Activated EphA4 receptor in *Xenopus laevis* acts through src-like tyrosine kinase p59fyn to inhibit RhoA GTPase

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ACTIVATED EphA4 RECEPTOR IN XENOPUS LAEVIS ACTS THROUGH Src-LIKE TYROSINE KINASE p59fyn TO INHIBIT RhoA GTPase

by

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ABSTRACT

Eph receptors and their ligands, ephrins, have been implicated in many important developmental processes and in tumorigenesis. The activation of EphA4 receptors in the frog, *Xenopus laevis*, causes loss of cell adhesion, tight junctions, and stress fibers. Previous studies revealed that these receptors achieve their cellular effects at least in part by inhibiting RhoA GTPase. However, what links these GTPases with the EphA4 receptor remained unknown.

In an attempt to investigate involvement of the Src-like tyrosine kinase p59fyn in EphA4 signaling, a series of experiments was planned using the chimeric receptor EPP. The findings presented in this thesis indicate that the Src-like tyrosine kinase, p59fyn, acts as a functional link between the EphA4 receptor and RhoA GTPase in *Xenopus laevis* embryonic cells. Moreover, an activated EphA4 receptor achieves its cellular effects through activation of p59fyn, which in turn inhibits RhoA.
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INTRODUCTION AND BACKGROUND

Each multicellular organism starts off as a single cell, and then, the progeny of that cell form complex structures such as tissues and organs. Organs are composed of different types of tissues that are arranged in a precise manner. The coordination of cells to form organs and the organism is due to the receptors on the embryonic cell surfaces that respond to inducer proteins, ligands, initiating a cascade of interactive proteins that transmit the signal from receptor to the nucleus (Gilbert 2000). Receptor tyrosine kinases are important components of signal transduction pathways that control cell shape, proliferation, differentiation, and migration. This family is divided into 14 subfamilies, including the Eph family, the largest subfamily of receptor tyrosine kinases. Based on sequence homology, the Eph family is further divided in two subclasses: EphA and EphB (Knoll and Dresher 2002). The ligands for the Eph receptors are membrane-bound molecules, ephrins A and ephrins B (Flanagan and Vanderhaeghen 1998). Eph receptors and their ligands are dynamically expressed during the development of various vertebrate species, and their functions are diverse. Interactions between Eph receptors and ephrins have been implicated in many vital processes of embryonic development. They play important roles in body segmentation by segmental patterning of the hindbrain (the neural tube) and the paraxial mesoderm (Holder and Klein 1999). Further, Eph receptors and their ligands are also involved in neuronal network formation by guiding axons to follow the correct pathway, reach the target region, and within this target region, find and recognize the correct synaptic partner (Nakamoto 2000). Other evidence suggests that Eph
receptors together with their ligands control blood vessel formation (Dodelet and Pasquale 2000). The experiments performed by several groups (Adams and others 1999; Gerety and others 1999) showed that both mice lacking ephrin-B2 and a number of double mutants deficient in EphB2 and EphB3 display severe defects in the remodeling of the embryonic vascular system. In addition, Eph signaling is involved in controlling the migration of neural crest cells along particular routes to reach the locations of their differentiation and regulating cell migration in embryos during gastrulation (Holder and Klein 1999). Interrupting normal signaling of EphA4 and ephrin-B1 in Xenopus can lead to abnormal adhesion of blastomeres during gastrulation, leading to the dissociation of blastomeres and to the abortion of gastrulation (Jones and others 1998; Winning and others 1996). Overexpression of dominant negative forms of both receptor and ligand (ephrin) causes blastomeres to dissociate and gastrulation to abort. The crucial roles of Eph receptors and ephrins during development suggest that disruption of their normal functions can cause congenital disorders affecting the nervous system and other tissues. It has also been suggested that Eph receptors and ephrins may be involved in carcinogenesis. The overexpression of several Eph receptors of both the A and B classes have been observed in various tumor types, such as malignant melanoma, colon cancer, gastric tumors, and human breast carcinomas (Dodelet and Pasquale 2000). Moreover, Eph receptors may affect cell adhesion and migration, which is involved in tumor invasion and metastasis.
Structure of Eph receptor

The Eph receptors are transmembrane proteins with an extracellular ligand-binding domain, a transmembrane domain, and an intracellular catalytic domain. The intracellular side is further subdivided into four separate parts, each with its own signaling potential: a juxtamembrane region, a conserved kinase domain, a sterile-_-motif (SAM) domain, and a PSD95/Dlg/ZO1 (PDZ)-binding motif (Kalo and Pasquale 1999). The juxtamembrane region contains two conserved tyrosine residues, which are the major autophosphorylation sites, and when phosphorylated, they become docking sites for multiple SH-2 domain (Src homology 2 domain) containing effectors (Bruckner and Klein 1998). The SH-2 domain is found in many intracellular signaling proteins including Src-like family kinases, in which these domains were first discovered (Janeway and others 2001). The other part of the intracellular domain, the tyrosine kinase domain, provides catalytic activity of the receptor and may also be a binding site for adaptor proteins. The SAM domain is a potential dimerization domain, which upon phosphorylation interacts with a distinct set of proteins (Smalla and others 1999). Finally, the PDZ binding motif plays an important role in recruiting several PDZ domain-containing proteins, which may have roles in targeting Eph receptors to subcellular sites, in receptor clustering and signaling (Bruckner and Klein 1998).

Role of Eph receptor in bidirectional signaling

An interesting feature of Eph receptors is their involvement in bidirectional
signaling (Holland and others 1996; Kullander and Klein 2002). Both Eph receptors and their ligands ephrins are membrane-bound, and their interactions at sites of cell-to-cell contact initiate signal transduction not only downstream of Eph receptors into receptor-bearing cells but also downstream of the ephrin ligands into the ephrin-bearing cells. The first step in the initiation of Eph-mediated signaling is the recognition and binding of Eph receptors and ephrins and formation of high-affinity heterodimers. Crystallographic studies of the EphB2 and EphrinB2 complex (Himanen and others 2001) revealed the formation of a tetrameric complex where each receptor interacted with two ligands and each ligand interacted with two receptors. The dimerization allows the EphB2 receptor kinase domains to phosphorylate each other and then to initiate forward signaling by phosphorylating other intracellular molecules (Schmucker and Zipursky 2001). At the same time, the B-ephrin transmembrane and cytoplasmic domains are converted into an active configuration, and cytoplasmic tails then are phosphorylated by Src-family tyrosine kinase (Palmer and others 2002). As a result, a reverse signal is transduced. Other evidence shows that the glycosylphosphatidylinositol (GPI) anchored A-ephrins also transduce reverse signals (Knoll and others 2001) as transmembrane tethered B-ephrins do. Interestingly, the heterodimers of Eph receptors and ephrins are further arranged into clusters observed in vivo at the sites of cell-to-cell contact. It is likely that receptor ligand tetramers are necessary for receptor phosphorylation, but the formation of the clusters is essential for physiological signaling. Moreover, the size of these aggregates may define the nature of the signal (Boyd and
A few Eph and ephrin regions could be involved in the clustering process, but the Eph SAM domain is of particular interest because this conserved region is present in all Eph receptors. It also known that SAM domain mediates protein–protein interactions, including oligomerization, in many other receptors (Behlke and others 2001).

Involvement of Eph receptor in both repulsion and attraction

The Eph receptors and their ligands ephrins have been described as repulsive cues involved in the guidance of axonal growth, cell migration, boundary formation, body segmentation, and vasculogenesis (Dodelet and Pasquale 2000). However, recent studies suggest that some of the effects of Eph receptors and ephrins are the result of cellular attraction and adhesion but not repulsion (Holmberg and others 2000). Experiments performed with mice lacking ephrin-A5 revealed the importance of ephrin-A5-mediated repulsion in establishing neuronal connections. Interestingly, these mice also developed neural-tube defects that clearly were the result of a lack of adhesion. The development of such phenotype indicated that ephrin-A5 could mediate both cell repulsion as well as adhesion. The work of Holmberg and coworkers (2000) provided one possible explanation for how cells expressing the same sets of receptors respond differently to a particular stimulus. In these experiments mutant mice lacking either the EphA7 receptor or its ligand, ephrin-5, were used. The analysis of the neural fold cells at the site of the defect, showed the presence of three different splice forms of the EphA7—two truncated forms
lacking the intracellular tyrosine kinase domain and one full-length receptor—in a single cell. To investigate further, cell lines expressing only the full-length form of a receptor or coexpressing the full-length and truncated splice forms of a receptor, EphA7-T1, were generated. Cells expressing the ligand, ephrin-A5, repelled cells expressing only the full-length receptor. However, cells coexpressing both the full-length and truncated forms of the receptor exhibited significant adhesion to ephrin-A5-expressing cells. This study demonstrated that different splice forms of an Eph receptor could determine how the cell will respond to the ligand, with either repulsion or attraction. Another research paper (Stein and others 1998) reported that the degree of ephrin oligomerization might also influence the response of receptor-expressing cells. Results presented by this research group show that tetramers of ephrins increased the attraction and/or adhesion but dimers of ephrins induced repulsion. Another example of attraction and/or adhesion between Eph or ephrin-expressing cells is the vomeronasal axon pathfinding in the accessory olfactory bulb, where axons expressing large amounts of A-ephrins specifically project to regions of increased EphA receptor expression (Knoll and others 2001). The hypothesis is that axonally localized EphAs and ephrin-As exercise opposite functions; activation of EphAs leads to repulsion, and activation of Ephrin-As leads to increased attraction of growth cones (Knoll and Drescher 2002). In short, increasing evidence points out that the Eph subfamily is involved in both repulsive and attractive guidance mechanisms, at least during the establishment of neuronal connections.
Regulation of cellular adhesion by Eph receptors

In spite of the significance of Eph receptors in embryogenesis as well as in tumorigenesis, the molecular mechanisms underlying the specific roles of Eph receptors are not well understood. However, recently the ability of Eph receptors to affect cell-matrix attachment by altering integrin activity was highlighted (Miao and others 2000). Eph receptors may also influence cell–cell attachment by interacting with other adhesion molecules, cadherins (Orsulic and Kemler 2000). Indeed, the ectopic expression of EphA4 in early Xenopus embryos disrupted cadherin-dependent cell adhesion (Winning and others 1996). Even though cell adhesion is a crucial aspect of cell motility, other aspects are no less important. The general process of cell guidance during migration also involves reorganization of the cytoskeleton to allow for changes in the cell morphology during movement. Analysis by scanning electron microscopy (SEM) of embryonic cells of Xenopus expressing the activated EphA4 receptor has revealed that in addition to loss of adhesion, the cells change their shape and lose their polarity (Winning and others 2001).

Rho family GTPases

The small GTPases of the Rho family have been implicated in a variety of processes linking signaling pathways to the actin cytoskeleton (Dickson 2001). Like other small monomeric GTPases, the Rho family cycles between an active state (GTP bound) and an inactive state (GDP bound). Rho, Cdc42, and Rac are three of the best-characterized members of this family. Constitutively activated
mutants of Rho and Rac were found to induce the assembly of contractile actin
and myosin filaments (stress fibers) and actin-rich surface protrusions
(lamellipodia), respectively, when introduced into fibroblasts (Ridley and Hall
1992a; Ridley and others 1992b). Cdc42 was shown to promote the formation of
actin-rich filopodia (Nobes and Hall 1995). Rho GTPases are also able to
influence cell polarity by participating in establishment and promotion of tight and
adherens junctions (Van Aelst and Symons 2002; Rojas and others 2001). The
importance of the Rho family of GTPases in regulating cell motility and axon
guidance is also well established (Luo 2000). Recently, a link between Eph
receptors and these GTPases has been established. The inhibition of RhoA by
EphA4 in early *Xenopus* embryos disrupted cell adhesion and caused cellular
dissociation (Winning and others 2002). On the other hand, Rho activation by
ephrinA in mammalian axonal growth cones caused a loss of cell adhesion and
growth cone collapse (Wahl and others 2000). Thus, Rho protein activation or
inactivation by Eph receptors may contribute to cell migration during embryonic
development as well as during tumor invasion and metastasis.

*Signaling downstream of Eph receptor*

Nevertheless, the intracellular signaling mechanisms that transduce
signals into changes in axon growth cone or in cell motility are poorly understood.
The signal transduction events triggered by activated Eph receptors are very
complex. The receptors cytoplasmic domain is subdivided into four separate
entities, each with its own signaling potential (Kullander and others 2001). But
recently, light was shed on the molecular pathway involved in ephrin/Eph signaling when Shamah and coworkers (Shamah and others 2001) identified the protein ephexin acting in neuronal cells as a direct link between EphA4 receptors and Rho GTPases. When EphA4 receptors are stimulated, ephexin activates RhoA and growth cone collapse is induced. However, other data (Winning and others 2002) suggest that in *Xenopus* embryos EphA4 manifests its downstream effects through inhibition of RhoA and via an ephexin-independent pathway. The contradiction observed here might represent a difference in EphA4 functions in neuronal and non-neuronal cells. Several other cytoplasmic proteins that might interact with activated Eph receptors also have been identified. These include proteins containing SH2 domains such as Src-like tyrosine kinases p59fyn and p60src, which bind to conserved tyrosine phosphorylation sites in the juxtamembrane region of EphA4 (Ellis and others 1996) and EphA8 (Choi and Park 1999) and also to EphB2 (Zisch and others 1998). The experiments functionally linking these kinases with Eph receptors have yet to be done.

**EphA4 signaling in Xenopus laevis**

The EphA4 receptor has a specific pattern of expression during *Xenopus laevis* embryogenesis. Transcripts encoding the receptor can be detected in the forebrain, rombomere r3 and r5 of the hindbrain, in visceral arch 3, in mesoderm during gastrulation, in the otic vesicle and in the pronephros (Winning and others 1996). To investigate the developmental role of the signaling through the EphA4 receptor in *Xenopus laevis*, a chimeric form of the EphA4 receptor was created.
by Winning and his colleagues (1996). This chimeric molecule, named EPP, consists of the extracellular domain (ligand-binding) of human epidermal growth factor receptor (EGFR) joined to the transmembrane and intracellular (catalytic) domains of EphA4. Therefore, it can be activated by the ligands of EGFR, epidermal growth factor (EGF), and transforming growth factor alpha (TGF-α), whereas the cellular effects are activated by the EphA4 intracellular domain. In order to observe the effects of activated EPP in blastulae, the ectopic expression of this receptor was induced by injection of the RNA-encoding EPP and its ligand into fertilized eggs (one-cell stage) of *Xenopus laevis* and allowing the embryos to develop until blastula stage. By analyzing these effects of EPP on embryonic morphology, the function of EphA4 during normal development could be postulated. EPP expression and activation in blastula stage *Xenopus* embryos resulted in cellular dissociation of animal pole cells, and as a consequence, occlusion of blastocoels by dissociated cells (Winning and others 1996). Analysis of affected cells by SEM revealed other manifestations of this so-called “EPP phenotype”, such as change in cell shape, loss of cell polarity, and apical modifications, microvilli. This suggests involvement of the actin cytoskeleton in EphA4 signaling (Winning and others 2001), as both the loss and gain of epithelial morphology must involve actin reorganization. The molecular mechanisms that link EphA4 to the actin cytoskeleton in *Xenopus* cells remain unclear, yet recent evidence suggests that one mechanism by which EphA4 manifests downstream effects in *Xenopus* embryos is through inhibition of RhoA GTPase (Winning and others 2002). The other signaling molecules that interact
with activated receptors have been difficult to identify, but they may involve the Src-like tyrosine kinase p59fyn (Ellis and others 1996). The purpose of the work presented here is to link p59fyn with the EphA4 receptor as a downstream element in its signaling pathway. Therefore, the following hypothesis was proposed.

**Hypothesis:** Activated EphA4 receptor in *Xenopus laevis* embryonic cells inhibits RhoA GTPase through activation of the Src-like tyrosine kinase p59fyn.

To test this hypothesis part-by-part, three sets of experiments were designed by using the previously described experimental model and the construct of the chimeric receptor, EPP (Winning and others 1996). In this study I show that either activated p59fyn or activated EphA4 receptor produce the same cell phenotype, the EPP phenotype. I also demonstrate that activated EphA4 achieves its cellular effects through activation of p59fyn because dominant negative p59fyn was able to rescue embryos from the EPP phenotype. The data collected and presented in this work suggest that p59fyn acts as a link between EphA4 receptors and RhoA GTPases. This conclusion is consistent with the proposed hypothesis.
MATERIALS AND METHODS

In vitro transcription

RNA encoding the chimeric receptor EPP, its ligand TGF-β, and constitutively active (ca) *Xenopus* RhoA (V14XRhoA) were generously provided by R. S. Winning. The construction and synthesis of EPP and the synthesis of caRhoA RNA is described elsewhere (Winning and others 1996 and Winning and others 2002, respectively).

RNA encoding constitutively active (ca) *Xenopus* fyn (Xld-fyn) and dominantly negative (dn) *Xenopus* fyn (Theta-fyn MT) was obtained as follows. Plasmids containing the mentioned clones were purified from transformed DHS_bacteria, using the Wizard Plus SV Minipreps Purification system’s vacuum protocol, and linearized using *Eco* Rfl. The restriction digest was terminated and the DNA was purified by phenol/chloroform extraction. The DNA was then precipitated with isopropanol. The concentration of the DNA was quantified by ultraviolet (UV) absorbance spectrophotometry at 260 nm and confirmed by 1% agarose (Sigma) gel electrophoresis. Transcription was carried out using an Ambion mMessage mMachine SP6 transcription kit and incubated at 37 °C for 2 h to achieve maximum RNA yield. Afterwards, the DNA template was removed by incubating the reaction with DNase I at 37 °C for 15 min., and a poly(A)tail was added to the newly synthesized RNA, using an Ambion poly(A)tailing kit. Once again the reaction was incubated at 37 °C for 1 h. The RNA was recovered from the transcription mix by Trizol (Gibco BRL) and chloroform extraction, then precipitated in isopropanol, resuspended in RNAse-free water, filtered through a
Durotore 0.22-μm filter (Millipore), and stored at -70 °C. The RNA concentration was measured by UV absorbance spectrophotometry at 260 nm and confirmed by denaturing 1% agarose gel electrophoresis, using a Northern Max Gly kit (Ambion) running buffer and agarose.

**Frog handling and fertilization**

Adult *Xenopus laevis* were purchased from Xenopus I (Dexter, MI). Approximately 12 hours before eggs were needed, female frogs were injected with 800 units of human chorionic gonadotropin (Sigma) per 100 g of frog weight in the dorsal lymph sac by a subcutaneous injection on one side of the midline of the lower back. Injected females were kept overnight at 18 °C in a constant-temperature room. Twelve hours later eggs were obtained by gently squeezing the females on the dorsal side from the midpoint toward the ovipositor. The eggs were deposited directly into a 60-mm Petri dish containing 0.3 X Marc’s Modified Ringer’s (MMR) solution. 1 X MMR contains 100 mM NaCl, 2.0 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES pH 7.4. A male frog was anaesthetized in 0.5% ethyl-m aminobenzoic acid (Sigma) for 20 min. Symmetrical incisions were made on the ventral side just above the legs. The fat bodies were pulled through the openings and attached testes were exposed. Testes were removed from the body by cutting them from the fat bodies, and then the separated testes were placed in cold 1 X MMR in a 25-mm Petri dish on ice. To fertilize the eggs, one testis was macerated with a blunt forceps in a small dish containing 400 μl of 1 X MMR in order to release the sperm into solution. This sperm suspension was
added to the eggs. Then the eggs were fertilized for 20 min. on a shaker. When the eggs were fertilized, they rotated so that the animal poles (darkly pigmented side) were oriented upward. Fertilized eggs were dejellied by treating them with 2% L-cysteine (Sigma), pH 7.8-8.1 for 10 min., rinsed five times in distilled water, and then transferred to 0.1 X MMR containing Petri dish.

**Microinjection**

Injections were carried out using a Narishige IM300 microinjector. Needles for injection were pulled from 9-cm-long glass capillaries (Drummond), using a Narishige (model # PP-83) needle puller. For calibration purposes, 1-mm marks were placed along the outside of the needle, using an extra-fine marker. The end of the needle was polished for 2 min. at a speed of about 30-40 and angle setting of 27.5° on a needle grinder (Narishige, Model # EG-44). For calibration, the needle was placed in the needle holder on the micromanipulator of the microinjector, and the tip of the needle was positioned in the liquid to be injected. The needle was filled with the liquid until the sixth mark, and then the time needed for the liquid to travel 5 mm in the needle was measured. The volume of the needle contained within 1mm of the length was also measured. Finally, the time it would take to inject 10 nl or 20 nl of the liquid was calculated.

To prevent leakage of the cytoplasm through the injection wound, one-cell-stage embryos were placed into an injection dish containing 5% Ficoll (Sigma) in 1 X MMR and injected into animal poles with nuclease-free water or RNA, in 10-nl or 20-nl volumes. The RNAs injected consisted of 500 pg of EPP,
500 pg of TGF-_, 500 pg ca RhoA, 100 pg, 250 pg, 500 pg ca p59fyn, and 5 ng and 7 ng of dn p59fyn in different combinations. After injection, the embryos were transferred to a fresh dish containing 5% Ficoll in 1 X MMR and allowed to develop until the 32-cell stage. At this stage, the embryos were transferred into 1 X MMR lacking Ficoll and permitted to develop to mid-blastula stage. Embryos were staged according to Nieuwkoop and Faber (1994).

Light microscopy

After development to mid-blastula stage, when the effects of receptor activity become apparent, the embryos were fixed overnight at 4 °C in 2.5% glutaraldehyde (Sigma) in 1X Phosphate-buffered saline (PBS). After being washed three times in PBS, the embryos were bisected with a scalpel blade and examined under a dissecting microscope for any change in blastocoel and cellular morphology.

Scanning electron microscopy

After analysis by light microscopy, bisected embryos were postfixed in buffered 1% osmium tetroxide (Polysciences, Inc.) for 30 min. in a hood, washed twice in distilled water for 10 min. each time, then dehydrated through a graded series of ethanol to three changes of 100% for 10 min. each. An agitator was used for all of the aforementioned steps. Next, the embryos were treated with hexamethyldisalane (HMDS) twice for 2 min. each and a third time for 3 min. For these incubations just enough HMDS was added to cover the specimens and no
agitation was applied. After the HMDS treatment, the specimens were dried overnight in a fume hood. The dried specimens were positioned onto double-stick carbon-permeated tape attached to polished stubs, stabilized using colloidal graphite, and placed in a vacuum for one hour. They were then coated with gold and examined with an AMRay 1820I scanning electron microscope at 5 kV in order to better characterize the phenotype resulting from activation of the EphA4 receptor.
DATA ANALYSIS

Previous experiments (Winning and others 2002) revealed that activated EphA4 in *Xenopus laevis* manifests its downstream effects through inhibition of RhoA GTPase. However, what links these GTPases with the EphA4 receptor is unknown. In an attempt to investigate the possibility that the Src-like tyrosine kinase p59fyn could be this linking molecule, a series of experiments was planned using the chimeric receptor named EPP. This receptor consists of the extracellular (ligand-binding) domain of human epidermal growth factor receptor (EGFR) joined to the transmembrane and intracellular (catalytic) domains of EphA4 (Winning and others 1996). When activated by the EGFR ligand TGF-β, the catalytic domain of EPP initiates signaling cascade resulting in dissociation of animal pole cells and blastocoel occlusion by these cells (Winning and others, 1996). During normal development, a fertilized frog egg rapidly divides and, at the end of cleavage, takes the shape of a sphere known as a blastula. At this stage a fluid-filled cavity, the blastocoel, forms in the animal hemisphere (Figure 1A). When EPP and its ligand are introduced into one-cell-stage embryos of *Xenopus laevis*, normal embryonic development is aborted and a blastocoel is not present in blastula stage embryos (Figure 1B). Detailed analysis of affected cells by scanning electron microscopy (SEM) revealed other manifestations of this so-called “EPP phenotype”, such as change in cell shape, loss of cell polarity, and apical modifications, microvilli (Winning and others 2001). The EPP phenotype is easy to observe, so it became a useful assay tool for any changes induced in *Xenopus* embryos.
Figure 1  Scanning electron micrographs of blastula stage *Xenopus laevis* embryos. X120  **A.** Blastula stage embryo during normal development exhibits a well-formed blastocoel indicated by an *arrow*.  **B.** Embryos expressing activated EPP display an altered morphology (“EPP phenotype”); the blastocoel is not visible because rounded, dissociated cells occlude it. The *scale bar* represents 100 µm.
Constitutively active p59fyn causes cell dissociation in Xenopus blastulae

One prediction derived from the hypothesis is that if activated EphA4 acts through activation of Src-like tyrosine kinase p59fyn, then activation of either of them should stimulate the same changes in embryo and cell phenotypes. To test this, one-cell-stage embryos were injected with either 100, 250, or 500 pg of RNA encoding constitutively active (ca) Xenopus fyn. Control embryos were injected either with water or coinjected with EPP RNA and TGF-β RNA. After development to mid-blastula stage, when effects of receptor activity become apparent, the embryos were fixed in glutaraldehyde. Then the embryos were bisected, examined under a dissecting microscope for blastocoel morphology, and scored into one of three categories: “normal” blastocoel, “abnormal” blastocoel, and “no” blastocoel. Embryos with no cellular dissociation were identified as having a “normal” blastocoel; embryos that displayed some cellular dissociation to an extent insufficient to completely conceal the blastocoel were classified as having an “abnormal” blastocoel; embryos exhibiting the EPP phenotype with a blastocoel completely occluded with dissociated cells were referred to as having “no” blastocoel. The pooled results of three experiments are shown in Table 1; a comparison of the effects of injected RNAs is presented in Figure 2. More than 80% of the water-injected embryos exhibited a normal phenotype. In contrast, over 90% of the embryos coinjected with EPP and TGF-β RNAs developed some aspects of the EPP phenotype; almost 60% of these embryos had a completely occluded blastocoel. Ca Xenopus fyn (p59fyn) exhibited its effect on embryo development in a dose-dependent manner.
Injection of 100 pg of ca p59fyn RNA induced considerable cellular dissociation, and development of the EPP phenotype in 30% of the embryos, whereas injection of 250 pg of ca p59fyn RNA induced the same effect in 50% of the injected embryos. Injection of 500 pg of ca p59fyn RNA had an even greater effect on normal embryo development as 66% of the embryos had completely occluded blastocoels. Interestingly, embryos injected with 100 pg or 250 pg of ca p59fyn RNA exhibited a higher percentage of intermediate phenotype, “abnormal” blastocoel, as compared to those injected with 500 pg of the same RNA (Table 1 and Figure 2). To better characterize changes in morphology, the embryos were further examined by SEM at low magnification (120X). Figure 3 shows representative embryos from each sample group. Water-injected embryos showed normal morphology, with a well-formed blastocoel and well-ordered, multilayered cell arrangement in the animal hemisphere. (Figure 3A). In contrast, embryos expressing activated EPP exhibited an altered morphology characterized by an extensive dissociation of internal cells, the absence of the blastocoel, and rounded morphology of cells (Figure 3B). Similarly to embryos expressing activated EPP, embryos injected with either 250 pg or 500 pg of ca p59fyn RNA developed a phenotype that resulted in loss of cell–cell adhesion and blastocoel obstruction (Figures 3D and 3E). The intermediate phenotype of the embryo injected with 100 pg of ca p59fyn is presented in Figure 3C.
Table 1  Injection of constitutively active p59fyn causes loss of cell-cell adhesion.

Pooled data of three experiments is presented.

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>RNA injected</th>
<th>Water</th>
<th>100 pg ca*</th>
<th>250 pg ca</th>
<th>500 pg ca</th>
<th>EPP TGF-β</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>p59fyn</td>
<td>p59fyn</td>
<td>p59fyn</td>
<td>p59fyn</td>
<td>p59fyn</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>81% ± 5</td>
<td>52% ± 9</td>
<td>28% ± 13</td>
<td>26% ± 24</td>
<td>7% ± 9</td>
</tr>
<tr>
<td>Blastocoel</td>
<td>(95)</td>
<td>(56)</td>
<td>(41)</td>
<td>(22)</td>
<td>(2)</td>
<td></td>
</tr>
<tr>
<td>Abnormal blastocoel</td>
<td>7% ± 8</td>
<td>18% ± 17</td>
<td>22% ± 5</td>
<td>9% ± 0.6</td>
<td>35% ± 21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>(22)</td>
<td>(24)</td>
<td>(5)</td>
<td>(5)</td>
<td></td>
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<tr>
<td>No</td>
<td>12% ± 9</td>
<td>30% ± 19</td>
<td>50% ± 15</td>
<td>66% ± 23</td>
<td>58% ± 12</td>
<td></td>
</tr>
<tr>
<td>blastocoel</td>
<td>(12)</td>
<td>(23)</td>
<td>(57)</td>
<td>(33)</td>
<td>(14)</td>
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<td>Total</td>
<td></td>
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<td>embryos injected</td>
<td>115</td>
<td>101</td>
<td>122</td>
<td>58</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>

* Constitutively active.

Percentages indicate the fraction of embryos exhibiting each phenotype.

Errors are indicated as ± SD (standard deviation).

In parentheses are the numbers of embryos exhibiting each phenotype.
Figure 2  Comparison of the effects of the injection of constitutively active p59fyn RNA on the development of one of the three phenotypes: “normal blastocoel”, “abnormal blastocoel”, and “no blastocoel”. The RNA was injected into one-cell-stage embryos; blastocoel morphology and cellular dissociation were assessed at mid-blastula stage. Injection of constitutively active p59fyn causes loss of cell–cell adhesion. Pooled data of three experiments are presented.
Figure 3  Injection of constitutively active p59fyn causes loss of cell-cell adhesion. Scanning electron micrographs (120 X) show a typical embryo from different samples.  

**A.** Water-injected embryo.  

**B.** Embryo injected with EPP RNA and TGF-αRNAs.  

**C.** Embryo injected with 100 pg of ca p59fyn RNA.  

**D.** Embryo injected with 250 pg of ca p59fyn RNA.  

**E.** Embryo injected with 500 pg of ca p59fyn RNA. The *scale bar* in **E** represents 100 µm. *Arrow* in **A** points to blastocoel.
Dominant negative p59fyn rescues embryos that express activated EPP from loss of cell adhesion

As mentioned previously, ectopic expression of activated EPP in Xenopus embryos results in loss of cell adhesion of animal hemisphere cells, and as a consequence, in blastocoel occlusion by dissociated cells. If activated EPP receptor manifests its downstream effects by activating the Src-like tyrosine kinase p59fyn, the expression of a dominant negative (dn) form of p59fyn should rescue embryos from the EPP phenotype. To investigate this, one-cell-stage embryos were injected with two doses of RNA encoding dn p59fyn (5 ng and 7 ng) with coinjection of RNA encoding EPP and TGF-_. For the EPP phenotype, control embryos were injected with EPP and TGF-_- RNAs. For control of normal development, embryos were injected either with water or dn p59fyn RNA alone. Blastula-stage embryos were fixed in glutaraldehyde, bisected, and scored under a dissecting microscope. The pooled results of two experiments are presented in Table 2 and Figure 4. Over 60% of the embryos injected with water displayed normal morphology, with well-formed blastocoels. Conversely, over 60% of the embryos expressing activated EPP experienced massive cellular dissociation. The comparison of embryos by SEM supported that observation (Figures 5A and 5B). The injection of dn p59fyn RNA, either 5 ng or 7 ng, along with RNA encoding EPP and TGF-_, rescued more than 40% of the embryos from complete cellular dissociation and restored the blastocoel (Figures 5C and 5D). The similar numbers of rescued embryos suggest that there is no distinct difference in rescue ability between these two concentrations. However, a
detailed examination of the rescued embryos by SEM at slightly higher magnification revealed that even though blastocoel morphology was restored in embryos injected with 5 ng of dn p59fyn RNA, cells of the animal pole retained some characteristics of the EPP phenotype, particularly a rounded shape with numerous bulging processes (Figure 6C). Injection of 7 ng of dn p59fyn RNA more effectively rescued embryos (Figure 6D) because animal hemisphere cells had the typical morphology exhibited by the animal pole cells in normally developing *Xenopus* embryos. The important observation is that the embryos injected with 7 ng of dn p59fyn exhibited a higher percentage of intermediate phenotype, “abnormal” blastocoel, and a smaller percentage of “no” blastocoel phenotype as compared to the embryos injected with 5 ng of dn p59fyn RNA (Table 2 and Figure 3).

*Src-like tyrosine kinase p59fyn is a link between EPP receptor and RhoA GTPase*

It is known that RhoA is a part of the EphA4 pathway (Winning et al., 2002). According to the previous experiments, p59fyn is a part of the EphA4 pathway also. It is not known, however, where they occur in the pathway relative to each other. If RhoA is downstream of p59fyn, then one would predict that constitutively active (ca) RhoA should rescue embryos from the effects of ca p59fyn. To test whether p59fyn and RhoA are in the same pathway, one-cell-stage embryos were injected with 500 pg of ca p59fyn plus 500 pg of ca RhoA. Once again, two sets of embryos were injected with water and ca RhoA RNA for
Table 2  Dominant negative p59fyn rescues embryos from the loss of cell-cell adhesion. Combined results of two experiments are presented.

<table>
<thead>
<tr>
<th>Phenotypes</th>
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<th></th>
<th></th>
<th></th>
<th></th>
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<tr>
<td></td>
<td>Water</td>
<td>EPP TGF_</td>
<td>EPP TGF_-</td>
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<td>EPP TGF_-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 ng dn*</td>
<td>7 ng dn</td>
<td>p59fyn</td>
<td>p59fyn</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>62% ± 11</td>
<td>43% ± 14</td>
<td>47% ± 1</td>
<td>50% ± 18</td>
<td>19% ± 8</td>
<td></td>
</tr>
<tr>
<td>Blastocoel</td>
<td>(36)</td>
<td>(43)</td>
<td>(49)</td>
<td>(34)</td>
<td>(16)</td>
<td></td>
</tr>
<tr>
<td>Abnormal</td>
<td>21% ± 4</td>
<td>18% ± 1</td>
<td>34% ± 13</td>
<td>16% ± 5</td>
<td>17% ± 14</td>
<td></td>
</tr>
<tr>
<td>blastocoel</td>
<td>(12)</td>
<td>(15)</td>
<td>(30)</td>
<td>(15)</td>
<td>(17)</td>
<td></td>
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<tr>
<td>No</td>
<td>18% ± 14</td>
<td>39% ± 15</td>
<td>19% ± 14</td>
<td>34% ± 13</td>
<td>64% ± 23</td>
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<td>blastocoel</td>
<td>(15)</td>
<td>(25)</td>
<td>(27)</td>
<td>(32)</td>
<td>(40)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>63</td>
<td>83</td>
<td>106</td>
<td>81</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>

* Dominant negative.

Percentages indicate the fraction of embryos exhibiting each phenotype.

Errors are indicated as ± SD (standard deviation).

In parentheses are the numbers of embryos exhibiting each phenotype.
Figure 4  The comparison of the effects of 5 ng and 7 ng of dominant negative p59fyn RNA on the development of one of the three phenotypes: “normal blastocoel”, “abnormal blastocoel”, and “no blastocoel”. The RNA was injected into one-cell-stage embryos; blastocoel morphology and cellular dissociation was assessed at mid-blastula stage. Pooled data of two experiments is presented.
Figure 5  Injection of dominant negative p59fyn rescues embryos from loss of cell–cell adhesion. Scanning electron micrographs (120 X) show a typical embryo from different samples. **A.** Water-injected embryo. **B.** Embryo injected with EPP RNA and TGF-β RNA. **C.** Embryo injected with 5 ng of dn p59fyn RNA with coinjection of EPP RNA and TGF-β RNA. **D.** Embryo injected with 7 ng of dn p59fyn RNA with coinjection of EPP RNA and TGF-β RNA. **E.** Embryo injected with 7 ng of dn p59fyn RNA alone. The *scale bar* in **E** represents 100 µm. *Arrow* in **A** points to blastocoel.
Figure 6  SEM micrograph demonstrating that injection of 5 ng of dn p59fyn with coinjection of EPP and TGF-α incompletely rescues embryos from the EPP phenotype. X250. **A.** Embryo injected with water. **B.** Embryo injected with EPP and TGF-α RNAs. **C.** Embryo injected with 5 ng of dn fyn with coinjection of EPP and TGF-α. **D.** Embryo injected with 7 ng of dn fyn with coinjection of EPP and TGF-α. *Arrows in B and C points to rounded protrusions.*
control of blastocoel development, and two more sets of embryos were injected with ca p59fyn RNA alone or EPP RNA coinjected with TGF-β RNA for control of the development of EPP phenotype. As previously mentioned, after development to mid-blastula stage, the embryos were fixed in glutaraldehyde, bisected, and examined under a dissecting microscope for blastocoel morphology. Table 3 and in Figure 7 show the results of these injections. As predicted, coinjection of ca p59fyn RNA with ca RhoA RNA reduced cellular dissociation in 78% of the embryos. Scanning electron micrographs in Figure 8 present the examples of the embryonic phenotypes from each sample group. Suprisingly, only 42% of the embryos displaying reduced cellular dissociation were rescued completely, and, in fact, more than 30% of the embryos developed the intermediate phenotype with blastocoel defects. Nonetheless, the examination by SEM revealed that rescued embryos were completely restored to a normal phenotype with nicely formed blastocoel and angularly shaped cells (Figure 8 E) without displaying any visible abnormalities.
Table 3  Data demonstrating that p59fyn is a direct link between EPP receptor and RhoA GTPase. Constitutively active RhoA is able to rescue embryos from the EPP phenotype when coinjected with constitutively active p59fyn. Presented are pooled results of two experiments.

<table>
<thead>
<tr>
<th>Phenotypes</th>
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<th></th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>500 pg ca*</td>
<td>500 pg</td>
<td>500 pg ca</td>
<td>EPP</td>
</tr>
<tr>
<td></td>
<td>p59fyn</td>
<td>ca RhoA</td>
<td>p59 fyn</td>
<td>TGF-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>_</td>
<td>500 pg ca RhoA</td>
</tr>
<tr>
<td>Normal</td>
<td>70% ± 19</td>
<td>8% ± 4</td>
<td>61% ± 19</td>
<td>42% ± 12</td>
<td>3% ± 1</td>
</tr>
<tr>
<td>Blastocoel</td>
<td>(40)</td>
<td>(6)</td>
<td>(54)</td>
<td>(39)</td>
<td>(3)</td>
</tr>
<tr>
<td>Abnormal</td>
<td>20% ± 4</td>
<td>18 % ± 20</td>
<td>27% ± 6</td>
<td>35% ± 7</td>
<td>21% ± 5</td>
</tr>
<tr>
<td>blastocoel</td>
<td>(12)</td>
<td>(14)</td>
<td>(26)</td>
<td>(33)</td>
<td>(22)</td>
</tr>
<tr>
<td>No</td>
<td>11% ± 16</td>
<td>75 % ± 16</td>
<td>13% ± 13</td>
<td>24% ± 9</td>
<td>7% ± 4</td>
</tr>
<tr>
<td>blastocoel</td>
<td>(8)</td>
<td>(48)</td>
<td>(14)</td>
<td>(24)</td>
<td>(78)</td>
</tr>
<tr>
<td>Total</td>
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<td></td>
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<tr>
<td>embryos injected</td>
<td>60</td>
<td>68</td>
<td>94</td>
<td>96</td>
<td>103</td>
</tr>
</tbody>
</table>

* Constitutively active.

Percentages indicate the fraction of embryos exhibiting each phenotype.

Errors are indicated as ± SD (standard deviation).

In parentheses are the numbers of embryos exhibiting each phenotype.
Figure 7  Graphic comparison of data demonstrating that constitutively active RhoA rescues embryos from the EPP phenotype when coinjected with constitutively active p59fyn. RNA was injected into one-cell stage embryos; embryos were grouped into one of three phenotypes: “normal blastocoel”, “abnormal blastocoel”, and “no blastocoel”. Pooled data of two experiments are presented.
Figure 8 Coinjection of constitutively active RhoA RNA with constitutively active p59fyn RNA rescues embryos from loss of cellular adhesion. Scanning electron micrographs (120 X) show a typical embryo from different samples. A. Water-injected embryo. B. Embryo injected with EPP RNA and TGF-β RNA. C. Embryo injected with 500 pg of ca RhoA alone. D. Embryo injected with 500pg of ca p59fyn RNA alone. E. Embryo injected with 500 pg of ca RhoA plus 500 pg of ca p59fyn. The scale bar in E represents 100 µm. Arrow in A points to blastocoe
DISCUSSION

The findings presented herein indicate that the Src-like tyrosine kinase p59fyn acts as a functional link between the EphA4 receptor and RhoA GTPase in *Xenopus laevis* embryonic cells. Moreover, activated EphA4 receptor achieves its cellular effect through activation of p59fyn, which in turn inhibits RhoA (Figure 9). Three lines of evidence support these conclusions.

First, evidence has been provided that activation of p59fyn results in a phenotype very similar to the phenotype produced by activation of the chimeric receptor EPP. Expression of constitutively active (ca) p59fyn in blastula-stage *Xenopus* embryos resulted in loss of cell adhesion, cellular dissociation, and blastocoel occlusion. Likewise, embryos expressing EPP exhibited an altered morphology also characterized by an extensive dissociation of internal cells in the animal hemisphere and absence of the blastocoel (Winning and others 1996). This so-called “EPP phenotype” was described previously in detail by Winning and colleagues (2001). They also demonstrated that one mechanism by which EPP manifests its downstream effects in *Xenopus* blastula is through inhibition of RhoA GTPase (Winning and others 2002). In their experiments, inhibition of Rho-family GTPases by the bacterial proteins C3 transferase and ToxinA disrupted cell adhesion and produced a phenotype similar to that resulting from EPP activation. In addition, expression of constitutively active RhoA concurrent with activated EPP rescued embryos from cellular dissociation and blastocoel occlusion. On the basis of these results and on my observations, one might speculate that the loss of cell adhesion in embryos injected with
Figure 9  Proposed model of EphA4 receptor signaling. P- phosphotyrosine; ?- unknown protein; + indicates activation; - indicates inhibition.
ca p59fyn is due to RhoA inhibition. However, the results of my experiments involving injection of ca p59fyn do not directly implicate this protein in inhibiting RhoA and do not show that these two molecules are linked.

The evidence that p59fyn and RhoA operate in the same pathway came from experiments where ca p59fyn and ca RhoA were coexpressed. Embryos expressing both of these proteins displayed normal morphology with well-formed blastocoels. In contrast, injection of 500 pg of ca p59fyn alone greatly affected normal embryo development, and a high percentage of embryos had completely occluded blastocoels. Once again, embryos injected with 500 pg of ca RhoA alone exhibited normal morphology. All together these data illustrate that expression of ca RhoA is able to rescue embryos from the phenotype produced by activation of p59fyn. Thus, it can be concluded that RhoA and p59fyn are linked in the same signaling pathway. If this were not true, embryos coinjected with ca p59fyn and ca RhoA RNAs could not be rescued, and instead of developing normally, they would develop the EPP phenotype. Therefore, the results presented here strongly support the hypothesis, which states that when activated, p59fyn causes cellular dissociation by inhibiting RhoA. It should be mentioned that a high number of the embryos injected with ca p59fyn and ca RhoA developed the intermediate phenotype with blastocoel defects. One possible explanation is that this is due to the ability of ca RhoA at dosages of 500 pg and higher to cause embryo abnormalities manifesting in blastocoel defects that are typically due to cytoskeletal problems and not to loss of cell–cell adhesion (Winning and others 2002). The question remains: What activates
p59fyn? On the basis of evidence that both EPP and p59fyn generate similar embryonic phenotypes through the inhibition of RhoA, I hypothesized that p59fyn is a downstream target of EPP (and therefore of EphA4).

Confirmation that the EPP receptor and p59fyn are linked was obtained by coexpressing dominant negative (dn) p59fyn with activated EPP. Injection of 7 ng of RNA encoding dn p59fyn resulted in the rescue of many embryos from the EPP phenotype. This dominant negative approach revealed that p59fyn signaling plays an important role in linking EphA4 signaling to RhoA GTPase and, consequently, to cell-attachment responses. Consistent with my data, coexpression of kinase-inactive p59fyn with the EphA8 receptor greatly increased the ability of mammalian 293T cell lines to strongly adhere to the culture dish (Choi and Park 1999). The results of my experiments using a dominant negative approach strongly support the hypothesis that p59fyn is activated by EPP and demonstrate a functional link between EPP and p59fyn. However, there is no evidence presented in this work of a physical association between p59fyn and EPP in Xenopus laevis embryonic cells.

Even though the EphA4 receptor manifests its downstream signals by activating p59fyn, the other signaling proteins linking p59fyn and the EphA4 receptor may also be involved. It may be true that upon its autophosphorylation, EphA4 binds this unknown molecule, which in turn activates p59fyn. Earlier studies showed that tyrosine-phosphorylated Eph receptors are able to directly interact with the SH2 domains of Src-family proteins. Research by Ellis and coworkers (1996) using an in vitro binding assay and BIAcore analysis in fact
demonstrated specific and high-affinity interactions between p59fyn SH2 domains and conserved tyrosine phosphorylation sites in the juxtamembrane region of the EphA4 receptor. Choi and Park (1999) found that like EphA4, the EphA8 receptor also formed a stable complex with p59fyn SH2 domain. Moreover, this binding was preferential to the p59fyn SH2 domain rather than to the p60src or other proteins-SH2 domains. These experiments were extended further, and the importance of p59fyn signaling in linking EphA8 signaling to cell attachment was investigated. The coexpression of dominant negative p59fyn with EphA8 resulted in increased cell adhesion. Both of these works suggested that p59fyn is the principal target of the EphA8 and EpA4 receptors in signal transduction. Experiments physically linking the EphA4 receptor with p59fyn in \textit{Xenopus} have yet to be done. It will be interesting to further investigate whether p59fyn in fact forms a stable complex with EphA in \textit{Xenopus laevis} embryonic cells.

It should be mentioned that in all my experiments, changes in embryo morphology were assessed mostly on the basis of cellular dissociation. However, other manifestations of the EPP phenotype, such as change in cell shape, loss of cell polarity, and loss of microvilli are no less important. Cells in embryos expressing EPP usually lack the characteristic angularity; they acquire a rounded morphology and often exhibit multiple rounded protrusions (Winning and others 2001). After thorough examination of the embryos injected with ca p59fyn and after their comparison with the embryos injected with activated EPP, I made an interesting observation. Even though the embryos in both conditions exhibited
the EPP phenotype with completely occluded blastocoels, the dissociating cells in embryos injected with cap59fyn exhibited more angular morphology and lacked the rounded protrusions. There is one possible explanation for these different outcomes of EPP and p59fyn activation. It is known from the literature that Eph receptors can initiate not one but several different signaling cascades simultaneously by binding and activating variety of downstream targets (Bruckner and Klein 1998), such as p59fyn, p60src, and RasGAP, a negative regulator of RhoGTPase (Holland and others 1997). Furthermore, Eph receptors can initiate signaling cascades by binding the adaptor proteins Grb2 and Grb10 (Stein and others 1996). Both of these adaptors have been shown to bind to the EphB1 receptor. The hypothesis exists that all of these different pathways, activated by Eph, converge to regulate rearrangements in the actin cytoskeleton. So, it is possible that activation of the EPP receptor initiates two or more signaling pathways involving numerous intracellular signaling proteins and, in turn, more deeply affects the changes in embryo and/or cell morphology and more completely reproduces the EPP phenotype than does p59fyn activation alone.

Eph receptors and their ligands ephrins play vital roles in targeting axons and migrating cells during early developmental processes, but it is clear now that they are also involved in the function of the adult organism (Kullander and Klein 2002). Disruption of their normal functions can cause congenital disorders. Increased evidence also implicates Eph-family proteins in cancer (Dodelet and Pasquale 2000). This underscores the importance of identifying the signaling processes downstream of the EphA4 receptor. Manipulation of Eph signaling
may be able to modify unwanted processes. On the basis of their biological functions in axons and cell targeting and on the recent evidence implicating the effects of Eph receptors on actin rearrangement, signaling by the Eph-ephrin system may provide a link to understanding the machinery of the cell cytoskeleton. This would be of great significance to biologists because the regulation of the actin cytoskeleton is the basis for many vital biological processes.
REFERENCES


APPENDIX: IACUC APPROVAL

EASTERN MICHIGAN UNIVERSITY
OFFICE OF RESEARCH DEVELOPMENT
Starkweather Hall

TO: Robert Winning  
Biology  
FROM: Brian Anderson  
ex officio  
Institutional Animal Care and Use Committee

DATE: 10/04/01

Eastern Michigan University’s Institutional Animal Care and Use Committee (IACUC) has reviewed your Application To Use Animals In Research or Instruction referenced below. This project has been approved. The proposed animal use procedures are in compliance with University guidelines, State and Federal regulations and the standards of the “Guide for the Care and Use of Laboratory Animals.”

When communicating with the IACUC Office, please refer to the Approval Number referenced below. The appropriate Approval Number must accompany all requisitions for animals and pharmaceuticals. No research, testing or instructional use of vertebrate animals may be initiated without an Approval Number.

The Approval Period for your Approval Number is also indicated below. However, the United States Department of Agriculture (USDA) requires an annual review of applications to use animals. Therefore, each year of this application, prior to the anniversary of its approval date, you will receive a short Annual Review Form. Your continued animal use approval is contingent upon the completion and return of this form. You will also be notified prior to the expiration of the Approval Period so that any renewal application can be prepared, submitted and reviewed in a timely manner and an interruption in the approval status of this project avoided.

Committee approval must be obtained prior to changes in procedures that could affect the humane use of animals. If changes are contemplated, a revised Animal Use Form (with the changes highlighted) must be submitted and approved prior to initiation of the modified procedures. Contact the Associate Provost’s Office for more information.

| TITLE: “Pagliaccio Function in Embryonic Development” SEE ATTACHED. |
| APPROVAL PERIOD: 10/04/01 to 10/03/04 |
| IACUC APPROVAL NO.: 01-007 |

cc: Committee