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REGULATION OF PROCESS RETRACTION AND CELL MIGRATION BY EPHA3 IS MEDIATED BY THE ADAPTOR PROTEIN NCK1†

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Abstract

The Eph family of tyrosine kinase receptors and their ligands, the ephrins, participates in the regulation of a wide variety of biological functions under normal and pathological conditions. During embryonic development, interactions between the ligands and receptors define tissue boundaries, guide migrating axons, and regulate angiogenesis, as well as bone morphogenesis. These molecules have also been shown to modify neural activity in the adult nervous system, and influence tumor progression. However, the molecular mechanisms underlying these diverse functions are not completely understood. In the present study, a yeast two-hybrid screen has been conducted to identify molecules that physically interact with Eph receptors using the cytoplasmic domain of EphA3 as “bait”. This study identified Nck1 as a strong binding partner of EphA3 as assayed using both GST-fusion protein pull down and co-immunoprecipitation techniques. The interaction is mediated through binding of the Nck1 SH2 domain to the phosphotyrosine residue at position 602 (Y602) of EphA3 receptor. The removal of the SH2 domain or the mutation of the Y602 residue abolishes the interaction. It is further demonstrated that EphA3 activation inhibits cell migration and process outgrowth, and these inhibiting effects are partially alleviated by dominant-negative Nck1 mutants that lack functional SH2 or SH3 domains, but not by the wild type Nck1 gene. These results suggest that Nck1 interacts with EphA3 to regulate cell migration and process retraction.

Keywords

Eph; ephrin; tyrosine kinase receptors; SH2 domains; Cell migration; yeast-two-hybrid; Dominant-negative mutants

Eph receptors make up the biggest group of receptor tyrosine kinases, and a total of 16 different receptors have been identified in vertebrates (1). These receptors can be grouped into two

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subclasses based on their sequence homology and their specificity of ligand interactions (2). The EphA receptors (EphA1 to EphA10) bind to glycosylphosphatidyl-inositol (GPI) linked ligands (ephrin-A1 to ephrin-A6), and the EphB receptors (EphB1 to EphB6) interact with transmembrane ligands (ephrin-B1 to ephrin-B3) (1,3). In general, ligands and receptors interact promiscuously within each subclass (2). However, exceptions do exist to this subclass specificity. For example, EphA4 interacts with both the A- and the B-ephrins, while EphB2 can also bind ephrin-A5 in addition to the B-ephrins (2,4). It was also reported that ephrin-A1/A3/A4 can bind to EphB1 with low affinity (5), although it was not clear whether these interactions would lead to any significant biological functions.

Members of the Eph receptor family and their ligands are highly expressed in a wide variety of tissues in developing and adult organisms (3). Consistent with their wide expression, Eph receptors and their ligands are found to play important roles in many developmental processes, including tissue morphogenesis, vascular network formation, neural crest cell migration, axon fasciculation, axon guidance, and topographic neural map formation (6). Ephrin-Eph interaction usually produces repulsive effects that lead to tissue boundary formation and axon retraction (7). However, attractive effects were also observed in certain biological functions (8-12). Although most of these functions depend on the kinase activity of the receptors, kinase independent signaling also exists through the receptors (13,14), or through reverse signaling of the ligands (15). In addition to the roles during development, Eph receptors have also been shown to regulate adult functions such as learning and memory (16,17), and pathological conditions such as tumorigenesis (18,19).

Several downstream molecules that mediate Eph receptor functions have been identified. They include the Rho family small GTPases, namely RhoA, Rac1, and Cdc42, as well as the guanine-nucleotide exchange factors (GEFs), such as kalirin, Ephexin, Vav, and the FERM domain including RhoGEFs (FIR) (20). The GEFs usually bind to the activated Eph receptors, and the binding leads to RhoA activation and Cdc42/Rac1 inhibition. In addition, the activation of Eph receptors has been shown to recruit GTPase-activating proteins (GAPs) for Rac and lead to the inactivation of Rac (21-23). These modulations of small GTPase activity trigger changes in cytoskeleton dynamics, causing growth cone collapse and dendritic spine remodeling (20). There is also evidence that RasGAP and p62 (dok) associate with activated EphB2 to mediate inhibition of Erk-MAP kinase activity induced by ephrin-B1 (24). Furthermore, activation of EphA2 recruits protein tyrosine phosphatase Shp2 and triggers dephosphorylation of FAK, which also forms a protein complex with EphA2 (25). Several other protein tyrosine phosphatases (PTPs), including the low molecular weight (LMW) PTPs (26,27), the receptor-type phosphatase PTPO (28), and Src family kinases (29-32), have been reported to associate with Eph receptors and modulate their activity as well. Eph receptors were also found to interact with neurotransmitter receptors. For example, EphB2 binds to NMDA receptor to promote synaptogenesis (33,34).

In spite of the progress made so far, the understanding of signaling mechanisms underlying different Eph receptor functions remains incomplete. To further identify potential signaling molecules downstream of the Eph receptors, a yeast two-hybrid screen using an EphA receptor intracellular domain as “bait” was conducted. In this study, the Src homology (SH) 2 and SH3 domain-containing adaptor protein Nck1 was identified as a strong EphA3 binding protein. We show here that this interaction is mediated through binding of Nck1 SH2 domain to the tyrosine residue 602 of the EphA3 receptor, and that blocking Nck1 function also abrogates the inhibition on process outgrowth and cell migration induced by EphA3 activation. These studies indicate that Nck1 is a key downstream signaling molecule that mediates EphA3 functions.

MATERIALS AND METHODS

Reagents and antibodies

Ephrin-A5-Fc fusion protein was purchased from R&D systems (Minneapolis, MN). The ligand was clustered by antibody cross-linking using anti-human Fc (Jackson ImmunoResearch, West Grove, PA) at an antibody to ephrin ratio of 1 to 5 in weight (1:15 in molar ratio). The mixture was incubated at 4 °C for 8 hours and used to stimulate cells at 1 – 2 µg/ml concentration. Mouse anti-Nck1 monoclonal antibody was purchased from BD Biosciences (San Jose, CA). The rabbit anti-EphA3 polyclonal antibody and mouse anti-*myc* monoclonal antibody were purchased from Santa Cruz (Santa Cruz, CA). The phosphotyrosine antibody used in analyzing the phosphorylation of the EphA3 receptors was purchased from Cell Signaling Technology (Danvers, MA). For Western blot analyses, these antibodies were used at 1:1000. Secondary antibodies used in western blotting were acquired from Sigma-Aldrich (St. Louis, MO). When re-blotting was required during western blot, the nitrocellulose membrane was washed briefly and incubated in western-blot re-strip buffer from G-Biosciences for 30 minutes (St. Louis, MO).

Yeast two-hybrid screen

The Yeast two-hybrid screen was performed with DupLex-A system from Origene (Rockville, MD) according to the instructions. In brief, the intracellular domain of EphA3 receptor was cloned into pEG202-NLS vector, fused to DNA binding protein LexA to generate the “bait” plasmid pEG202-NLS-EphA3intra. This plasmid was then transformed into yeast strain EGY188, along with a reporter plasmid carrying a LacZ gene and an embryonic mouse brain cDNA library cloned in the target plasmid pJG4–5. The transformed yeast cells were plated and screened for LacZ transcription through X-gal reaction. Plasmid DNA from the positive clones were then extracted, amplified in *E. coli*, and sequenced. The identity of the positive clones was determined using BLAST searches against public databases. The specificity of the interaction between the identified cDNA products and the “bait” protein was further confirmed using yeast mating test. Briefly, the isolated target plasmids carrying the cDNA fragments were transformed into EGY188 and the resulting cells mated with cells of EGY40 strain containing both the reporter plasmid and pEG202-NLS-EphA3intra. The mated cells were lysed and incubated with X-gal solution for 2 hours. Negative controls for mating test were generated by using an empty bait plasmid (pBait) or an irrelevant control plasmid in place of pEG202-NLS-EphA3intra in the test.

GST fusion protein pull-down

. 293A cells transfected with various EphA3 constructs were stimulated with cross-linked ephrin-A5-Fc (1 µg/ml) for 30 minutes at 37 °C. The cells were washed once with ice cold PBS and lysed in lysis buffer (10 mM Hepes, pH 7.4, 1% Triton X-100, 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin, 1 mM sodium orthovanadate). The lysed cells were collected and centrifuged at 13,000 rpm for 10 minutes in an Eppendorf microcentrifuge. After centrifugation to remove cell debris, Glutathione Sepharose beads conjugated with the desired GST fusion proteins were added to the supernatant and the mixture was incubated at 4 °C for 2 hours. The beads were then collected by centrifugation and washed 3 times with PBS supplemented with 0.05% Triton X-100, each for 10 minutes. The beads were boiled in 2X Laemmli buffer for 5 minutes before analysis through Western blot technique.

Co-immunoprecipitation assay

Transfected 293A cells were stimulated with cross-linked ephrin-A5-Fc for 30 minutes at 37 °C. The cells were washed gently with ice cold PBS once and lysed in cell lysis buffer. The cell lysate was then cleared by centrifugation and the protein concentration of the supernatant

determined. A small fraction of the lysate was used later for analysis of protein expression levels. For immunoprecipitation, desired antibody was added to 2 mg of each lysate and incubated at 4°C for 4 hours. The immunoprecipitates were collected with protein A sepharose beads and washed with PBS with 0.05% Triton X-100 for 3 times. The immunocomplexes were boiled in 2X Laemmli buffer for 5 minutes and analyzed with SDS-PAGE coupled with Western blot analysis using different antibodies.

Cellular process retraction assay

HEK293A cells were plated sparsely on culture dishes to allow examination of individual cells and their processes. The cells were transfected with desired plasmids and 48 hours after transfection, stimulated with or without cross-linked ephrin-A5 for 1 hour at 37 °C. Pictures of transfected cells (N>30) identified with EGFP expression, were taken before and after the treatment. The length of processes was measured from where they exit the cell body to the end of the process using ImagePro software. The difference between the length of the processes before and after incubation was then divided by the length before treatment and expressed as percentage change.

Cell migration assay

To prepare for this assay, transwell inserts (20 mm diameter, 8 µm pore diameter) purchased from Corning Labware (Corning, NY) were coated on the underside with fibronectin to facilitate attachment of migrated cells. HEK293A cells were transfected with desired genes using Lipofectamine 2000 reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, the cells were trypsinized and washed with PBS. A portion of the cells was plated in 6 well dishes for determination of transfection efficiency. An equal number of the transfected cells were plated on the transwell inserts in DMEM without serum and the lower compartment in the setup was filled with complete culture medium (DMEM supplemented with 10% FBS) with or without 2 µg/ml cross-linked ephrin-A5. The transwell dishes were then incubated overnight at 37 °C with 5% CO₂. On the second day, cells on the inserts were fixed and all the cells that haven't migrated were cleaned off using Q-tips. Cells on the underside of the inserts were stained with DAPI to allow cell counting under a fluorescence microscope. Five to ten random fields were chosen and the number of all cells that migrated through the membrane (T2) and the number of the transfected cells that migrated (T1) were both quantified. In addition, the transfection efficiency was individually determined and expressed as a percentage of total cells ($R = \text{transfected cells} / \text{all cells}$). The migration ability (M) of cells expressing different genes was calculated using the following formula: $M = T1 / (T2 \times R)$. When the data were used in the graph, M was compared with EGFP transfected controls and converted as a percentage of the M value of EGFP control, which is considered to be 100%.

RESULTS

Nck1 is an EphA receptor interacting protein

In the yeast two-hybrid screen, the intracellular domain of EphA3 was inserted into the bait plasmid and a whole mouse embryonic cDNA library was screened. In all, about 300 positive clones were isolated and sequenced. Among them, one clone that showed the strongest binding encoded for a part of the mouse Nck1 protein, including the second and third SH3 domain and the complete SH2 domain (Fig. 1). When this clone was further examined in a yeast mating test, the cDNA protein product had the ability to interact with EphA3 "bait" protein and turn on LacZ transcription, while neither an empty bait plasmid pBait nor a negative control plasmid could do so (Fig. 1A), suggesting a specific interaction between Nck1 and EphA3.

Coincidentally, when the intracellular domain of EphA5 was used in a yeast two-hybrid screen, Nck1 was also one of the strongest interacting proteins, as determined by the LacZ colorimetric assay (data not shown). These findings collectively show that Nck1 interacts with both EphA3 and EphA5 and probably mediates critical functions of EphA receptors.

Interaction between Nck1 and EphA3 is mediated by binding of the Nck1 SH2 domain to tyrosine 602 (Y602) of EphA3

To map the binding site of Nck1, wild type EphA3 (EphA3WT) was transfected into HEK293A cells, and 48 hours after transfection, the cells were stimulated with cross-linked ephrin-A5 for 30 minutes. The lysates were then incubated with GST fusion proteins that contained either full length (GST-Nck1), SH2 domain (GST-Nck1SH2) or the SH3 domains (GST-Nck1SH3) of mouse Nck1 (Fig. 1B). Both GST-Nck1 full length and GST-Nck1SH2 proteins precipitated EphA3 (Fig. 1C), while GST control and GST-Nck1SH3 fusion protein did not. Experiments performed with cell lysates of unstimulated EphA3 expressing 293A cells showed a much reduced EphA3 binding by GST-Nck1SH2 (Fig. 1D). These pull-down studies indicate that the interaction with EphA3 is mediated by the SH2 domain of Nck1.

SH2 protein domains typically bind to phosphorylated tyrosine residues (35). To identify the tyrosine residues on EphA3 that Nck1 SH2 domain binds, several cytoplasmic tyrosine residues of EphA3 known to be phosphorylated upon activation (Y596, Y602, Y736, Y779, Y937) (G Shi and R Zhou, unpublished data) were mutated to phenylalanine using *in vitro* mutagenesis method. In addition, a mutant containing a lysine to arginine mutation at amino acid position 653, which was known to inactivate EphA3 kinase activity, was used as a negative control (K653R). These EphA3 mutants, along with wild type EphA3, were each transiently expressed in HEK293A cells, and the cell lysates were then incubated with GST-Nck1SH2 protein. Among the tyrosine mutants, both Y596F and Y602F showed no binding to Nck1 SH2 domain, while the others displayed clear binding (Figure 2A, lane 1 to 5). The kinase dead mutant EphA3-K653R also failed to bind Nck1 SH2 domain compared to wild type EphA3 (Fig. 2A, lane 6 & 7). Since Y596 and Y602 may regulate EphA3 kinase activity, the loss of binding we observed could be due to either a complete loss of all tyrosine phosphorylation, or the absence of the key tyrosine residues. To differentiate between these two possibilities, two additional mutants (Y596E and Y602E) were generated, with Y596 and Y602 being replaced by glutamic acid, respectively. This glutamic acid replacement was shown previously to mimic both the size and charge of a phosphorylated tyrosine and restore kinase activity of similar mutants (36). Pull-down studies using these mutants showed that only Y602E failed to bind GST-Nck1SH2 protein (Fig. 2A, lane 8–10).

To further establish the loss of Y602 phosphorylation, not the loss of kinase activity, is responsible for the loss of the binding, we examined the ability of EphA3 mutants to autophosphorylate. Wild type and mutant EphA3 constructs were transiently transfected into 293A cells. Two days after transfection, the cells were treated with ephrin-A5, lysed, and EphA3 proteins immunoprecipitated with a rabbit polyclonal anti-EphA3 antibody. The immunoprecipitates were further analyzed for tyrosine phosphorylation using western blot technique with a monoclonal anti-phosphotyrosine antibody. This analysis showed that indeed both Y596F and K653R mutants lacked kinase activity (Fig. 2B, lane 1 and 6), correlating with their inability to bind to Nck1 SH2 domain. Replacement of Y596 with glutamic acid in Y596E mutant restored both kinase activity and Nck1 SH2 domain binding, suggesting that Y596 is required for EphA3 kinase activity, but not SH2 binding. In contrast, both Y602F and Y602E mutants maintained kinase activity (Fig. 2B, Lane 2 and 9), but lost the ability to interact with Nck1 SH2 domain (Fig. 2A). Thus we conclude that Y602 is the critical residue, which serves as the Nck1 docking site when phosphorylated.

Co-immunoprecipitation of EphA3 and Nck1

To examine whether Nck1 interacts with EphA3 in mammalian cells, EphA3 and *myc* tagged wild type or mutant Nck1 were transiently transfected into HEK293A cells. The transfected cells were stimulated with 2 $\mu\text{g/ml}$ cross-linked ephrin-A5 for 15 minutes, and lysed with lysis buffer. Nck1 protein was then precipitated from the cell lysates with a rabbit polyclonal anti-*myc* antibody. The immunoprecipitated proteins were analyzed using Western blot to determine whether EphA3 was co-precipitated. The analysis revealed that a significant amount of EphA3 receptor co-precipitated only with Nck1 containing wild type SH2 domain (Fig. 3A). Mutations in SH2 domain of Nck1 (Nck1R308K) abolished the interaction while mutations in SH3 domains (Nck1W38/143/229K) had no effect. This observation indicates that Nck1 binds to the activated EphA3 through its SH2 domain in living cells.

To determine whether EphA3 and Nck1 also interact in primary cells, we examined if Nck1 can be immunoprecipitated with EphA3 from the lysates of embryonic cortical neurons, since both proteins are highly expressed in these cells (37,38). Indeed EphA3 could be co-precipitated by an anti-Nck1 antibody (Fig. 3B, lane 2), but not by a control mouse IgG (Fig. 3B, lane 3). Moreover, it was noted that in the absence of ephrin-A5 stimulation, no co-precipitation occurred (Fig. 3B, lane 1). Together, these observations provide further support for EphA3-Nck1 interaction.

Nck1 mutants inhibit ephrin-A5-induced cellular process retraction

Activation of EphA receptors by ephrin-A5 in 293 cells has been shown to lead to the retraction of cellular processes when EphA3 receptor is expressed [(39), Fig. 4A]. This provides a convenient assay to examine whether Nck1 is required for EphA3 function. In this assay, HEK293A cells were plated sparsely on 35 mm tissue culture dishes and transfected with various EphA3 mutants as described in the methods. Forty-eight hours after transfection, cells were treated with 2 $\mu\text{g/ml}$ cross-linked ephrin-A5 for one hour. Parallel cultures were treated without ephrin-A5 as controls. Select cells with processes were photographed before and after treatment. The length of the processes before or after treatment was measured and expressed as a quantitative index of the extent of process retraction. Ephrin-A5 treatment of cells transfected with an empty vector expressing EGFP only led to minimal length change of the cellular processes (Fig. 4). Treatment of cells transfected with kinase-dead EphA3 also did not lead to any deleterious effects on the processes (Fig. 4). However, when the cells were transfected with EphA3WT, ephrin-A5 treatment led to a significant retraction of the processes. More importantly, when either EphA3Y602F or EphA3Y602E was expressed in these cells, process retraction was also minimal, unlike cells expressing wild type EphA3 (Fig. 4). We conclude, therefore, that the kinase activity of EphA3 is required for ephrin-A5-induced retraction and that an intact tyrosine residue at 602 is critical. These findings are consistent with the notion that Nck1 binding to EphA3 is important for EphA3 function.

In order to further confirm roles of Nck1 in EphA3 mediated cell retraction, effect of two dominant-negative mutants Nck1R308K and Nck1W38/143/229K were analyzed (Fig. 5A and 5B). Both of these mutants have been previously reported to inhibit functions of the wild type Nck1 (40). The R308K mutation disrupts the ability of the SH2 domain to bind to phosphotyrosine residues, while the W38/143/229K mutations abolish the ability of all three SH3 domains to interact with downstream targets (41). As shown in Fig. 5A, co-transfection of Nck1WT with EphA3WT did not inhibit process retraction of EphA3-expressing cells following ephrin-A5 challenge. The process length was shortened by about 22.0% (+/- 3.0%) similar to cells expressing only EphA3WT. In contrast, when either Nck1R308K, or Nck1W38/143/229K, was co-expressed with EphA3WT, the process retraction induced by ephrin-A5 was significantly inhibited (Fig. 5). In the presence of Nck1R308K, or Nck1W38/143/229K, the decrease of process length of cells expressing EphA3WT was only

9.2 % (+/- 3.9%) and 6.5% (+/- 2.1%), respectively. These results indicate that both the interaction of EphA3-SH2 domain and the interaction between SH3 domains and other downstream targets are required for EphA3 function.

Nck1 mutants down-modulate ephrin-A5-mediated inhibition of cell migration

To further examine roles of Nck1, we tested the effects of different Nck1 mutants on the regulation of cell migration by EphA3, using the Transwell assay. HEK293A cells were transfected and twenty-four hours later, the cells were plated on the Transwell inserts in serum free medium. The lower compartment of the transwell apparatus was filled with serum containing culture medium supplemented with or without cross-linked ephrin-A5. After culturing overnight, cells that migrated through the pores onto the underside of the insert were counted. This analysis showed that expression of EphA3WT caused a sharp decline in cell migration compared to EGFP controls or EphA3KD (Fig. 6A). Thus, EphA3 inhibits cell migration and the kinase activity is indispensable to this regulation. In comparison, neither EphA3Y602F nor EphA3Y602E had any effects (Fig. 6A), indicating that tyrosine 602 is required for EphA3 function.

To examine whether Nck1 is required for inhibition of cell migration by EphA3, we examined the effects of various Nck1 mutants. Expression of Nck1WT, Nck1R308K or Nck1W34/143/229K alone showed no significant effects on cell migration (Fig. 6B). However, when Nck1R308K or Nck1W34/143/229K mutants were co-expressed with EphA3WT, these mutants significantly alleviated the inhibition on cell migration by ephrin-A5 (Fig. 6B). In contrast, Nck1WT had no effect. Collectively, these observations indicate that interactions through both SH2 and SH3 domains of Nck1 are necessary for regulation of cell migration by EphA3.

Nck1 acts as a key downstream factor in EphA3 mediated signaling

The function of Nck1 in EphA3 signaling was further investigated by using Nck-null mouse embryonic fibroblasts (MEF), which lack both Nck1 and Nck2 expression, in the transwell cell migration assay. These MEF cells were transfected with wild type Nck1, or EphA3, or both. In the mock transfected cells, no expression of Nck1 or EphA3 proteins were detected (Fig. 7A). Nck1 and EphA3 proteins were detected in cells transfected with respective cDNAs (Fig. 7A), showing efficient expression of the transfected genes. In the absence of ephrin-A5 stimulation, expressed EphA3 was partially autophosphorylated (Fig. 7A), indicating that overexpression of EphA3 lead to partial tyrosine phosphorylation in MEF cells. However, ephrin-A5 stimulation increased EphA3 phosphorylation significantly (Fig. 7A) and induced the strongest inhibition of cell migration when both wild type EphA3 and Nck1 were present in the cell (Fig. 7B).

When the Nck-null cells were transfected with EphA3 or Nck1 alone, Ephrin-A5 induced only a slight inhibition on cell migration (Fig. 7B). However, when both EphA3 WT and Nck1 WT were expressed, the migration of these cells was drastically inhibited by ephrin-A5. In contrast, co-transfection of EphA3 or Nck1 mutants that disrupt the interaction failed to induce strong inhibition by ephrin-A5. When wild type EphA3 is co-transfected with Nck1 mutants (Nck1R308K, Nck1W38/143/229K), or EphA3Y602F with wild type Nck1, ephrin-A5 failed to strongly inhibit migration of the transfected cells (Fig. 7B). Taken together, these results further confirm that Nck1 is a critical downstream molecule in mediating the responses to ephrin-A5.

DISCUSSIONS

The present study identified Nck1 as a strong binding partner of the EphA receptors, and showed that the binding was mediated by interaction of the Nck1 SH2 domain to the phosphotyrosine residue at position 602 of EphA3. Inhibition of Nck1 function using dominant-negative mutants tempered inhibitory effects of EphA3 on cell migration and process extension, and loss of Nck function leads to a significant loss of inhibition of cell migration by ephrin-A5, suggesting that Nck1 is a key downstream mediator of EphA3 functions.

Nck1 interacts with activated EphA3

Using EphA3 and EphA5 intracellular domains as “baits” in a yeast two-hybrid screen, we found that Nck1 displayed strong interaction with both receptors. An earlier study by Stein et al. showed that Nck1 interacted with the intracellular domain of EphB1 (42). It was also reported that EphB2 bound to p62 dok after activation and formed a protein complex that included both RasGAP and Nck1 (24). These data together support the notion that Nck1 functions downstream of both EphA and EphB receptor.

Nck1 has no known enzymatic activity and appears to serve as an adaptor protein to link tyrosine phosphorylation signals to downstream effectors. In addition to the SH2 domain, Nck1 also contains three SH3 domains, which mediate binding to multiple downstream molecules that regulate actin cytoskeleton (43). Consistent with the observation in the yeast two-hybrid experiments, we found that Nck1 protein was co-precipitated with EphA3 from both transfected cells and primary neurons. The interaction was mediated by Nck1 SH2 domain binding to the receptor. These findings indicate that the interaction between Nck1 and EphA3 is similar to its interaction with EGF receptor and Crk-associated substrate (p130^{Cas}) in PDGF-stimulated cells (44-46), but different from its binding to DCC and Robo, which takes place through the SH3 domains instead (47). Mutagenesis coupled with *in vitro* pull-down experiments conducted in this study allowed us to localize the key interaction site of EphA3 for Nck1. Indeed, EphA3-Y596F and EphA3-Y602F failed to bind to Nck1. However, EphA3-Y596F mutant lacks kinase activity for auto-phosphorylation as well as phosphorylation of exogenous substrate (Fig. 2A and data not shown). Replacement of Y596 with glutamic acid restored the kinase activity and interaction with Nck1, showing that Y596 is required for activation of the receptor but not for Nck1 binding. On the other hand, neither EphA3-Y602F nor EphA3-Y602E has any Nck1 binding activity, although both mutants have normal kinase activity as measured by autophosphorylation and phosphorylation of exogenous substrate (Fig. 2A and G. Shi and R. Zhou, unpublished observations). Thus, tyrosine residue 602 of EphA3 is indispensable for interaction with Nck1. In addition, Nck1 SH2 domain has been shown to bind strongly to the sequence of “pY-hydrophilic-hydrophilic-I/P” through an unbiased screen of a degenerate phosphopeptide library (48). EphA3 amino acid sequence after tyrosine 602 (pYEDP) but not 596 (pYVDP) fits this description. Earlier studies showed that Nck1 interacted with EphB1, through the SH2 domain binding to Y594 (pYIDP, the equivalent of Y596 of EphA3) (49), indicating that there are variations in molecular interactions among different Eph receptors.

We also showed that activation of EphA3 by ephrin-A5 treatment enhanced the interaction in primary neurons. A small amount of EphA3 was precipitated with GST-Nck1SH2 from protein lysates of transfected but unstimulated 293A cells (Fig. 1D). This was probably due to the presence of activated EphA3 in transfected cells because of high levels of expression, which has been shown to activate EphA3 receptor (39).

Nck1 mediates regulation of process retraction and cell migration by EphA3 activation

In both process retraction assay and cell migration assay, we showed that introduction of either of the dominant negative Nck1 mutants, Nck1R308K and Nck1W38/143/229K, was able to inhibit effects of EphA3 activation, while the Nck1 wild type had no effects. Since these two dominant negative genes were deficient in either SH2-mediated binding or SH3-mediated binding (40,41), these observations suggest that both the recruitment of Nck1 onto activated EphA3 through the SH2 domain binding and the interaction with downstream effectors through the SH3 domain are important steps in the transduction of EphA3 signals. A potential concern in using dominant negative mutants in this study is that the effects observed were due to non-specific inhibition of binding of other SH2 or SH3 domain-containing proteins by the mutants. However, if the effects of the mutants were non-specific, and Nck1 had no roles in EphA3 signaling, one would expect expression of the wild type Nck1 should also block EphA3 function. Our observation that wild type Nck1 did not block EphA3 function indicates that the mutants specifically inhibit Nck1 function. Therefore, the fact that both mutants inhibited effects of EphA3 suggests Nck1 is an important signaling intermediate in the pathway of Eph receptor signaling. Analysis using Nck-null MEF cells further confirms this conclusion since ephrin-A5 has minimal effects on the migration of these cells.

In the cell migration assays, we found no increase in inhibition by EphA3 when exogenous Nck1 is also expressed. Since HEK293 cells do express significant levels of endogenous Nck1, it is possible that this level was already sufficient in mediating EphA3 effects, which may explain why further increase in levels did not lead to any enhancement. Introduction of wild type Nck1, not the SH2 or SH3 mutants, into the Nck-null MEF cells restores the inhibitory effects of ephrin-A5 on cell migration. This observation indicates that interaction with Nck1 leads to the recruitment of a signal transduction cascade leading to the inhibition cell migration. Notably, in the absence of either Nck1 or EphA3, ephrin-A5 stimulation can still mildly inhibit cell migration. This is probably due to other Eph receptors present in MEF cells. In addition, studies in our group have shown that the completed inhibition effect requires the cooperation of Nck1 and at least one other effector (unpublished data). The disruption of either of these two effectors leads to only a partial deficiency in EphA3 function.

From Nck to cytoskeletal regulations

Nck1 has been shown to interact with the p21-activated kinase (PAK1) via its second SH3 domain (50,51) and translocate PAK1 from the cytosol to plasma membrane. As a result, PAK1 is brought to close proximity of Rac1 and Cdc42, and becomes activated (52). Active PAK1 phosphorylates substrates including LIM (Lin-11, Isl-1 and Mec-3 domain) kinase, which in turn inactivates actin depolymerizing factor (ADF) family proteins such as cofilin, and myosin light chain kinase (MLCK), important regulators of actin/myosin organization (53,54). Inactivation of PAK1 leads to cofilin dephosphorylation and actin depolymerization. Indeed PAK1 activity is reduced in ephrin-A1 treated cortical neurons (55), although it is not known if ADF/cofilin activity is increased. The existence of a Nck1-PAK1 pathway is also supported by genetic data from *Drosophila*: the guidance and targeting of photoreceptor growth cones requires activity of both Nck1 and PAK *Drosophila* homologs (56). It would be interesting in future experiments to examine whether ephrin-induced inhibition of cell migration and process outgrowth is mediated through the Nck1-PAK1 pathway.

Another important group of Nck1 binding proteins are the WASP family proteins including WASP and N-WASP, via interaction with its SH3 domains (57-59). WASP mutations were first discovered in an X chromosome linked immuno-deficiency disease (Wiskott-Aldrich syndrome) (60), which was characterized by cell abnormalities and actin cytoskeleton defects. The WASP family proteins possess a C-terminal motif that binds to Arp2/3 and activate actin re-organization (59). When Nck1 SH3 domain was overexpressed in cells along with N-WASP

and Arp2/3, an unusually high rate of actin nucleation was observed (57). Indeed, Clustering of Nck1 SH3 domain alone is sufficient to induce localized actin polymerization (46), suggesting that the SH3 domains mediate actin cytoskeleton regulation. N-WASP is highly enriched in the brain and regulates neurite growth (59). Thus, Nck1 may function through the WASP pathway to regulate cell morphology and migration following EphA receptor activation.

It has been reported that EphB stimulation led to the formation of a protein complex that included Nck1, NIK, Dok1, RasGAP and the activation of the Nck1-interacting kinase (NIK), which in turn activated the c-Jun amino-terminal kinase (JNK) (61). Interestingly, when the *Drosophila* NIK homolog, Misshapen, was mutated, the photoreceptor axon targeting was disrupted (62), in a manner similar to the phenotype of Dock (Nck1 homolog in *Drosophila*) knockout (63). Nck1 has been shown to interact with more than 30 proteins, most of which have been implicated in actin cytoskeleton reorganization (64). Although the Nck1 downstream proteins that are responsible for the effects of ephrin-A5 are not known, our data strongly support for a critical role of Nck in regulating cell migration and process outgrowth by Eph receptors, a notion reinforced by recent findings that Nck-null mice have phenotypes similar to that of EphA4-null mice (65).

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ABBREVIATIONS

ADF, actin depolymerizing factor
DCC, deleted in colorectal cancer
DMEM, Dulbecco's modified Eagle's medium
Dock, *Drosophila* Nck1 homolog
EGF, epidermal growth factor
EGFP, enhanced green fluorescence protein
Eph, Erythropoietin-producing hepatocellular carcinoma kinase
ephrin, Eph family receptor interacting proteins
FBS, fetal bovine serum
GAPs, GTPase-activating proteins
GEF, guanine-nucleotide exchange factor
GST, glutathione S-transferase
GPI, glycosylphosphatidyl-inositol
HEK293A, human embryonic kidney 293A cells
HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonate
JNK, c-Jun amino-terminal kinase
KD, kinase-dead
LIM, lin-11, Isl-1, and Mec-3 domain
LMW-PTP, low-molecular weight phosphotyrosine phosphatase
MLCK, myosin light chain kinase
Nck, non-catalytic region of tyrosine kinase adaptor protein
NIK, nick-interacting kinase
PAK1, p21-activated kinase 1
PBS, phosphate-buffered saline
PDGF, platelet-derived growth factor
PMSF, phenylmethanesulfonyl fluoride
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH2, Src homology 2

SH3, Src homology 3
(N)WASP, (Neuronal) Wiskott-Aldrich syndrome protein
WT, wild type

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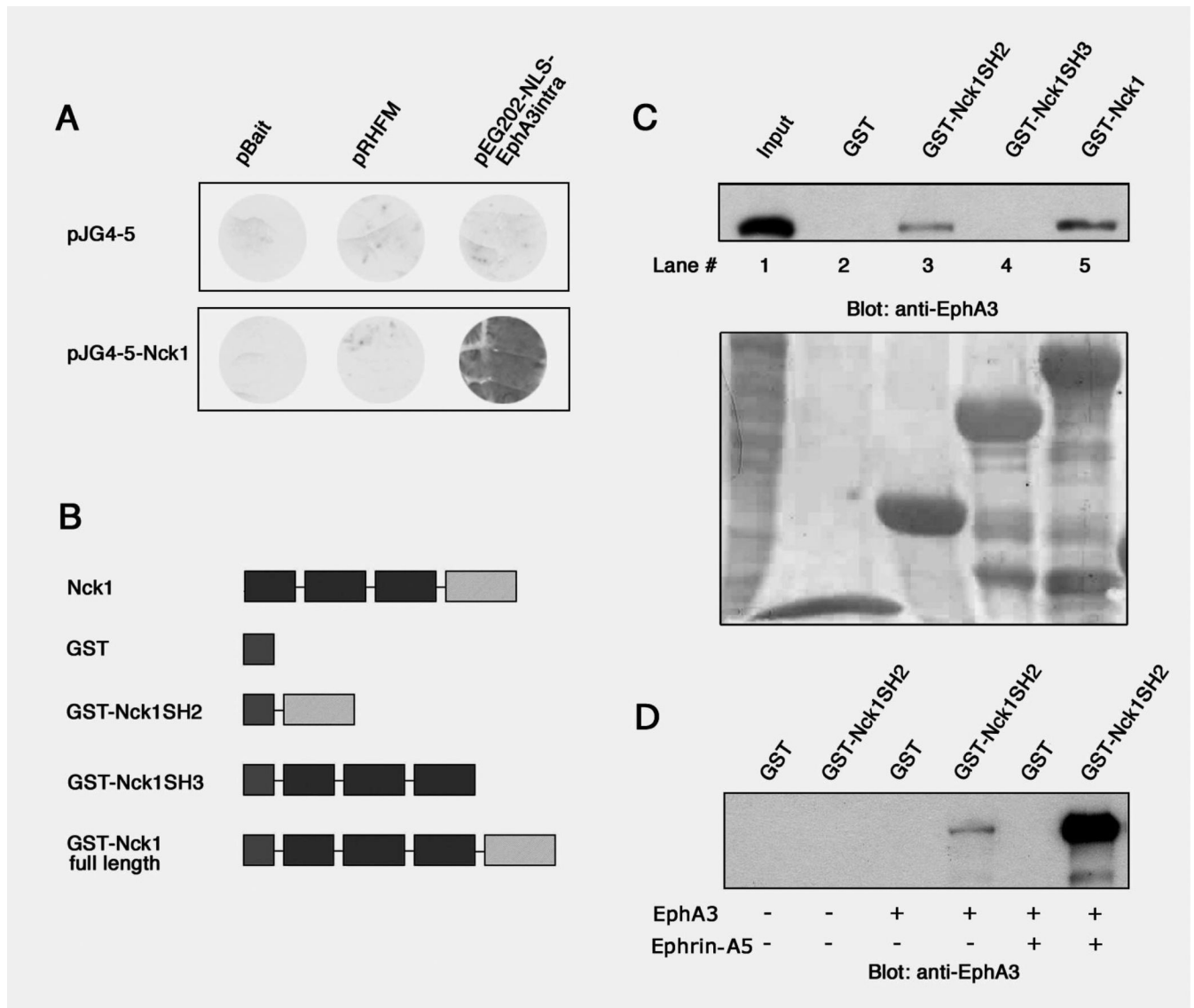


Figure 1. Identification of Nck1 as a major binding protein of EphA3

(A) Yeast mating test. Yeast cells carrying pJG4-5-Nck1 cDNA, the LacZ reporter plasmid, and either an irrelevant control plasmid pRHFM, an empty bait plasmid pBait, or pEG202-NLS-EphA3intra were lysed and incubated with LacZ substrate X-Gal. Only the transformation of both pJG4-5-Nck1 and pEG202-NLS-EphA3intra was capable of turning on the LacZ gene expression. (B) Domain structures of wild type Nck1 protein and Nck1 fusion proteins used in GST pull-down assay. (C) Identification of the EphA3 binding domain of Nck1. The GST fusion proteins were incubated with the lysates of HEK293A cells expressing EphA3 protein. The precipitated protein complex was subjected to Western blot analysis with anti-EphA3 antibody. "Input" shows the expression of EphA3 in the cell lysate of transfected HEK 293A cells. The blot was stained with ponceau S solution to show the levels of GST fusion proteins used in the assay (lower panel). (D) Demonstration of the interaction between auto-phosphorylated EphA3 and Nck1. GST fusion proteins were incubated with cell lysates from stimulated or un-stimulated EphA3 transfected 293A cells and the pull-down complex was analyzed by Western blot using anti-EphA3 antibody.

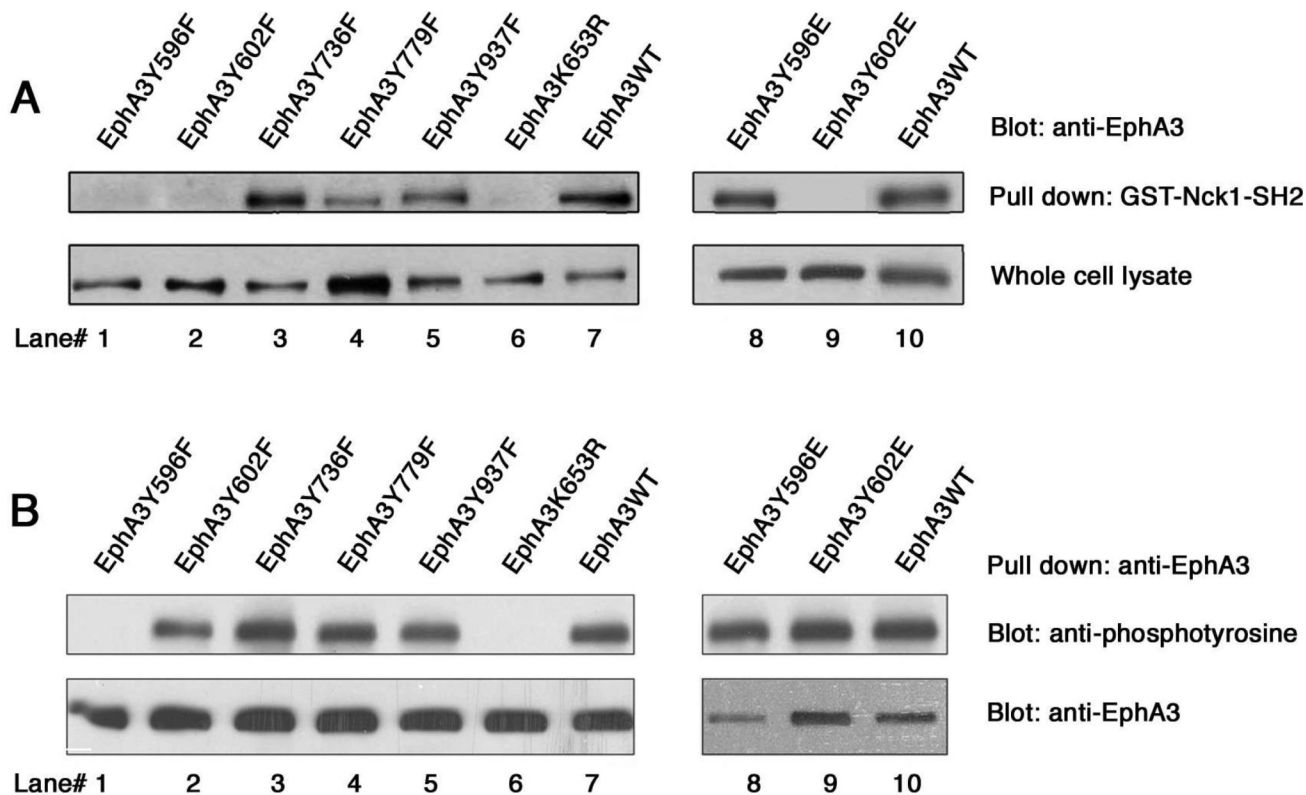


Figure 2. Identification of the Nck1 binding tyrosine residue of EphA3

(A) Y602 mutation inactivated Nck1 binding, as shown through GST fusion protein pull down assay. The GST-Nck1SH2 fusion protein was incubated with the lysate of HEK293A cells expressing wild type or mutant EphA3 proteins. The transfected cells were stimulated with cross-linked ephrin-A5-Fc for 30 minutes (1 μ g/ml) before cell lysis. The precipitated protein complex was subjected to Western blot analysis with anti-EphA3 antibody. (B) Auto-phosphorylation of EphA3 mutants used to map the binding site. Five tyrosine-to-phenylalanine mutants (Y596F, Y602F, Y736F, Y779F, Y937F), two tyrosine-to-glutamic acid mutants (Y596E, Y602E), a kinase inactive mutant (K653R) and wild type EphA3 (WT) were expressed individually in 293A cells. EphA3 proteins were immunoprecipitated from ephrin-A5 treated 293A cell lysates and analyzed with Western blot technique.

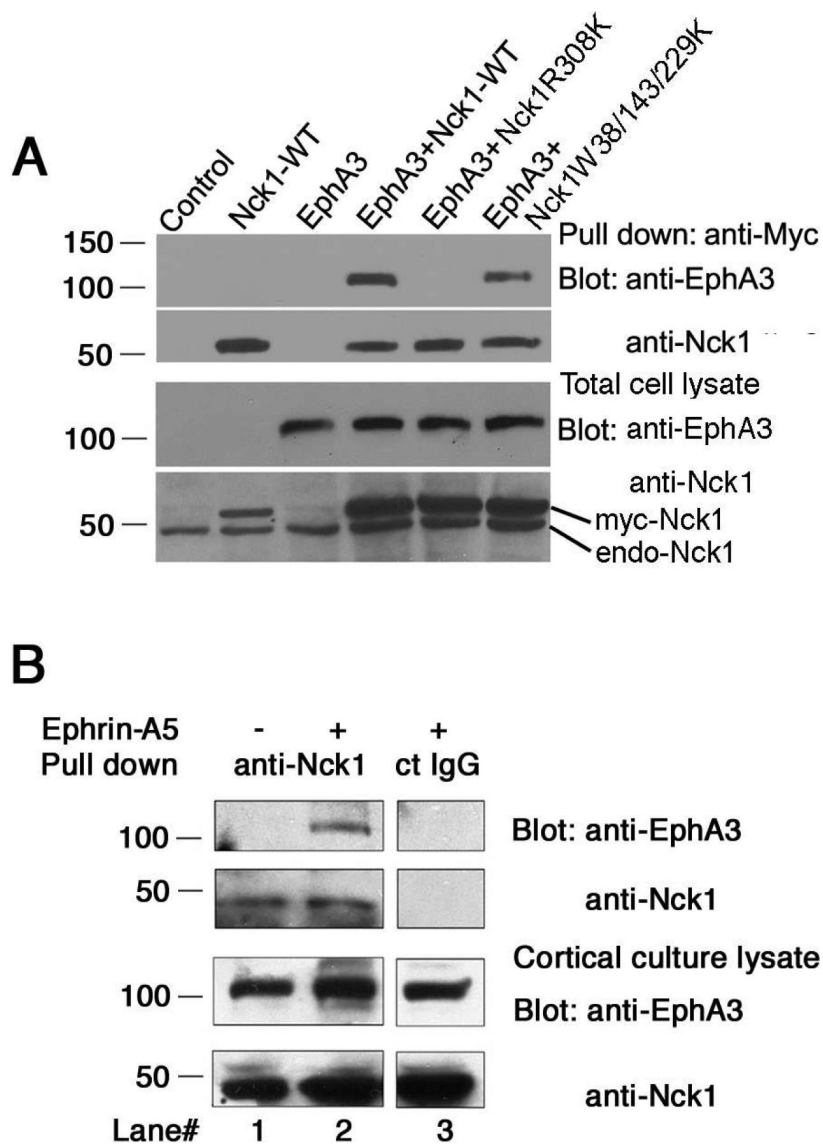


Figure 3. EphA3 interacts with Nck1 in both 293A cells and cultured primary neuron
 (A) Co-immunoprecipitation of EphA3 and Nck1 in 293A cells. EphA3 and *myc*-tagged Nck1 were co-transfected into 293A cells and the Nck1 protein complex was pulled down with a monoclonal anti-*myc* antibody. The immunoprecipitates were then analyzed with anti-EphA3 or anti-*myc* Western blots. Expression levels of endogenous (endo-Nck1) and transfected (*myc*-Nck1) Nck1 in transfected 293A cells are shown in the bottom two panels. (B) Co-immunoprecipitation of Nck1 and EphA3 from primary rat embryonic neuron extracts. E18 cortical neuron cultures were treated with cross-linked ephrin-A5 and the cell lysates were precipitated with either anti-Nck1 antibody or control IgG. Total expression levels of both proteins in the cortical culture lysates were also shown in the bottom two panels.

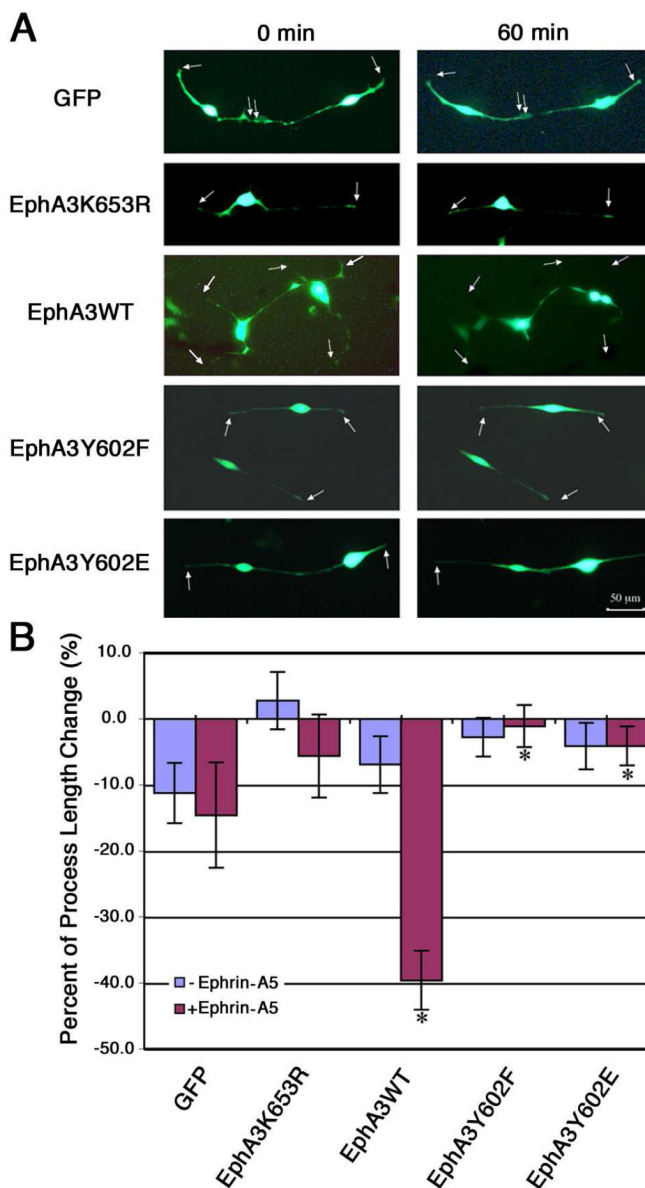


Figure 4. Ephrin-A5 induces cellular process retraction in EphA3-transfected cells
 (A) Effects of EphA3 mutants on cellular process growth. HEK293A cells were transfected with EGFP or various EphA3 mutants and treated with cross-linked ephrin-A5. Select cells were photographed before and after the treatment and the length of all identifiable processes on all cells was measured. Arrows indicate the end of the processes before treatment. (B) Quantification of the effects of EphA3 mutants. Plotted are changes of process length after treatment, compared to the length before the treatment. A positive number indicates process extension during treatment, while a negative value shows retraction. Asterisks indicate statistical significance, when compared to the control with the same treatment (EphA3K653R; student t-test, $p < 0.05$).

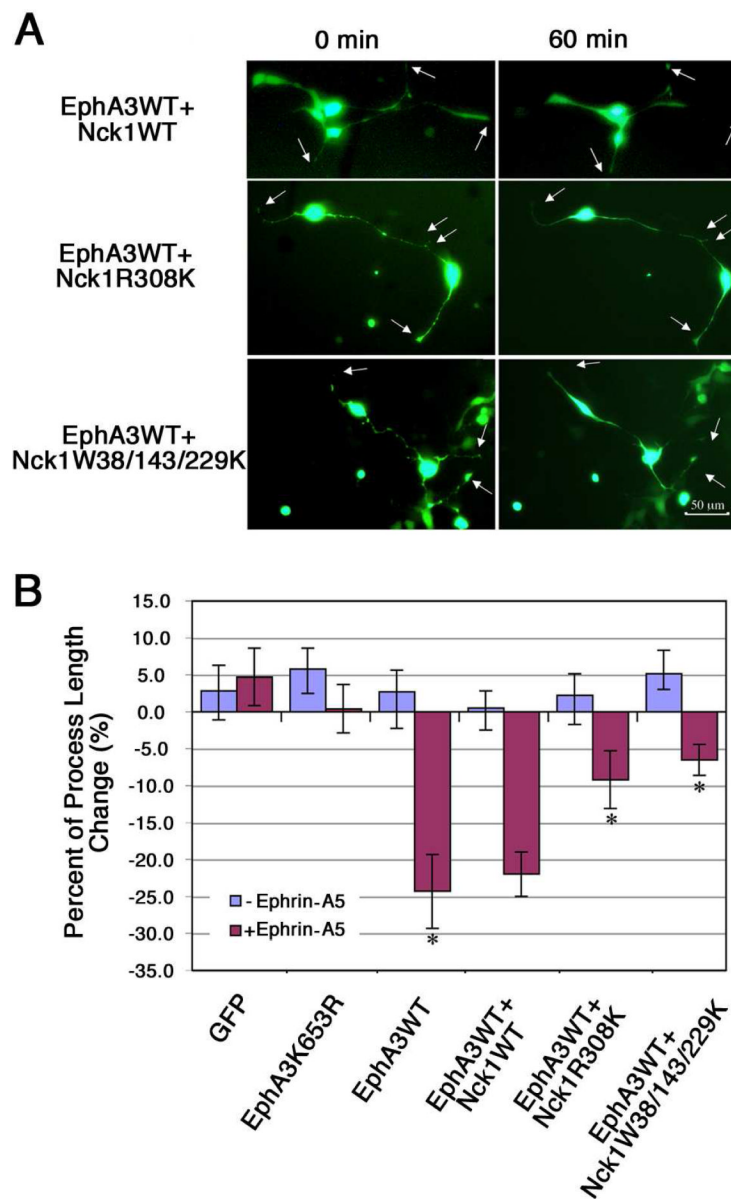


Figure 5. Nck1 mutants block EphA3-induced process retraction

(A) Various Nck1 mutants were co-transfected with EphA3WT to examine their effects on process retraction induced by ephrin-A5 stimulation. Transfected cells were treated and quantified as described in Fig. 4 legend. Arrows show the ends of cellular processes before ligand addition. (B) Quantitative analysis of EphA3-induced process retraction, plotted in a fashion similar to Fig. 4B. Asterisks show statistical significance, when results were compared to their proper controls with the same treatment (EphA3KD for EphA3WT, EphA3WT+Nck1WT for EphA3WT+Nck1R308K and EphA3WT+Nck1W38/143/229K; student t-test, $p < 0.05$).

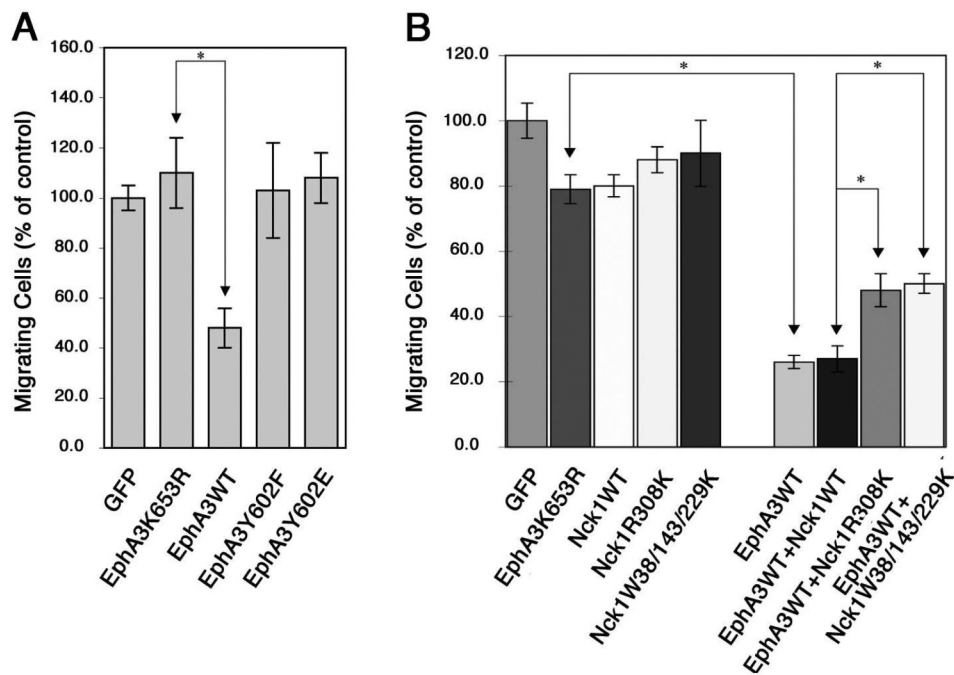


Figure 6. Roles of kinase activity, Y602, and Nck in EphA3-induced inhibition of cell migration
 HEK293 cells were transfected with various constructs as indicated, and the transfected cells were then assayed for cell migration using transwell tissue culture cells in the presence of 2 μ g/ml of cross-linked ephrin-A5-Fc in the bottom chamber. (A) EphA3 kinase activity and Y602 are required for inhibition of cell migration by ephrin-A5. (B) Dominant-negative Nck1 mutants reduces EphA3 inhibition on cell migration. Asterisks indicate statistical significance (student t-test, $p < 0.01$), compared to their proper controls.

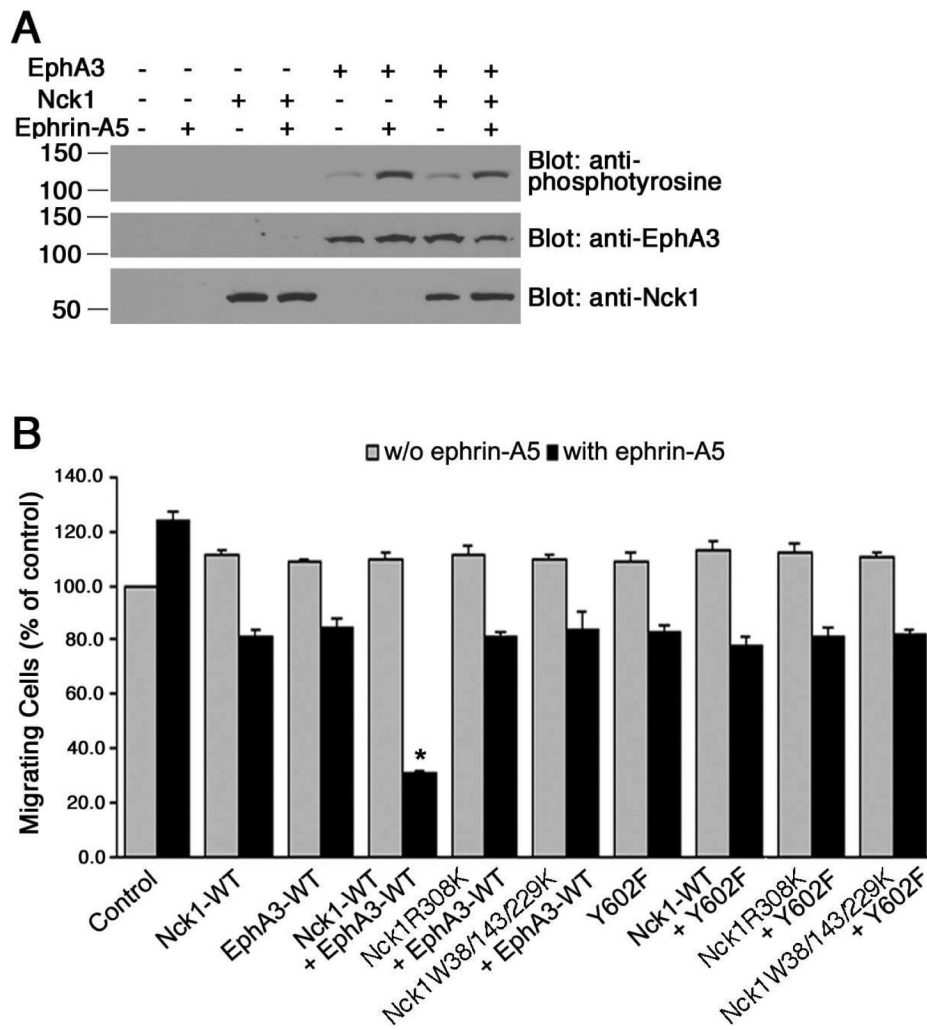


Figure 7. Nck1 acts as a downstream mediator in EphA3-induced inhibition of cell migration
 (A) Expression of EphA3 and Nck1 in transfected cells. MEF cells were transfected with the specified cDNAs and the lysates were analyzed for EphA3 and Nck1 expression. In addition, EphA3 phosphorylation was analyzed using an anti-phosphotyrosine antibody. (B) The expression of wild type Nck1 enables EphA3-mediated inhibition of cell migration. Asterisks indicate statistical significance (student t-test, $p < 0.05$), when compared to their respective controls.