating an *in vivo* system to screen candidate molecules for Eph-ephrin signal transduction. In this system, I have taken advantage of the tetracycline-inducible system by generating mice that express the reverse tetracycline transactivator, rtTA2S-M2, under promoter control of particular Ephs and ephrins for which physiological roles are well characterized. This was achieved by using bacterial homologous recombination (recombineering) to insert the rtTA2S-M2 into the open reading frame (ORF) of BACs containing genomic sequences for a particular *Eph* or *ephrin*, and then performing pronuclear injections of fertilized oocytes with this targeted BAC to generate transgenic mice. The fidelity of rtTA2S-M2 activation in Eph- or ephrin-expressing cells can then be verified by crossing BAC-Tg-*Eph*^{rttA} or BAC-Tg-*ephrin*^{rtTA} mice to a TRE-reporter line such as TRE-GFP or TRE-lacZ. In the case of BAC-Tg-*EphB3*^{rtTA}, as shown in Chapter 3, this has worked well. When attempted with BAC-Tg-*ephrin-B2*^{rtTA}, I did not see full recapitulation of ephrin-B2 expression; I therefore performed an alternate plan in which I targeted an *IRES-rtTA2S-M2* cassette into the 3′ UTR of the *ephrin-B2* locus, which properly mimicked endogenous ephrin-B2 expression. As of now, I have rtTA2S-M2-targeted BACs for *ephrin-B1* and *ephrin-B3*,

along with *EphB1*, *EphB3*, *EphB4*, and *EphA4* genomic sequences. BAC-Tg-*EphB1*^{rtTA} and BAC-Tg-*ephrin-B1*^{rtTA} have recently been injected to derive transgenic animals.

The manner in which I am planning to use this system to screen candidate Eph-ephrin signaling molecules *in vivo* is to generate transgenic mice expressing dominant-negative isoforms of these candidate molecules under TRE control. For example, Grb4 is an important candidate for B-ephrin reverse signaling that has yet to show any functional relevance in B-ephrin physiological roles. I will create transgenic mice with a dominant-negative isoform of Grb4 under TRE control, an allele in which I have either deleted the three SH3 domains of Grb4 or mutated these domains with point amino acid substitutions to prevent SH3 domain interactions, so that Grb4 can interact with the ephrin cytoplasmic tail through its SH2 domain, but cannot recruit any of the effector molecules the adaptor protein utilizes to establish a cytoskeletal response. This has already been shown in the Henkemeyer laboratory to act in a dominant-negative fashion to block ephrin-B reverse signals (Cowan and Henkemeyer, 2001; Xu and Henkemeyer, 2009). I will then cross this *TRE-Grb4*^{DN} animal to the *ephrin-B2*^{rtTA} mouse, which now gives me the flexibility to induce the expression of this dominant-negative allele of *Grb4* in ephrin-B2 expressing cells at any given timepoint in embryonic development. I can then induce the expression of dominant-negative Grb4 in ephrin expressing cells over the course of embryonic development when any of a number of developmental events involving ephrin-B reverse signaling take place, such as hindgut and foregut septation (Figure 9.1). I can then easily score these mice for defects in these septation events, which would provide some of the first *in vivo* evidence for functional relevance of Grb4 or any other tested molecule in these different developmental processes. Similar processes could be used to drive the

expression of dominant-negative isoforms for candidates of Eph receptor forward signaling, as well.

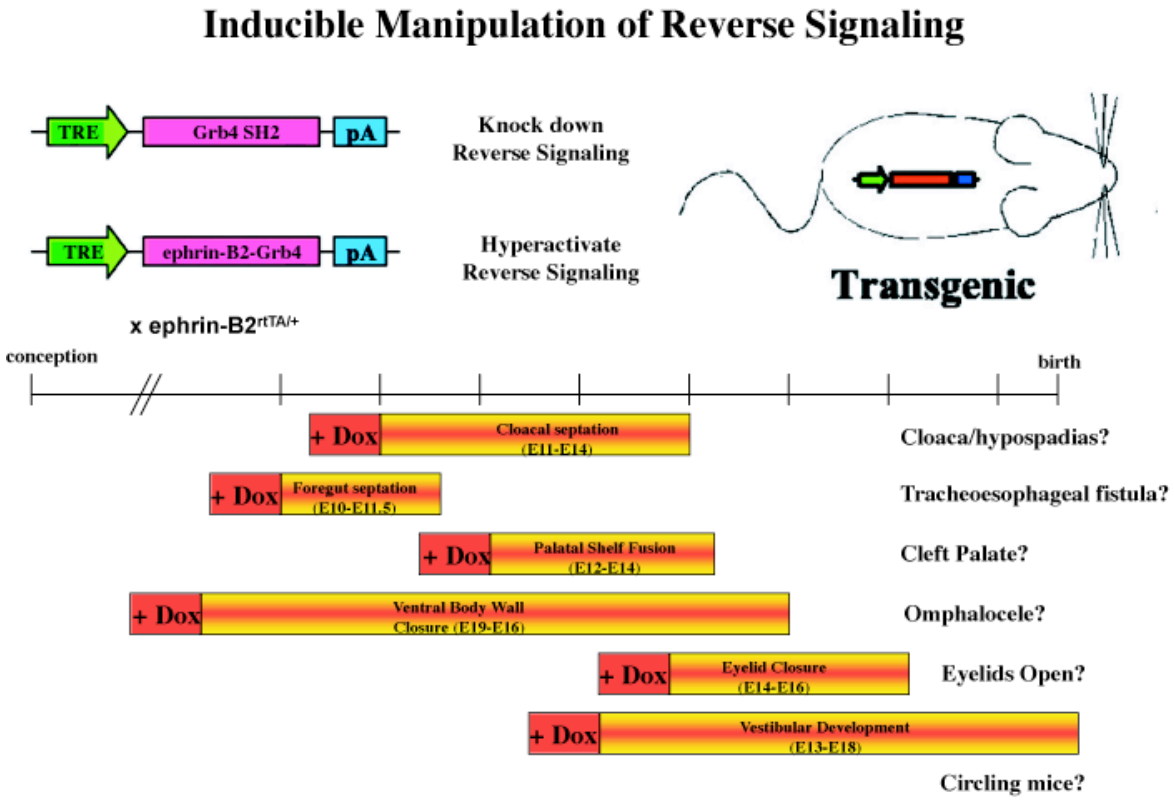


Figure 9.1

Inducible manipulation of reverse signaling. The expression of dominant-negative or hyperactivated alleles of mediators of ephrin-B reverse signaling could be driven using the tetracycline-inducible system over the window of different developmental events herein linked to ephrin-B reverse signaling, to potentially link candidate effectors molecules to ephrin reverse signaling *in vivo*.

This system has additional potential uses as well. This system could be used to transiently rescue phenotypes in null animals, to allow for the identification of novel physiological roles for particular Ephs and ephrins that are masked by embryonic lethality, as with EphB4. In this case, I would express wild-type cDNA for *EphB4* under TRE promoter control, then cross this to BAC-Tg-*EphB4*^{rtTA} animals. Doxycycline (Dox) induction would rescue the embryonic lethality, and after these mice are born the Dox can be removed to cease the rescue and determine what these Ephs and ephrins are doing outside of the embryo. Similarly, this can be repeated with mutated alleles of *EphB4* in which different sites of molecular interaction are disrupted, to determine which protein interactions are necessary for embryonic survival.

There are a number of other applications in which this system could be used, but the final manner in which I would like to use this system is to address the relevance of EphB-ephrin-B co-expression. As theorized above, the co-expression of Eph and ephrin is unique to the sites of adhesion I have characterized, leading to the suggestion that Eph-ephrin co-expression might function to elicit cell-cell adhesion. Here I have a perfect system now to drive ectopic co-expression *in vivo*. I can simply place wild-type *EphB* or *ephrin-B* cDNA under TRE-control, and then cross these mice to either BAC-Tg-*ephrin-B*^{rtTA} or BAC-Tg-*EphB*^{rtTA} animals, respectively. All ephrin-B positive cells will then co-express EphB, and I can then address the relevance of this either by looking for malformations *in vivo*, or isolating these cells in primary cultures to more closely examine changes in cytoskeletal structure *in vitro*.

Discover novel pathways for ephrin-B2 reverse signaling

One of the more surprising finds from my data was that *ephrin-B2*$^{6YF\Delta V}$ mice do not phenocopy *ephrin-B2*^{lacZ} mice, suggesting that B-subclass ephrin reverse signaling is more intricate than just molecular interactions based on SH2 and PDZ domain interactions. Identifying and characterizing these alternate signaling pathways represents a tremendous opportunity to provide novel advancement to the field. The Henkemeyer laboratory has had great success using targeted germline mutations in mice to deduce signaling roles for Eph and ephrin molecules, and that expertise could be easily used to determine the functional relevance of signaling through putative SH3 domain binding and serine phosphorylation *in vivo*. Existing targeting vectors for manipulating ephrin-B2 could be easily modified to create mutations that convert prolines to alanines within the P-P-x-x-P domain of ephrin-B2 to eliminate SH3 domain interactions, either alone, or in concert with the tyrosine to phenylalanine and valine deletion mutations. It would be interesting to determine if mice in which SH2, SH3, and PDZ domain interactions via ephrin-B2 were specifically abolished would phenocopy *ephrin-B2*^{lacZ} mutants. Similarly, phosphorylated serines on the ephrin cytoplasmic tail could be mutated to alanine to determine the functional relevance of serine phosphorylation *in vivo*.

Coincidental with these *in vivo* studies, the Henkemeyer laboratory has also had success using *in vitro* and biochemical assays to piece together the roles of these molecules. A postdoctoral fellow in our laboratory, Nan-Jie Xu, has recently used neurite retraction assays with the neuroblastoma cell line NG-108 to decipher roles for ephrin-B3 reverse signaling (Xu and Henkemeyer, 2009). The same assay could be performed comparing the ability of NG-108 cells to undergo EphB2-Fc induced neurite retraction

when they express either wild-type *ephrin-B2* or mutated alleles of *ephrin-B2* in which SH3 domain binding or serine phosphorylation are compromised. Developing a cell adhesion assay has proven difficult so far, but once created, mutated alleles of ephrin-B2 could be similarly used to explore any roles of SH3 domain binding or serine phosphorylation in mediating cell adhesion.

Similarly, cell assays should be used to determine how this SH3 domain might influence Grb4 interaction with the ephrin-B cytoplasmic tail. Our classical understanding was that Grb4 bound phosphorylated tyrosine residues through SH2 domain interactions, and then used its three SH3 domains to recruit various effector molecules. The identification of the SH3 binding site on ephrin suggests Grb4 can interact with ephrin through one of its SH3 domains as well. This needs to be clarified further. Does Grb4 bind ephrin at the same time through both SH2 and SH3 domain interactions? Are there instances where Grb4 interacts with ephrin through only SH2 or only SH3 domain interactions, and is there functional relevance to this? Key cytoskeletal regulators such as Abi-1, axin, CAP, Dock180, and PAK1 have been shown to bind Grb4 SH3 domains and have been proposed as key mediators of ephrin reverse signaling. Is the interaction between Grb4 and ephrin-B through SH3 domain interaction necessary for their recruitment to Grb4 and ephrin? Might the proline rich region of ephrin compete with the SH3 binding domains of these effector molecules for access to Grb4? These are questions that can be addressed using the same straightforward assays Chad Cowan employed to identify Grb4—simple biochemistry, along with co-transfection experiments using various alleles of ephrin, Grb4, and effector molecules, and looking to see how the recruitment of these molecules into EphB-Fc induced signaling centers occurs.

Explore the role of claudin in ephrin-B2 reverse signaling

As discussed above, the role of claudin as an adhesion molecule, the fact that claudin interacts with B-subclass ephrins, and my discovery that the lack of B-ephrin reverse signaling results in failed cell adhesion, suggests that claudin and ephrin might be working together to mediate these adhesion events. To address this, I plan to perform IF on sections from wild-type and *ephrin-B2*$^{lacZ/lacZ}$ embryos at sites of adhesion, probing with antibodies against claudin and other markers of cell adhesion. If I can demonstrate that the localization of claudin in these *ephrin-B2*$^{lacZ/lacZ}$ embryos is abnormal compared to the wild-type, that is claudin does not get to the cell surface or to AJs, for example, that would further strengthen the link between ephrin and claudin, and suggest that ephrin is either necessary for recruiting claudin to sites of adhesion or plays some role in regulating the cytoskeleton at AJs where claudin becomes localized. Coincidentally, IF looking at adhesion morphology could strength the argument that the defects in *ephrin-B2*$^{lacZ/lacZ}$ mice are the consequence of failed cell adhesion. Finally, I have taken protein lysates from both wild-type and *ephrin-B2*$^{lacZ/lacZ}$ embryos, and plan to perform the same biochemical protein-protein interactions used to identify the interaction of ephrin and claudin, to determine if claudin can interact with the ephrin-B2-βgal fusion protein, as it does wild-type ephrin-B2.

My initial data has already indicated that claudin-1 and claudin-4 are specifically localized along the septating cloaca and the leading edge of the palatal shelf where adhesion will take place, which further raises the anticipation for this future work.

Continue to explore the adhesion defects in ephrin-B2 mutants

Another area of focus is to continue to better characterize what is going wrong in the embryonic malformations I have found in *ephrin-B2*^{lacZ} embryos. It would be great to know what is going awry at the level of the cytoskeleton. I have already mentioned above the need to immunostain wild-type and *ephrin-B2*$^{lacZ/lacZ}$ embryos and look at adhesion and cytoskeletal markers, to determine what ephrin-B2 reverse signaling is doing in the cell at these sites of adhesion. Another strategy is to perform electron microscopy on these adhesion sites. In particular, this could be used to visualize the cytoskeleton of these adhering cells. Do wild-type embryos send out filopodia-like extensions that facilitate adhesion at these sites of septation, as is the case in *Drosophila* dorsal closure, while *ephrin-B2*$^{lacZ/lacZ}$ embryos do not, and might this relate to the failure in adhesion in these mutants? Are there structural deficiencies at AJs in *ephrin-B2*$^{lacZ/lacZ}$ mice? These same IF and EM assays could be performed on *ephrin-B2*$^{\Delta V}$ and *ephrin-B2*$^{6YF\Delta V}$ embryos as well. These mice do not present with any of the defects found in mice, but the presence of midline malformations in *ephrin-B2*$^{lacZ/6YF\Delta V}$ mice suggests important roles through SH2 and PDZ domain interactions. Therefore, there may only be subtle defects in *ephrin-B2*$^{\Delta V}$ and *ephrin-B2*$^{6YF\Delta V}$ mice, which are not sufficient to elicit the full scale failure in midline adhesion found *ephrin-B2*$^{lacZ/lacZ}$ mice, but may be tangible enough to be detected through IF or electron microscopy.

Hyperactivate ephrin-B reverse signaling

To date, my work on trying to identify the roles and mechanisms of ephrin-B2 reverse signaling has focused on the consequences stemming from a loss of this signal

transduction. A counter approach is to hyperactivate ephrin-B2 reverse signaling, and using either *in vivo* or *in vitro* models, analyze what has gone awry to determine functional roles for these reverse signals. However, it is not intuitive as to how to hyperactive ephrin reverse signaling, as these molecules do not possess any catalytic domain. Hyperactivating EphB2 forward signaling on the other hand was relatively straightforward, in producing a mutation that yielded a constitutively active kinase domain. Recently, the Henkemeyer laboratory demonstrated that it is possible to hyperactive ephrin reverse signaling by producing a fusion protein between ephrin-B and the SH3 domains of Grb4 (Xu and Henkemeyer, 2009). This fusion protein mimics the recruitment of Grb4 to ephrin, and so functionally ephrin is constitutively phosphorylated. I have already made a similar fusion protein between Grb4 and ephrin-B2, and it would be of tremendous interest to knock this fusion protein into the *ephrin-B2* locus to produce mice with constitutively active ephrin-B2 reverse signaling. Alternatively, the tetracycline inducible system I have created for ephrin-B2 could be utilized to drive the expression of this fusion protein *in vivo*, as well. This fusion protein could also be transfected into various cell lines, looking for EphB-independent induction of cytoskeletal remodeling events consistent with cell adhesion.

Fill the slack with Rho and Rac

As I have described in detail above, the balance of activity between Rho family members Rho, Rac, and Cdc42 appears to make all the difference in whether a cell extends protrusions or retracts them. Determining how this differential activation of Rho is achieved by Eph-ephrin signaling to elicit repulsive and adhesive behavior remains a

major point of emphasis for me and for the field. In particular, I would like to address the consequence of a cell receiving both forward and reverse signals, as appears to be the case in co-expressing adherent epithelia, in comparison to similar cells receiving only forward or only reverse signals, in terms of the physiological behavior of the cell and the balance of Rho/Rac/Cdc42 activity. If I stimulate an ephrin-expressing cell with EphB-Fc and ephrin-B-Fc to activate reverse signaling, what does the cell do and what is the readout of Rho/Rac/Cdc42? If I then initiate co-expression of Eph, perhaps through a tetracycline-inducible system, and the cell now receives forward and reverse signals what does the cell now do, and again how does the balance of Rho/Rac/Cdc42activity change? This can be addressed using established cell culture lines, or using primary cells and explants from my inducible transgenic mice, and could be very instructive in helping us understanding more precisely the regulation of adhesion vs. repulsion by the Ephs and ephrins.

Deconstructing EphB2 forward signaling

While the focus of my work was initially geared toward uncovering novel physiological roles for ephrin-B2 reverse signaling, my data has also implicated EphB2 forward signaling in these midline adhesion events as well. Just as with the reverse signal, the signaling pathways and molecular interactions mediated by this EphB2 forward signal have not been defined. The Henkemeyer laboratory has attempted to dissect this EphB2 forward signaling by creating several germline mutations in mice that specifically disrupt signaling avenues for the receptor tyrosine kinase. A former postdoctoral fellow in the laboratory, Michael Halford, created an *EphB2*^{K661R} mutation that specifically replaces a lysine residue with arginine to render the catalytic domain of EphB2 inactive; an

EphB2$^{\Delta VEV}$ mutation that specifically deletes three C-terminal residues to eliminate PDZ domain interactions, and an *EphB2*$^{K661R;\Delta VEV}$ mutation that combines both the kinase inactive and PDZ dead mutations. Disappointingly, in the sense that it would have made my studies less complex, homozygous mutants for all three of these mutations do not present with defects in urorectal septation, palatal shelf fusion, or omphalocele, even in an *EphB3*$^{\Delta}$ null background.

Analysis of these mice therefore suggests that EphB2 forward signaling is more complex than just the phosphorylation of effector molecules through the kinase domain of the Eph receptor or through protein interactions via the PDZ binding motif of the receptor. That should not come as a big surprise. PDZ domain interactions do not appear to be a focal part of Eph receptor forward signaling, so the lack of any obvious phenotypes in *EphB2*$^{\Delta VEV}$ mice is understandable. Further, while the kinase activity of the Eph receptor is likely very important in forward signaling, it does not appear to be essential. After all, one of the Eph receptors, EphB6 possesses a functionally inactive kinase domain, yet still participates in a variety of functions (Matsuoka et al., 2005). The kinase domain functions to phosphorylate other Eph receptors and effector molecules; however, effector molecules can also be phosphorylated by Src family kinases, which are activated by ephrin-binding, and Eph receptors can also be phosphorylated either by these same Src family kinases or by other Eph receptors *in trans*. It would seem therefore that there is a contingency plan in effect for when kinase activity is lost, which could explain why *EphB2*$^{K661R;\Delta VEV}$ mice present without a phenotype at the midline.

It should also be stated that these are large molecules, with a number of potential interaction sites that were left untouched in any of these *EphB2* point mutants. Of

particular note, nothing was done to disrupt the phosphorylation of the juxtamembrane tyrosines that are major sources of SH2 domain interaction in all Eph receptors. Interactions through the SAM domain of EphB2 were left unabated, as well. Future work should explore the function of protein interactions through these SH2 and SAM domains. The same genomic sequences that we have used to generate previous *EphB2* mutant mice could be altered to convert these juxtamembrane tyrosines into SH2 dead phenylalanines, or to delete the SAM domain. Producing these mutations either alone or in tandem with the kinase dead and PDZ dead mutations would be very instructive in determining how cell autonomous signals through EphB2 play a number of physiological roles. Further, as EphB2 has clearly defined roles in both midline adhesion and in repulsive behavior, the potential exists to link different signaling avenues with different cytoskeletal responses.

Is cell repulsion the dominant outcome from Eph-ephrin signaling?

My data has demonstrated that Eph-ephrin signaling apparently mediates cell-cell adhesion in a number of different morphogenetic structures during embryonic development, which has challenged the paradigm that signaling through these molecules results in cell repulsion. This then raises the question of whether or not some of these physiological roles that were presumed to be cell-repulsion outcomes from Eph-ephrin signaling are perhaps actually cell adhesion events? The segregation of Eph- and ephrin-expressing cells is a well-defined physiological role for these molecules thought to proceed through cell repulsion. Interestingly though, in epithelial cell lines, this phenomenon has been shown to require E-cadherin, suggesting that perhaps this process requires some combination of cell adhesion as well (Cortina et al., 2007). It might be

worthwhile to then revisit some of the boundary or segmentation processes mediated by Eph-ephrin signaling, such as somite or rhombomere segregation, to determine what role cell adhesion might play in these events. Cadherin has also been shown to regulate dendritic spine morphogenesis, a process the Ephs and ephrins are instrumental in regulating, which has not been historically considered a cell adhesion process but perhaps should be revisited in that light (Togashi et al., 2002).

Focus on candidate Eph-ephrin effector molecules that phenocopy Eph-ephrin mutants

One of the best ways to identify which effector molecules are mediating the forward and reverse signals involved in these midline events is to knockout these potential effectors and look to see if they phenocopy the same defects seen in *EphB2;EphB3* null or *ephrin-B2*^{lacZ} mice. Unfortunately, that is not a realistic proposition for a single laboratory, given the manpower and costs of generating a knockout, and the expansive list of candidate effector molecules for the Ephs and ephrins. Fortunately however, some other laboratories have generated knockout animals for some of these effectors, and interestingly, some do phenocopy our Eph/ephrin mutant animals. As mentioned above, *Rock-I* knockout mice present with EOB and omphalocele. *Abl;Arg* knockout mice present with neural tube defects, while *Abi-2* knockout mice present with defects in eye developmental and spine morphogenesis and is involved in adherens junction formation (Grove et al., 2004; Koleske et al., 1998). GRIP1 binds to the PDZ binding domains of both EphB2 and ephrin-B2, and *Grip1* knockout mice present with Fraser syndrome-like defects, which is associated with defects in eye and urogenital development (Takamiya et

al., 2004). These would therefore seem prudent effector molecules to examine in closer detail in relation to the adhesion defects seen in *EphB2;EphB3* null or *ephrin-B2*^{lacZ} mice. In particular, future work should use IF to determine whether these molecules show any aberrant localization at the sites of adhesion in *ephrin-B2*^{lacZ} mutant animals, and biochemistry should be performed to determine if there is abnormal tyrosine phosphorylation of these molecules, or in the case of Abl and Rock the phosphorylation of their targets, in *ephrin-B2*^{lacZ} mice as well.

Conclusion

Here I have detailed my vision for future work to build upon the discoveries I have outlined in the first seven chapters of this thesis. These include descriptions of novel mouse models that I have either generated or are at various states of creation, and a number of experiments that are either planned or in progress. It is my hope that these ideas, findings, and tools might facilitate ongoing research in the Henkemeyer laboratory, as we strive to build a better understanding for all aspects of Eph-ephrin signaling.

Methods

Chapter 2

Generation of *ephrin-B2*^{lacZ} and *ephrin-B2*^{T} mutations

To construct the targeting vector, a 8.5 Kb BamHI restriction fragment of mouse genomic DNA containing exons 3 (codons 135-170), 4 (codons 170-208) and 5 (codons 208-336) of the *ephrin-B2* gene was modified to create a SalI restriction enzyme site immediately downstream of codon 263, with the resulting 2.6 Kb BamHI-SalI fragment being used as the 5' arm for homologous recombination. A *lacZ* cassette was flanked with *loxP* sequences and then inserted in-frame at the introduced SalI site in the modified *ephrin-B2* exon to delete codons 264-336 and create an ephrin-B2-βgal fusion (with a short peptide linker provided by the SalI-*loxP* sequences: RRHITSYSIHYTKLS). An *frt*-flanked *PGK-neo* cassette for positive selection was inserted 3' of the *loxP-lacZ-loxP* cassette, and a 4.1 kb EcoRI genomic fragment was then added for the 3'arm for homologous recombination. The entire construct was subcloned into pPNT to provide the HSV-*tk* cassette used for negative selection. The targeting vector was electroporated into ES cells and 20 out of 1,632 total cell lines screened exhibited homologous recombination as identified by Southern blot analysis using 5' and 3' external probes (Nagy et al., 1993). Chimeric mice were made from two *ephrin-B2*$^{lacZ/+}$ heterozygous targeted ES cell lines by blastocyst injection and both transmitted the mutation through the germline. The *ephrin-B2*^{T} allele was subsequently obtained by genetic crosses to a mouse, which expresses Cre recombinase in the germline to delete the *lacZ* cassette. This generates a carboxy-terminal truncated form of ephrin-B2 that retains the SalI-*loxP* peptide

linker (RRHITSYSIHYTKL), but does not have β-gal attached. The *ephrin-B2*$^{+}$ and *ephrin-B2*^{lacZ} alleles were genotyped by PCR with three oligonucleotides; EB2-1 (TCTGTCAAGTTCGCTCTGAGG), EB2-2N (CTTGTAGTAAATGTTGGCAGGACT), and LZ (AGGCGATTAAGTTGGGTAACG). EB2-1 + EB2-2N yields the wild-type product of 500 bp, and EB2-1 + LZ yields the *ephrin-B2*^{lacZ} product of 400 bp. The *ephrin-B2*^{T} allele was identified by a SalI digest following PCR using oligonucleotides EB2-1 and EB2-T (GAATTCCTGCAGCCCG), yielding a 300 bp product. The frt-flanked *neomycin* resistance cassette that is inserted at the 3' end of the *ephrin-B*2 gene was not deleted in this study as it did not affect the normal expression of the mutant proteins. Mice used in this analysis were of the 129 inbred background or on mixed backgrounds following backcrossing to C57BL6 or CD1 mice, with no difference in phenotype noted. Males were identified by PCR using oligonucleotides specific for the Y-chromosome. (Nobuhiko Yokoyama)

Biochemical studies

COS-7 cells maintained in Dulbeco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco) were transfected (Fugene-6; Roche) with pCDNA plasmids containing either the full-length *ephrin-B2* cDNA to express wild-type protein or a chimeric *ephrin-B2-lacZ* cDNA to express the same ephrin-B2-βgal fusion protein encoded by the *ephrin-B2*^{lacZ} allele. After 48 hours, cells were split and serum starved overnight in DMEM followed by a 30 minute stimulation with 4 μg/ml of aggregated EphB2-Fc (R&D Systems). Equal amounts of whole-cell protein lysates were separated by SDS-PAGE, transferred to Protran nitrocellulose membranes (Schleicher & Schuell), and then immunoblotted with

antibodies raised against the phosphorylated tail of B-class ephrins (Cell Signaling Technologies), the extracellular domain of ephrin-B2 (biotinylated BAF496, R&D Systems) and the C-terminal domain of B-class ephrins (C-18, Santa Cruz). Immunoblots were visualized using HRP-conjugated secondary antibodies (Jackson Laboratories) and streptavidin-HRP (Pierce) with Super Signal West Dura Substrate (Pierce) and X-Omat film (Kodak). (Michael Chumley)

Immunofluorescence

Embryos were collected in 0.1 M phosphate buffer, fixed at RT for two hours in 4% paraformaldehyde in 0.1 M phosphate buffer, washed 3x20' in cold 0.1M phosphate buffer and then cryoprotected at 4°C in 15% sucrose in 0.1M phosphate buffer for one hour followed by 30% sucrose overnight. Embryos were embedded in OCT, cryosectioned at 14μm and allowed to air dry at RT. Sections were then washed 3x10' at RT in PBS-T (PBS with 0.25% Triton-X100), blocked for 1 hour at RT with 5% normal donkey serum (Jackson ImmunoResearch) in PBS-T and then incubated with primary antibodies diluted in 5% Normal Donkey Serum in PBS-T overnight at 4°C. Sections were returned to RT and washed 3x20' at RT in PBS-T, incubated with species specific secondary antibodies conjugated to Cy2 or Cy3 for 45', washed 3x5' in PBS-T, 1x5' in PBS and then mounted in Aqua Polymount (Polysciences). Primary antibodies used were goat anti-EphB2 (AF467, R&D Systems) and rabbit anti-β-Gal (Cappel). Secondary antibodies were Cy2 donkey anti-goat and Cy3 donkey anti-rabbit (Jackson ImmunoResearch). Fluorescence was visualized using a Zeiss LSM 510 confocal microscope.

X-gal stain of lacZ tissues

Whole-mount embryos were X-gal stained by dissecting the embryos, washing them in 0.1M phosphate buffer, fixing them for 15-45' based on size in lacZ fixative (2% glutaraldehyde, 5mM EGTA pH 7.3, 2mM $MgCl_2$ in 0.1M phosphate buffer), washing them in wash buffer (8mM $MgCl_2$, .08% Nonidet-P40 in 0.1M phosphate buffer) 3 x 5', and staining them in X-gal stain (2.0 mL of 25 mg/mL X-gal, .106 g potassium ferrocyanide, .082 g potassium ferricyanide in 50 mL wash buffer) overnight at 37 °C. The next day, embryos were washed in wash buffer and post-fixed in 4% paraformaldehyde. To obtain X-gal stained sections, some whole-mounts were then paraffin or OCT embedded and sectioned following X-gal staining and post-fixation. In other cases, sections were directly X-gal stained. In these instances, embryos were collected, fixed first in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1M phosphate buffer, and then fixed in lacZ fixative. Embryos were then washed 3 x 5' in 0.1M phosphate buffer, cryoprotected in 15% sucrose for 45' and 30% sucrose overnight at 4 °C, embedded in OCT, and cryosectioned. Cryosections were then washed 3 x 5' in PBS, fixed in 0.2% glutaraldehyde in 0.1M phosphate buffer for 5', washed 2 x 5' in wash buffer, and stained overnight at 37 °C in X-gal stain. The next day, sections were washed 2 x 5' in wash buffer, fixed for 10' in 3% formalin, washed 2 x 5' in PBS, 1 x 5' in H_2O, stained 30 seconds in Nuclear Fast Red, washed 1 x 5' in H_2O, then mounted following ethanol dehydration and xylene clearing.

Generation of *ephrin-B2*$^{\Delta V}$ and *ephrin-B2*^{rtTA} mice

The BAC RP23-328F4, which contains genomic sequences for exon 5 of *ephrin-B2*, was obtained from the BACPAC resources center at the Children's Hospital of Oakland

Research Institute. To create *ephrin-B2*$^{\Delta V}$ germline mutants, exon 5 of *ephrin-B2* in RP23-328F4 was targeted using bacterial homologous recombination in E.coli with a pL452-based minitargeting vector containing ephrin-B2 homology arms of 500 bp in length, in which the three nucleotides encoding the C-terminal valine of ephrin-B2 were deleted directly in front of the stop codon. To create *ephrin-B2*^{rtTA} knock-in animals, exon 5 of *ephrin-B2* in RP23-328F4 was targeted using bacterial homologous recombination in E.coli with a pL452-based minitargeting vector containing ephrin-B2 homology arms of 500 bp in length, in which an *IRES-rtTA-2SM2* cassette was inserted shortly after the stop codon. Targeting vectors were produced by homologous recombination-mediated retrieval into pL254, which carries diphtheria toxin and HSV-TK expression cassettes (Liu et al., 2003). The respective targeting vectors were linearized with a unique AscI site in the pL254 backbone, and the purified DNA was then used to target RI ES cells, as described above. ES cell colonies that were resistant for neomycin and gangcyclovir were screened through Southern blotting to verify proper 5' and 3' integration. Positive ES cell colonies were used to generate chimeric mice as described above. The LoxP-flanked Neo cassette was excised using *Krox-20* Cre recombinase-expressing males.

Chapters 3-6

Animals

The *EphB2 (Henkemeyer et al., 1996)* and *EphB3 (Orioli et al., 1996)* mutations used in this analysis have been described. The *EphB2* and *EphB3* mutations have been maintained on an inbred 129 background and have also been backcrossed to the CD1 strain for eleven generations (~99.95% CD1). The *ephrin-B2*^{lacZ} mutation is maintained on a mixed 129/CD1 background. Induction of the tet-on system was achieved by supplying pregnant females with drinking water containing 0.5mg/ml doxycycline and 5% sucrose for 16 hours.

Generation of BAC-Tg-*EphB3*^{rtTA} and BAC-Tg-*EphB3*^{YFP} mice

BAC recombineering was utilized to generate the BAC-Tg-*EphB3*^{rtTA} and BAC-Tg-*EphB3*^{YFP} mice (Lee et al., 2001). The BAC RP23-213M14 was obtained from the BACPAC resources center at the Children's Hospital of Oakland Research Institute. RP23-213M14 comes from a C57BL/6J-derived library, and contains the start codon for EphB3. RP23-213M14 is 208 kb long, with 42 kb of that sequence 5′ of the EphB3 ORF. The second generation reverse tetracycline transactivator, *rtTA-2SM2,* was targeted into the ORF of RP23-213M14 in the following manner: *rtTA-2SM2* cDNA was cloned into *pL451*, a vector containing an Frt-flanked Neo^{R}/Kan^{R} cassette. At the same time, a 2 kb fragment containing the start codon for *EphB3* was PCR amplified off of RP23-213M14 and cloned into pBluescript (pBS) in the recombination competent DY380 bacterial strain. PCR was then performed on these pl451 based cassettes to generate an *rtTA-2SM2-Frt-*

Neo^{R}/*Kan*^{R}-*Frt* fragment with 50 bp homology arms, which was then used to target *rtTA-2SM2* into the ORF of EphB3 contained in the *pBS-EphB3-2KB* plasmid. The targeted plasmid was then purified, and restriction endonuclease digestion was performed to liberate a fragment containing the *rtTA-2SM2-Frt-Neo*^{R}/*Kan*^{R}-*Frt* cassette flanked by 1kb homology arms. This fragment was then used to target RP23-213M14 in the recombination competent EL250 strain. Candidate colonies showing both kanamycin and chloramphenicol resistance were isolated and screened through PCR. Positive colonies were then induced with L-(+)-arabinose to induce Flipase activity and remove the *Frt-Neo*^{R}/*Kan*^{R}-*Frt* cassette. Positive colonies were then further verified by PCR and sequence analysis to insure proper targeting of the *rtTA-2SM2* into the ORF of EphB3, and by restriction endonuclease analysis to verify the structural integrity of the BAC had not been compromised by the recombineering. The targeted BAC, *EphB3*^{rtTA}, was then used in pronuclear injections from B6D2F1 donor eggs to generate transgenic mice in established protocols. Uptake and transmission of the *EphB3*^{rtTA} allele was confirmed through PCR. Protocols and more detailed descriptions of the plasmids and bacterial strains utilized in recombineering can be found at http://recombineering.ncifcrf.gov/. *EphB3*^{rtTA} here refers to the BAC-Tg-*EphB3*^{rtTA} animal referenced in this thesis. To generate BAC-Tg-*EphB3*^{YFP}, the same strategy was utilized, with the only exception being that cDNA encoding a destabilized variant of eYFP, *pd2EYFP-1* from Clontech, was used in place of *rtTA-2SM2*.

Immunofluorescence

Immunofluorescence on embryonic tissue was performed as described (Chapter 2).

As a phosphotyrosine control sections were treated with λ-protein phosphatase (NEB) for 45' prior to primary application. Primary antibodies used: goat anti-EphB2 (R&D Systems), rabbit anti-β-Gal (Cappel), rabbit anti-phospho-ephrin-B (Cell Signaling Technology), rabbit anti-phospho-ephrin-B [Tyr298] recognizing mouse Y317 (Novus Biologicals), rabbit anti-ephrin-B1 (C-18, Santa Cruz), and rabbit anti-GFP (Molecular Probes). Secondary antibodies were Cy2 conjugated donkey anti-goat, Cy3 conjugated donkey anti-rabbit, and Cy2 conjugated donkey anti-rabbit (Jackson ImmunoResearch). Images were visualized on a Zeiss 510 LSM confocal microscope.

Histochemical staining for expression of β-gal

X-gal and BluO-gal stains for whole mount embryos and tissue sections were performed as described (Chapter 2 Methods) and counterstained with nuclear fast red. For whole-mount analysis, embryos were dehydrated through sequential ethanol washes and cleared in methyl salicylate.

***In vitro* palatal shelf fusion assays**

In vitro palatal shelf fusion assays were performed as described in (Risley et al., 2008).

Chapter 7

Mice

The *EphB2* (Henkemeyer et al., 1996) and *EphB3* (Orioli et al., 1996) mutations used in this analysis have been described. *EphB2* and *EphB3* mutations have been

backcrossed to the CD1 strain for eleven generations (~99.95% CD1) as previously described (Cowan et al., 2000). The *ephrin-B2* results in Table 1 were greater obtained after two or more backcrosses to CD1 (> 75% CD1) and in Table 2 were after four backcrosses (~ 94% CD1). A mouse was scored to have vestibular dysfunction if it was observed to exhibit the classic *Waltzer* continuous head-bobbing and rapid circling locomotion on three separate occasions (approximately 3, 5, and 8 weeks of age) during active periods. No apparent differences were noticed in the degree of vestibular dysfunction between individual animals classified as circling, regardless of genotype. To determine if genetic background and *EphB*/*ephrin-B2* mutations influenced this circling phenotype, Fisher's exact tests where carried out and statistical significance set at $P<0.05$

Immunofluorescence

For immunofluorescent localizations in embryonic ears, animals were collected from timed pregnancies at day E17.5 and decapitated after spending 20 minutes on ice. The skull cavity was pierced with forceps to expose the brain to the fixative, and the heads were fixed at 4 degrees with shaking for 2 hours in 4% paraformaldehyde in phosphate buffer. The heads were next washed 3x 20 minutes in cold phosphate buffer, then cryoprotected by treatment with 15% sucrose in phosphate buffer for 1 hour, and 30% sucrose in phosphate buffer for 36 hours. The heads were then embedded in OCT, and sectioned at 16 microns. Tissue was reacted with anti-ephrin-B at 1:100 (C-18, Santa Cruz), anti-EphB2 at 1:200 (R & D Systems), anti-phospho-ephrinB at 1:500 (Cell Signaling), and anti-βGal (Cappel) antibodies using described methods (previous chapter). For immunolocalizations in adults, the inner ear was dissected out of CO_2-euthanized

mice, perfused briefly with 4% paraformaldehyde, and then post-fixed for 30 minutes at RT. The inner ear was then demineralized by treatment with 10% EDTA for 36 hours, cryoprotected, sectioned, and treated with above antibodies.

Paint filling of inner ears

The method for filling the lumen of the vestibular apparatus with latex paint is described elsewhere (Bissonnette and Fekete, 1996; Morsli et al., 1998). Briefly, newborn mice were harvested and fixed overnight in Bodian's fixative. Specimens were then dehydrated in ethanol and cleared in methyl salicylate prior to injecting the inner ears with 0.1% white latex paint in methyl salicylate into the lumen of the membranous labyrinth. (Doris Wu)

Measurement of endolymphatic [K^+] and utricular potential

The methods for recording K^+ concentration and endolymphatic potential were described previously and conformed to protocols approved by the Institutional Animal Care and Use Committee of Kansas State University (Marcus et al., 2002). Briefly, the bulla of the mouse temporal bone was exposed in anesthetized mice and a small (~30 μm dia.) hole was made in the lateral bony wall of the utricle for insertion of a double-barrel electrode tip into the endolymph. Each microelectrode barrel was connected to an input of a dual channel electrometer and signals were digitized (16-bit) at 480 samples/s and averaged at 0.5 s intervals. Data are expressed as the mean ± S.E.M. (n = number of ears) and analyzed for significance ($P < 0.05$) with one-way ANOVA and Dunnett's post test. (Tao Wu and Daniel Marcus)

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