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Ephrin-B2 reverse signaling is required for axon pathfinding and cardiac valve formation but not early vascular development

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Abstract

Vascular development begins with the formation of a primary vascular plexus that is rapidly remodeled by angiogenesis into the interconnected branched patterns characteristic of mature vasculature. Several receptor tyrosine kinases and their ligands have been implicated to control early development of the vascular system. These include the vascular endothelial growth factor receptors (VEGFR-1 and VEGFR-2) that bind VEGF, the Tie-1 and Tie-2 receptors that bind the angiopoietins, and the EphB4 receptor that binds the membrane-anchored ligand ephrin-B2. Targeted mutations in the mouse germline have revealed essential functions for these molecules in vascular development. In particular, protein-null mutations that delete either EphB4 or ephrin-B2 from the mouse have been shown to result in early embryonic lethality due to failed angiogenic remodeling. The venous expression of EphB4 and arterial expression of ephrin-B2 has lead to the speculation that the interaction of these two molecules leads to bidirectional signaling into both the receptor-expressing cell and the ligand-expressing cell, and that both forward and reverse signals are required for proper development of blood vessels in the embryo. Indeed, targeted removal of the ephrin-B2 carboxy-terminal cytoplasmic tail by another group was shown to perturb vascular development and result in the same early embryonic lethality as the null mutation, leading the authors to propose that ephrin-B2 reverse signaling directs early angiogenic remodeling of the primary vascular plexus [Cell 104 (2001) 57]. However, we show here that the carboxy-terminal cytoplasmic domain of ephrin-B2, and hence reverse signaling, is not required during early vascular development, but it is necessary for neonatal survival and functions later in cardiovascular development in the maturation of cardiac valve leaflets. We further show that ephrin-B2 reverse signaling is required for the pathfinding of axons that form the posterior tract of the anterior commissure. Our results thus indicate that ephrin-B2 functions in the early embryo as a typical instructive ligand to stimulate EphB4 receptor forward signaling during angiogenic remodeling and that later in embryonic development ephrin-B2 functions as a receptor to transduce reverse signals involved in cardiac valve maturation and axon pathfinding.
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Introduction

The large family of Eph receptor tyrosine kinases and their membrane-anchored ephrin ligands have been implicated to control a diverse array of cell movements by regulating repulsion and adhesion events such as those involved in axon guidance and cell migration (Cowan and Henkemeyer, 2002; Frisen et al., 1999; Gale and Yancopoulos, 1999; Holmberg and Frisen, 2002; Kullander and Klein, 2002; Wilkinson, 2001). They accomplish this by transducing bidirectional tyrosine kinase-mediated signals

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that are propagated into both the receptor-expressing cell and the ligand-expressing cell in what has been termed forward and reverse signaling, respectively (Cowan and Henkemeyer, 2002). Thus, Eph molecules are thought to act as both receptors to transduce cell autonomous forward signals into their cell and as ligands to stimulate noncell autonomous reverse signaling into adjacent ephrin-expressing cells. Likewise, the ephrins are thought to function as ligands to stimulate noncell autonomous forward signaling in adjacent Eph-expressing cells and as receptors to transduce cell autonomous reverse signals into their cell.

Development of the vascular system begins with the formation of a primary vascular plexus that is rapidly remodeled by angiogenesis into the interconnected branched patterns characteristic of mature blood vessels (Risau, 1997; Risau and Flamme, 1995). A number of different receptor tyrosine kinase families and their ligands have been implicated to play important roles in the early development of blood vessels and formation of the cardiovascular system (Adams and Klein, 2000; Flamme et al., 1997; Gale and Yancopoulos, 1999; Merenmies et al., 1997; Risau, 1997; Tallquist et al., 1999; Yancopoulos et al., 2000). Two key players are EphB4, which is specifically expressed in veins, and ephrin-B2, which is specifically expressed in arteries and the perivascular mesenchyme (Adams et al., 1999; Gale et al., 2001; Gerety et al., 1999; Wang et al., 1998). Prior studies determined that targeted, protein-null alleles of murine *EphB4* or *ephrin-B2* lead to early embryonic lethality due to a complete arrest of angiogenesis and failed cardiovascular development (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). Additional genetic studies in mice determined that the arterial expression of ephrin-B2 is essential for its function in angiogenesis (Gerety and Anderson, 2002).

Given the restricted expression of EphB4 to veins and ephrin-B2 to arteries, it has been tempting to speculate that the roles for these two receptor-ligand pairs in the cardiovascular system is to regulate blood vessel development by transducing bidirectional tyrosine kinase signals into both the veins (forward signals) and the arteries (reverse signals) as these two cell types come in contact with each other (Adams, 2002; Adams, 2003; Adams and Klein, 2000; Augustin and Reiss, 2003; Cheng et al., 2002; Gale and Yancopoulos, 1999; Yancopoulos et al., 2000). Consistent with the idea that ephrin-B2 can function as a receptor to transduce important reverse signals into arteries, it was reported that truncation of the majority of the ephrin-B2 carboxy-terminal cytoplasmic tail results in the same embryonic lethality as the null allele, leading the authors to conclude that reverse signals stemming from this ephrin are important for early cardiovascular development (Adams et al., 2001). We have also generated two targeted mutations in mice that delete the cytoplasmic domain of ephrin-B2 (Dravis et al., 2004). Both mutations delete the same cytoplasmic residues as the Adams et al. (2001) study (amino acids 264–336), with one allele conjugating beta-

galactosidase (βgal) to the truncated tail (the ephrin-B2– βgal fusion protein) and the other allele producing an unconjugated truncation (the ephrin-B2-ΔC protein) similar to the previous study, which added a short HA epitope tag to the truncated ephrin-B2 molecule (ephrin-B2-ΔC-HA). Although we find that our ephrin-B2-ΔC protein results in the same early embryonic lethality with severe defects in vascular development as Adams et al. (2001) found for their ephrin-B2-ΔC-HA protein, we find that the ephrin-B2–βgal fusion protein allows for normal angiogenesis, full embryonic development, and the birth of live pups. The ability of the ephrin-B2–βgal fusion protein to produce fully developed embryos that are live born demonstrates that the carboxy-terminal cytoplasmic domain of ephrin-B2 is not essential for vascular development and indicates that reverse signaling is dispensable for angiogenesis. Consistent with this functional data, we find that our truncated ephrin-B2-ΔC protein, and likely the ephrin-B2-ΔC-HA protein reported by Adams et al. (2001), does not traffic to the plasma membrane but rather becomes trapped in the trans-Golgi network and thus acts essentially like a protein-null. As the ephrin-B2–βgal fusion protein does traffic to the cell surface, it can function as a proper ligand to bind EphB receptors and stimulate forward signaling.

Methods

Mice

Mice carrying mutations in the *ephrin-B2* gene are described in the accompanying article (Dravis et al., 2004). X-gal staining of *ephrin-B2*[lacZ] animals to detect expression of the ephrin-B2–βgal fusion protein is described elsewhere (Henkemeyer et al., 1996).

Flow cytometry

Mouse embryo fibroblasts were isolated at E9 from *ephrin-B2*^{+/+}, *ephrin-B2*[lacZ/+], *ephrin-B2*[lacZ/lacZ], *ephrin-B2*[T/+], and *ephrin-B2*[T/T] animals using 0.05% trypsin and 1 mg/ml collagenase at 37°C for 2–5 min with titration. Cell suspensions were washed and expanded in vitro in DMEM supplemented with 15% fetal bovine serum, penicillin or streptomycin, L-glutamine, and nonessential amino acids. Following expansion, fibroblasts were isolated with 0.5 mM EDTA/0.5 mM EGTA, washed with staining buffer (PBS + 2% BSA + 0.2% sodium azide), and incubated for 1 h at 4°C with rabbit anti-ephrin-B2 extracellular domain antibodies (P-20 or H-83, Santa Cruz Biotechnology, Santa Cruz, CA). Cells were then washed with staining buffer and incubated with biotinylated goat anti-rabbit IgG(H+L) secondary antibody (Caltag, Burlingame, CA) for 45 min at 4°C. Finally, cells were washed with staining buffer and incubated for 30 min with streptavidin-PE (Caltag). Control stains were performed always and included secondary

antibody and streptavidin-PE but excluded the primary antibody for ephrin-B2. Cell surface expression of ephrin-B2 was analyzed on a FACScan (BD Biosciences, San Jose, CA). Forward and side scatter gates were adjusted to include only nucleated cells. Postexperiment analysis was performed using CellQuest Pro software (BD Biosciences).

Immunofluorescence

Mouse embryo yolk sacs isolated at E10 from *ephrin-B2*$^{+/+}$, *ephrin-B2*$^{lacZ/+}$, *ephrin-B2*$^{lacZ/lacZ}$, *ephrin-B2*$^{T/+}$, and *ephrin-B2*$^{T/T}$ animals were fixed in 4% paraformaldehyde in PBS for 30 min at 4°C, washed in PBS, and then blocked in PBS containing 0.1% Tween-20, 2% BSA, and 1% normal goat serum. Yolk sacs were incubated with goat anti-mouse ephrin-B2 extracellular domain antibodies (AF496, R&D Systems, Minneapolis, MN), rat anti-mouse PECAM-1 (CD31) monoclonal antibodies (clone MEC13.3, BD Biosciences), and either mouse anti-Syntaxin 6 monoclonal antibodies (clone 3D10/VAM-SV025, StressGen Biotechnologies, Victoria, BC, Canada), mouse anti-Membrin monoclonal antibodies (clone 4HAD6/VAM-PT046, StressGen Biotechnologies), or rabbit anti-Calnexin polyclonal antibodies (SPA-860, StressGen Biotechnologies). Yolk sacs were then washed extensively in PBS, incubated with appropriate fluorescent-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA), washed, mounted, and images were acquired with a confocal microscope (Zeiss LSM 510) and processed with Adobe Photoshop.

Horizontal cryosections bisecting the anterior commissure of *ephrin-B2*$^{+/+}$ wild-type and *ephrin-B2*$^{lacZ/lacZ}$ mutant brains collected at E18 were fixed in 4% paraformaldehyde in PBS for 30 min at 4°C, washed in PBS, and then blocked in PBS containing 0.1% Tween-20, 2% BSA, and 1% normal goat serum. Brain sections were incubated with Tag1 and L1 antibodies (Developmental Studies Hybridoma Bank, University of Iowa), washed, incubated with appropriate fluorescent-conjugated secondary antibodies (Jackson ImmunoResearch), washed, mounted, and images were acquired with a confocal microscope (Zeiss LSM 510) and processed with Adobe Photoshop.

Results and discussion

Ephrin-B2$^{lacZ/lacZ}$ *homozygotes survive embryonic development and are live born*

Homologous recombination in murine embryonic stem cells was used to target the *ephrin-B2* gene (see Dravis et al., 2004). 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 an ephrin-B2-βgal fusion protein. By specifically deleting only the cytoplasmic domain, the ephrin-B2-

βgal protein will traffic to the membrane (see below) and interact with Eph receptors on adjacent cells to activate forward signaling, but it will not be able to interact with intracellular SH2 and PDZ domain-containing proteins, and is therefore unable to participate in reverse signaling. The second mutation, termed *ephrin-B2*^{T}, produces a protein (termed ephrin-B2-ΔC) that truncates the cytoplasmic domain of ephrin-B2 at the same residue 264 as the *ephrin-B2*^{lacZ} allele; however, it does not have β-gal attached to the tail.

When *ephrin-B2*$^{T/+}$ heterozygous mice were intercrossed, no *ephrin-B2*$^{T/T}$ homozygous animals were observed at birth. Further analysis of the lethal phenotype at early gestation stages (E9 and E10 days development) revealed that *ephrin-B2*$^{T/T}$ homozygotes exhibited a failure in cardiovascular development (Table 1). Mutant embryos were smaller, had enlarged pericardia, and displayed abnormal heart and vascular structures, consistent with disrupted angiogenic remodeling of the primary capillary plexus (Fig. 1A). Similar remodeling defects were also observed in the blood vessels within the yolk sacs of the *ephrin-B2*$^{T/T}$ homozygotes (Fig. 2G). These results are identical to what has been reported for *ephrin-B2* protein-null mice (Adams et al., 1999; Wang et al., 1998) and animals harboring the truncation mutation in *ephrin-B2* described by Adams et al. (2001).

However, quite surprisingly, when *ephrin-B2*$^{lacZ/+}$ heterozygous animals were intercrossed, live *ephrin-B2*$^{lacZ/lacZ}$ homozygous mutant newborns were observed, although they died within the first day (Table 1). Analysis throughout gestation (E9 until birth) revealed that *ephrin-B2*$^{lacZ/lacZ}$

Table 1
Ephrin-B2$^{lacZ/lacZ}$ homozygotes survive embryonic development

Cross	Stage	+/+	+/−	−/−
ephrin-B2$^{T/+}$ × *ephrin-B2*$^{T/+}$	E9	10	20	8[a]
	E10	25	46	21[a]
ephrin-B2$^{lacZ/+}$ × *ephrin-B2*$^{lacZ/+}$	E9	3	9	3
	E10	23	30	11
	E11	30	56	13
	E12	17	43	13
	E13–E18	105	178	43
	neonate	11	12	5[b]
	adult	153	170	0

Fertile, heterozygous males and females of the indicated genotypes were mated and resulting offspring were scored at the various embryonic, neonatal, and adult stages. Note that only approximately 50% of the expected number of *ephrin-B2*$^{lacZ/lacZ}$ homozygous embryos were recovered when compared to the numbers of *ephrin-B2*$^{lacZ/+}$ and *ephrin-B2*$^{+/+}$ embryos obtained. This is because approximately 50% of the *ephrin-B2*$^{lacZ/lacZ}$ homozygotes exhibit peri-implantation lethality that may be due to defects in placentation.
[a] All *ephrin-B2*$^{T/T}$ homozygotes collected were visibly abnormal, appearing growth-retarded and necrotic. All showed obvious defects in both embryonic (cardiovascular) and extraembryonic (yolk sac) blood vessels. No viable *ephrin-B2*$^{T/T}$ homozygotes were present at later stages of development.
[b] The *ephrin-B2*$^{lacZ/lacZ}$ homozygotes were born alive and started breathing; however, they expired within 24 h.
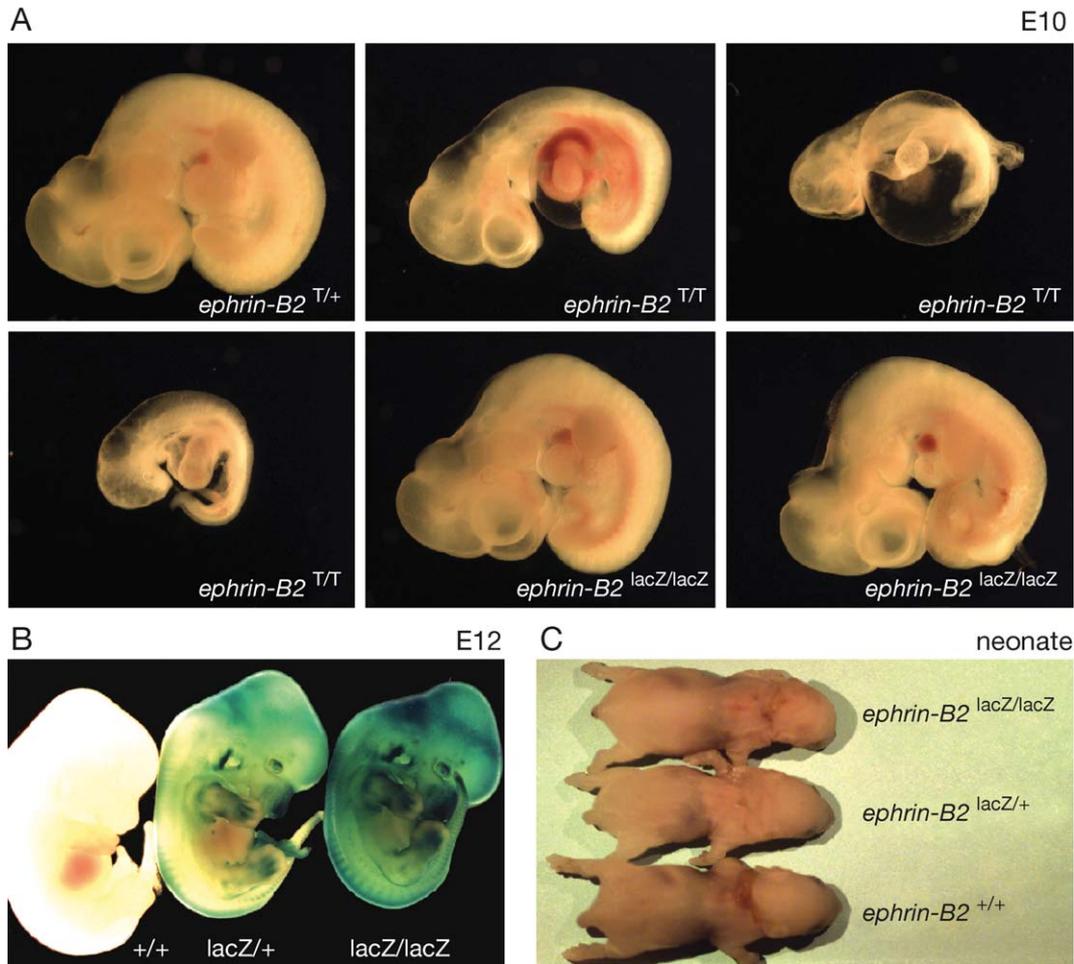
Fig. 1. The ephrin-B2 carboxy-terminus is not required for embryonic development. (A) Whole-mount embryos of the indicated genotypes collected at E10 show that *ephrin-B2*^T/T^ homozygotes expressing a truncated, unconjugated ephrin-B2 display delayed development and embryonic lethality due to defective cardiovascular development. In contrast, *ephrin-B2*^lacZ/lacZ^ homozygotes that express a similarly truncated ephrin-B2–βgal fusion protein appear normal. (B) Whole-mount embryos stained at E12 with X-gal to reveal expression of the ephrin-B2-βgal fusion protein. The *ephrin-B2*^lacZ/lacZ^ homozygote appears normal and, as expected, stains twice as strongly as the *ephrin-B2*^lacZ/+^ heterozygote. (C) The *ephrin-B2*^lacZ/lacZ^ homozygotes survive embryonic development and are live born.

homozygotes were grossly normal and indistinguishable from wild-type littermates (Fig. 1). Importantly, these animals exhibited none of the early vascular defects associated with the truncated *ephrin-B2*^T^ allele (Fig. 2). The observation of live-born *ephrin-B2*^lacZ/lacZ^ mutants indicates the cytoplasmic domain of ephrin-B2 is not required for early vascular development or embryonic survival as previously proposed (Adams et al., 2001), and suggests the truncated protein produced by the *ephrin-B2*^T^ allele is nonfunctional.

The ephrin-B2-βgal fusion protein is localized to the plasma membrane but the ephrin-B2-ΔC protein is trapped in the trans-Golgi network

To gain insight into the observed phenotypic differences between the *ephrin-B2*^T^ and *ephrin-B2*^lacZ^ alleles, we performed a series of experiments to characterize the gene products of the two mutant alleles. We analyzed the cell-surface expression of the various ephrin-B2 proteins on murine embryo fibroblasts derived from E9 wild-type, *ephrin-B2*^lacZ^, and *ephrin-B2*^T^ heterozygous and homozygous animals by fluorescence-activated cell sorting using antibodies raised against the extracellular region of ephrin-B2 (Fig. 3A). This analysis revealed that 9.5% of the wild-type cells were positive for cell-surface ephrin-B2. Similarly, cell-surface ephrin-B2 protein was observed in 14.3% of the *ephrin-B2*^lacZ/+^ heterozygous cells, 16.6% of the *ephrin-B2*^lacZ/lacZ^ homozygous cells, and 12.5% of the *ephrin-B2*^T/+^ cells. In stark contrast, the cells from *ephrin-B2*^T/T^ homozygous embryos showed no detectable cell-surface ephrin-B2 protein. These results indicate that the truncated ephrin-B2-ΔC protein does not become properly localized to the cell surface. The higher percentage of the ephrin-B2-βgal fusion protein on the cell surface may be due to an increase in protein stability conferred by the β-gal moiety and/or a reduction in protein turnover.
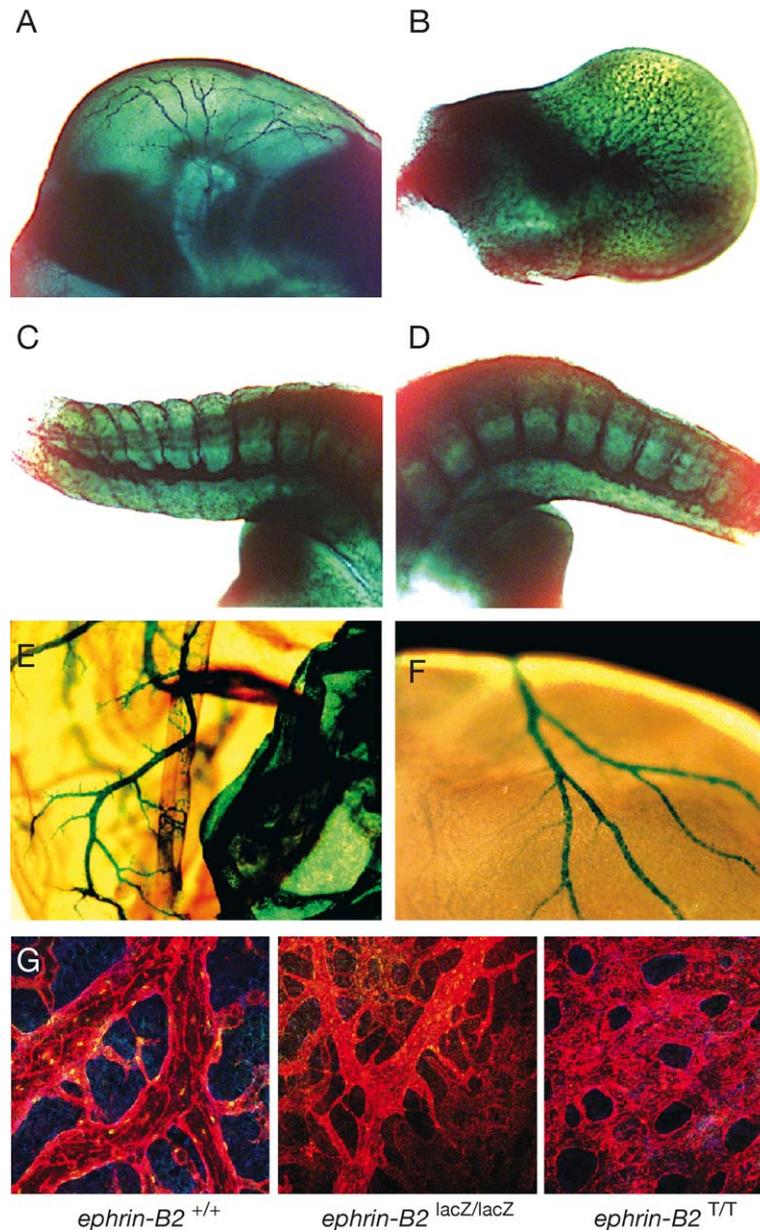
Fig. 2. The ephrin-B2 carboxy-terminus is not required for blood vessel development. (A–F) Whole mounts of *ephrin-B2*^lacZ/lacZ homozygotes collected at E11 (A to E) or E12 (F) and stained with X-gal reveals strong expression of the ephrin-B2-βgal fusion protein in arteries. Note normal branching of blood vessels forming the carotid artery in the brain (A), blood vessels in the limb bud (B), in the dorsal aorta and its intersegmental branches (C and D), and in the yolk sac (E and F). (G) Yolk sacs collected from the indicated genotypes at E10 were stained with anti-PECAM antibodies to reveal blood vessel architecture. The *ephrin-B2*^T/T homozygotes show an abnormal primitive honeycombed organization of similar-sized vessels. In contrast, blood vessels in the *ephrin-B2*^lacZ/lacZ mutant yolk sac appear normal and consist of both large and small vessels.

We also carried out whole-mount immunofluorescence analysis of E10 yolk sacs from wild-type, *ephrin-B2*^lacZ, and *ephrin-B2*^T embryos using antibodies raised against the extracellular region of ephrin-B2. Expression of each gene product was detected in PECAM positive yolk sac endothelial cells (Figs. 3B–D). Wild-type ephrin-B2 protein was observed in small discrete spots on the plasma membrane of these cells (Fig. 3B). Similarly, the ephrin-B2-βgal fusion protein was also observed in distinct spots on the cell surface (Fig. 3C). Intriguingly, the ephrin-B2-ΔC

protein did not cluster to spots on the plasma membrane, but rather appeared to reside in irregularly shaped intracellular structures (Fig. 3D). Biochemical analysis of the previously reported truncated ephrin-B2-ΔC-HA protein showed that this molecule could be precipitated by wheat-germ agglutinin due to posttranslational covalent addition of carbohydrate moieties to the extracellular portion of the protein (Adams et al., 2001). As carbohydrates are added to proteins in the endoplasmic reticulum (ER) and Golgi apparatus of cells, it seemed likely that the
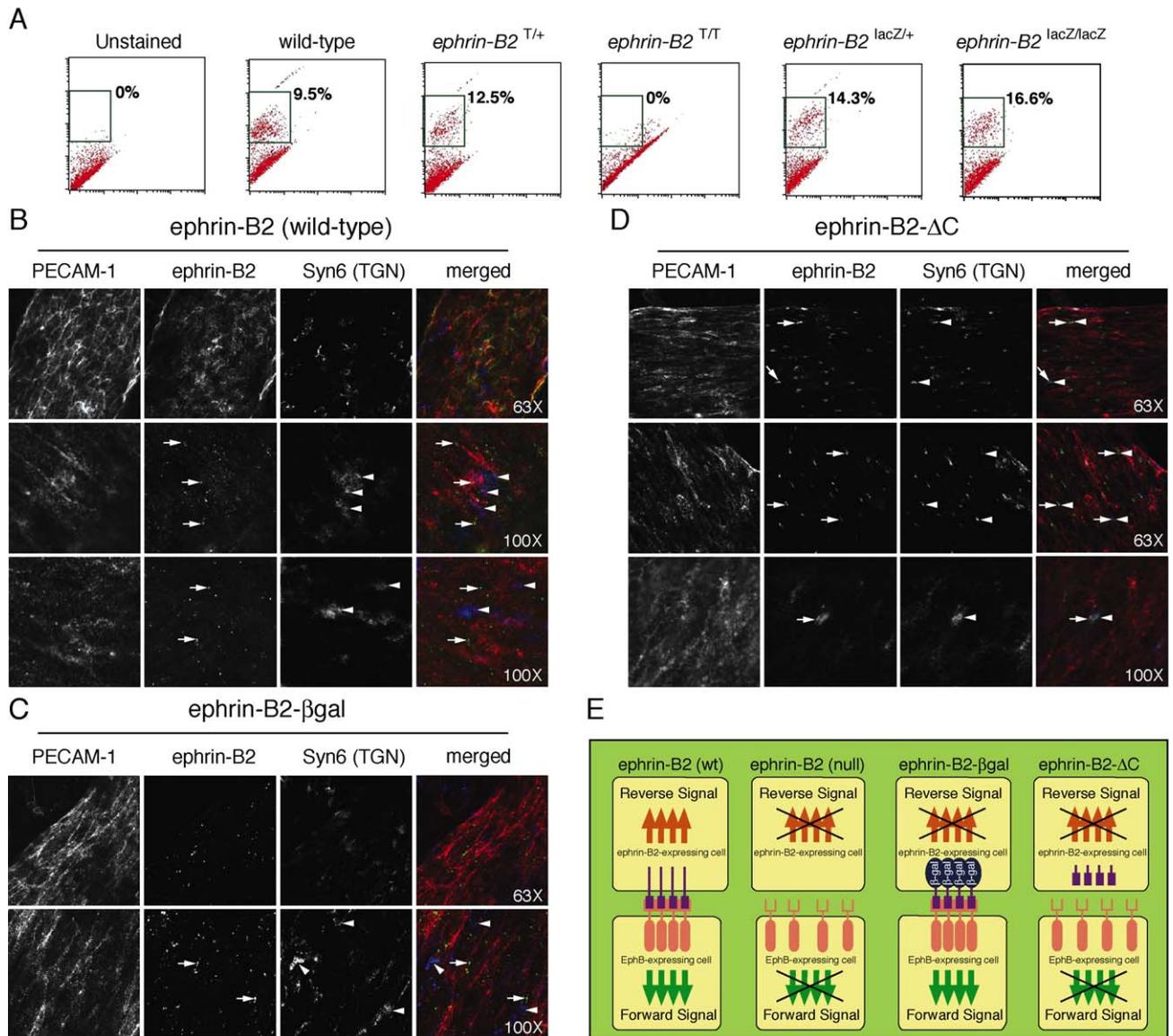
Fig. 3. The truncated ephrin-B2-ΔC protein is not localized to the plasma membrane. (A) Flow cytometry was used to measure the surface expression of ephrin-B2 protein in embryonic fibroblasts obtained from the indicated genotypes. Shown is a representative example of experiments using four different cell lines for each genotype class and two different antibodies directed against the ephrin-B2 extracellular domain. Note that the *ephrin-B2*[T/T] homozygote cells show only background levels of cell-surface ephrin-B2 (compare to the unstained control where the primary antibody was omitted from wild-type cells). All other genotypes showed strong expression of ephrin-B2 protein on the cell surface, including the *ephrin-B2*[lacZ/lacZ] homozygotes. (B–E) An antibody raised against the extracellular domain of ephrin-B2 was used along with anti-PECAM-1 (blood vessels) and anti-Syn6 (trans-Golgi network) antibodies to triple label yolk sacs from *ephrin-B2*^{+/+} wild-type (B), *ephrin-B2*[lacZ/lacZ] homozygote (C), and *ephrin-B2*[T/T] homozygote (D) embryos collected at E10. The *ephrin-B2*[lacZ]-encoded ephrin-B2-βgal fusion protein shows normal localization to distinct spots on the surface of endothelial cells just like what is observed for the wild-type protein, while the truncated, unconjugated ephrin-B2-ΔC protein encoded by the *ephrin-B2*[T] allele is trapped in the trans-Golgi network. Note in the merged images the complete overlap of the ephrin-B2 staining (arrows) with the Syn6 marker for the trans-Golgi network (arrowheads) in the *ephrin-B2*[T/T] homozygotes. (E) Summary of results.

truncated ephrin-B2-ΔC protein might be aberrantly mis-localized to one of these subcellular compartments. We therefore performed triple immunofluorescent labeling of E10 wild-type, *ephrin-B2*[lacZ], and *ephrin-B2*[T] yolk sacs with antibodies directed against ephrin-B2, PECAM, and either an ER resident protein (Calnexin), a cis-Golgi marker (Membrin), or a marker of the trans-Golgi network (Syntaxin 6). This analysis revealed the truncated ephrin-B2-ΔC protein specifically colocalized with the trans-Golgi

network marker Syntaxin 6 (Fig. 3D). The wild-type ephrin-B2 protein and the ephrin-B2-βgal fusion protein did not colocalize with any of these ER and Golgi markers, indicating proper transport of these molecules through the intracellular compartments and a normal cell-surface posi-tioning (Figs. 3B, C). These data show that the ephrin-B2-ΔC protein is inappropriately trapped within the trans-Golgi network and is not properly localized to the surface of the cell.

The results described above indicate that the *ephrin-B2*[T] allele functionally mimics a protein null as the truncated ephrin-B2-ΔC protein is both incapable of transducing reverse signals and unable to bind Eph receptors and activate forward signaling due to its abnormal intracellular location in the trans-Golgi network (Fig. 3E). Thus, the *ephrin-B2*[lacZ] allele provides a better genetic model to understand the biological functions of ephrin-B2 reverse signaling as this protein is properly localized to the cell surface and is therefore able to act as a ligand to interact with Eph receptors on adjacent cells and activate forward signaling while the replacement of the cytoplasmic tail by β-gal selectively disrupts reverse signals (Fig. 3E). Notably, the absence of early lethality associated with defects in vascular development in *ephrin-B2*[lacZ/lacZ] homozygotes indicates that reverse signaling by ephrin-B2 is not required for morphogenesis of the embryonic vasculature.

Ephrin-B2[lacZ/lacZ] homozygotes exhibit thickened cardiac valves

Our initial observation that *ephrin-B2*[lacZ/lacZ] homozygotes died within the first day after birth indicated that cell autonomous ephrin-B2 mediated reverse signaling, while not required for embryonic development, is required for neonatal survival. To investigate the consequences of disrupting ephrin-B2 reverse signaling, we performed a detailed histological examination of *ephrin-B2*[lacZ/lacZ] homozygotes at late gestational stages. Transverse sections of the heart at E18 and P0 revealed normal chamber formation and vascular connections in the *ephrin-B2*[lacZ/lacZ] homozygotes. However, closer inspection revealed a marked thickening of the aortic and pulmonary valves in all mutant animals examined ($n = 10$; Fig. 4A). Cell counting revealed that there were approximately twice as many mesenchymal cells in the mutant aortic and pulmonary valves compared to the wild type. Furthermore, the mitral valve leaflets were slightly hyperplastic with approximately 30% more mesenchymal cells, while the tricuspid valve exhibited no significant change in leaflet size. X-gal staining of *ephrin-B2*[lacZ/+] heterozygotes at E14 showed strong expression of ephrin-B2-βgal in the epithelia of the valve leaflets in the developing heart (Fig. 4B). This endocardial epithelial layer receives signals from TGFβ family members from the myocardium that instruct them to transform into mesenchymal cells, which in turn then detach and migrate into the underlying extracellular matrix to form the valve tissue (Barnett and Desgrosellier, 2003; Eisenberg and Markwald, 1995; Schroeder et al., 2003). Our data thus indicate ephrin-B2 functions in a cell autonomous fashion in the epithelial cells of the developing valve leaflets to transduce reverse signals that help regulate the growth, transformation, or migration of cells that form valve tissue. It is interesting to note that possible roles for ephrin-B2 reverse signaling in mesenchymal-to-epithelial transitions (e.g., somite formation) have recently been appreciated (Barrios et al., 2003).
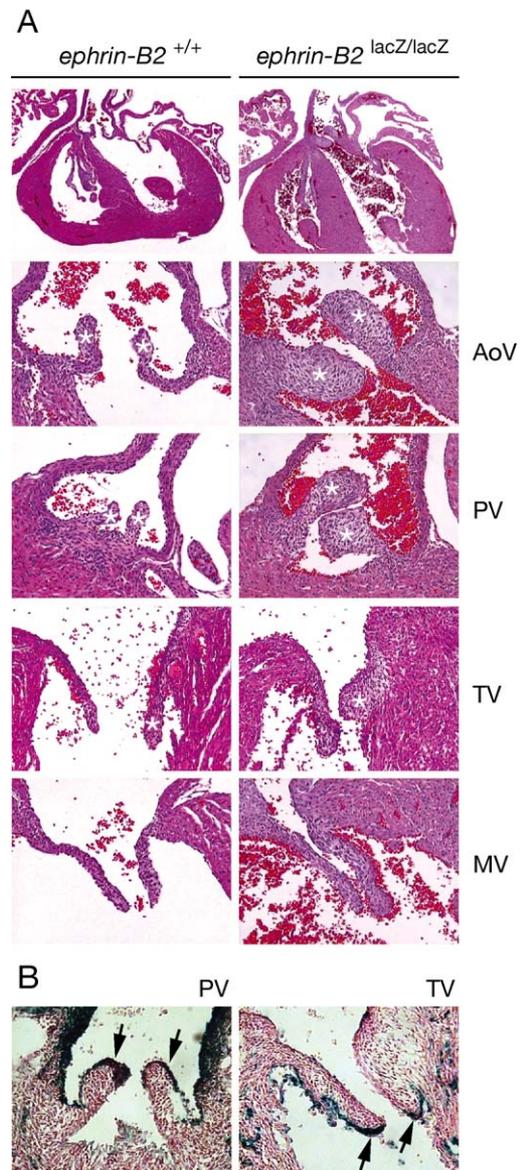

Fig. 4. The ephrin-B2 cytoplasmic domain is required for normal cardiac valve development. (A) Hematoxylin–eosin stained transverse sections of wild-type (left panels) and *ephrin-B2*[lacZ/lacZ] homozygote (right panels) hearts collected at birth. The low magnification images (upper panels) indicate an overall normal chamber formation and vascular connections in the mutants. However, high magnification images of the valve regions (lower panels) reveal enlarged hyperplastic aortic and pulmonary valves (AoV and PV) in the mutants (asterisks). The mitral valve (MV) leaflets were slightly thicker in the mutants. (B) X-gal staining of *ephrin-B2*[lacZ/+] heterozygotes collected at E14 shows strong expression of ephrin-B2-βgal in the epithelial cell layer of the developing valve leaflets (arrows).

Disrupted pathfinding of anterior commissure axons in ephrin-B2[lacZ/lacZ] homozygotes

We also asked whether ephrin-B2 reverse signaling was required for the pathfinding of axons that form the posterior tract of the anterior commissure (acP), a major forebrain commissure that connects the two temporal lobes of the cortex. We previously determined that the EphB2 receptor

tyrosine kinase acted in a noncell autonomous manner as a ligand to direct the pathfinding of acP axons across the midline of the brain (Henkemeyer et al., 1996). As ephrin-B molecules were found to be expressed in the axons forming

A *ephrin-B2* ^{lacZ/+} E16

B *ephrin-B2* ^{lacZ/+} *ephrin-B2* ^{lacZ/lacZ} E16

C *ephrin-B2* ^{+/+} E18

D *ephrin-B2* ^{lacZ/lacZ} E18

the acP tract, the data led to the hypothesis that the ephrins can also function as receptors to transduce cell autonomous reverse signals that help guide axons across the midline of the brain (Henkemeyer et al., 1996). In an effort to confirm this hypothesis, we investigated the pathfinding of acP axons in *ephrin-B2*^{lacZ} animals. Coronal cryosections of *ephrin-B2*^{lacZ/+} heterozygous brains dissected at E16 and stained with X-gal showed a normal anterior commissure that strongly expressed the ephrin-B2-βgal fusion protein in axons of the acP tract (Fig. 5A). Horizontal cryosections of the forebrain of *ephrin-B2*^{lacZ/+} heterozygous confirmed that ephrin-B2 was expressed in axons of the acP as well as axon fibers of the anterior tract of this commissure (acA) (Fig. 5B). Further histological examination showed that the formation of the acP tract was severely compromised in all *ephrin-B2*^{lacZ/lacZ} homozygotes analyzed ($n > 15$) as illustrated by the failure of these axons to extend across the midline of the brain (Figs. 5B–D). The observed axon pathfinding defects in *ephrin-B2*^{lacZ/lacZ} homozygotes strongly suggest that ephrin-B2 is functioning as a receptor in the acP axons to transduce reverse signals that are required for the guidance of these nerve fibers across the midline of the brain.

Conclusion

Our results indicate that ephrin-B2 reverse signals are not required for early cardiovascular development as previously described (Adams et al., 2001), but that this protein must be present at the cell surface to act as a ligand for Eph receptors to properly remodel the primary capillary plexus. When these data are combined with the findings that mice lacking the EphB4 receptor exhibit embryonic lethality due to a similar failure in cardiovascular development (Gerety et al.,
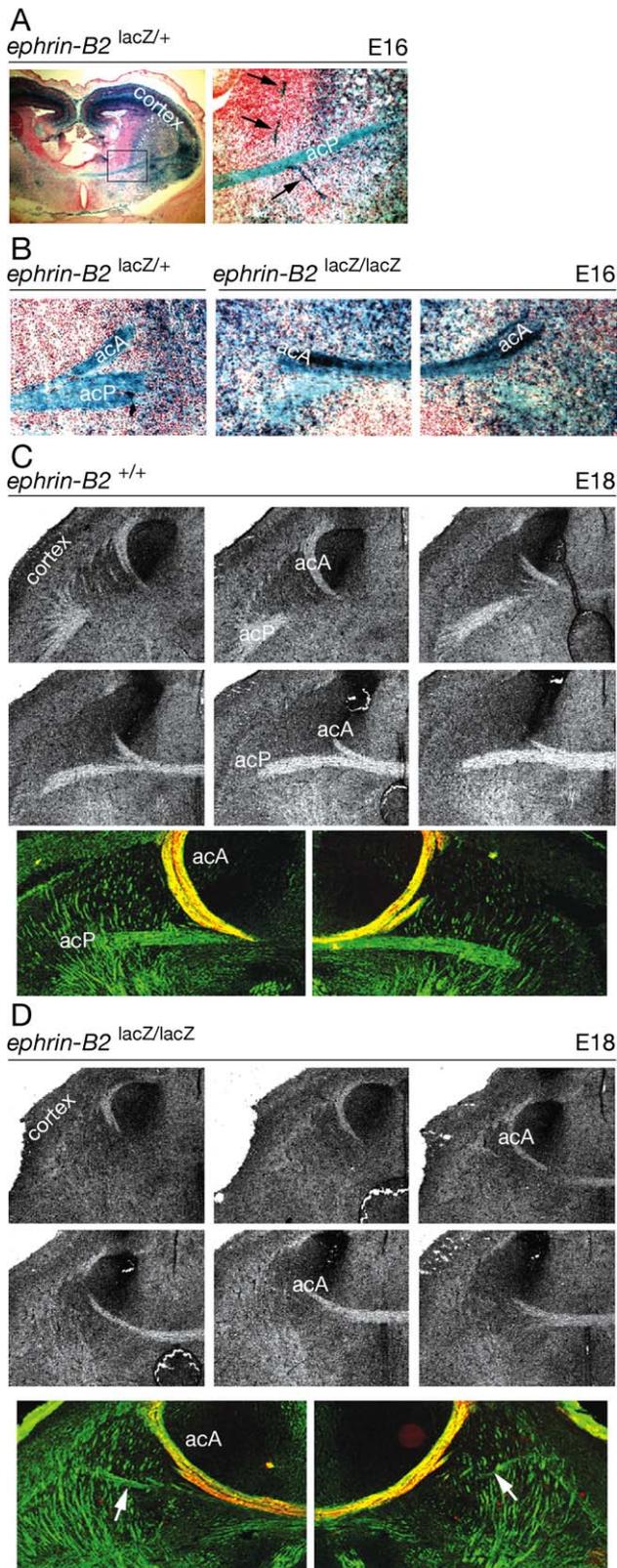
Fig. 5. The ephrin-B2 cytoplasmic domain is required for pathfinding of anterior commissure axons. (A) Low (left) and high (right) magnification of a coronal section from an E16 day *ephrin-B2*^{lacZ/+} heterozygote forebrain stained with X-gal. Note strong expression of ephrin-B2-βgal in the cortex and in the axons of the posterior tract of the anterior commissure (acP). The anterior tract (acA) is not visible in this section. Note that blood vessels in the brain also stain strongly for ephrin-B2 (arrows). (B) High magnification of horizontal sections from E16 day forebrains stained with X-gal. The staining for ephrin-B2-βgal in an *ephrin-B2*^{lacZ/+} heterozygote brain is localized to axons that form both the acA and acP tracts. In *ephrin-B2*^{lacZ/lacZ} homozygote brains, the acP tract is much reduced, while the acA tract appears normal. Also, note that the acA tract stains approximately twice as much for the ephrin-B2-βgal fusion protein in the homozygotes, indicating the increased gene dosage of the *lacZ* allele. (C and D) The upper panels show dark field images of serial horizontal sections (starting from the ventral floor of the left forebrain) of an *ephrin-B2*^{+/+} wild-type (C) and *ephrin-B2*^{lacZ/lacZ} homozygote (D) to reveal the acA and acP tracts of the anterior commissure. The acP axons are severely reduced in the mutant. The bottom panels show confocal images of horizontal sections of *ephrin-B2*^{+/+} wild-type (C) and *ephrin-B2*^{lacZ/lacZ} homozygote (D) forebrains double labeled with L1 antibodies to detect major axon bundles including the acA and acP tracts (green signal) and with TAG-1 to detect the acA tract (red signal). The acP tract is greatly reduced in the mutants (arrows).

1999), it becomes likely that forward signaling from EphB4 is responsible for directing early remodeling of the capillary plexus. While our results indicate ephrin-B2 is acting as a typical ligand to stimulate EphB4 forward signaling in developing blood vessels, ephrin-B2 does appear to act as a receptor to transduce reverse signals later in cardiovascular development during the formation of cardiac valve leaflets. Indeed, the massive hyperplastic thickening of the aortic and pulmonary valves likely leads to the neonatal lethality associated with the *ephrin-B2*$^{lacZ/lacZ}$ homozygotes. Our data also indicate ephrin-B2 reverse signals are required for the pathfinding of axons that form a major forebrain commissure, the posterior tract of the anterior commissure. This result validates the original hypothesis that ephrin ligands are capable of acting like receptors to transduce reverse signals into their own cell as first proposed following observations of a similar defect in the anterior commissure in *EphB2*null animals (Henkemeyer et al., 1996). Thus, our *ephrin-B2*^{lacZ} mice provide a model for the investigation of B-ephrin reverse signaling.

Acknowledgments

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