Regulation of Avian Cranial Neural Crest Cell Migration by Eph Receptors and Ephrin Ligands

by

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ABSTRACT

Eph receptors and their ephrin ligands play important roles in guiding mouse and *Xenopus* cranial neural crest (CNC) cells to their destinations. My objective was to determine if Ephs and ephrins also regulate avian CNC pathfinding. By double labeling for Eph or ephrin RNA and a neural crest marker protein, I was able to clearly distinguish neural crest from ectoderm and head mesenchyme and show that avian CNC cells express EphA3, 4, and 7 and EphB1 and 3 and migrate along pathways bordered by non-neural crest cells expressing ephrin-B1. Surprisingly, avian CNC cells also express ephrin-B2 and migrate along pathways bordered by non-neural crest cells expressing EphB2. Consistent with these findings, explanted avian CNC cells are labeled by both ephrin-B1 and EphB2 Fc fusion proteins. Given the choice between growing out onto substrate-bound fibronectin (FN) or FN plus clustered Fc protein in the stripe assay, these cells show no preference for either condition. Conversely, given the choice between FN or FN plus clustered ephrin-B1 or EphB2 Fc fusion protein, the cells strongly localize to stripes containing only FN. This response is mitigated in the presence of soluble ephrin-B1/Fc or EphB2/Fc, but not in the presence of soluble Fc alone. These findings show that avian CNC cells have a mutually exclusive distribution with non-neural crest cells expressing ephrin-B1 and EphB2 RNA *in situ* and are repelled from ephrin-B1 and EphB2 protein *in vitro*, suggesting that their migration is guided by both forward signaling through a variety of Eph receptors as stimulated by ephrin-B1 and reverse signaling through ephrin-B2 as stimulated by EphB2. I further explore the phylogeny of Ephs and ephrins and show that these genes diversified at different times in evolutionary history, such that the ancestral chordate likely had a single receptor for two different ligands.

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List of Abbreviations

acP, posterior tract of the anterior commissure

AOB, accessory olfactory bulb

BAI, branchial arch one

BAII, branchial arch two

BAIII, branchial arch three

BAIV, branchial arch four

BCIP, 5-bromo-4-chloro-3-indoyl phosphate

BS, bootstrap

BSA, bovine serum albumin

CAP, Cbl-associated protein

cDNA, complementary deoxyribonucleic acid

Ce, *Caenorhabditis elegans*

Ci, *Ciona intestinalis*

CNC, cranial neural crest

CO_2, carbon dioxide

DAPI, 4',6-diamidino-2-phenylindole

Dsh, Dishevelled

Ec, ectoderm

ECM, extracellular matrix

Efn, ephrin

EGF, epidermal growth factor

FAK, focal adhesion kinase

FBS, fetal bovine serum

FGF, fibroblast growth factor

FITC, fluorescein isothiocyanate

FN, fibronectin

GAP, GTPase activating protein

GDI, guanine nucleotide dissociation inhibitor

GDP, guanosine diphosphate

GEF, guanine nucleotide exchange factor

Gg, *Gallus gallus*

GPI, glycosylphosphatidylinositol

GTP, guanosine triphosphate

hr, hour

Hs, *Homo sapiens*

IgG, immunoglobulin G

ISH, in situ hybridization

JM, juxtamembrane

LBD, ligand binding domain

LMCl, lateral motor column

ME, minimum evolution

mg, milligram

min, minute

ml, millilitre

ML, maximum likelihood

mm, millimeter

mM, millimolar

MMCm, medial motor column

MP, maximum parsimony

mRNA, messenger ribonucleic acid

My, mesenchyme

NBT, nitro blue tetrazolium

NC, neural crest

ng, nanogram

NJ, neighbour joining

NRTK, non-receptor tyrosine kinase

NT, neural tube

OV, otic vesicle

p, probability value

PBS, phosphate buffered saline

PBST, phosphate buffered saline with Tween

PCR, polymerase chain reaction

PFA, paraformaldehyde

PLL, poly-L-lysine

r2, rhombomere two

r3, rhombomere three

r4, rhombomere four

r5, rhombomere five

r6, rhombomere six

RGC, retinal ganglion cell

RNA, ribonucleic acid

RTK, receptor tyrosine kinase

RT-PCR, reverse transcription polymerase chain reaction

n, sample size

s, somite

SAM, sterile alpha motif

SC, superior colliculus

SFK, Src family kinase

SH2, Src homology 2

Sp, *Strongylocentrotus purpuratus*

TBST, Tris buffered saline with Tween

TKD, tyrosine kinase domain

TMD, transmembrane domain

TNC, trunk neural crest

tRNA, transfer ribonucleic acid

VNO, vomeronasal organ

w/v, weight/volume

Xl, *Xenopus laevis*

°C, degrees Celsius

µg, microgram

µl, microlitre

µm, micrometer

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CHAPTER 1

INTRODUCTION

1.1 The Neural Crest

One of the defining features of vertebrates is the neural crest (NC), a transient group of precursor cells that originate within the dorsal neuroectoderm of the neural tube (NT) during embryonic development (Le Douarin and Kalcheim, 1999). Following an epithelial to mesenchyme transition in which they lose affinity for their neighbours in the neuroectodermal epithelium, NC cells individualize and gain the capacity to migrate over long distances through the underlying mesodermal layer. After dispersing to peripheral locations, NC cells differentiate into a variety of cell types, including the neurons and glia of sensory ganglia and the entire autonomic nervous system, endocrine cells, and melanocytes.

The NC can be broadly divided into two different subpopulations. Cranial neural crest (CNC) cells migrate ventrally from the hindbrain region of the NT to the branchial arches. CNC derivatives include cranial ganglia, the mesenchymal component and calcitonin-producing cells of the pharyngeal glands, the walls of the great arteries emanating from the heart as well as the aorticopulmonary septum of the heart, and, perhaps most notable in the context of vertebrate evolution, most of the skeleton, cartilage, and connective tissue of the face, jaw, and neck (Kirby *et al.*, 1983, Le Lievre and Le Douarin, 1975, Lumsden, 1991, Lumsden and Guthrie, 1991, and Polak *et al.*, 1974). The trunk neural crest (TNC) migrates ventrally from the trunk region of the NT through the somites and gives rise to spinal ganglia, the adrenal medulla, and most of the body's pigmentation (Bronner-Fraser *et al.*, 1980 and Serbedzija *et al.*, 1989).

In addition to their lineage, NC cells are a popular subject of research because of the manner in which they migrate. Rather than migrating in broad waves along the length of an embryo's rostrocaudal axis, both cranial and trunk NC cells travel in discrete streams separated from one another by crest-free zones. In the head of the early avian embryo, for example, streams of CNC cells are aligned between the branchial arches and alternating segments, or rhombomeres, of the hindbrain (Lumsden and Keynes, 1989). One stream extends from the second rhombomere (r2) to the first branchial arch (BAI). A second stream skirts the rostral margin of a structure known as the otic vesicle (OV), which is a hollow ball of invaginated ectoderm lateral to the mid hindbrain that contributes to inner ear development, while traveling from the fourth rhombomere (r4) to the second branchial arch (BAII). Just caudal to the OV, a third stream emerges from the sixth rhombomere (r6) and splits into two branches that invest in the third and fourth branchial arches (BAIII and IV). As such, CNC cells are absent from the areas lateral to the third and fifth rhombomeres (r3 and 5). In the trunk region, streams of TNC cells migrate exclusively through the rostral half of each somite (Rickmann *et al.*, 1985).

1.1.1 Segregated Migration

This stream-like pattern of NC cell migration is attributable to extrinsic guidance cues. In the head, the dorsal neuroectoderm of r3 and 5 shows elevated levels of apoptosis around the onset of NC migration, which could explain the absence of CNC cells from the areas lateral to these segments (Graham *et al.*, 1993). However, cell labeling studies have shown that r3 and 5 in fact produce small numbers of CNC cells that, rather than migrating laterally, are diverted rostrally or caudally into the CNC streams emanating from the even-numbered rhombomeres (Sechrist *et al.*, 1993). This effect is not a function of cell number, since even-numbered rhombomeres grafted in place of odd-numbered ones produce CNC cells undepleted by apoptosis that are similarly diverted into the major CNC streams (Farlie *et al.*, 1999). These findings suggest the presence of an exclusion zone in the crest-free regions adjacent to r3 and 5 and/or an attractive signal that is localized to the pathways taken by CNC cells. Further evidence for the necessity of extrinsic guidance cues to CNC pathfinding comes from *Xenopus*, where there is no focal depletion of CNC cells from specific rhombomeres (Hensey and Gautier, 1998). Moreover, *Xenopus* CNC streams become separated only as they enter the branchial arches, and yet do not intermingle beforehand (Sadaghiani and Thiebaud, 1987).

In the trunk, the segmental migration of NC cells is intimately tied to the segmentation of the adjacent mesoderm. Newly emerged TNC cells have an initially even distribution along the NT, but assume their characteristic stream-like pattern of migration as the somites develop (Tosney, 1978 and Weston, 1963). This pattern can be specifically attributed to differences between the rostral and caudal somite halves. For instance, if the rostrocaudal polarity of the somites is reversed, the spinal ganglia switch their orientation to project through the caudal somites halves, which represent the original rostral somite halves (Keynes and Stern, 1984). Replacing intact somites with a series of rostral somite halves results in TNC cells migrating in a broad wave and giving rise to fused spinal ganglia (Kalcheim and Teillet, 1989). When the same experiment is performed with caudal somite halves, TNC migration is prevented. These findings suggest that repulsive signals present in the caudal somite halves, perhaps in conjunction with attractive signals present in the rostral somite halves, are responsible for the segmental migration of TNC cells.

1.1.2 Mechanisms of Segregation

There are a number of ways in which NC cells could be directed to their targets. For one, NC cell pathfinding could be regulated by the differential distribution of extracellular matrix (ECM) molecules. Both cranial and trunk NC cells express integrins (Duband *et al.*, 1986), which are cell surface receptor proteins that mediate adhesion to the ECM protein fibronectin (FN). Cultured NC cells adhere to and grow out on FN-coated substrates, but not in the presence of an antibody that blocks the interaction between integrins and FN (Bronner-Fraser, 1985). NC migration *in vivo* is also disrupted by the same antibody, indicating that FN plays a key role in this process. However, the finding that FN is broadly expressed wherever NC cells are found (Krotoski *et al.*, 1986) suggests that other factors are responsible for confining these cells to stereotypical pathways and that FN instead acts as a substrate protein that is permissive for their migration.

Alternatively, NC cells may be attracted to their targets by the process of chemotaxis. For example, fibroblast growth factor (FGF) receptors are expressed by CNC cells, whereas expression of the FGF ligands for these receptors is concentrated in the branchial arches (Kubota and Ito, 2000). Consistent with a chemotactic guidance mechanism, FGF receptor-expressing CNC cells are attracted to FGFs *in vitro*. In addition, the melanocyte precursors of the TNC appear to be attracted to the dermis by long range signals (Tosney, 2004) and tissue grafting experiments have shown that ectopic OVs, which result from the inversion of r3 and 4, can attract CNC cells (Sechrist *et al.*, 1994). On the other hand, the finding that NC cells transplanted to a medial position in the trunk can migrate both towards and away from the NT (Erickson, 1985) argues against the existence of a chemoattractant emanating from a ventral target site and instead suggests that these cells move passively along a substrate that is permissive for their migration. Even if chemotaxis does play a role in NC migration, it is difficult to conceive of how a hypothetical chemoattractant that is presumably capable of diffusing not only along the stereotypical pathways of migration, but also into the adjacent crest-free regions could promote the segregation of NC cells into discrete streams. Alternatively, both cranial (Kulesa and Fraser, 1998) and trunk NC cells (Krull *et al.*, 1995) have been found to migrate in chain-like units of multiple cells held together *via* intracellular connections, which could account for the cohesiveness of cells within a given NC stream. However, this cannot account for how adjacent streams are kept separate from one another.

The remaining possibility is that NC cells are actively excluded from crest-free regions by repulsive guidance cues and thereby channeled into their stereotypical pathways of migration. There are a number of cues that could serve this purpose in both the head and the trunk. In the head, the ErbB4 receptor tyrosine kinase (RTK) is expressed in r3 and 5 (Gassmann *et al.*, 1995), whereas a ligand for this receptor, neuregulin-1, is expressed in r2 and 4 (Meyer *et al.*, 1997). When the gene encoding ErbB4 is knocked out, a subset of the CNC emerging from r4 mismigrates rostrally into the area lateral to r3 (Golding *et al.*, 2000). While CNC cells transplanted from ErbB4 knockout embryos to wild-type embryos migrate normally, wild-type CNC cells transplanted into an ErbB4-negative background do not. ErbB4 is therefore thought to function in a non-CNC cell autonomous fashion, perhaps, as a result of interacting with the neuregulin-1 ligands produced in the hindbrain, by inducing the expression of inhibitory signals in the areas lateral to the junctions between r2 and 3 and r3 and 4 or by effecting the secretion of inhibitory signals into these areas. A second type of RTK, neuropilin-1, is expressed by both cranial and trunk NC cells (Eickholt *et al.*, 1999). A ligand for this receptor, Sema 3A, is expressed in r3 and 5 (and secreted into the areas lateral to these segments) and the caudal half of each somite. *In vitro*, cultured NC cells round up in the presence of soluble Sema 3A and avoid substrates containing immobilized Sema 3A. *In vivo*, the presence of ectopic Sema 3A results in the disruption/displacement of CNC streams (Osborne *et al.*, 1995). In the trunk, a host of proteins are expressed exclusively in the caudal half of each somite, including peanut agglutinin-binding glycoprotein (Bagnall and Sanders, 1989 and Krull *et al.*, 1995), T-cadherin (Ranscht and Bronner-Fraser, 1991), and ECM molecules such as tenascin (Halfter *et al.*, 1989 and Stern *et al.*, 1989), versican (Landolt *et al.*, 1995 and Perris *et al.*, 1996), collagen IX (Ring *et al.*, 1996), and F-spondin (Debby-Brafman *et al.*, 1999). Many of these proteins have been shown to interfere with the adhesion and outgrowth of cultured TNC cells onto otherwise permissive substrates *in vitro*.

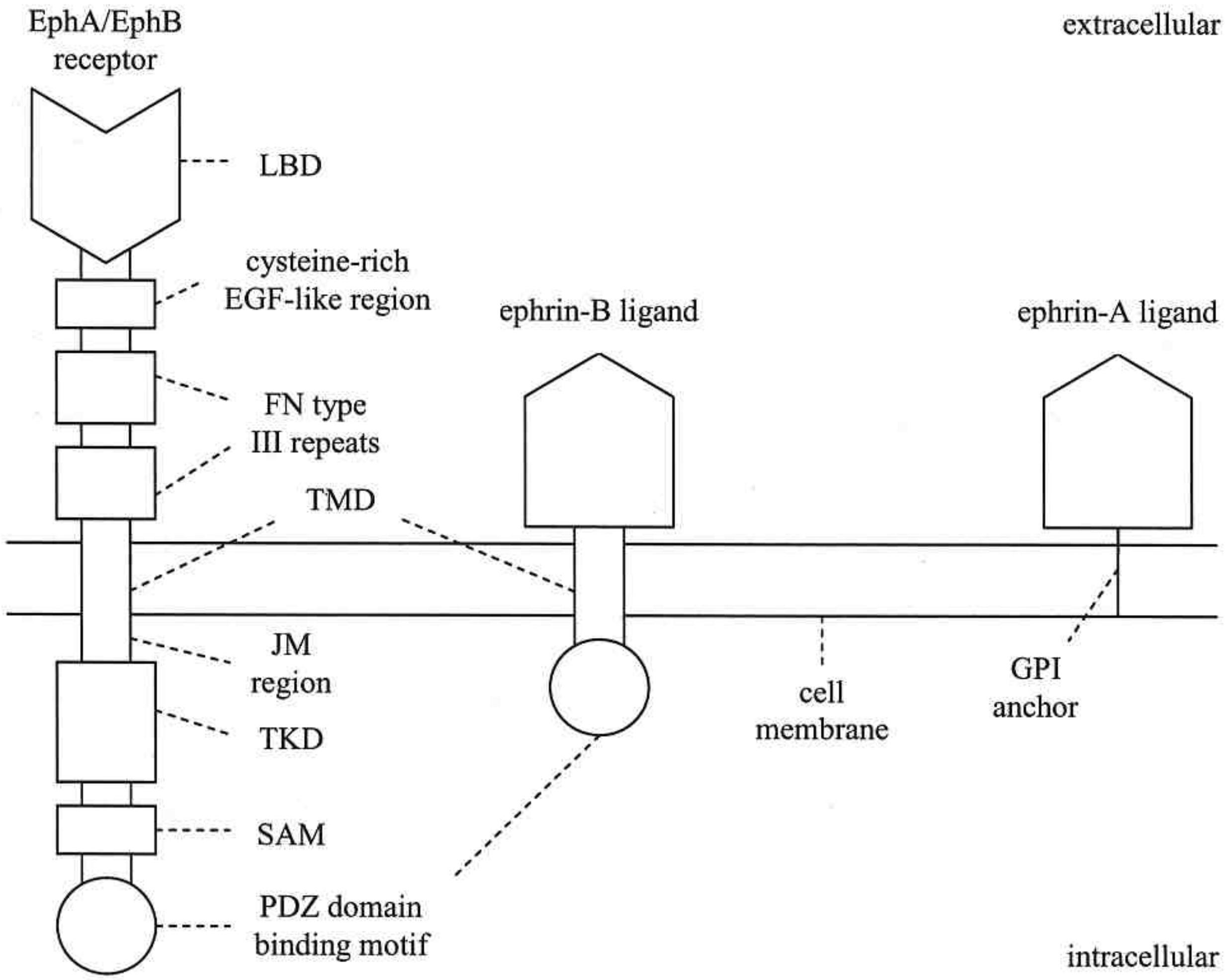
1.2 Eph Receptors and Ephrin Ligands

Eph family RTKs and their membrane-anchored protein ligands, the ephrins, also play important roles in establishing the relative positions of cells and tissues in developing embryos. The Eph family represents the largest subgroup of RTKs, with 14 members having been discovered so far in vertebrates (Murai and Pasquale, 2003). Eph receptors can be divided into two classes based on sequence similarity and ligand binding affinity (Eph Nomenclature Committee, 1997 and Gale *et al.*, 1996). A-type Eph receptors (EphA1-8,10) bind promiscuously to glycosylphosphatidylinositol (GPI)-linked ephrin-A ligands (ephrin-A1-6), whereas B-type Eph receptors (EphB1-4, 6) bind promiscuously to transmembrane ephrin-B ligands (ephrin-B1-3). The only known exceptions to this rule are EphA4, which can bind ephrin-B2 and 3 in addition to the ephrin-As (Gale *et al.*, 1996), and EphB2, which can bind ephrin-A5 in addition to the ephrin-Bs (Himanen *et al.*, 2004). In going from amino to carboxy-terminus, Eph receptors are comprised of an extracellular domain with a globular ligand binding domain (LBD), a cysteine-rich epidermal growth factor (EGF)-like region, and two FN type III repeats, a transmembrane domain (TMD), and an intracellular domain with a juxtamembrane (JM) region, a protein tyrosine kinase domain (TKD), a sterile alpha motif (SAM), and a PDZ domain binding motif (Kullander and Klein, 2002) (Fig. 1).

The interaction between Eph and ephrin results in the phosphorylation of two tyrosine residues in the receptor's JM region (Kalo and Pasquale, 1999). These phosphotyrosines are required for full activation of the receptor's tyrosine kinase activity and also act as docking sites for Src homology 2 (SH2) domain-containing proteins (Zisch *et al.*, 2000), which in turn act to transmit the signal initiated by ephrin binding to intracellular effectors (Kullander and Klein, 2002). Unlike the ligands for other RTKs, ephrins are membrane-bound and fail to elicit a response from their receptors when presented in a soluble form (Davis *et al.*, 1994). On the other hand, soluble ephrins that have been artificially clustered are functional, indicating that Ephs and ephrins mediate cell contact-dependent interactions and that membrane anchorage facilitates the dimerization/multimerization of receptor-ligand complexes. This leads to activation of receptor catalytic activity and cross-phosphorylation of the JM tyrosine residues.

Figure 1. Domain structure of Eph receptors and ephrin ligands.

Eph receptors are comprised of a series of domains, whereas the simpler ephrins can be distinguished by how they are anchored to the cell membrane. EGF, epidermal growth factor; FN, fibronectin; GPI, glycosylphosphatidylinositol; JM, juxtamembrane; LBD, ligand binding domain; SAM, sterile alpha motif; TKD, tyrosine kinase domain; TMD, transmembrane domain.


EphA/EphB receptor
extracellular
LBD
cysteine-rich EGF-like region
ephrin-B ligand
ephrin-A ligand
FN type III repeats
TMD
JM region
TKD
SAM
PDZ domain binding motif
cell membrane
GPI anchor
intracellular

1.2.1 Functions

Eph and ephrin function is exemplified by the roles that these proteins play in visual topographic map formation and hindbrain segmentation. Retinal ganglion cell (RGC) axons make connections to the optic tectum in birds, fish, and amphibians or the superior colliculus (SC) in mammals in the same order in which they project from the retina, thus preserving nearest neighbour relationships and enabling spatially intact visual images to be transmitted to the brain (O'Leary and Wilkinson, 1999). EphA3 shows increasing expression along the nasal to temporal axis of the retina and is also expressed by RGC axons (Cheng *et al.*, 1995), whereas ephrin-A2 and 5 are expressed in a low anterior to high posterior gradient across the tectum (Monschau *et al.*, 1997). The fact that temporal RGC axons project to the anterior tectum and nasal RGC axons project to the posterior tectum suggests that axons expressing high levels of EphA3 are excluded from tectal regions expressing high levels of ephrin-A2/5 by a receptor-mediated repulsive guidance mechanism (O'Leary and Wilkinson, 1999). In support of this, temporal, but not nasal RGC axons strongly avoid growing out onto ephrin-A2/5-containing substrates *in vitro* (Nakamoto *et al.*, 1996 and Monschau *et al.*, 1997). *In vivo*, patches of ectopic ephrin-A2 expression in the anterior tectum are similarly avoided by just temporal RGC axons (Nakamoto *et al.*, 1996). Ectopic expression of EphA3 in a subset of RGCs causes their axonal connections in the tectum to shift anteriorly (Brown *et al.*, 2000). On the other hand, gene knockout of ephrin-A5 in mouse (Frisen *et al.*, 1998) or ectopic expression of cytoplasmic domain-truncated EphA3 in the chicken retina (Feldheim *et al.*, 2004) results in temporal RGC axons projecting aberrantly to the posterior SC/tectum.

In the hindbrain, EphA4 and EphB2 and 3 are expressed by cells in r3 and 5, whereas ephrin-B1-3 have a complementary distribution in r2, 4, and 6 (Xu *et al.*, 2000). Lineage studies have demonstrated that cells can intermingle within a given rhombomere, but movement beyond that rhombomere's boundaries is restricted (Fraser *et al.*, 1990). Intriguingly, there are large intracellular spaces between the boundaries of adjacent rhombomeres (Heyman *et al.*, 1993), perhaps indicating that a repulsive interaction between Eph-expressing cells in odd-numbered rhombomeres and ephrin-expressing cells in even-numbered rhombomeres contributes to hindbrain segmentation. In *Xenopus* and zebrafish embryos expressing cytoplasmic domain-truncated EphA4, cells with an r3/5 identity appear in the adjacent even-numbered rhombomeres (Xu *et al.*, 1995). When cytoplasmic domain-truncated ephrin-B2 is expressed in a mosaic fashion in the zebrafish embryo, the expressing cells become randomly distributed in r2/4/6, but sort to the boundaries of r3/5 (Xu *et al.*, 1999). These findings indicate that Eph signaling in the hindbrain functions to restrict receptor-expressing cells to the odd-numbered rhombomeres by effecting their repulsion from ligand-expressing cells. Conversely, mosaic expression of truncated EphA4 or EphB2 results in the expressing cells becoming randomly distributed within r3/5 and sorting to the boundaries of r2/4/6. This surprising finding indicates that the ephrin-B2 ligands expressed by even-numbered rhombomere cells can themselves effect the repulsion of Eph-expressing cells, presumably as a result of a 'reverse' signaling mechanism. As such, hindbrain segmentation appears to depend on bidirectional signaling from ephrin-Eph complexes formed between cells at the interfaces of even and odd-numbered rhombomeres. Further support for the existence of bidirectional signaling comes from the finding that intermingling between adjacent cell populations in culture is severely restricted when one group expresses EphA4 or EphB2 and the other expresses ephrin-B2, but not when one group expresses wild-type EphA4/EphB2 and the other expresses truncated ephrin-B2 or *vice versa* (Mellitzer *et al.*, 1999).

1.2.2 Reverse Signaling

An important implication from the above is that ephrins appear to function not only as ligands that stimulate signaling from Eph receptors, but also as receptors in their own right that transmit intracellular signals when engaged by Eph 'ligands'. Powerful evidence in support of this idea comes from the finding that exposure of cells expressing ephrin-B1 or 2 to clustered EphB2 extracellular domains results in these ligands becoming tyrosine-phosphorylated (Holland *et al.*, 1996). This phosphorylation has been mapped to three tyrosine residues in the intracellular domain of ephrin-B1 (Kalo *et al.*, 2001) and is positively regulated by Src family non-receptor tyrosine kinases (NRTKs), which are recruited to and transiently activated in ephrin-B expression domains following engagement with EphB receptors (Palmer *et al.*, 2002). Ephrin-Bs also possess a putative carboxy-terminal PDZ domain binding motif that has been shown to bind to PDZ domain-containing proteins (Lin *et al.*, 1999). There are numerous examples of signaling pathways downstream of both the SH2 domain-containing proteins and PDZ domain-containing proteins that interact with ephrin-Bs (Cowan and Henkemeyer, 2002 and Kullander and Klein, 2002). Despite the fact that ephrin-A ligands lack an intracellular domain, they have also been shown to transmit intracellular signals. Stimulation of ephrin-A5-expressing cells with soluble EphA5 results in the tyrosine phosphorylation of cytoplasmic proteins in a Src family kinase (SFK)-dependent fashion (Davy *et al.*, 1999). This signal, which is thought to be transmitted to the cytoplasm through the association of ephrin-A5 with a transmembrane adaptor protein, functions to regulate the activity of integrins (Davy *et al.*, 2000).

1.2.3 Regulation of Directed Neural Crest Cell Migration

Eph receptors and ephrin ligands are among the most well studied regulators of NC pathfinding. In mouse, EphA4 and EphB1 and 3 are expressed by all three streams of CNC cells, whereas ephrin-B2 has a mutually exclusive distribution around the clefts dividing each branchial arch (Adams *et al.*, 2001). When ephrin-B2 is knocked out, these streams become scattered and CNC cells invade the regions where ephrin-B2 is normally expressed. However, correct targeting is restored by expression of cytoplasmic domain-truncated ephrin-B2, indicating that this ligand contributes to pathfinding in a non-CNC cell autonomous manner. In *Xenopus*, EphA4 and EphB1 are expressed by CNC cells destined for BAIII, whereas ephrin-B2 is expressed by cells along the adjacent BAII pathway (Smith *et al.*, 1997). Expression of cytoplasmic domain-truncated EphA4 and/or EphB1 results in the BAIII CNC mismigrating rostrally into BAII territory. Ephs and ephrins also contribute to NC pathfinding in the trunk region. In mouse, ephrin-B1 and 2 are expressed in caudal somite halves, whereas TNC cells migrating through the rostral somite halves express EphB2 (Wang and Anderson, 1997). Similarly in chicken, EphB3 is expressed by TNC cells and ephrin-B1 is expressed in the caudal half of each somite (Krull *et al.*, 1997). NC cells growing out from the trunk region of isolated mouse or chicken NTs in culture show a repulsive response to ephrin-B ligands. Together, these findings suggest that Eph-expressing NC cells are restricted to stereotypical pathways of migration by receptor-mediated exclusion from regions expressing ephrin ligands.

1.3 Objectives

A number of Eph and ephrin genes are expressed in the hindbrain region of the early chicken embryo. EphA4 is expressed in r3 and 5 and by CNC cells emerging from the NT at a point just caudal to the OV (Hirano *et al.*, 1998). EphB3 expression is prominent in the branchial arches (Baker *et al.*, 2001). Ephrin-A5, ephrin-B2, EphA3, 7, and 9, and EphB1 all show varying distributions in and around the hindbrain (Baker and Antin, 2003). Based on these observations, I set out to determine if Eph receptors and ephrin ligands regulate the directed migration of avian CNC cells. I started by performing a comprehensive survey of Eph and ephrin gene expression patterns during the course of CNC migration in the chicken embryo. I then used my findings to develop and test specific hypotheses on how these genes function. While there is a large body of research dealing with where Eph receptors and ephrin ligands are expressed and how they function, relatively little is known about their evolution. Thus I have also conducted a phylogenetic analysis of these proteins in order to address this issue.

CHAPTER 2

MATERIALS AND METHODS

2.1 Probe Plasmids

Table 1 summarizes where the probes prepared for use in this study align with the corresponding genes in the GenBank database. The identity of each probe sequence was confirmed through BLAST searches (National Center for Biotechnology Information). Specific details for how the various probe plasmids were prepared are as follows.

2.1.1 EphB1 and Eph2

Small blocks of tissue bracketing the OV region were excised from stage 13-15 chicken embryos. Poly(A)+ mRNA was extracted from this tissue with a MicroPoly(A) Pure kit (Ambion) and first strand cDNA was generated from the mRNA with a ProSTAR RT-PCR kit (Stratagene). Degenerate codehop primers (Rose *et al.*, 1998) designed against conserved protein sequences of B-type Eph receptors, EphB-F1 (5'-CATCCTGGTGAACTCCAACCTNGTNTGYAARG-3') and EphB-R1 (5'-TGGATGTGGTTCAGGATCTTCTTYTGRTGNCC-3'), were then used to amplify fragments of EphB receptors from the first strand cDNA by PCR. These fragments were cloned with a TOPO-TA Cloning kit (Invitrogen). Two of the resulting clones were identified as fragments of the chicken EphB1 and 2 genes. In a second round of PCR, primers EphB1-F1 (5'-GAATTCGGATCTTCTTCTGGTGCCCA-3') and EphB1-R1 (5'-GAATTCGGATCTTCTTCTGGTGCCCA-3') were used to add EcoRI and PstI cut sites to the ends of the EphB1 fragment. This reaction product was subsequently cloned between the EcoRI and PstI sites of pBluescript II SK +. Likewise, primers EphB2-F1 (5'-CTCGAGTGGACAGCGCCTGAGGCAAT-3') and EphB2-R1 (5'-ACTAGTGTTCAGAATTTTCTTCTGGT-3') were used to add SpeI and XhoI sites to the EphB2 fragment so that it could be cloned between the same sites in pBluescript II KS +.

Table 1. Alignment of the probe sequences prepared for use in this study with the corresponding genes in the GenBank database.

Gene	Accession no.	Nucleotide range covered by probe	Domains included
ephrin-B1	NM_205035	388-814	most of the extracellular domain
ephrin-B2	AF180729	582-889	TMD and most of the intracellular domain
EphA3	M68514	365-814	most of the LBD
EphA4	D38174	2182-2692	most of the TKD
EphA7	Y14271	819-1278	region between the LBD and the 1st FN type III domain
EphB1	XM_422685	3400-3970	most of the TKD and SAM
EphB2	NM_206951	2429-2943	most of the TKD and SAM
EphB3	Z19061	291-756	part of the LBD

2.1.2 EphA7, EphA7, and EphB3

NTs from stage 10-12 chicken embryos were isolated and grown overnight in culture (see below). cDNA was extracted from the NTs and their associated cell outgrowths with a Cells-to-cDNA II kit (Ambion) and used as template in PCRs with gene-specific primers. The products from these reactions were cloned with a TOPO-TA Cloning kit. Primers EphA3-F1 (5'-GTCGACCTGGGCACTTGCAAAGAG-3') and EphA3-R1 (5'-GGTACCGCCAGCATTACACAAGCAC-3') were used to amplify a fragment of the chicken EphA3 gene that was subsequently subcloned between the BamHI and EcoRV sites of pBluescript II SK +. Primers EphA7-F1 (5'-GAGCTCTACTACAAGAAGTGCTGGTC-3') and EphA7-R1 (5'-GGTACCGAGGACTCCACTCTAAACTC-3') were used to amplify a fragment of the chicken EphA7 gene that was subsequently subcloned between the KpnI and SacI sites of pBluescript II SK +. Finally, primers EphB3-F1 (5'-GAGCTCCCTGCAAAGAGACCTTCAAC-3') and EphB3-R1 (5'-GGTACCTTCATGGCTGGCTCGTAC-3') were used to amplify a fragment of the chicken EphB3 gene that was subsequently subcloned between the EcoRV and PstI sites of pBluescript II SK +.

2.1.3 Ephrin-B1 and EphA4

Plasmids containing chicken ephrin-B1 and EphA4 sequences (provided by E. Pasquale, see Holash *et al.*, 1997 and Sajjadi and Pasquale, 1993) were used as templates in PCRs to isolate shorter fragments of the same sequences. The products from these reactions were cloned with a TOPO-TA Cloning kit. The ephrin-B1 fragment amplified by primers ephrin-B1-F1 (5'-ATCGATGAGTGGGAAAGGGTTGGTC-3') and ephrin-B1-R1 (5-GTCGACGTTGTCTGCCTCCTTGCTG-3') was cloned between the ClaI and SalI sites of pBluescript II SK +. The EphA4 fragment amplified by primers EphA4-F1 (5'-GAGCTCTTCGTGGCATCGGCTCAGGA-3') and EphA4-R1 (5'-

GAATTCCTGGAGCTCTCGCTGCCTGT-3') was cloned between the EcoRI and SacI sites of pBluescript II SK +.

2.1.4 Ephrin-B2

Plasmid containing a chicken ephrin-B2 sequence was provided by K. Patel (Othman-Hassan *et al.*, 2001). A PstI/SacI restriction digest fragment of this sequence was cloned between the same cut sites in pBluescript II SK +.

2.1.5 Ephrin-A5 and EphA1

Probe plasmids for ephrin-A5 and EphA1 (sometimes referred to as EphA9) were provided by P. Antin (Baker and Antin, 2003).

2.2 *In Situ* Hybridization

RNA *In situ* hybridizations (ISHs) were carried out essentially as described in Streit and Stern (2001). Digoxigenin-labeled sense and anti-sense RNA probes were generated by linearizing each probe plasmid with the appropriate restriction enzyme and then transcribing with T3 or T7 RNA polymerase (Promega) according to the manufacturer's instructions. Dissected stage 11-15 chicken embryos were fixed for 2 hrs with ice-cold 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) and then stored overnight in methanol at 4°C.

The next day, the embryos were rehydrated in 75% methanol in PBS with 0.05% Tween (PBST), then 50% methanol in PBST, and finally 25% methanol in PBST before being washed two times for 5 min each in PBST. The embryos were then bleached in 6% hydrogen peroxide in PBST for 1 hr, washed three times for 5 min each in PBST, treated with 10 µg/ml proteinase K (Roche) in PBST for 30 min, rinsed with PBST, fixed again with a solution of 4% PFA with 0.1% glutaraldehyde in PBST for 20 min, rinsed twice with PBST, and incubated in hybridization buffer (50% formamide, 1.3X SSC, 50 µg/ml yeast tRNA from Invitrogen, 100 µg/ml heparin, 0.2%

Tween-20, 0.5% CHAPS, 5 mM EDTA) for 3 hrs at 70°C. Hybridizations were carried out overnight at 70°C with probes diluted to approximately 500 ng/ml in fresh hybridization buffer.

The next day, the embryos were rinsed three times with pre-warmed hybridization buffer, washed two times for 30 min each at 70°C in hybridization buffer, washed for 20 min in a pre-warmed solution of 1:1 hybridization buffer and Tris buffered saline with Tween (TBST) (137 mM NaCl, 2.7 mM KCl, 25 mM Tris, 0.05% Tween, pH 7.6), rinsed three times with TBST, and washed three times for 30 min each in TBST. The embryos were then incubated in a blocking buffer containing 5% lamb serum and 1 mg/ml bovine serum albumin (BSA) (Sigma) in TBST for 3 hrs before being left overnight at 4°C in fresh blocking buffer containing alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Roche) that had been pre-adsorbed against chicken embryo powder and diluted to 1:5000.

The next day, the embryos were rinsed three times with TBST, washed three times for 1 hr each in TBST, washed two times for 5 min each in alkaline phosphatase buffer (100mM NaCl, 100 mM Tris, 5 mM $MgCl_2$, 0.05% Tween, pH 9.5), and finally incubated in a solution containing 120 μg/ml of BCIP and 300 μg/ml of NBT (both from Roche) in alkaline phosphatase buffer. Once staining could be detected, the reaction was stopped by washing the embryos two times for 10 min each in TBST.

2.3 Antibody Labeling and Sectioning of Stained Embryos

Embryos stained by the ISH procedure were incubated overnight at 4°C with the HNK-1 primary antibody (American Type Culture Commission) diluted to 1:100 in a blocking buffer containing 5% lamb serum in TBST. After being washed five to eight times for one hour each in TBST, the embryos were incubated overnight at 4°C with Alexa Fluor 568 goat anti-mouse secondary antibody (Molecular Probes) diluted to 1:400 in blocking buffer. Embryos exhibiting strong HNK-1 and probe labeling after being washed another five to eight times for 1 hr each in

TBST were prepared for sectioning by being embedded in Optimal Cutting Temperature Compound (Sakura) and flash frozen in liquid nitrogen. 10 μm cross sections were collected with a cryostat, placed onto gelatin-coated slides, and rehydrated with a mounting medium comprised of 1:1 PBS and glycerol. Sections from along the length of the rostrocaudal axis of these embryos were thoroughly examined for the presence of probe and/or HNK-1 staining. In the interest of conserving space however, only those deemed to be the most representative examples of the staining patterns observed are presented in the results section.

2.4 Cell Culture

Chicken NT explants were prepared largely according to Newgreen and Murphy (2000). Stage 10-12 chicken embryos were harvested in PBS and dissected such that the extraembryonic membranes, head, and tail were removed, leaving only a small block of tissue around the OV. Tissue pieces were rinsed briefly with PBS, transferred to a solution of 400 μg/ml Dispase I neutral protease (Roche) in PBS, incubated at 37°C for 30 min, and then transferred to a 35 mm Petri dish flooded with PBS containing 5% fetal bovine serum (FBS) (Gibco). Using fine tungsten needles, the NTs were gently teased away from the surrounding tissue, including endoderm, ectoderm, somites, and head mesenchyme (although whenever possible, the notochord was left intact to mark the ventral surface of the NT). The isolated NTs were then rinsed briefly in a culture medium consisting of D-MEM/F-12 with 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM MEM sodium pyruvate, 2 mM L-glutamine, 5% FBS (Gibco), and 1% OPI media supplement (Sigma) and transferred in a single 100 μl volume of fresh medium onto an 18 mm glass coverslip affixed to the center of a new 35 mm Petri dish with a small drop of Matrigel (BD Biosciences). The Petri dish contained an additional 900 ul of medium and the coverslip had pre-coated with 0.1% (w/v) poly-L-lysine (PLL) (Sigma) according to Nguyen-Ba-Charvet *et al.* (1999) and subsequently coated with substrate proteins (details for specific experiments are given below). After the NTs were positioned on the substrate (ideally lying flat on their sides), 500 μl of medium was immediately withdrawn from the dish to strand them in place. The dish was then carefully transferred to a tissue culture incubator (containing 5% CO_2 and heated to 37°C) and left there undisturbed for 1 hr to give the NTs a chance to adhere to the substrate, after which the dish was topped up with 500 μl of fresh medium and the NTs were left to incubate overnight.

2.5 Fc Fusion Protein Staining

The protocol used for staining cultured chicken CNC cells with Fc fusion proteins was adapted from Prin *et al.* (2005). NT explant cultures were grown overnight on a FN-coated coverslip. The next day, the NTs and the cells that had grown out from them were blocked with culture medium containing 1 mg/ml BSA for 30 min in a tissue culture incubator. After being rinsed with Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, pH 7.2), the cells were incubated for 1 hr at 4°C in fresh Ringer's containing 1 mg/ml BSA and 10 μg/ml of the Fc domain of human immunoglobulin G (IgG) or a chimeric protein made up of the extracellular domain of mouse ephrin-B1 or EphB2 fused to the Fc domain of human IgG (all from R&D Systems). The cells were then rinsed with Ringer's, fixed with 2% PFA in Ringer's for 10 min, and rinsed again with Ringer's. Finally, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated, Fc specific anti-human IgG (Sigma) diluted 1:200 in Ringer's with 1 mg/ml BSA for 30 min, rinsed with Ringer's, briefly incubated in 4',6-diamidino-2-phenylindole (DAPI) nuclear stain diluted 1:1000 in Ringer's, and rinsed again in Ringer's. The stained cells were then prepared for imaging by being inverted onto a drop of mounting medium on a microscope slide.

2.6 Preparation of Stripe Assay Substrates

Stripe assay substrates were prepared with purified proteins as in Hornberger *et al.* (1999) and Weinl *et al.* (2003). The template matrix used for applying stripes of protein to culture substrates was supplied by S. Lang (Vielmetter *et al.*, 1990). In brief, 64 μg/ml of ephrin-B1 or

EphB2 Fc fusion protein or Fc protein alone was pre-clustered for 1 hr with 640 μg/ml of Fc specific anti-human IgG (Sigma) and 110 μg/ml of an FITC marker conjugated to BSA (Sigma). A PLL-coated coverslip was then firmly pressed onto the stripe assay template and the Fc protein solution was drawn into the channel system with a micropipette. After 1 hr in a tissue culture incubator, the channels were flushed with PBS and the coverslip was gently removed from the template and affixed stripe side up to the center of a 35 mm Petri dish with Matrigel. The coverslip was then blanketed with a solution of 50 mg/ml FN (Sigma) in PBS and incubated again for 1 hr in a tissue culture incubator. Once NT explants (see above) were ready to be transferred to the dish, the coverslip was rinsed with PBS and the dish was flooded with 900 μl of culture medium. The NT explants were ideally positioned on these substrates such that they were perpendicular with the stripes.

2.7 Soluble Competitor Experiments

Substrates with stripes of immobilized ephrin-B1 or EphB2 Fc fusion protein were prepared as described above. Chicken NT explants were isolated and placed on top of these substrates. After withdrawing enough medium to strand the explants in place, soluble Fc proteins were added to the remaining medium to a final concentration of 10 μg/ml. With the ephrin-B1/Fc substrate, either soluble ephrin-B1/Fc or soluble Fc alone was added. Likewise with the EphB2/Fc substrate, either soluble EphB2/Fc or soluble Fc alone was added. The medium added to the dish after the NT explants had been given time to attach to the substrate also contained 10 μg/ml of soluble Fc protein.

2.8 Antibody Labeling of Outgrowth Cultures

NT explant cultures that showed exuberant cell outgrowth on stripe assay substrates after the overnight incubation period were processed for immunolocalization of HNK-1. First, cultures were rinsed with PBS and fixed with ice-cold methanol for 10 min. Fixed cultures were rinsed successively with PBS and TBST, then incubated in blocking buffer (5% FBS in TBST). After 1 hr, the blocking buffer was replaced with HNK-1 antibody diluted 1:100 in fresh blocking buffer and the cultures were incubated in a humidified chamber at 4°C overnight. In some cases where the signal from the FITC-BSA marking the Fc protein stripes was weak, FITC-conjugated anti-human IgG was also added at a 1:100 dilution to make the stripes more apparent. The next day, the cultures were rinsed thoroughly with TBST and incubated with secondary antibody diluted to 1:400 in blocking buffer. After 90 min, the cultures were again rinsed thoroughly with TBST and incubated briefly with DAPI diluted 1:1000 in TBST. The coverslip on which the explants were cultured was then gently removed from the dish and inverted onto a drop of mounting medium on a microscope slide.

2.9 Quantification of Outgrowth Cultures

For any given field of cell outgrowth, three images were taken: one each for HNK-1 and DAPI staining and a third for the underlying FITC-marked protein stripes. Layered composites of these images were assembled in Adobe Photoshop 6.0.1 (Adobe Systems Inc.), such that the total HNK-1-positive cell outgrowth was represented in each composite. The HNK-1 layer was used to identify and remove from the composite image HNK-1-negative cells (i.e. those cells labeled by DAPI alone) as well as HNK-1-positive cells that were extensively intermingled with HNK-1-negative cells. The remaining cells in the DAPI layer were then divided according to their positions relative to the FITC-marked stripes, resulting in a set of two images for each explant representing outgrowth on stripes and outgrowth between stripes. On stripe and between stripe cell counts were obtained with Image-Pro Plus (Media Cybernetics, Inc.), converted to percentages of the total outgrowth, averaged for each treatment, and graphed with SigmaPlot (SPSS Inc.). Statistical measurements (standard deviations and contingency table analyses by Fisher's exact test) were done with GraphPad InStat (GraphPad Software, Inc.).

2.10 Image Capturing and Figure Preparation

ISH images were captured with a Zeiss epifluorescence microscope (Carl Zeiss, Inc.) and DAGE-MTI 3CCD camera (DAGE-MTI, Inc.) using Scion Image 4.0.3 (Scion Corporation). Cell culture images were captured with a Leica DM 6000 B epifluorescence microscope (Leica Microsystems) and Hamamatsu Orca-ER camera (Hamamatsu Photonics) using Openlab 4.0.4 (Improvision Ltd.). Images were captured as tif files and imported into Adobe Photoshop, where they were adjusted (e.g. brightness/contrast enhancement, cropping) and subsequently assembled into figures.

2.11 Preparation of Phylogenetic Trees

Full length Eph and ephrin amino acid sequences were retrieved (from http://www.ncbi.nlm.nih.gov/Genomes/ and http://genome.jgi-psf.org/Cioin2/Cioin2.home.html, see Appendix for aligned sequences) for organisms whose genomes have been completely sequenced. Note that if sequences from fully sequenced genomes were available but fragmentary, these organisms were not included in the analysis (e.g. the sequences for zebrafish ephrins are intact, but the receptors were found to be in fragments that could not be easily assembled). As such, the final organism list is comprised of *Caenorhabditis elegans* (nematode), *Drosophila melanogaster* (insect), *Strongylocentrotus purpuratus* (echinoderm), *Ciona intestinalis* (urochordate), *Xenopus laevis* (amphibian), *Gallus gallus* (bird), and *Homo sapiens* (mammal). Sequences were aligned in MEGA 3.1 and assembled into trees using the maximum parsimony, neighbour joining, and minimum evolution tree building methods. Maximum likelihood trees were derived from these sequences using the PHYLIP program. Trees were created under the Jones-Taylor-Thornton model of amino acid substitution (where appropriate) using 100 bootstrap replications.

CHAPTER 3

EPH AND EPHRIN-MEDIATED REGULATION OF NEURAL CREST MIGRATION

CNC cells migrate to their destinations in segregated streams. Evidence from mouse and *Xenopus* indicates that Eph receptors and their ephrin ligands contribute to this behaviour. Ephs and ephrins have also been shown to be expressed in the chicken embryo, which prompted me to ask whether these proteins regulate the directed migration of avian CNC cells. As it turns out, avian CNC cells do indeed express Eph receptors and ephrin ligands, both of which appear to be responsible for confining these cells to stereotypical pathways of migration.

3.1 Results

3.1.1 Co-localization of Eph/Ephrin RNA and Neural Crest

Avian Eph and ephrin gene expression patterns were evaluated by RNA ISH. Based on findings from previous studies (Baker, 2001, Baker and Antin, 2001, and Hirano *et al.*, 1998) as well as my own preliminary cloning experiments, I focused my analysis on ephrin-A5, ephrin-B1 and 2, EphA1, 3, 4, and 7, and EphB1-3. CNC cells were co-localized by double labeling with an antibody that recognizes a NC antigen, HNK-1. This allowed me to clearly distinguish expression in CNC cells from expression in surrounding cells and tissues, such as ectoderm and head mesenchyme. The stages of development examined range from 11-15, during which most of the CNC cell migration in the chicken embryo takes place (Le Douarin and Kalcheim, 1999). However, only representative examples are shown here. Embryos probed with control sense transcripts developed weak background staining or no colour at all (data not shown). Analysis of the CNC subpopulation associated with BAI has been excluded due to technical problems with resolving NC staining from staining in the hindbrain. ISHs for ephrin-A5 and EphA1 failed to detect anything other than background staining.

3.1.1a EphA3, EphA7, and EphB3

The mRNAs for EphA3 and 7 and EphB3 have a common distribution in streams of cells on either side of the OV. One of these streams reaches from BAII to the upper rostral face of the OV (arrow 1 in Fig. 2Ai, iii, v). A second reaches from BAIII to the upper caudal face of the OV (arrow 2 in Fig. 2Ai, iii, and v). A third reaches from BAIV to a point in the second stream around the lower level of the OV (arrow 3 in Fig. 2Ai, iii, v). The HNK-1 antibody marks streams of cells that have a very similar distribution to those labeled by the EphA3 and 7 and EphB3 probes (Fig. 2Aii, iv, vi). In cross-section, these probes localize to narrow bands of cells in the mesenchyme just underlying the ectoderm that reach from the NT to the branchial arches (Fig. 2Bi, iii, v). HNK-1 staining again shows a very similar distribution (Fig. 2Bii, iv, vi), such that the same cells that are labeled by the EphA3 or 7 or EphB3 probe also appear to be labeled by the HNK-1 antibody (arrowheads). These results indicate that EphA3 and 7 and EphB3 are all expressed by avian CNC cells destined for BAII, III, and IV.

3.1.1b EphA4 and EphB1

RNA probes for EphA4 and EphB1 hybridize most strongly to cells in r3 and 5 (Fig. 3Ai, iii). These probes also hybridize to a sharp streak of cells (arrows in Fig. 3Ai, iii) that curls around the caudal margin of the OV and reaches into BAIII. For both EphA4 and EphB1, probe staining appears to coincide only with the stream of HNK-1-positive cells associated with BAIII, but not the closely related branch associated with BAIV (Fig. 3Aii, iv). Cross-sections reveal that both probes hybridize to a narrow band of cells in the mesenchyme immediately underlying the ectoderm between the NT and BAIII (Fig. 3Bi, iii). HNK-1-positive cells have a very similar distribution (Fig. 3Bii, iv), such that EphA4 and EphB1 probe appears to co-localize with the HNK-1 antibody (arrowheads). From these results, I have concluded that CNC in the chicken embryo expresses EphA4 and EphB1, but this expression is restricted to just those cells that migrate to BAIII.

3.1.1c Ephrin-B1

Figure 2. EphA3, EphA7, and EphB3 are expressed by avian cranial neural crest cells. Distribution of EphA3, EphA7, and EphB3 mRNA and HNK-1 protein in the hindbrain region of the stage 14 chicken embryo. (A) Wholemount images. EphA3 probe labels streams of cells (arrows) rostral and caudal to the OV, as does the HNK-1 antibody (i, ii). EphA7 (iii, iv) and EphB3 (v, vi) probe-binding cells have a similar distribution. Insets show the same embryos at lower magnification. (B) Cross section images. Cells labeled by EphA3 probe and HNK-1 antibody (arrowheads) form a narrow, sub-ectodermal band that reaches from the NT to the branchial arches (i, ii). This is also the case with EphA7 (iii, iv) and EphB3 (v, vi). Insets show the same sections at lower magnification. Ec, ectoderm; My, mesenchyme.

Fig. 2A

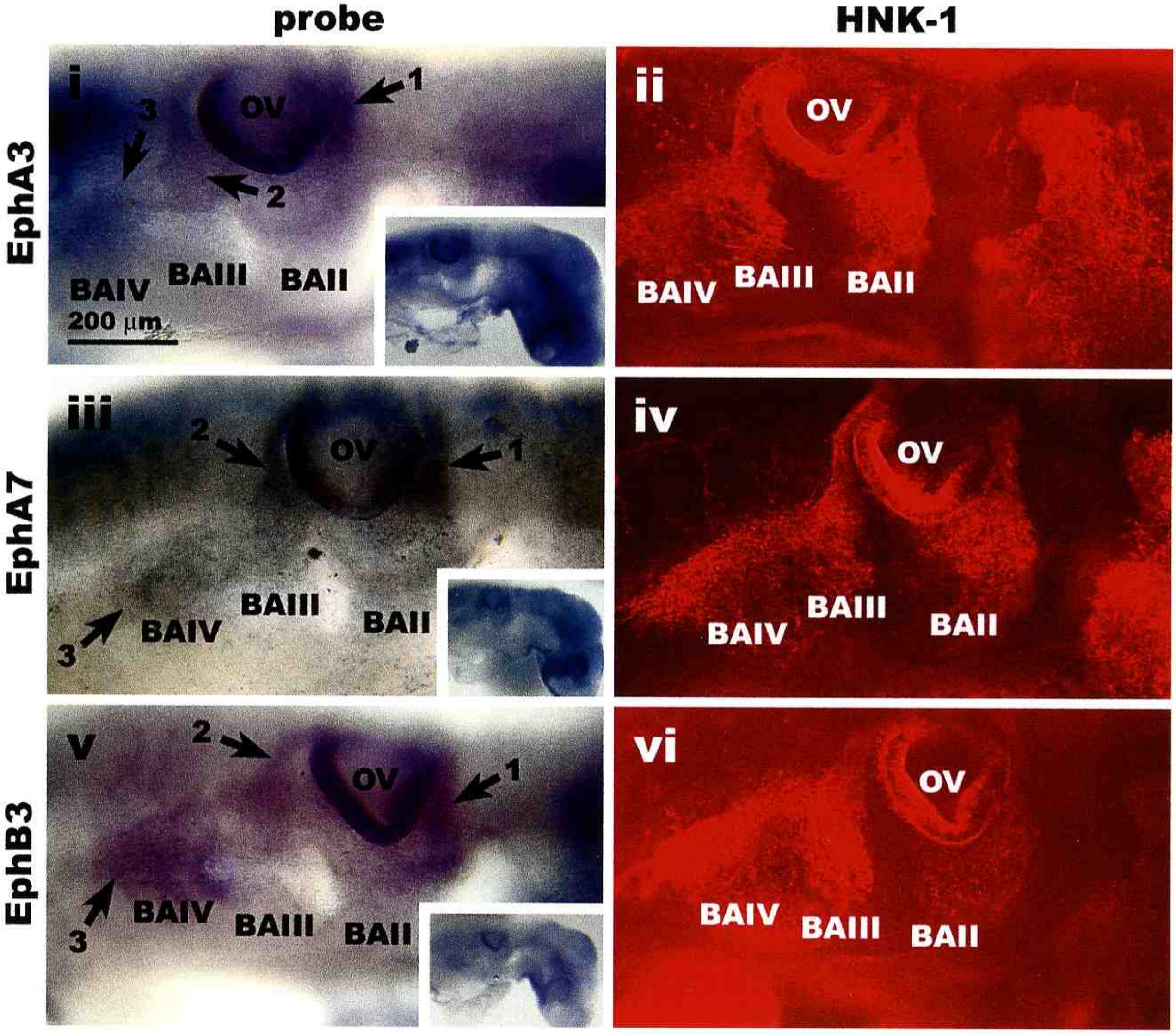

probe
HNK-1
EphA3
EphA7
EphB3
i
ii
iii
iv
v
vi
OV
1
2
3
BAIV
BAIII
BAII
200 µm

Fig. 2B

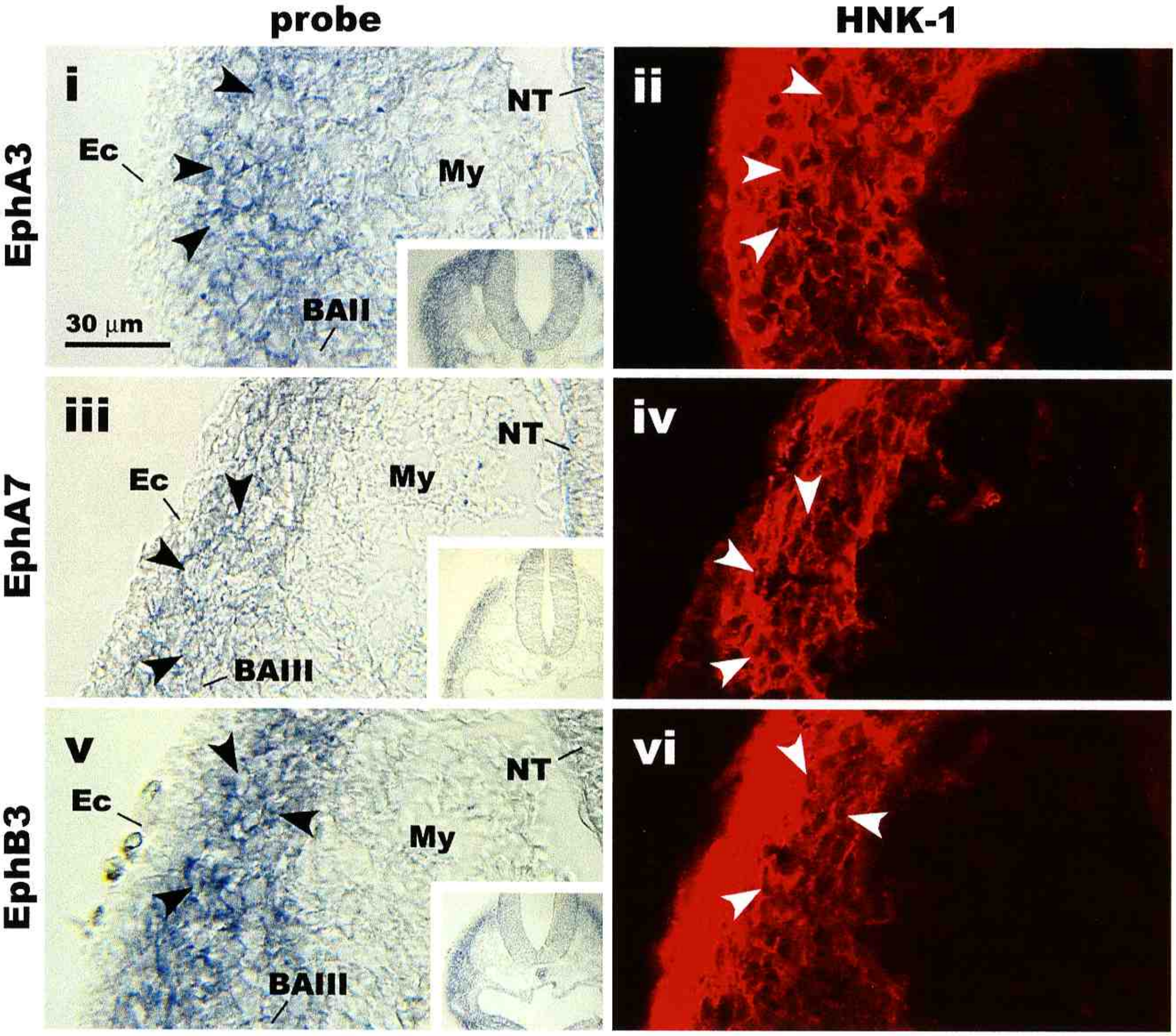

probe
HNK-1
EphA3
EphA7
EphB3
i
ii
iii
iv
v
vi
Ec
NT
My
BAII
BAIII
30 μm

Figure 3. EphA4 and EphB1 are expressed by a subset of avian cranial neural crest cells. Distribution of EphA4 and EphB1 mRNA and HNK-1 protein in the hindbrain region of the stage 14 chicken embryo. (A) Wholemount images. EphA4 probe hybridizes to r3 and 5 and a streak of cells caudal to the OV (arrow) that coincides with the stream of HNK-1-positive cells associated with BAIII (i, ii). EphB1 probe-binding cells have an identical distribution (iii, iv). Insets show the same embryos at lower magnification. (B) Cross section images. EphA4 probe-binding cells overlap with cells stained by HNK-1 (arrowheads in i, ii). This is also the case with EphB1 (iii, iv). Insets show the same sections at lower magnification. Ec, ectoderm; My, mesenchyme.

Fig. 3A

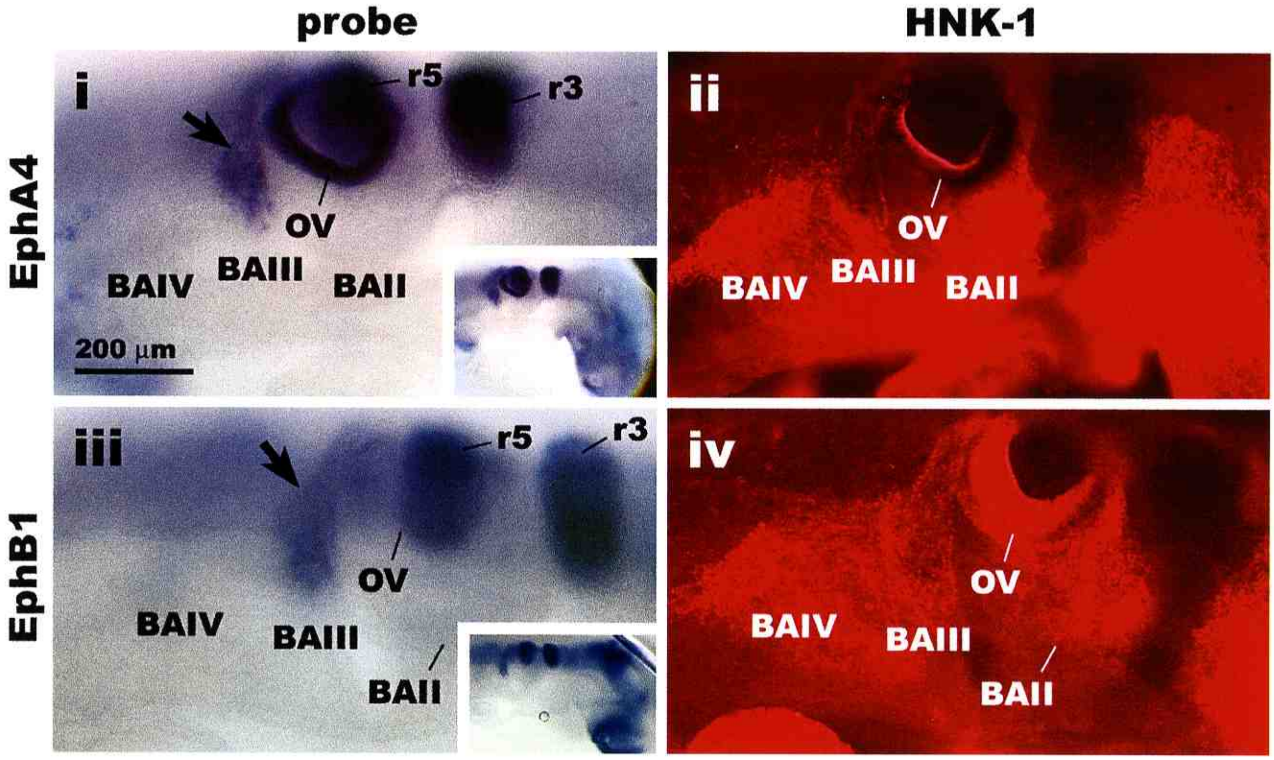
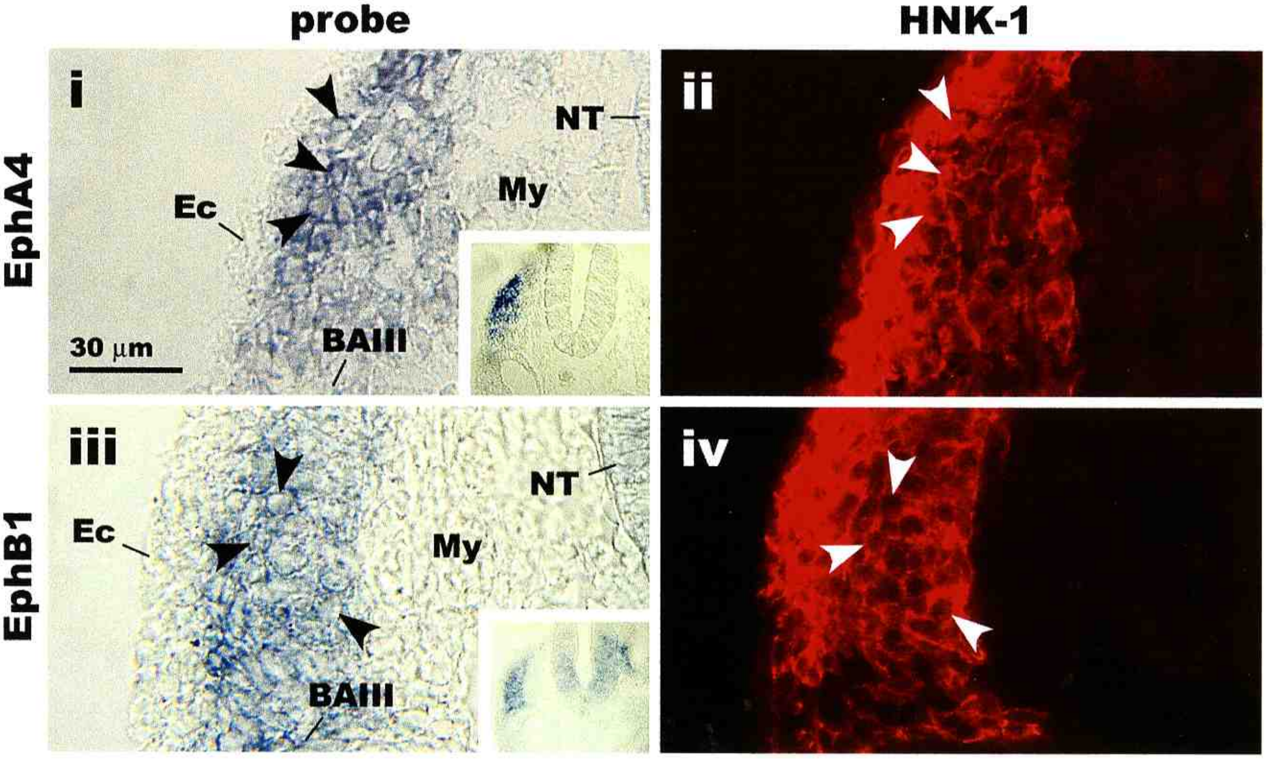

probe
HNK-1
EphA4
EphB1
i
r5
r3
OV
BAIV
BAIII
BAII
200 μm
ii
OV
BAIV
BAIII
BAII
iii
r5
r3
OV
BAIV
BAIII
BAII
iv
OV
BAIV
BAIII
BAII

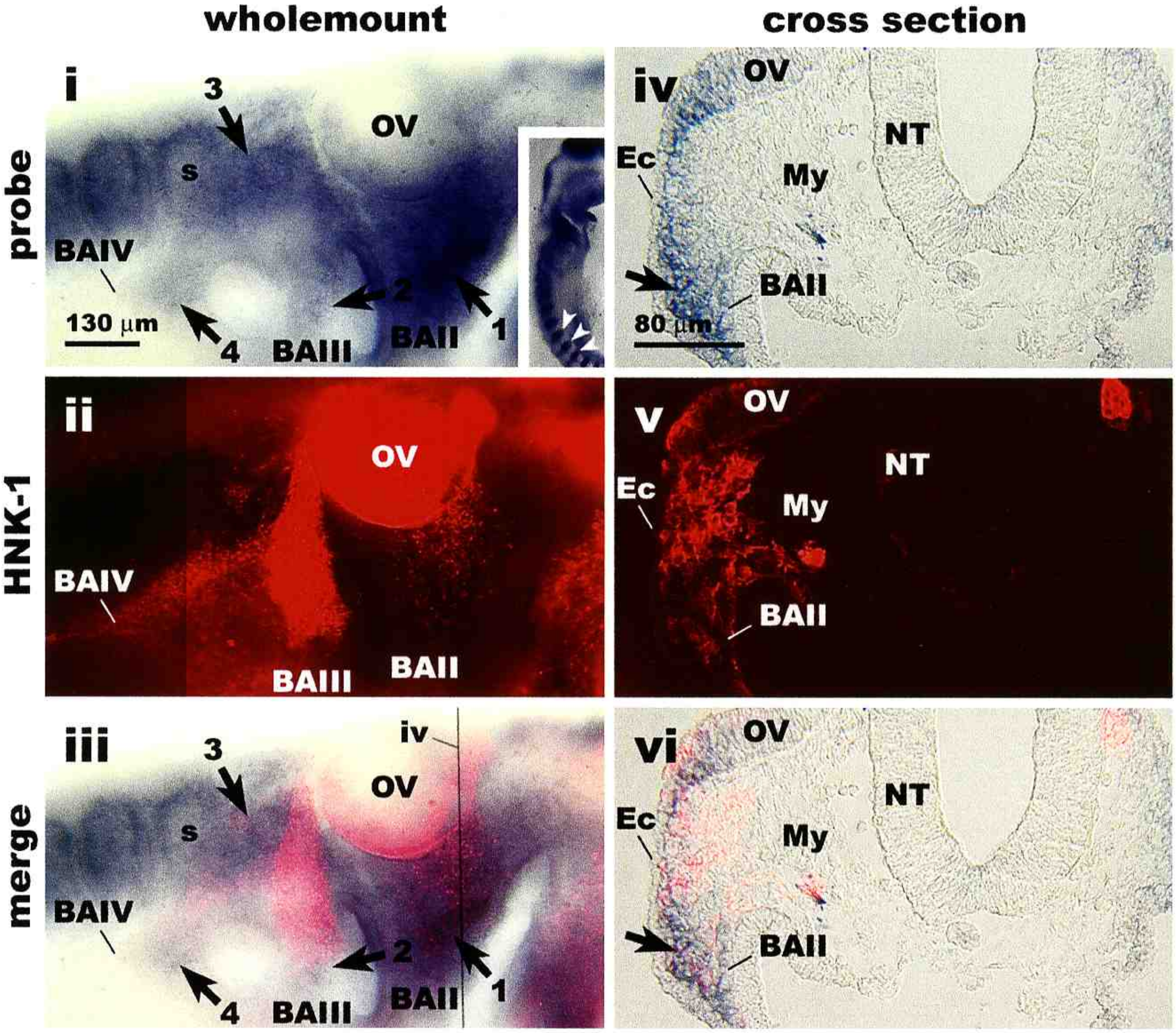
Fig. 3B


probe
HNK-1
EphA4
EphB1
i
ii
iii
iv
NT
My
Ec
BAIII
30 μm
NT
My
Ec
BAIII

Ephrin-B1 probe hybridizes most prominently to the caudal halves of somites (arrowheads, Fig. 4i inset). Intense probe staining is also found in cells within BAII, particularly in a dense band around the rostral and dorsal margin of the arch (arrow 1 in Fig. 4i). This region roughly corresponds to the same point where the stream of HNK-1-positive cells just rostral to the OV (Fig. 4ii) comes to an end (arrow 1 in Fig. 4iii). Ephrin-B1 probe also hybridizes to a thin, diffuse strip of cells that extends from the lower caudal surface of the OV to the cleft between BAII and BAIII and into the rostral side of BAIII for a short distance before bending back in the opposite direction - resulting in a roughly 'j'-shaped pattern (arrow 2 in Fig. 4i). A second strip of diffuse staining angles ventrally away from around the mid caudal face of the OV, such that it overlaps with the first somites, to a point just dorsal to the cleft between BAIII and BAIV (arrow 3 in Fig. 4i). In combination, these two strips resemble an inverted 'v'. Lastly, probe hybridizes weakly to a small circle of cells around the opening to BAIV (arrow 4 in Fig. 4i). The dorsal half of the stream of HNK-1-positive cells just caudal to the OV (Fig. 4ii) is bracketed by the ephrin-B1 probe-binding cells highlighted by arrows 2 and 3 in Fig. 4iii. As is the case in BAII, the ventral limit of this stream roughly coincides with the position of a cluster of ephrin-B1 probe-binding cells within BAIII (arrow 2 in Fig. 4iii). Finally, the branch of HNK-1-positive cells that extends to BAIV (Fig. 4ii) is positioned between the groups of probe-binding cells indicated by arrows 3 and 4 in Fig. 4iii. In a sample cross-section (position indicated by the transparent black line in Fig. 4iii), the ephrin-B1 probe-binding cells indicated by arrow 1 in Fig. 4i and iii are concentrated within the ventral mesenchyme of BAII (arrow in Fig. 4iv). The HNK-1 antibody binds to a band of mesenchymal cells distributed between the lower margin of the OV and the opening of BAII (Fig. 4v). These cells are found no further ventrally than the point where ephrin-B1 probe staining becomes prominent within BAII (arrow in Fig. 4vi). I have therefore concluded that cells expressing ephrin-B1 in the chicken embryo border the pathways taken by and have a complementary distribution

Figure 4. Ephrin-B1 is expressed by cells that have a mutually exclusive distribution with the avian cranial neural crest.

Distribution of ephrin-B1 mRNA and HNK-1 protein in the hindbrain region of the stage 14 chicken embryo. (i-iii) Wholemount images. The stream of HNK-1-positive cells rostral to the OV comes to an end at the point where strong ephrin-B1 probe staining appears in BAII (arrow 1). The stream of HNK-1-positive cells caudal to the OV is bordered by groups of cells (arrows 2-4) labeled by the ephrin-B1 probe. Inset shows the same embryo at lower magnification. (iv-vi) Cross section images. Probe (arrow) and HNK-1 stained cells have a reciprocal distribution. Inset shows the same section at lower magnification. Ec, ectoderm; My, mesenchyme; s, somite.


wholemount
cross section
probe
HNK-1
merge
i
3
OV
s
BAIV
2
1
130 μm
4
BAIII
BAII
ii
iii
iv
iv
Ec
My
NT
BAII
80 μm
v
vi

with CNC cells.

3.1.1d Ephrin-B2

Cells labeled by ephrin-B2 probe have a distribution similar to that of cells labeled by EphA3 or 7 or EphB3 probe. Ephrin-B2 probe stains one stream of cells that spans the distance from the upper rostral face of the OV to BAII (arrow 1 in Fig. 5Ai). A second reaches from a point around the upper caudal margin of the OV to BAIII (arrow 2 in Fig. 5Ai). A shorter streak of probe-binding cells branches off of this second stream around the lower level of the OV and extends to BAIV (arrow 3 in Fig. 5Ai). HNK-1 is similarly distributed in the same region (Fig. 5Aii). In cross-section, the ephrin-B2 probe and HNK-1 antibody both label thin bands of cells just beneath the ectoderm that reach from the NT to the branchial arches (arrowheads in Fig. 5Bi, ii). Therefore, ephrin-B2 is expressed by the CNC cells in the chicken embryo that are destined for BAII, III, and IV, as was the case with EphA3 and 7 and EphB3.

3.1.1e EphB2

EphB2 probe-binding cells, like ephrin-B1 probe-binding cells, have a patchy distribution. Rostral to the OV, EphB2 probe hybridizes to a narrow band of cells that extends dorsally from the cleft between BAI and II, such that a thin space of unstained tissue is left between it and the OV (arrow 1 in Fig. 6i). Just beneath the OV, a broader, flatter band of probe staining is positioned just over the cleft between BAII and III (arrow 2 in Fig. 6i). A third, roughly triangular patch of labeled cells is found over the cleft between BAIII and IV (arrow 3 in Fig. 6i), while a longer streak of probe staining that descends ventrally at an angle from a point just overlapping the first somites, a short distance from the caudal margin of the OV, is separated from this patch by a thin space of unstained tissue (arrow 4 in Fig. 6i). The rostral margin of the OV in combination with the band of EphB2 probe-binding cells marked by arrow 1 in Fig. 6iii forms a channel around the stream of HNK-1-positive cells associated with BAII (Fig. 6ii). Similarly, the spaces between the OV and the

Figure 5. Ephrin-B2 is expressed by avian cranial neural crest cells.

Distribution of ephrin-B2 mRNA and HNK-1 protein in the hindbrain region of the stage 14 chicken embryo. (A) Wholemount images. Ephrin-B2 probe labels streams of cells rostral and caudal to the OV (arrows), as does HNK-1. Inset shows the same embryo at lower magnification. (B) Cross section images. Ephrin-B2 probe-binding cells again co-localize with cells stained by HNK-1 (arrowheads). Inset shows the same section at lower magnification. Ec, ectoderm; My, mesenchyme.

Fig. 5A

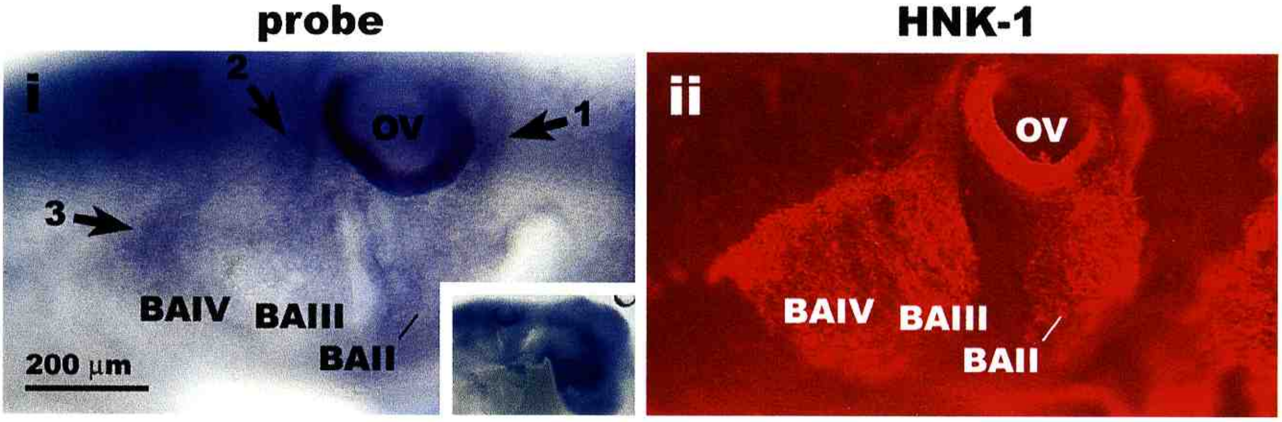

probe
HNK-1
i
2
OV
1
3
BAIV
BAIII
BAII
200 µm
ii
OV
BAIV
BAIII
BAII

Fig. 5B

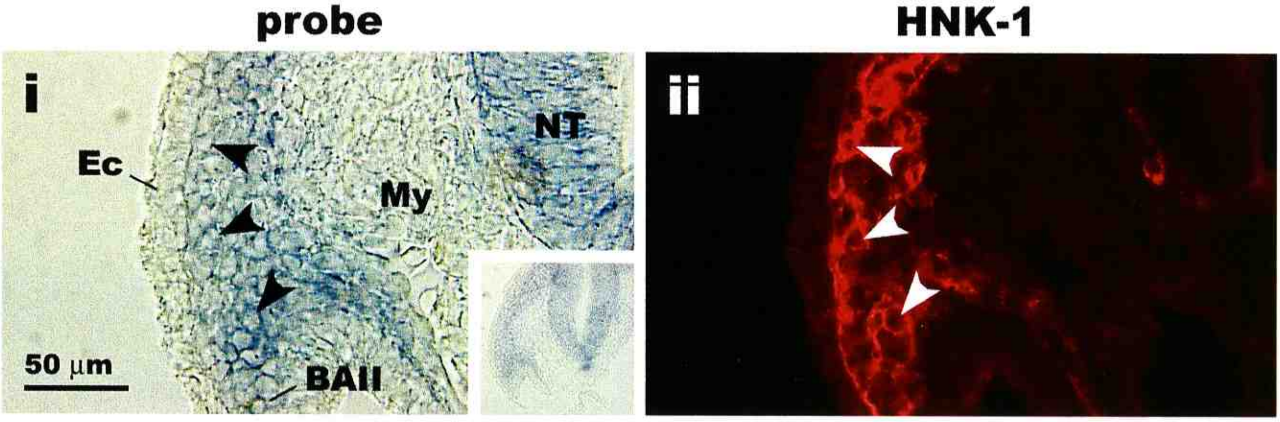
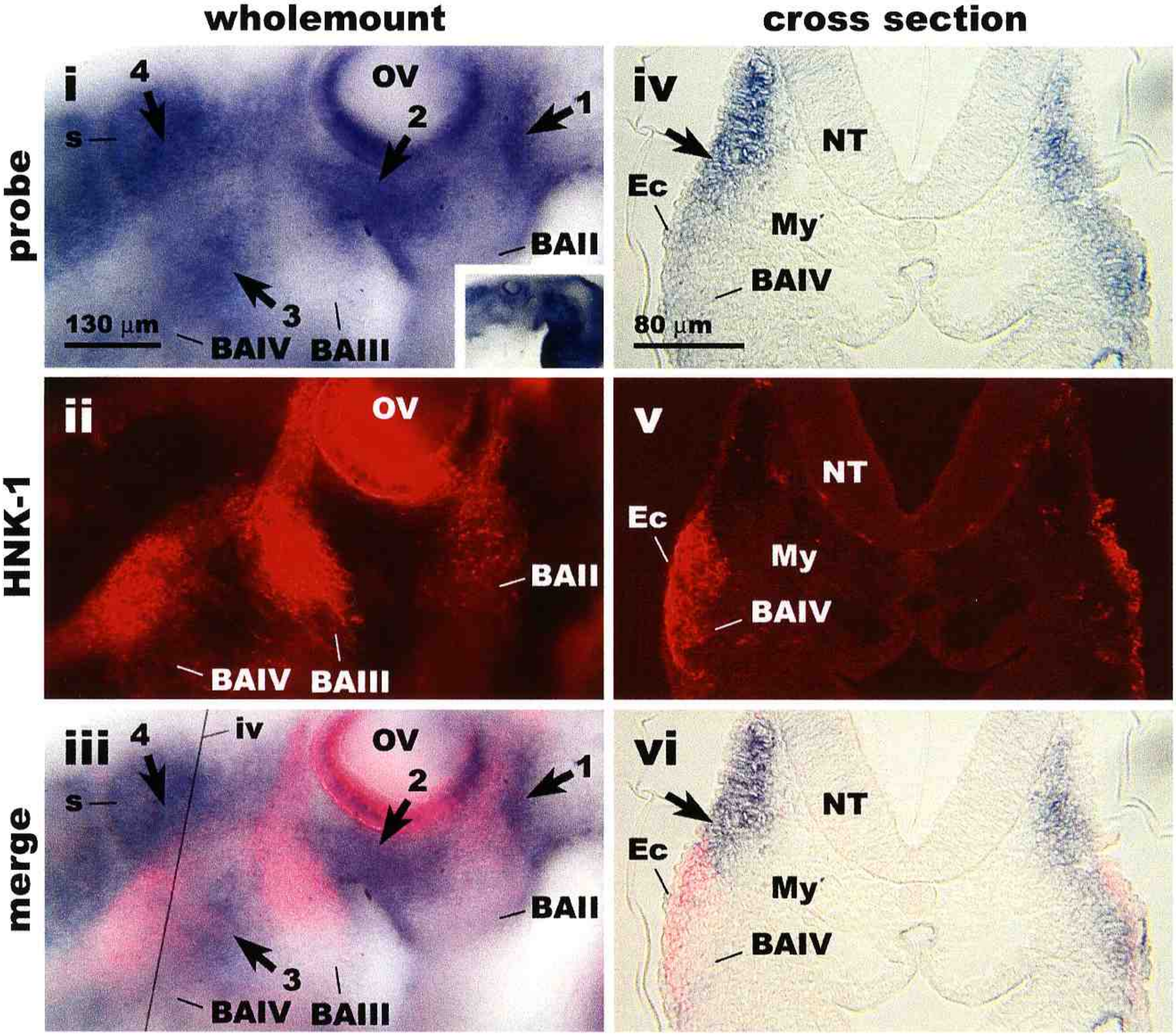

probe
HNK-1
i
Ec
NT
My
50 µm
BAII
ii

Figure 6. EphB2 is expressed by cells that have a mutually exclusive distribution with the avian cranial neural crest.

Distribution of EphB2 mRNA and HNK-1 protein in the hindbrain region of the stage 14 chicken embryo. (i-iii) Wholemount images. EphB2 probe staining is found in patches (arrows) bordering cells labeled by HNK-1. Inset shows the same embryo at lower magnification. (iv-vi) Cross section images. Probe (arrow) and HNK-1 stained cells have a non-overlapping distribution. Inset shows the same section at lower magnification. Ec, ectoderm; My, mesenchyme; s, somite.


wholemount
cross section
probe
HNK-1
merge
i
4
OV
1
2
s
BAII
3
130 μm
BAIV
BAIII
ii
OV
BAII
BAIV
BAIII
iii
4
iv
OV
2
1
s
BAII
3
BAIV
BAIII
iv
NT
Ec
My
BAIV
80 μm
v
NT
Ec
My
BAIV
vi
NT
Ec
My
BAIV

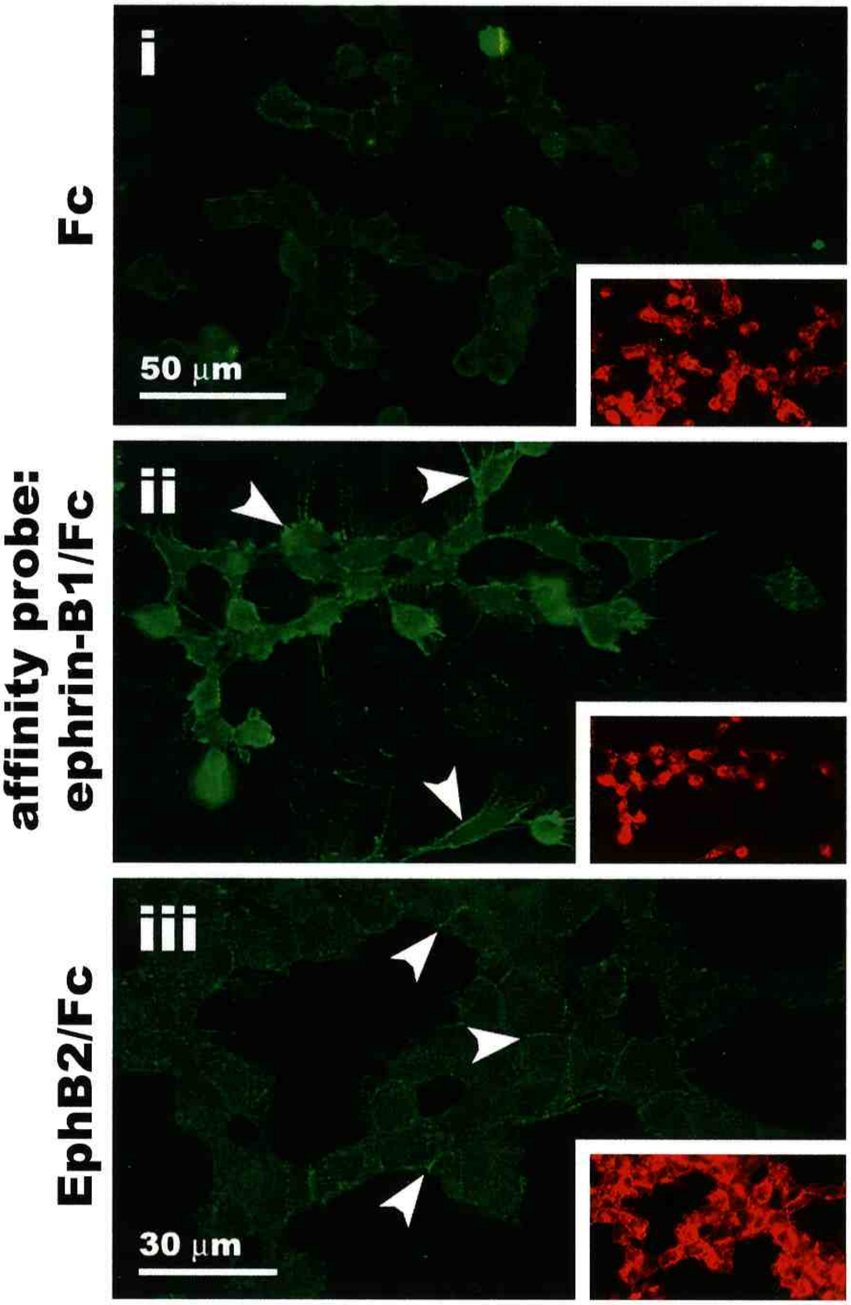
clusters of EphB2 probe-binding cells marked by arrows 2, 3, and 4 in Fig. 6iii approximate the shape of an upside down 'y' into which the bifurcating tract of HNK-1-positive cells caudal to the OV (Fig. 6ii) fits perfectly. In a sample cross-section (position indicated by the transparent black line in Fig 6iii), the patch of probe staining highlighted by arrow 4 in Fig. 6i and iii is located in the mesenchyme just underlying the ectoderm proximal to the NT (arrow in Fig. 6iv). HNK-1 staining in the same section appears in the mesenchyme in and around BAIV, distal to the NT (Fig. 6v). As such, HNK-1 is least concentrated where the EphB2 probe staining is most prominent and *vice versa* (Fig. 6vi). As was the case with ephrin-B1, CNC cells in the chicken embryo therefore have a non-overlapping distribution with cells expressing EphB2, such that they appear to migrate along pathways bordered by EphB2-expressing cells.

However, it is interesting to note that there are subtle differences in the expression patterns for these two genes. For instance, ephrin-B1 expression is concentrated inside of BAII (arrow 1 in Fig. 4iii), whereas EphB2 has a more dorsal distribution in the same region (arrow 1 in Fig. 6iii). Likewise, ephrin-B1 expression just caudal to the OV is focused around the dorsal half of the CNC stream in that region (arrows 2, 3, and 4 in Fig. 4iii), whereas EphB2 expression is more prominent around BAIII and IV (arrows 2, 3, and 4 in Fig. 6iii).

3.1.2 Cell Culture Localizations

My survey of Eph and ephrin gene expression in the hindbrain region of the chicken embryo shows that avian CNC cells express both Eph receptors and ephrin ligands and appear to migrate along pathways bordered by cells that express ephrin-B1 or EphB2. In order to confirm that avian CNC cells express proteins that bind to ephrin-B1 and EphB2, I exposed the outgrowth from stage 10-12 chicken NT explants to affinity probes comprised of the extracellular domain of either ephrin-B1 or EphB2 fused to the Fc domain of human IgG and then stained them with an FITC-conjugated antibody specific for the Fc domain of human IgG. In the control experiment, where

Figure 7. Avian cranial neural crest cells express proteins that bind to ephrin-B1 and EphB2. Binding of ephrin-B1 and EphB2 affinity probes to cultured chicken CNC cells. (i) Cells exposed to Fc protein are not labeled by an anti-Fc antibody. (ii) Cells exposed to ephrin-B1/Fc protein exhibit bright peripheral spots of anti-Fc staining (arrowheads). (iii) Cells exposed to EphB2/Fc protein exhibit weaker anti-Fc staining at points of contact between neighbouring cells (arrowheads). Insets show HNK-1 staining for each field of cells.


Fc
affinity probe:
ephrin-B1/Fc
EphB2/Fc
i
ii
iii
50 µm
30 µm

cells were exposed to the Fc domain of human IgG alone, cells labeled by the HNK-1 antibody fail to be stained by the anti-Fc antibody (Fig. 7i). Conversely, exposure to ephrin-B1/Fc fusion protein results in HNK-1-positive cells showing small and intense foci of anti-Fc staining around the cell periphery (arrowheads in Fig. 7ii). HNK-1-positive cells exposed to EphB2/Fc also exhibit anti-Fc staining, but in this case it is fainter than with ephrin-B1/Fc and most prominent at the interfaces between adjacent cells (arrowheads, Fig. 6iii). Thus avian CNC cells express proteins that bind to ephrin-B1 and EphB2, which is in agreement with the *in situ* localization data.

3.1.3 Stripe Assay Experiments

3.1.3a Without Soluble Competitor

I next wanted to directly test whether avian CNC cells could respond to ephrin-B1 or EphB2 in a manner that is consistent with their distributions *in situ*. I did this by way of the stripe assay, which is a type of experiment in which cells are cultured on a surface patterned with two sets of alternating lanes, each consisting of a different substrate protein composition, and then analyzed for preferential localization to one set of lanes or the other (Walter *et al.*, 1987). In the control experiment, stage 10-12 chicken NT explants were cultured on a substrate made up of lanes of FN alternating with lanes of FN plus an FITC marker and Fc protein pre-clustered with an anti-Fc antibody. Under these conditions, HNK-1-positive cell outgrowth is spread evenly across the two sets of lanes (Fig. 8Ai). In contrast, cell outgrowth is very strongly localized to the lanes between the stripes of FITC marker when ephrin-B1/Fc takes the place of Fc in the stripe assay (Fig. 8Aii). In the corresponding experiment with EphB2/Fc, cell outgrowth is similarly concentrated in the lanes between the FITC stripes (Fig. 8Aiii). In each case, cells labeled by HNK-1 make up a large proportion of the total cell outgrowth, as revealed by DAPI nuclear staining (Fig. 7A insets).

The average distribution of HNK-1-positive cell outgrowth between the two sets of lanes in the three experimental conditions described above is summarized in Fig. 8B. Outgrowth on the

Figure 8. Ephrin-B1 and EphB2 act as repellents for avian cranial neural crest cells. Outgrowth of CNC cells from stage 10-12 chicken NT explants on stripe assay substrates. (A) Representative results from the three experimental conditions. Cells have been double labeled with HNK-1 antibody (red) and DAPI nuclear stain (blue). The position of the first set of lanes is revealed by an FITC marker (faint green stripes) and further highlighted by short white bars on the left side of each panel. (i) Clustered Fc + FN vs. FN (n = 10). Cell outgrowth shows no bias for either set of lanes. (ii) Clustered ephrin-B1/Fc + FN vs. FN (n = 9) and (iii) clustered EphB2/Fc + FN vs. FN (n = 7). Cell outgrowth is almost entirely restricted to the lanes between the FITC marked stripes of ephrin-B1/Fc or EphB2/Fc protein. Insets show total cell outgrowth. (B) Bar graph summarizing the quantified stripe assay results. As in (A), cell outgrowth is abundant on lanes of Fc protein, but much scarcer on lanes of ephrin-B1/Fc or EphB2/Fc protein. Error bars represent standard deviation.

Fig. 8A

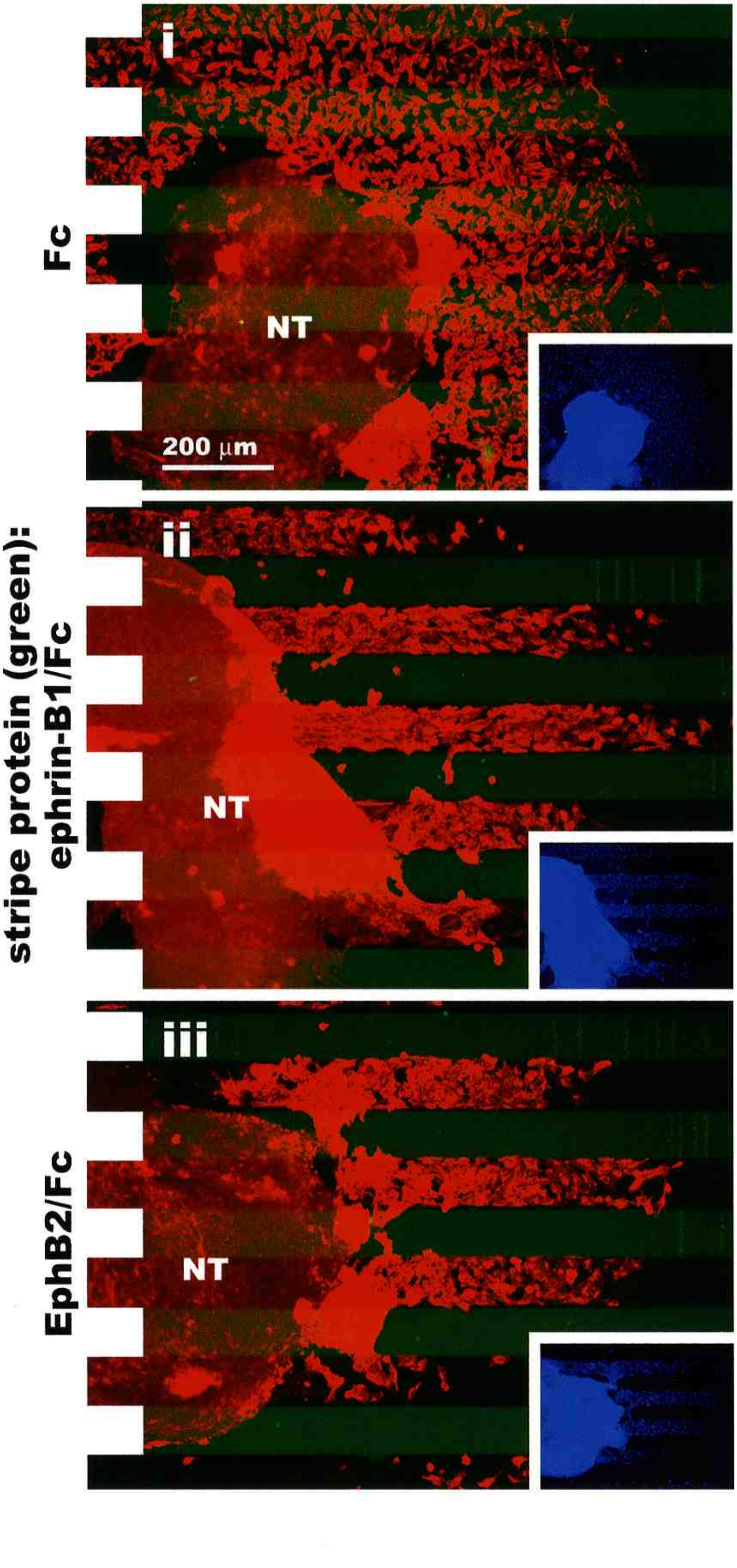
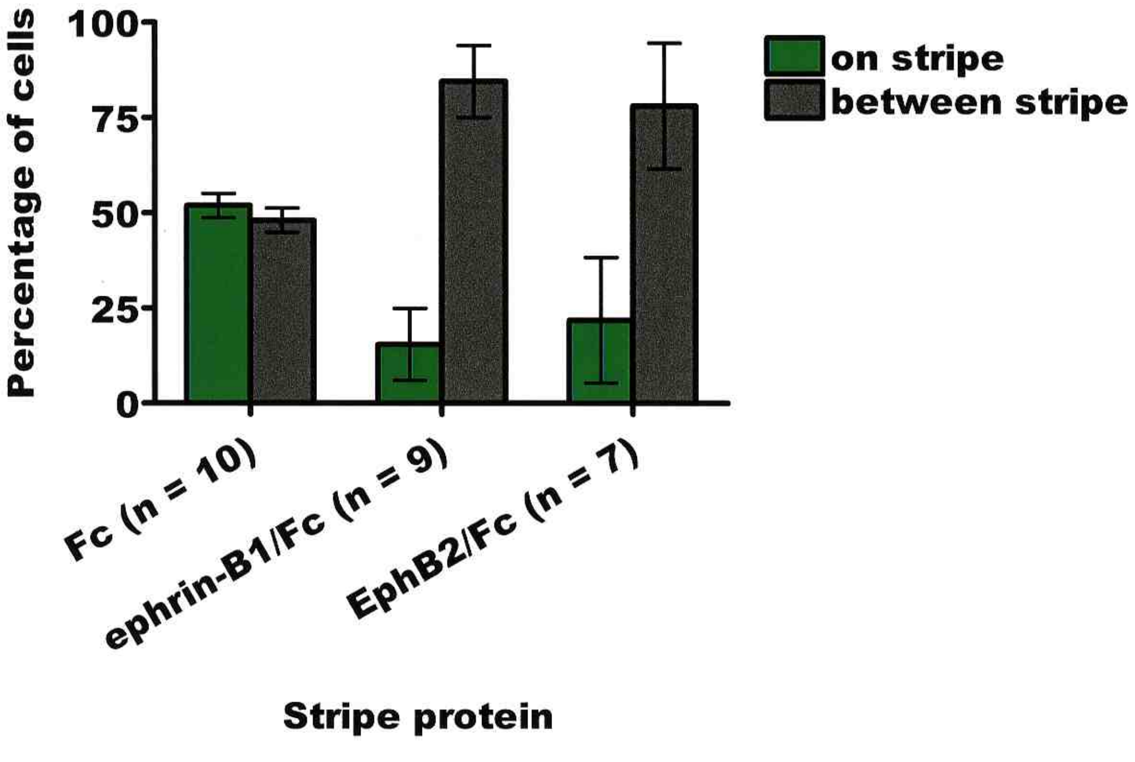

stripe protein (green):
Fc
ephrin-B1/Fc
EphB2/Fc
i
ii
iii
NT
NT
NT
200 μm

Fig. 8B


Percentage of cells
100
75
50
25
0
Fc (n = 10)
ephrin-B1/Fc (n = 9)
EphB2/Fc (n = 7)
Stripe protein
on stripe
between stripe

control substrate has an essentially even distribution, with 52% of the cells being found on the FITC stripes and 48% between them (n = 10). Conversely, outgrowth is highly skewed on the substrate containing lanes of ephrin-B1/Fc, with 15% of the cells being found on the FITC stripes and 85% between them (n = 9). Outgrowth on the substrate containing lanes of EphB2/Fc has a similar distribution, with 22% of the cells being found on the FITC stripes and 78% between them (n = 7). The difference between the control and ephrin-B1/Fc or EphB2/Fc results, as determined by contingency table analysis with Fisher's exact test, is extremely significant ($p < 0.0001$), whereas the difference between the ephrin-B1/Fc and EphB2/Fc results is not significant ($p = 0.2745$). I have therefore concluded that HNK-1-positive cells growing out from chicken NT explants are indifferent to Fc alone in the stripe assay, but avoid both ephrin-B1/Fc and EphB2/Fc strongly and to a similar extent.

3.1.3b With Soluble Competitor

In order to demonstrate that the response of avian CNC to ephrin-B1/Fc and EphB2/Fc in the stripe assay is mediated by the Eph receptors and ephrin ligands that these cells express, I next asked the question of what would happen if the ability of these proteins to associate with substrate bound ephrin-B1/Fc or EphB2/Fc was compromised by the addition of soluble competitors to the culture medium. In the first control experiment, stage 10-12 chicken NT explants were cultured in the presence of soluble Fc protein on a substrate composed of lanes of clustered ephrin-B1/Fc plus FN and an FITC marker alternating with lanes of FN alone. The resulting HNK-1-positive outgrowth is localized to the lanes between the FITC marked stripes of ephrin-B1/Fc protein, such that there are almost no cells on these stripes (Fig. 9Ai). When the same experiment is performed with soluble ephrin-B1/Fc added to the culture medium rather than the control Fc protein, there is a marked increase in the number of cells on the lanes of substrate-bound ephrin-B1/Fc (Fig. 9Aii). In the corresponding experiments with EphB2/Fc, there is a similar increase in the number of cells on

Figure 9. The repellent effect of ephrin-B1 and EphB2 on avian cranial neural crest cells is mitigated by inhibition of Eph or ephrin function.

Outgrowth of CNC cells from stage 10-12 chicken NT explants on stripe assay substrates in the presence of soluble competitors. (A) Representative results from the four experimental conditions. Cells have been double labeled with HNK-1 antibody (red) and DAPI nuclear stain (blue). The position of the first set of lanes is revealed by an FITC marker (faint green stripes) and further highlighted by short white bars on the left side of each panel. (i) Clustered ephrin-B1/Fc + FN vs. FN with soluble Fc ($n = 9$). Cell outgrowth from the NT is strongly localized to the lanes between stripes of FITC-marked ephrin-B1/Fc. (ii) Clustered ephrin-B1/Fc + FN vs. FN with soluble ephrin-B1/Fc ($n = 7$). Substitution of the soluble Fc with ephrin-B1/Fc results in an increase in the number of cells found on the stripes of immobilized ephrin-B1/Fc. (iii) Clustered EphB2/Fc + FN vs. FN with soluble Fc ($n = 4$) and (iv) clustered EphB2/Fc + FN vs. FN with soluble EphB2/Fc ($n = 7$). As with the ephrin-B1/Fc experiments, there is a significant increase in the number of cells on lanes of substrate-bound EphB2/Fc in going from adding soluble Fc to soluble EphB2/Fc to the culture medium. Insets show total cell outgrowth. (B) Bar graph summarizing the quantified stripe assay results. As in (A), there are significantly more cells found growing on lanes of substrate bound ephrin-B1/Fc or EphB2/Fc protein in the presence of soluble ephrin-B1/Fc or EphB2/Fc than in the presence of soluble Fc. Error bars represent standard deviation.

Fig. 9A

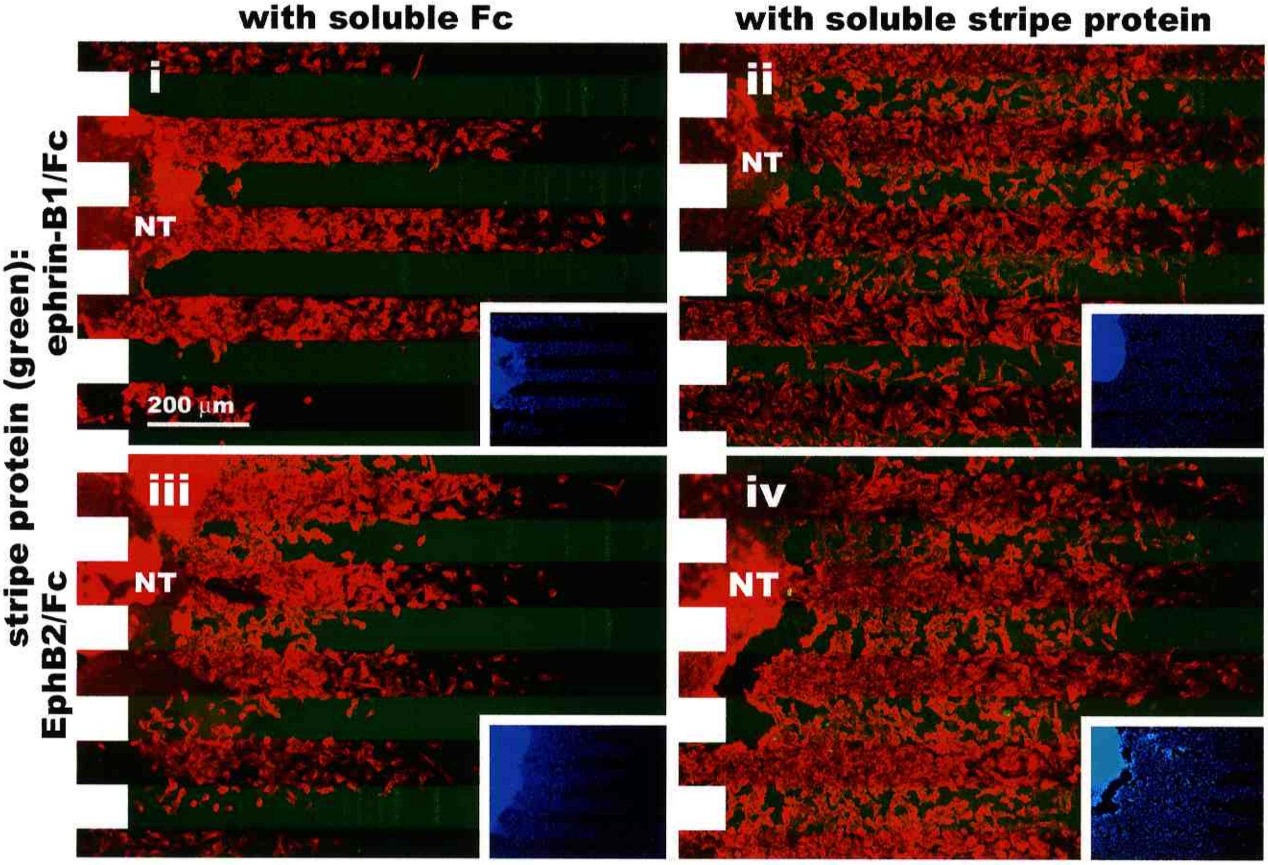
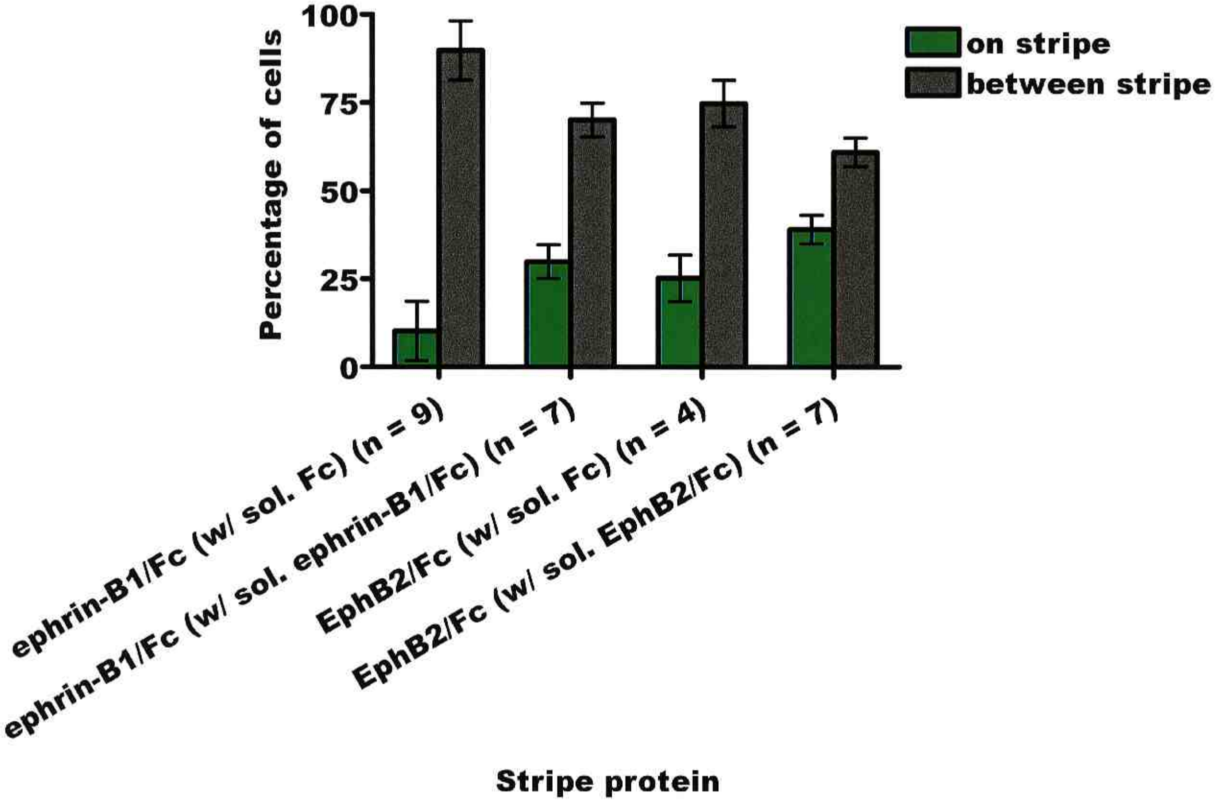

with soluble Fc
with soluble stripe protein
stripe protein (green):
ephrin-B1/Fc
EphB2/Fc
i
ii
iii
iv
NT
NT
NT
NT
200 μm

Fig. 9B


100
75
50
25
0
Percentage of cells
on stripe
between stripe
ephrin-B1/Fc (w/ sol. Fc) (n = 9)
ephrin-B1/Fc (w/ sol. ephrin-B1/Fc) (n = 7)
EphB2/Fc (w/ sol. Fc) (n = 4)
EphB2/Fc (w/ sol. EphB2/Fc) (n = 7)
Stripe protein

the lanes of substrate-bound EphB2/Fc in going from adding soluble Fc (Fig. 9Aiii) to EphB2/Fc (Fig. 9Aiv) to the culture medium. With both sets of experiments, the results from the control are similar to the assays in which no soluble protein was added to the culture medium (compare Fig. 8Aii with Fig. 9Ai and Fig. 8Aiii with Fig. 9Aiii). It should also be noted that the presence of soluble ephrin-B1/Fc or EphB2/Fc did not completely eliminate the striped outgrowth seen in the presence of soluble Fc alone, but rather made it less conspicuous. As in the experiments where no soluble proteins were added to the culture medium, the total cell outgrowth (Fig. 8A insets) from the explanted chicken NTs is homogeneous, being comprised almost exclusively of cells labeled by the HNK-1 antibody.

The average distribution of HNK-1-positive cell outgrowth between the two sets of lanes in the four experimental conditions described above is summarized in Fig. 9B. When chicken NT explants are cultured on the clustered ephrin-B1/Fc plus FN and FITC *versus* FN substrate in the presence of soluble Fc protein, 10% of the outgrowing cells are found on the FITC stripes while 90% are found between them (n = 9). In the corresponding experiment with soluble ephrin-B1/Fc, this distribution shifts to 30% of the cells being found on the stripes and 70% between them (n = 7). When explants are cultured on the clustered EphB2/Fc plus FN and FITC *versus* FN substrate in the presence of soluble Fc, 25% of the outgrowing cells are found on the FITC stripes while 75% are found between them (n = 4). In the presence of soluble EphB2/Fc, this distribution shifts to 39% of the cells being found on the stripes and 61% between them (n = 7). The difference between the absence and presence of soluble Fc in the culture medium is not significant for either the ephrin-B1/Fc (p = 0. 3928) or EphB2/Fc (p = 0.7390) experiments. For the soluble competitor assays, however, the difference between the control and experimental treatments is extremely significant (p = 0.0004) for the ephrin-B1/Fc assays and also significant (p = 0.0483) for the EphB2/Fc assays. These results indicate that addition of soluble Fc to the culture medium has no effect on the outgrowth of CNC cells from avian NT explants onto the stripe assay substrates described above, but addition of soluble forms of ephrin-B1/Fc and EphB2/Fc does.

3.2 Discussion

I have shown that a suite of Eph receptors and ephrin ligands are expressed in the hindbrain region of the chicken embryo at the time of CNC cell migration. RNA probes for EphA3, 4, and 7 and EphB1 and 3 label segregated streams of cells that span the distance from the hindbrain to the branchial arches. Importantly, these cells are also labeled by an antibody against a NC marker, HNK-1. I have therefore concluded that avian CNC cells express EphA3, 4, and 7 and EphB1 and 3. Streams of cells in the hindbrain region are also double labeled by the HNK-1 antibody and a probe against ephrin-B2, which led me to the surprising conclusion that avian CNC cells express not only an assortment of Eph receptors, but also an ephrin ligand. The CNC streams in the chicken embryo are positioned between HNK-1-negative groups of cells labeled by probes for ephrin-B1 or EphB2. Thus, avian CNC cells migrate along pathways bordered by cells expressing potential binding partners for the Eph receptors and ephrin ligands that they express. This led me to hypothesize that CNC cells in the chicken embryo are channeled into streams and guided to their destinations by being excluded from ephrin-B1 or EphB2-expressing territories.

To test this hypothesis, I performed a series of cell culture experiments. It was first necessary to confirm that avian CNC cells express proteins that bind to ephrin-B1 and EphB2, which was demonstrated by the finding that these cells are labeled by ephrin-B1 and EphB2 affinity probes in culture. I next investigated whether avian CNC cells could respond directly to ephrin-B1 or EphB2 in the *in vitro* stripe assay in a manner that is consistent with their distributions *in situ*. While these cells showed an even distribution on a control substrate, they strongly avoided growing out onto lanes of substrate-bound ephrin-B1 or EphB2, indicating that both of these proteins can act as repellents for avian CNC. Finally, in order to determine what role the Ephs and ephrins expressed by avian CNC cells play in this response, I asked the question of what would happen if these proteins had to compete for binding to the substrate-bound ephrin-B1 or EphB2. In both cases, addition of soluble competitor resulted in a significantly more even pattern of outgrowth than in controls, presumably because the Ephs and ephrins expressed by the outgrowing cells were rendered less sensitive to the substrate-bound ephrin-B1 or EphB2 through their interactions with the same proteins present in solution. Taken together, these results suggest that avian CNC cells express Eph receptors and ephrin ligands that mediate their repulsion from ephrin-B1 or EphB2-expressing territories, respectively.

This repulsion could explain how avian CNC cells are guided to their targets *in vivo*, given that the stereotypical pathways through which they migrate are bordered by cells expressing ephrin-B1 or EphB2. Furthermore, while the paradigm for Eph/ephrin function in directed cell migration is that Eph-expressing migratory cells/outgrowing axons are guided to their targets by receptor-mediated repulsion from ephrin-expressing territories, my results suggest that the opposite can also hold true. Thus the novel finding presented here is that co-expressed Ephs and ephrins can similarly regulate the pathfinding behaviour of migrating cells.

3.2.1 Reverse Signaling and Pathfinding

My findings add to the limited body of evidence that ephrin-expressing migratory cells can be guided to their targets by ligand-mediated repulsion from regions where complementary Eph receptors are expressed. The best example of this comes from studies on the formation of a large forebrain axon tract known as the anterior commissure. In EphB2-null mice, the majority of axons forming the posterior tract of the anterior commissure (acP) migrate aberrantly (Henkemeyer *et al.*, 1996). This phenotype is rescued by expression of cytoplasmic domain-truncated EphB2, indicating that signaling from EphB2 is dispensable in guiding acP axons. In agreement, EphB2 is not expressed by acP axons, but rather by the cells underlying them. Because acP axons invade the

EphB2 expression domain in null, but not wild-type embryos, EphB2 is thought to normally function in repelling these axons. On the other hand, ephrin-B2 is expressed by acP axons and the loss of its cytoplasmic domain results in a phenotype similar to that of EphB2-null embryos, suggesting that this ligand mediates the guidance of acP axons away from EphB2 expression domains in a cell autonomous fashion (Cowan *et al.*, 2004).

Reverse signaling may also be at work during CNC pathfinding in other species. As discussed previously, *Xenopus* CNC cells destined for BAIII express EphA4 and EphB1 and are targeted to their destination at least in part by receptor-mediated repulsion from ephrin-B2 ligands expressed by mesoderm along the adjacent BAII pathway (Smith *et al.*, 1997). Intriguingly, CNC cells destined for BAII also express ephrin-B2 while mesoderm along the BAIII pathway also expresses EphA4 and EphB1, which raises the possibility that signaling from these ephrin-B2 ligands is responsible for the exclusion of the BAII CNC from the neighbouring EphA4/EphB1-expressing mesoderm. In mouse, ephrin-B1 is detected in both the branchial arches and in migrating CNC cells (Davy *et al.*, 2004). Conditional deletion of the ephrin-B1 gene from just the CNC leads to the mismigration of these cells into areas that they normally avoid as well as the development of craniofacial defects, indicating that ephrin-B1 normally acts in a cell autonomous fashion to regulate CNC pathfinding in mouse. Reverse signaling involving a PDZ domain-containing effector protein appears to be required for this function, since deletion of just the PDZ domain binding motif from ephrin-B1 results in a very similar phenotype. Interestingly, craniofacial defects in ephrin-B1-null mice are more severe than those observed in embryos where ephrin-B1 has been conditionally deleted from the CNC. This raises the possibility that ephrins might be acting both CNC cell autonomously and non-CNC cell autonomously here, similar to what I have observed in the chicken embryo.

3.2.2 Ephrin-A Ligands

An immediate question raised by my *in situ* localization data is whether a binding partner for the EphA3, 4, and 7 receptors expressed by avian CNC cells is also expressed in the hindbrain region of the chicken embryo. Orthologues for ephrin-A1, 3, and 4 have not been found in the chicken genome. A fourth ligand, ephrin-A6, is expressed in the retina (Menzel *et al.*, 2001), which leaves only ephrin-A2 and 5 to consider. Previous research has shown that these two ligands are indeed expressed in the head of the early chicken embryo (Baker and Antin, 2003). While ephrin-A5 is expressed in the neural folds, head mesenchyme, and patches of cells lateral to the forming NT, ephrin-A2 has a more diffuse and non-specific pattern of expression. Based on these observations, I ruled out the possibility that ephrin-A2 could be involved in CNC migration and instead focused on ephrin-A5. Unfortunately, I was not able to detect ephrin-A5 expression in any of the stages examined, which would seem to leave the EphA receptors expressed by avian CNC cells as 'orphans'. On the other hand, there are examples of cross-class receptor-ligand interactions, namely EphA4 with ephrin-B2 and 3 (Gale *et al.*, 1996) and EphB2 with ephrin-A5 (Himanen *et al.*, 2004). The possibility therefore exists that EphA3, 4, and 7 may in fact be interacting with the ephrin-B1 ligands that are expressed by cells bordering the streams of CNC.

3.2.3 Potential Significance of Receptor-ligand Co-expression

3.2.3a Sensitization

The major implication from my findings is that avian CNC cells express both Eph receptors and ephrin ligands. Previous work has shown that co-expressed EphA4 and ephrin-A2 can associate directly with one another in *cis*, resulting in the formation of complexes that fail to transmit intracellular signals (Yin *et al.*, 2004). This also impairs the ability of these proteins to interact with their binding partners on other cells and thus blocks *trans*-activated signaling. Receptor-ligand co-expression in a cell could therefore function to modulate the sensitivity of that cell to external cues. For example, ephrin-A2 and 5 are expressed not only in the optic tectum, but are also co-expressed with EphAs in RGC axons (Hornberger *et al.*, 1999). Axons projecting from the temporal side of cultured retinas express high levels of EphAs and low levels of ephrin-As and normally avoid growing out onto fragments of cell membranes from the posterior tectum (where ephrin expression is abundant) in the stripe assay. This response is abolished by over-expression of ephrin-A5 in the temporal axons. On the other hand, nasal axons normally express high levels of ephrin-As and low levels of EphAs and are indifferent to ephrin-A2 in the stripe assay, but avoid growing out onto lanes of this protein after enzymatic removal of the axonal ephrin-As. These results indicate that the responsiveness of RGC axons to ephrin-As is a function of the ratio of axonal EphA to ephrin-A expression, perhaps because the greater the amount of ligand co-expression, the more likely that *cis* interactions can interfere with the receptor-mediated response to *trans* signals. Interestingly, EphA receptor phosphorylation is also associated with responsiveness, since temporal axons show low levels of phosphorylation and nasal EphA receptors are highly phosphorylated. Perhaps a constitutive *cis* association between ephrin-As and EphAs in the nasal axons leads to chronic receptor phosphorylation and adaptation, whereas the temporal EphA receptors are only weakly phosphorylated (if at all) and thus remain competent to transduce extracellular signals.

While ephrin-B2 and the majority of Eph receptors investigated in this study appear to have a CNC-wide distribution in the chicken embryo, EphA4 and EphB1 expression is restricted to the stream of CNC cells associated with BAIII. Given that CNC cells destined for BAIII and IV share a common point of origin in the hindbrain (Lumsden and Keynes, 1989), it is surprising that EphA4 and EphB1 are not also expressed in the BAIV CNC stream. The possibility exists that this restricted expression has something to do with why one group of CNC cells emerging from r6 migrates to BAIII while another is diverted into BAIV. Although both express ephrin-B2, EphA3 and 7, and EphB3, the cells destined for BAIII additionally express EphA4 and EphB1. As such, they have a greater level of total Eph expression and thus any potential *cis* inhibitory interactions with ephrin-B2 might be expected to have less of an effect on their responsiveness to *trans* signals present along the migratory pathway. Therefore, differential targeting of CNC migrating from r6 could occur if the cells destined for BAIII are, by virtue of their higher level of Eph expression, more sensitive to *trans* ephrins and thus strictly confined to a pathway bounded by these ligands, whereas the cells destined for BAIV are less sensitive to the same signals and thus migrate to their target in a more passive fashion. However, the fact that CNC cells migrating to BAIV, rather than being diffuse and scattered, appear in a compact stream that is, like the BAIII CNC, bordered by expression domains for ephrin-B1 argues against this idea. Furthermore, the implication from my stripe assay results that ephrin-B2 itself mediates a repulsive response to an external cue (EphB2) suggests that this ligand does more than potentially acting to titrate out signaling from co-expressed Eph receptors. Thus the significance of the BAIII CNC-exclusive expression of EphA4 and EphB1 is unclear.

3.2.3b Cell-cell Adhesion

An alternative possibility is that co-expression of ephrin ligands and Eph receptors by cells within a stream of CNC promotes intercellular adhesion. While the interaction between Eph-expressing cells and ephrin-expressing cells classically leads to repulsion, the association between receptor and ligand is an inherently adhesive one, as revealed by a study on the function of Ephs and ephrins during NT formation in mouse (Holmberg *et al.*, 2000). The NT derives from a thickened region of ectoderm known as the neural plate. It forms when the edges of the neural plate elevate to become the neural folds, which subsequently move towards the dorsal midline, juxtapose, and fuse, resulting in tubularization of the neural plate. In a subpopulation of ephrin-A5 or EphA7-null mice, the neural folds juxtapose, but fail to fuse. Intriguingly, cells in the neural folds express both full-length and cytoplasmic domain-truncated forms of EphA7. Neural fold cells expressing full-length EphA7 in culture are repelled from ephrin-A5-expressing cells. However, when full-length and truncated EphA7 are co-expressed, the receptor-expressing cells adhere to ligand-expressing cells.

Recent evidence indicates that Ephs and ephrins also promote cell-cell adhesion during hindbrain segmentation in the zebrafish embryo (Cooke *et al.*, 2005). Recall that Ephs and ephrins have a reciprocal distribution in alternating segments of the embryonic hindbrain, such that bidirectional repulsive signals transmitted at the interfaces between adjacent segments act to establish and maintain rhombomere boundaries. As such, mosaic inhibition of receptor or ligand function within a given rhombomere would be expected to result in the affected cells becoming randomly distributed among the wild-type ones. In fact, EphA4-negative cells sort robustly to the edges of EphA4-expressing rhombomeres, whereas EphA4-expressing cells transplanted into an EphA4 negative host from tight aggregates with one another. These results suggest that EphA4 mediates cell-cell adhesion within rhombomeres and that cells which have lost the expression of this receptor are excluded from associating with cells that do express it. Adhesion in this case is probably based upon the interaction of EphA4 with ephrin-A2 and/or 5, both of which show low levels of expression throughout the embryonic zebrafish hindbrain.

As discussed previously, CNC cells typically migrate in loose aggregates (Kulesa and Fraser, 1998). In *Xenopus*, both EphA2 and its ligands (as revealed by EphA2 affinity probe staining) appear to share a common distribution with the CNC in streams of cells distributed between the hindbrain and the branchial arches (Helbling *et al.*, 1998). When EphA2 function is disrupted, CNC cells destined for BAIII mismigrate caudally and the BAIII CNC fails to separate from the BAIV CNC. It is therefore possible that the contact between Eph receptors and ephrin ligands is responsible for the cohesiveness of the cells within a given CNC stream, which may in turn prevent intermingling with cells from adjacent streams. In this respect, it is interesting to note that my affinity probe staining experiments showed that ephrin-B1-binding proteins localize to the periphery of individual avian CNC cells in culture and are often concentrated in membrane processes, whereas EphB2-binding proteins are more commonly observed at the interfaces between aggregated cells. Perhaps this indicates that ephrin-B1 functions as a guidance cue, whereas the ephrin-B2 ligands expressed by CNC cells act to promote adhesion to other CNC cells.

3.2.3c Attraction versus Repulsion

Yet another possibility is that co-expressed Ephs and ephrins mediate different responses to external cues. For example, chicken spinal motor neurons express EphA4 in addition to ephrin-A2 and 5 and respond differently to *trans* EphAs and ephrin-As: EphAs induce spreading of the neuronal tip, whereas ephrin-As induce collapse (Marquardt *et al.*, 2005). These findings indicate that Ephs are not always inhibited by co-expressed ephrins. In agreement, cells co-expressing EphA3 and ephrin-A5 in culture show no difference in receptor phosphorylation after exposure to *trans* ephrin-A5 from cells expressing just EphA3. Furthermore, EphA and ephrin-A affinity probes can bind simultaneously to the surface of spinal motor neuron growth cones. On the other hand, expression of GPI-linked EphA extracellular domains or chimeric ephrin-As that are anchored to the plasma membrane by EphA transmembrane domains results in impaired spreading or collapse responses. Together with the finding that EphA and ephrin-A affinity probes label different sub-domains of motor neuron plasma membranes, these results show that the GPI anchors of ephrin-As and the transmembrane domains of EphAs target these proteins to different regions of the cell surface, which prevents co-expressed Ephs and ephrins from interacting with one another in *cis*. This differential localization makes it possible for Ephs and ephrins that are expressed by the same cell to mediate distinct responses to extracellular signals.

Another implication from the above study is that ephrins can mediate attractive responses, which is further supported by findings from other works. In *Xenopus*, for example, axons projecting from the dorsal retina express high levels of ephrin-B ligands and map to the ventral tectum, where EphB1 expression is also high (Mann *et al.*, 2002). These axons show preferential outgrowth onto lanes of EphB1 in the stripe assay and exposure to soluble EphB protein or expression of dominant-negative ephrin-B2 *in vivo* causes their connection points in the tectum to shift dorsally. On the other hand, ventral retinal axons ectopically expressing ephrin-B2 project to more ventral locations in the tectum than they would normally. Similarly, axons projecting from the apical region of the vomeronasal organ (VNO) in mouse, which is primarily responsible for the detection of pheromones, express high levels of ephrin-A5 and map to the anterior accessory olfactory bulb (AOB), where expression of EphA6 is also high (Knoll *et al.*, 2001). VNO axons preferentially grow out onto lanes of EphA protein in the stripe assay and when ephrin-A5 is knocked out *in vivo*, there is an increase in the number of these axons that project to the posterior AOB. Therefore, ephrin-expressing cells can be guided to their targets through ligand-mediated attraction to cells expressing complementary receptors.

While Eph signaling classically leads to cell repulsion, there are examples of these receptors also mediating attractive responses. Motor neurons from both the lateral motor column (LMCl) and medial motor column (MMCm) express EphA4, whereas ephrin-A5 is expressed in the ventral limb mesoderm as well as the rostral half of each somite (Eberhart *et al.*, 2004). Both types of motor neurons extend ventrally from the NT together, but LMCl neurons innervate the dorsal limb mesoderm while MMCm neurons innervate rostral somite halves. These observations suggest that LMCl neurons are guided to the dorsal limb mesoderm by EphA4-mediated repulsion from ephrin-A5 ligands expressed in the ventral limb mesoderm, whereas MMCm neurons are targeted to the rostral somite halves by EphA4-mediated attraction to the ephrin-A5 ligands expressed there. In agreement, the presence of ectopic ephrin-A5 prevents LMCl neurons from entering the dorsal limb mesoderm and MMCm neurons grow aberrantly into caudal somite halves when EphA4 signaling is blocked by dominant-negative inhibition or when ephrin-A5 is ectopically expressed there.

A similar example comes from avian TNC cells, which, as discussed previously, express EphB3 and migrate exclusively through rostral somite halves because of receptor-mediated repulsion from ephrin-B1 ligands expressed in the caudal somite halves (Krull *et al.*, 1997). Intriguingly, the later-migrating TNC cells that differentiate into melanocytes also express EphB receptors, but travel through a more superficial pathway that is dense with ephrin-B ligands (Santiago and Erickson, 2002). Addition of soluble ephrin-B1 inhibits migration through this pathway, suggesting that targeting in this case is based on an attractive guidance mechanism. In agreement, ephrin-B1 has a positive effect on the late cells in transwell migration assays, but a negative effect on early ones. Moreover, late cells adhere strongly to an ephrin-B1-coated substrate and exhibit a spread morphology, whereas early cells are rounded and weakly attached.

Yet another example comes from retinocolliclular mapping, where RGC axons projecting from the same part of the retina enter the SC over a broad extent across the lateral-to-medial axis and subsequently extend interstitial branches in a directional fashion towards a central termination zone. These axons express EphB2, whereas ephrin-B1 is expressed in a low lateral to high medial gradient in the SC (Hindges *et al.*, 2002). In EphB2-null mutants, branch extension has an aberrant lateral bias, suggesting that in the absence of EphB2 function, branches are no longer attracted to the ephrins concentrated medially (McLaughlin *et al.*, 2003). Ectopic expression of ephrin-B1 similarly results in a lateral shift in branching, indicating that ephrin-B1 can also have a repulsive effect on interstitial RGC axon branches.

Thus the response of Eph-expressing migratory cells or outgrowing axons to ephrins appears to be a function of ephrin concentration. Indeed, axons growing out from retinal explants show increased outgrowth on substrates containing low concentrations of ephrin-A2, whereas higher ephrin-A2 concentrations have an inhibitory effect on outgrowth (Hansen *et al.*, 2004). Attraction *versus* repulsion responses could be attributed to different signaling pathways. A simpler explanation, however, is that sparsely expressed ephrins interact with their receptors without inducing signal transduction, whereas more abundantly expressed ephrins can promote receptor oligomerization and thus the activation of signaling pathways that ultimately trigger cell repulsion. As seen in NT formation in the mouse embryo, Eph receptors that are incapable of transducing a signal can mediate adhesion to other cells through binding to a complementary ephrin (Holmberg *et al.*, 2000). Such adhesion could potentially be translated into an attractive force for axon outgrowth or cell migration. Further support for a concentration-dependent mechanism of Eph function comes from the finding that clustered multimeric, but not dimeric forms of ephrin-B1 trigger the recruitment of intracellular effector proteins to EphB1 or EphB2 (Stein *et al.*, 1998). Likewise, low concentrations of ephrin-B2 are associated with increased adhesion and migration of EphB6-expressing cells, whereas high concentrations have the opposite effect (Matsuoka *et al.*, 2005). Low ligand concentrations do not trigger EphB6 phosphorylation in these cells, whereas higher concentrations induce phosphoryation of the receptor and a constitutively-associated NRTK. Similarly, the tips of LMCl neurons, which are excluded from the ventral limb mesoderm by the ephrins expressed there, are highly phosphorylated, whereas the tips of MMCm neurons, which are targeted to ephrin-rich somitic mesoderm, are not (Eberhart *et al.*, 2004).

The switch from attraction to repulsion may therefore be based upon the concentration-dependent switch from passive adhesion between engaged receptors and ligands to the activation of intracellular signaling pathways. Directed migration to a non-diffusible cue, such as an ECM protein, can occur if cells migrate along a gradient of that cue, such that the ECM ahead of any given cell is more attractive than that around or behind it. Attractiveness in this case is the result of the cue stimulating increased adhesion (be it cell-substrate or cell-cell) and/or cell migration.

However, the fact that avian CNC cells do not appear to migrate over a gradient of either ephrin-B1 or EphB2, but rather have a mutually exclusive distribution with these cues would seem to argue against this idea. In this respect, it is interesting to note that my ephrin-B2 probe staining of avian CNC cells *in situ* was somewhat weak, particularly in comparison to the EphA4 or EphB1 staining. While these differences could be attributed to the hybridization efficiency of the probes themselves, it is also possible that ephrin-B2 is only weakly expressed by avian CNC cells and thus may not be abundant enough to do anything other than participate in mediating cell-cell adhesion within the CNC streams (as discussed previously). Alternatively, Eph signaling may be associated with repulsion and ephrin signaling may be associated with attraction (or *vice versa*), such that correct targeting results from the balance of these activities. For example, CNC pathfinding in the chicken embryo could be the result of a 'push-pull' mechanism in which cells are channeled into segregated streams by a marginal repulsive signal, while an attractive signal that is also at the margin of these streams simultaneously controls their breadth.

3.2.3d Spatial Differences

On the other hand, my stripe assay results indicate that both the Eph receptors and ephrin-B2 ligands expressed by avian CNC cells mediate the exclusion of these cells from substrates containing ephrin-B1 or EphB2, respectively. In agreement, my localization analyses demonstrate that EphA3, 4, and 7 and EphB1 and 3 have a mutually exclusive distribution with ephrin-B1, as is also the case with ephrin-B2 and EphB2. While the concentrations of ephrin-B1 and EphB2 used in the stripe assays may not be representative of those encountered *in vivo*, the last and seemingly most likely potential explanation for why these cells co-express Eph receptors and ephrin ligands is therefore that both mediate repulsive guidance responses. Strikingly, the distributions of ephrin-B1 and EphB2 are similar, but not identical. Ephrin-B1-expressing cells bracket the dorsal extent of the CNC stream caudal to the OV that invests in BAIII and IV and are also found in the ventral half of BAII. In contrast, EphB2-expressing cells border the dorsal part of the BAII CNC stream and the ventral branches of the post-otic stream. In combination, the ephrin-B1-expressing cells and EphB2-expressing cells completely bound the streams of CNC associated with BAII, III, and IV. Individually, they do not. Thus the position of CNC cells migrating to BAII appears to be defined by EphB2 dorsally and ephrin-B1 ventrally while the position of CNC cells migrating to BAIII or IV appears to be defined by ephrin-B1 dorsally and by EphB2 ventrally. Why such a guidance mechanism should exist is not obvious.

3.2.4 Eph and Ephrin Signaling Mechanisms

In order to understand the potential mechanisms by which the signals transmitted through ephrin ligands or Eph receptors could lead to CNC cell repulsion, it is useful to first briefly review how cell migration works. The migration of an individual cell (or the functionally and structurally analogous growth cone at the tip of an outgrowing axon) is a cyclical process in which a foot-like protrusion extends from the leading edge of the plasma membrane and forms new traction-generating attachments to the ECM *via* integrins, then intracellular contractile forces cause the breaking of older attachments, retraction of the trailing edge, and translocation of the cell body, resulting in net forward movement.

Cell migration is driven by dynamic changes in the actinomyosin cytoskeleton as regulated by proteins from the Ras superfamily of low molecular weight GTPases (Ridley, 2001). GTPases possess an intrinsic enzymatic activity that catalyzes the hydrolysis of bound guanosine triphosphate (GTP) to guanosine diphosphate (GDP). They function as molecular switches that are able to interact with and regulate the activity of effector proteins in their GTP-bound form, but are inactive when associated with GDP. The signaling activity of GTPases is regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs), which are themselves regulated by extracellular signals transduced by cell surface receptor proteins. GEFs promote GTPase signaling by stimulating the exchange of GDP for GTP. In contrast, GAPs suppress GTPase signaling by activating the GTP-hydrolyzing activity of these proteins. GDIs perform a similar function by preventing the dissociation of bound GDP.

The cell migration cycle is largely under the control of two members of the Rho subfamily of GTPases, Rac1 and RhoA (Ridley, 2001). Rac1 signaling leads to the polymerization and assembly of actin into a meshwork of filaments that push the cell membrane outwards to form broad, flat processes known as lamellipodia, which are the 'feet' that pull migrating cells forward. Conversely, RhoA regulates the contraction of actin filaments by the myosin motor protein and is thus critical to translocation of the cell proper. Impairment of Rac function has a negative effect on cell migration as a result of inhibited lamellipodia formation. Unchecked Rho signaling similarly results in the inhibition of lamellipodia formation and thus the inhibition of cell migration, suggesting that Rho-mediated contractility, while necessary for locomotion, can also have an antagonistic effect on Rac-mediated protrusion. As such, one way in which ephrin/Eph signaling could regulate directed cell migration is through the local modulation of Rac1 and/or RhoA activity. By this mechanism, increased RhoA activity would be expected to result in the contraction of membrane processes while decreased Rac1 activity would be expected to result in a block to the actin polymerization required to generate these processes.

3.2.4a Signaling to Rho Family GTPases

There are a number of signaling pathways linking ephrins and Ephs to Rho family GTPases. For instance, exposure of RGC axons to ephrin-A5 leads to RhoA activation and growth cone collapse (Wahl *et al.*, 2000). Inhibition of RhoA or its immediate effector, Rho kinase, strongly reduces this response, indicating that EphA receptors can signal to cell repulsion responses through this GTPase. Another key component of this pathway is ephexin, a GEF that associates with

EphA4 and increases the activity of RhoA (Shamah *et al.*, 2001). Ephrin-A1-induced RGC growth cone collapse is enhanced by over-expression of wild-type ephexin, but inhibited by dominant-negative ephexin, indicating that this GEF lies downstream of EphA4 in mediating the response to ephrin-A1. EphA4 signaling results in ephexin becoming phosphorylated on tyrosine, which is a requirement for the ephrin-A1-induced increase in RhoA activity and RGC growth cone collapse (Sahin *et al.*, 2005). Interestingly, ephexin can act on both RhoA and Rac1, but phosphorylation specifically enhances its exchange activity towards Rho while leaving its exchange activity towards Rac unaltered, resulting in a balance of GTPase signaling that promotes growth cone collapse. This phosphorylation is mediated by SFKs, which associate with EphA4 in RGC axons (Knoll and Drescher, 2004). SFKs also phosphorylate cortactin, another protein that interacts with EphA4. Phosphorylation of cortactin attenuates its ability to promote actin polymerization (Lua and Low, 2005).

While ephexin represents the best characterized effector of EphA receptors in the signaling pathways stimulated by ephrin-As that lead to cell repulsion, an alternative route may be through the CrkII adaptor protein. Exposure of non-neuronal cells to ephrin-A5 results in the recruitment of this adaptor to phosphorylated EphA3, RhoA activation, and retraction of cellular processes (Lawrenson *et al.*, 2002). This response is blocked by dominant-negative CrkII or by inhibitors of RhoA or Rho kinase. Similarly, the Abl NRTK has been shown to associate with activated EphA4 and EphB2 (Yu *et al.*, 2001) and appears to play a role in ephrin-A5-induced RGC growth cone collapse, since inhibitors of Abl block this response (Harbott and Nobes, 2005). Because Abl associates with and phosphorylates CrkII (Chodniewicz and Klemke, 2004), these two proteins may operate in conjunction with one another to mediate cell repulsion downstream of activated Eph receptors. Phosphorylation of CrkII results in the displacement of an associated RacGEF, DOCK180, and down-regulation of Rac1 activity. In addition, CrkII can bind a GDI specific for

RhoA and has thus been speculated to indirectly activate RhoA by displacing it from this GDI (Tsuda *et al.*, 2004).

With the possible exception of Abl, B-type Ephs and ephrins signal to a different set of effector proteins than EphA receptors. Activated EphB2 forms a complex with the p62dok-1 adaptor protein and p120-RasGAP, a GAP specific for the prototypical small GTPase Ras (Holland *et al.*, 1997). In neuronal cells, activation of EphB2 by ephrin-B1 results in decreased Ras signaling and growth cone collapse (Elowe *et al.*, 2001). This response is blocked by expression of a dominant-negative form of p120-RasGAP or a constitutively-active form of Ras, indicating that EphB2-mediated growth cone collapse is the result of reduced Ras activity. Because Ras is a positive regulator of Rac signaling (Sarner *et al.*, 2000), growth cone collapse in this instance may be the result of an indirect block to Rac-mediated actin polymerization and membrane protrusive activity through the inactivation of Ras. Alternatively, Dishevelled (Dsh), an important intermediate in the canonical Wnt signaling pathway that regulates formation of the dorsal-ventral body axis, interacts directly with ephrin-B1 in a non-phosphotyrosine-dependent manner and indirectly with activated EphB2 *via* adaptor proteins such as Nck and Grb4 (Tanaka *et al.*, 2003). Dominant-negative forms of Dsh or RhoA impair the sorting of cultured *Xenopus* cells expressing ephrin-B1 or EphB2 from one another and co-expression of ephrin-B1 or EphB2 with Dsh results in the activation of RhoA and Rho kinase. These findings indicate that, as is the case with EphAs, signals from ephrin-B ligands and EphB receptors that lead to cell repulsion are relayed through RhoA. A protein known as Daam1 may be a part of this pathway, since it can bind to both Dsh and Rho and is required for Dsh-mediated activation of RhoA downstream of Wnt (Habas *et al.*, 2001). Activation of RhoA is likely mediated by a Rho-specific GEF such as xNET1, which can also associate with both Dsh and Rho (Miyakoshi *et al.*, 2004). As discussed previously, mouse CNC cells appear to be guided to their targets by PDZ domain-dependent reverse signaling from ephrin-

B1 (Davy *et al.*, 2004). A potential effector in this signaling pathway is PDZ-RGS3, a PDZ domain-containing protein that has been shown to associate with ephrin-B1 and inhibit chemotaxis downstream of heterotrimeric G protein-coupled receptors in response to EphB2 binding (Lu *et al.*, 2001).

3.2.4b Regulation of Cell-substrate Adhesions

A second manner in which ephrin or Eph signaling could lead to cell repulsion is through the differential regulation of cell-substrate adhesions. More specifically, CNC cells could be confined to stereotypical pathways of migration if the interaction between Eph and ephrin resulted in them becoming less adherent to the ECM outside of these pathways. This could happen through regulated disassembly of the integrin adhesion complexes that link the ECM to the cytoskeleton and thereby provide the traction needed for cell movement. These multimolecular complexes result when ligated integrin receptors recruit a NRTK known as focal adhesion kinase (FAK), which subsequently autophosphorylates and itself recruits linker proteins that bind to actin filaments (Petit and Thiery, 2000). Disassembly can occur when the FAK autophosphorylation site is dephosphorylated or when FAK is displaced from the complex as a result of SFK-mediated phosphorylation of a negative regulatory site (Wozniak *et al.*, 2004).

Signaling from ephrin ligands and Eph receptors has been implicated in de-adhesion responses in non-neuronal cells. Ephrin-B1 signaling has been shown to result in cell rounding and the loss of cell-substrate adhesions (Cowan and Henkemeyer, 2001). This response is dependent on the Grb4 adaptor protein, which associates with the intracellular domain of ephrin-B1 in a phosphotyrosine-dependent fashion and is capable of binding to an assortment of effector proteins that could potentially link ephrin-B1 to changes in integrin activity. For example, one of these effectors, Cbl-associated protein (CAP), has a positive effect on adhesion complex formation through its interaction with FAK (Ribon *et al.*, 1998). It is possible that the indirect association of

CAP with an ephrin-B ligand results in its sequestration from adhesion complexes, which may have a negative effect on integrin function. Activation of EphA2 can similarly lead to impaired adhesion (Miao *et al.*, 2000). In this case, exposure to ephrin-A1 results in the tyrosine phosphatase SHP2 being recruited to EphA2 and dephosphorylating FAK, which presumably leads to de-adhesion through cross-talk with integrin receptors. Alternatively, EphA8 signaling can promote de-adhesion in a Fyn SFK-dependent fashion (Choi and Park, 1999). EphB signaling can also regulate integrin function, as EphB2 activation leads to tyrosine phosphorylation of R-Ras, a relative of Ras that is a positive regulator of FAK (Kwong *et al.*, 2003), which results in poor adhesion to substrates coated with integrin ligands (Zou *et al.*, 1999). The adaptor protein SHEP1 binds a JM phosphotyrosine of EphB2, may itself be phosphorylated by this receptor, and can bind R-Ras (Dodelet *et al.*, 1999). As such, it may represent an intermediate in this pathway.

3.2.4c Regulation of Cell-cell Adhesions

While the interaction between Eph-expressing cells and ephrin-expressing cells classically leads to repulsion, it is interesting to note that the association between receptor and ligand is an inherently adhesive one. This is exemplified by the previously discussed function of Ephs and ephrins during NT closure in mouse (Holmberg *et al.*, 2000). One way in which the adhesion between Eph and ephrin could be converted to repulsion is through the proteolytic cleavage of receptor-ligand complexes. Indeed, ephrin-A2 associates with the ADAM10 protease *via* its extracellular domain and binding to EphA3 triggers its removal from the cell membrane (Hattori *et al.*, 2000). Axon withdrawal from cells expressing uncleavable ephrin-A2 is slower than axon withdrawal from cells expressing wild-type ephrin-A2, indicating that severing the bond between paired Ephs and ephrins can be an important component of cell repulsion responses. ADAM10 has also been reported to associate with the extracellular domain of EphA3 and cleave bound ephrin-A5 in *trans* (Janes *et al.*, 2005).

While a similar mechanism is not known to exist for ephrin-Bs, an alternative possibility is that the contacts between receptor-expressing cells and ligand-expressing cells are effectively severed by internalization of receptor-ligand complexes. In fact, expression of EphB4 and ephrin-B2 in adjacent cells leads to the retraction of those cells from one another concomitant with endocytosis of phosphorylated EphB4 and *trans*-endocytosis of ephrin-B2 (Marston *et al.*, 2003). This response is dependent on actin polymerization and Rac1 signaling. Other experiments have shown that endocytosis of receptor-ligand complexes can also occur between contacting cells expressing ephrin-B1 or EphB2, only in this case internalization occurs bidirectionally since it is blocked by truncated forms of ephrin-B1 or EphB2 (Zimmer *et al.*, 2003). A similar mechanism appears to be at work in EphA signaling as well, since Rac1 is required for endocytosis of the plasma membrane during ephrin-A2-induced RGC growth cone collapse (Jurney *et al.*, 2002). The link between Eph signaling and Rac-dependent endocytosis appears to be the Vav2 GEF, which is recruited to the intracellular domain of EphA4 or EphB1 upon receptor activation and activated by phosphorylation (Cowan *et al.*, 2005). Vav2 function is required for internalization of receptor-ligand complexes and ephrin-A1-induced RGC growth cone collapse.

CHAPTER 4

PHYLOGENY OF EPH RECEPTORS AND EPHRIN LIGANDS

Ephs and ephrins are found throughout the metazoans. While vertebrate Ephs and ephrins have a rigid classification, the relationship of these proteins to their invertebrate counterparts is not as clear. The increasing availability of genomic data, particularly for primitive deuterostomes such as urochordates and echinoderms, presented me with the opportunity to more clearly establish the phylogenetic relationships of metazoan Ephs and ephrins and perhaps shed some light on how they evolved.

Phylogenetic trees can be constructed by a number of different methods (Page and Holmes, 1998). These methods can be distinguished by the types of data that they use: discrete or distance-based. Discrete data is a direct representation of individual character states (i.e. nucleotide or amino acid sites), whereas distance data reflects the similarities between sequences as revealed by pairwise alignments. Tree building methods can be further differentiated by the manner in which they construct phylogenies. Clustering methods yield a single tree by using a series of steps to progressively group sequences together, whereas optimality methods use a criterion to choose from a set of possible trees. There are advantages and disadvantages associated with the type of data or approach used. For example, discrete data is computationally expensive to manipulate, but retains the information present in individual sites that is lost when the relationship between sequences is converted to a distance. While clustering methods can give an unambiguous answer to a question in the form of a single tree, they do not allow for competing hypotheses to be evaluated as they can with optimality methods.

Therefore, no one tree building method can be considered superior to the others and thus I have employed four different methods to investigate the phylogenies of Eph receptors and ephrin ligands. The neighbour joining (NJ) method arranges sequences based on the clustering of distance data. The minimum evolution (ME) method also deals with distance data, but uses optimality to select trees with the shortest possible branch lengths. The maximum parsimony (MP) and maximum likelihood (ML) methods also use optimality criteria, but deal with discrete data rather than distances. The MP method operates under the assumption that trees requiring the least number of sequence substitutions are most likely to be correct, whereas the ML method asks the question of which trees are most likely to produce the observed data. Using this strategy with protein sequences for the Ephs and ephrins from an assortment of vertebrate and invertebrate species, I have found that different tree building methods produce trees with a number of subtle differences, but also some important common features that have surprising implications for the evolution of Eph receptors and ephrin ligands.

4.1 Results

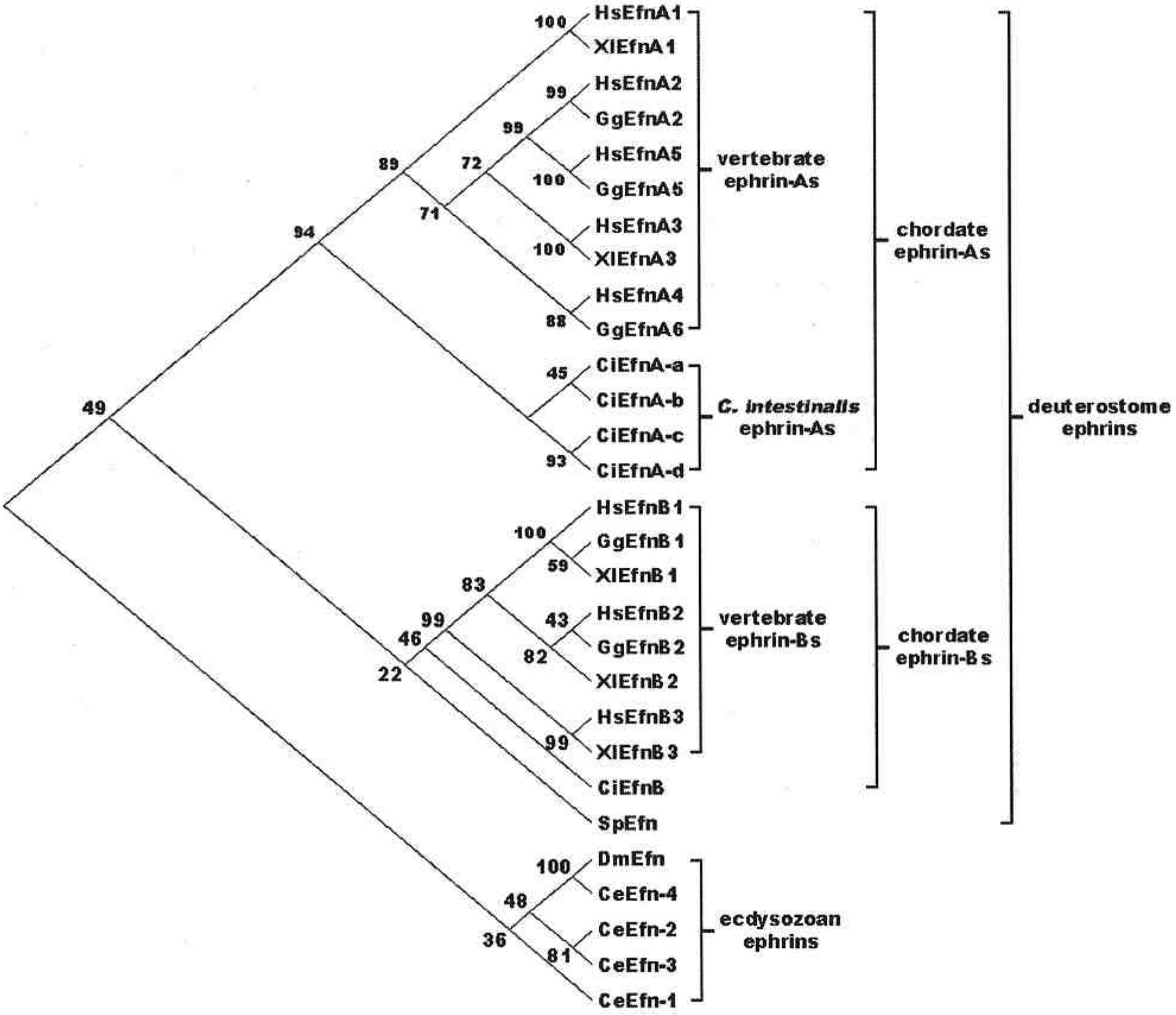
4.1.1 Ephrin Ligands

The four ephrin trees are all made up of three major clades (Fig. 10). The first of these is comprised of a vertebrate ephrin-A subclade supported by bootstrap (BS) values ranging from 83 to 99% and a *C. intestinalis* ephrin-A subclade with a BS of 79-96%, which group together with strong support (BS = 79-96%). Within the vertebrate ephrin-A subclade, ephrin-A2-6 form a group (BS = 48-92%) which is further divided into two subgroups, one comprised of ephrin-A2, 3, and 5 (BS = 72-85%) and another comprised of ephrin-A4 and 6 (BS = 81-99%). Within the *C. intestinalis* ephrin-A subclade, CiEfnA-c and d are always most closely related (BS = 92-100%) and the ML and ME trees place CiEfnA-a and b together in a subgroup (BS = 45 and 52%, respectively). The second major ephrin clade consists of a vertebrate ephrin-B subclade (BS = 79-100%) that is grouped together with the single *C. intestinalis* ephrin-B, though with only moderate support (BS = 36-66%). The last major ephrin clade is comprised of the four *C. elegans* ephrins and the single *D. melanogaster* ephrin, which group together with wide ranging support (BS = 18-72%). Within this

Figure 10. Phylogenetic trees for vertebrate and invertebrate ephrin ligands.

Numbers represent bootstrap values for 100 replications. The maximum likelihood (A), maximum parsimony (B), neighbour-joining (C), and minimum evolution trees (D) each have three major clades: one made up of the vertebrate and *C. intestinalis* ephrin-As, another made up of the vertebrate ephrin-Bs and the single *C. intestinalis* ephrin-B, and a third made up of the ecdysozoan ephrins. There is no consensus on the placement of the single *S. purpuratus* ephrin within these trees. Efn, ephrin; Ce, *C. elegans*; Ci, *C. intestinalis*; Gg, *G. gallus*; Hs, *H. sapiens*; Sp, *S. purpuratus*; Xl, *X. laevis*.

Fig. 10A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49
93
100
59
83
99
43
46
82
22
99
100
48
36
81
vertebrate ephrin-As
C. intestinalis ephrin-As
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vertebrate ephrin-Bs
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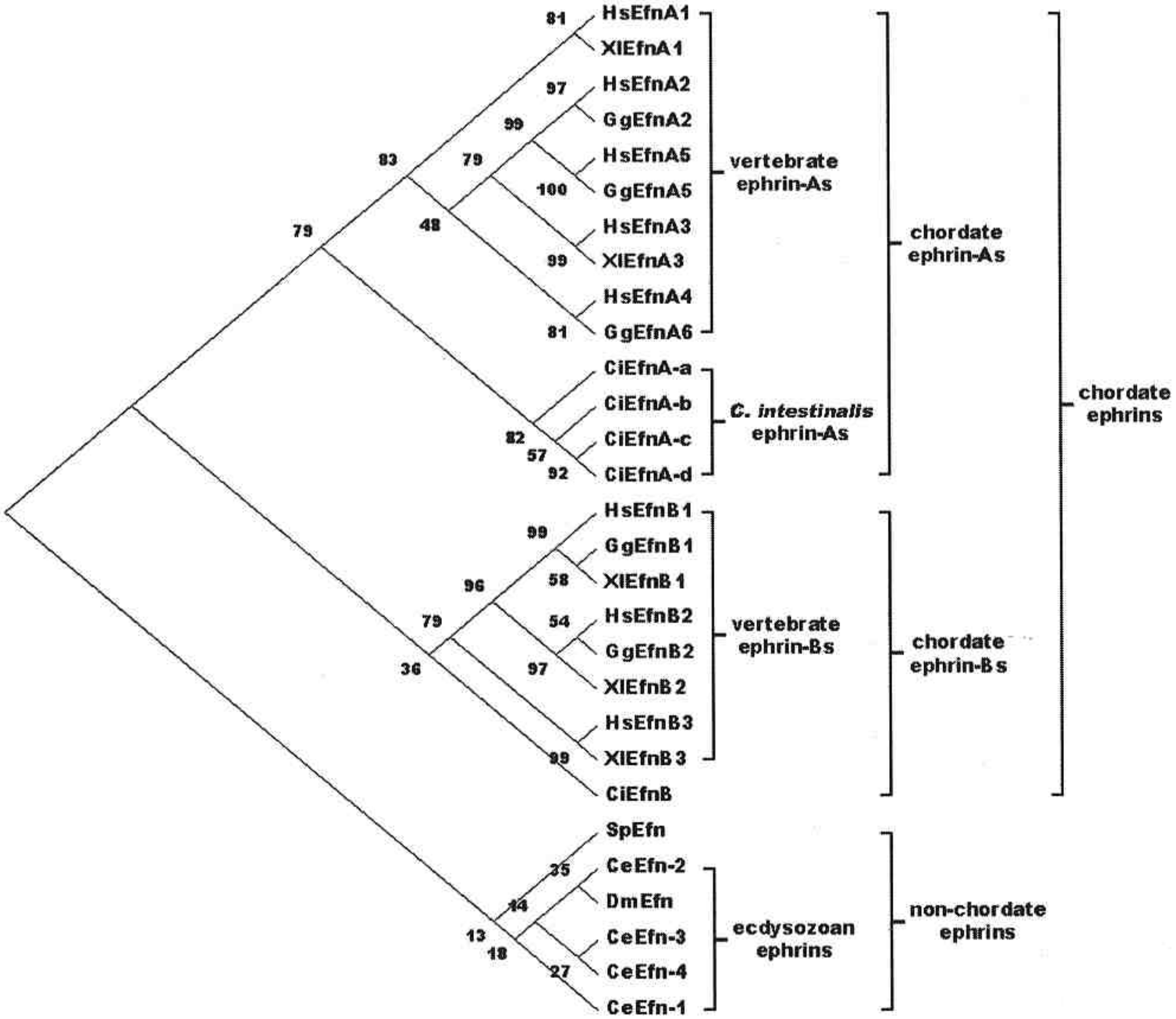
deuterostome ephrins
ecdysozoan ephrins

Fig. 10B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XlEfnA3
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GgEfnA6
CiEfnA-a
CiEfnA-b
CiEfnA-c
CiEfnA-d
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GgEfnB1
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HsEfnB3
XlEfnB3
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DmEfn
CeEfn-3
CeEfn-4
CeEfn-1
vertebrate ephrin-As
C. intestinalis ephrin-As
chordate ephrin-As
vertebrate ephrin-Bs
chordate ephrin-Bs
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ecdysozoan ephrins
non-chordate ephrins
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83
79
100
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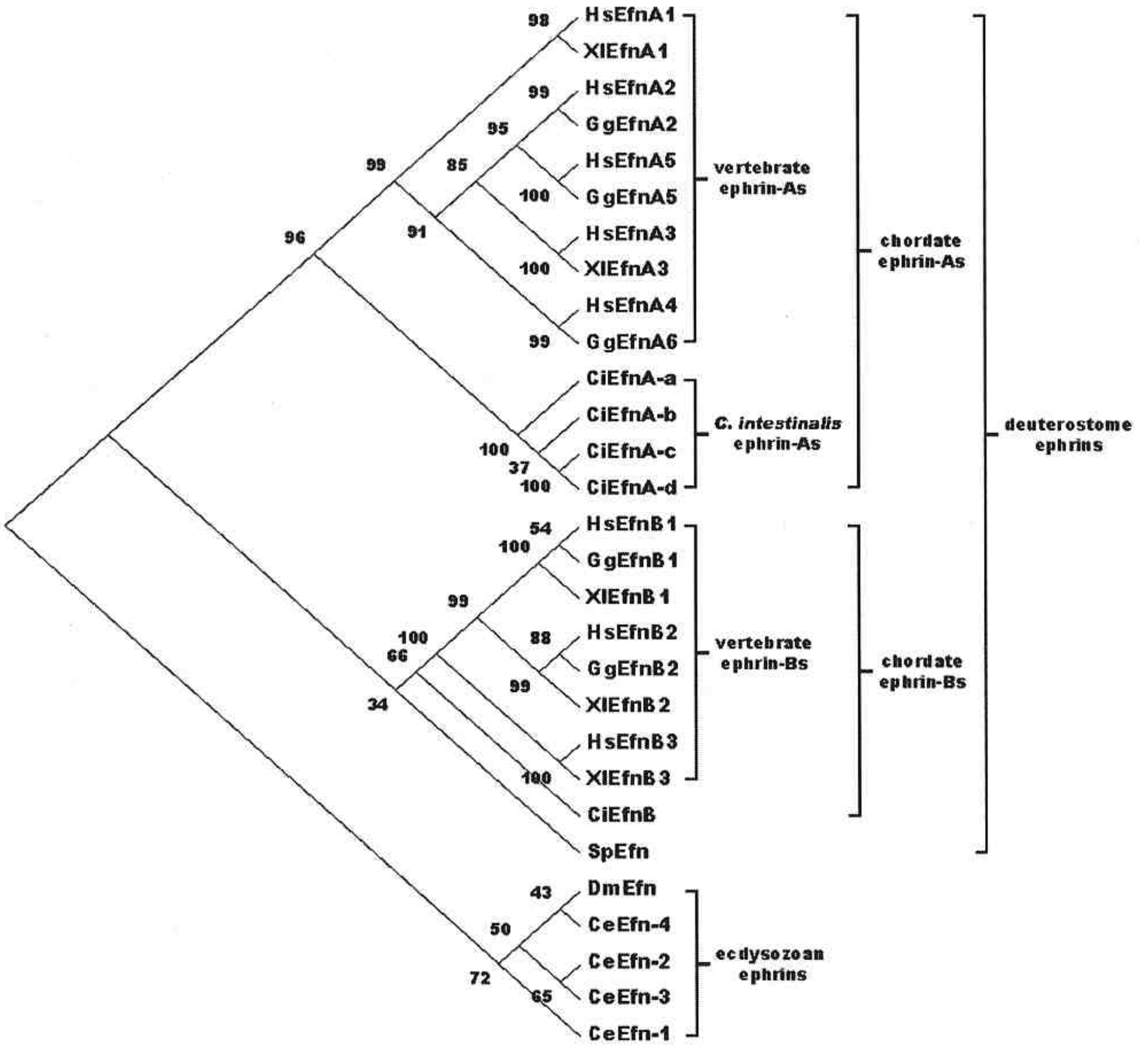
Fig. 10C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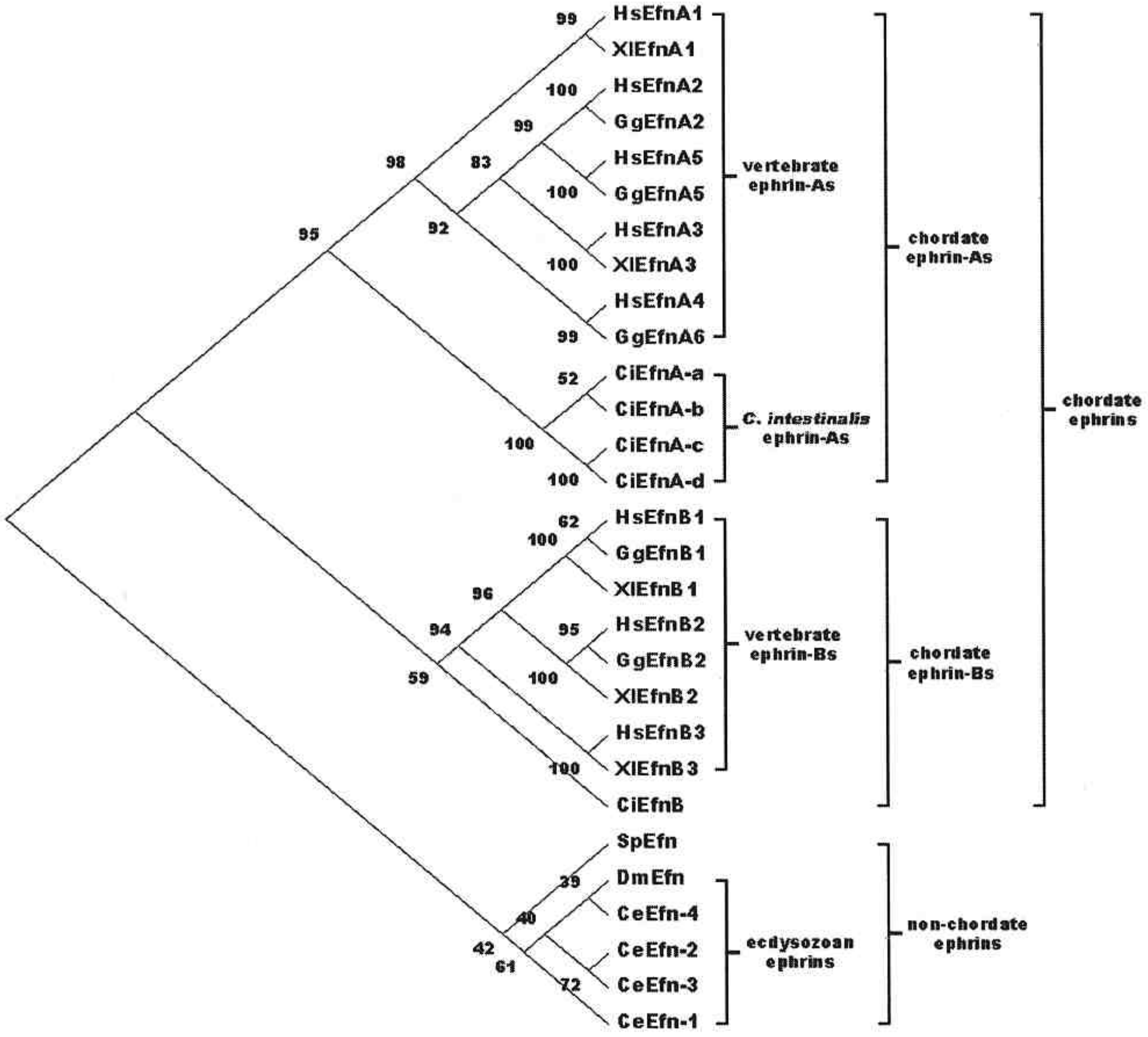
GgEfnA6
CiEfnA-a
CiEfnA-b
CiEfnA-c
CiEfnA-d
HsEfnB1
GgEfnB1
XlEfnB1
HsEfnB2
GgEfnB2
XlEfnB2
HsEfnB3
XlEfnB3
CiEfnB
SpEfn
DmEfn
CeEfn-4
CeEfn-2
CeEfn-3
CeEfn-1
98
99
95
99
85
100
96
91
100
99
100
37
100
54
100
99
100
66
88
99
34
100
43
50
72
65
vertebrate ephrin-As
C. intestinalis ephrin-As
chordate ephrin-As
vertebrate ephrin-Bs
chordate ephrin-Bs
deuterostome ephrins
ecdysozoan ephrins

Fig. 10D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CiEfnA-b
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CiEfnA-d
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GgEfnB1
XlEfnB1
HsEfnB2
GgEfnB2
XlEfnB2
HsEfnB3
XlEfnB3
CiEfnB
SpEfn
DmEfn
CeEfn-4
CeEfn-2
CeEfn-3
CeEfn-1
99
100
99
98
83
100
95
92
100
99
52
100
100
62
100
96
94
95
59
100
100
39
40
42
61
72
vertebrate ephrin-As
C. intestinalis ephrin-As
chordate ephrin-As
vertebrate ephrin-Bs
chordate ephrin-Bs
chordate ephrins
ecdysozoan ephrins
non-chordate ephrins

ecdysozoan clade, CeEfn-2-4 and DmEfn always form a weakly supported subclade (BS = 14-50%). Within this subclade, three of the four trees (NJ, ME, and ML) group CeEfn-2 with CeEfn-3 (BS = 65-81%) and CeEfn-4 with DmEfn (BS = 39-100%). One point that remains unclear from this analysis is the placement of the single *S. purpuratus* ephrin. In the ML and NJ trees, it groups with the *C. intestinalis* and vertebrate ephrin-Bs with weak support (BS = 22 and 34%, respectively). In the MP and ME trees, however, it groups with the other non-chordate ephrins, though again with weak support (BS = 13 and 42%, respectively).

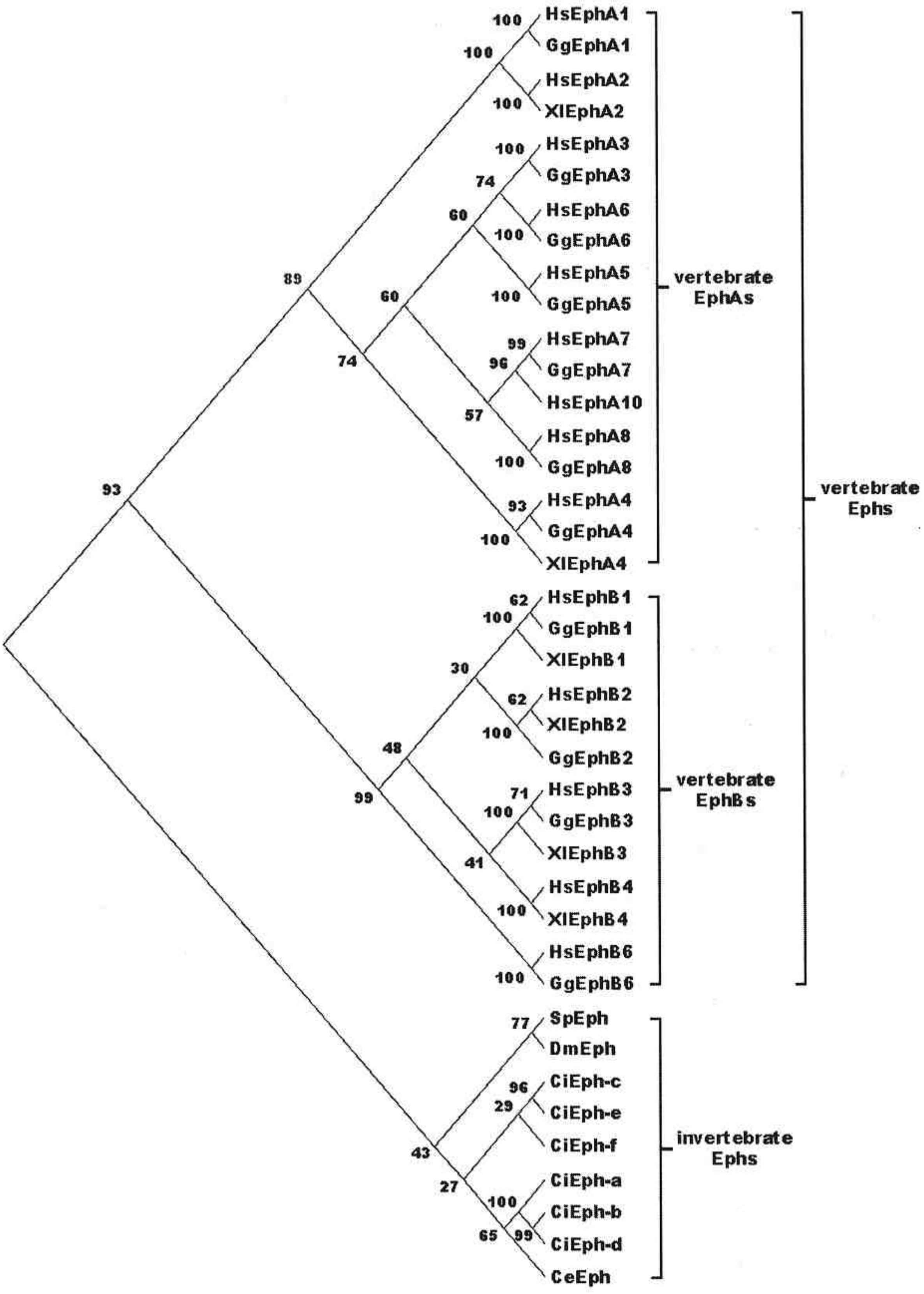
4.1.2 Eph Receptors

The four Eph trees have a similar arrangement (Fig. 11). All vertebrate EphBs are grouped together in one clade with strong support (BS = 92-100%). In three of the four trees (NJ, ML, and MP), all vertebrate EphAs are similarly placed together in a monophyletic clade (BS = 58-89%). The ME tree, on the other hand, places the EphB group together with one made up of EphA3-8 and 10 with a BS value of 59%. This clade is nested within one comprised of EphA1 and 2. Despite this difference, there are many common features in the relationships of the vertebrate EphAs. For one, EphA1 and 2 are always closely grouped (BS = 95-100%). The remaining EphAs form a monophyletic clade (BS = 65-95%) that contains at least two subgroups. One of these is made up of EphA7 and 10 (BS = 55-96%) and in all but the MP tree, EphA8 (BS = 46-58%). The second is made up of EphA3, 5, and 6 (BS = 49-96%). Where things become unclear is over the placement of EphA4. The NJ and ME trees place it just outside of the group containing EphA3, 5, and 6 (BS = 78 and 36%, respectively). On the other hand, the ML and MP trees place it outside of a clade containing EphA3, 5-8, and 10 (BS = 60 and 29%, respectively). Finally, the third major clade is comprised of the invertebrate Ephs, which group together with widely ranging support (BS = 43-100%). In all four trees, there are subgroups comprised of CiEph-a, b, and d (BS = 66-100%), CiEph-c and e (BS = 96-100%), and SpEph and DmEph (BS = 53-89%). In two of the four trees

Figure 11. Phylogenetic trees for vertebrate and invertebrate Eph receptors.

Numbers represent bootstrap values for 100 replications. The maximum likelihood (A), maximum parsimony (B), and neighbour-joining trees each have three major clades: one made of the vertebrate EphAs, another made up of the vertebrate EphBs, and a third made up of the invertebrate Ephs. The minimum evolution tree (D) has a slightly different arrangement in which the vertebrate Ephs are not divided into two major clades. Ce, *C. elegans*; Ci, *C. intestinalis*; Gg, *G. gallus*; Hs, *H. sapiens*; Sp, *S. purpuratus*; Xl, *X. laevis*.

Fig. 11A



100
HsEphA1
GgEphA1
100
HsEphA2
100
XlEphA2
100
HsEphA3
GgEphA3
74
HsEphA6
60
100
GgEphA6
89
HsEphA5
60
100
GgEphA5
99
HsEphA7
74
96
GgEphA7
HsEphA10
57
HsEphA8
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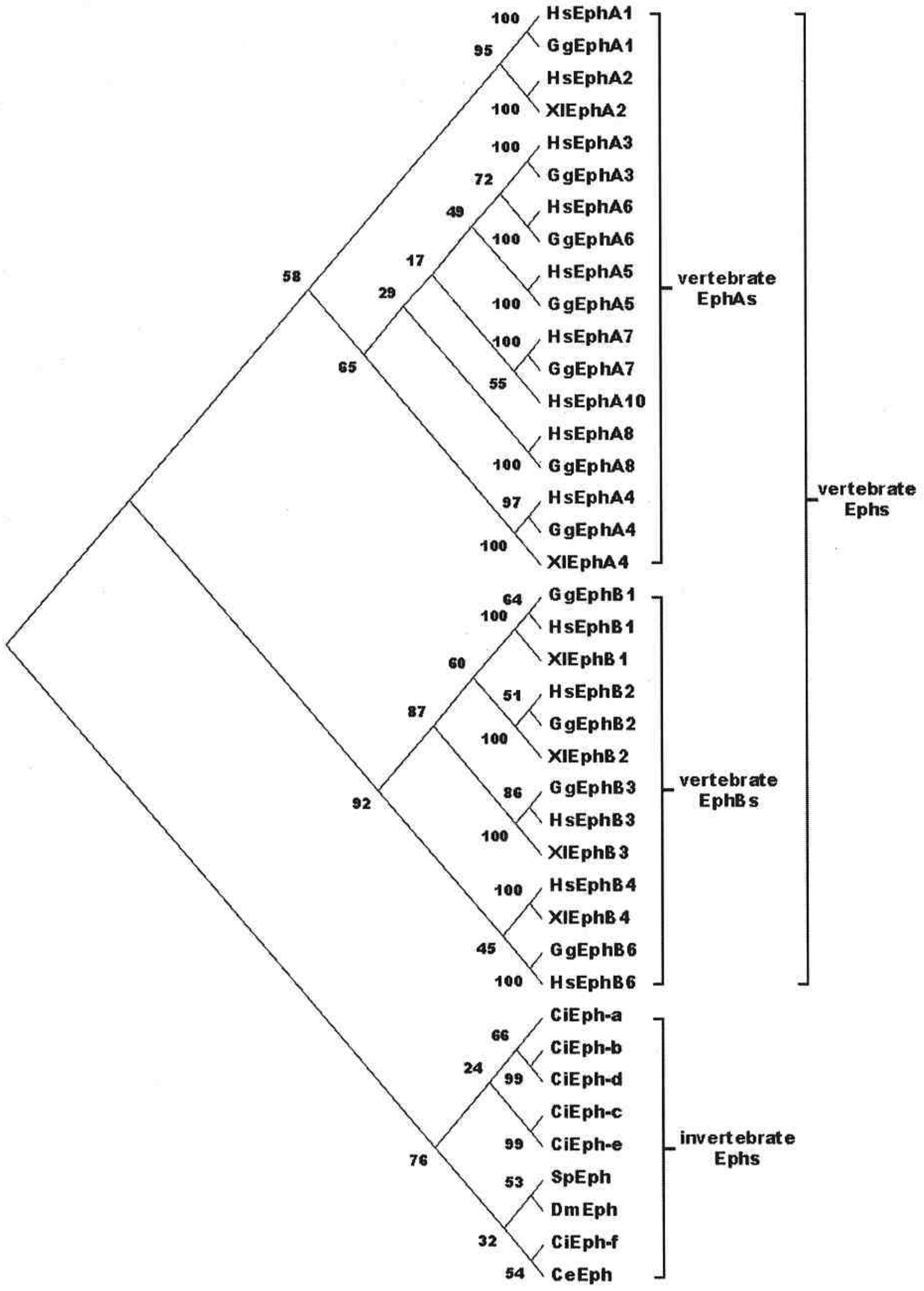
GgEphA8
93
93
HsEphA4
GgEphA4
100
XlEphA4
62
HsEphB1
100
GgEphB1
XlEphB1
30
62
HsEphB2
XlEphB2
100
GgEphB2
48
71
HsEphB3
99
100
GgEphB3
XlEphB3
41
HsEphB4
100
XlEphB4
HsEphB6
100
GgEphB6
77
SpEph
DmEph
96
CiEph-c
29
CiEph-e
CiEph-f
43
CiEph-a
27
100
CiEph-b
65
99
CiEph-d
CeEph
vertebrate EphAs
vertebrate EphBs
invertebrate Ephs
vertebrate Ephs

Fig. 11B


HsEphA1
GgEphA1
HsEphA2
XlEphA2
HsEphA3
GgEphA3
HsEphA6
GgEphA6
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GgEphA5
HsEphA7
GgEphA7
HsEphA10
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GgEphA8
HsEphA4
GgEphA4
XlEphA4
GgEphB1
HsEphB1
XlEphB1
HsEphB2
GgEphB2
XlEphB2
GgEphB3
HsEphB3
XlEphB3
HsEphB4
XlEphB4
GgEphB6
HsEphB6
CiEph-a
CiEph-b
CiEph-d
CiEph-c
CiEph-e
SpEph
DmEph
CiEph-f
CeEph
vertebrate EphAs
vertebrate EphBs
invertebrate Ephs
vertebrate Ephs
100
95
100
100
72
49
100
17
58
29
100
100
65
55
100
97
100
64
100
60
51
87
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86
92
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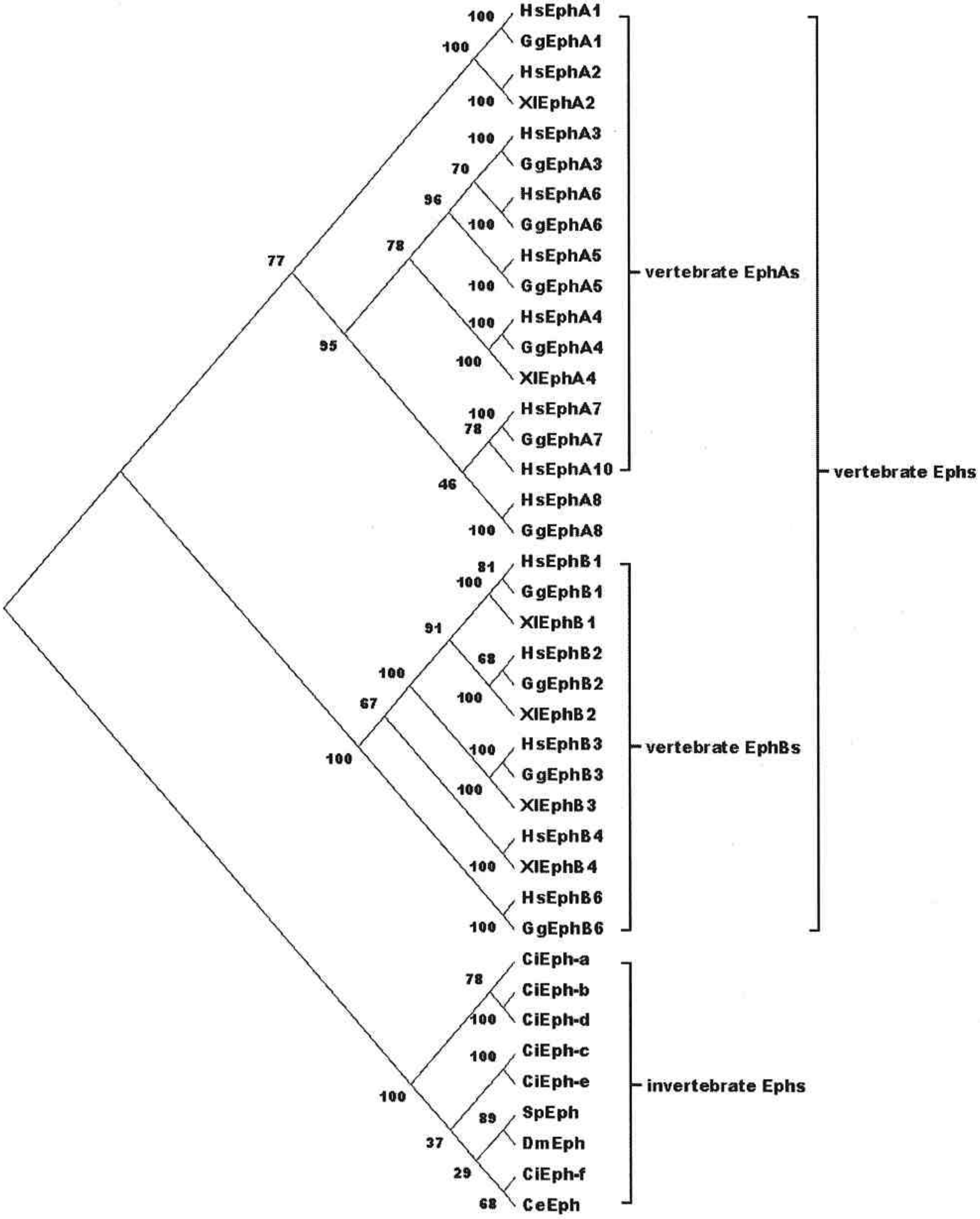
45
100
66
24
99
99
76
53
32
54

Fig. 11C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GgEphA3
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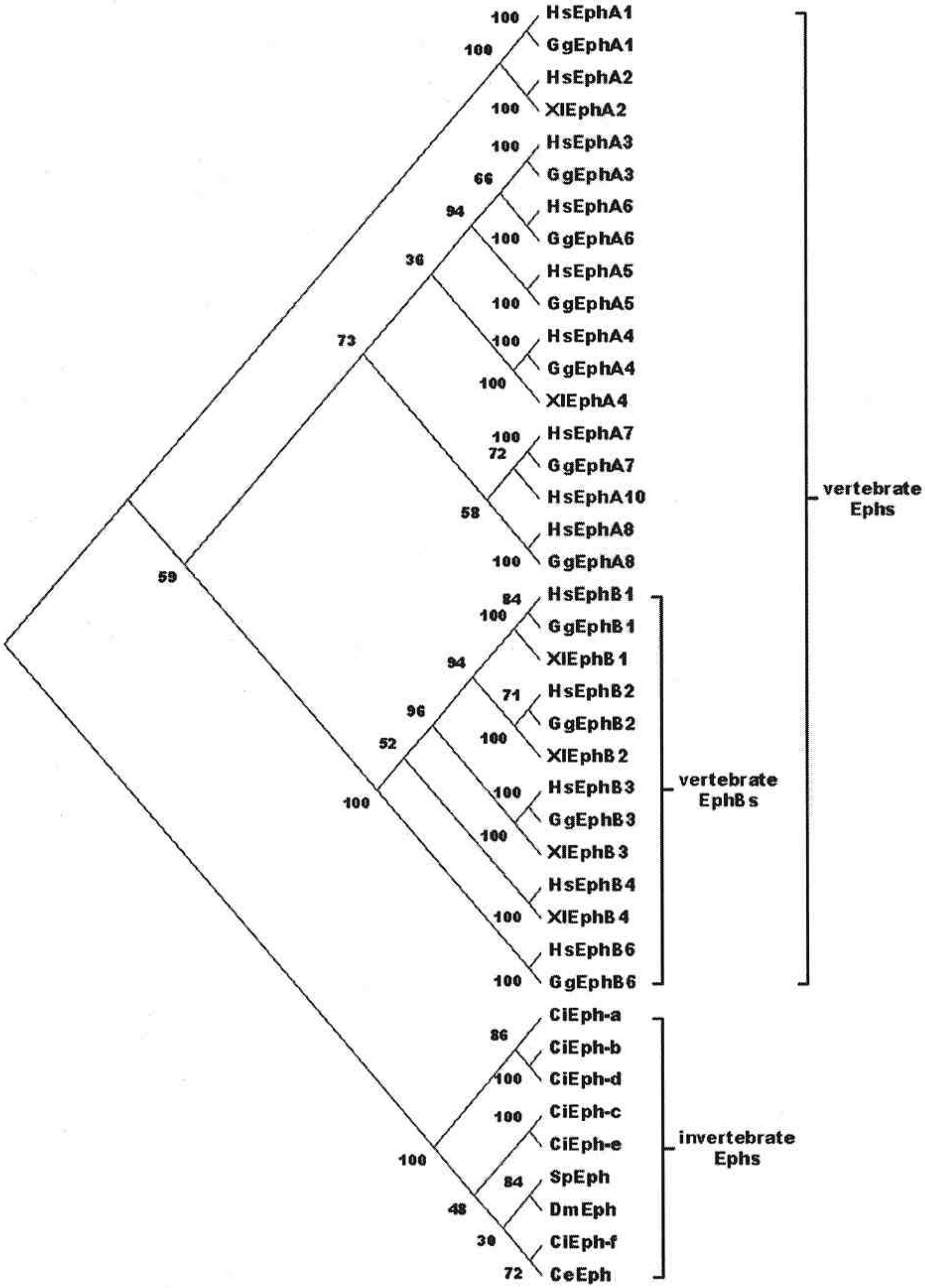
GgEphA6
78
77
HsEphA5
100
GgEphA5
vertebrate EphAs
100
HsEphA4
95
GgEphA4
100
XlEphA4
100
HsEphA7
78
GgEphA7
HsEphA10
46
HsEphA8
100
GgEphA8
vertebrate Ephs
81
HsEphB1
100
GgEphB1
XlEphB1
91
68
HsEphB2
100
GgEphB2
67
100
XlEphB2
100
HsEphB3
vertebrate EphBs
100
GgEphB3
100
XlEphB3
HsEphB4
100
XlEphB4
HsEphB6
100
GgEphB6
CiEph-a
78
CiEph-b
100
CiEph-d
100
CiEph-c
CiEph-e
invertebrate Ephs
100
89
SpEph
37
DmEph
29
CiEph-f
68
CeEph

Fig. 11D


100
HsEphA1
100
GgEphA1
HsEphA2
100
XlEphA2
100
HsEphA3
66
GgEphA3
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HsEphA6
100
GgEphA6
36
HsEphA5
100
GgEphA5
73
100
HsEphA4
GgEphA4
100
XlEphA4
100
HsEphA7
72
GgEphA7
HsEphA10
58
HsEphA8
100
GgEphA8
59
84
HsEphB1
100
GgEphB1
XlEphB1
94
71
HsEphB2
96
GgEphB2
100
52
XlEphB2
100
HsEphB3
100
GgEphB3
100
XlEphB3
HsEphB4
100
XlEphB4
HsEphB6
100
GgEphB6
CiEph-a
86
CiEph-b
100
CiEph-d
100
CiEph-c
CiEph-e
100
84
SpEph
48
DmEph
30
CiEph-f
72
CeEph
vertebrate Ephs
vertebrate EphBs
invertebrate Ephs

(NJ and ME), CiEph-a, b, and d group outside of a clade containing the remaining invertebrate Ephs (BS = 37 and 48%, respectively). SpEph, DmEph, CeEph, and CiEph-f form a weakly supported subclade (BS = 29-32%) in all but the ML tree. Within this subclade, CeEph and CiEph-f group together with moderate support (BS = 54-72%), again in all but the ML tree.

4.2 Discussion

As mentioned above, the relationship of the single *S. purpuratus* ephrin to the other ephrins is unclear. In two of the trees, it is grouped with the ecdysozoan ephrins to form a non-chordate clade. In the two other trees, however, it is grouped with the chordate ephrin-Bs. BLAST searches reveal that the closest matches to SpEfn are vertebrate ephrin-Bs, which would seem to identify these proteins as the more ancient of the two types of chordate ephrins. However, SpEfn is decidedly an ephrin-A in structure, in that it lacks a cytoplasmic domain, but possesses a putative GPI anchor site. Given that the BS support for either scenario is quite low, it is ultimately difficult to say one way or another where SpEfn belongs.

A second observation from these trees is that the *C. elegans* ephrins and *C. intestinalis* Ephs fail to form monophyletic clades. If the single *D. melanogaster* ephrin is included, then the four *C. elegans* ephrins do form a monophyletic clade. It is worth noting, however, that DmEfn has a highly unusual domain structure in which a long and unique amino-terminal sequence leads to a transmembrane domain, a central ephrin domain, a second long and unique sequence, and finally two carboxy-terminal transmembrane domains (Bossing and Brand, 2002). I conducted extensive database searches to ensure that this peculiar structure was not owing to a cloning artifact. Indeed, the same sequence of domains can be assembled from various expressed sequence tags, indicating that this is in fact the structure of the protein. As such, the relationships within the ecdysozoan clade may have something to do with how the unique segments in DmEfn are aligned with the amino acid sequences of the other ephrins. To evaluate this possibility, trees were assembled using just the receptor binding domain of the proteins in the data set, which is a common feature. As it turned out, the same relationship within the ecdysozoans was observed (data not shown), indicating that the *C. elegans* ephrins do indeed group with DmEfn. As for the six *C. intestinalis* Ephs, five of them group outside of a clade containing CiEph-f and the other invertebrate Ephs. These observations suggest that the common ancestor for *C. elegans* and *D. melanogaster* possessed multiple ephrins, some of which were lost in the lineage to *D. melanogaster*. Further, *C. intestinalis* appears to have evolved receptors in addition to an ancestral one (represented by CiEph-f). On the other hand, the BS values supporting the relationships within these invertebrate clades tend to be low and there is rarely a consensus on topology between the four trees. As such, the possibility exists that there simply is not enough information available in the sequence data to order these proteins definitively.

The most important implication from these trees is that ephrins and Ephs diversified at different times in evolutionary history. With the ephrins, there is an ancestral clade comprised of the ecdysozoan ephrins, an ephrin-A clade comprised of the vertebrate and *C. intestinalis* ephrin-As, and an ephrin-B clade comprised of the vertebrate and *C. intestinalis* ephrin-Bs. With the Ephs, on the other hand, there is an ancestral clade comprised of all invertebrate Ephs (both *C. intestinalis* and non-chordate), a vertebrate EphA clade, and a vertebrate EphB clade. This indicates that the diversification of the Ephs followed the diversification of the ephrins and further suggests that the ancestral chordate had just one receptor for two different ligands (an ephrin-A and an ephrin-B).

The possibility exists that EphA4 and EphB2 have some special relation to this ancestral Eph, given that they are uniquely capable of interacting with both types of ephrin ligands (Gale *et al.*, 1996 and Himanen *et al.*, 2004). EphA4 and EphB2 may therefore represent the most ancient vertebrate Eph receptors. By this scenario, the ancestral Eph gave rise to receptors that retained the ability to interact with both ephrin-As and ephrin-Bs, but at the same time were more specialized to interact with one type of ligand or the other. The ancestral EphA gave rise to EphA4 and a group of EphAs that lost the ability to interact with both types of ligands. Likewise, the ancestral EphB gave rise to EphB2 and a group of EphBs with more restricted binding specificities. Our trees, however, fail to support this idea since neither EphA4 nor EphB2 is placed outside of a subclade containing the remaining EphA or EphB receptors. This suggests that vertebrate Eph receptors randomly lost the ability to interact with both ephrin-As and ephrin-Bs as they diversified.

It is interesting to speculate that the diversification of Eph receptors is associated with a diversification of function. The functions performed by Ephs and ephrins typically fall into one of three basic categories: regulation of axon guidance (e.g. in the formation of visual topographic maps), directed cell migration (e.g. NC cell pathfinding), or tissue segregation (e.g. hindbrain segmentation). Do EphA receptors perform different functions from EphB receptors? A survey of the literature suggests that while some specific roles are unique to each class of receptor, both EphAs and EphBs perform a wide range of functions. For example, EphAs are uniquely involved in tissue segregation events that are part of somite segmentation (Barrios *et al.*, 2003 and Durbin *et al.*, 1998) and limb development (Araujo *et al.*, 1998, Ganju *et al.*, 1994, Patel *et al.*, 1996, and Wada *et al.*, 2003), as well as motor neuron axon guidance (Araujo and Nieto, 1997, Eberhart *et al.*, 2002, Eberhart *et al.*, 2004, Helmbacher *et al.*, 2000, Iwamasa *et al.*, 1999, Kury *et al.*, 2000, Olivieri and Miescher, 1999). On the other hand, both EphAs and EphBs contribute to hindbrain segmentation (Becker *et al.*, 1994, Nieto *et al.*, 1992, Henkemeyer *et al.*, 1994, Xu *et al.*, 1995, and Xu *et al.*, 1999), segregation of veins from arteries during angiogenesis (Adams *et al.*, 1999, Brantley-Sieders *et al.*, 2004, Gerety *et al.*, 1999, and Wang *et al.*, 1998), patterning of the inner ear (Bianchi and Liu, 1999 and Pickles *et al.*, 2002), directed migration of NC cells (Helbling *et al.*, 1998, Krull *et al.*, 1997, Smith *et al.*, 1997, and Wang and Anderson, 1997), and guidance of RGC axons to their targets (Feldheim *et al.*, 2004, Hindges *et al.*, 2002, and Walkenhorst *et al.*, 2000). Thus it is difficult to ascribe one set of functions to EphAs and another to EphBs.

While both EphAs and EphBs perform a wide range of functions, it is possible that receptors within these classes perform specific functions. Indeed, my analysis shows that there are several subgroups of ephrin-A ligands and EphA receptors, although this does not appear to be the case with the B-type ephrins or Ephs. For the ligands, ephrin-A1 always groups outside of a clade containing the other ephrin-As. Within this clade, there are two subgroups, one consisting of ephrin-A2, 3, and 5 and another consisting of ephrin-A4 and 6 (it should be noted, however, that within my data set, ephrin-A4 is unique to *H. sapiens* and ephrin-A6 is unique to *G. gallus*). For the receptors, there is one group consisting of EphA1 and 2, a second consisting of EphA7, 8, and 10, and a third consisting of EphA3, 5, and 6. The relationship of EphA4 to these groups is ambiguous, although it is certainly not a part of the EphA1 and 2 group.

These groupings may reflect a sharing of functions. A notable example comes from ephrin-A3 and 5, which are both important regulators of retinal axon guidance during the formation of visual topographic maps (Cheng *et al.*, 1995, Ciossek *et al.*, 1998, Drescher *et al.*, 1995, and Monschau *et al.*, 1997). On the other hand, there is evidence that receptors from all three EphA subgroups can perform similar functions. For instance, EphA2, EphA4, and EphA7 may all have roles in regulating neural crest cell migration (Araujo and Nieto, 1997, Helbling *et al.*, 1998, and Smith *et al.*, 1997) and brain segmentation (Araujo and Nieto, 1997, Becker *et al.*, 1994, and Ganju *et al.*, 1994). As such, the significance behind these subgroupings is unclear. They may reflect the duplication and diversification of ancestral receptors to perform similar, yet subtly different functions. Alternatively, functional redundancy of these receptors may act as a kind of fail-safe against deleterious mutations.

Another intriguing question is if one of the three prototypical functions performed by Eph receptors can be regarded as the ancestral one. In *C. elegans*, Ephs and ephrins are expressed in adjacent populations of neural precursor cells and when either receptor or ligand function is disrupted, the neural precursors become disorganized and the ventral epidermis fails to close over these cells (George *et al.*, 1998 and Chin-Sang *et al.*, 1999). While the Ephs and ephrins are expressed in neural cells, the function performed here is more consistent with a tissue segregation mechanism. On the other hand, defective Eph function in *C. elegans* also results in axons extending beyond their expected target sites (Mohammed and Chin-Sang, 2006), indicating that these receptors can contribute to axon guidance in invertebrates as well. In *D. melanogaster*, Ephs and ephrins function similarly to guide cortical axons to the optic ganglion (Dearborn *et al.*, 2002) and restrict interneuronal axons from either crossing the midline or exiting the central nervous system (Bossing and Brand, 2002). In *S. purpuratus*, Ephs and ephrins do not appear to function in axon guidance, but rather contribute to the formation of a ring of tissue known as the ciliary band (Jones *et al*, 2006).

Notably absent from these examples is a role for invertebrate Ephs and ephrins in regulating cell migration. While this could simply represent a lack of research on the subject, the possibility also exists that a cell migration function arose somewhere in the lineage to the vertebrates. Given the wider variety of Eph receptors and ephrin ligands in *C. intestinalis* as compared to the non-chordate invertebrate examples used here, perhaps this function originated in a primitive chordate. Unfortunately, there is no research on what Ephs and ephrins do in *C. intestinalis* to substantiate this. Indeed, my phylogenetic analysis indicates that the *C. intestinalis* Ephs do not have vertebrate orthologues, which argues that the ability of Ephs to regulate cell migration developed within the vertebrates.

CONCLUSIONS

I have shown that Eph receptors and their ephrin ligands contribute to CNC pathfinding in the chicken embryo, as is the case in mouse and *Xenopus*. However, the guidance mechanism appears to be more complex than one in which Eph-expressing cells are excluded from ephrin-expressing territories by receptor-mediated repulsion. Avian CNC cells do express an assortment of Eph receptors and migrate along pathways bordered by cells expressing ephrin-B1. However, while some of these receptors are expressed CNC-wide, others have a more restricted distribution. In addition, avian CNC cells express ephrin-B2 and migrate along pathways bordered by cells expressing EphB2. My functional analyses show that both ephrin-B1 and EphB2 have a repulsive effect on avian CNC cells and further suggest that these effects are mediated by the Ephs and ephrins that these cells express. These findings suggest that a migrating cell can be guided to its destination in a cell autonomous fashion by the joint action of co-expressed Eph receptors and ephrin ligands.

I have also shown through phylogenetic analysis that there are three major groups of Eph receptors and ephrin ligands. For the ligands, there is a ecdysozoan ephrin clade, a vertebrate and *C. intestinalis* ephrin-A clade, and a vertebrate and *C. intestinalis* ephrin-B clade. For the receptors, there is an invertebrate Eph clade, a vertebrate EphA clade, and a vertebrate EphB clade. Ephrins and Ephs therefore diversified at different points in evolution, such that the ancestral chordate possessed a single receptor, but two ligands. This receptor diversification does not appear to be related to a diversification of function within the vertebrates, since both EphAs and EphBs perform a wide range of similar functions. However, it may be associated with the development of novel functions not performed by invertebrate Ephs.

A number of questions remain. Whether or not an ephrin-A ligand that could interact with the EphA3, 4, and 7 receptors expressed by avian CNC cells is itself expressed in the crest-free zones adjacent to these cells remains an issue. It would also be useful to confirm that the same CNC cells actually do express both Eph receptors and ephrin ligands, perhaps through two colour ISH. Does this apparent co-expression have an effect on *trans*-activated signaling or are forward and reverse signaling pathways both active? What effect would disruption of Eph or ephrin function (e.g. by dominant-negative inhibition, application of soluble competitors, or mis-expression of wild-type proteins) have on CNC migration *in vivo*? What signaling pathways lie downstream of ephrin-B2 and the assortment of Ephs expressed by avian CNC cells? From an evolutionary standpoint, it would be interesting to find out exactly what Ephs and ephrins do in basal chordates. Also, genome sequence data from lophotrochozoans such as mollusks might allow for a considerable gap in the phylogenetic analysis presented here to be filled in.

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APPENDIX

A. Aligned Ephrin Sequences

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            ....|....| ....|....| ....|....| ....|....| ....|....|
                    10         20         30         40         50
CeEfn-1     ---------- ---------- ---------- ---------- ----------
CeEfn-2     ---------- ---------- ---------- ---------- ----------
CeEfn-3     ---------- ---------- ---------- ---------- ----------
CeEfn-4     ---------- ---------- ---------- ---------- ----------
DmEfn       MQERSKQLRL TVSWTQSKSQ IHDSCQRRSM LACKRRLTTS KVLEDSHPPV
SpEfn       ---------- ---------- ---------- ---------- ----------
CiEfnA-a    ---------- ---------- ---------- ---------- ----------
CiEfnA-b    ---------- ---------- ---------- ---------- ----------
CiEfnA-c    ---------- ---------- ---------- ---------- ----------
CiEfnA-d    ---------- ---------- ---------- ---------- ----------
CiEfnB      ---------- ---------- ---------- ---------- ----------
XlEfnA1     ---------- ---------- ---------- ---------- ----------
XlEfnA3     ---------- ---------- ---------- ---------- ----------
XlEfnB1     ---------- ---------- ---------- ---------- ----------
XlEfnB2     ---------- ---------- ---------- ---------- ----------
XlEfnB3     ---------- ---------- ---------- ---------- ----------
GgEfnA2     ---------- ---------- ---------- ---------- ----------
GgEfnA5     ---------- ---------- ---------- ---------- ----------
GgEfnA6     ---------- ---------- ---------- ---------- ----------
GgEfnB1     ---------- ---------- ---------- ---------- ----------
GgEfnB2     ---------- ---------- ---------- ---------- ----------
HsEfnA1     ---------- ---------- ---------- ---------- ----------
HsEfnA2     ---------- ---------- ---------- ---------- ----------
HsEfnA3     ---------- ---------- ---------- ---------- ----------
HsEfnA4     ---------- ---------- ---------- ---------- ----------
HsEfnA5     ---------- ---------- ---------- ---------- ----------
HsEfnB1     ---------- ---------- ---------- ---------- ----------
HsEfnB2     ---------- ---------- ---------- ---------- ----------
HsEfnB3     ---------- ---------- ---------- ---------- ----------


            ....|....| ....|....| ....|....| ....|....| ....|....|
                    60         70         80         90        100
CeEfn-1     ---------- ---------- ---------- ---------- ----------
CeEfn-2     ---------- ---------- ---------- ---------- ----------
CeEfn-3     ---------- ---------- ---------- ---------- ----------
CeEfn-4     ---------- ---------- ---------- ---------- ----------
DmEfn       AFPNCKSHRH QQQKEKHKVQ LYSGKPLSIK LYVPGSIESI PKIRHKALTT
SpEfn       ---------- ---------- ---------- ---------- ----------
CiEfnA-a    ---------- ---------- ---------- ---------- ----------
CiEfnA-b    ---------- ---------- ---------- ---------- ----------
CiEfnA-c    ---------- ---------- ---------- ---------- ----------
CiEfnA-d    ---------- ---------- ---------- ---------- ----------
CiEfnB      ---------- ---------- ---------- ---------- ----------
XlEfnA1     ---------- ---------- ---------- ---------- ----------
XlEfnA3     ---------- ---------- ---------- ---------- ----------
XlEfnB1     ---------- ---------- ---------- ---------- ----------
XlEfnB2     ---------- ---------- ---------- ---------- ----------
XlEfnB3     ---------- ---------- ---------- ---------- ----------
GgEfnA2     ---------- ---------- ---------- ---------- ----------
GgEfnA5     ---------- ---------- ---------- ---------- ----------
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GgEfnA6       ---------- ---------- ---------- ---------- ----------
GgEfnB1       ---------- ---------- ---------- ---------- ----------
GgEfnB2       ---------- ---------- ---------- ---------- ----------
HsEfnA1       ---------- ---------- ---------- ---------- ----------
HsEfnA2       ---------- ---------- ---------- ---------- ----------
HsEfnA3       ---------- ---------- ---------- ---------- ----------
HsEfnA4       ---------- ---------- ---------- ---------- ----------
HsEfnA5       ---------- ---------- ---------- ---------- ----------
HsEfnB1       ---------- ---------- ---------- ---------- ----------
HsEfnB2       ---------- ---------- ---------- ---------- ----------
HsEfnB3       ---------- ---------- ---------- ---------- ----------


              ....|....| ....|....| ....|....| ....|....| ....|....|
                     110        120        130        140        150
CeEfn-1       ---------- ---------- ---------- ---------- ----------
CeEfn-2       ---------- ---------- ---------- ---------- ----------
CeEfn-3       ---------- ---------- ---------- ---------- ----------
CeEfn-4       ---------- ---------- ---------- ---------- ----------
DmEfn         TNKQQPAMHR KSKSKSKFQG FNNLKPLYSP SKRQPPEKHS SVLVEAGIES
SpEfn         ---------- ---------- ---------- ---------- ----------
CiEfnA-a      ---------- ---------- ---------- ---------- ----------
CiEfnA-b      ---------- ---------- ---------- ---------- ----------
CiEfnA-c      ---------- ---------- ---------- ---------- ----------
CiEfnA-d      ---------- ---------- ---------- ---------- ----------
CiEfnB        ---------- ---------- ---------- ---------- ----------
XlEfnA1       ---------- ---------- ---------- ---------- ----------
XlEfnA3       ---------- ---------- ---------- ---------- ----------
XlEfnB1       ---------- ---------- ---------- ---------- ----------
XlEfnB2       ---------- ---------- ---------- ---------- ----------
XlEfnB3       ---------- ---------- ---------- ---------- ----------
GgEfnA2       ---------- ---------- ---------- ---------- ----------
GgEfnA5       ---------- ---------- ---------- ---------- ----------
GgEfnA6       ---------- ---------- ---------- ---------- ----------
GgEfnB1       ---------- ---------- ---------- ---------- ----------
GgEfnB2       ---------- ---------- ---------- ---------- ----------
HsEfnA1       ---------- ---------- ---------- ---------- ----------
HsEfnA2       ---------- ---------- ---------- ---------- ----------
HsEfnA3       ---------- ---------- ---------- ---------- ----------
HsEfnA4       ---------- ---------- ---------- ---------- ----------
HsEfnA5       ---------- ---------- ---------- ---------- ----------
HsEfnB1       ---------- ---------- ---------- ---------- ----------
HsEfnB2       ---------- ---------- ---------- ---------- ----------
HsEfnB3       ---------- ---------- ---------- ---------- ----------


              ....|....| ....|....| ....|....| ....|....| ....|....|
                     160        170        180        190        200
CeEfn-1       ---------- ---------- ---------- ---------- -MHPPIKIQT
CeEfn-2       ---------- ---------- ---------- ---------- -----MQIAT
CeEfn-3       ---------- ---------- ---------- ---------- ----MSSSWA
CeEfn-4       ---------- ---------- ---------- ---------- ----MKQFFE
DmEfn         KASRHFVGKK RIKNRNCLLS SPQPSPMRCK MMIPFPKFGA TSFVTLLTLI
SpEfn         ---------- ---------- ---------- -------MAL PKAKSGIIFF
CiEfnA-a      ---------- ---------- ---------- ---------- --MLIHQCVF
CiEfnA-b      ---------- ---------- ---------- --MPLSKVNR MILSLFMCYL
CiEfnA-c      ---------- ---------- ---------- ---------- MIRSLAICFS
CiEfnA-d      ---------- ---------- ---------- ---------M ATQFTYYLFT
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CiEfnB      ---------- ---------- ---------- ---MTTVKMD KLTIFVAALS
XlEfnA1     ---------- ---------- ---------- ------MMEL YRAAVQLIVG
XlEfnA3     ---------- ---------- ---------- ----MAVVFV LLFPLLFLFP
XlEfnB1     ---------- ---------- ---------- -------MEG ---LRRLLG-
XlEfnB2     ---------- ---------- ---------- ---MALSG-- DSVWKYSLG-
XlEfnB3     ---------- ---------- ---------- --------MF SRECALYIR-
GgEfnA2     ---------- ---------- ---------- ----MPRWEA AALLAAIVG-
GgEfnA5     ---------- ---------- ---------- ----MPHVEM LLLAVAALW-
GgEfnA6     ---------- ---------- ---------- ---------- --LLGLLLWA
GgEfnB1     ---------- ---------- ---------- ----MARPRG ---GRWLLG-
GgEfnB2     ---------- ---------- ---------- ---MAARRRD ASACKYCWG-
HsEfnA1     ---------- ---------- ---------- -------MEF LWAPL-----
HsEfnA2     ---------- ---------- ---------M APAQRPLLPL LLLLLPLPPP
HsEfnA3     ---------- ---------- ---------- ----MAAAPL LLLLLLVPVP
HsEfnA4     ---------- ---------- ---------- -------MRL LPLLRTVLWA
HsEfnA5     ---------- ---------- ---------- ----MLHVEM LTLVFLVLW-
HsEfnB1     ---------- ---------- ---------- ----MARPGQ RWLGKWLVAM
HsEfnB2     ---------- ---------- ---------- ---MAVRR-- DSVWKYCWG-
HsEfnB3     ---------- ---------- ---------- -----MGPPH SGPGGVRVG-


            ....|....| ....|....| ....|....| ....|....| ....|....|
                   210        220        230        240        250
CeEfn-1     ILLFILTTVH CSAKRLPQIY WNSTNPLVER ---------- -YAAIG----
CeEfn-2     FILLSLFPFI GWARKIPDIN WISSNPIFDV SN----TDHV ISVHIG----
CeEfn-3     LFLLLFVAPL VSCRNTHDVM WNSKQFPVAF K------TTP IKVDLG----
CeEfn-4     FLITTFLLLG LAAADEHIVY WNSTNSLFRN R------QPT IEVRMG----
DmEfn       CMETVLLSTM SSCAKTFYMH WNTSNSIFRI DN----TDHI IDVNKGNLAF
SpEfn       VNAVLLLWSV TLAKTLQPIA WDKNNSNFTS ------TGLN ISVEIN----
CiEfnA-a    LLTCLSLVKL VISRTRHVVF WDRSINSSLT ------TELR LQVQIN----
CiEfnA-b    LFDGVRSKRF NSGTITHEVM WDPRENGGFA FG----NEFS IEVHMR----
CiEfnA-c    LLLFQGEGN- -AKRRHHVIY WNTVMNPGLL S----TSEHT YHVKIG----
CiEfnA-d    LCLICVNSQ- -ATVDRHVLY WNSVMTPGLK -----KNDYV MQVKMG----
CiEfnB      IVIWGPESVT VEGKTREPIY WNSANPLWDS ------ETRL INVAIN----
XlEfnA1     VGLGVGLWLR EAQGERHIVF WNSSNYRFMQ ------EDYT VQVQLN----
XlEfnA3     RS-----G-- ---ANRHSVY WNSSNYHLRR ------DGYT VQVDVN----
XlEfnB1     LLLVLYRLCS ALGKNLEPVT WNSQNPRFIS G-----KGLV LYPEIG----
XlEfnB2     VCILLLRTAI SWSTVLDPIY WNSSNARFLP G-----QGLI LYPKIG----
XlEfnB3     MLFTLWDFCS ISALSLDPIY WNSSNKRFED T-----EGYV LYPQIG----
GgEfnA2     VCVWSDDPG- KVISDRYAVY WNRSNPRFHR ------GDYT VEVSIN----
GgEfnA5     VCVRGQEPGR KAVADRYAVY WNSTNPRFQQ ------GDYH IDVCIN----
GgEfnA6     PLLWAPPPP- -VRGRRHGVY WNGSNPRFLQ ------DDYS IQVSIN----
GgEfnB1     VLLALCRLAA PLAKSLEPVS WSAGNPKFMS G-----KGLV IYPEIG----
GgEfnB2     ALMVLWRTAL AKSIVLDPIY WNSSNPKFLP G-----QGLV LYPQIG----
HsEfnA1     ----LGLCCS LAAADRHTVF WNSSNPKFRN ------EDYT IHVQLN----
HsEfnA2     PFARAEDAA- RANSDRYAVY WNRSNPRFHA GAGDDGGYT VEVSIN----
HsEfnA3     LLPLLAQGPG GALGNRHAVY WNSSNQHLRR ------EGYT VQVNVN----
HsEfnA4     AFLGSPLRG- -GSSLRHVVY WNSSNPRLLR ------GDAV VELGLN----
HsEfnA5     MCVFSQDPGS KAVADRYAVY WNSSNPRFQR ------GDYH IDVCIN----
HsEfnB1     VVWALCRLAT PLAKNLEPVS WSSLNPKFLS G-----KGLV IYPKIG----
HsEfnB2     VLMVLCRTAI SKSIVLEPIY WNSSNSKFLP G-----QGLV LYPQIG----
HsEfnB3     ALLLLGVLGL VSGLSLEPVY WNSANKRFQA E-----GGYV LYPQIG----


            ....|....| ....|....| ....|....| ....|....| ....|....|
                   260        270        280        290        300
CeEfn-1     --DTLDIVCP FFDENSDE-- -------LTE QSIIYRVTEE EYENCERRSK
CeEfn-2     --DRVSIRCP KSDETG---- -------KYE YSYIYMVSDE EYDHCFLSKP
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CeEfn-3       --DQLTIICP KAYG------ ------MTYE YAKLYWVGET EWSQCWLHEP
CeEfn-4       --DVVRFVCP DNEEGR---- ------NDGE YLIVYEVTEF AMDDCALESH
DmEfn         EFDQVHIICP VYEPGTFEN- -------ETE KYIIYNVSKV EYETCRITNA
SpEfn         --DLIDIQCP QTENTTEGR- ------LKAQ YLKFMQVSYQ GYEQCSLSAR
CiEfnA-a      --EWMDILCP KPARNDEKR- ------GEEL YIDVFNVTAA QYHRCN--TQ
CiEfnA-b      --DYMNIHCP QYDQDD---- ------KAKL SFIIYNVSEQ SYNSCSLTDM
CiEfnA-c      --EFLDILCP QNSLMGITD- ----GLEEPE TFNLYNVTQT DFEQCSHGDN
CiEfnA-d      --QFMDILCP QRSLMGIT-- ----GPKEPT TFDLYNVTED NYQQCNTGGH
CiEfnB        --DRLDIICP RRGRDE---- -----GEELF YYKLYLVSKE DFKRCNASNN
XlEfnA1       --DYLDIVCP YYEEGSVAG- ------HTVE RYTLFLVDYE EYETCKPKSK
XlEfnA3       --DYLDIYCP HYNESVVEH- -------KME QYILYMVSYE GYRTCN-ISQ
XlEfnB1       --DRLDIICP KG-------- ----LFQPYE YYKLYMVR-R DQLEACSTVI
XlEfnB2       --DKLDIICP KVDSK----- ----STGQYE YYKIYLVD-K EQADSCTIK-
XlEfnB3       --DRLDLLCP RSEPQGPF-- ----SSSPYE YYKLYLVGTK EEMSSCSILR
GgEfnA2       --DYLDIYCP HY-EEPLP-- -----AERME RYVLYMVNYE GHASCDHRQK
GgEfnA5       --DYLDVFCP HY-EDSVP-- -----EDKTE RYVLYMVNFD GYSSCDHISK
GgEfnA6       --DHLDIYCP HY-SAPTP-- -------WAE SFTLFMVDEE GYRGCS-ETP
GgEfnB1       --DKLDIICP KAE------- ----PSKPYD YYKLYLVK-K DQADACSTVM
GgEfnB2       --DKLDIICP KVDSK----- ----TVGQYE YYKVYMVD-K DQADSCAIRK
HsEfnA1       --DYVDIICP HYEDHSVAD- ------AAME QYILYLVEHE EYQLCQPQSK
HsEfnA2       --DYLDIYCP HY-GAPLPP- -----AERME HYVLYMVNGE GHASCDHRQR
HsEfnA3       --DYLDIYCP HYNSSGVGPG AGPGPGGGAE QYVLYMVSRN GYRTCN-ASQ
HsEfnA4       --DYLDIVCP HY-EGPGPP- ------EGPE TFALYMVDWP GYESCQAEGP
HsEfnA5       --DYLDVFCP HY-EDSVP-- -----EDKTE RYVLYMVNFD GYSACDHTSK
HsEfnB1       --DKLDIICP RAE------- ----AGRPYE YYKLYLVR-P EQAAACSTVL
HsEfnB2       --DKLDIICP KVDSK----- ----TVGQYE YYKVYMVD-K DQADRCTIKK
HsEfnB3       --DRLDLLCP RARPPGPH-- ----SSPNYE FYKLYLVGGA QGRR-CEAPP


              ....|....| ....|....| ....|....| ....|....| ....|....|
                      310        320        330        340        350
CeEfn-1       A-KELGRCTQ PYQEE----- ---------- --------KL KVAFRLMSPN
CeEfn-2       R--LVGACDN QTIN------ ---------- -------ASI NIVFRSFTPT
CeEfn-3       VWLGVCATEN YTT------- ---------- --------EV KLIFRQTNPI
CeEfn-4       SREVIRCAPE GTAEKVLRTQ QLSGGRREDW KKQKVPPKNV AQLIRQLNPI
DmEfn         DPRVIAICDK PQKL------ ---------- -------MFF TITFRPFTPQ
SpEfn         N-RHLLSCNR PYVQN----- ---------- --------RL TLLFQEHTPY
CiEfnA-a      KIQRVLRCSD PKRE------ ---------- -------IKL TTKFQERSPS
CiEfnA-b      K-TAIFKCDN PVKGR----- ---------- --------KL TTKFQRRSPN
CiEfnA-c      K-NFIFACDR PEREN----- ---------- --------KL TIKFQTISPS
CiEfnA-d      K-NFIFACDR PEREN----- ---------- --------KL TIKFQTISPS
CiEfnB        DRRRLITCNV PDREK----- ---------- --------KY TFYFQEISPS
XlEfnA1       D-QVRWECNK PFAPHGP--- ---------- -------EKF CEKFQKFTPF
XlEfnA3       G-FKRWECNR PHVPHSP--- ---------- -------IKF SEKFQRYSAF
XlEfnB1       DPNVLVTCNQ PGKE------ ---------- -------YRF TIKFQEFSPN
XlEfnB2       DRTPLLNCAK PDQD------ ---------- -------VKF TIKFQEFSPN
XlEfnB3       TPNLLLTCDR PSQD------ ---------- -------LRF TIKFQEFSPN
GgEfnA2       G-FKRWECNR PDSPSGP--- ---------- -------LKF SEKFQLFTPF
GgEfnA5       G-FKRWECNR PHSPNGP--- ---------- -------LKF SEKFQLFTPF
GgEfnA6       GAFKRWECNK PFAPFVP--- ---------- -------VRF SEKIQRFTPF
GgEfnB1       DPNVLVTCNR PEQE------ ---------- -------IRF TIKFQEFSPN
GgEfnB2       DNTPLLNCAK PDQD------ ---------- -------VKF TIKFQEFSPN
HsEfnA1       D-QVRWQCNR PSAKHGP--- ---------- -------EKL SEKFQRFTPF
HsEfnA2       G-FKRWECNR PAAPGGP--- ---------- -------LKF SEKFQLFTPF
HsEfnA3       G-FKRWECNR PHAPHSP--- ---------- -------IKF SEKFQRYSAF
HsEfnA4       RAYKRWVCSL PFG---H--- ---------- -------VQF SEKIQRFTPF
HsEfnA5       G-FKRWECNR PHSPNGP--- ---------- -------LKF SEKFQLFTPF
HsEfnB1       DPNVLVTCNR PEQE------ ---------- -------IRF TIKFQEFSPN
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HsEfnB2          ENTPLLNCAK PDQD------ ---------- -------IKF TIKFQEFSPN
HsEfnB3          APNLLLTCDR PDLD------ ---------- -------LRF TIKFQEYSPN


                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         360        370        380        390        400
CeEfn-1          PSGLDYRPGV TYYFISTSTG SRKGLYNEQG GLCASHNLKM VIHITDRNGD
CeEfn-2          PGGFEFQPGK NYFLISTSDG TLEGIDRKKD GLCTAKQMKI KFEVGQDRRG
CeEfn-3          PDGMDFQVGK TYYIISTSTG DIEGINQAVG GLCKYHHMKL AISVVGYEKQ
CeEfn-4          PNGKEYQPGQ TYYYMTTSTG KANGTNHRMY GLCESQNMRL SMKVSASQPH
DmEfn            PGGLEFLPGN DYYFISTSS- -KDDLYRRIG GRCSTNNMKV VFKVCCAPED
SpEfn            PRGPTFRHGE EYYFITTSNG SANGIDNSRG GLCESHYMKL RVYVKPKPTA
CiEfnA-a         PLGFVFRPGQ SYYYISKPS- --KGGTRGCS TDTLRLVVEV ESQHGRVEAN
CiEfnA-b         PLGFVYQPNK DYFFMAFKKD EPQNCKSAMK MKVHVLPKRL HEHDRKPVTA
CiEfnA-c         PLGFRFQYCS EYYFVAASRD PNK-RTNGCN KNSMRLRIFV ECRETRHHKH
CiEfnA-d         PLGFRFQYCS EYYFVAVPRN GRRGKQSGCH KQSTRLRISV GCHETTTTST
CiEfnB           PWGIEFQPDE TYYVISTSDG TKSGIESLQG GVCTRMNMKL ELRVHPEDTP
XlEfnA1          TLGTEFREGR TYYYISKP-- ----IHY-HG ETCMRLRVHV SGRTTPPPVN
XlEfnA3          SLGYEFHAGH EYYYISTP-- ----THN-HR RSCLKMKVFV CCASTSHSGE
XlEfnB1          YMGLEFRRNQ DYYITSTSNS TLQGLENREG GVCQTRSMKI IMKVGQDPNA
XlEfnB2          LWGLEFQRDK DYYIISTSNG SIEGVDNQEG GVCVTKAMKI LMKVGQDPD-
XlEfnB3          LWGHEFQSQR DYYIIATSDG TMDGIETLQG GVCETKGMKV TLKVGQSPNG
GgEfnA2          SLGFEFRPGH EYYYISAS-- ----PLNVVD RPCLKLKVYV RPTN------
GgEfnA5          SLGFEFRPGR EYFYISSA-- ----IPDNGR RSCLKLKVFV RPANSCMKTI
GgEfnA6          SLGFEFRPGE TYYYISVP-- ----TPG-SA GRCLKLRVSV CCRASTPEP-
GgEfnB1          YMGLEFKRQQ DYFITSTSNG TLDGLENREG GVCQTRSMKI VMKVGQDPNA
GgEfnB2          LWGLEFQKNK DYYVISTSNG SLEGLNNQEG GVCQTKTMKI LMKVGQDPNS
HsEfnA1          TLGKEFKEGH SYYYISKP-- ----IHQ-HE DRCLRLKVTV SGKITHSPQA
HsEfnA2          SLGFEFRPGH EYYYISAT-- ----PPNAVD RPCLRLKVYV RPTN------
HsEfnA3          SLGYEFHAGH EYYYISTP-- ----THN-LH WKCLRMKVFV CCASTSHSGE
HsEfnA4          SLGFEFLPGE TYYYISVP-- ----TPE-SS GQCLRLQVSV CCKERKSE--
HsEfnA5          SLGFEFRPGR EYFYISSA-- ----IPDNGR RSCLKLKVFV RPTNSCMKTI
HsEfnB1          YMGLEFKKHH DYYITSTSNG SLEGLENREG GVCRTRTMKI IMKVGQDPNA
HsEfnB2          LWGLEFQKNK DYYIISTSNG SLEGLDNQEG GVCQTRAMKI LMKVGQDASS
HsEfnB3          LWGHEFRSHH DYYIIATSDG TREGLESLQG GVCLTRGMKV LLRVGQSPRG


                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         410        420        430        440        450
CeEfn-1          IGPHHHRHHH KKTTTTTTTS TSTSTPKTIP PVVEMDSSAE KLWEQFYEKV
CeEfn-2          IENPKFAART LKKDRDAEHS TPVMYVVHD- ---------- ----------
CeEfn-3          SHSK------ ------SEIT EKNFAHGIG- ---------- ----------
CeEfn-4          PTRRAPTRRQ EDFVTTASAE LMGGQEDEDS DNDNAHLLPR DLEGSTN---
DmEfn            NNKTTALSNS KSVTDTGGAI NVNIANNDES HVNSHGNNIA IGTNIGINGG
SpEfn            SPTTGGPPTT PSNSSTPPDI SRAATSMTSR TTTRATTTLR TTSLPPTTTR
CiEfnA-a         KHTRFDPWLD QSEFGMKHNK RHHHLKNAEN ELEKREPQEP VVLSQKNSAA
CiEfnA-b         GTTRSSTPRT LSSTTTTTTI ATTTTTTPRT SQKPHIPRQS STRNTPPTKT
CiEfnA-c         DKKTTTASTT TTTTLPTTTI TKVRTTTSRK PRIVLPNVEH SNTTKS---I
CiEfnA-d         TTTTTTTTTT TTTTTTPKPT TSTANPDYEF IKKLLASTKD TQSSRDETFV
CiEfnB           IVDNRVMVDT TEHNLPKPHL PPKVTDRKPI IDPNPPTHYN PVVVPPGTPK
XlEfnA1          VH-------- ------TPR- SHIQSDEPE- ---------- ----------
XlEfnA3          KHSPTLPQFT IGPEVNIEDL DNF---NPE- ---------- ----------
XlEfnB1          VPPEQLT--T TRPSKEADNT GKIATFGPWN --GPVQNPGK SDTNLSDKPT
XlEfnB2          FHNHRGSSST RRPDHESGTN GKSSTTSPHV N----GPEGS STDGKNAGHS
XlEfnB3          ATPPRRP--S SAG---KDSG ISPSVPNPDI P----NVGET SGNATKTGEN
GgEfnA2          ---------- ---------- -DSLYESP-- ---------- ----------
GgEfnA5          GVHDRVFDVN DKVENSLEPA DDTVRESA-- ---------- ----------
GgEfnA6          ---------- ------LTEV PNSQPRGRG- ---------- ----------
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GgEfnB1     VIPEQLT--T SRPSKEADNT VKIVTQSPRH KVPTVEEPGK PGSVNQNGQE
GgEfnB2     AGLPRSTDPT KRPEQEAGTN GKSSTTSPFV K----DHSGS STDGSKAGHS
HsEfnA1     HD-------- ------NPQE KRLAADDPE- ---------- ----------
HsEfnA2     ---------- ---------- -ETLYEAP-- ---------- ----------
HsEfnA3     KPVPTLPQFT MGPNVKINVL EDFEGENPQ- ---------- ----------
HsEfnA4     ---------- ---------- -SAHPVGSP- ---------- ----------
HsEfnA5     GVHDRVFDVN DKVENSLEPA DDTVHESA-- ---------- ----------
HsEfnB1     VTPEQLT--T SRPSKEADNT VKMATQAPGS RGSLGDSDGK HETVNQEEKS
HsEfnB2     AGSTRNKDPT RRPELEAGTN GRSSTTSPFV K----PNPGS STDGNSAGHS
HsEfnB3     GAVPRKP--V SEMPMERDRG AAHSLEPGKE N----LPGDP TSNATSRGAE


            ....|....| ....|....| ....|....| ....|....| ....|....|
                    460        470        480        490        500
CeEfn-1     MPIDN----- ---------- TWPEITRGER VTLYQGNKKD EYEQVPAEVV
CeEfn-2     ---------- ---------- ------ESDI DDDDDGSNAC SYFVSIVVLL
CeEfn-3     ---------- ---------- ---YEIHEVG QLVSSGHQNF TLLTTTSLLF
CeEfn-4     ---------- ---------- ---PKFRRPS QLETAGVENQ QFMKVVQMAQ
DmEfn       QIIGGPQSAG --------IP INPLSGNNNI NGIPTTINSN IDQFNRIPIQ
SpEfn       RTTRYTTG-- --------GK IKSTEEGNNI PDNMTRGAAN RRHVSTTLLL
CiEfnA-a    P--------- ---------- --PDGGNSAV GIHSRRHDTR LLALITSSLL
CiEfnA-b    LKP------- ---------- --GYVRGDGE GDGPGGGVGR ITCATTWMLV
CiEfnA-c    PDKK-----P SED------P ETLRQIKNRR GNNSSSCLSF SINTWTLTFF
CiEfnA-d    PKLKSLLIKS SEKNSPVVLA SSLDQTSDNK GNAAPSHTSV FSTTLLVMLT
CiEfnB      NTPPQSIN-- -------PVD PPVDPVAPTQ SENSTSVGSN GLVIGVVLGA
XlEfnA1     ---------- ---------- --VPLPGVMK SVAGNSAAPG TPCTLYGLLL
XlEfnA3     ---------- ---------- --IPKLEKSV SGSSPKRKHL HLTVAVCLLI
XlEfnB1     GRWGV----- ---------- --DGFFNSKI AVFAAIGAGC VIFILIIIFL
XlEfnB2     ---------- ---------- ---SILGSEV ALFAGIASGC IIFIVIIITL
XlEfnB3     G--------- ---------- ---PLPISHV PLVAGAAGGA ALLLL-VFGV
GgEfnA2     ---------- ---------- --EPIFTSNN SCCSLAVPRA VLVA--APVF
GgEfnA5     ---------- ---------- --EPS-RGEN AAQTPRIPIR LLAT--LLFL
GgEfnA6     ---------- ---------- --GPEGDAGS PRDAAPIPQR SRTRLVALAV
GgEfnB1     TQGPS----- ---------- --DGFLSSKV AVFAAIGAGC VIFILIIIFL
GgEfnB2     ---------- ---------- ---SILGSEV ALFAGIASGC IIFIVIIITL
HsEfnA1     ---------- ---------- --VR---VLH SIGHSAAPRL FPLAWTVLLL
HsEfnA2     ---------- ---------- --EPIFTSNN SCSSPGGCRL FLST--IPVL
HsEfnA3     ---------- ---------- --VPKLEKSI SGTSPKREHL PLAVGIAFFL
HsEfnA4     ---------- ---------- --GESGTSGW RGGDTPSP-L CLLLLLLLLI
HsEfnA5     ---------- ---------- --EPS-RGEN AAQTPRIPSR LLAI--LLFL
HsEfnB1     GPGASGGS-- --------SG DPDGFFNSKV ALFAAVGAGC VIFLLIIIFL
HsEfnB2     G--------- ---------- --NNILGSEV ALFAGIASGC IIFIVIIITL
HsEfnB3     G--------- ---------- ---PLPPPSM PAVAGAAGGL ALLLLGVAGA


            ....|....| ....|....| ....|....| ....|....| ....|....|
                    510        520        530        540        550
CeEfn-1     DFEIHEIGDV ESLYSSSGRL RYVLLLPALL LLRIF----- ----------
CeEfn-2     ASRYLL---- ---------- ---------- ---------- ----------
CeEfn-3     CTMFLSGVLF ---------- ---------- ---------- ----------
CeEfn-4     AGKTGTFENE KEAIAQKSSE KDGWHPVNVQ YVADLMNNAY QNADERISYQ
DmEfn       PNIIGNHVGT NAVGTGIVGG GGIILTPGHA HGNINMLQPG RGGINGAYPG
SpEfn       ASLLVAVVRW LHP------- ---------- ---------- ----------
CiEfnA-a    MVLFTKWV-- ---------- ---------- ---------- ----------
CiEfnA-b    ALVLTVLLQN ---------- ---------- ---------- ----------
CiEfnA-c    VYVITTWVMS ---------- ---------- ---------- ----------
CiEfnA-d    SWVLRSLLLV ---------- ---------- ---------- ----------
CiEfnB      CAVLFIVLLG FLGYKVYRRR RHMKKYQSPP MTPTRGATPL HQVTLLPIST
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XlEfnA1         AALLLRL--- ---------- ---------- ---------- ----------
XlEfnA3         MTLLAS---- ---------- ---------- ---------- ----------
XlEfnB1         VVLLIKIRKR HRKHTQQ-AA ALSLSTLASP K-CSGNA--- -GSEPSDIII
XlEfnB2         VVLLLKYRRR HRKHSPQHTT TLSLSTLATP KRSGNNN--- -GSEPSDIII
XlEfnB3         VGWVCHRRRQ AKHSDTRHP- PLSLGSITSP KRGGNN---- NGHEPSDIIM
GgEfnA2         WTLLGS---- ---------- ---------- ---------- ----------
GgEfnA5         LAMLLIL--- ---------- ---------- ---------- ----------
GgEfnA6         VAVLWV---- ---------- ---------- ---------- ----------
GgEfnB1         VVLLIKIRKR HRKHTQQRAA ALSLSTLASP K-CSGNA--- -GSEPSDIII
GgEfnB2         VVLLLKYRRR HRKHSPQHTT TLSLSTLATP KRSGNNN--- -GSEPSDIII
HsEfnA1         PLLLLQTP-- ---------- ---------- ---------- ----------
HsEfnA2         WTLLGS---- ---------- ---------- ---------- ----------
HsEfnA3         MTFLAS---- ---------- ---------- ---------- ----------
HsEfnA4         LRLLRIL--- ---------- ---------- ---------- ----------
HsEfnA5         LAMLLTL--- ---------- ---------- ---------- ----------
HsEfnB1         TVLLLKLRKR HRKHTQQRAA ALSLSTLASP KGGSGTA--- -GTEPSDIII
HsEfnB2         VVLLLKYRRR HRKHSPQHTT TLSLSTLATP KRSGNNN--- -GSEPSDIII
HsEfnB3         GGAMCWRRRR AKPSESRHPG PGSFGRGGSL GLGGGGGMGP REAEPGELGI


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       560        570        580        590        600
CeEfn-1         ---------- ---------- ---------- ---------- ----------
CeEfn-2         ---------- ---------- ---------- ---------- ----------
CeEfn-3         ---------- ---------- ---------- ---------- ----------
CeEfn-4         RDFEIHEEND LAVKSLEYSS SSTSLSTNFA ILLAVIYVLY ----------
DmEfn           HHHIQTGIRI NNVPTQHNYP SHKGNANSNI NGNDDHHHYN KHPNEVVKNE
SpEfn           ---------- ---------- ---------- ---------- ----------
CiEfnA-a        ---------- ---------- ---------- ---------- ----------
CiEfnA-b        ---------- ---------- ---------- ---------- ----------
CiEfnA-c        ---------- ---------- ---------- ---------- ----------
CiEfnA-d        ---------- ---------- ---------- ---------- ----------
CiEfnB          PHGHHARIQS EHERHRTGTM SSHSERPPPS YNESFLDNGG PVVAV-----
XlEfnA1         ---------- ---------- ---------- ---------- ----------
XlEfnA3         ---------- ---------- ---------- ---------- ----------
XlEfnB1         PLRTTENN-- -YCPHYEKVS GDYGHPVYIV QEMPPQSPAN IYYKV-----
XlEfnB2         PLRTAEGV-- -FCPHYEKVS GDYGHPVYIV QEMPPQSPAN IYYKV-----
XlEfnB3         PLRPSEAG-- AFCPHYEKVS GDYGHPVYIV QDMASQSPAN IYYKV-----
GgEfnA2         ---------- ---------- ---------- ---------- ----------
GgEfnA5         ---------- ---------- ---------- ---------- ----------
GgEfnA6         ---------- ---------- ---------- ---------- ----------
GgEfnB1         PLRTTENN-- -YCPHYEKVS GDYGHPVYIV QEMPPQSPAN IYYKV-----
GgEfnB2         PLRTADSV-- -FCPHYEKVS GDYGHPVYIV QEMPPQSPAN IYYKV-----
HsEfnA1         ---------- ---------- ---------- ---------- ----------
HsEfnA2         ---------- ---------- ---------- ---------- ----------
HsEfnA3         ---------- ---------- ---------- ---------- ----------
HsEfnA4         ---------- ---------- ---------- ---------- ----------
HsEfnA5         ---------- ---------- ---------- ---------- ----------
HsEfnB1         PLRTTENN-- -YCPHYEKVS GDYGHPVYIV QEMPPQSPAN IYYKV-----
HsEfnB2         PLRTADSV-- -FCPHYEKVS GDYGHPVYIV QEMPPQSPAN IYYKV-----
HsEfnB3         ALRGGGAADP PFCPHYEKVS GDYGHPVYIV QDGPPQSPPN IYYKV-----


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       610        620        630        640        650
CeEfn-1         ---------- ---------- ---------- ---------- ----------
CeEfn-2         ---------- ---------- ---------- ---------- ----------
CeEfn-3         ---------- ---------- ---------- ---------- ----------
```

```
CeEfn-4     ---------- ---------- ---------- ---------- ----------
DmEfn       ELTYNSGAAT SDGNIFALWI WILSIFPLLS IQSCHLSSYW ISASFLVSTI
SpEfn       ---------- ---------- ---------- ---------- ----------
CiEfnA-a    ---------- ---------- ---------- ---------- ----------
CiEfnA-b    ---------- ---------- ---------- ---------- ----------
CiEfnA-c    ---------- ---------- ---------- ---------- ----------
CiEfnA-d    ---------- ---------- ---------- ---------- ----------
CiEfnB      ---------- ---------- ---------- ---------- ----------
XlEfnA1     ---------- ---------- ---------- ---------- ----------
XlEfnA3     ---------- ---------- ---------- ---------- ----------
XlEfnB1     ---------- ---------- ---------- ---------- ----------
XlEfnB2     ---------- ---------- ---------- ---------- ----------
XlEfnB3     ---------- ---------- ---------- ---------- ----------
GgEfnA2     ---------- ---------- ---------- ---------- ----------
GgEfnA5     ---------- ---------- ---------- ---------- ----------
GgEfnA6     ---------- ---------- ---------- ---------- ----------
GgEfnB1     ---------- ---------- ---------- ---------- ----------
GgEfnB2     ---------- ---------- ---------- ---------- ----------
HsEfnA1     ---------- ---------- ---------- ---------- ----------
HsEfnA2     ---------- ---------- ---------- ---------- ----------
HsEfnA3     ---------- ---------- ---------- ---------- ----------
HsEfnA4     ---------- ---------- ---------- ---------- ----------
HsEfnA5     ---------- ---------- ---------- ---------- ----------
HsEfnB1     ---------- ---------- ---------- ---------- ----------
HsEfnB2     ---------- ---------- ---------- ---------- ----------
HsEfnB3     ---------- ---------- ---------- ---------- ----------


            ....|....| ....|....| ....|....| ....|....| ....|..
                   660        670        680        690
CeEfn-1     ---------- ---------- ---------- ---------- -------
CeEfn-2     ---------- ---------- ---------- ---------- -------
CeEfn-3     ---------- ---------- ---------- ---------- -------
CeEfn-4     ---------- ---------- ---------- ---------- -------
DmEfn       AILGIHYLIQ ITLQTTVQRY SPGMVEITAT SMNGMFDQNA GTIEYDR
SpEfn       ---------- ---------- ---------- ---------- -------
CiEfnA-a    ---------- ---------- ---------- ---------- -------
CiEfnA-b    ---------- ---------- ---------- ---------- -------
CiEfnA-c    ---------- ---------- ---------- ---------- -------
CiEfnA-d    ---------- ---------- ---------- ---------- -------
CiEfnB      ---------- ---------- ---------- ---------- -------
XlEfnA1     ---------- ---------- ---------- ---------- -------
XlEfnA3     ---------- ---------- ---------- ---------- -------
XlEfnB1     ---------- ---------- ---------- ---------- -------
XlEfnB2     ---------- ---------- ---------- ---------- -------
XlEfnB3     ---------- ---------- ---------- ---------- -------
GgEfnA2     ---------- ---------- ---------- ---------- -------
GgEfnA5     ---------- ---------- ---------- ---------- -------
GgEfnA6     ---------- ---------- ---------- ---------- -------
GgEfnB1     ---------- ---------- ---------- ---------- -------
GgEfnB2     ---------- ---------- ---------- ---------- -------
HsEfnA1     ---------- ---------- ---------- ---------- -------
HsEfnA2     ---------- ---------- ---------- ---------- -------
HsEfnA3     ---------- ---------- ---------- ---------- -------
HsEfnA4     ---------- ---------- ---------- ---------- -------
HsEfnA5     ---------- ---------- ---------- ---------- -------
HsEfnB1     ---------- ---------- ---------- ---------- -------
HsEfnB2     ---------- ---------- ---------- ---------- -------
```

```
HsEfnB3      ---------- ---------- ---------- ---------- -------
```

B. Aligned Eph Sequences

```
             ....|....| ....|....| ....|....| ....|....| ....|....|
                     10         20         30         40         50
CeEph        ---------- ---------- ---------- ---------- ----------
DmEph        ---------- ---------- ---------- ---------- ----------
SpEph        ---------- ---------- ---------- ---------- ----------
CiEph-a      ---------- ---------- ---------- ---------- ----------
CiEph-b      ---------- ---------- ---------- ---------- ----------
CiEph-c      ---------- ---------- ---------- ---------- ----------
CiEph-d      ---------- ---------- ---------- ---------- ----------
CiEph-e      ---------- ---------- ---------- ---------- ----------
CiEph-f      ---------- ---------- ---------- ---------- ----------
XlEphA2      ---------- ---------- ---------- ---------- ----------
XlEphA4      ---------- ---------- ---------- ---------- ----------
XlEphB1      ---------- ---------- ---------- ---------- ----------
XlEphB2      ---------- ---------- ---------- ---------- ----------
XlEphB3      ---------- ---------- ---------- ---------- ----------
XlEphB4      ---------- ---------- ---------- ---------- ----------
GgEphA1      ---------- ---------- ---------- ---------- ----------
GgEphA3      ---------- ---------- ---------- ---------- ----------
GgEphA4      ---------- ---------- ---------- ---------- ----------
GgEphA5      ---------- ---------- ---------- ---------- ----------
GgEphA6      ---------- ---------- ---------- ---------- ----------
GgEphA7      ---------- ---------- ---------- ---------- ----------
GgEphA8      ---------- ---------- ---------- ---------- ----------
GgEphB1      ---------- ---------- ---------- ---------- ----------
GgEphB2      ---------- ---------- ---------- ---------- ----------
GgEphB3      ---------- ---------- ---------- ---------- ----------
GgEphB6      ---------- ---------- ---------- ---------- ----------
HsEphA1      ---------- ---------- ---------- ---------- ----------
HsEphA2      ---------- ---------- ---------- ---------- ----------
HsEphA3      ---------- ---------- ---------- ---------- ----------
HsEphA4      ---------- ---------- ---------- ---------- ----------
HsEphA5      ---------- ---------- ---------- ---------- ----------
HsEphA6      MQFPSPPAAR SSPAPQAASS SEAAAPATGQ PGPSCPVPGT SRRGRPGTPP
HsEphA7      ---------- ---------- ---------- ---------- ----------
HsEphA8      ---------- ---------- ---------- ---------- ----------
HsEphA10     ---------- ---------- ---------- ---------- ----------
HsEphB1      ---------- ---------- ---------- ---------- ----------
HsEphB2      ---------- ---------- ---------- ---------- ----------
HsEphB3      ---------- ---------- ---------- ---------- ----------
HsEphB4      ---------- ---------- ---------- ---------- ----------
HsEphB6      ---------- ---------- ---------- ---------- ----------


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     60         70         80         90        100
CeEph        ---------- ---------- ---------- ---------- MRLYNSRILN
DmEph        ---------- ---------- ---------- ---------- --------MS
SpEph        ---------- ---------- ---------- ---------- --------MD
CiEph-a      ---------- ---------- ---------- ---------- ----------
CiEph-b      ---------- ---------- ---------- ---------- ----------
CiEph-c      ---------- ---------- ---------- ---------- ----------
CiEph-d      ---------- ---------- ---------- ---------- -MEQKYTRCF
CiEph-e      ---------- ---------- ---------- ---------- ----------
```

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CiEph-f         ---------- ---------- ---------- ---------- --MVIPAKMF
XlEphA2         ---------- ---------- ---------- ---------- --------MK
XlEphA4         ---------- ---------- ---------- ---------- --------MA
XlEphB1         ---------- ---------- ---------- ---------- --------ME
XlEphB2         ---------- ---------- ---------- ---------- ----------
XlEphB3         ---------- ---------- ---------- ---------- -------MLP
XlEphB4         ---------- ---------- ---------- ---------- --------MD
GgEphA1         ---------- ---------- ---------- ---------- --------ME
GgEphA3         ---------- ---------- ---------- ---------- --------MD
GgEphA4         ---------- ---------- ---------- ---------- --------MA
GgEphA5         ---------- -------MGL RGGGGR---A GG-------- ----------
GgEphA6         ---------- ---------- ---------- ---------- --------MA
GgEphA7         ---------- ---------- ---------- ---------- -----MVLRS
GgEphA8         ---------- ---------- ---------- ----MAWAQG DLTSSLSIVI
GgEphB1         ---------- ---------- ---------- ---------- --------MA
GgEphB2         ---------- ---------- ---------- ---------- -MPGPERTMG
GgEphB3         ---------- ---------- ---------- ---------- ------GVSS
GgEphB6         ---------- ---------- ---------- ----MDSNAD ISARRVSGMD
HsEphA1         ---------- ---------- ---------- ---------- --------ME
HsEphA2         ---------- ---------- ---------- ---------- --------ME
HsEphA3         ---------- ---------- ---------- ---------- --------MD
HsEphA4         ---------- ---------- ---------- ---------- --------MA
HsEphA5         ---------- ----MRGSGP RGAGHRRPPS GGGDTPITPA SLAGCYSAPR
HsEphA6         AGRVEEEEEE EEEDVDKDPH PTQNTCLRCR HFSLRERKRE PRRTMGGCEV
HsEphA7         ---------- ---------- ---------- ---------- -----MVFQT
HsEphA8         ---------- ---------- ---------- ----MAPARG RLPPALWVVT
HsEphA10        ---------- ---------- ---------- ---------- ---METCAGP
HsEphB1         ---------- ---------- ---------- ---------- --------MA
HsEphB2         ---------- ---------- ---------- ---------- ---MALRRLG
HsEphB3         ---------- ---------- ---------- ---------- --------MA
HsEphB4         ---------- ---------- ---------- ---------- --------ME
HsEphB6         ---------- ---------- ---------- ---------- -------MVC


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
CeEph           PHQSIFILVL QCLITIVT-- ---------- ----SHQEVL FDLSKVGSDL
DmEph           LLRTILLIII SIHFKL---- ---------- ---AHADQVV LLDTTREATL
SpEph           NLRNCIIFIV LCAWT----- ---------- ---SYAKEVK LYDSNAFSGL
CiEph-a         MDYLAVLIPL IIGLTNLQ-- ---------- -SISSEEVTI LDTTQATSDL
CiEph-b         ---------- ---------- ---------- ---MFVSEIL LDTRTALSDL
CiEph-c         ------MFFL FIVFYCIS-- ---------- -IVTGEIHVL YNTKVATSDL
CiEph-d         LCNKMIWIII ITGFLAER-- ---------- -HVIAEEAIL MDTRTAISDI
CiEph-e         ---------- ---------- ---------- -------VDL YNSKSATTDL
CiEph-f         PVSSLLILFA NFVHFVTS-- ---------- ----YQVEIL NTRNATSDLI
XlEphA2         MRAERIVPVL TL-ILSNV-- -------FIV LQT--KEVVL LDFEKTQGEL
XlEphA4         GIVHGILFCG LFGLCWAV-- -------TGS RIYPASEVTL LDSRSVQGEL
XlEphB1         LNVLLLLLCL SGG------- ---------- -QVGAVEETL MDTRTATAEL
XlEphB2         ---------- ---------- ---------- ---------- ----------
XlEphB3         AVCVILALSA VQG------- ---------- -----LEETL MDTKWTTSEL
XlEphB4         LWTLFLWLPL SLA------- ---------- -----LEVNL MNTKLETSDL
GgEphA1         LRMGRIELSL G-ILLFGS-- -------LLP AEVLGKEVNL LDTSTAQGEL
GgEphA3         RRR-LPLLLL CAALGSA--- -------GRL SARPGNEVNL LDSKTIQGEL
GgEphA4         GVPVGALLPL LVGVCGAV-- -------TGS RVYPANEVTL LDSRSVQGEL
GgEphA5         PAPGWTCLLL CAALR----- --------SL LASPGSEVNL LDSRTVMGDL
GgEphA6         RRCVVTTGLY C--------- ---------C TWCP------ ----------
GgEphA7         RLPPWIMLCS VWLLRFAH-- -------TG- EAQAAKEVIL LDSKAQQTEL
GgEphA8         ALAACLSATT SEGLLIFI-- -------TQR SAVPTPKRVL GTGSSILPPF
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GgEphB1     ADLVVLLCLA A--------- ---------- -AAAAVEETL MDTRTATAEL
GgEphB2     PLWFCCLPLA LLP------- ---------- -LLAAVEETL MDSTTATAEL
GgEphB3     RARRPPGSSR SS-------- ---------- ---------- --RRGVTSEL
GgEphB6     WLWLVCFFHL VTS------- ---------- -----LEEIL LDTTGETSEI
HsEphA1     R---RWPLGL GLVLLLCA-- -------PLP PGARAKEVTL MDTSKAQGEL
HsEphA2     LQAARACFAL LWGCALAA-- -------AAA AQG--KEVVL LDFAAAGGEL
HsEphA3     CQLSILLLLS CSVLDSF--- -------GEL IPQPSNEVNL LDSKTIQGEL
HsEphA4     GIFYFALFSC LFGICDAV-- -------TGS RVYPANEVTL LDSRSVQGEL
HsEphA5     RAPLWTCLLL CAALR----- --------TL LASPSNEVNL LDSRTVMGDL
HsEphA6     REFLLQFGFF LPLLTAWP-- -------GDC SHVSNNQVVL LDTTTVLGEL
HsEphA7     RYPSWIILCY IWLLRFAH-- -------TG- EAQAAKEVLL LDSKAQQTEL
HsEphA8     AAAAAATCVS ---------- ---------- -AARGEVNLL DT-STIHGDW
HsEphA10    HPLRLFLCRM QLCLALLL-- -------GPW RPGTAEEVIL LDSKASQAEL
HsEphB1     LDYLLLLLLA S--------- ---------- -AVAAMEETL MDTRTATAEL
HsEphB2     ------AALL LLP------- ---------- -LLAAVEETL MDSTTATAEL
HsEphB3     RARPPPPPSP PPGLLPLLPP LLLLPLLLLP AGCRALEETL MDTKWVTSEL
HsEphB4     LRVLLCWASL AAA------- ---------- -----LEETL LNTKLETADL
HsEphB6     SLWVLLLVSS VLA------- ---------- -----LEEVL LDTTGETSEI


            ....|....| ....|....| ....|....| ....|....| ....|....|
                   160        170        180        190        200
CeEph       KWDQVSLRHD ---------- ---------- ---------- ----------
DmEph       EWTRYP-YGP QA-------- ---------- ---------- ----------
SpEph       YWTVYPPQDP DS-------- ---------- ---------- ----------
CiEph-a     GWTAS-GTG- ---------- ---------- ---------- ----------
CiEph-b     GWTKSQGSE- ---------- ---------- ---------- ----------
CiEph-c     DWALHPTYG- ---------- ---------- ---------- ----------
CiEph-d     GWAMSPGSD- ---------- ---------- ---------- ----------
CiEph-e     KWTPFSTGIG ---------- ---------- ---------- ----------
CiEph-f     GWNMTTYVNN ---------- ---------- ---------- ----------
XlEphA2     GWLTHP---- ---------- ---------- ---------- ----------
XlEphA4     GWIASP---- ---------- ---------- ---------- ----------
XlEphB1     GWTANP---- ---------- ---------- ---------- ----------
XlEphB2     ---VFA---- ---------- ---------- ---------- ----------
XlEphB3     AWVAYP---- ---------- ---------- ---------- ----------
XlEphB4     KWTSYP---- ---------- ---------- ---------- ----------
GgEphA1     GWLPDP---- ---------- ---------- ---------- ----------
GgEphA3     GWISY----- ---------- ---------- ---------- ----------
GgEphA4     GWIASP---- ---------- ---------- ---------- ----------
GgEphA5     GWIAY----- ---------- ---------- ---------- ----------
GgEphA6     ---------- ---------- ---------- ---------- ----------
GgEphA7     EWISSP---- ---------- ---------- ---------- ----------
GgEphA8     CYARQPQQAR CRCPCRQQRP LGPGVSIVPA VSRADKACAG SLSLPLSHSP
GgEphB1     GWTANP---- ---------- ---------- ---------- ----------
GgEphB2     GWMVHP---- ---------- ---------- ---------- ----------
GgEphB3     AWTTHP---- ---------- ---------- ---------- ----------
GgEphB6     GWTSHP---- ---------- ---------- ---------- ----------
HsEphA1     GWLLDP---- ---------- ---------- ---------- ----------
HsEphA2     GWLTHP---- ---------- ---------- ---------- ----------
HsEphA3     GWISY----- ---------- ---------- ---------- ----------
HsEphA4     GWIASP---- ---------- ---------- ---------- ----------
HsEphA5     GWIAF----- ---------- ---------- ---------- ----------
HsEphA6     GWKTY----- ---------- ---------- ---------- ----------
HsEphA7     EWISSP---- ---------- ---------- ---------- ----------
HsEphA8     GWLTYP---- ---------- ---------- ---------- ----------
HsEphA10    GWTALP---- ---------- ---------- ---------- ----------
HsEphB1     GWTANP---- ---------- ---------- ---------- ----------
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HsEphB2    GWMVHP---- ---------- ---------- ---------- ----------
HsEphB3    AWTSHP---- ---------- ---------- ---------- ----------
HsEphB4    KWVTFP---- ---------- ---------- ---------- ----------
HsEphB6    GWLTYP---- ---------- ---------- ---------- ----------


           ....|....| ....|....| ....|....| ....|....| ....|....|
                  210        220        230        240        250
CeEph      ---------- --------RD DVWMEETWRN PAATDEKHAN QRAYVTCNYD
DmEph      ---------- --------QT PGWVEESFTD FVKGINWRSY VVCDVAYHN-
SpEph      ---------- --------QT YVGWTESGAS YER-----IF QTCHVSDP--
CiEph-a    ---------- ---------- --WTELTNHD ETD-ADLRTF QVCHIR----
CiEph-b    ---------- ---------- --WMEVTGDD ENQ-NERRML FVCQIT----
CiEph-c    ---------- ---------- -AWEELSGLD VDG-NTIRYH QVCNTG----
CiEph-d    ---------- ---------- --WTEANGPH PDG-RDRRML VVCQIK----
CiEph-e    ---------- ---------- VSWEEVSSYD EGHRTTIRSY QVCPQEDT--
CiEph-f    ---------- ---IEDFSPE VKWDEVTFHQ LNP--SVRGF QICPDSQDTQ
XlEphA2    ---------- --------YG KGWDLLQNVM N-G-SLIYIY SVCSVQ----
XlEphA4    ---------- --------LE GGWEEVSIMD EKN-TPIRTY QVCNVM----
XlEphB1    ---------- -------SSG --WEEVSGYD ENL-NTIRTY QVCNVF----
XlEphB2    ---------- -------PGQ --WEEVSGYD ENM-NTIRTY QVCNVF----
XlEphB3    ---------- -------DSG --WEEVSGYD EAS-NPIRTY QVCNVL----
XlEphB4    ---------- -------KVD GQWDEMSGLD DEG-TPVRTY EICNAHLS--
GgEphA1    ---------- --------PE IGWSEVQQMI N-G-TPVYMY QDCSVLS---
GgEphA3    ---------- --------PS HGWEEISGVD EHY-TPIRTY QESNVM----
GgEphA4    ---------- --------LE GGWEEVSIMD EKN-TPIRTY QVCNVM----
GgEphA5    ---------- --------PK NGWEEIGEVD ENY-APIHTY QVCKVM----
GgEphA6    ---------- ---------- -QWDAITEMD EHN-HPIHTY QVCNVM----
GgEphA7    ---------- --------PN G-WEEISGLD ENY-TPIRTY QVCQVM----
GgEphA8    AAIPAEASPP HAEQIMAMGC HGWDSINEMD EFF-SPIHTY QVCNVM----
GgEphB1    ---------- -------PSG --WEEVSGYD ENL-NTIRTY QVCNVF----
GgEphB2    ---------- -------PSG --WEEVSGYD ENM-NTIRTY QVCNVF----
GgEphB3    ---------- -------ETG --WEEVSGYD EAM-NPIRTY QVCNVR----
GgEphB6    ---------- -------PDG --WEEVSVRD DKE-RQIRTF QVCNMD----
HsEphA1    ---------- --------PK DGWSEQQQIL N-G-TPLYMY QDCPMQG---
HsEphA2    ---------- --------YG KGWDLMQNIM N-D-MPIYMY SVCNVM----
HsEphA3    ---------- --------PS HGWEEISGVD EHY-TPIRTY QVCNVM----
HsEphA4    ---------- --------LE GGWEEVSIMD EKN-TPIRTY QVCNVM----
HsEphA5    ---------- --------PK NGWEEIGEVD ENY-APIHTY QVCKVM----
HsEphA6    ---------- --------PL NGWDAITEMD EHN-RPIHTY QVCNVM----
HsEphA7    ---------- --------PN G-WEEISGLD ENY-TPIRTY QVCQVM----
HsEphA8    ---------- ---------A HGWDSINEVD ESF-QPIHTY QVCNVM----
HsEphA10   ---------- ---------S NGWEEISGVD EHD-RPIRTY QVCNVL----
HsEphB1    ---------- -------ASG --WEEVSGYD ENL-NTIRTY QVCNVF----
HsEphB2    ---------- -------PSG --WEEVSGYD ENM-NTIRTY QVCNVF----
HsEphB3    ---------- -------ESG --WEEVSGYD EAM-NPIRTY QVCNVR----
HsEphB4    ---------- -------QVD GQWEELSGLD EEQ-HSVRTY EVCDVQRAP-
HsEphB6    ---------- -------PGG --WDEVSVLD DQR-RLTRTF EACHVAGAPP


           ....|....| ....|....| ....|....| ....|....| ....|....|
                  260        270        280        290        300
CeEph      --MINPSNWL FSHFIEVK-T ARRIYIELLF NTRDCDAYLN -----PKSCK
DmEph      -----VNNWL WSPFIDRG-S ANRLYIEIQF TIRDCSLFPG N----ALSCK
SpEph      -----RDNWL RMPYIERQ-G ANRIHVEVKF TMYSCTGIVD -----AQLCK
CiEph-a    --SPHQNNWL KTNFINVT-T TQRVYVEIKF TIRSCDGIR- ----GVVSCK
CiEph-b    --QPNQNNWL RTQYIRVD-G ARRIYVEVQF TMRSCEDVP- ----NVATCK
CiEph-c    --MDEQDNWV RSPFIDAK-S AQRIYMDIEF SV-------- ---MKCEECR
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CiEph-d        --KPHQNNWA RTPYIPVD-G AERIYVEFEF TIRSCEDVP- ----NVATCK
CiEph-e        --TVESDNWI RSPYIDAR-D GKLIHLDVEF SINHCSHDTL PDNMKKTQCK
CiEph-f        SYKTNVQNWL RSPYMKLI-G RSRLYVELNF WMRECENKDT -------ICP
XlEphA2        --EGEQDNWL RTNWIYRS-E AHGIFVELKF TVRDCNSFPG ----GSGTCK
XlEphA4        --ESSQNNWL RTDWIPRS-G AQRVYVEIKF TLRDCNSLPG ----VMGTCK
XlEphB1        --GPKQNNWL LTTFIPRR-G AHRVYVEMRF TVRDCSSLPN ----VPGSCK
XlEphB2        --AANQNNWL RTKFIRRR-G AHRIHVEMKF SVRDCSSIPS ----VPGSCK
XlEphB3        --DSNQNNWL RTQFIPRQ-D VQRVYVELKF TVRDCNSLPN ----IPGSCK
XlEphB4        ----NQNNWL RTNYIQRG-L ASNAYVEITF TMLECSFLSR ----AGRSCK
GgEphA1        --EGDTDHWL RTNWIYRGEA ASRIYVELKF TVRDCKSFKG ----EAVTCK
GgEphA3        --DHSQNNWL RTNWIPRN-S AQKIYVELKF TLRDCNSIPL ----VLGTCK
GgEphA4        --EPSQNNWL RTDWIPRE-G AQRVYIEIKF TLRDCNSLPG ----VMGTCK
GgEphA5        --EQNQNNWL LTSWISNE-G RPASSFELKF TLRDCNSLPG ----GLGTCK
GgEphA6        --EPNQNNWL RTNWISRD-A AQKIYVEMKF TLRDCNSIPW ----VVGTCK
GgEphA7        --ESNQNNWL RTNWIAKS-N AQRIFVELKF TLRDCNSLPG ----VLGTCK
GgEphA8        --TPNQNNWL RTNWVQRD-G ARRVYAEIKF TLRDCNSMPG ----VLGTCK
GgEphB1        --EPNQNNWL LTTFINRR-G AHRIYTEMRF TVRDCSSLPN ----VPGSCK
GgEphB2        --ESSQNNWL RTKYIRRR-G AHRIHVEMKF SVRDCSSIPN ----VPGSCK
GgEphB3        --EANQNNWL RTKFIQRQ-D VQRVYVELKF TVRDCNSIPN ----IPGSCK
GgEphB6        --EPGQNNWL RTHFIERR-G AHRVHVRLHF SVRDCASMRT ----VASTCK
HsEphA1        --RRDTDHWL RSNWIYRGEE ASRVHVELQF TVRDCKSFPG GA--GPLGCK
HsEphA2        --SGDQDNWL RTNWVYRG-E AERIFIELKF TVRDCNSFPG ----GASSCK
HsEphA3        --DHSQNNWL RTNWVPRN-S AQKIYVELKF TLRDCNSIPL ----VLGTCK
HsEphA4        --EPSQNNWL RTDWITRE-G AQRVYIEIKF TLRDCNSLPG ----VMGTCK
HsEphA5        --EQNQNNWL LTSWISNE-G ASRIFIELKF TLRDCNSLPG ----GLGTCK
HsEphA6        --EPNQNNWL RTNWISRD-A AQKIYVEMKF TLRDCNSIPW ----VLGTCK
HsEphA7        --EPNQNNWL RTNWISKG-N AQRIFVELKF TLRDCNSLPG ----VLGTCK
HsEphA8        --SPNQNNWL RTSWVPRD-G ARRVYAEIKF TLRDCNSMPG ----VLGTCK
HsEphA10       --EPNQDNWL QTGWISRG-R GQRIFVELQF TLRDCSSIPG ----AAGTCK
HsEphB1        --EPNQNNWL LTTFINRR-G AHRIYTEMRF TVRDCSSLPN ----VPGSCK
HsEphB2        --ESSQNNWL RTKFIRRR-G AHRIHVEMKF SVRDCSSIPS ----VPGSCK
HsEphB3        --ESSQNNWL RTGFIWRR-D VQRVYVELKF TVRDCNSIPN ----IPGSCK
HsEphB4        ----GQAHWL RTGWVPRR-G AVHVYATLRF TMLECLSLPR ----AGRSCK
HsEphB6        --GTGQDNWL QTHFVERR-G AQRAHIRLHF SVRACSSLGV ----SGGTCR


               ....|....| ....|....| ....|....| ....|....| ....|....|
                      310        320        330        340        350
CeEph          ETFSVYLKQF KTSRPG---- STKIEKERFS EDIDNWKNIG RLARSN----
DmEph          ETFSLLFYEF D--AATREPP PWQTDSYRLI ARIAAGEGRF NQNSDVD---
SpEph          ETFDVYFYQS DTDEATETSP DWTAPPYQKV ARIAA-EGRF SDPDTLD---
CiEph-a        ETFNLYYLES NHKNDRRSLR -LNEDTYTKV DTIAADERFK PDTR------
CiEph-b        ETFNLYYYET DRDEATSTFP PWGEGAYVKV DTIAADQRFR PGSD------
CiEph-c        ETFALYYYPS SSDTATTTFP PWRENPYIKI DTLAAGERFD SETVGA----
CiEph-d        ETFNLYYYET DRDEATSTFP PWREGAYIKV DTIAADKRFK PGS-------
CiEph-e        ETFNMFYYEA DGDVATSTFP PWREKPYVKI DTIAANS--- -----V----
CiEph-f        ETFKIYYAIT SHQSEVP--- QLNESVFTKI DTVAAEDQFA DSNSDWTEVL
XlEphA2        ETFNLYYMES DIDYGT---- NFQKRQFRKI DTIAPDDITV PADFAS----
XlEphA4        ETFNLYYYES NNDKER---- FIRETQYVKI DTIAADESFT QVDIGD----
XlEphB1        ETFNLYYYET DSNIENKIST FWNESPYLKV DTIAADESFS QVDFGG----
XlEphB2        ETFNLYYYES DFDSATKNFP SWMENPWVKV DTIAADESFS QVDLGG----
XlEphB3        ETFNFFYYES DSDSASADSP FWMENPYIKV DTIAPDESFS RRDAG-----
XlEphB4        ETFNLYYYQA NHDLANDHFP PWMENPWVRV DTVAADFLGR PNRRVSS---
GgEphA1        ETFNLYYMES EQDVGI---- QFRRPMFIKL NTVAADRSFT NRDIES----
GgEphA3        ETFNLYYMES DDDHLA---- KFREHQFTKI DTIAADESFT QMDLGD----
GgEphA4        ETFNLYYYES NNDKER---- FIRESQFAKI DTIAADESFT QVDIGD----
GgEphA5        ETFNMYYFES DDEDGR---- NIRENQYIKI DTIAADESFT ELDLGD----
GgEphA6        ETFNLYYMES DESHGL---- KFKPNQYTKI DTIAADESFT QMDLGD----
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GgEphA7           ETFNLYYYET DYDTGR---- NIRENQYVKI DTIAADESFT QGDLGE----
GgEphA8           ETFNLYYLES DRDLGT---- STRESQFMKI DTIAADESFT NVDLGV----
GgEphB1           ETFNLYYYET DSVIATKKSA FWTEAPYLKV DTIAADESFS QVDFGG----
GgEphB2           ETFNLYYYES DFDSATKTFP NWMENPWMKV DTIAADESFS QVDLGG----
GgEphB3           ETFNLFYYES DTDSASANSP FWMENPYIKV DTIAPDESFS KLESG-----
GgEphB6           ETFTLYYHQS DVDIASQELP EWHEGPWTKV DTIAADESFS QVDRTG----
HsEphA1           ETFNLLYMES DQDVGI---- QLRRPLFQKV TTVAADQSFT IRDLAS----
HsEphA2           ETFNLYYAES DLDYGT---- NFQKRLFTKI DTIAPDEITV SSDFEA----
HsEphA3           ETFNLYYMES DDDHGV---- KFREHQFTKI DTIAADESFT QMDLGD----
HsEphA4           ETFNLYYYES DNDKER---- FIRENQFVKI DTIAADESFT QVDIGD----
HsEphA5           ETFNMYYFES DDQNGR---- NIKENQYIKI DTIAADESFT ELDLGD----
HsEphA6           ETFNLFYMES DESHGI---- KFKPNQYTKI DTIAADESFT QMDLGD----
HsEphA7           ETFNLYYYET DYDTGR---- NIRENLYVKI DTIAADESFT QGDLGE----
HsEphA8           ETFNLYYLES DRDLGA---- STQESQFLKI DTIAADESFT GADLGV----
HsEphA10          ETFNVYYLET EADLGRG-RP RLGGSRPRKI DTIAADESFT QGDLGE----
HsEphB1           ETFNLYYYET DSVIATKKSA FWSEAPYLKV DTIAADESFS QVDFGG----
HsEphB2           ETFNLYYYEA DFDSATKTFP NWMENPWVKV DTIAADESFS QVDLGG----
HsEphB3           ETFNLFYYEA DSDVASASSP FWMENPYVKV DTIAPDESFS RLDAG-----
HsEphB4           ETFTVFYYES DADTATALTP AWMENPYIKV DTVAAEHLTR -KRPGAE---
HsEphB6           ETFTLYYRQA EEPDSPDSVS SWHLKRWTKV DTIAADESFP SSSSSSSSSS


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                          360        370        380        390        400
CeEph             ---------- -----SNMTT ETLGMEIDSD TKTIRIAFEE QGICLSLLNV
DmEph             ---------- -----INTEV KSIA----VN KKGVYFAFRD QGACISVLAV
SpEph             ---------- -----EEVVN VRIENFGPVT ANGFYIAFRD QGACMALLQV
CiEph-a           ---------- ------VEAN TETRMISPIR SRGLYIAFQD TGACMSIMHV
CiEph-b           ---------- ------KVVN FERRNIGPIS KNGIYLAIQD TGACVAIMHV
CiEph-c           ---------- ------EGIN KKTLVIGPLS RRGFYIAAQD QGACMSIMSL
CiEph-d           ---------- ------AERN FETRDIGPLT KRGVYLAIQD TGACMALMHV
CiEph-e           ---------- ------NRVN TKTFSFGPIT RRGFYVSVQD QGACMSLISL
CiEph-f           N--------- -----TEIVN IEIPQSIDIS HAGVYVAIVD KGACIALVSV
XlEphA2           ---------- ----RNVKVN VEVRSVGALS RKGFYLAFQD IGACVALLSV
XlEphA4           ---------- ----RIMKLN TEVRDVGPLS KKGFYLAFQD VGACIALVSV
XlEphB1           ---------- ----RLMKVN TEVRSFGPLT RSGFYLAFQD YGACMSLLSV
XlEphB2           ---------- ----RVMKIN TEVRSFGPVS KSGFYLAFQD YGGCMSLIAV
XlEphB3           ---------- -------RVN TKLRSFGPLS RTGFYLAFQD LGACVSLISV
XlEphB4           ---------- ----GSTRIN VKTLRIGPLT GDGFYLAFQD QGACMALLAV
GgEphA1           ---------- ----GAMQLN TEVCPIGKLS RRGFYLAFQN SGACVAMVSV
GgEphA3           ---------- ----RILKLN TEVREVGPVS KKGFYLAFQD VGACVALVSV
GgEphA4           ---------- ----RIMKLN TEVRDVGPLS KKGFYLAFQD VGACIALVSV
GgEphA5           ---------- ----RVMKLN TEVRDVGPLT KKGFYLAFQD VGACIALVSV
GgEphA6           ---------- ----RILKLN TEVREVGPIN KKGFYLAFQD IGACIALVSV
GgEphA7           ---------- ----RKMKLN TEVREIGPLS KKGFYLAFQD VGACIALVSV
GgEphA8           ---------- ----RRLKLN TEVRGVGPLS KRGFYLAFQD IGACIAIVSV
GgEphB1           ---------- ----RLMKVN TEVRSFGPLT RNGFYLAFQD YGACMSLLSV
GgEphB2           ---------- ----RVMKIN TEVRSFGPVS KNGFYLAFQD YGGCMSLIAV
GgEphB3           ---------- -------RVN TKVRSFGPLS KNGFYLAFQD LGACMSLISV
GgEphB6           ---------- ----KVVRMN VKVRSFGPLT KHGFYLAFQD SGACMSLVAV
HsEphA1           ---------- ----GSVKLN VERCSLGRLT RRGLYLAFHN PGACVALVSV
HsEphA2           ---------- ----RHVKLN VEERSVGPLT RKGFYLAFQD IGACVALLSV
HsEphA3           ---------- ----RILKLN TEIREVGPVN KKGFYLAFQD VGACVALVSV
HsEphA4           ---------- ----RIMKLN TEIRDVGPLS KKGFYLAFQD VGACIALVSV
HsEphA5           ---------- ----RVMKLN TEVRDVGPLS KKGFYLAFQD VGACIALVSV
HsEphA6           ---------- ----RILKLN TEIREVGPIE RKGFYLAFQD IGACIALVSV
HsEphA7           ---------- ----RKMKLN TEVREIGPLS KKGFYLAFQD VGACIALVSV
HsEphA8           ---------- ----RRLKLN TEVRSVGPLS KRGFYLAFQD IGACLAILSL
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HsEphA10      ---------- ----RKMKLN TEVREIGPLS RRGFHLAFQD VGACVALVSV
HsEphB1       ---------- ----RLMKVN TEVRSFGPLT RNGFYLAFQD YGACMSLLSV
HsEphB2       ---------- ----RVMKIN TEVRSFGPVS RSGFYLAFQD YGGCMSLIAV
HsEphB3       ---------- -------RVN TKVRSFGPLS KAGFYLAFQD QGACMSLISV
HsEphB4       ---------- ----ATGKVN VKTLRLGPLS KAGFYLAFQD QGACMALLSL
HsEphB6       SAAWAVGPHG AGQRAGLQLN VKERSFGPLT QRGFYVAFQD TGACLALVAV


              ....|....| ....|....| ....|....| ....|....| ....|....|
                     410        420        430        440        450
CeEph         KIYYRICDEF TDQLVYFRPQ VTGPKE-TDM VRMNGSCIPN ASKKIPG---
DmEph         KVYYITCPAV TENFAHFNET PTGREI-TII EKQNGTCVD- -NAEPY----
SpEph         RIFYQVCPQV VRDFAIFNQT NEGPET-HTL VTVPGTCVK- -NAQPVPPG-
CiEph-a       RVYYKSCQET LRGLAIFPPT VSGPMM-TSL VDVPGQCVPN SATMKNT---
CiEph-b       KVYYKYCGET LKNLASFSRT ISGPQV-TDL VKTRGSCVRN -AITEDG---
CiEph-c       KLYYYHCEET THNLAYFPNT ISGGGI-AEL VSQSGKCVSN SVYAN-----
CiEph-d       RVYYKYCAPT ESNLANFGRT TAGPYM-ASL VEANGTCVDN -SLADND---
CiEph-e       QIYYFYCGET WKNLAVFPKT VSGDSM-ASL VEVVGSCVDH AVQSTNL---
CiEph-f       RVYSIVCPME IQSLAIFPET LTGELD-TSL VKVNGSCVER SHPANSEVS-
XlEphA2       RIYYKKCPSV VQGMAQFPET VAGADS-QSL AKVSGKCVNN AVS-VNRDD-
XlEphA4       RVFYKKCPLT VRNLAQFPDT ITGSDT-SSL VEVRGSCVD- -NSEEKD---
XlEphB1       RVFFKEMPSV VQNLLVFPET MTGAES-TSL VIARGTCIP- -NAEEVD---
XlEphB2       RVYDRKCPRI IQNGAIFQET LSGAES-TSL VAARGNCMP- -NAEEVD---
XlEphB3       RVFFKKCPQT IAGFASFPET ITGAEP-TSL VIAPGTCVP- -NALEVS---
XlEphB4       RVFYRVCPAV VASLASFPKT VSEGLV---- VSAEGSCVE- -GAEGPQ---
GgEphA1       RVYYKTCPEA VQGLARFPET LAGLEG---L TEVPGVCVEY AAEETGSP--
GgEphA3       RVYFKKCPFT VKNLAMFPDT VP-MDS-QSL VEVRGSCVN- -HSKEEE---
GgEphA4       RVFYKKCPLT VRNLAQFPDT ITGADT-SSL VEVRGSCVN- -NSEEKD---
GgEphA5       RVYYKKCPSV IRNLARFPDT ITGADS-SQL LEVSGVCVN- -HSVTDE---
GgEphA6       RVYYKKCPFT VRNLAMFPDT IPRVDS-SSL VEVRGSCVK- -SAEERD---
GgEphA7       KVYYKKCWSI IENLAIFPDT VTGSEF-SSL VEVRGTCVS- -SAEEEAEN-
GgEphA8       RVYYKKCPAM VRNLASFSEA VTGADS-SSL VEVRGECVG- -HSEERD---
GgEphB1       RVFFKKCPSV VQNFAIFPET MTGAES-TSL VTARGTCIP- -NAEEVD---
GgEphB2       RVFYRKCPRV IQNGAVFQET LSGAES-TSL VAARGTCIS- -NAEEVD---
GgEphB3       RAFYKKCSNT IAGFAIFPET LTGAEP-TSL VIAPGTCIP- -NAVEVS---
GgEphB6       QVFFYKCPAV VKGFASFPET FAGGER-TSL VESLGTCVA- -NAEEAS---
HsEphA1       RVFYQRCPET LNGLAQFPDT LPGPAG---L VEVAGTCLPH ARASPRPSG-
HsEphA2       RVYYKKCPEL LQGLAHFPET IAGSDA-PSL ATVAGTCVDH AVVPPGGEE-
HsEphA3       RVYFKKCPFT VKNLAMFPDT VP-MDS-QSL VEVRGSCVN- -NSKEED---
HsEphA4       RVFYKKCPLT VRNLAQFPDT ITGADT-SSL VEVRGSCVN- -NSEEKD---
HsEphA5       RVYYKKCPSV VRHLAVFPDT ITGADS-SQL LEVSGSCVN- -HSVTDE---
HsEphA6       RVFYKKCPFT VRNLAMFPDT IPRVDS-SSL VEVRGSCVK- -SAEERD---
HsEphA7       KVYYKKCWSI IENLAIFPDT VTGSEF-SSL VEVRGTCVS- -SAEEEAEN-
HsEphA8       RIYYKKCPAM VRNLAAFSEA VTGADS-SSL VEVRGQCVR- -HSEERD---
HsEphA10      RVYYKQCRAT VRGLATFPAT AAESAF-STL VEVAGTCVA- -HSEGEPGS-
HsEphB1       RVFFKKCPSI VQNFAVFPET MTGAES-TSL VIARGTCIP- -NAEEVD---
HsEphB2       RVFYRKCPRI IQNGAIFQET LSGAES-TSL VAARGSCIA- -NAEEVD---
HsEphB3       RAFYKKCAST TAGFALFPET LTGAEP-TSL VIAPGTCIP- -NAVEVS---
HsEphB4       HLFYKKCAQL TVNLTRFPET VPRELV---- VPVAGSCVV- -DAVPAP---
HsEphB6       RLFSYTCPAV LRSFASFPET QASGAGGASL VAAVGTCVA- -HAEPEEDGV


              ....|....| ....|....| ....|....| ....|....| ....|....|
                     460        470        480        490        500
CeEph         ------VDLI GLCMSTGSGI KT-SGECVCD SGYSQIADSN GARCESCPTN
DmEph         ------ETPT YLCKGDGKWT IL-TGGCRCK AGYEPNYTNK TCTE-CPLGT
SpEph         ------STPQ YICQNEGIWS LN-QGGCGCS PGYEASADRT RCEE-CQIGT
CiEph-a       ------KAPS LHCNGEGEWL VP-TGSCSCN PGYEPNNQLT ECNQ-CSVGR
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CiEph-b     ------QYPI YHCGGIGNWT VP-TGTCFCT AGYEPNYSRT QCNA-CARNR
CiEph-c     ------EVPK YRCNIYGEWQ VP-TGSCQCR AGYEPNTQLT ACTG-CMVGK
CiEph-d     ------DRAT FRCNSEGEWI VP-KGSCLCD RGYGPDYTST SCQE-CGRGF
CiEph-e     ------GSPE YRCNSNGRWQ IN-TGGCVCD VGFQPNNQQT RCQP-CPDGT
CiEph-f     ------SQPV NHCTSDGRWN VR-TGACYCE AGYQPDHNMR TCQP-CPRNM
XlEphA2     --------PT MHCNTDGEWL VP-IGHCLCQ PGYEKVGD-- TCQA-CQPGF
XlEphA4     -------VPK MYCGADGEWL VP-IGNCLCN AGFEEHNG-- GCQA-CKVGY
XlEphB1     ------VPIK LYCNGDGEWM VP-IGKCTCK AGYE-PENHV VCKA-CPAAM
XlEphB2     ------VPIK LYCNGDGEWL VP-IGRCLCK AGYESVENGT VCRD-VPVGM
XlEphB3     ------VPLK LYCNGDGDWM VP-VGACTCA AGFEPAEKDT QCQA-CKRGT
XlEphB4     -----GRRPT MYCREDGEWA KPAVGECACV AGREEKDGRT KCAA-CVPGY
GgEphA1     --------PR MHCSTDGEWL VP-MGRCLCA VGFEEVDG-- SCVA-CQRGF
GgEphA3     -------PPK MYCSTEGEWL VP-IGKCLCN AGYEERGF-- ACQA-CRPGF
GgEphA4     -------VPK MYCGADGEWL VP-IGNCLCN AGYEERNG-- ECQA-CKIGY
GgEphA5     -------APK MHCSAEGEWL VP-IGKCLCK AGYEEKNN-- TCQV-CRPGF
GgEphA6     -------TPK LYCGADGDWL VP-LGRCICS VGYEELDG-- SCHA-CRPGF
GgEphA7     -------SPK MHCSAEGEWL VP-IGKCICK AGYQQKGD-- TCEP-CGRGF
GgEphA8     -------TPK MYCSAEGEWL VP-IGKCVCS AGYEEQRD-- SCMAACQLGF
GgEphB1     ------VPIK LYCNGDGEWM VP-IGRCTCK AGYE-PENNV ACRA-CPAGT
GgEphB2     ------VPIK LYCNGDGEWL VP-IGRCMCR PGYESVENGT VCRG-CPSGT
GgEphB3     ------VPLK LYCNGDGEWM VP-VGACTCA AGYEPAMKDT QCQA-CGPGT
GgEphB6     --TTGSSGVR LHCNGEGEWM VA-TGRCSCK AGYQSVDNEQ ACQA-CPIGS
HsEphA1     -------APR MHCSPDGEWL VP-VGRCHCE PGYEEGGSGE ACVA-CPSGS
HsEphA2     --------PR MHCAVDGEWL VP-IGQCLCQ AGYEKVED-- ACQA-CSPGF
HsEphA3     -------PPR MYCSTEGEWL VP-IGKCSCN AGYEERGF-- MCQA-CRPGF
HsEphA4     -------VPK MYCGADGEWL VP-IGNCLCN AGHEERSG-- ECQA-CKIGY
HsEphA5     -------PPK MHCSAEGEWL VP-IGKCMCK AGYEEKNG-- TCQV-CRPGF
HsEphA6     -------TPK LYCGADGDWL VP-LGRCICS TGYEEIEG-- SCHA-CRPGF
HsEphA7     -------APR MHCSAEGEWL VP-IGKCICK AGYQQKGD-- TCEP-CGRGF
HsEphA8     -------TPK MYCSAEGEWL VP-IGKCVCS AGYEERRD-- ACVA-CELGF
HsEphA10    -------PPR MHCGADGEWL VP-VGRCSCS AGFQERGD-- FCEA-CPPGF
HsEphB1     ------VPIK LYCNGDGEWM VP-IGRCTCK PGYE-PENSV ACKA-CPAGT
HsEphB2     ------VPIK LYCNGDGEWL VP-IGRCMCK AGFEAVENGT VCRG-CPSGT
HsEphB3     ------VPLK LYCNGDGEWM VP-VGACTCA TGHEPAAKES QCRP-CPPGS
HsEphB4     -----GPSPS LYCREDGQWA EQPVTGCSCA PGFEAAEGNT KCRA-CAQGT
HsEphB6     GGQAGGSPPR LHCNGEGKWM VA-VGGCRCQ PGYQPARGDK ACQA-CPRGL


            ....|....| ....|....| ....|....| ....|....| ....|....|
                   510        520        530        540        550
CeEph       TYKPKGQSLC KSCPSNS--- -ISS--EAAS SCRCLNGYFR AEDELISM-P
DmEph       FKSPE-VTKC TPCPPNS--- NASK--TGSP FCKCASGYYR HPNDGRHM-P
SpEph       FKSDIGTTEC SPCPAKS--- HADT--VAVT RCTCIPDYYR APGEEASS-P
CiEph-a     FKHEVGNVPC QPCPARS--- --FNVNFAST MCMCISGYYR SPGDRPDA-P
CiEph-b     YKSVVKNVAC RRCPAYS--- --SSSNRGAT VCDCMNGYFR AHEDTADQ-P
CiEph-c     YKSTNGNTPC QVCPQHS--- VTHSTSAS-- HCTCVAGHYR AENDPISQ-A
CiEph-d     YKSSVGNGGC VRCPAYS--- --ESQSDGST ECQCMPGYYR ALSDDAAK-P
CiEph-e     YKVSNTISEC LPCPNNSGFN ISHTNHASNN ECLCNTGYAR ASGETVNT-P
CiEph-f     YKPTSGNLNC TKCPSVS--- --YTHHVGSI SCVCSDGHLV STSYLDGCGV
XlEphA2     YKSETSNGPC QLCPDHT--- EPTS--QAAT FCPCNDGFFR STSDPSSD-P
XlEphA4     YKALSTDAAC SKCPPHS--- YALR--EGST SCTCDRGYFR ADTDPASM-P
XlEphB1     FKANQGMGIC AQCPANS--- RSTS--EASP ICICRNGYYR ADFDTPEA-P
XlEphB2     FKANQGDDSC LHCPINS--- RTTS--EGAT NCVCRTGYYR SDSDHLQM-H
XlEphB3     YKSKQGEGSC MPCPINS--- RAIS--SAAT ICSCQNGYYR AEGESAET-A
XlEphB4     FKASVGEKPC QPCPANS--- LSTG--PGAT VCACKPGYYR ASTDPPTA-A
GgEphA1     YRHSLETKRC LKCPPNS--- LSNE--LGAT SCSCNAGFYR APSEGQNI-A
GgEphA3     YKASAGNVKC AKCPPHS--- STYE--DASL NCRCEKNYFR SEKDPPSM-A
GgEphA4     YKALSTDVAC AKCPPHS--- YSIW--EGST SCTCDRGFFR AENDAASM-P
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GgEphA5     FKASPHSPSC SKCPPHS--- YTLD--EAST SCLCEEHYFR RESDPPTM-A
GgEphA6     YKAFAGNVKC SKCPPHS--- STYV--EATS VCQCEKGYFR AEKDPPTM-A
GgEphA7     YKSSSQDLQC SRCPTHS--- FSDK--EGSS RCDCEDSYYR APSDPPYV-A
GgEphA8     YKSDPGDQLC AKCPPHS--- HSES--RAAR VCRCDSSFYR AVQDPPSA-A
GgEphB1     FKASQGAGLC ARCPPNS--- RSSA--EASP LCACRNGYFR ADLDPPTA-A
GgEphB2     FKASQGDEGC VHCPINS--- RTTS--EGAT NCVCRNGYYR ADADPVDM-P
GgEphB3     FKSKQGEGPC SPCPPNS--- RTTA--GAAT VCICRSGFFR ADADPADS-A
GgEphB6     FKASVGDDPC LLCPAHS--- HAPLPLPGSI ECVCQSHYYR SASDNSDA-P
HsEphA1     YRMDMDTPHC LTCPQQS--- TAES--EGAT ICTCESGHYR APGEGPQV-A
HsEphA2     FKFEASESPC LECPEHT--- LPSP--EGAT SCECEEGFFR APQDPASM-P
HsEphA3     YKALDGNMKC AKCPPHS--- STQE--DGSM NCRCENNYFR ADKDPPSM-A
HsEphA4     YKALSTDATC AKCPPHS--- YSVW--EGAT SCTCDRGFFR ADNDAASM-P
HsEphA5     FKASPHIQSC GKCPPHS--- YTHE--EAST SCVCEKDYFR RESDPPTM-A
HsEphA6     YKAFAGNTKC SKCPPHS--- LTYM--EATS VCQCEKGYFR AEKDPPSM-A
HsEphA7     YKSSSQDLQC SRCPTHS--- FSDK--EGSS RCECEDGYYR APSDPPYV-A
HsEphA8     YKSAPGDQLC ARCPPHS--- HSAA--PAAQ ACHCDLSYYR AALDPPSS-A
HsEphA10    YKVSPRRPLC SPCPEHS--- RALE--NAST FCVCQDSYAR SPTDPPSA-S
HsEphB1     FKASQEAEGC SHCPSNS--- RSPA--EASP ICTCRTGYYR ADFDPPEV-A
HsEphB2     FKANQGDEAC THCPINS--- RTTS--EGAT NCVCRNGYYR ADLDPLDM-P
HsEphB3     YKAKQGEGPC LPCPPNS--- RTTS--PAAS ICTCHNNFYR ADSDSADS-A
HsEphB4     FKPLSGEGSC QPCPANS--- HSNT--IGSA VCQCRVGYFR ARTDPRGA-P
HsEphB6     YKSSAGNAPC SPCPARS--- HAPN--PAAP VCPCLEGFYR ASSDPPEA-P


            ....|....| ....|....| ....|....| ....|....| ....|....|
                   560        570        580        590        600
CeEph       CTQPPSRPIK LVANAITAT- -STRLSWNEP -SSLGGRP-- -----EIWYE
DmEph       CYSPPAAPTN LTLLFVDQTS AIISWSAPAK NESFSSETNS KIYHSDIVYK
SpEph       CTAPPSAPTS LT-AVVDEKS SVVTLTWRNP -ENLGGRT-- -----DIDFR
CiEph-a     CTRTPSKPST LESSVNK--- TSVKLTWVEP -RKSGGRT-- -----DITYS
CiEph-b     CTKPPTKVVN LYAAVNQS-- TSVTLKWERP -LNEGGRT-- -----DLTYS
CiEph-c     CTRPPTKPRN VTHVQNK--- TSLLLSWVPP -STTGGRT-- -----DIYYS
CiEph-d     CSQPPSKPVN VLAIVNQ--- TKITLTWERP -FNDGGRD-- -----DITYR
CiEph-e     CTAAPSSPLN LLASEHDG-- HAVTLTWLPP -VHLGGRK-- -----DIFYK
CiEph-f     CPQLPSPPLK LKVIKSTTVP GAARLEWEKP -QDFGNCQ-- -----KIFYC
XlEphA2     CTSFPSAPRD LTAVDLGS-- -KVMLRWLPP -SNSGGRS-- -----DITYT
XlEphA4     CTRPPSAPQN LISNVNET-- -SVNLEWSPP -QNSGGRP-- -----DVSYN
XlEphB1     CTSVPSGPRN VISIVNET-- -AITLEWHPP -RETGGRD-- -----DVDYN
XlEphB2     CTTIPSAPQN VISSVKQT-- -SLMLEXAPP -RDSGGRE-- -----DLVYH
XlEphB3     CTSVPSAPRH VISNVNET-- -SVVLEWAEP -GHLGGRG-- -----DILYN
XlEphB4     CSTPPSAPRS IVARVNGS-- -MVDLEWSEP -LDAGGRG-- -----DLYYE
GgEphA1     CTRPPSAPRN VSFSLTGT-- -QLSLWWQPP -SDHGGRK-- -----DLTYT
GgEphA3     CTRPPSAPRN VISNINET-- -SVILDWSWP -LDTGGRK-- -----DVTFN
GgEphA4     CTRPPSAPQN LISNVNET-- -SVNLEWSAP -QNKGGRD-- -----DISYN
GgEphA5     CTRPPSAPRS AISNVNET-- -SVFLEWIPP -ADTGGRK-- -----DVSYY
GgEphA6     CTRPPSAPRN VIFNINET-- -ALMLEWSPP -SDTGGRK-- -----DLTYS
GgEphA7     CTRPPSAPQN LIFNINQT-- -TVSLEWSPP -ADNGGRN-- -----DVTYR
GgEphA8     CTRPPSAPVN LVSSVNGT-- -SVTLEWGPP -LDKGGRS-- -----DVVYN
GgEphB1     CTSVPSGPRN VISIVNET-- -SIILEWNPP -RETGGRD-- -----DVTYN
GgEphB2     CTTIPSAPQA VISSVNET-- -SLMLEWTPP -RDSGGRE-- -----DLVYN
GgEphB3     CTSVPSAPRS VISNVNET-- -SLVLEWSEP -QDAGGRD-- -----DLLYN
GgEphB6     CTGIPSAPRD LSYEIVGS-- -NVLLTWRLP -KDLGGRK-- -----DVFFN
HsEphA1     CTGPPSAPRN LSFSASGT-- -QLSLRWEPP -ADTGGRQ-- -----DVRYS
HsEphA2     CTRPPSAPHY LTAVGMGA-- -KVELRWTPP -QDSGGRE-- -----DIVYS
HsEphA3     CTRPPSSPRN VISNINET-- -SVILDWSWP -LDTGGRK-- -----DVTFN
HsEphA4     CTRPPSAPLN LISNVNET-- -SVNLEWSSP -QNTGGRQ-- -----DISYN
HsEphA5     CTRPPSAPRN AISNVNET-- -SVFLEWIPP -ADTGGRK-- -----DVSYY
HsEphA6     CTRPPSAPRN VVFNINET-- -ALILEWSPP -SDTGGRK-- -----DLTYS
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HsEphA7       CTRPPSAPQN LIFNINQT-- -TVSLEWSPP -ADNGGRN-- -----DVTYR
HsEphA8       CTRPPSAPVN LISSVNGT-- -SVTLEWAPP -LDPGGRS-- -----DITYN
HsEphA10      CTRPPSAPRD LQYSLSRSP- LVLRLRWLPP -ADSGGRS-- -----DVTYS
HsEphB1       CTSVPSGPRN VISIVNET-- -SIILEWHPP -RETGGRD-- -----DVTYN
HsEphB2       CTTIPSAPQA VISSVNET-- -SLMLEWTPP -RDSGGRE-- -----DLVYN
HsEphB3       CTTVPSPPRG VISNVNET-- -SLILEWSEP -RDLGGRD-- -----DLLYN
HsEphB4       CTTPPSAPRS vVSRLNGS-- -SLHLEWSAP -LESGGRE-- -----DLTYA
HsEphB6       CTGPPSAPQE LWFEVQGS-- -ALMLHWRLP -RELGGRG-- -----DLLFN


              ....|....| ....|....| ....|....| ....|....| ....|....|
                     610        620        630        640        650
CeEph         VKCSGRGECG ---------- ---------- -TVVMTP--G DKKLS-----
DmEph         IKCNICSP-- ---------- ---------- -NVVYNP--S TDTFN-----
SpEph         IDCKRCPA-- ---------- ---------- -DVQFSS--I QRIGQ-----
CiEph-a       VTCERCDA-- ---------- TYTLCSRCGE -GADFIP--G RTRLT-----
CiEph-b       IECFQCQP-- ---------- GFVDCTPCTE -RADYEP--Q KTGLV-----
CiEph-c       ISCELCDS-- ---------- EHENCQPCNV -DVQYRP-SN HQHTT-----
CiEph-d       IECQRCQV-- ---------- GFDHCAPCPQ -GVDFEP--R QRDLV-----
CiEph-e       IGCKSTPLW- ---------- ELEQFISCSN -ALVYQPHLN EGKIF-----
CiEph-f       VLCQVHSINH ASTSLSKQNN NSSLFKQSDP TQPSYTVDAC DGVLYYPHQC
XlEphA2       VTCEKCAPEA ---------- --SECTPCDH NSIRFSE--N PLELK-----
XlEphA4       LVCKRCGS-- ---------- DLTRCRPCGS -GVHYSP--Q QNGLK-----
XlEphB1       IVCKKCRADR ---------- --RACSRCDD -NVDFVP--R QLGLT-----
XlEphB2       IICKSCGSGR ---------- --GAYTHCGD -NVQFAP--A QLGLM-----
XlEphB3       VICKKC---- L--------- -ERLCSRCGD -NVQFWP--R QLGVT-----
XlEphB4       VQCMEC---- ---------- -AGPCIPCS- -RLNYSP--R PQRLE-----
GgEphA1       VFCQRCHFLS ---------- ----CDPCEP -GVVFSP--S SSGLT-----
GgEphA3       IICKKCGG-- ---------- SSKICEPCSD -NVRFLP--R QTGLT-----
GgEphA4       VVCKRCGAG- ---------- EPSHCRSCGS -GVHFSP--Q QNGLK-----
GgEphA5       IACKKCNS-- ---------- HSGLCEACGS -HVRYLP--Q QTGLK-----
GgEphA6       VICKKCGS-- ---------- DASQCEICGG -GIRFIP--R HTGLI-----
GgEphA7       ILCKRCSW-- ---------- EQGECVPCGS -NIGYMP--Q QTGLV-----
GgEphA8       VQCRRCGA-- ---------- DAGQCEACGG -GIRFVP--Q QMSLV-----
GgEphB1       IVCKKCRADR ---------- --RACSRCDD -NVEFVP--R QLGLT-----
GgEphB2       IICKSCGSGR ---------- --GACTRCGD -NVQFAP--R QLGLT-----
GgEphB3       VICKKCSVER ---------- --RLCSRCDD -NVEFVP--R QLGLTG----
GgEphB6       VICKECPTR- ---------- SAGTCVRCGD -NVQFEP--R QVGLT-----
HsEphA1       VRCSQCQGTA Q--------- DGGPCQPCGV -GVHFSP--G ARGLT-----
HsEphA2       VTCEQCWPES ---------- --GECGPCEA -SVRYSE--P PHGLT-----
HsEphA3       IICKKCGW-- ---------- NIKQCEPCSP -NVRFLP--R QFGLT-----
HsEphA4       VVCKKCGAG- ---------- DPSKCRPCGS -GVHYTP--Q QNGLK-----
HsEphA5       IACKKCNS-- ---------- HAGVCEECGG -HVRYLP--R QSGLK-----
HsEphA6       VICKKCGL-- ---------- DTSQCEDCGG -GLRFIP--R HTGLI-----
HsEphA7       ILCKRCSW-- ---------- EQGECVPCGS -NIGYMP--Q QTGLE-----
HsEphA8       AVCRRCPW-- ---------- ALSRCEACGS -GTRFVP--Q QTSLV-----
HsEphA10      LLCLRCGREG ---------- PAGACEPCGP -RVAFLP--R QAGLR-----
HsEphB1       IICKKCRADR ---------- --RSCSRCDD -NVEFVP--R QLGLT-----
HsEphB2       IICKSCGSGR ---------- --GACTRCGD -NVQYAP--R QLGLT-----
HsEphB3       VICKKCHGAG G--------- -ASACSRCDD -NVEFVP--R QLGLT-----
HsEphB4       LRCRECRP-- ---------- -GGSCAPCGG -DLTFDP--G PRDLV-----
HsEphB6       VVCKECEGRQ EPASG----- GGGTCHRCRD -EVHFDP--R QRGLT-----


              ....|....| ....|....| ....|....| ....|....| ....|....|
                     660        670        680        690        700
CeEph         ---TRSVQIN GLRPSSDYTF LVFAKNKVSA QFPEFSE--- ----------
DmEph         ---ETKITLT NLEPVTTYTV QIHAINSVSH INEFKRH--- --------SN
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SpEph               ---GYEVNVS RLSSHTTYEF KVHSYNEVSS QGTVDNY--- --------AG
CiEph-a             ---KGSLLVR DLAPHTDYKF KVYSFNGVSG VSGLR----- ----------
CiEph-b             ---ATHVTVK NLRLFAFYKF KVIATNGVTD IAAASGQ--- --------QL
CiEph-c             ---TTYMEVS NLNPFSCYKF KVSSSNGVSR VSVE------ ---------P
CiEph-d             ---VTKVVVR DLSSYAYYRF KIFAINGVTW VAQLS-P--- --------SE
CiEph-e             ---STTLQIS NMSPYTQYML SIQAANGVSG IIDNY-D--- --------VT
CiEph-f             SLKTNFVDMH NLTGHGLYTF QVVATNAVTH KHADSITGQD AVRNKMLNPK
XlEphA2             ---GTTITIM DLEPHLNYSF TVEARNGVSG SGSS------ ----------
XlEphA4             ---TTKVSIN DLQAHTNYTF EVWAINGVSK QNPEQ----- ----------
XlEphB1             ---DTRVFIS NLWAHTPYTF ETQAVNGVTN KSPFP----- ----------
XlEphB2             ---EPRVYMS DLLAHTQYTF EIQAVNGVTD MSPYS----- ----------
XlEphB3             ---QRLVSVS HLQAHTKYSF EIQAVNGVSG KSPHI----- ----------
XlEphB4             ---DRRLRVS GLRPRVTYNF RVLALNGVSE RSSGA----- ----------
GgEphA1             ---KTAVDVD GLEAYTNYTF AVEAHNGVSG MGPAPQ---- ----------
GgEphA3             ---NTTVTVV DLLAHTNYTF EIDAVNGVSD LSTLS----- ----------
GgEphA4             ---TTKVSIT DLLAHTNYTF EVWAVNGVSK HNPSQ----- ----------
GgEphA5             ---NTSVMMV DLLAHTNYTF EIEAVNGVSD QNPGA----- ----------
GgEphA6             ---NSSVTVL DFVSHVNYTF EIEATNGVSE LSFSP----- ----------
GgEphA7             ---DNYVTVM DLLAHANYTF EVEAVNGVSD LSRSQ----- ----------
GgEphA8             ---QGALTVT NLLAHTNYSF WVEAVNGVSD LSVEA----- ----------
GgEphB1             ---ETRVFIS SLWAHTPYTF EIQAVNGVSN KSPFP----- ----------
GgEphB2             ---EPRIYIS DLLAHTQYTF EIQAVNGVTD QSPFS----- ----------
GgEphB3             -LTERRIYIS KVMAHPQYTF EIQAVNGISS KSPYP----- ----------
GgEphB6             ---ESRVQVS NLLARVQYTF EIQAVNLVTE LSSEA----- ----------
HsEphA1             ---TPAVHVN GLEPYANYTF NVEAQNGVSG LGSS-G---- ----------
HsEphA2             ---RTSVTVS DLEPHMNYTF TVEARNGVSG LVTS------ ----------
HsEphA3             ---NTTVTVT DLLAHTNYTF EIDAVNGVSE LSSPP----- ----------
HsEphA4             ---TTKVSIT DLLAHTNYTF EIWAVNGVSK YNPNP----- ----------
HsEphA5             ---NTSVMMV DLLAHTNYTF EIEAVNGVSD LSPGA----- ----------
HsEphA6             ---NNSVIVL DFVSHVNYTF EIEAMNGVSE LSFSP----- ----------
HsEphA7             ---DNYVTVM DLLAHANYTF EVEAVNGVSD LSRSQ----- ----------
HsEphA8             ---QASLLVA NLLAHMNYSF WIEAVNGVSD LSPEP----- ----------
HsEphA10            ---ERAATLL HLRPGARYTV RVAALNGVSG PAAAAG---- ----------
HsEphB1             ---ECRVSIS SLWAHTPYTF DIQAINGVSS KSPFP----- ----------
HsEphB2             ---EPRIYIS DLLAHTQYTF EIQAVNGVTD QSPFS----- ----------
HsEphB3             ---ERRVHIS HLLAHTRYTF EVQAVNGVSG KSPLP----- ----------
HsEphB4             ---EPWVVVR GLRPDFTYTF EVTALNGVSS LATGP----- ----------
HsEphB6             ---ESRVLVG GLRAHVPYIL EVQAVNGVSE LSPDP----- ----------


                    ....|....| ....|....| ....|....| ....|....| ....|....|
                           710        720        730        740        750
CeEph               --KNAVIDIR TRSEEEDVPP VSHLRVDASQ ---------- ----------
DmEph               ESSLVAVSDI VFSNTSLLNI PLDLNEVKTG QAEIVFTTES VLLSTVFNLR
SpEph               ------VNAT TFS--SVPSA VRDLKVVED- ---------- ----------
CiEph-a             ---PAFAEIR IRTNQAAPSA VQPVRVVMAT ---------- ----------
CiEph-b             ---YNFALVN VERNQAAPSP VLNVRSSEID ---------- ----------
CiEph-c             ---EQFELIK ICTNAAAPSA VTGLKFIWIG ---------- ----------
CiEph-d             ---VEYAEVT VETNEAAPSM VLGVRVTDLS ---------- ----------
CiEph-e             ---NSIATVT VNTTQSTPSS VYGIETSDIE ---------- ----------
CiEph-f             VSNTSSAFVT FSIDKFHPST PTNLHMIHSN ---------- ----------
XlEphA2             ---RSYATLR ISLNQTEPPK VTFITLDNQG ---------- ----------
XlEphA4             ---DQAVSVT VTTNQAAPST VTQIQPKEIT ---------- ----------
XlEphB1             ---PQHVSVN ITTNQAAPSS VPIMHQVKAT ---------- ----------
XlEphB2             ---PHFSSVN ITTNQAAPSS VSIMHLVTRA ---------- ----------
XlEphB3             ---PNYYTVN ITTNQAAPSA VPMVQSHGSS ---------- ----------
XlEphB4             ---VG-EEVN VTTDRDVPLP VSGIQQMAAT ---------- ----------
GgEphA1             ---RTAPAVW VAVGHAAPVT VSQFILTQRD ---------- ----------
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GgEphA3         ---RQFAAVS ITTNQAAPSP ITVIRKDRTS ---------- ----------
GgEphA4         ---DQAVSVT VTTNQAAPSP IALIQAKEIT ---------- ----------
GgEphA5         ---RQFVSVN VTTNQAAPSP VSSVKKGKIT ---------- ----------
GgEphA6         ---KQFTAIT VTTDQDAPSL IGMVRKDWAS ---------- ----------
GgEphA7         ---RLFAAVS ITTGQAAPSQ VSGVMKERVL ---------- ----------
GgEphA8         ---RRAAVAN ITTNQAAPSQ VVAVRHESTG ---------- ----------
GgEphB1         ---PQHVSVN ITTNQAAPST VPIMHQVSAT ---------- ----------
GgEphB2         ---PQFASVN ITTNQAAPSA VSIMHQVSRT ---------- ----------
GgEphB3         ---PHFASVN ITTNQAAPSA VPTMHLHSST ---------- ----------
GgEphB6         ---PQYATIN VSTSQSVPSA IPMMHQVSRA ---------- ----------
HsEphA1         ---HASTSVS ISMGHAESLS GLSLRLVKKE ---------- ----------
HsEphA2         ---RSFRTAS VSINQTEPPK VR---LEGRS ---------- ----------
HsEphA3         ---RQFAAVS ITTNQAAPSP VLTIKKDRTS ---------- ----------
HsEphA4         ---DQSVSVT VTTNQAAPSS IALVQAKEVT ---------- ----------
HsEphA5         ---RQYVSVN VTTNQAAPSP VTNVKKGKIA ---------- ----------
HsEphA6         ---KPFTAIT VTTDQDAPSL IGVVRKDWAS ---------- ----------
HsEphA7         ---RLFAAVS ITTGQAAPSQ VSGVMKERVL ---------- ----------
HsEphA8         ---RRAAVVN ITTNQAAPSQ VVVIRQERAG ---------- ----------
HsEphA10        ---TTYAQVT VSTGPGAPWE EGEIRRDRVE ---------- ----------
HsEphB1         ---PQHVSVN ITTNQAAPST VPIMHQVSAT ---------- ----------
HsEphB2         ---PQFASVN ITTNQAAPSA VSIMHQVSRT ---------- ----------
HsEphB3         ---PRYAAVN ITTNQAAPSE VPTLRLHSSS ---------- ----------
HsEphB4         ---VPFEPVN VTTDREVPPA VSDIRVTRSS ---------- ----------
HsEphB6         ---PQAAAIN VSTSHEVPSA VPVVHQVSRA ---------- ----------


                ....|....| ....|....| ....|....| ....|....| ....|....|
                       760        770        780        790        800
CeEph           -----SDGIT IAWSVSD--- -------SDV SDFEVEVRPA IV--------
DmEph           ILAITNKDAD LEWDKPV--- -QSD---FPL EFYEVRWFPK VELDA-----
SpEph           --DVNDESIK LRWRQPL--- -FPN---GEI TNYQVIYRTS AETQEN----
CiEph-a         -----SAEVL LQWSPPR--- -YSSN---PI LDYQIQ---- ----------
CiEph-b         -----ATSVL IRWDAPT--- -LING---HI LDYEVTAQHS KTRMV-----
CiEph-c         -----EVSAT LSWLPPT--- -HSN----KI VGYEVQ---- ----------
CiEph-d         -----ETSAI LTWDTPA--- -KVNG---LI RSYVVS---- ----------
CiEph-e         -----PTSAT LSWHVPR--- -YPNG---EI IEYVVK---- ----------
CiEph-f         -----YTSVT IQWNPPVYTS -------GDQ ISYKIVWVEN QARN------
XlEphA2         -----TNSLS MSWSVP---- PRQQ---TRV WKYEVTYSKK H---------
XlEphA4         -----RHSVS LTWPEPE--- -RAN---GVI LEYEVKYYEK D---------
XlEphB1         -----MKSIT LSWPQQE--- -QPN---GII LDYEIRYYEK ----------
XlEphB2         -----IDSIT LSWSQPD--- -QPN---GII LDYELLYDEK ----------
XlEphB3         -----ANSLT LSWAPPE--- -SPN---GII LDYEIKYYAK G---------
XlEphB4         -----ETTLT LRWATPL--- -HSAPTKGNI LDYEVKYYEK DQ--------
GgEphA1         -----DSSLS VSWLAPR--- QRS----RTS VEYEVMFFEK G---------
GgEphA3         -----RNSVS LSWQEPE--- -HPN---GII LDYEVKYYEK Q---------
GgEphA4         -----RHSVA LAWLEPD--- -RPN---GVI LEYEVKYYEK D---------
GgEphA5         -----KNSIS LSWQEPD--- -RPN---GII LEYEIKYFEK ----------
GgEphA6         -----QNSIA LSWQEPD--- -FPH---GAI LDYEIKYYEK VYPRIAPAFW
GgEphA7         -----QRSVE LSWQEPE--- -HPN---GVI TEYEIKYYEK D---------
GgEphA8         -----QNSVT LLWQEPD--- -QPN---GII LEYEIKYYEK D---------
GgEphB1         -----MRSIT LSWPQPE--- -QPN---GII LDYELRYYEK LSRICTPDVS
GgEphB2         -----VDSIT LSWSQPD--- -QPN---GVI LDYELQYYEK ----------
GgEphB3         -----GNSMT LSWTPPE--- -RPN---GII LDYEIKYSEK QG--------
GgEphB6         -----TSSIT LSWPQPD--- -QPN---GVI LDYQLRYFDK AE--------
HsEphA1         -----PRQLE LTWAGSR--- PRSP---GAN LTYELHVLNQ D---------
HsEphA2         -----TTSLS VSWSIP---- PPQQ---SRV WKYEVTYRKK G---------
HsEphA3         -----RNSIS LSWQEPE--- -HPN---GII LDYEVKYYEK Q---------
HsEphA4         -----RYSVA LAWLEPD--- -RPN---GVI LEYEVKYYEK D---------
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HsEphA5     -----KNSIS LSWQEPD--- -RPN---GII LEYEIKYFEK ----------
HsEphA6     -----QNSIA LSWQAPA--- -FSN---GAI LDYEIKYYEK ----------
HsEphA7     -----QRSVE LSWQEPE--- -HPN---GVI TEYEIKYYEK D---------
HsEphA8     -----QTSVS LLWQEPE--- -QPN---GII LEYEIKYYEK D---------
HsEphA10    -----PQSVS LSWREPIPA- GAPG---AND TEYEIRYYEK G---------
HsEphB1     -----MRSIT LSWPQPE--- -QPN---GII LDYEIRYYEK ----------
HsEphB2     -----VDSIT LSWSQPD--- -QPN---GVI LDYELQYYEK ----------
HsEphB3     -----GSSLT LSWAPPE--- -RPN---GVI LDYEMKYFEK S---------
HsEphB4     -----PSSLS LAWAVPR--- -APS---GAV LDYEVKYHEK GA--------
HsEphB6     -----SNSIT VSWPQPD--- -QTN---GNI LDYQLRYYDQ AE--------


            ....|....| ....|....| ....|....| ....|....| ....|....|
                    810        820        830        840        850
CeEph       ---------- -KKRTFETRH VNMTYTTFIG LNPETVYQFR VRIRDDLRWS
DmEph       ---------- ---INKSALN TKETKAHIVG LLENTEYGFQ VRCKTNNG--
SpEph       ---------- VKYHYQAVLD GEVQETTVHG LVPGTEYVFE VAANTASG--
CiEph-a     ---------Y SSIHGLFTNV TTKTTYKLSN LSPSTSYIVQ VRARSKVG--
CiEph-b     ---LEHIASG VSKSSFKTNR TSNRYWRIVG LEPSNDYLIK VRARTMAG--
CiEph-c     -----LFQNN QRLKLTKIME VSTPNVTING LNPGWKYVVM VRACNNDG--
CiEph-d     ---LGLVYQG QVTDMRMFNS TSKRSMRLDG LKQNNMYAVK VRASTDAG--
CiEph-e     -----LQYGG -HSNTPTLVK TSDTTVHLDG LLSNTNYIVL IHARTVAG--
CiEph-f     --KTAILQLK STTLPTSHML VNVTRAHLAQ LNSGVNYYIA VQALSTAG--
XlEphA2     ---------- -DANSYSVQR CEGNSVTLLK LTPGTTYVVR VQALTQEG--
XlEphA4     ---------- QNERSYRIVK TASRSADIKG LNPLTGYVFH VRARTAAG--
XlEphB1     ---------D HHEFNSSLAR SQTNTARRTG GRVWMFMSVQ VRARTVAG--
XlEphB2     ---------D LTEHNSSRGQ SSTNTVLVPG LRAGTIYVFQ VLSRRMAG--
XlEphB3     ---------- -HSGAGNTVT SQRTTVRMEE MTPDTVYVVQ VRARTVAG--
XlEphB4     ---------- EKP-LIMFLK TPSNEARLLE LHAGTVYVVQ VRARTEAG--
GgEphA1     ---------- -EEARYTVKH LLEPNVTLTD LQPDTTYLLR VRSITPLG--
GgEphA3     ---------- EQETSYTILR AKSTNVTISG LKPDTTYVFQ IRARTAAR--
GgEphA4     ---------- QNERSYRIVK TASRNTDIKG LNPLTSYVFH VRARTAAG--
GgEphA5     ---------- DQETSYTIIK SKETAITADG LKPGSAYVFQ IRARTAAG--
GgEphA6     HYLRVE---E HEQLSYSSTR SKAPSVIITG LKPATKYIFH IRVRTAAG--
GgEphA7     ---------- QRERTYSTVK TKSTSASINN LKPGTVYVFQ IRAFTAAG--
GgEphA8     ---------- KEMQSYSTLK SKGTAATISG LKPATRYIFQ VRARTSAG--
GgEphB1     GTVGSRPAAD HNEYNSSVAR SQTNTARLEG LRPGMVYVVQ VRARTVAG--
GgEphB2     ---------N LSELNSTAVK SPTNTVTVQN LKAGTIYVFQ VRARTVAG--
GgEphB3     ---------- QGDGIANTVT SQKNSVRLDG LKANARYMVQ VRARTVAG--
GgEphB6     ---------- -DEDNSFTLT SETNMATILN LSPGKIYVFQ VRARTAVG--
HsEphA1     ---------- -EER---YQM VLEPRVLLTE LQPDTTYIVR VRMLTPLG--
HsEphA2     ---------- -DSNSYNVRR TEGFSVTLDD LAPDTTYLVQ VQALTQEG--
HsEphA3     ---------- EQETSYTILR ARGTNVTISS LKPDTIYVFQ IRARTAAG--
HsEphA4     ---------- QNERSYRIVR TAARNTDIKG LNPLTSYVFH VRARTAAG--
HsEphA5     ---------- DQETSYTIIK SKETTITAEG LKPASVYVFQ IRARTAAG--
HsEphA6     ---------E HEQLTYSSTR SKAPSVIITG LKPATKYVFH IRVRTATG--
HsEphA7     ---------- QRERTYSTVK TKSTSASINN LKPGTVYVFQ IRAFTAAG--
HsEphA8     ---------- KEMQSYSTLK AVTTRATVSG LKPGTRYVFQ VRARTSAG--
HsEphA10    ---------- QSEQAYSMVK TGAPTVTVTN LKPATRYVFQ IRAASPGPSW
HsEphB1     ---------E HNEFNSSMAR SQTNTARIDG LRPGMVYVVQ VRARTVAG--
HsEphB2     ---------E LSEYNATAIK SPTNTVTVQG LKAGAIYVFQ VRARTVAG--
HsEphB3     ---------- --EGIASTVT SQMNSVQLDG LRPDARYVVQ VRARTVAG--
HsEphB4     ---------- EGPSSVRFLK TSENRAELRG LKRGASYLVQ VRARSEAG--
HsEphB6     ---------- -DESHSFTLT SETNTATVTQ LSPGHIYGFQ VRARTAAG--


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                    860        870        880        890        900
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CeEph        QPISYQLGRG LMSSPSSNEV EESQ-----F LNQTG----- ----------
DmEph        -FGSYS--NM IYAQTLQSVG --------SV YDDSVQ---- ----------
SpEph        -VGAYS--QQ VLATSSGSGG QSVP---TVI LAAAVS---- ----------
CiEph-a      -YGLYSRASA FRTKPVKSST AHYV----LT NPHGASVDID GGNDNMVHDT
CiEph-b      -FGPYS--VP IKVKTLNKST TTVA----IN STAGS----- ----------
CiEph-c      -CGQFS--EK LQLVTYDKGS EPES----IV EGSTT----- ----------
CiEph-d      -FGPYS--VP LKFSPLESTT SSPN----TN DHVG------ ----------
CiEph-e      -FGPFS--KP ISFKTLTPAT IAKP----VT VSPTV----- ----------
CiEph-f      -QSNYSSVLE VRTMSSDLTS KPTG----KP NNSND----- ----------
XlEphA2      -VGVYS--RD YEFETLSVEE ---------- -SSNK----- ----------
XlEphA4      -YGEFS--GP FEFTTNTVPS ------PMIG EGTSP----- ----------
XlEphB1      -YGKFS--SK CGFQTLTAED YKS------E LREQL----- ----------
XlEphB2      -YGRYS--GK MYFQTMTEAE YQS------S FQEKL----- ----------
XlEphB3      -YGAYS--EP REFQTIAEDG DRS------S LQEQV----- ----------
XlEphB4      -YGAFS--GD TLFQTLTQDP EKP------- -HPQV----- ----------
GgEphA1      -PGPYS--QE QEFRTLPPGT ---------- -GALS----- ----------
GgEphA3      -YGTSS--RK FEFETSPDSF SIS------- SENSQ----- ----------
GgEphA4      -YGDFS--GP FEFTTNTVPS ------PIIG DGTNP----- ----------
GgEphA5      -YGGFS--RR FEFETSP-VL AAS------- SDQSQ----- ----------
GgEphA6      -YSGYS--QK FEFETGDETS DMA------- AEQGQ----- ----------
GgEphA7      -YGNYS--PR LDVATLEEAT -----ATAVS SEQNP----- ----------
GgEphA8      -CGRFS--QT VEVETGKPVS ---------- -LRYD----- ----------
GgEphB1      -YGKYS--GK MCFQTLTDDD YKS------E LREQL----- ----------
GgEphB2      -YGRYS--GK MYFQTMTEAE YQT------S VQEKL----- ----------
GgEphB3      -YGRYS--LP TEFQTTAEDG STSK-----T FQE-L----- ----------
GgEphB6      -YGPYS--GK MYFQTLMAGE HSE------M AQDRL----- ----------
HsEphA1      -PGPFS--PD HEFRTSPPVS ---------- -RGLT----- ----------
HsEphA2      -QGAGS--KV HEFQTLSPEG ---------- -SGN------ ----------
HsEphA3      -YGTNS--RK FEFETSPDSF SIS------- GESSQ----- ----------
HsEphA4      -YGDFS--EP LEVTTNTVPS ------RIIG DGANS----- ----------
HsEphA5      -YGVFS--RR FEFETTP-VF AAS------- SDQSQ----- ----------
HsEphA6      -YSGYS--QK FEFETGDETS DMA------- AEQGQ----- ----------
HsEphA7      -YGNYS--PR LDVATLEEAT GKMFEATAVS SEQNP----- ----------
HsEphA8      -CGRFS--QA MEVETGKPR- ---------- -PRYD----- ----------
HsEphA10     EAQSFN--PS IEVQTLGEAA SGS------- RDQSP----- ----------
HsEphB1      -YGKFS--GK MCFQTLTDDD YKS------E LREQL----- ----------
HsEphB2      -YGRYS--GK MYFQTMTEAE YQT------S IQEKL----- ----------
HsEphB3      -YGQYS--RP AEFETTSERG SGAQ-----Q LQEQL----- ----------
HsEphB4      -YGPFG--QE HHSQTQLDES EGW------- -REQL----- ----------
HsEphB6      -HGPYG--GK VYFQTLPQGE LSS------Q LPERL----- ----------


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                    910        920        930        940        950
CeEph        ---------- SALLIIIALI LIVIAVALCM IVVQKKSKNR KQMSDLDVLD
DmEph        ---------- IRFIAGAIVT GVLFLVIF-- --IIATVYFM RS--------
SpEph        ---------- AIFIIVVLVA VVIFLVFWRR WKAKQQLYYQ IST-------
CiEph-a      TVKPSSNHNR LVVAVCVACS FLVITLCALL ILCGRRNRY- ----------
CiEph-b      -------GPD YLLFVAIGGG ILFVVAIVIV IVIFRRRRR- ----------
CiEph-c      ---------W IGGVIGGV-- LVIVIIIIVV MIKRRQTD-- ----------
CiEph-d      ---------T IQLIIAVIGG CSIVIAIVLI VFCCRRKSH- ----------
CiEph-e      ---------N VPVIAGAVCG LALVIILIVV VLRCRKTGNF L---------
CiEph-f      ---------- SLYVYLVAVV SVLLFGGFVF FFLWRRCRG- ----------
XlEphA2      ---------- AAVIGGAIAG SIIIAIFVGV IIFMHR---- ----------
XlEphA4      ---------- TVLLVSVAGS -IVLVVILIA AFVISR---- ----------
XlEphB1      ---------- PLTG-SAAAG VVFIVSLVAI SIVCSRK--- ----------
XlEphB2      ---------- PLIIGSSAAG LVFLIALVVI IIVCSRR--- ----------
XlEphB3      ---------- PMVVGSVTAG LIFIIAVVII VIVCFSR--- ----------
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XlEphB4          ---------- ALIAGTAGVG TLLILAVIIT AVVCIRK--- ----------
GgEphA1          ---------- GGVMASIIFG VLLFIGLL-L GILFFR---- ----------
GgEphA3          ---------- VVMIAISAAV AIILLTVVVY VLIG------ ----------
GgEphA4          ---------- TVLLVSVAGS -VVLVVILIA AFVISR---- ----------
GgEphA5          ---------- IPIIVVSVTV GVILLAVVIG FLLSGSCCDH GCGWASSLRA
GgEphA6          ---------- ILVIVTAAVG GFTLLVILTL FFLITG---- ----------
GgEphA7          ---------- VIIIAVVAVA GTIILVFMVF GFIIGR---- ----------
GgEphA8          ---------- TMTIVWICLT LITGLVALLV VLICKK---- ----------
GgEphB1          ---------- PLIAGSAAAG VVFIVSLVAI SIVCSRK--- ----------
GgEphB2          ---------- PLIIGSSAAG LVFLIAVVVI IIVCNRR--- ----------
GgEphB3          ---------- PLIVGSATAG LLFVIVVVII AIVCFRKGMV TE--------
GgEphB6          ---------- PLIVGSALGG LAFLVIAAIA ILAIIFK--- ----------
HsEphA1          ---------- GGEIVAVIFG LLLGAALL-L GILVFR---- ----------
HsEphA2          ---------- LAVIGGVAVG VVLLLVLAGV GFFIHR---- ----------
HsEphA3          ---------- VVMIAISAAV AIILLTVVIY VLIG------ ----------
HsEphA4          ---------- TVLLVSVSGS -VVLVVILIA AFVISR---- ----------
HsEphA5          ---------- IPVIAVSVTV GVILLAVVIG VLLSGSCCEC GCGRASSLCA
HsEphA6          ---------- ILVIATAAVG GFTLLVILTL FFLITG---- ----------
HsEphA7          ---------- VIIIAVVAVA GTIILVFMVF GFIIGR---- ----------
HsEphA8          ---------- TRTIVWICLT LITGLVVLLL LLICKK---- ----------
HsEphA10         ---------- AIVVTVVTIS ALLVLGSVMS VLAIWR---- ----------
HsEphB1          ---------- PLIAGSAAAG VVFVVSLVAI SIVCSRK--- ----------
HsEphB2          ---------- PLIIGSSAAG LVFLIAVVVI AIVCNRR--- ----------
HsEphB3          ---------- PLIVGSATAG LVFVVAVVVI AIVCLRK--- ----------
HsEphB4          ---------- ALIAGTAVVG VVLVLVVIVV AVLCLRK--- ----------
HsEphB6          ---------- SLVIGSILGA LAFLLLAAIT VLAVVFQ--- ----------


                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         960        970        980        990       1000
CeEph            TYKQDSMTPD YHTTSRHHHH QGNLPATLHE QLRSTTKLNA PLIPSFGSPI
DmEph            ---------- KHQDDLDKKS TNHLPLP--- ----LDYA-- ----------
SpEph            --------LP PRKKEVPPSE NGHLIYANGT NGEAITFG-- ----------
CiEph-a          -----AKAVE SDLEKYPGFI N------SSV DL-------- ----------
CiEph-b          -----RTVKS KKLKSDKSMY NGVSVTFNSS FQ-------- ----------
CiEph-c          ---------- --KKKRKEIQ ARTKLNENTQ QL-------- ----------
CiEph-d          -----QNKSK LDMEKLPMFV MGHLKNGSIG YM-------- ----------
CiEph-e          -----CFTCK RINTKDMEMS LDVKSDPMIS ER-------- ----------
CiEph-f          ---------- TSGKQKSKPC LDKPLQSSSE ECDG------ ----------
XlEphA2          ---------S RRNPNIRHSE EDIYFS--KP DQ-------- ----------
XlEphA4          -------RRS KYSKAKQEAD EEK-----HL N--------- ----------
XlEphB1          ---------- RTYS-KEAVY SDKLQHYSTG RG-------- ----------
XlEphB2          ---------- RGFERADYEY TDKLQHYTSG HM-------- ----------
XlEphB3          ---------- KQRNDSESEY TEKLQQY--- -M-------- ----------
XlEphB4          ---------- -HGSTNEPEY CDKPGQY--- LI-------- ----------
GgEphA1          ---------R KKAHR-RQAR PDYSTSGFDR E--------- ----------
GgEphA3          -------RFC GYKKSKHGTD EKR-LHFGNG HL-------- ----------
GgEphA4          -------RRS KYSKAKQEAD EEK-----HL N--------- ----------
GgEphA5          VAYPSLIWRC GYSKAKQDPE EEK-MHFHNG HI-------- ----------
GgEphA6          -------RCQ WYIKAKMKSE EKRRAHLQNG NL-------- ----------
GgEphA7          -------RHC GYSKADQEGD EEL-----YF HF-------- ----------
GgEphA8          -------RHC GYSKAFQDSD EEK-MHYQNG QV-------- ----------
GgEphB1          ---------- RAYS-KEVVY SDKLQHYSTG RG-------- ----------
GgEphB2          ---------- RGFERADSEY TDKLQHYTSG HM-------- ----------
GgEphB3          -QLLSSPLGR KQRNSTDPEY TEKLQQY--- -V-------- ----------
GgEphB6          ---------- --SKRRETPY TDRLQQY-IS TR-------- ----------
HsEphA1          ---------S RRAQRQRQQR QRDRATDVDR ED-------- ----------
HsEphA2          ---------R RKNQRARQSP EDVYFS--KS EQ-------- ----------
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HsEphA3         -------RFC GYK-SKHGAD EKR-LHFGNG HL-------- ----------
HsEphA4         -------RRS KYSKAKQEAD EEK-----HL N--------- ----------
HsEphA5         VAHPSLIWRC GYSKAKQDPE EEK-MHFHNG HI-------- ----------
HsEphA6         -------RCQ WYIKAKMKSE EKRRNHLQNG HL-------- ----------
HsEphA7         -------RHC GYSKADQEGD EEL-----YF HF-------- ----------
HsEphA8         -------RHC GYSKAFQDSD EEK-MHYQNG QAPPPVFL-- ----------
HsEphA10        -------RPC SYGKGGGDAH DEE---ELYF HF-------- ----------
HsEphB1         ---------- RAYS-KEAVY SDKLQHYSTG RG-------- ----------
HsEphB2         ---------- RGFERADSEY TDKLQHYTSG HM-------- ----------
HsEphB3         ---------- -QRHGSDSEY TEKLQQY--- -I-------- ----------
HsEphB4         ---------- -QSNGREAEY SDKHGQY--- LI-------- ----------
HsEphB6         ---------- --RKRRGTGY TEQLQQY-SS P--------- ----------


                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1010       1020       1030       1040       1050
CeEph           SQPPPYYGGV HPNSGKYKTY VDPTTYEDPY QA----LIEF TFDISPNDVF
DmEph           -----SNEVT TPLFGNSRSY VDPHTYEDPN QA----IREF AREIDANYIT
SpEph           -----HSTVI LPHIKKVKTY VDPFTYEDPN QA----VKEF ATEIDATQIR
CiEph-a         ---------- ----LSTRTY VDPSTYEDPY NA----VKEF AKEIDFKLVN
CiEph-b         ---------- ----SNHKTY IDPTTYEDPQ RA----VSEF TKEIDATYIK
CiEph-c         ---------- ----NQRRTY VD---YRDPH NG----VKEI ATEIDQTRIK
CiEph-d         ---------- ----PGHKTY IDPTTYEDPA RA----VLDF TKEIDASYIG
CiEph-e         ---------- ----LIDRIY VK---YPNPQ TA----LAKF KCEINPSAIT
CiEph-f         ---------- ---FTVCTEL SDQRTYVDPF SCGPIQTHKF SCETDPDLVS
XlEphA2         ---------- ---LKPLKTY VDPHTYEDPN KA----VLKF TMRFRPTPSR
XlEphA4         ---------- ----QGVKTY VDPFTYEDPN QA----VREF AKEIDASCIK
XlEphB1         ---------- ---SPGMKIY IDPFTYEDPN EA----VREF AKEIDVSFVK
XlEphB2         ---------- ---TPGMKIY IDPFTYEDPN EA----VREF AKEIDISCVK
XlEphB3         ---------- ---VPGTKLY IDPFTYEDPN EA----VRDF AKEIDISSVK
XlEphB4         ---------- ---GHNSKVY IDPFTYEDPN EA----VREF AKEIDVSCVK
GgEphA1         ---------- ---KVWLKPY VDLQPYDDPS RG----VLEF IKELDVSCVT
GgEphA3         ---------- --KLPGLRTY VDPHTYEDPN QA----VHEF AKELDASNIS
GgEphA4         ---------- ----QGVRTY VDPFTYEDPN QA----VREF AKEIDASCIK
GgEphA5         ---------- --KLPGVRTY IDPHTYEDPN QA----VHEF AKEIEASCIT
GgEphA6         ---------- --RFPGIKTY IDPDTYEDPS LA----VHEF AKEIDPSRIR
GgEphA7         ---------- --KFPGTKTY IDPETYEDPN RA----VHQF AKELDASCIK
GgEphA8         ---------- --KFPESKFY VDPHTYEDPC QA----VHEF TREIEASRIK
GgEphB1         ---------- ---SPGMKIY IDPFTYEDPN EA----VREF AKEIDVSFVK
GgEphB2         ---------- ---TPGMKIY IDPFTYEDPN EA----VREF AKEIDISCVK
GgEphB3         ---------- ---TPGMKVY IDPFTYEDPN EA----VREF AKEIDISCVK
GgEphB6         ---------- ---GLGVKYY IDPSTYEDPN EA----IREF AKEIDVSFIK
HsEphA1         ---------- ---KLWLKPY VDLQAYEDPA QG----ALDF TRELDPAWLM
HsEphA2         ---------- ---LKPLKTY VDPHTYEDPN QA----VLKF TTEIHPSCVT
HsEphA3         ---------- --KLPGLRTY VDPHTYEDPT QA----VHEF AKELDATNIS
HsEphA4         ---------- ----QGVRTY VDPFTYEDPN QA----VREF AKEIDASCIK
HsEphA5         ---------- --KLPGVRTY IDPHTYEDPN QA----VHEF AKEIEASCIT
HsEphA6         ---------- --RFPGIKTY IDPDTYEDPS LA----VHEF AKEIDPSRIR
HsEphA7         ---------- --KFPGTKTY IDPETYEDPN RA----VHQF AKELDASCIK
HsEphA8         -----PLHHP PGKLPEPQFY AEPHTYEEPG RA----GRSF TREIEASRIH
HsEphA10        ---------- --KVPTRRTF LDPQSCGDLL QA----VHLF AKELDAKSVT
HsEphB1         ---------- ---SPGMKIY IDPFTYEDPN EA----VREF AKEIDVSFVK
HsEphB2         ---------- ---TPGMKIY IDPFTYEDPN EA----VREF AKEIDISCVK
HsEphB3         ---------- ---APGMKVY IDPFTYEDPN EA----VREF AKEIDVSCVK
HsEphB4         ---------- ---GHGTKVY IDPFTYEDPN EA----VREF AKEIDVSYVK
HsEphB6         ---------- ---GLGVKYY IDPSTYEDPC QA----IREL AREVDPAYIK
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        1060       1070       1080       1090       1100
CeEph           ITQVIGGGEF GDVCLGGLSK NSPAAAKWSV SNTTMGRGGG GGGYESEPYE
DmEph           IEAIIGGGEF GDVCRGRLKI PP-------- ---------- ----NFVQDI
SpEph           ILEVIGGGEF GDVCSGLMLM PD-------- ---------- ------KTTI
CiEph-a         IEQVIGRGEF GEVCKG--TF FN-------- ---------- ---------K
CiEph-b         IEQVIGAGEF GEVCRGRLQL PN-------- ---------- ------KPES
CiEph-c         IDSVIGRGEF GEVCRG--KM LT-------- ---------- -----GKTTT
CiEph-d         IEEVIGGGEF GEVCLGRMRL PN-------- ---------- -----SKKEH
CiEph-e         IEDVIGSGEF AEVCCG--RL VH-------- ---------- -----ENKTI
CiEph-f         INKVIGCGEF GEVYQGSLTL TDTG------ ---------- ----GKRKVI
XlEphA2         AKKVIGAGEF GEVFKGILKL P--------- ---------- -----GKKES
XlEphA4         IEKVIGVGEF GEVCSGRLKV P--------- ---------- -----GKREI
XlEphB1         IEEVIGAGEF GEVYKGRLKL P--------- ---------- -----SKREI
XlEphB2         IEQVIGAGEF GEVCSGHLKL P--------- ---------- -----XXREI
XlEphB3         IEEVIGAGEF GEVCRGRLKQ A--------- ---------- -----GRREQ
XlEphB4         IEEVIGAGEF GEVCRGRLKV P--------- ---------- -----GKKEN
GgEphA1         MENVIGEGEF GEVYRGSLRL P--------- ---------- -----GKERI
GgEphA3         IDKVVGAGEF GEVCSGRLKL P--------- ---------- -----SKKEI
GgEphA4         IEKVIGVGEF GEVCSGRLKV P--------- ---------- -----GKREI
GgEphA5         IERVIGAGEF GEVCSGRLKL Q--------- ---------- -----GKREF
GgEphA6         IERVIGAGEF GEVCSGRLKT P--------- ---------- -----GKREI
GgEphA7         IERVIGAGEF GEVCSGRLKL P--------- ---------- -----GKRDV
GgEphA8         IEKVIGSGES GEVCYGRLKL P--------- ---------- -----GKREI
GgEphB1         IEEVIGAGEF GEVYKGRLKL P--------- ---------- -----GKREI
GgEphB2         IEQVIGAGEF GEVCSGHLKL P--------- ---------- -----GKREI
GgEphB3         IEEVIGAGEF GEVCRGRLKL P--------- ---------- -----GRREI
GgEphB6         IEEVIGSGEF GEVCFGRLKH P--------- ---------- -----GKREY
HsEphA1         VDTVIGEGEF GEVYRGTLRL P--------- ---------- -----SQDCK
HsEphA2         RQKVIGAGEF GEVYKGMLKT SS-------- ---------- -----GKKEV
HsEphA3         IDKVVGAGEF GEVCSGRLKL P--------- ---------- -----SKKEI
HsEphA4         IEKVIGVGEF GEVCSGRLKV P--------- ---------- -----GKREI
HsEphA5         IERVIGAGEF GEVCSGRLKL P--------- ---------- -----GKREL
HsEphA6         IERVIGAGEF GEVCSGRLKT P--------- ---------- -----GKREI
HsEphA7         IERVIGAGEF GEVCSGRLKL P--------- ---------- -----GKRDV
HsEphA8         IEKIIGSGDS GEVCYGRLRV P--------- ---------- -----GQRDV
HsEphA10        LERSLGGGRF GELCCGCLQL P--------- ---------- -----GRQEL
HsEphB1         IEEVIGAGEF GEVYKGRLKL P--------- ---------- -----GKREI
HsEphB2         IEQVIGAGEF GEVCSGHLKL P--------- ---------- -----GKREI
HsEphB3         IEEVIGAGEF GEVCRGRLKQ P--------- ---------- -----GRREV
HsEphB4         IEEVIGAGEF GEVCRGRLKA P--------- ---------- -----GKKES
HsEphB6         IEEVIGTGSF GEVRQGRLQP R--------- ---------- -----GRREQ


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                        1110       1120       1130       1140       1150
CeEph           TVAIKTLKSG SSAKAKAEFL TEATIMGQFS HPNVIRLIGV VTS-------
DmEph           DVAIKTLKPG SSEKARCDFL TEASIMGQFD HPNVIYLQGV VTR-------
SpEph           KVAVKTLKTG ATDKDRSDFL SEASIMGQFD HPNVIKLLGV VTK-------
CiEph-a         CVAIKTLKVG YNTQERSDFL GEASIMGQFD HPNVIRLEGV VTKS------
CiEph-b         DVAIKTLKTG YSTQQKLDFL GEASIMGQFD HPNVIRLEGV VTKS------
CiEph-c         SVAVKRLKHG ASLIDHTNFL REACTMAQFK DPNIIQLKGV VTKSNFALLF
CiEph-d         DVAIKTLKAG YSTQQKLDFL GEASIMGQFD HPNVIRLEGV VTKS------
CiEph-e         KVAVKRLKDS ATSVDETNFL QEGVTLTQFD DANVVKIQGV VTSS------
CiEph-f         DVAIKQLHAQ CQPNEQIDFL HEAKALERFQ HPNIVKLEAV VMAS------
XlEphA2         TVAIKTLKAG YTEKQRNDFL SEASIMGQFC HHNIIRLEG- WSK-------
XlEphA4         YVAIKTLKAG YTDKQRRDFL SEASIMGQFD HPNIIHLEGV VTK-------
XlEphB1         SVAIKTLKAG YSEKQRRDFL SEASIMGQFD HPNIIRLEGV VTK-------
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XlEphB2     FVAIKTLKSG YTEKQRRDFL SEASIMGQFD HPNVIHLEGV VTK-------
XlEphB3     LVAIKTLKAG YTEQQRRDFL GEASIMGQFD HPNIIRLEGV VTR-------
XlEphB4     YVAIKTLKGG YTERQRKEFL SEASIMGQFN HPNIIHLEGV ITN-------
GgEphA1     VVAIKTLKST YSDSQWWNFL REATIMGQFN HPNIVHLEGV VTK-------
GgEphA3     SVAIKTLKAG YTEKQRRDFL GEASIMGQFD HPNIIRLEGV VTK-------
GgEphA4     CVAIKTLKAG YTDKQRRDFL SEASIMGQFD HPNIIHLEGV VTK-------
GgEphA5     PVAIKTLKVG YTEKQRRDFL GEASIMGQFD HPNIIHLEGV VTK-------
GgEphA6     PVAIKTLKGG YMDRQRRDFL REASIMGQFD HPNIIRLEGV VTKRSFPTIG
GgEphA7     AVAIKTLKVG YTEKQRRDFL CEASIMGQFD HPNVVHLEGV VTR-------
GgEphA8     PVAIKALKAG YTEKQRRDFL SEASIMAQFD HPNVIHLEGV VTR-------
GgEphB1     YVAIKTLKAG YSEKQRRDFL SEASIMGQFD HPNIIRLEGV VTK-------
GgEphB2     FVAIKTLKSG YTEKQRRDFL SEASIMGQFD HPNVIHLEGV VTK-------
GgEphB3     FVAIKTLKVG YTERQRRDFL SEASIMGQFD HPNIIHLEGV VTK-------
GgEphB6     TVAIKTLKSG YTDEQRREFL SEASIMGQFE HPNVIHLEGV VTK-------
HsEphA1     TVAIKTLKDT SPGGQWWNFL REATIMGQFS HPHILHLEGV VTK-------
HsEphA2     PVAIKTLKAG YTEKQRVDFL GEAGIMGQFS HHNIIRLEGV ISK-------
HsEphA3     SVAIKTLKVG YTEKQRRDFL GEASIMGQFD HPNIIRLEGV VTK-------
HsEphA4     CVAIKTLKAG YTDKQRRDFL SEASIMGQFD HPNIIHLEGV VTK-------
HsEphA5     PVAIKTLKVG YTEKQRRDFL GEASIMGQFD HPNIIHLEGV VTK-------
HsEphA6     PVAIKTLKGG HMDRQRRDFL REASIMGQFD HPNIIRLEGV VTKRSFPAIG
HsEphA7     AVAIKTLKVG YTEKQRRDFL CEASIMGQFD HPNVVHLEGV VTR-------
HsEphA8     PVAIKALKAG YTERQRRDFL SEASIMGQFD HPNIIRLEGV VTR-------
HsEphA10    LVAVHMLRDS ASDSQRLGFL AEALTLGQFD HSHIVRLEGV VTR-------
HsEphB1     YVAIKTLKAG YSEKQRRDFL SEASIMGQFD HPNIIRLEGV VTK-------
HsEphB2     FVAIKTLKSG YTEKQRRDFL SEASIMGQFD HPNVIHLEGV VTK-------
HsEphB3     FVAIKTLKVG YTERQRRDFL SEASIMGQFD HPNIIRLEGV VTK-------
HsEphB4     CVAIKTLKGG YTERQRREFL SEASIMGQFE HPNIIRLEGV VTN-------
HsEphB6     TVAIQALWAG GAESLQMTFL GRAAVLGQFQ HPNILRLEGV VTK-------


            ....|....| ....|....| ....|....| ....|....| ....|....|
                  1160       1170       1180       1190       1200
CeEph       ---------- ---------- ---------- -----AEPVM IVAEYMANGS
DmEph       ---------- ---------- ---------- -----SNPVM IITEYMENGS
SpEph       ---------- ---------- ---------- -----TRPAM IVTEFMENGS
CiEph-a     ---------- ---------- ---------- ------RPLM IITEFMENGS
CiEph-b     ---------- ---------- ---------- ------RPLM IITEFMENGS
CiEph-c     KLIRY----- -----KFILK IISTKYISTN LPEVLCIPAM IITEFMEHGS
CiEph-d     ---------- ---------- ---------- ------QPHM IITEYMENGS
CiEph-e     ---------- ---------- ---------- ------PNIM IVFEYMENGA
CiEph-f     ---------- ---------- ---------- ------RPFM IVTELMVNGC
XlEphA2     ---------- ---------- ---------- -----YKPMM IVTEHMENGA
XlEphA4     ---------- ---------- ---------- -----CKPVM IITEYMENGS
XlEphB1     ---------- ---------- ---------- -----SRPVM IITEFMENGA
XlEphB2     ---------- ---------- ---------- -----SSPVM IITEFMENGS
XlEphB3     ---------- ---------- ---------- -----SRPVM ILTEFMENGA
XlEphB4     ---------- ---------- ---------- -----NCPVM IITEYMENGA
GgEphA1     ---------- ---------- ---------- -----RRPMM IITEYMENGA
GgEphA3     ---------- ---------- ---------- -----SKPVM IVTEYMENGS
GgEphA4     ---------- ---------- ---------- -----CKPVM IITEYMENGS
GgEphA5     ---------- ---------- ---------- -----SKPVM IVTEYMENGS
GgEphA6     VKSLS-RFLR AGFLNSILTS HPVSGGGSLP PSISSGRPVM IVVEYMENGS
GgEphA7     ---------- ---------- ---------- -----GKPVM IVIEYMENGA
GgEphA8     ---------- ---------- ---------- -----SKLVM IVTEYMENGS
GgEphB1     ---------- ---------- ---------- -----SRPVM IITEFMENGA
GgEphB2     ---------- ---------- ---------- -----SSPVM IITEFMENGS
GgEphB3     ---------- ---------- ---------- -----SRPVM IITEFMENCA
GgEphB6     ---------- ---------- ---------- -----SRPVM IVTEFMENGS
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HsEphA1         ---------- ---------- ---------- -----RKPIM IITEFMENGA
HsEphA2         ---------- ---------- ---------- -----YKPMM IITEYMENGA
HsEphA3         ---------- ---------- ---------- -----SKPVM IVTEYMENGS
HsEphA4         ---------- ---------- ---------- -----CKPVM IITEYMENGS
HsEphA5         ---------- ---------- ---------- -----SKPVM IVTEYMENGS
HsEphA6         VEAFCPSFLR AGFLNSIQAP HPVPGGGSLP PRIPAGRPVM IVVEYMENGS
HsEphA7         ---------- ---------- ---------- -----GKPVM IVIEFMENGA
HsEphA8         ---------- ---------- ---------- -----GRLAM IVTEYMENGS
HsEphA10        ---------- ---------- ---------- -----GSTLM IVTEYMSHGA
HsEphB1         ---------- ---------- ---------- -----SRPVM IITEFMENGA
HsEphB2         ---------- ---------- ---------- -----STPVM IITEFMENGS
HsEphB3         ---------- ---------- ---------- -----SRPVM ILTEFMENCA
HsEphB4         ---------- ---------- ---------- -----SMPVM ILTEFMENGA
HsEphB6         ---------- ---------- ---------- -----SRPLM VLTEFMELGP


                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1210       1220       1230       1240       1250
CeEph           LDQFLRNTDQ RGEKVHWEKI TEMLYGIASG MKYLTDMGYV HRVSFLRDLA
DmEph           LDTFLRVND- --GKFQTLQL IVMLRGIASG MSYLSDMNYV H-----RDLA
SpEph           LDKYLKEND- --GRFTVTQL LGMMRGVGFG MRFLSEMNFV H-----RDLA
CiEph-a         LDAFLRNQE- --GKIPSAQL TEMLLSIAQG MEYLSNMGYV H-----RDLA
CiEph-b         LDAFLRNHD- --NGFTVIQL VGILRNIAAG MKYLSDMGYV H-----RDLA
CiEph-c         LDKFLQARS- --GQLTVLQL LEMLRGIASG MKYLSSMKYV H-----RDLA
CiEph-d         LDTFLRKHD- --GEFTVIQL VGILRNIAAG MKYLSDMGYV H-----RDLA
CiEph-e         LDKYLQLHK- --NDITLFQL LEMLRGIASG MKYLSSMKYV H-----RDLA
CiEph-f         LGKFLRHHRG AKRYFKPTVL VRMLHGVAKG MAYLSKMCYV H-----RDLA
XlEphA2         LDKFLKDND- --GEFSPIQL VGMLRGIAAG MKYLSEMNYV H-----RDLA
XlEphA4         LDAFLRKND- --GRFTVIQL VGMLRGIGSG MKYLSDMSYV H-----RDLA
XlEphB1         LDSFLRQND- --GQFTVIQL VGMLRGIAAG MKYLSEMNYV H-----RDLA
XlEphB2         LDSFLRKND- --GQFTVIQL VSMLRGIAAG MKYLANMNYV H-----RDLA
XlEphB3         LDSFLRMND- --GQFTVIQL VGILRGIASG MKYLSEMNYV H-----RDLA
XlEphB4         LDSFLRQND- --GQFTPIQL VGMLRGIASG MRYLSEMSFV H-----RDLA
GgEphA1         LDTFLRENE- --EKFSPVQL VNMLQGIASG MTYLSEHNYV H-----RDLA
GgEphA3         LDSFLRKHD- --AQFTVIQL VGMLRGIASG MKYLSDMGYV H-----RDLA
GgEphA4         LDAFLRKND- --GRFTVIQL VGMLRGIGSG MKYLSDMSYV H-----RDLA
GgEphA5         LDTFLKKND- --GQFTVIQL VGMLRGIASG MKYLSDMGYV H-----RDLA
GgEphA6         LDSFLRKHD- --GHFTVIQL VGMLRGIASG MKYLSDMGYV H-----RDLA
GgEphA7         LDAFLRKHD- --GQFTVIQL VGMLRGIAAG MRYLADMGYV H-----RDLA
GgEphA8         LDTFLRKHD- --GQFTIIQL VGMLRGIGAG MRYLSDLGYV H-----RDLA
GgEphB1         LDSFLRQND- --GQFTVIQL VGMLRGIAAG MKYLAEMNYV H-----RDLA
GgEphB2         LDSFLRQND- --GQFTVIQL VGMLRGIAAG MKYLADMNYV H-----RDLA
GgEphB3         LDSFLRLND- --GQFTVIQL VGMLRGIAAG MKYLSEMNYV H-----RDLA
GgEphB6         LDSFLRQKE- --GQFSVLQL VGMLRGIAAG MRYLSDMNYV H-----RDLA
HsEphA1         LDAFLRERE- --DQLVPGQL VAMLQGIASG MNYLSNHNYV H-----RDLA
HsEphA2         LDKFLREKD- --GEFSVLQL VGMLRGIAAG MKYLANMNYV H-----RDLA
HsEphA3         LDSFLRKHD- --AQFTVIQL VGMLRGIASG MKYLSDMGYV H-----RDLA
HsEphA4         LDAFLRKND- --GRFTVIQL VGMLRGIGSG MKYLSDMSYV H-----RDLA
HsEphA5         LDTFLKKND- --GQFTVIQL VGMLRGISAG MKYLSDMGYV H-----RDLA
HsEphA6         LDSFLRKHD- --GHFTVIQL VGMLRGIASG MKYLSDMGYV H-----RDLA
HsEphA7         LDAFLRKHD- --GQFTVIQL VGMLRGIAAG MRYLADMGYV H-----RDLA
HsEphA8         LDTFLRTHD- --GQFTIMQL VGMLRGVGAG MRYLSDLGYV H-----RDLA
HsEphA10        LDGFLRRHE- --GQLVAGQL MGLLPGLASA MKYLSEMGYV H-----RGLA
HsEphB1         LDSFLRQND- --GQFTVIQL VGMLRGIAAG MKYLAEMNYV H-----RDLA
HsEphB2         LDSFLRQND- --GQFTVIQL VGMLRGIAAG MKYLADMNYV H-----RDLA
HsEphB3         LDSFLRLND- --GQFTVIQL VGMLRGIAAG MKYLSEMNYV H-----RDLA
HsEphB4         LDSFLRLND- --GQFTVIQL VGMLRGIASG MRYLAEMSYV H-----RDLA
HsEphB6         LDSFLRQRE- --GQFSSLQL VAMQRGVAAA MQYLSSFAFV H-----RSLS
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            ....|....| ....|....| ....|....| ....|....| ....|....|
                  1260       1270       1280       1290       1300
CeEph       ARNVLLDMEL RCKIADFGLS RGVRSEGSSV EPEYTTN--- ----------
DmEph       ARNVLVNAQL ICKIADFGLS REIEN----A SDAYTTR--- ----------
SpEph       ARNILVNEHL VCKVADFGLS RRKE-----L DGAYETR--- ----------
CiEph-a     ARNILVNSNL VCKVSDFGLS RVLEQLEEDD VAMYTTR--- ----------
CiEph-b     ARNILVNAQL ICKVSDFGMS RVLE---EDS DAAYTAR--- ----------
CiEph-c     ARNILVNSQL VCKVSDFGLS RTLE---NDP QATYTTQ--- ----------
CiEph-d     ARNILVNAQL ICKVSDFGLS RVLE---ESS DSTYTTR--- ----------
CiEph-e     ARNILVNSQL VCKVSDFGLS RTLE---NDP QATYTTQ--- ----------
CiEph-f     ARNVLINNEL VCKVADFGLI RHIG----EK KSTVNTK--- ----------
XlEphA2     ARNILVNSQL VCKVSDFGLS RVLE---DDP EATYTTS--- ----------
XlEphA4     ARNILVNSNL VCKVSDFGMS RVLE---DDP EAAYTTR--- ----------
XlEphB1     ARNILVNSNL VCKVSDFGLS RYLQ---DDT SDPTYTS--- ----------
XlEphB2     XXNILVNSNL VCKVSDFGLS RFLE---DDT SDPTYTS--- ----------
XlEphB3     ARNILVNSNL VCKVSDFGLS RFLE---NSR SDPTYTS--- ----------
XlEphB4     ARNILVNSNL VCKVSDFGLS RFLQ---EGS TDPTYTS--- ----------
GgEphA1     ARNILVTRSL QCKVSDFGLS RILE---NDA EGTYETK--- ----------
GgEphA3     ARNILINSNL VCKVSDFGLS RVLE---DDP EAAYTTR--- ----------
GgEphA4     ARNILVNSNL VCKVSDFGMS RVLE---DDP EAAYTTR--- ----------
GgEphA5     ARNILINSNL VCKVSDFGLS RVLE---DDP EAAYTTR--- ----------
GgEphA6     ARNILVNSNL MCKVSDFGLS RVLE---DDP EAAYTTSTVT LGDKKSVYWF
GgEphA7     ARNILVNSNL VCKVSDFGLS RVIE---DDP EAVYTTT--- ----------
GgEphA8     ARNILVNSNL VCKVSDFGLS RILE---DDP DAAYTTT--- ----------
GgEphB1     ARNILVNSNL VCKVSDFGLS RYLQ---DDT SDPTYTS--- ----------
GgEphB2     ARNILVNSNL VCKVSDFGLS RFLE---DDT SDPTYTS--- ----------
GgEphB3     ARNILVNSNL VCKVSDFGLS RFLE---DDP ADPTYTS--- ----------
GgEphB6     ARNILVNSNL VCKVSDFGLS RFLE---DDA SNPTYTG--- ----------
HsEphA1     ARNILVNQNL CCKVSDFGLT RLLD----DF DGTYETQ--- ----------
HsEphA2     ARNILVNSNL VCKVSDFGLS RVLE---DDP EATYTTS--- ----------
HsEphA3     ARNILINSNL VCKVSDFGLS RVLE---DDP EAAYTTR--- ----------
HsEphA4     ARNILVNSNL VCKVSDFGMS RVLE---DDP EAAYTTR--- ----------
HsEphA5     ARNILINSNL VCKVSDFGLS RVLE---DDP EAAYTTR--- ----------
HsEphA6     ARNILVNSNL VCKVSDFGLS RVLE---DDP EAAYTTT--- ----------
HsEphA7     ARNILVNSNL VCKVSDFGLS RVIE---DDP EAVYTTT--- ----------
HsEphA8     ARNVLVDSNL VCKVSDFGLS RVLE---DDP DAAYTTT--- ----------
HsEphA10    ARHVLVSSDL VCKISGFGRG --PR---DRS EAVYTTMS-- ----------
HsEphB1     ARNILVNSNL VCKVSDFGLS RYLQ---DDT SDPTYTS--- ----------
HsEphB2     ARNILVNSNL VCKVSDFGLS RFLE---DDT SDPTYTS--- ----------
HsEphB3     ARNILVNSNL VCKVSDFGLS RFLE---DDP SDPTYTS--- ----------
HsEphB4     ARNILVNSNL VCKVSDFGLS RFLE---ENS SDPTYTS--- ----------
HsEphB6     AHSVLVNSHL VCKVARLGHS ---------- ---------- ----------


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                  1310       1320       1330       1340       1350
CeEph       ------GGKI PVRWTAPEAI THRKFTPSSD VWSFGVVIWE VCSFGERPYW
DmEph       ------GGKI PVRWTAPEAI AFRKFTSASD VWSYGVVLWE VMSYGERPYW
SpEph       ------GGKI PIRWTAPEAI GYKKFTSASD VWSLGVVMWE IMSYGERPYW
CiEph-a     ------GGMI PIRWTSPEAI TMRTFSTSSD VWSFGIVMWE VMSFGERPYW
CiEph-b     ------CGKI PIRWTAPEAF TYRKFTSASD VWSYGIVMWE VMSYGERPYW
CiEph-c     ------GGKI ALRWTAPESI RCRQFTSASD VWSYGIVMWE VMSYGEKPYW
CiEph-d     ------GGKI PIRWTAPEAI NYRKFTSASD VWSYGIVMWE VMSYGERPYW
CiEph-e     ------GGKI AIRWTAPESI LFRQFTSASD VWSYGIVMWE VMSYGEKPYW
CiEph-f     ------GGKI SLRWTAPEAV AYQAYSQASD VWSFGVFAWE VITYGEKPYW
XlEphA2     ------GGKI PIRWTAPEAI SYRKFTSASD VWSYGIVMWE VMSYGERPYW
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XlEphA4       ------GGKI PIRWTAPEAI AYRKFTSASD VWSYGIVMWE VMSYGERPYW
XlEphB1       ----SLGGKI PVRWTAQEAI AYRKFTSASD VWSYGIVMWE VMSYGERPYW
XlEphB2       ----SLGGKI PIRWKAPEAI QYRKFTSASD VWSYGIVMWE VMSYGERPYW
XlEphB3       ----ALGGKI PIRWTAPEAI SYRKFTSASD VWSYGIVMWE VMSYGERPYW
XlEphB4       ----CLGGKI PIRWTAPEAI AFRKFTSASD VWGYGIVMWE VMSFGERPYW
GgEphA1       ------GGKI PIRWTAPEAI AHRIFTSASD VWSFGIVMWE VLSFGDKPYG
GgEphA3       ------GGKI PIRWTSPEAI AYRKFTSASD AWSYGIVLWE VMSYGERPYW
GgEphA4       ------GGKI PIRWTAPEAI AYRKFTSASD VWSYGIVMWE VMSYGERPYW
GgEphA5       ------GGKI PIRWTAPEAI AFRKFTSASD VWSYGIVMWE VMSYGERPYW
GgEphA6       VTTAAKGGKI PIRWTAPEAI AYRKFSSASD AWSYGIVMWE VMSYGERPYW
GgEphA7       ------GGKI PVRWTAPEAI QYRKFTSASD VWSYGIVMWE VMSYGERPYW
GgEphA8       ------GGKI PIRWTAPEAI AYRKFSSASD VWSYGIVMWE VLAYGERPYW
GgEphB1       ----SLGGKI PVRWTAPEAI AYRKFTSASD VWSYGIVMWE VMSFGERPYW
GgEphB2       ----ALGGKI PIRWTAPEAI QYRKFTSASD VWSYGIVMWE VMSYGERPYW
GgEphB3       ----SLGGKI PIRWTAPEAI AYRKFTSASD VWSYGIVMWE VMSYGERPYW
GgEphB6       ----ALGCKI PIRWTAPEAV QYRKFTSSSD VWSYGIVMWE VMSYGERPYW
HsEphA1       ------GGKI PIRWTAPEAI AHRIFTTASD VWSFGIVMWE VLSFGDKPYG
HsEphA2       ------GGKI PIRWTAPEAI SYRKFTSASD VWSFGIVMWE VMTYGERPYW
HsEphA3       ------GGKI PIRWTSPEAI AYRKFTSASD VWSYGIVLWE VMSYGERPYW
HsEphA4       ------GGKI PIRWTAPEAI AYRKFTSASD VWSYGIVMWE VMSYGERPYW
HsEphA5       ------GGKI PIRWTAPEAI AFRKFTSASD VWSYGIVMWE VVSYGERPYW
HsEphA6       ------GGKI PIRWTAPEAI AYRKFSSASD AWSYGIVMWE VMSYGERPYW
HsEphA7       ------GGKI PVRWTAPEAI QYRKFTSASD VWSYGIVMWE VMSYGERPYW
HsEphA8       ------GGKI PIRWTAPEAI AFRTFSSASD VWSFGVVMWE VLAYGERPYW
HsEphA10      -------GRS PALWAAPETL QFGHFSSASD VWSFGIIMWE VMAFGERPYW
HsEphB1       ----SLGGKI PVRWTAPEAI AYRKFTSASD VWSYGIVMWE VMSFGERPYW
HsEphB2       ----ALGGKI PIRWTAPEAI QYRKFTSASD VWSYGIVMWE VMSYGERPYW
HsEphB3       ----SLGGKI PIRWTAPEAI AYRKFTSASD VWSYGIVMWE VMSYGERPYW
HsEphB4       ----SLGGKI PIRWTAPEAI AFRKFTSASD AWSYGIVMWE VMSFGERPYW
HsEphB6       ----PQGPSC LLRWAAPEVI AHGKHTTSSD VWSFGILMWE VMSYGERPYW


              ....|....| ....|....| ....|....| ....|....| ....|....|
                    1360       1370       1380       1390       1400
CeEph         DWTNQKVISE VMIGYRLPPP MDCPMGLYRI AQWCWKMERH ERPTFTQLLA
DmEph         NWSNQDVIKS IEKGYRLPAP MDCPEALYQL MLDCWQKQRT HRPTFASIVS
SpEph         NWPNQDVIKA VEKGYRLPPP MECPEAIHQL MLDCWQRDRN HRPTFTTIVS
CiEph-a       EMSNQDVVQF VDSGYRLPPP CYCPRIIHSV MLECWKRDRT QRPNFSHLVT
CiEph-b       GMSNRDVMNA VETGYRLPAP MDCPQVQHKL MLDCWKKDRN QRPKFGQIVS
CiEph-c       DMSNEVVTEV LEDGYRLPSP EGCPTPVHSL MLKCWSYEPK RRPTLLEIIK
CiEph-d       GMSNQDVIHA VGTGYRLPAP MDCPQVQHEL MLDCWKKDRN ERPKFSQVVA
CiEph-e       DMSNKQVMES IQKGMRLPSP MNCPKPLHDL MLECWFAEVK DRPSFLQIFQ
CiEph-f       SLTNQQVLQA INDGYRLPAP EDCPSVLHQL MLECWHRDKN TRPTFEQVVT
XlEphA2       EMSNQEVMKA INEGFRLPAP MDCPSAIYQL MMQCWQQERN RRPKFPDIVS
XlEphA4       DMSNQDVIKA IEEGYRLPPP MDCPIALHQL MLDCWQKDRS DRPKFGQIVS
XlEphB1       TMSNQDVINA IEQDYRLPPP MDCPAALHQL MLDCWQKDRN SRPRLAEIVN
XlEphB2       YMTNQSAIHA IVQDYRLPPP MDCPNALHQL LLDCWQKDRN HRPKFGQNVN
XlEphB3       DMSNQDVINA IEQDYRLPPP MDCPSALHQL MLDCWLRDRN LRPKFSQIVS
XlEphB4       DMSNQDVINA IEQDYRLPAP PDCPTALHQL MLDCWQRDRA LRPRFADIVS
GgEphA1       NMTNQEVMKS LEDGYRLPPP VDCPSILYEL MKSCWSHDRM RRPHFQEIRA
GgEphA3       EMSFQDVIKA VDEGYRLPPP MDCPAALYQL MLDCWQKDRN NRPKFEQIVS
GgEphA4       DMSNQDVIKA IEEGYRLPPP MDCPIALHQL MLDCWQKERS DRPKFGQIVN
GgEphA5       EMTNQDVIKA VEEGYRLPSP MDCPAALYQL MLDCWQKDRN SRPKFDEIVS
GgEphA6       EMSNQDVILS IEEGYRLPAP MGCPASLHQL MLHCWQKERN HRPKFSDIVS
GgEphA7       DMSNQDVIKA IEEGYRLPAP MDCPAGLHQL MLDCWQKERG ERPKFEQIVG
GgEphA8       NMTNRDVINS VEEGYRLPAP MGCPTALHQL MLDCWQKDRS ERPRFSQIVG
GgEphB1       DMSNQDVINA IEQDYRLPPP MDCPAALHQL MLDCWQKDRN TRPRLAEIVN
GgEphB2       DMTNQDVINA IEQDYRLPPP MDCPNALHQL MLDCWQKDRN HRPKFGQIVN
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GgEphB3         DMSNQDVINA VEQDYRLPPP MDCPTALHQL MLDCWVRDRN LRPKFAQIVN
GgEphB6         DMSNQDVINA IDQDYRLPPP PDCPTVLHLL MLDCWQKDRV QRPKFEQIVS
HsEphA1         EMSNQEVMKS IEDGYRLPPP VDCPAPLYEL MKNCWAYDRA RRPHFQKLQA
HsEphA2         ELSNHEVMKA INDGFRLPTP MDCPSAIYQL MMQCWQQERA RRPKFADIVS
HsEphA3         EMSNQDVIKA VDEGYRLPPP MDCPAALYQL MLDCWQKDRN NRPKFEQIVS
HsEphA4         DMSNQDVIKA IEEGYRLPPP MDCPIALHQL MLDCWQKERS DRPKFGQIVN
HsEphA5         EMTNQDVIKA VEEGYRLPSP MDCPAALYQL MLDCWQKERN SRPKFDEIVN
HsEphA6         EMSNQDVILS IEEGYRLPAP MGCPASLHQL MLHCWQKERN HRPKFTDIVS
HsEphA7         DMSNQDVIKA IEEGYRLPAP MDCPAGLHQL MLDCWQKERA ERPKFEQIVG
HsEphA8         NMTNRDVISS VEEGYRLPAP MGCPHALHQL MLDCWHKDRA QRPRFSQIVS
HsEphA10        DMSGQDVIKA VEDGFRLPPP RNCPNLLHRL MLDCWQKDPG ERPRFSQIHS
HsEphB1         DMSNQDVINA IEQDYRLPPP MDCPAALHQL MLDCWQKDRN SRPRFAEIVN
HsEphB2         DMTNQDVINA IEQDYRLPPP MDCPSALHQL MLDCWQKDRN HRPKFGQIVN
HsEphB3         DMSNQDVINA VEQDYRLPPP MDCPTALHQL MLDCWVRDRN LRPKFSQIVN
HsEphB4         DMSNQDVINA IEQDYRLPPP PDCPTSLHQL MLDCWQKDRN ARPRFPQVVS
HsEphB6         DMSEQEVLNA IEQEFRLPPP PGCPPGLHLL MLDTWQKDRA RRPHFDQLVA


                ....|....| ....|....| ....|....| ....|....| ....|....|
                      1410       1420       1430       1440       1450
CeEph           TFHKYILQPT LIEHDPGELP RRVQ------ ---------- ----------
DmEph           TLDNLARQPQ SLLTTRPSPE SDGN------ ---------- ----------
SpEph           TLDRLIRNPN ALRPLAKNGS PLG------- ---------- ----------
CiEph-a         LLDRFLLEAD CLTDTINNRP FR-------- ---------- ----------
CiEph-b         SLDKMLRDPS QLKAKANEEG SRPVGGNTAL RTAPPQFTAN GHANRRSHTI
CiEph-c         TLDHFIKQPS SLQDDMEADA SAP------- ---------- ----------
CiEph-d         TLDRMIREPE QLKVIAGHDK NR----NGCL LNE------R GSLRRPSFQG
CiEph-e         KLNRLLR--D WVEQPVASVA SKY------- ---------- ----------
CiEph-f         RLDAMINQRG VLDTLASPAS TGED------ ---------- ----------
XlEphA2         ILDKLIRAPD SLK-TLADFD PRIS------ ---------- ----------
XlEphA4         MLDKLIRNPN SLKRTGLENS SRTN------ ---------- ----------
XlEphB1         TLRPMIRNPA SLK-TVATIP AVPS------ ---------- ----------
XlEphB2         TLDKMIRNPN TIK-AVAPLS SGVT------ ---------- ----------
XlEphB3         SLDKLIRNAA SLK-VTSPGQ AGVS------ ---------- ----------
XlEphB4         SLDKLIRNPA SLK-ITTREQ LGTS------ ---------- ----------
GgEphA1         QLQHFSSSPH LLR-CVADFD PRVT------ ---------- ----------
GgEphA3         ILDKLIRNPS SLK-IITNAA ARPS------ ---------- ----------
GgEphA4         MLDKLIRNPN SLKRTGSE-S SRPS------ ---------- ----------
GgEphA5         MLDKLIRNPS SLK-TLVNAS SRVS------ ---------- ----------
GgEphA6         FLDKLIRNPS TLH-TLVEDV LVMP------ ---------- ----------
GgEphA7         ILDKMIRNPN SLK-TPLGTC SRPI------ ---------- ----------
GgEphA8         ILDKLIRNPD NLK--CTATV SRFT------ ---------- ----------
GgEphB1         TLDKMIRNPA SLK-TVATIT AVPS------ ---------- ----------
GgEphB2         TLDKMIRNPN SLK-AMAPLS SGVN------ ---------- ----------
GgEphB3         TLDKLIRNAA SLK-VIASVQ SGVS------ ---------- ----------
GgEphB6         ALDKMIRKPS ALK-ATGTGS SRPS------ ---------- ----------
HsEphA1         HLEQLLANPH SLR-TIANFD PRVT------ ---------- ----------
HsEphA2         ILDKLIRAPD SLK-TLADFD PRVS------ ---------- ----------
HsEphA3         ILDKLIRNPG SLK-IITSAA ARPS------ ---------- ----------
HsEphA4         MLDKLIRNPN SLKRTGTE-S SRPN------ ---------- ----------
HsEphA5         MLDKLIRNPS SLK-TLVNAS CRVS------ ---------- ----------
HsEphA6         FLDKLIRNPS ALH-TLVEDI LVMP------ ---------- ----------
HsEphA7         ILDKMIRNPN SLK-TPLGTC SRPI------ ---------- ----------
HsEphA8         VLDALIRSPE SLR--ATATV SRCP------ ---------- ----------
HsEphA10        ILSKMVQDPE PPK-CALTTC PRPP------ ---------- ----------
HsEphB1         TLDKMIRNPA SLK-TVATIT AVPS------ ---------- ----------
HsEphB2         TLDKMIRNPN SLK-AMAPLS SGIN------ ---------- ----------
HsEphB3         TLDKLIRNAA SLK-VIASAQ SGMS------ ---------- ----------
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HsEphB4     ALDKMIRNPA SLK-IVAREN GGAS------ ---------- ----------
HsEphB6     AFDKMIRKPD TLQ-AGGDPG ERPS------ ---------- ----------


            ....|....| ....|....| ....|....| ....|....| ....|....|
                   1460       1470       1480       1490       1500
CeEph       ----SQSALN TYGSVNVGVV PTPPSSAAPM PSLDDFLRQI GLNHVYGQLV
DmEph       ---------- -HILDGQRGQ NIFI------ -STDLWLEHI KMSRYCHHFK
SpEph       ---------- --LSSSLNDL SRFN------ -SVSDWLDSL KMGRYKDSFT
CiEph-a     ---------- -DQDDCIPEM LDLS------ -SLGQWLEQM HMLKYKQTMM
CiEph-b     TNQAPDSGYI EETDEEVPLV TSNGDNEPRH TELDVWLERR GFSQFKDNFI
CiEph-c     ---------- LLKPDSPNSI QDVS------ -TLDEWLDMV KLGRYRRSFH
CiEph-d     PNGISET-MT LLMDDRTRCT SLLRHQVP-- SSLGVWLDGL GLGNYKENFV
CiEph-e     ---------- VERP-TPRLH RDIT------ -NVQQWLDKL CMSEYSETFN
CiEph-f     ---------V LFFTDSPPHT PCP------- --VPTVTDGQ SHHNYATCRI
XlEphA2     ---------I RLPSTSGSEG MPFR------ -TIAEWLDSI KMQQYTEFFM
XlEphA4     ---------T ALLDPSSPEW SQV------- ASVLDWLQAI KMERYKDNFT
XlEphB1     ---------Q PLLDRSIPDI SAFT------ -SVDDWLSAI KMGQYRDNFL
XlEphB2     ---------L PLLDRTLPAY TSLS------ -RGDEWLDAI KMAQYKESFA
XlEphB3     ---------Q QLLDRTVPDY TTFP------ -TVSDWLEAI KMGQYQENFL
XlEphB4     ---------Q PLLDQRTPHY SSFS------ -SVSEWLHAI KMGRYEDGFR
GgEphA1     ---------L RLPSCSGSDG IPYR------ -SIPEWLESI PMKRYISNFR
GgEphA3     ---------N LLLDQSNIDI SAF------- RTAGDWLNGF RTGQCKGIFT
GgEphA4     ---------T ALLDPSSPEF SAV------- VSVSDWLQAI KMERYKDNFT
GgEphA5     ---------N LLVEHSPVGS GAY------- RSVGEWLEAI KMGRYTEIFM
GgEphA6     ---------D SPGEVP--EF PLF------- VSVSDWLDSI KMGQYKSNFM
GgEphA7     ---------S PLLDQNTPDF TTF------- CSVGEWLQAI KMERYKDNFT
GgEphA8     ---------Q TPFNRTLLDF SS-------C LTVEDWLDSI RLGHYRDNFA
GgEphB1     ---------Q PLLDRSIPDF TAFT------ -SVEDWLSAV KMSQYRDNFL
GgEphB2     ---------L PLLDRTIPDY TSFN------ -TVDEWLDAI KMSQYKESFA
GgEphB3     ---------Q PLLDRTVPDY TTFT------ -TVGDWLDAI KMGRYKENFV
GgEphB6     ---------Q PLLSNSPPDF PSLS------ -NAHEWLDAI KMGRYKENFD
HsEphA1     ---------L RLPSLSGSDG IPYR------ -TVSEWLESI RMKRYILHFH
HsEphA2     ---------I RLPSTSGSEG VPFR------ -TVSEWLESI KMQQYTEHFM
HsEphA3     ---------N LLLDQSNVDI TTF------- RTTGDWLNGV WTAHCKEIFT
HsEphA4     ---------T ALLDPSSPEF SAV------- VSVGDWLQAI KMDRYKDNFT
HsEphA5     ---------N LLAEHSPLGS GAY------- RSVGEWLEAI KMGRYTEIFM
HsEphA6     ---------E SPGEVP--EY PLF------- VTVGDWLDSI KMGQYKNNFV
HsEphA7     ---------S PLLDQNTPDF TTF------- CSVGEWLQAI KMERYKDNFT
HsEphA8     ---------P PAFVRSCFDL RGGSGGGG-G LTVGDWLDSI RMGRYRDHFA
HsEphA10    ---------T PLADRAFSTF PSFG------ -SVGAWLEAL DLCRYKDSFA
HsEphB1     ---------Q PLLDRSIPDF TAFT------ -TVDDWLSAI KMVQYRDSFL
HsEphB2     ---------L PLLDRTIPDY TSFN------ -TVDEWLEAI KMGQYKESFA
HsEphB3     ---------Q PLLDRTVPDY TTFT------ -TVGDWLDAI KMGRYKESFV
HsEphB4     ---------H PLLDQRQPHY SAFG------ -SVGEWLRAI KMGRYEESFA
HsEphB6     ---------Q ALLTPVALDF PCLD------ -SPQAWLSAI GLECYQDNFS


            ....|....| ....|....| ....|....| ....|....| ....|....|
                   1510       1520       1530       1540       1550
CeEph       SNNIHSVSDL ANTSHLDLLA YGLMSAECST VRDGLNGRIS GSPPGSSGTI
DmEph       EANLINAQQI SRLTAQQLSD MGITLVGHQK KILHQARQLD TII-------
SpEph       AAGYVRLEDI ARLSQSDLPR LGVTLAGHQK KIMKGIHSIR AQLEQ-----
CiEph-a     RHGCVTAEQI IRLTASGLAH MGFSDMNDVN TVLREADLLR ---------Q
CiEph-b     RNGCNCVEQI QQLTHSDLDR MGITSAEDQD VLLSAIQSS- --SNVPDIPN
CiEph-c     NNGINDLESL AHISESELDR LGIASPSHRT RLQGGINTLR ---------Q
CiEph-d     SAGYSSLDQV LQMSPSDLAS LGIVETNQQN AVMESISSIR YKTNPGRKPP
CiEph-e     ENEIKNLHLV STLTASDLKA MGISNEDHKR VILDGVLALK ---------R
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CiEph-f       ADNDSPLGNL IRGETPDPAE VTEPYVESND LVDGSPSDDL LSHHS-----
XlEphA2       ASPYNSMDKI ILMNQEDIKH LGIRQTGHQK RIAFSILGLK EHAST-----
XlEphA4       AAGYTSLEAV VHVNQDDLTR IGISSPSHQN KILSSVQGMR TQLQQ-----
XlEphB1       SSGFTSLQLV AQMTSEDLLR IGITLAGHQK KILNSIQSMR VQITQ-----
XlEphB2       NAGFTSFDVI SQMCMEDILR VGVTLAGHQK KILNSLQVMR AQMNQ-----
XlEphB3       SAGFTSFHLV AQMTAEDLLR IGVTLAGHQK KLLNSVQDMR LQMSQ-----
XlEphB4       NAGFTTFSRV QNISTEDLLR MGVTLAGHQK KILSSLQQIL PSEKS-----
GgEphA1       TAGLDTMESI LDLTAEDLKQ MGVSLPGHQK RILCSIQGFK E---------
GgEphA3       GVEYSSCDTI AKISTDDMKK VGVTVVGPQK KIVSSIKTLE THTKN-----
GgEphA4       AAGYTTLEAV VHMNQDDLAR IGITAITHQN KILSSVQAMR SQMQQ-----
GgEphA5       ENGYSSMDSV AQVTLEDLRR LGVTLVGHQK KIMNSLQEMK VQLVN-----
GgEphA6       AAGFTTLDMV SRMSIDDIRR IGVVLIGHQR RIVSSLQTLR LHMMH-----
GgEphA7       AAGYNSLESV ARMTIEDVMS LGITLVGHQK KIMSSIQTMR AQMLH-----
GgEphA8       MAGYSSLGMV MSMNIEDVRS LGITMMGHQK KILSSIQAMR SQLLN-----
GgEphB1       SAGFTSLQLV AQMTSEDLLR IGVTLAGHQK KILNSIQSMR VQMSQ-----
GgEphB2       SAGFTTFDIV SQMTVEDILR VGVTLAGHQK KILNSIQVMR AQMNQ-----
GgEphB3       NAGFASFDLV AQMTAEDLLR IGVTLAGHQK KILSSIQDMR LQMNQ-----
GgEphB6       QAGLITFDVI SRMTLEDLQR IGITLVGHQK KILNSIQLMK VHLNQ-----
HsEphA1       SAGLDTMECV LELTAEDLTQ MGITLPGHQK RILCSIQGFK D---------
HsEphA2       AAGYTAIEKV VQMTNDDIKR IGVRLPGHQK RIAYSLLGLK DQVNT-----
HsEphA3       GVEYSSCDTI AKISTDDMKK VGVTVVGPQK KIISSIKALE TQSKN-----
HsEphA4       AAGYTTLEAV VHVNQEDLAR IGITAITHQN KILSSVQAMR TQMQQ-----
HsEphA5       ENGYSSMDAV AQVTLEDLRR LGVTLVGHQK KIMNSLQEMK VQLVN-----
HsEphA6       AAGFTTFDLI SRMSIDDIRR IGVILIGHQR RIVSSIQTLR LHMMH-----
HsEphA7       AAGYNSLESV ARMTIEDVMS LGITLVGHQK KIMSSIQTMR AQMLH-----
HsEphA8       AGGYSSLGMV LRMNAQDVRA LGITLMGHQK KILGSIQTMR AQLTS-----
HsEphA10      AAGYGSLEAV AEMTAQDLVS LGISLAEHRE ALLSGISALQ ARVLQ-----
HsEphB1       TAGFTSLQLV TQMTSEDLLR IGITLAGHQK KILNSIHSMR VQISQ-----
HsEphB2       NAGFTSFDVV SQMMMEDILR VGVTLAGHQK KILNSIQVMR AQMNQ-----
HsEphB3       SAGFASFDLV AQMTAEDLLR IGVTLAGHQK KILSSIQDMR LQMNQ-----
HsEphB4       AAGFGSFELV SQISAEDLLR IGVTLAGHQK KILASVQHMK SQAKPGTPGG
HsEphB6       KFGLCTFSDV AQLSLEDLPA LGITLAGHQK KLLHHIQLLQ QHLRQ-----


              ....|....| ....|....| ....|....| ....|....| ....|....|
                    1560       1570       1580       1590       1600
CeEph         HATTRGTRTT RPPREEGFFV ---------- ---------- ----------
DmEph         ---------- ---------- ---------- ---------- ----------
SpEph         -AAETLV--- ---------- ---------- ---------- ----------
CiEph-a       KIEEVGNVGV AV-------- ---------- ---------- ----------
CiEph-b       YGEACRTAAI SV-------- ---------- ---------- ----------
CiEph-c       HLVEVNEVQS SSNAPYDAHA STLPIAHGKP SNPVAV---- ----------
CiEph-d       QYRDERITGI PV-------- ---------- ---------- ----------
CiEph-e       RFAQEV---- ---------- ---------- ---------- ----------
CiEph-f       -SYEVSTM-- ---------- ---------- ---------- ----------
XlEphA2       MGIPI----- ---------- ---------- ---------- ----------
XlEphA4       MQGRMVPV-- ---------- ---------- ---------- ----------
XlEphB1       -SPTSIA--- ---------- ---------- ---------- ----------
XlEphB2       -IQSVEV--- ---------- ---------- ---------- ----------
XlEphB3       -TLPVQV--- ---------- ---------- ---------- ----------
XlEphB4       TPIPDHCY-- ---------- ---------- ---------- ----------
GgEphA1       ---------- ---------- ---------- ---------- ----------
GgEphA3       SPVPV----- ---------- ---------- ---------- ----------
GgEphA4       MHGRMVPV-- ---------- ---------- ---------- ----------
GgEphA5       GMVPL----- ---------- ---------- ---------- ----------
GgEphA6       IQEKGFHTTY TDVLGYVSKL LHEICAARNW DTSACLQAGE VARRSTCMSH
GgEphA7       LHGTGIQV-- ---------- ---------- ---------- ----------
GgEphA8       TNGPRRHL-- ---------- ---------- ---------- ----------
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GgEphB1         -SPTSMA--- ---------- ---------- ---------- ----------
GgEphB2         -IQSVEV--- ---------- ---------- ---------- ----------
GgEphB3         -TLPVQV--- ---------- ---------- ---------- ----------
GgEphB6         -LEPVEV--- ---------- ---------- ---------- ----------
HsEphA1         ---------- ---------- ---------- ---------- ----------
HsEphA2         VGIPI----- ---------- ---------- ---------- ----------
HsEphA3         GPVPV----- ---------- ---------- ---------- ----------
HsEphA4         MHGRMVPV-- ---------- ---------- ---------- ----------
HsEphA5         GMVPL----- ---------- ---------- ---------- ----------
HsEphA6         IQEKGFHV-- ---------- ---------- ---------- ----------
HsEphA7         LHGTGIQV-- ---------- ---------- ---------- ----------
HsEphA8         TQGPRRHL-- ---------- ---------- ---------- ----------
HsEphA10        LQGQGVQV-- ---------- ---------- ---------- ----------
HsEphB1         -SPTAMA--- ---------- ---------- ---------- ----------
HsEphB2         -IQSVEV--- ---------- ---------- ---------- ----------
HsEphB3         -TLPVQV--- ---------- ---------- ---------- ----------
HsEphB4         TGGPAPQY-- ---------- ---------- ---------- ----------
HsEphB6         -QGSVEV--- ---------- ---------- ---------- ----------


                ....|....| ....|....| ....|....
                        1610      1620
CeEph           ---------- ---------- ---------
DmEph           ---------- ---------- ---------
SpEph           ---------- ---------- ---------
CiEph-a         ---------- ---------- ---------
CiEph-b         ---------- ---------- ---------
CiEph-c         ---------- ---------- ---------
CiEph-d         ---------- ---------- ---------
CiEph-e         ---------- ---------- ---------
CiEph-f         ---------- ---------- ---------
XlEphA2         ---------- ---------- ---------
XlEphA4         ---------- ---------- ---------
XlEphB1         ---------- ---------- ---------
XlEphB2         ---------- ---------- ---------
XlEphB3         ---------- ---------- ---------
XlEphB4         ---------- ---------- ---------
GgEphA1         ---------- ---------- ---------
GgEphA3         ---------- ---------- ---------
GgEphA4         ---------- ---------- ---------
GgEphA5         ---------- ---------- ---------
GgEphA6         LVNQIVFPDS QKGYRCILVA KMEKQLGKD
GgEphA7         ---------- ---------- ---------
GgEphA8         ---------- ---------- ---------
GgEphB1         ---------- ---------- ---------
GgEphB2         ---------- ---------- ---------
GgEphB3         ---------- ---------- ---------
GgEphB6         ---------- ---------- ---------
HsEphA1         ---------- ---------- ---------
HsEphA2         ---------- ---------- ---------
HsEphA3         ---------- ---------- ---------
HsEphA4         ---------- ---------- ---------
HsEphA5         ---------- ---------- ---------
HsEphA6         ---------- ---------- ---------
HsEphA7         ---------- ---------- ---------
HsEphA8         ---------- ---------- ---------
HsEphA10        ---------- ---------- ---------
HsEphB1         ---------- ---------- ---------
```

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HsEphB2         ---------- ---------- ---------
HsEphB3         ---------- ---------- ---------
HsEphB4         ---------- ---------- ---------
HsEphB6         ---------- ---------- ---------
```