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### Lab Alleles

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### Mode of Inheritance

Autosomal Recessive

### Local Stock

Sperm, gDNA

### Repository

MMRRC: 032237-UCD

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Phenotypic Description

The *frog* phenotype was identified in the G3 population of ENU-induced germline mutants. The index mouse displayed a gait defect in which it displayed a hopping motion when prodded.

Nature of Mutation

The *frog* mutation was mapped to Chromosome 1, and corresponds to a T to C transition in the donor splice site of intron 6 of the *Epha4* gene (position 113891 in Genbank genomic region NC_000067 for linear genomic DNA sequence of *Epha4*). *Epha4* contains 18 exons encoding 986 translated amino acids. The effect of the mutation at the cDNA and protein level has not been determined. One possibility, shown below, is that aberrant splicing results in skipping of exon 6, and a frameshift that creates a premature stop codon early in exon 7 (aberrant amino acids after position 439, truncation after position 442).

\[
\begin{align*}
\text{correct} & \quad \text{deleted} & \quad \text{aberrant} \\
88529 & \text{AACCAAGCAG} & \text{TATGAAAAG} & \text{GTACTCTTTTC} & \text{GATCAGAATGA} & 115302 \\
437 & \text{\_\_\_-Q\--A--\_\_\_--\_-Y\--E--\_\_K} & & & \text{G\--S\--E\--*} & 442 \\
\end{align*}
\]

The donor splice site of intron 6, which is destroyed by the *frog* mutation, is indicated in blue; the mutated nucleotide is indicated in red.

Protein Prediction

EphA4 is one of 16 receptor tyrosine kinases (RTKs) related to Eph (now known as EphA1), a receptor originally named for its expression in an erythropoietin-producing human hepatocellular...
carcinoma cell line (1). Eph receptors are activated by binding to membrane-anchored ligands called ephrins, which are divided into two classes based on their means of membrane attachment, the ephrin-A proteins being glycosylphosphatidylinositol (GPI)-linked and ephrin-B proteins anchored through a transmembrane domain (2). Eph receptors also comprise two classes (A and B) defined by sequence conservation in the extracellular domain, which generally corresponds to binding preference for either ephrin-A or ephrin-B ligands. In mammals, there are ten EphA receptors and six EphB receptors that bind to six ephrin-A and three ephrin-B ligands. EphA4 is unique among class A Eph receptors in its ability to bind with relatively high affinity to both ephrin-A and ephrin-B ligands (3). The Eph receptor subfamily, the largest among RTKs, is thought to represent the earliest evolutionary split within the RTK superfamily (4).

Figure 1. EphA4 domain structure. The frog mutation, in the donor splice site of intron 6, is predicted to result in truncation of the protein after amino acid 442 (indicated by an X), located in the membrane-proximal FN III domain. LBD, ligand binding domain; Cys-rich, cysteine-rich domain; FNIII, fibronectin type III domain; TM, transmembrane domain; JMS, juxtamembrane segment; SAM, sterile ? motif.

Like all Eph receptors, EphA4 is a type I transmembrane protein with a conserved modular organization (Figure 1). The extracellular N terminal region consists of a globular ligand-binding domain (LBD) followed by a cysteine-rich region and two fibronectin type III domains. On the cytoplasmic side of a single transmembrane domain are a juxtamembrane region, an uninterrupted tyrosine kinase domain, a sterile ? motif (SAM), and a PDZ-binding motif. Both ligand binding and kinase activation of Eph receptors are relatively well understood at the structural level and are briefly discussed here.
The LBD of Eph receptors is necessary and sufficient for ephrin recognition and binding (5), and is composed of 11-12 antiparallel \( \beta \)-strands (designated A-M) arranged in a \( \beta \)-sandwich topology (6-11). The \( \beta \)-strands are connected by loops of varying length (named for the \( \beta \)-strands they connect), with loops D-E, J-K, and G-H especially important for ligand recognition and binding. These loops, together with a convex sheet of four \( \beta \)-strands, form a hydrophobic channel into which the solvent-exposed G-H loop of an ephrin inserts to form the “high affinity interface” that mediates receptor-ligand dimerization. The high affinity interface is common to all Eph-ephrin complexes, consistent with the conserved overall structure of the LBDs of all Eph receptors and the ectodomains of all ephrins. However, specific residues within and outside the high affinity interface mediate the selectivity of individual receptors for their cognate ephrins. These include two lower affinity sites in the LBD and cysteine-rich region that may facilitate receptor oligomerization (8), and another site in the membrane-proximal fibronectin type III domain.
Eph receptors interact relatively indiscriminately with ephrins of the same class, but they only rarely interact with ephrins of the other class. Only EphB2 and EphA4 have demonstrated interclass binding. Evidence suggests that residues within the H-I loop of Eph receptors provide some determinants of class specificity (6-11). Two recent crystal structures of an EphA4-ephrin-B2 complex have revealed sequence and structural features that allow EphA4 to bind with significant affinity to both class A and class B ephrins, and that likely contribute to ligand class selectivity in other Eph receptors (Figure 2, PDB ID 3GXU and 2WO2) (12;13). First, EphA4 has substantial structural plasticity in areas of ligand contact, in particular the D-E and J-K loops (11;13), that allows EphA4 to accommodate B class ephrins, which require considerable receptor

Figure 2. Crystal structure of the EphA4 ligand binding domain in complex with ephrin-B2. Specific loops important for complex formation are indicated. The ephrin-B2 G-H loop inserts into a hydrophobic channel on EphA4. Surface polar contacts involving EphA4 residues Gln12 and Glu14 and ephrin-B2 residues Gln109 and Lys 112 also contribute to complex formation. UCSF Chimera structure is based on PDB 3GXU, Qin et al, J Biol Chem 285, 644-654 (2010). Click on the 3D structure to view it rotate.
rearrangements, and A class ephrins, which do not (7). EphA4 also makes polar contacts with ephrin-B2 using surface residues Gln12 and Glu14 that lie outside the high affinity interface (12). These interactions supplement the weaker association, relative to EphB4-ephrin-B2 or EphB2-ephrin-B2 interactions, of EphA4 and ephrin-B2 resulting from less extensive binding between the EphA4 J-K loop and the ephrin-B2 G-H loop. Mutation of Gln12 and Glu14 reduces by 10-fold the affinity of EphA4 for ephrin-B ligands, but not ephrin-A ligands, indicating that these surface contacts are important for interclass and not intraclass ephrin binding.

Upon ephrin binding, Eph receptors oligomerize, an event required for receptor activation. Receptor oligomerization is induced by engagement with pre-clustered ephrin ligands (14), and only membrane-bound or clustered soluble ephrins can fully activate Eph receptors (15; 16). The structure of the EphB2 LBD in complex with ephrin-B2 revealed that stable heterotetramers (dimers of ligand-receptor dimers) form in crystals, mediated by the high affinity interface and by a low affinity interface on the opposite side of the receptor, in which each ligand interacts with two receptors and each receptor with two ligands (8). Eph receptors lacking the LBD can also oligomerize, and contacts in the cysteine-rich region and fibronectin type III domains have been implicated in ephrin-independent oligomerization (14; 17). These contacts may particularly facilitate Eph cluster propagation that appears to occur after some initial ephrin-mediated clustering (14). Finally, the SAM domain has been proposed to contribute to Eph receptor oligomerization (18; 19), although the SAM-SAM interaction is relatively low affinity and may be secondary to stronger initial oligomerization interactions.
Figure 3. Crystal structure of the EphB2 juxtamembrane and kinase domains in the autoinhibited state. The juxtamembrane segment (pink) adopts a helical conformation that associates with the kinase domain. Autophosphorylation of Tyr604 and Tyr610 (Tyr596 and Tyr602 in mouse EphA4), here mutated to Phe, permits kinase activation. UCSF Chimera structure is based on PDB 1JPA, Wybenga-Groot et al, *Cell* **106**, 745-757 (2001). Click on the 3D structure to view it rotate.

The kinase domains of Eph receptors adopt the canonical bilobal fold characteristic of most protein kinases. As observed in kinase domain crystal structures of EphB2 (20), EphA2 (21), and EphA4 (22), the smaller N-lobe consists of a twisted five-stranded antiparallel ?-sheet and a single helix (? C); the larger C-lobe contains two ?-strands and six ?-helices (Figure 3, PDB ID 2HEL (EphA4) and 1JPA (EphB2)). In the autoinhibited, unphosphorylated state, the juxtamembrane segment adopts a helical conformation that associates with the kinase domain and prevents the activation loop from adopting an ordered activated conformation (20; 22). Ephrin binding to the extracellular domain of Eph receptors leads to receptor oligomerization and autophosphorylation on multiple residues leading to kinase domain activation.

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activation. In particular, autophosphorylation of two tyrosines (Y596 and Y602 in mouse EphA4) within a highly conserved juxtamembrane domain motif relieves autoinhibition \( (23-25) \) by promoting dissociation of the juxtamembrane segment from the kinase domain, allowing increased inter-lobe flexibility and sampling of catalytically competent conformations for helix \( \alpha C \) and the activation loop \( (20;22) \). The N-lobe assists in the binding and coordination of ATP for productive transfer of the \( \gamma \)-phosphate to a substrate oriented in the catalytic cleft between lobes. The phosphorylated juxtamembrane segment also serves as a binding site for the SH2 domains of downstream signaling proteins \( (24-27) \).

The \textit{frog} mutation occurs in the donor splice site of intron 6, and is predicted to result in skipping of exon 6 (encoding amino acids 440-481), a frameshift, and a premature stop after codon 442. The translated protein would be truncated at the start of the membrane-proximal fibronectin type III domain.

**Expression/Localization**

During embryonic development, EphA4 expression is spatially and temporally restricted within the developing forebrain, hindbrain, and mesoderm \( (28-30) \). In the final stages of embryogenesis, EphA4 is expressed predominantly within the central nervous system, including the cerebral cortex, striatum, thalamus, hippocampus, and ventral spinal cord. EphA4 is expressed in corticospinal tract neurons of the somatomotor cortex and on their axons \( (31) \). In the hindbrain, EphA4 expression is restricted to rhombomeres 3 and 5 \( (28) \).

EphA4 is detected by \textit{in situ} hybridization and immunohistochemistry in the cortical epithelial cells of the thymus, mainly in the cortex with some staining in the medulla \( (32;33) \). EphA4 is also found by RT-PCR in all fetal and adult B-lineage cells, with high levels of expression in peripheral blood B cells \( (34) \). Dendritic cells derived from GM-CSF- and TNF-\( \gamma \)-treated CD34\( ^+ \) progenitors from human umbilical cord expressed EphA4 as detected by RT-PCR \( (35) \).

**Background**

Eph-ephrin signaling is distinct from signaling by other RTK systems in several ways \[reviewed in \( (36) \)]. First, whereas all other RTKs bind to soluble, diffusible ligands, Eph engagement requires membrane attachment, so that productive signaling requires cell-cell contact. Second,
whereas classical RTKs become activated upon dimerization, functional Eph-ephrin signaling requires higher order clusters (see Protein Prediction) (37). Furthermore, despite high affinity multimeric binding sites between Ephs and ephrins, the cellular response to ligand-receptor interaction is often repulsion between the two cells. For example, in the central nervous system (CNS) where Eph-ephrin signaling plays an important role in axon guidance, ephrins not only serve as repellents, but can also act as attractive cues for navigating axons (31;38-40) (see Ephs and ephrins in the CNS below). This raises the question of how ligand-receptor engagement, which necessarily brings cells into contact, can mediate cell repulsion.

Adhesion versus repulsion mediated by Eph-ephrin signaling

Whether Eph-ephrin interactions result in cell adhesion or repulsion depends in large part upon the mechanism of signal termination, and specifically on whether the two interacting cells can easily separate following cell-cell contact. Two mechanisms are known to terminate Eph-ephrin signaling: proteolytic cleavage of either Ephs or ephrins by transmembrane proteases, and endocytosis of vesicles containing Eph-ephrin complexes from the cell surface (Figure 4) (36; 41). The metalloprotease A-Disintegrin-And-Metalloprotease (ADAM)-10 has been shown to cleave ephrin-A2 in vivo (42), by a mechanism proposed to involve ephrin cleavage in trans (43).
Figure 4. Negative regulation of Eph-ephrin signaling. (A) Proeolytic cleavage. Upon binding of EphA3 to ephrin-A5, ADAM10 is activated to cleave the stem region of ephrin-A5 on the surface of the opposing cell, terminating signaling and releasing adhesive cell-cell interactions. (B) Trans-endocytosis. EphB-ephrin-B interactions can result in formation of intracellular vesicles containing EphB-ephrin-B complexes in both cells. Endocytosis of either protein into the opposing cell likely requires remodeling of the actin cytoskeleton through RhoGTPase action. (C) Eph-ephrin interactions in cis. EphA and A class ephrins on the same cell can interact through their extracellular binding domains, preventing their subsequent activation by engagement with ephrins and Ephs on an opposing cell.

Before cell-cell contact, ADAM10 is constitutively associated with EphA3. Contact with ephrin-A5 presented by an opposing cell repositions ADAM10 and activates its N-terminal protease domain for cleavage of the ephrin stem region on the surface of the opposing cell. B-class ephrins and Ephs have also been shown to be cleaved by a presenilin ?-secretase activity (44-46). In the case of B-ephrins, they are first cleaved by an ADAM protease upon Eph binding. The remaining membrane-attached ephrin fragment is further processed by a ?

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secretase to release an intracellular signaling peptide that binds Src and inhibits its association with the inhibitory kinase Csk (46).

Endocytosis is another means by which Eph-ephrin signaling is terminated. In cell culture assays, cell-cell contact upon EphB-ephrin-B interaction results in the rapid formation of intracellular vesicles containing full length EphB-ephrin-B complexes in both cells in a manner dependent on the intracellular domains of the molecules (47) and on Rac signaling (48). Notably, this mechanism of signal termination requires that one of the proteins is trans-endocytosed from one cell into the opposing cell. How this occurs remains incompletely understood, although cytoskeletal regulatory molecules, including the Rac guanine nucleotide exchange factor Vav, have been proposed to play a role (49). Trans-endocytosis of neuronal material into glial cells has been observed at the neuron-to-glia interface between hippocampal neurons exogenously expressing EphB2 and co-cultured glial cells (50).

Eph-ephrin interactions in cis, that is, between receptors and ligands expressed on the same cell, are also believed to contribute to negative regulation of signaling (Figure 4) (51). Two types of cis interactions have been identified. EphA receptors and co-expressed ephrin-A ligands can interact directly via their functional binding domains, and this interaction does not seem to mediate intracellular signals, but has an inhibitory effect on trans interactions (52). EphA3 and ephrin-A5 can also interact in cis via the EphA3 fibronectin type III domain, inhibiting cellular responses to ephrins presented in trans (53).

Bidirectional Eph-ephrin signaling
Another distinctive feature of Eph-ephrin signaling, in contrast to that of classical RTKs, is that the ligands for Eph receptors, the ephrins, can themselves serve as receptors that are activated by Ephs to initiate signaling in their own cells (36;54;55). Eph receptor-activated “forward” signaling requires kinase activation, autophosphorylation and phosphorylation of targets, and recruitment of effectors to the receptor. Ephrin-activated “reverse” signaling for B class ephrins also requires tyrosine phosphorylation of their cytoplasmic tails, mediated by Src family kinases and other RTKs. Reverse signaling for A class ephrins is not well understood and may depend on associated transmembrane proteins, including integrins and the p75 low affinity nerve growth factor receptor, as well as Src family kinases. The C-terminal PDZ-binding motif of both Ephs and ephrins is also important for forward and reverse signaling.
Eph receptors play a prominent role in the regulation of the cytoskeletal organization and dynamics that underlie cellular adhesion, migration, and morphogenesis, and thus a main target of signaling from both Eph receptors and ephrins is the Rho family of GTPases, master regulators of the actin cytoskeleton. Rho GTPases, including RhoA, Rac, and Cdc42, function as molecular switches that cycle between an inactive GDP-bound and an active GTP-bound state that binds and activates specific effectors. Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by catalyzing the binding of GTP to the GTPase, while GTPase activating proteins (GAPs) inactivate Rho GTPase signaling by accelerating the hydrolysis of GTP to GDP.

Eph receptors can directly recruit GEFs to control cytoskeletal remodeling. For example, the Rho GEF ephexin1 binds specifically and constitutively to EphA receptors including EphA4 (56;57), whereas another GEF, Vav2, binds preferentially to activated Eph receptors of both classes (58). In neurons, in the absence of ephrin engagement, EphA-bound ephexin1 activates RhoA, Rac, and Cdc42 in growth cones. Upon ephrin binding, ephexin1 becomes tyrosine phosphorylated resulting in preferential activation of ephexin1 exchange activity towards RhoA but not Rac1 and Cdc42, and leading to growth cone collapse (57). Eph-induced tyrosine phosphorylation of Vav2 promotes local Rac1-dependent endocytosis of Eph-ephrin complexes, thereby terminating signaling and promoting a cellular repulsion (58). Other GEFs including intersectin-1, Kalirin-7, and Tiam have also been reported to function in signaling downstream of Ephs (59). Finally, the Rac1 GAP ?2-chimaerin has been implicated as a critical EphA4 effector in vivo (60-63). Activation of EphA4 by ephrin-B3 leads to ?2-chimaerin binding, phosphorylation, and activation of GAP activity, which stimulates axonal growth cone collapse through downregulation of Rac1 activity.

Ephs and ephrins in the CNS
Eph receptors and ephrins are highly expressed in the developing nervous system, where their functions have been most extensively characterized. Eph-ephrin signaling was first shown to be required for the establishment of topographically organized neuronal connections in the developing visual system, and its role in this system is described here as an illustration of the types of cellular responses choreographed by Ephs and ephrins. Eph receptors and ephrins are expressed throughout the developing embryo, and are well known for their involvement in organ development and patterning of the vascular (64), skeletal, and nervous systems. In addition, Eph receptors have been shown to regulate functions in the adult, including learning and memory (65), bone homeostasis (66), intestinal homeostasis (67-69), and insulin secretion (70); their
involvement in immune functions is discussed in the following section. Eph receptors and ephrins are often upregulated in cancer tissue and their effects on oncogenesis are the subject of an expansive field of investigation (54).

Topographically organized connections from the retina to the optic tectum (in frogs, fish, and chicks) or the superior colliculus (in mammals) form during embryonic visual system development [reviewed in (71;72)]. Topographic mapping depends on the specification of topographic cellular identities in the retina and tectum/collliculus, which establishes the pattern of expression of numerous guidance molecules that direct axons as they make connections in their target field. It was originally observed that certain Ephs and ephrins have complementary and graded expression patterns in the retina and the optic tectum/superior colliculus (73-77), and that axons of temporally localized retinal ganglion cells avoid trajectories expressing ephrin-A2 and ephrin-A5 (78-80). These findings suggested that ephrins provide positional information to Eph-expressing axons, and specifically repel them from their vicinity. Many further studies using knockouts of different Ephs and ephrins have shown that in general, EphAs and ephrin-As control RGC axon mapping along the anterior-posterior axis of the optic tectum/superior colliculus by inhibiting interstitial branching of RGC axons posterior to their correct target zone. EphBs and ephrin-Bs act bifunctionally (in either attractant or repellent modes) to define connections along the dorsoventral axis by directing the extension of branches that then arborize in the vicinity of their correct dorsal-ventral and anterior-posterior position (71;72).

In addition to controlling axon guidance in the visual system, ephrins and their receptors play important roles in the guidance of axons across the midline of the nervous system (81) (see Physiological functions of EphA4 below), segmentation of the embryonic hindbrain (82-84), the growth of axons and dendrites, and synaptogenesis (65).

Ephs and ephrins in immune function
Most Ephs and ephrins are expressed in the thymus, on all thymocyte subsets, cortical and medullary thymic epithelial cells, and on T cells. Several studies have shown that interference with Eph-ephrin signaling impairs thymocyte survival and maturation by disrupting the structural organization of the thymus and guidance of thymocyte movement through the thymic compartments that support their maturation into T cells [reviewed in (85)]. For example, addition of either EphB2-Fc or ephrin-B1-Fc fusion proteins to fetal thymic organ cultures decreased the numbers and altered the ratios of double positive (DP) and single positive (SP) thymocytes,
increased their apoptosis, and resulted in disorganization of the three-dimensional epithelial network supporting T cell maturation (86;87). EphB2 forward signals and EphB2-induced reverse signals are required for organizing thymic epithelial cells; EphB3 also plays a role in this process (88).

Eph receptors modulate T cell receptor (TCR) responses (89). TCR engagement results in EphB receptor migration to lipid rafts (90-92). EphB6, which is highly expressed in the thymus and peripheral T cells, is reported to function as a co-stimulatory receptor for T cell activation. Treatment with solid-phase antibodies against EphB6 dramatically increases T cell responses (CD25 and CD69 expression; interferon-?, transforming growth factor-?, tumor necrosis factor-?, and interleukin-6 secretion; proliferation) to suboptimal TCR stimulation (90-93). Conversely, T cells from Ephb6-/- mice respond poorly to solid-phase anti-CD3/CD28 stimulation, and the animals exhibit compromised induction of delayed-type hypersensitivity and experimental autoimmune encephalitis (90). These findings suggest that T cell responses to antigens depend not only on interactions with antigen presenting cells, but also on T cell-T cell interactions mediated in part by EphB6 and its ephrin ligands. Several studies also demonstrate that thymocyte and T cell migratory responses to chemokines may be modulated by EphA receptors (94-96).

Ephrin-B2 and ephrin-B3 have been demonstrated to serve as the cell entry receptors for Nipah and Hendra viruses, two emerging paramyxoviruses comprising the newly defined Henipavirus genus (97;98). The natural host for henipaviruses is the fruit bat, but they can infect a wide range of species including humans. In humans, these viruses are extremely lethal and are classified as category 4 containment pathogens. The viruses bind to the same region of ephrin-B2 and ephrin-B3 that mediates high affinity binding to EphB receptors (99-101).

**Physiological functions of EphA4**

EphA4 plays an important role in the guidance of axons in the corticospinal (CST) and anterior commissure (AC) tracts. Mice with a targeted deletion of EphA4 are born in expected Mendelian frequencies (102;103). However, they exhibit locomotor abnormalities, in particular impaired coordinated movement of the limbs, that result in hesitation in initiating locomotion and a lack of normal synchronous movement of each forelimb with the contralateral hindlimb (102;103). Epha4-/- mice display a synchronous, kangaroo-like movement of the hindlimbs while reciprocal movement of the forelimbs is maintained. Analysis of the corticospinal tract, which

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extends from layer V neurons in the motor cortex through the forebrain, midbrain, and hindbrain to terminate at various levels of the spinal cord, revealed inappropriate terminations at the level of the medulla and spinal cord in EphA4/- mice. The reduced innervation of lower spinal cord segments resulting from axonal terminations is consistent with the more marked motor defect in the lower limbs of EphA4/- mice. No defects were observed in the in the targeting of spinal motor neuron projections to muscle. A knock-in mutant mouse expressing a kinase defective EphA4 (EphA4K653M) displayed the same locomotor and CST abnormalities as EphA4 null mutants, demonstrating that CST formation requires intact kinase activity, i.e. forward signaling, in EphA4-expressing cells (31). EphA4 expression was detected on CST fibers, supporting a cell-autonomous role for EphA4 in neurons. In addition to inappropriate terminations, a significant number of CST axons also crossed back across the spinal cord midline aberrantly in mutant mice. Ephrin-B3 was demonstrated to act as the midline repulsive cue preventing EphA4-expressing CST fibers from recrossing the midline (104;105). Deletion of ephrin-B3 in mice results in a CST phenotype similar to that of Epha4/- mice, which may be rescued by expression of a mutated ephrin-B3 lacking the cytoplasmic domain, indicating that EphA4 forward signaling is sufficient to inhibit aberrant midline recrossing.

The AC is a major forebrain axon tract consisting of an anterior (aAC) and posterior branch (pAC) that connects the two olfactory bulbs and the two lobes of the temporal cortex, respectively. Epha4/- mice display an absence of or disruptions in the projection of both aAC and pAC tracts across the midline, with both branches reduced in diameter and shifted ventrally away from the third ventricle and striatum (31;103;106). These defects are similar to those observed in EphB2 and EphB3 knockout animals (107;108). In contrast to CST axons, AC tract projections are normal in knock-in mice expressing kinase defective EphA4, indicating that reverse signaling in which EphA4 acts as a ligand regulates pathfinding of ephrin-expressing axons (31). EphA4 mRNA is neither expressed in olfactory bulbs, which contain the cell bodies of neurons that form the aAC branch, nor in the aAC itself, and is found only at very low levels in the pAC. Instead, EphA4-positive cells are found dorsal of both branches of the anterior commissure, immediately adjacent to AC axons, suggesting that ephrin-expressing AC axons respond to EphA4 as they navigate across the forebrain midline. EphA4 binds to ephrin-B2 and ephrin-B3, but not other B-ephrins, in addition to A-ephrins. Although ephrin-B2 and ephrin-B3 are found on aAC and pAC axons, the AC phenotypes of mice lacking these molecules are different from that of Epha4/- mice (104;105;109), raising the possibility that EphA4 may act as a ligand for A-ephrin-expressing axons of the AC.
As indicated above, several molecules have been implicated in EphA4-mediated signaling, including the GEF ephexin1, and the GAP ? 2-chimaerin. Ephexin1-deficient mice displayed normal locomotor behavior and normal retinocollicular and CST axon guidance (57). In contrast, ? 2-chimaerin-deficient mice exhibited the same hopping gait and CST midline axon guidance defects as Epha4/- mice, indicating a key role for ? 2-chimaerin in EphA4-directed axon guidance. EphA4 has been shown to regulate neuronal morphology through spine-associated RapGAP (SPAR)-mediated inactivation of Rap GTPases (110), and to control neuronal dendritic spine remodeling through ? 1-integrin signaling pathways (111).

In the immune system, EphA4-deficient mice display defects in thymocyte maturation (112). Thymi of Epha4/- mice have reduced cellularity and reduced percentages of DP cells, an increased proportion of which were apoptotic relative to those of wild type mice. These defects are proposed to stem from abnormal development of stromal cells of the thymic cortex, which support thymocyte survival and maturation. It has been reported that T cells from humans with Sézary syndrome, a malignancy of CD4+ memory skin homing T cells, express elevated amounts of EphA4 (113).

**Putative Mechanism**

Although the phenotype has not been extensively characterized, the altered gait of frog homozygotes is similar to that of Epha4/- mice. Based on the predicted effect of the mutation and the mutant phenotype, it is likely that aberrant early termination and degradation of the translated protein result in absence of the expressed protein in frog mice. However, it is not definitively known whether the frog mutation is hypomorphic or functionally null.

**Genotyping**

*Frog* genotyping is performed by amplifying the region containing the mutation using PCR, followed by sequencing of the amplified region to detect the single nucleotide insertion.

**Primers**

frog (F): 5’- ACACATTTCCATGCCCAATTTTGCAAT -3’
frog (R): 5’- ACCCACACCTTTATTTTGACAGCAGT -3’

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PCR program
1) 95°C 2:00
2) 95°C 0:30
3) 56°C 0:30
4) 72°C 1:00
5) repeat steps (2-4) 29X
6) 72°C 7:00
7) 4°C

Primers for sequencing
frog_seq(F): 5’- GTACTGTTGATCCAGACTCAGGC -3’
frog_seq(R): 5’- GTGTGTTTCAAGATAACGAGCC -3’

The following sequence of 1250 nucleotides (from Genbank genomic region NC_000067 for linear genomic sequence of *Epha4*, sense strand) is amplified:

```
aca catttccatg cccaaattttq caatggccat caggtatagatccactgt
tcctacttt aactgttggtg tccatattcag tgaagagaca ccattgacaaaggaatcatttcttt
atggcatgacg gctttggtgcttgcttgccttgctgctgcacatcgtggggtgttgaggtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtgttacgtgtg
```
Mutagenetix Phenotypic Mutation 'frog'

tgcagagcaa
113521 gccctaccca cagcgttttg tcttcagtct cctcacaatt gatcttttaa
agacaaacc
113581 taaacaaagg aaggtctgga gataatggta ataataaatg ctcttgcttg
aaaaagggca
113641 acgtgaccaa caggaaggca tgggactctg aagtcagttg aaatttttga
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113701 ctaaaatatg tttctttctta aagtaagatt attatctcag tctttctgc
tttctcccc
113761 atacgacat catccattgc tttgttcag gctaaagaag ttacaagata
tagcctagct
113821 ctggctttggc tggaccagaa tcgacaaat ggagctcatc tagaatatga
agtcaaatat
113881 tatgaatagc taccttttctt tttctttcttt gcttttccttc ttctttctct
tcttttcccc
113941 tcttttcttc tttctttttc tctgtgtgttc tcaagtatta gacagttcta
aatcatgaa
114001 cagcagcaga agaaggtcct aatgcatctt gattccccac aaatctgatt
atgagatgta
114061 atatggcctc gtttacttttg aaacacacac acaatgtctct aatataatta
tattttcttt
114121 cgaatataag aatcaagac tcaatctata tacctttctt cacagagcat
gctcataaagc
114181 tgcataata atgatattgt taagcagatc actggttgc aaaataaagg
tgtgggt

Primer binding sites are underlined; sequencing primer binding sites are highlighted in gray; the mutated T is indicated in red.

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