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, participate 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 pulldown 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. Furthermore, we showed that in the absence of Nck function, ephrin-A5 promotes cell migration. These results suggest that Nck1 interacts with EphA3 to regulate cell migration and process retraction.

KEY WORDS: 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 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, 4*). 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, 5*). It was also reported that ephrin-A1/A3/A4 can bind to EphB1 with low affinity (*6*), 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, 7*). 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 (*8-14*). Ephrin-Eph interaction usually produces repulsive effects that lead to tissue boundary formation and axon retraction (*9, 11*). However, attractive effects were also observed in certain biological functions (*15-20*). Although most of these functions depend on the kinase activity of the receptors, kinase independent signaling also exists through the receptors (*21, 22*), or through reverse signaling of the ligands (*23-25*). In addition to the roles during development, Eph receptors have also been shown to regulate adult functions such as learning and memory (*24, 26-28*), and pathological conditions such as tumorigenesis (*29, 30*).

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) (*31-35*). 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 R-Rap or Rac (*36, 37*). These modulations of small GTPase activity trigger changes in cytoskeleton dynamics, causing growth cone collapse and dendritic spine remodeling (*32, 34*). 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 (*38*). Furthermore, activation of EphA2 recruits protein tyrosine phosphatase Shp2 and triggers dephosphorylation of FAK, which also forms a protein complex with EphA2 (*39*). Several other protein tyrosine phosphatases (PTPs), including the low molecular weight (LMW) PTPs (*40, 41*), the receptor-type phosphatase PTPO (*42*), and Src family kinases (*43*), 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 (*24*).

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. Moreover, the complete loss of Nck1 function converts inhibition of cell migration by ephrin-A5 to promotion in mouse embryonic fibroblasts (MEFs). 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-HA 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 with a cell scraper 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% CO_2. 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 (R=transfected cells/all cells). The migration ability (M) of cells expressing different genes was calculated using the following formula: M=T1/(T2 x 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 bind to the 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), confirming the direct interaction of Nck1 with 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 the 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 similar results, except that the

amount of precipitated EphA3 by GST-Nck1 and GST-Nck1SH2 was reduced (data not shown). 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 (*44-47*). 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). 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 (*48*). 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 auto-phosphorylate. Wild type and mutant EphA3 constructs were transiently transfected into 293A cells. Two days after transfection, the cells were 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 HA tagged Nck1 were transiently expressed in HEK293A cells. The expression of both proteins was confirmed by Western blot analysis using anti-EphA3 antibody and anti-HA monoclonal antibody (Fig. 3A). To confirm interaction through co-immunoprecipitation, the transfected cells were stimulated either with 2 μg/ml cross-linked ephrin-A5 or control IgG for 15 minutes, and lysed with lysis buffer. Nck1 protein was then precipitated from the cell lysates with a mouse monoclonal anti-HA 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 with Nck1 only from cell lysates with ephrin-A5 treatment, but not with control stimulation (Fig. 3A, lane 4 and 5). This observation indicates that EphA3 activation by ephrin-A5 is required for Nck1 binding in living cells.

To determine whether EphA3 and Nck1 also interact in primary cells, we examined if Nck1 can be immunoprecipitated from the lysates of embryonic cortical neurons, since both proteins are highly expressed in these cells (*49, 50*). 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 [(*51*), 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 μg/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 concluded, 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 (*52*). 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 (*53*). 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 switch 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. Interestingly, regardless of ephrin-A5 stimulation, expressed EphA3 was highly phosphorylated (Fig. 7A), indicating that overexpression of EphA3 lead to tyrosine phosphorylation in MEF cells.

Surprisingly, in the absence of Nck1 expression, ephrin-A5 promoted the migration of the Nck-null cells. Similarly, expression of EphA3 alone also had a positive effect on cell migration. These observations indicate that in the absence of Nck1, activation of the Eph receptor system has a chemotactic effect on migrating cells. Expression of Nck1 clone also improved cell migration, consistent with previous finding (*49*). However, when Nck1 was expressed, the migration of these cells was now inhibited by ephrin-A5. Similarly, ephrin-A5 inhibited the migration of MEF cells when both EphA3 WT and Nck1 WT are expressed. Taken together, these results suggest that Nck1 switches the responses of MEF cells to ephrin-A5. In the absence of Nck1 function, activation of Eph receptors leads to chemotactic effects for cell migration. Thus, Nck1 acts as a switch of cell migration response to ephrin-A5, as the expression of Nck1 dramatically changes the behavior of the MEF cells from being attracted to repelled by 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 complete loss of inhibition of cell migration by ephrin-A5, suggesting that Nck1 is a key downstream mediator of EphA3 functions. In addition, in the absence of Nck1, ephrin-A5 promotes cell migration, indicating that Nck switches Eph signals from chemotactic to chemorepulsive for cell migration.

Nck1 interacts with activated EphA3. Yeast two-hybrid technique has been widely used to identify protein-protein interactions (*54-56*). Using EphA3 and EphA5 intracellular domains as "baits", 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 (*57*). It was also reported that EphB2 bound to p62 dok after activation and formed a protein complex that included both RasGAP and Nck1 (*38*). 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 (*58-60*). 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 (*61-63*), but different from its binding to DCC

and Robo, which takes place through the SH3 domains instead (*64*). 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 (*65*). 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) (*66*), 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 the absence of ephrin-A5 stimulation, little EphA3 was co-immunoprecipitated by anti-Nck1 antibody from transfected 293A cells or primary neurons. However, we did notice that a small amount of EphA3 could be precipitated with GST-Nck1SH2 and GST-Nck1 from protein lysates of transfected but unstimulated 293A cells (data not shown). This was probably due to the presence of some activated EphA3 in transfected cells because of high levels of expression, which has been shown to activate EphA3 receptor (*67*).

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 (*52, 53*), 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 no longer inhibits migration of these cells.

Different signaling pathways control effects of ephrin-A5 on cell migration. An interesting and unexpected observation in this study is that in the absence of both Nck1 and Nck2, mouse embryo fibroblasts are attracted by ephrin-A5, suggesting that an Nck-independent pathway mediates positive effects on cell migration. The nature of this pathway is not known at present. It has been reported that Eph receptors can mediate either repulsion or attraction of migrating cells, depending on ligand concentration or cellular content (*68-70*). Another possibility is that Eph receptors may interact with other chemoattractive signaling system such as ErbB2 signaling to promote chemotaxis (*71*). Further studies will determine if any of these pathways are indeed responsible for the attractive activity of ephrin-A5. Introduction of Nck1 expression in 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.

Nck1 has been shown to interact with the p21-activated kinase (PAK1) via its second SH3 domain (*72-74*) and translocate PAK1 from the cytosol to plasma membrane. As a result, PAK1 is brought to close proximity of Rac1 and Cdc42, and activated (*75*). 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 (*76, 77*). Inactivation of PAK1 leads to cofilin dephosphorylation and actin depolymerization. Indeed PAK1 activity is reduced in ephrin-A1 treated cortical neurons (*32*), 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 (*78, 79*). 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 (*80-83*). WASP mutations were first discovered in an X chromosome linked immuno-deficiency disease (Wiskott-Aldrich syndrome) (*84*), which was characterized by cell abnormalities and actin cytoskeleton defects. The WASP family proteins possess a C-terminal motif that binds to Arp2/3 (*82, 85, 86*) and activate actin re-organization (*87*). When Nck1 SH3 domain was overexpressed in cells along with N-WASP and Arp2/3, an unusually high rate of actin nucleation was observed (*82*). Indeed, Clustering of Nck1 SH3 domain alone is sufficient to induce localized actin polymerization (*63*), suggesting that the SH3 domains mediate actin cytoskeleton regulation. N-WASP is highly enriched in the brain and regulates neurite growth (*81*). Thus, Nck1 may function through the WASP-WIP 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) (*88*). Interestingly, when the Drosophila NIK homolog, Misshapen, was mutated, the photoreceptor axon targeting was disrupted (*89*), in a manner similar to the phenotype of Dock (Nck1 homolog in Drosophila) knockout (*90*). Nck1 has been shown to interact with more than 30 proteins, most of which have been implicated in actin cytoskeleton re-organization (*59*). 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 (*91*).

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(1) Pasquale, E. B. (2005) Eph receptor signalling casts a wide net on cell behaviour. *Nature Reviews Molecular Cell Biology 6*, 462-75.

(2) Gale, N. W., Holland, S. J., Valenzuela, D. M., Flenniken, A., Pan, L., Ryan, T. E., Henkemeyer, M., Strebhardt, K., Hirai, H., Wilkinson, D. G., Pawson, T., Davis, S., and Yancopoulos, G. D. (1996) Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. *Neuron 17*, 9-19.

(3) Zhou, R. (1998) The Eph family receptors and ligands. *Pharmacol Ther 77*, 151-81.

(4) Kullander, K., and Klein, R. (2002) Mechanisms and functions of Eph and ephrin signalling. *Nat Rev Mol Cell Biol 3*, 475-86.

(5) Himanen, J. P., Chumley, M. J., Lackmann, M., Li, C., Barton, W. A., Jeffrey, P. D., Vearing, C., Geleick, D., Feldheim, D. A., Boyd, A. W., Henkemeyer, M., and Nikolov, D. B. (2004) Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling. *Nat Neurosci 7*, 501-9. Epub 2004 Apr 25.

(6) Beckmann, M. P., Cerretti, D. P., Baum, P., Vanden Bos, T., James, L., Farrah, T., Kozlosky, C., Hollingsworth, T., Shilling, H., Maraskovsky, E., and et al. (1994) Molecular characterization of a family of ligands for eph-related tyrosine kinase receptors. *Embo J 13*, 3757-62.

(7) Boyd, A. W., and Lackmann, M. (2001) Signals from Eph and ephrin proteins: a developmental tool kit. *Science's Stke [Electronic Resource]: Signal Transduction Knowledge Environment 2001*, RE20.

(8) Krull, C. E. (1998) Inhibitory interactions in the patterning of trunk neural crest migration. *Annals of the New York Academy of Sciences 857*, 13-22.
(9) Flanagan, J. G., and Vanderhaeghen, P. (1998) The ephrins and Eph receptors in neural development. *Annu Rev Neurosci 21*, 309-45.
(10) Brantley-Sieders, D. M., and Chen, J. (2004) Eph receptor tyrosine kinases in angiogenesis: from development to disease. *Angiogenesis 7*, 17-28.
(11) Poliakov, A., Cotrina, M., and Wilkinson, D. G. (2004) Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. *Developmental Cell 7*, 465-80.
(12) McLaughlin, T., and O'Leary, D. D. (2005) Molecular gradients and development of retinotopic maps. *Annual Review of Neuroscience 28*, 327-55.
(13) Chin-Sang, I. D., George, S. E., Ding, M., Moseley, S. L., Lynch, A. S., and Chisholm, A. D. (1999) The ephrin VAB-2/EFN-1 functions in neuronal signaling to regulate epidermal morphogenesis in C. elegans. *Cell 99*, 781-90.
(14) George, S. E., Simokat, K., Hardin, J., and Chisholm, A. D. (1998) The VAB-1 Eph receptor tyrosine kinase functions in neural and epithelial morphogenesis in C. elegans. *Cell 92*, 633-43.
(15) Magal, E., Holash, J. A., Toso, R. J., Chang, D., Lindberg, R. A., and Pasquale, E. B. (1996) B61, a ligand for the Eck receptor protein-tyrosine kinase, exhibits neurotrophic activity in cultures of rat spinal cord neurons. *J Neurosci Res 43*, 735-44.
(16) Gao, P. P., Sun, C. H., Zhou, X. F., DiCicco-Bloom, E., and Zhou, R. (2000) Ephrins stimulate or inhibit neurite outgrowth and survival as a function of neuronal cell type [In Process Citation]. *J Neurosci Res 60*, 427-36.
(17) Zhou, X., Suh, J., Cerretti, D. P., Zhou, R., and DiCicco-Bloom, E. (2001) Ephrins stimulate neurite outgrowth during early cortical neurogenesis. *J Neurosci Res 66*, 1054-63.
(18) Hindges, R., McLaughlin, T., Genoud, N., Henkemeyer, M., and O'Leary, D. (2002) EphB Forward Signaling Controls Directional Branch Extension and Arborization Required for Dorsal-Ventral Retinotopic Mapping. *Neuron 35*, 475.
(19) Holmberg, J., and Frisen, J. (2002) Ephrins are not only unattractive. *Trends Neurosci 25*, 239-43.
(20) Eberhart, J., Barr, J., O'Connell, S., Flagg, A., Swartz, M. E., Cramer, K. S., Tosney, K. W., Pasquale, E. B., and Krull, C. E. (2004) Ephrin-A5 exerts positive or inhibitory effects on distinct subsets of EphA4-positive motor neurons. *Journal of Neuroscience 24*, 1070-8.
(21) Matsuoka, H., Obama, H., Kelly, M. L., Matsui, T., and Nakamoto, M. (2005) Biphasic functions of the kinase-defective Ephb6 receptor in cell adhesion and migration. *Journal of Biological Chemistry 280*, 29355-63.
(22) Miao, H., Strebhardt, K., Pasquale, E. B., Shen, T. L., Guan, J. L., and Wang, B. (2005) Inhibition of integrin-mediated cell adhesion but not directional cell migration requires catalytic activity of EphB3 receptor tyrosine kinase. Role of Rho family small GTPases. *J Biol Chem 280*, 923-32.
(23) Davy, A., and Soriano, P. (2005) Ephrin signaling in vivo: look both ways. *Dev Dyn 232*, 1-10.
(24) Grunwald, I. C., Korte, M., Wolfer, D., Wilkinson, G. A., Unsicker, K., Lipp, H. P., Bonhoeffer, T., and Klein, R. (2001) Kinase-Independent Requirement of EphB2 Receptors in Hippocampal Synaptic Plasticity. *Neuron 32*, 1027-40.
(25) Lu, Q., Sun, E. E., Klein, R. S., and Flanagan, J. G. (2001) Ephrin-B reverse signaling is mediated by a novel PDZ-RGS protein and selectively inhibits G protein-coupled chemoattraction. *Cell 105*, 69-79.
(26) Dalva, M. B., Takasu, M. A., Lin, M. Z., Shamah, S. M., Hu, L., Gale, N. W., and Greenberg, M. E. (2000) EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. *Cell 103*, 945-56.
(27) Henderson, J. T., Georgiou, J., Jia, Z., Robertson, J., Elowe, S., Roder, J. C., and Pawson, T. (2001) The Receptor Tyrosine Kinase EphB2 Regulates NMDA-Dependent Synaptic Function. *Neuron 32*, 1041-56.
(28) Takasu, M. A., Dalva, M. B., Zigmond, R. E., and Greenberg, M. E. (2002) Modulation of NMDA

receptor-dependent calcium influx and gene expression through EphB receptors. *Science 295*, 491-5.
(29) Surawska, H., Ma, P. C., and Salgia, R. (2004) The role of ephrins and Eph receptors in cancer. *Cytokine Growth Factor Rev 15*, 419-33.
(30) Brantley-Sieders, D., Schmidt, S., Parker, M., and Chen, J. (2004) Eph receptor tyrosine kinases in tumor and tumor microenvironment. *Curr Pharm Des 10*, 3431-42.
(31) Penzes, P., Beeser, A., Chernoff, J., Schiller, M. R., Eipper, B. A., Mains, R. E., and Huganir, R. L. (2003) Rapid induction of dendritic spine morphogenesis by trans-synaptic ephrinB-EphB receptor activation of the Rho-GEF kalirin. *Neuron 37*, 263-74.
(32) Shamah, S. M., Lin, M. Z., Goldberg, J. L., Estrach, S., Sahin, M., Hu, L., Bazalakova, M., Neve, R. L., Corfas, G., Debant, A., and Greenberg, M. E. (2001) EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin. *Cell 105*, 233-44.
(33) Kubo, T., Yamashita, T., Yamaguchi, A., Sumimoto, H., Hosokawa, K., and Tohyama, M. (2002) A novel FERM domain including guanine nucleotide exchange factor is involved in Rac signaling and regulates neurite remodeling. *J Neurosci 22*, 8504-13.
(34) Sahin, M., Greer, P. L., Lin, M. Z., Poucher, H., Eberhart, J., Schmidt, S., Wright, T. M., Shamah, S. M., O'Connell, S., Cowan, C. W., Hu, L., Goldberg, J. L., Debant, A., Corfas, G., Krull, C. E., and Greenberg, M. E. (2005) Eph-dependent tyrosine phosphorylation of ephexin1 modulates growth cone collapse.[see comment]. *Neuron 46*, 191-204.
(35) Cowan, C. W., Shao, Y. R., Sahin, M., Shamah, S. M., Lin, M. Z., Greer, P. L., Gao, S., Griffith, E. C., Brugge, J. S., and Greenberg, M. E. (2005) Vav family GEFs link activated Ephs to endocytosis and axon guidance.[see comment]. *Neuron 46*, 205-17.
(36) Iwasato, T., Katoh, H., Nishimaru, H., Ishikawa, Y., Inoue, H., Saito, Y. M., Ando, R., Iwama, M., Takahashi, R., Negishi, M., and Itohara, S. (2007) Rac-GAP alpha-chimerin regulates motor-circuit formation as a key mediator of EphrinB3/EphA4 forward signaling. *Cell 130*, 742-53.
(37) Wegmeyer, H., Egea, J., Rabe, N., Gezelius, H., Filosa, A., Enjin, A., Varoqueaux, F., Deininger, K., Schnutgen, F., Brose, N., Klein, R., Kullander, K., and Betz, A. (2007) EphA4-dependent axon guidance is mediated by the RacGAP alpha2-chimaerin. *Neuron 55*, 756-67.
(38) Holland, S. J., Gale, N. W., Gish, G. D., Roth, R. A., Songyang, Z., Cantley, L. C., Henkemeyer, M., Yancopoulos, G. D., and Pawson, T. (1997) Juxtamembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells. *Embo J 16*, 3877-88.
(39) Miao, H., Burnett, E., Kinch, M., Simon, E., and Wang, B. (2000) Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation. *Nat Cell Biol 2*, 62-9.
(40) Parri, M., Buricchi, F., Taddei, M. L., Giannoni, E., Raugei, G., Ramponi, G., and Chiarugi, P. (2005) EphrinA1 repulsive response is regulated by an EphA2 tyrosine phosphatase. *J Biol Chem 280*, 34008-18.
(41) Kikawa, K. D., Vidale, D. R., Van Etten, R. L., and Kinch, M. S. (2002) Regulation of the EphA2 kinase by the low molecular weight tyrosine phosphatase induces transformation. *J Biol Chem 277*, 39274-9.
(42) Shintani, T., Ihara, M., Sakuta, H., Takahashi, H., Watakabe, I., and Noda, M. (2006) Eph receptors are negatively controlled by protein tyrosine phosphatase receptor type O. *Nat Neurosci 9*, 761-9.
(43) Knoll, B., and Drescher, U. (2004) Src family kinases are involved in EphA receptor-mediated retinal axon guidance. *J Neurosci 24*, 6248-57.
(44) Frese, S., Schubert, W. D., Findeis, A. C., Marquardt, T., Roske, Y. S., Stradal, T. E., and Heinz, D. W. (2006) The phosphotyrosine peptide binding specificity of Nck1 and Nck2 Src homology 2 domains. *J Biol Chem 281*, 18236-45.
(45) Wunderlich, L., Farago, A., Downward, J., and Buday, L. (1999) Association of Nck with tyrosine-phosphorylated SLP-76 in activated T lymphocytes. *Eur J Immunol 29*, 1068-75.
(46) Tanaka, M., Gupta, R., and Mayer, B. J. (1995) Differential inhibition of signaling pathways by dominant-negative SH2/SH3 adapter proteins. *Mol Cell Biol 15*, 6829-37.
(47) Pawson, T. (1994) SH2 and SH3 domains in signal transduction. *Adv Cancer Res 64*, 87-110.

(48) Zisch, A. H., Pazzagli, C., Freeman, A. L., Schneller, M., Hadman, M., Smith, J. W., Ruoslahti, E., and Pasquale, E. B. (2000) Replacing two conserved tyrosines of the EphB2 receptor with glutamic acid prevents binding of SH2 domains without abrogating kinase activity and biological responses. *Oncogene 19*, 177-87.

(49) Bladt, F., Aippersbach, E., Gelkop, S., Strasser, G. A., Nash, P., Tafuri, A., Gertler, F. B., and Pawson, T. (2003) The murine Nck SH2/SH3 adaptors are important for the development of mesoderm-derived embryonic structures and for regulating the cellular actin network. *Mol Cell Biol 23*, 4586-97.

(50) Kudo, C., Ajioka, I., Hirata, Y., and Nakajima, K. (2005) Expression profiles of EphA3 at both the RNA and protein level in the developing mammalian forebrain. *J Comp Neurol 487*, 255-69.

(51) Lawrenson, I. D., Wimmer-Kleikamp, S. H., Lock, P., Schoenwaelder, S. M., Down, M., Boyd, A. W., Alewood, P. F., and Lackmann, M. (2002) Ephrin-A5 induces rounding, blebbing and de-adhesion of EphA3- expressing 293T and melanoma cells by CrkII and Rho-mediated signalling. *J Cell Sci 115*, 1059-72.

(52) Tanaka, M., Gupta, R., and Mayer, B. J. (1995) Differential inhibition of signaling pathways by dominant-negative SH2/SH3 adapter proteins. *Molecular & Cellular Biology 15*, 6829-37.

(53) Gupta, R. W., and Mayer, B. J. (1998) Dominant-negative mutants of the SH2/SH3 adapters Nck and Grb2 inhibit MAP kinase activation and mesoderm-specific gene induction by eFGF in Xenopus. *Oncogene 17*, 2155-65.

(54) Fields, S., and Song, O. (1989) A novel genetic system to detect protein-protein interactions. *Nature 340*, 245-6.

(55) Keegan, L., Gill, G., and Ptashne, M. (1986) Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. *Science 231*, 699-704.

(56) Chien, C. T., Bartel, P. L., Sternglanz, R., and Fields, S. (1991) The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc Natl Acad Sci U S A 88*, 9578-82.

(57) Stein, E., Lane, A. A., Cerretti, D. P., Schoecklmann, H. O., Schroff, A. D., Van Etten, R. L., and Daniel, T. O. (1998) Eph receptors discriminate specific ligand oligomers to determine alternative signaling complexes, attachment, and assembly responses. *Genes Dev 12*, 667-78.

(58) McCarty, J. H. (1998) The Nck SH2/SH3 adaptor protein: a regulator of multiple intracellular signal transduction events. *Bioessays 20*, 913-21.

(59) Li, W., Fan, J., and Woodley, D. T. (2001) Nck/Dock: an adapter between cell surface receptors and the actin cytoskeleton. *Oncogene 20*, 6403-17.

(60) Buday, L., Wunderlich, L., and Tamas, P. (2002) The Nck family of adapter proteins. Regulators of actin cytoskeleton. *Cell Signal 14*, 723-31.

(61) Tang, J., Feng, G. S., and Li, W. (1997) Induced direct binding of the adapter protein Nck to the GTPase-activating protein-associated protein p62 by epidermal growth factor. *Oncogene 15*, 1823-32.

(62) Roche, S., McGlade, J., Jones, M., Gish, G. D., Pawson, T., and Courtneidge, S. A. (1996) Requirement of phospholipase C gamma, the tyrosine phosphatase Syp and the adaptor proteins Shc and Nck for PDGF-induced DNA synthesis: evidence for the existence of Ras-dependent and Ras-independent pathways. *EMBO Journal 15*, 4940-8.

(63) Rivera, G. M., Antoku, S., Gelkop, S., Shin, N. Y., Hanks, S. K., Pawson, T., and Mayer, B. J. (2006) Requirement of Nck adaptors for actin dynamics and cell migration stimulated by platelet-derived growth factor B. *Proceedings of the National Academy of Sciences of the United States of America 103*, 9536-41.

(64) Li, X., Meriane, M., Triki, I., Shekarabi, M., Kennedy, T. E., Larose, L., and Lamarche-Vane, N. (2002) The adaptor protein Nck-1 couples the netrin-1 receptor DCC (deleted in colorectal cancer) to the activation of the small GTPase Rac1 through an atypical mechanism. *Journal of Biological Chemistry 277*, 37788-97.

(65) Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., and et al. (1993) SH2 domains recognize specific phosphopeptide sequences. *Cell 72*, 767-78.

(66) Stein, E., Huynh-Do, U., Lane, A. A., Cerretti, D. P., and Daniel, T. O. (1998) Nck recruitment to Eph receptor, EphB1/ELK, couples ligand activation to c-Jun kinase. *J Biol Chem 273*, 1303-8.

(67) Lawrenson, I. D., Wimmer-Kleikamp, S. H., Lock, P., Schoenwaelder, S. M., Down, M., Boyd, A. W., Alewood, P. F., and Lackmann, M. (2002) Ephrin-A5 induces rounding, blebbing and de-adhesion of EphA3-expressing 293T and melanoma cells by CrkII and Rho-mediated signalling. *J Cell Sci 115*, 1059-72.

(68) Vindis, C., Cerretti, D. P., Daniel, T. O., and Huynh-Do, U. (2003) EphB1 recruits c-Src and p52Shc to activate MAPK/ERK and promote chemotaxis. *J Cell Biol 162*, 661-71.

(69) Matsuoka, H., Obama, H., Kelly, M. L., Matsui, T., and Nakamoto, M. (2005) Biphasic functions of the kinase-defective Ephb6 receptor in cell adhesion and migration. *J Biol Chem 280*, 29355-63.

(70) Yang, N. Y., Pasquale, E. B., Owen, L. B., and Ethell, I. M. (2006) The EphB4 receptor-tyrosine kinase promotes the migration of melanoma cells through Rho-mediated actin cytoskeleton reorganization. *J Biol Chem 281*, 32574-86.

(71) Brantley-Sieders, D. M., Zhuang, G., Hicks, D., Fang, W. B., Hwang, Y., Cates, J. M., Coffman, K., Jackson, D., Bruckheimer, E., Muraoka-Cook, R. S., and Chen, J. (2008) The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling. *J Clin Invest 118*, 64-78.

(72) Galisteo, M. L., Chernoff, J., Su, Y. C., Skolnik, E. Y., and Schlessinger, J. (1996) The adaptor protein Nck links receptor tyrosine kinases with the serine-threonine kinase Pak1. *Journal of Biological Chemistry 271*, 20997-1000.

(73) Lu, W., Katz, S., Gupta, R., and Mayer, B. J. (1997) Activation of Pak by membrane localization mediated by an SH3 domain from the adaptor protein Nck. *Curr Biol 7*, 85-94.

(74) Zhao, Z. S., Manser, E., and Lim, L. (2000) Interaction between PAK and nck: a template for Nck targets and role of PAK autophosphorylation. *Molecular & Cellular Biology 20*, 3906-17.

(75) Bokoch, G. M. (2003) Biology of the p21-activated kinases. *Annual Review of Biochemistry 72*, 743-81.

(76) Edwards, D. C., Sanders, L. C., Bokoch, G. M., and Gill, G. N. (1999) Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics.[see comment]. *Nature Cell Biology 1*, 253-9.

(77) Sanders, L. C., Matsumura, F., Bokoch, G. M., and de Lanerolle, P. (1999) Inhibition of myosin light chain kinase by p21-activated kinase. *Science 283*, 2083-2085.

(78) Garrity, P. A., Rao, Y., Salecker, I., McGlade, J., Pawson, T., and Zipursky, S. L. (1996) Drosophila photoreceptor axon guidance and targeting requires the dreadlocks SH2/SH3 adapter protein. *Cell 85*, 639-50.

(79) Hing, H., Xiao, J., Harden, N., Lim, L., and Zipursky, S. L. (1999) Pak functions downstream of Dock to regulate photoreceptor axon guidance in Drosophila. *Cell 97*, 853-63.

(80) Samarin, S. N. (2005) WASP family proteins act between cytoskeleton and cellular signaling pathways. *Biochemistry (Mosc) 70*, 1305-9.

(81) Takenawa, T., and Suetsugu, S. (2007) The WASP-WAVE protein network: connecting the membrane to the cytoskeleton. *Nat Rev Mol Cell Biol 8*, 37-48.

(82) Rohatgi, R., Nollau, P., Ho, H. Y., Kirschner, M. W., and Mayer, B. J. (2001) Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. *J Biol Chem 276*, 26448-52.

(83) Rivera, G. M., Briceno, C. A., Takeshima, F., Snapper, S. B., and Mayer, B. J. (2004) Inducible clustering of membrane-targeted SH3 domains of the adaptor protein Nck triggers localized actin polymerization. *Current Biology 14*, 11-22.

(84) Derry, J. M., Ochs, H. D., and Francke, U. (1994) Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. *Cell 78*, 635-44.

(85) Machesky, L. M., Mullins, R. D., Higgs, H. N., Kaiser, D. A., Blanchoin, L., May, R. C., Hall, M. E., and Pollard, T. D. (1999) Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex. *Proc Natl Acad Sci U S A 96*, 3739-44.

(86) Ibarra, N., Pollitt, A., and Insall, R. H. (2005) Regulation of actin assembly by SCAR/WAVE proteins. *Biochem Soc Trans 33*, 1243-6.

(87) Zalevsky, J., Lempert, L., Kranitz, H., and Mullins, R. D. (2001) Different WASP family proteins stimulate different Arp2/3 complex-dependent actin-nucleating activities. *Curr Biol 11*, 1903-13.

(88) Becker, E., Huynh-Do, U., Holland, S., Pawson, T., Daniel, T. O., and Skolnik, E. Y. (2000) Nck-interacting Ste20 kinase couples Eph receptors to c-Jun N-terminal kinase and integrin activation. *Mol Cell Biol 20*, 1537-45.

(89) Su, Y. C., Treisman, J. E., and Skolnik, E. Y. (1998) The Drosophila Ste20-related kinase misshapen is required for embryonic dorsal closure and acts through a JNK MAPK module on an evolutionarily conserved signaling pathway. *Genes Dev 12*, 2371-80.

(90) Ruan, W., Pang, P., and Rao, Y. (1999) The SH2/SH3 adaptor protein dock interacts with the Ste20-like kinase misshapen in controlling growth cone motility. *Neuron 24*, 595-605.

(91) Fawcett, J. P., Georgiou, J., Ruston, J., Bladt, F., Sherman, A., Warner, N., Saab, B. J., Scott, R., Roder, J. C., and Pawson, T. (2007) Nck adaptor proteins control the organization of neuronal circuits important for walking. *Proc Natl Acad Sci U S A 104*, 20973-8.

Figure 1

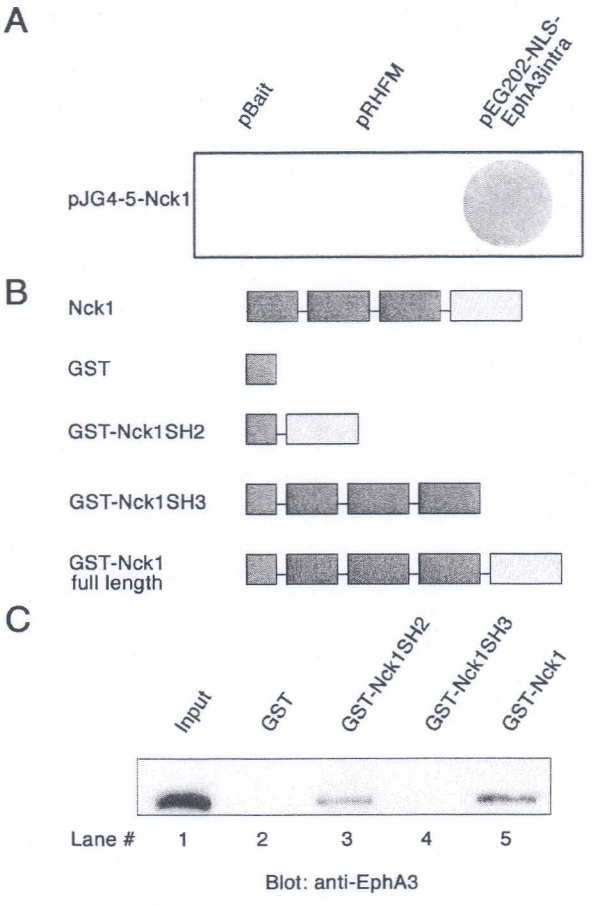

Figure 1. Identification of Nck1 as a major binding protein of EphA3. (A) Yeast mating test. Yeast cells carrying pJG4-5-Nck1 cDNA, the LacZ reporter plasmid, and either an irrelevant control plasmid pRHFM, an empty bait plasmid pBait, or pEG202-NLS-EphA3intra were lysed and incubated with LacZ substrate 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.

Figure 2

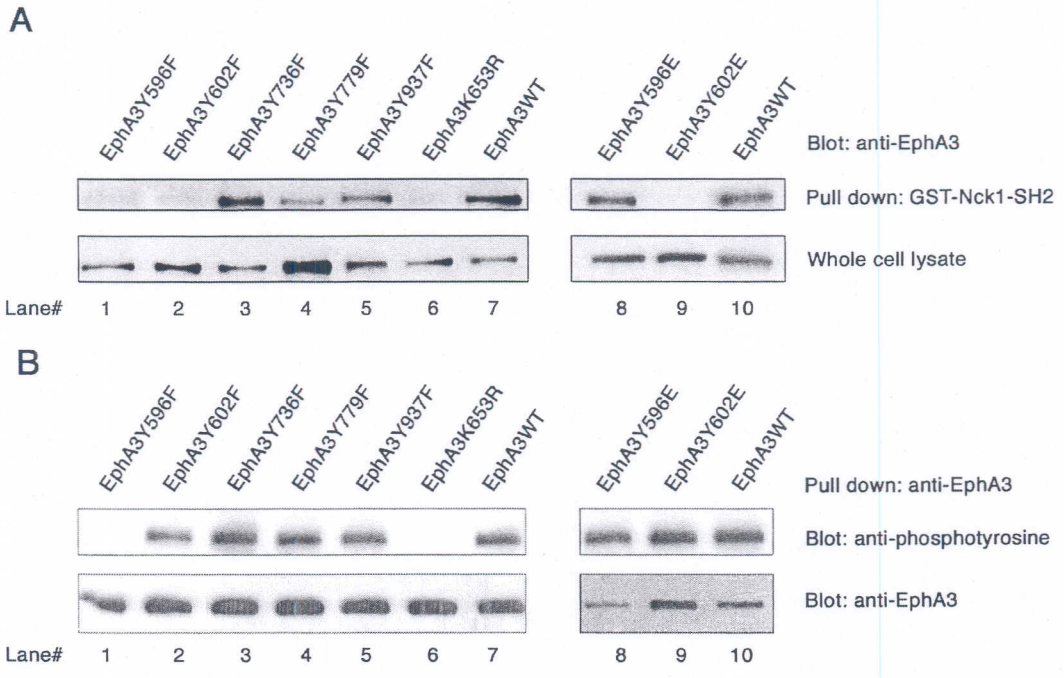

Figure 2. Identification of the Nck1 binding tyrosine residue of EphA3. (A) Auto-phosphorylation of EphA3 mutants used to map the binding site. Five tyrosine-to-phenylalanine mutants (Y596F, Y602F, Y736F, Y779F, Y937F), two tyrosine-to-glutamic acid mutants (Y596E, Y602E), a kinase inactive mutant (K653R) and wild type EphA3 (WT) were expressed individually in 293A cells. EphA3 proteins were immunoprecipitated and analyzed with Western blot technique. (B) Y602 mutation inactivated Nck1 binding, as shown through GST fusion protein pull down assay.

Figure 3

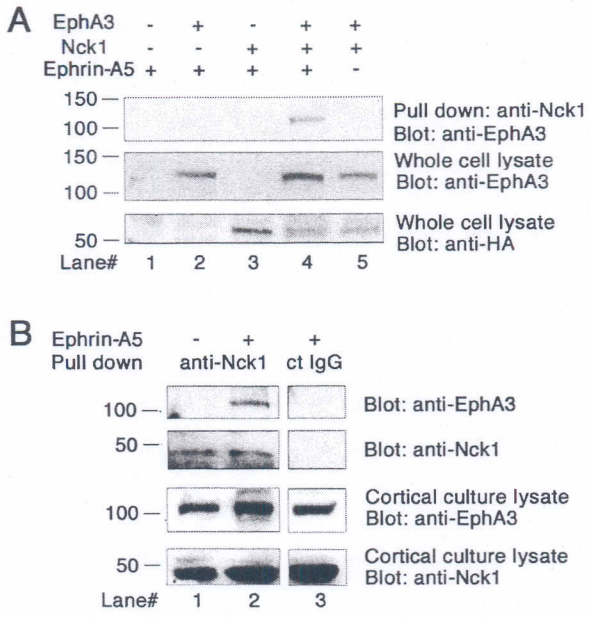

Figure 3. EphA3 interacts with Nck1 in both fibroblasts and primary neuron cultures. (A) EphA3 and Nck1 binding was confirmed in 293A cells through immunoprecipitation. EphA3 and Nck1 were co-transfected into 293A cells and the Nck1 protein complex was pulled down with a monoclonal anti-Nck1 antibody. The immunoprecipitates were then analyzed with anti-EphA3 Western blot. (B) Nck1 and EphA3 are co-immunoprecipitated from primary rat embryonic neurons. 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. The precipitated products were analyzed with Western blot using anti-EphA3 and anti-Nck1 antibodies. Total expression level of both proteins in the cortical culture lysates was also shown.

Figure 4

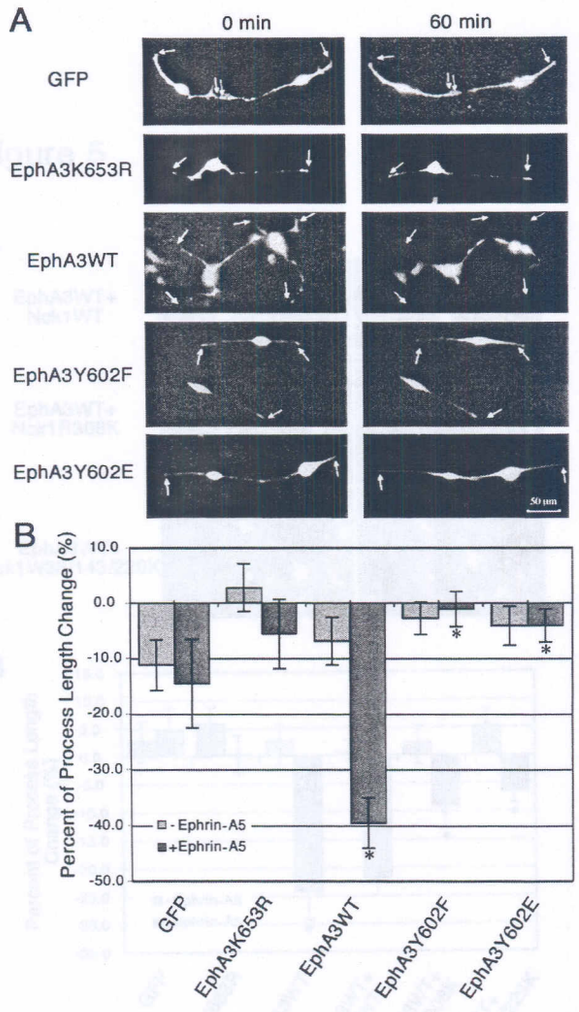

Figure 4. Activation of EphA3 induces cellular process retraction. (A) Effects of EphA3 mutants on cellular process growth. HEK293A cells were transfected with EGFP or various EphA3 mutants and treated with cross-linked ephrin-A5. Select cells were photographed before and after the treatment and the 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 6

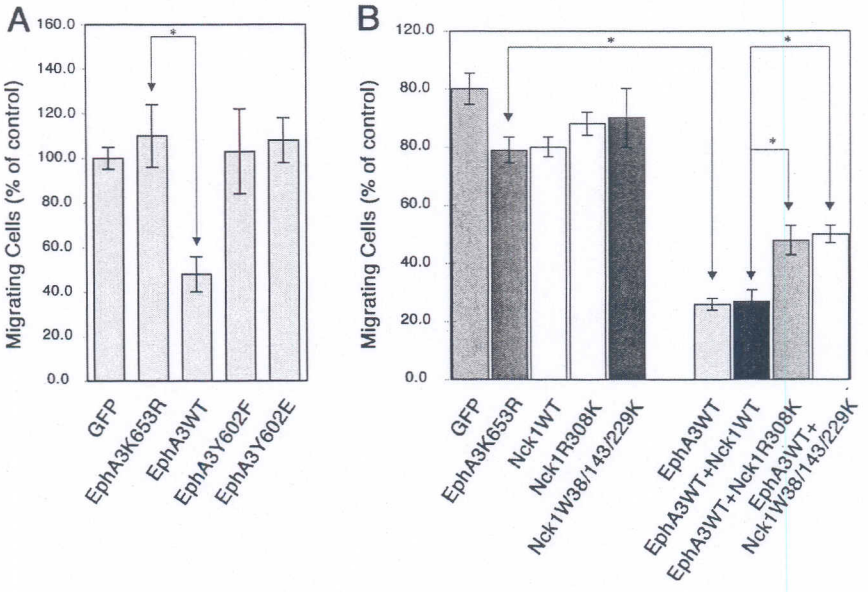

Figure 6. EphA3-induced inhibition of cell migration requires its kinase activity and an intact Y602. Nck1 mediates this function. (A) Various EphA3 clones were transiently transfected into HEK293A cells and tested for effects on cell migration in transwell assay. (B) Dominant-negative Nck1 mutants inhibit EphA3 inhibition on cell migration. Asterisks indicate statistical significance (student t-test, $p<0.01$), compared to their proper controls.

Figure 7

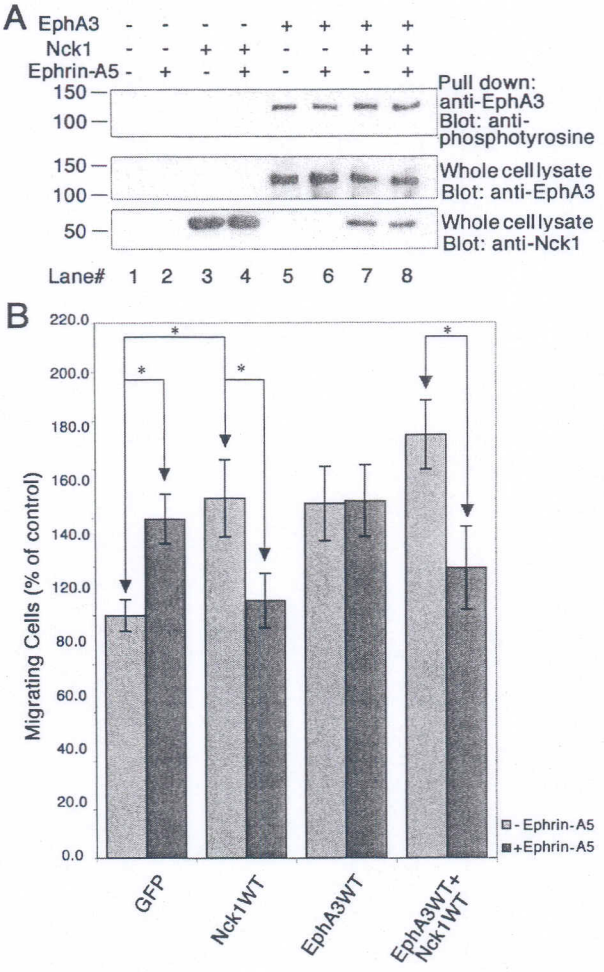

Figure 7. Nck1 acts as a switch in EphA3-mediated inhibition on cell migration. (A) Expression of EphA3 and Nck1 in transfected cells. MEF cells were transfected with specified cDNAs and the lysates were analyzed to show EphA3 and Nck1 expression. In addition, whole cell lysates of transfected cells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

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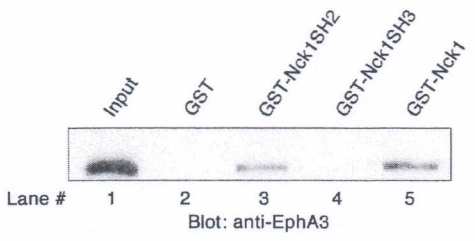

Title:

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

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