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Screening for EphB signaling effectors using SILAC with a linear ion trap-Orbitrap mass spectrometer

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Abstract

Eph receptors play important roles in development, neural plasticity and cancer. We used an Orbitrap mass spectrometer and Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) to identify and quantify 204 proteins with significantly changed abundance in anti-phosphotyrosine immunoprecipitates after ephrinB1-Fc stimulation. More than half of all known effectors downstream of EphB receptors were identified in this study, as well as numerous novel candidates for EphB signaling.

Keywords

EphB; SILAC; mass spectrometry; phosphoproteomics; Orbitrap; IP

Introduction

Erythropoietin-producing hepatocellular carcinoma (Eph) receptors form the largest family of receptor tyrosine kinases (RTKs). There are 16 Eph genes in vertebrate genomes and 14 of them are found in mammals^{1, 2}. Eph receptors are transmembrane proteins with conserved extra- and intracellular domains. The Eph family receptors are divided into two classes, EphA and EphB receptors, based on similarities in their extracellular domains and binding preference for either glycosylphosphatidylinositol linked ephrinA ligands or transmembrane ephrinB ligands.

Activation of Eph receptors generally leads to cytoskeleton rearrangement in the cell. However, recent studies have shown that Eph signaling can have diverse cellular effects in addition to changes in cytoskeleton dynamics depending on the cellular contexts¹. First identified as key regulators of axon guidance during development, Eph receptors have been found to play

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Supporting Information Available: Supporting Information Table 1 shows SILAC ratios and Pfam domain annotations for identified proteins. Supporting Information Table 2 shows the identified phosphopeptides and localization of phosphorylation sites using Ascore. Supporting Information Table 3 shows proteins that showed significantly different SILAC ratios in this study and a previous study that used QTOF mass spectrometry. Supporting Information Table 4 contains a list of all proteins with SILAC ratios showing more than 1.5-fold change after ephrin signaling from both replicates of the Orbitrap analysis (reported here) as well as those found in our previous QTOF study. Supporting Information Table 5 shows known effector proteins in EphB signaling. Supporting Information Figure 1 shows SILAC ratios measured by the QTOF and the Orbitrap of the proteins that were identified in both studies. Supporting Information Figure 2 and Figure 3 show the protein networks from the Ingenuity Pathways Analysis software. Supporting Information File1 and File2 show the annotated MS/MS spectra of identified phosphopeptides. This material is available free of charge via the Internet at <http://pubs.acs.org>.

important roles in tissue patterning, angiogenesis, cell morphogenesis, neural plasticity and cancer¹. Despite intensive studies over the last 20 years leading to more than 2000 publications in Pubmed, Eph signaling is still of a great research interest and new effectors and mechanisms continue to be discovered frequently.

Quantitative proteomics by stable isotope labeling with amino acids in cell culture (SILAC) has quickly become a major tool for high throughput screening analysis for signaling pathways in recent years³. Particularly, in combination with anti-phosphotyrosine (pY) immunoprecipitation (IP), SILAC has shown great promise for the investigation of signaling pathways downstream of RTKs^{4–6}. In these studies, cells are first metabolically labeled with light or heavy amino acids during cell culture. After labeling, in one cell population the RTK is activated to trigger tyrosine phosphorylation of downstream effectors. Then the lysates of the stimulated and the control cells are combined for anti-pY IP to pull down pY proteins together with their tight binding partners. The IPed proteins are then identified and quantified by MS and the relative abundance of the light and heavy versions of a protein is used to indicate whether the protein participates in the RTK pathway or not.

In a previous study, we used SILAC and quadrupole time-of-flight (QTOF) mass spectrometry (MS) to look for effectors in the EphB signaling pathway⁷. In that study, 46 out of 127 identified proteins were found to have changed abundance in pY IPs upon EphB receptor activation. Recently, the hybrid linear ion trap-Orbitrap (LTQ-OrbitrapTM) mass spectrometer has demonstrated great utility in proteomic research^{8–13}. With high speed and sensitivity in tandem mass spectrometry (MS/MS) mode by the linear ion trap, this instrument has been used to identify many more proteins from complex peptide mixtures than a QTOF instrument using data-dependent switching to and from MS/MS¹⁴. Moreover, the high resolution of the Orbitrap can increase the accuracy of peptide quantitation. In this study, we have repeated the SILAC experiment using an LTQ-Orbitrap with the aim of identify more candidate effectors in the EphB pathway.

Materials and Methods

Cell Culture and Cell Stimulation

Metabolic labeling and stimulation of cells were performed as previously described⁷. Briefly, ten 10 cm plates (approximately 10⁸ total cells)/condition NG108 cells (mouse neuroblastoma × rat glioma hybrid) stably overexpressing EphB2 receptor¹⁵ were differentially labeled in medium containing either normal or ¹³C₆ lysine and ¹³C₆ arginine (Cambridge Isotope Labs, Andover, MA). After five cell divisions to ensure nearly complete metabolic labeling, the cells were serum starved for 24 h. One cell population was treated with 2 µg/mL ephrinB1-Fc (Sigma-Aldrich) aggregated with anti-Fc IgG (Jackson ImmunoResearch) for 45 min while the other population was treated with anti-Fc IgG aggregated Fc as a control. Cells were lysed in lysis buffer containing 1% Triton X-100, 150 mM NaCl, 20 mM Tris, pH8, 0.2mM EDTA, 2 mM Na₃VO₄, 2mM NaF, and protease inhibitors (Complete tablet; Roche, Mannheim, Germany).

Immunoprecipitation (IP) and SDS-PAGE

The “light” and “heavy” lysates were mixed in a 1:1 ratio (v:v) and incubated with agarose-conjugated anti-pY antibody PY99 (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h, and the beads were washed 4 times with lysis buffer. Precipitated proteins were eluted with a low pH buffer (pH2) containing 0.2% TFA/1% SDS. The eluates were neutralized with 1M NH₄HCO₃ and separated by SDS-PAGE on a 7.5% Tris-HCl gel (Bio-rad). The gel was stained with Coomassie Blue and the gel lanes were cut horizontally into 10 sections for in-gel tryptic digestion.

In-gel Digestion

Gel bands were cut into small pieces and destained in 25 mM NH_4HCO_3 /50% acetonitrile, dehydrated with acetonitrile and dried. Then the gel pieces were rehydrated with 12.5 ng/ μl trypsin solution in 25 mM NH_4HCO_3 and incubated overnight at 37 °C. Peptides were extracted twice with 5% formic acid/50% acetonitrile followed by a final extraction with acetonitrile. Samples were concentrated by vacuum centrifugation to dryness and redissolved with 2% acetonitrile in 0.1% formic acid before further analysis.

Liquid Chromatography (LC)-MS/MS

An LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) equipped with a nano-ESI source (Jamie Hill Instrument Services) was used for all LC-MS/MS analyses. A Nano-Acquity UPLC system (Waters) equipped with a 100- $\mu\text{m} \times 15\text{-cm}$ reverse phase column (Symmetry C18, Waters) was coupled directly to the ion trap instrument via a 10- μm -inner diameter PicoTipTM nanoelectrospray emitter (New Objective). Samples were loaded onto a trap column (180 $\mu\text{m} \times 2\text{ cm}$ Symmetry C18, Waters) with 2% acetonitrile in 0.1% formic acid for 4 min at 5 $\mu\text{l}/\text{min}$. After sample loading, the flow rate was reduced to 0.4 $\mu\text{l}/\text{min}$ and directed through the analytical column, and peptides were eluted by a gradient of 6–40% acetonitrile in 0.1% formic acid over 120 min. Mass spectra were acquired in data-dependent mode with one 60,000 resolution MS survey scan by the Orbitrap and up to five concurrent MS/MS scans in the LTQ for the five most intense peaks selected from each survey scan. Automatic gain control was set to 500,000 for Orbitrap survey scans and 10,000 for LTQ MS/MS scans. Survey scans were acquired in profile mode and MS/MS scans were acquired in centroid mode. Mascot generic format files were generated from the raw data using DTASuperCharge (version 1.01) and Bioworks (version 3.2, Thermo Fisher Scientific) for database searching.

Protein Identification and Quantitation

Mascot software (version 2.1.0, Matrix Science, London, UK) was used for database searching. An IPI database containing mouse and rat protein sequences (downloaded January 01, 2007) was used. Peptide mass tolerance was 20 ppm, fragment mass tolerance was 0.6 Da, trypsin specificity was applied with a maximum of one missed cleavage, and variable modifications were $^{13}\text{C}_6$ Lys, $^{13}\text{C}_6$ Arg, oxidation of methionine, and phosphorylation of serine, threonine and tyrosine. To estimate the false positive rate for protein identification, a decoy database was created by reversing the protein sequences of the original database. Based on the decoy database search results, three filters for protein identification were applied: (1) Peptide score threshold was 20. (2) Protein score threshold was 40. (3) Each protein was identified based on at least two unique peptide sequences. The estimated false positive rate based on the decoy database search was 0.3%.

To merge the SILAC results of multiple gel fractions from the same sample preparation, all the identified peptide sequences from different gel fractions were combined and searched against the same IPI protein database to obtain the protein matches. Proteins identified based on the same set of peptides were grouped and reported as a single protein match. Proteins that were likely introduced during sample preparation were excluded from the reported protein list. These proteins included keratins and trypsin (from in-gel digestion), immunoglobins (from the PY99 antibody and Fc fusion protein), ephrinB1 (the stimulating ligand), ferritin, and serum albumin (from cell culture media).

SILAC quantitation was carried out using the open source software MSQuant (version 1.4.2a13) developed by Peter Mortensen and Matthias Mann at the University of Southern Denmark. The XIC intensities of the heavy and light peptides were measured, with the results verified by manual inspection of the MS spectra. The SILAC ratios of proteins were calculated by comparing the summed XIC intensities of all matched light peptides with those of the heavy

peptides. As a loading control, a small volume of the combined lysates was subjected to in-gel digestion, LC-MS/MS analysis and the identified proteins were also quantified. The average ratio for all quantified proteins was used as a correction for ratios of proteins identified from the IP.

Phosphorylation Analysis

Phosphopeptides were identified using Mascot. All matched MS/MS spectra were inspected manually. In cases where there were multiple possibilities for the localization of the phosphates on the peptide, a simple statistical model¹⁶ was used to calculate a score (Ascore) based on the number of matching ions for each possible localization using only the site determining ions¹⁷. We used an Ascore threshold of 19, which has been estimated to correspond to 99.5% confidence in site localization¹⁷. In addition, the results were filtered based on the intensity of the peak corresponding to the neutral loss of phosphoric acid (98 Da) from the precursor ion: The assignment was rejected if the peptide did not contain pS or pT and the intensity of the neutral loss peak was larger than 50% of the base peak.

Western Blotting

Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with Tris buffered saline with Tween 20 containing 2% bovine serum albumin, incubated with the corresponding primary and horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology), and visualized with ECL (Pierce Biotechnology, Rockford, IL). Anti-Hrs¹⁸ and anti-STAM2¹⁹ were kind gifts from Dr. Harald Stenmark. Anti-IRS2, anti-Erbin, PY99-HRP (Santa Cruz Biotechnology), anti-SASH1 (Abnova, Taiwan) and anti-EphB4 (R&D Systems, Minneapolis, MN) antibodies were used as indicated by the manufacturers.

Results and Discussion

Protein Identification and SILAC Quantitation

To screen for effectors in the EphB signaling pathway, we used a SILAC strategy that we employed in a previous study⁷. Briefly, two populations of NG108-EphB2 cells (NG108 cells that stably overexpress the EphB2 receptor) were differentially SILAC labeled with ¹³C₆ Lys / ¹³C₆ Arg or ¹²C₆ Lys / ¹²C₆ Arg. One cell population was treated with clustered ephrinB1-Fc to activate the EphB2 receptor while the other (control) population was treated with clustered Fc. After cell lysis, equal volumes of the two lysates were combined for anti-pY IP. Anti-pY Western blotting using the lysates and pY IPs indicated that several proteins were tyrosine phosphorylated after ligand stimulation (Figure 1). The IPed proteins were separated into 10 fractions using SDS-PAGE. Each fraction was digested with trypsin and analyzed by LC-MS with a hybrid LTQ-Orbitrap MS spectrometer for protein identification and quantitation. Coomassie staining of the gel before in-gel digestion revealed a faint haze of blue staining, but the gel contained insufficient protein for visualization of individual proteins (data not shown).

Two SILAC replicates (biological replicates) were carried out, in which 683 and 532 proteins were identified respectively. Figure 2A shows the number of protein identifications in the two replicates.

Of the 804 proteins identified in the two SILAC replicates, 777 (672 from replicate 1 and 513 from replicate 2, 408 from both) were quantified and 27 were not able to be confidently quantified due to poor MS spectral quality (Figure 2B). The protein ratios from the two individual replicates were consistent (Figure 3). A list of the 777 quantified proteins is shown in Supporting Information Table 1.

204 proteins increased at least 1.5-fold in abundance in pY IP after ephrinB1 stimulation and 12 showed at least 1.5-fold decreased abundance. To further remove from the protein list redundancy caused by orthologous proteins from mouse and rat, proteins corresponding to the same gene name were clustered into a single entry. After clustering, 194 proteins showed at least 1.5-fold increased ratios and 10 showed decreased ratios. Table 1 lists the proteins with changed ratios.

Western Blotting Verification

To further verify the SILAC result from the MS analysis, Western Blotting was carried out for five proteins with changed ratios whose antibodies were available. These proteins include: Hrs, STAM2, Erbin, IRS-2 and SASH1. None of these proteins have been previously reported to participate in Eph signaling except in our previous SILAC study⁷ that found Hrs, STAM2, IRS-2 and SASH1. As shown in Figure 4, the Western blotting results for all the selected proteins were consistent with the corresponding SILAC ratios.

The NG108-EphB2 cell line has been used frequently in previous studies of ephrin/Eph signaling^{15, 20–25}. We have observed from our previous SILAC study⁷ that the NG108-EphB2 cells express endogenous EphB4 and EphB3 receptors and upon ephrinB1 stimulation these receptors are activated along with EphB2. This observation was confirmed in this study. To verify that EphB receptors other than EphB2 are expressed in NG108 cells, wild type NG108 cells were treated with ephrinB1 using the same procedure as the SILAC experiment. Lysates and pY IPs were probed with anti-EphB4 and anti-pY antibodies. The anti-EphB4 blot (Figure 4) indicates that EphB4 is expressed in wild type NG108 cells and can be tyrosine phosphorylated upon ligand treatment. The anti-pY blot on the lysates and pY IPs did not show detectable difference between the control and stimulated cells due to interference from basal signals (data not shown), suggesting the effect of Eph signaling in wild type NG108 cells is much subtler than in the NG108-EphB2 cells. Based on these new findings, care should be taken when using NG108 cell lines for Eph signaling studies as well as interpreting results from previous studies in which these cell lines were used.

Phosphorylation Sites

The SILAC experiment identified 128 unique phosphopeptide sequences using Mascot. Because most of these phosphopeptides contain multiple serine/threonine/tyrosine residues, we tried to localize the phosphorylation sites using a simple statistical model for matching site-determining ions in the MS/MS spectra^{16, 17}.

Using this model, we localized 116 phosphorylation sites (38 pS, 12 pT and 66 pY) on 115 peptides. To determine how many of the identified phosphorylation sites are novel, the Swiss-prot knowledgebase (downloaded September 03, 2007) and datasets of phosphorylation sites from several major large scale proteomics studies^{26–30} were searched using in-house written Perl scripts. 67 of the 116 localized phosphorylation sites were not found in these databases (Supporting Information Table 2). The annotated MS/MS spectra of all identified phosphopeptides are included in Supporting Information File1 and File2. File1 contains spectra of phosphopeptides whose phosphorylation sites were localized by Ascores. File2 contains spectra of phosphopeptides whose phosphorylation sites could not be localized by Ascores. For these peptides, the phosphorylation sites corresponding to the best Ascores were used to annotate the fragment ions in the spectra. All the identified phosphopeptides together with their Ascores are listed in Supporting Information Table 2. It is possible that the phosphotyrosine sites on proteins with ratio changes are regulated by the EphB activity, but due to the fact that almost all phosphoproteins have multiple phosphorylation sites, a protein ratio change from anti-pY IP cannot be attributed to the change of a specific pY site. A quantitative experiment that does not involve pY protein IP (for example, phosphopeptide enrichment after digestion

of the whole cell lysate) would be needed to confirm the link between specific pY sites and EphB signaling.

Comparison between the LTQ-Orbitrap results and previous QTOF results

Previously we have carried out a similar SILAC study using an older QTOF instrument (Micromass, QTOF-Micro, installed in 2003)⁷. Figure 2B and 2C show a comparison of the numbers of protein identifications and phosphopeptide identifications in the two studies. Using approximately the same number of cells, the LTQ-Orbitrap analysis identified 5 times more proteins and 10 times more phosphopeptides than the QTOF Micro. This is largely attributed to the high sensitivity and sequencing speed afforded by the LTQ-Orbitrap as has been documented by previous studies^{10, 14}. When considering these results, it should be kept in mind that the Orbitrap SILAC experiment was performed twice, while the QTOF experiment was performed once, which would slightly exaggerate the difference in number of proteins quantified. 95 of the 127 proteins that were identified in the QTOF experiment were identified and quantified in the Orbitrap study. Supporting Information Figure 1 shows the protein ratios measured by the QTOF and the Orbitrap. While the majority of these proteins have consistent ratios in the two studies, we did observe that 13 of the 95 proteins changed by more than 1.5-fold in one experiment but not the other. These proteins are listed in Supporting Information Table 3. However for most of these proteins the two ratios are close to the cutoff ratio of 1.5. A summary comparison of the proteins identified in both Orbitrap experiments as well as the previous QTOF study is shown in Supporting Information Table 4.

Differences in SILAC ratios between the two studies can be attributed to two major reasons. First, although the same protocol for cell culture and treatment was used, the two experiments were performed more than two years apart and the intensities of Eph activation may have been slightly different in the two studies (biological variation). For example the protein Intersectin, which is a known effector downstream of EphB³¹, has a SILAC ratio of 0.99 in the QTOF study and 2.0 in the Orbitrap study. Second, the QTOF Micro and Orbitrap instruments have different dynamic ranges for quantitation. It was observed that the SILAC ratios measured by the Orbitrap were generally more dramatic than the ratio from the QTOF (Supporting Information Figure 1). We attribute this to the high dynamic range of Orbitrap³². Due to the different detection principles employed, the two instruments have quite different characteristics of spectral noise. As shown in Figure 5, the Orbitrap spectrum has lower background signal than the QTOF spectrum. When the peak intensity is low the background can contribute considerably to the ratio obtained by automated SILAC quantitation.

Comprehensiveness of the Screen

An extensive literature search using Pubmed found 42 key signaling proteins that are known to be close EphB binding partners or regulated by activation of EphB receptors (Supporting Information Table 5). 24 of these were identified in this study, and 17 of the 24 proteins had SILAC pY IP abundance changes of 1.5 or more. Seven proteins had pY IP abundance changes upon ephrin stimulation of less than 1.5-fold, but for most of them the directions of the subtle ratio changes were consistent with previous studies, such as PI3K, Grb2 and MAPK. The fact that more than half of all known effectors in EphB signaling were identified in this single study suggested the power of the strategy and the comprehensiveness of the screen. These known effectors only account for a small proportion (~10%) of the proteins with changed ratios identified in this study, suggesting many more novel candidates may participate in EphB signaling with their roles yet to be characterized.

The reasons why many known effectors were not identified in this screen may include: (1) Eph signaling has versatile functions. Many known effectors and signaling effects are only observed in specific cell types and cellular contexts, for example, the NMDA receptor in neurons³³ and

ZAP70 in T cells³⁴. (2) The known effectors are based on studies on any of the 6 EphB receptors while only three of them (EphB2, EphB3 and EphB4) are known to be expressed in the cell line that was used in this study. (3) It has been shown that different intensities of Eph signaling can produce very different cellular effects¹. Therefore regulation of effectors is dependent on intensity of Eph receptor activation, which is in turn dependent on level of receptor expression and concentration/affinity of stimulating ligands etc. (4) Different effectors may have different time courses of activation and the activation of specific effectors may only be observed at a specific time point. (5) Some effectors may not contain tyrosine phosphorylation sites or have low binding affinity/stoichiometry to other effectors. (6) Some effectors, for example, Src family kinases including Src, Fyn and Yes, are tyrosine phosphorylated at different sites both when activated and inhibited³⁵. In this case the overall protein phosphorylation level, i.e. the SILAC ratio, might not reflect the level of activation. (7) The amounts of the effector proteins, in the context of the other proteins being analyzed, were below the detection limit of the mass spectrometer.

Gene Ontology (GO) analysis

We performed a GO analysis of the quantified proteins using a commercial tool from ProteinCenter™ (Proxeon) for annotating and comparing protein datasets. The quantified proteins were first classified into two groups: proteins with ratio changes and proteins without ratio changes. GO analysis was performed for both groups using biological processes, cellular components and molecular functions classifications (Figure 6).

In theory, all proteins with changed ratios were pulled down in pY IPs due to specific binding and are specific to EphB signaling. Pulldown of proteins with no ratio changes could be due to either specific (some tyrosine phosphorylated proteins do not change their phosphorylation status after Eph activation) or non-specific binding during IP. Therefore enrichment of a specific GO annotation in one group can indicate a general difference between the Eph signaling specific/non-specific proteins or pY IP specific/non-specific proteins.

The GO cellular components analysis (Figure 6A) shows that proteins from ribosome, nucleus, mitochondrion, and endoplasmic reticulum (ER) are enriched in the unchanged protein group, suggesting that a considerable number of non-specific binding proteins from the pY IP came from these organelles. GO molecular functions analysis (Figure 6B) shows more nucleic acid/nucleotide binding proteins in the unchanged protein group, which also suggests the nucleus and ribosome as major sources of non-specific binding proteins. This implies that more vigorous clarification of lysate before pY IP, e.g. centrifugation at higher speed or for longer time, which can better remove these organelles, may reduce IP background and promote identification of more specific target proteins. Another observation is that membrane proteins are enriched in the changed groups, suggesting significant Eph signaling occurs on the cellular membrane.

From the GO molecular functions/biological processes analysis (Figure 6B and 6C), proteins in the categories of cell communication and signal transduction are enriched in the changed proteins, while proteins in the categories of metabolism and structural activity are enriched in the non-changed proteins. This is consistent with the established notion that the SILAC strategy we employed is an effective way to identify specific signaling target proteins from a background of non-specific interactions, which might be expected to be dominated by highly abundant housekeeping/structural proteins.

In all three GO analyses, the unannotated proteins are more enriched in the regulated proteins category, suggesting that many of the proteins with changed SILAC ratios have not been well studied.

Domain Analysis

The presence of conserved structural domains in a protein can suggest particular functions for the protein. Therefore domain analysis can be used as a preliminary search for potential protein groups with specific functions or interactions in a signaling pathway.

The cross-reference files for the IPI mouse and rat protein databases were searched to obtain the Pfam domain annotations for the all quantified proteins (Supporting Information Table 1). For the proteins with changed SILAC ratios upon ephrinB1 stimulation (shown in Figure 7), it is clear that several specific domains are overrepresented: (1) SH3 (including SH3_1 and SH3_2), PH, CH and RhoGAP domains are known to be indicative of a protein involved in signal transduction related to cytoskeletal organization, which is consistent with the consensus that cytoskeleton rearrangement is one major outcome of EphB signaling. (2) SH2 domains, which are important regulatory modules of tyrosine phosphorylation-dependent signaling cascades, are also overrepresented. (3) The EphB receptor has a PDZ binding motif at the C-terminus and is known to bind to PDZ-domain containing proteins including Syntenin, Afadin, and Grip1. All these proteins were identified in this SILAC study as proteins with changed ratios. In addition, nine proteins with changed SILAC ratios were found to contain PDZ domains, which are possible novel binding partners of EphB receptors through their PDZ domains. (4) UIM and VHS domains are also overrepresented, suggesting many effectors may be involved in vesicular trafficking / protein degradation triggered by ubiquitination of Eph receptors.

Biological Implications of the Novel Candidate Effectors

Of the proteins found to be involved in EphB signaling in this study but not our previous study, we validated Erbin by Western blotting (Fig. 4). Lending further support to the validity of our results, nine of the proteins found in this but not our previous study have been reported by others to be involved in ephrin signaling. These proteins include Intersectin³¹, PI3K^{36–39}, Shc⁴⁰, Vav2⁴¹, Cbl⁴², Syntenin⁴³, Grip1⁴⁴, Grb2⁴⁰, and Sos1²⁰. In addition, in-silico protein function and interaction analysis was performed by feeding the list of proteins of changed SILAC ratios into Ingenuity Pathways Analysis software (Ingenuity Systems, <http://www.ingenuity.com>), which searches existing literature and interaction databases (including Ingenuity curated findings, BIND, BIOGRID, DIP, INTACT, Interactome studies, MINT and MIPS, all downloaded 07/08/2008) for protein interaction and regulation networks. 98 of the 204 proteins with changed SILAC ratios could be assigned to a single interaction network based on previously reported direct protein-protein binding (Supporting Information Figure 2), thus supporting the ability of our screen to find functionally related proteins. The software was also able to classify the proteins into eleven major signaling networks (each network included a minimum of 10 proteins from our list of proteins that change in response to ephrinB addition) with relatively independent functions (Supporting Information Figure 3). These networks are mainly involved in regulation of cell morphology, cellular assembly and organization as well as development, which are consistent with known functions of EphB signaling. The same analysis was also carried out for the 46 proteins with changed SILAC ratios found in the QTOF analysis, which resulted in only two networks with 10 or more members from the list of changing proteins (Supporting Information Figure 3). It was noted that a considerable number of candidate effectors from the current study, which were missing in the previous QTOF analysis, were assigned into networks that participate in protein synthesis, indicating gene translation is quickly activated in response to EphB receptor activation. Another novel group of networks are involved in cell death and growth/proliferation, which is in line with the emerging discovery that Eph receptors play important roles in cancer.¹ Compared to the QTOF study, the much improved pY proteome coverage by the Orbitrap analysis greatly facilitated pathway analysis of EphB signaling.

Conclusions

We have used SILAC and LTQ-Orbitrap mass spectrometry to screen for novel effector proteins in the EphB signaling pathway. A considerable proportion of the tyrosine phosphoproteome was identified and quantified, allowing for a global view of the changes of a huge signaling network in response to EphB receptor activation. This study revealed an unprecedented large number of candidate effectors, which will greatly accelerate the achievement of our goal of a more complete understanding of the EphB signaling pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IP	immunoprecipitation
pY	phosphotyrosine
SILAC	stable isotope labeling with amino acids in cell culture
RTK	receptor tyrosine kinase
GO	gene ontology
IPI	international protein index
QTOF	quadrupole time-of-flight
LC	liquid chromatography
MS	mass spectrometry
MS/MS	tandem mass spectrometry
HRP	horseradish peroxidase

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Figure 1.

Tyrosine phosphorylation detected by anti-pY Western blotting of proteins in NG108 cell lysates and pY immunoprecipitates after ephrinB1 treatment. NG108-EphB2 cells were treated for 45 minutes with anti-Fc IgG aggregated ephrinB1-Fc or with anti-Fc IgG aggregated Fc as a control as indicated in the Materials and Methods. Total cell lysates and pY IPs were probed with PY99-HRP. Numbers to the left of the gel show $MW \times 10^{-3}$ based on protein MW standards.

Figure 2.

Identification of SILAC proteins and phosphopeptides from anti-pY IPs. (A) Venn diagram depicting the overlap in proteins identified in the two SILAC replicates by LTQ-Orbitrap. (B) Venn diagram depicting the overlap in proteins quantified in this study by Orbitrap and a previous study using a QTOF Micro mass spectrometer⁷. (C) Venn diagram depicting the overlap in phosphopeptides identified in this study by Orbitrap and the previous study using QTOF.

Figure 3.

SILAC protein ratios from the two SILAC replicates. 672 proteins from replicate 1 and 513 from replicate 2 were quantified. In total 777 proteins were quantified, and 408 of them were quantified in both replicates.

Figure 4. Western blotting analysis of selected candidate effector proteins

Cells were cultured and stimulated with ephrinB1 in the same way as in the SILAC experiments. The whole cell lysates and anti-pY IPs were probed with the indicated antibodies. For the EphB4 receptor, wild type NG108 cells were used. For all other proteins, NG108-EphB2 cells were used.

Figure 5.

Measurement of SILAC peptide ratios using LTQ-Orbitrap and QTOF Micro mass spectrometers. The identified peptide (LLVDNQGLSGR) was from SAM and SH3 domain-containing protein 1 (SASH1, IPI00338954). The peptide ratio was calculated as the sum of intensities of the first three isotopic peaks of the light peptide over the sum of the heavy peptide peak intensities. The arrow in each panel indicates the monoisotopic peak for the heavy peptide.

Figure 6 (A)

Figure 6 (B)

Figure 6 (C)

Figure 6.

GO analysis of proteins with changed and unchanged SILAC ratios. All quantified proteins were classified into two groups: proteins with ratio changes less than 1.5 fold (unchanged) and proteins without ratio changes greater 1.5 (changed). GO analysis was performed for both groups on cellular components (A), molecular functions (B) and biological processes (C).

Figure 7. Domain analysis for proteins with SILAC ratio changes

Domain information was obtained from the Pfam annotation in the IPI cross-reference files. Domains that occur in at least 5 (out of 204) proteins are included in the figure. The occurrence of all domains in the combined IPI mouse and rat database is used as a control.

Proteins with at least a 1.5 Change in Protein Abundance between Anti-phosphotyrosine Immunoprecipitates from ephrinB1 -Stimulated and Unstimulated NG108-EphB2 Cells ^a

Table 1

No.	accession	protein name	mean ratio	Rep. 1 CV	Rep. 2 CV	CV (reps 1,2)	Known EphB effector?
1	IP100319843	Beclin-1	509				no
2	IP100109667	nicotinamide nucleotide adenylyltransferase 1	255				no
3	IP100222366	sterile alpha motif domain containing 5	107	76	1	37	no
4	IP100556823	protein kinase, AMP-activated, alpha 1 catalytic subunit	106				no
5	IP100752269	novel protein (possible orthologue of human Src homology 2 domain containing F (SHF))	76	54	3	23	no
6	IP100330102	folliculin interacting protein 1	73	7			no
7	IP100115056	trafficking protein particle complex 3	58	33			no
8	IP100309259	partitioning-defective protein 3 homolog isoform 3	58	18	38	20	no
9	IP100416163	unnamed protein product	55	21	38	16	yes
10	IP100354665	apoptosis-stimulating protein of p53, 1	52	51	6	57	no
11	IP100408219	N-chimaerin	49	33	19	24	no
12	IP100749688	PREDICTED: similar to MGC114619 protein	46	15	36	28	no
13	IP100331766	putative C3orf6 protein	45	10	19	38	no
14	IP100127232	glutamate receptor interacting protein 1 isoform 1	39	8	31	36	yes ⁴⁴
15	IP100338954	SAM and SH3 domain-containing protein 1	36	20	26	8	no
16	IP100361275	PREDICTED: similar to TPR domain, ankyrin-repeat and coiled-coil-containing	35		16		no
17	IP100213347	afadin	33	22	22	0	yes ²⁴
18	IP100473693	Isoform 1 of Plakophilin-4	32	21			no
19	IP100753111	PREDICTED: similar to Afadin (Af-6 protein)	30	22	18	3	yes ²⁴
20	IP100359621	hypothetical protein LOC307833	30		13		no
21	IP100761456	chimerin (chimaerin) 2	29	8	19	17	no
22	IP100454039	Protein LAP2 (ErbB2-interacting protein) (Erbin)	27	12	16	2	no
23	IP100125855	protein kinase C, delta	26	3			no

No.	accession	protein name	mean ratio	Rep. 1 CV	Rep. 2 CV	CV (reps 1,2)	Known EphB effector?
24	IP100468418	signal transducing adaptor molecule2 (STAM2)	26	8	19	15	no
25	IP100108870	Eph receptor B2	26	11	17	13	yes
26	IP100565852	PREDICTED: similar to Eph receptor B3	25	12	15	3	yes
27	IP100408892	RAB7, member RAS oncogene family	21	7	15	14	no
28	IP100421832	dermcidin precursor	21	4	17	18	no
29	IP100471127	Cdc42 effector protein 1 (Binder of Rho GTPases 5)	20	4	13	9	no
30	IP100124742	eukaryotic translation initiation factor 4H	19	7	6	4	no
31	IP100322033	target of myb1-like 2 isoform a	19	9	11	4	no
32	IP100367930	PREDICTED: similar to ErbB2 interacting protein isoform 1	19		10		no
33	IP100125534	Docking protein 1 (Downstream of tyrosine kinase 1) (p62(dok))	18	10	8	0	yes ¹⁵
34	IP100316623	catenin, delta 1 isoform 2	18	7			no
35	IP100420753	PREDICTED: similar to SHB adaptor protein B	18	12	8	2	no
36	IP100312067	inositol polyphosphate phosphatase-like 1, isoform CRA_c	18	9	6	1	yes ⁴⁵
37	IP100315187	UPF0404 protein C11orf59 homolog	16	2	5	7	no
38	IP100343984	coiled-coil domain containing 85B	16	8			no
39	IP100187275	Carnitine deficiency-associated gene expressed in ventricle 3	16	2	5	15	no
40	IP100202691	cancer susceptibility candidate 3	15		8		no
41	IP100136475	Leucine-rich repeats and immunoglobulin-like domains protein 1 precursor (LIG-1)	14	7			no
42	IP100331568	HGF-regulated tyrosine kinase substrate (Hrs)	14	6	6	1	no
43	IP100153241	vacuolar protein sorting 37C	13		2	11	no
44	IP100228877	connector enhancer of kinase suppressor of Ras 2	13	6			no
45	IP100379844	Insulin receptor substrate 2 (IRS-2)	12	6	7	1	no
46	IP100132135	midline 2	12	6			no
47	IP100124298	Rho GTPase activating protein 5	12	6			no
48	IP100366801	YTH domain family 2	12	4			no

No.	accession	protein name	mean ratio	Rep. 1 CV	Rep. 2 CV	CV (reps 1,2)	Known EphB effector?
49	IP100134881	LIM domain-containing protein 1	11	4			no
50	IP100323349	Tight junction protein ZO-2	11	4	4	4	no
51	IP100137731	unnamed protein product	11	6	4	3	no
52	IP100347255	Protein KIAA1688	11	7	2	8	no
53	IP100336844	epsin 2	10	4			no
54	IP100135971	tight junction protein 1	10	5	6	3	no
55	IP100223987	leucyl/cystinyl aminopeptidase	10	4	4	4	no
56	IP100364933	similar to signal transducing adaptor molecule 1 (STAM1)	9.9	5.0	3.7	1.4	no
57	IP100130621	RAS p21 protein activator 1	9.6	1.4	3.4	1.9	yes ¹⁵
58	IP100660894	E3 ubiquitin-protein ligase CBL-B	9.5	3.8			no
59	IP100229955	Ras association (RalGDS/AF-6) domain family 8	9.3	5.8			no
60	IP100110435	nischarin	9.3	2.6	4.2	1.6	no
61	IP100120433	SH2B adapter protein 2	8.9	2.7	2.0	1.1	no
62	IP100480842	hypothetical protein LOC684097	8.6		2.4		no
63	IP100221581	Eukaryotic translation initiation factor 4B (eIF-4B)	8.5	2.7	4.6	2.4	no
64	IP100765594	non-catalytic region of tyrosine kinase adaptor protein 1 (predicted), isoform CRA_a	8.5	4.0	3.7	1.7	yes ¹⁵
65	IP100119809	lectin, galactoside-binding, soluble, 3 binding protein	8.3	3.9			no
66	IP100363834	PREDICTED: similar to plectstrin homology domain containing, family A member 6	8.2	3.0	2.8	2.9	no
67	IP100121319	LIM only protein HLP	7.6	1.1	2.4	0.0	no
68	IP100117375	syndecan binding protein isoform 1 (syntenin)	7.4	0.9	4.7	4.3	yes ⁴³
69	IP100123505	Synaptophysin	6.9			0.6	no
70	IP100116554	protein tyrosine phosphatase, non-receptor type 11	6.6	3.8	1.6	4.1	yes ⁴⁶
71	IP100154012	ubiquitin specific peptidase 15	6.5	3.0	1.4	2.9	no
72	IP100128454	seizure related 6 homolog like 2	6.5	0.3	1.2	2.1	no
73	IP100130185	protein phosphatase 1, catalytic subunit, alpha	6.4	1.4	2.5	1.8	no

No.	accession	protein name	mean ratio	Rep. 1 CV	Rep. 2 CV	CV (reps 1,2)	Known EphB effector?
74	IP100133679	hypothetical protein LOC73711	6.2	0.5	0.9	0.0	no
75	IP100117944	tumor susceptibility gene 101 protein	6.2	1.8	0.7	2.2	no
76	IP100205566	calponin 3, acidic	6.0				no
77	IP100229392	Ras-related GTP binding A	6.0	0.5	1.3	2.6	no
78	IP100229434	tumor protein p53 binding protein, 2	5.9	3.5			no
79	IP100323590	E3 ubiquitin-protein ligase CBL	5.8	1.4	1.3	0.2	yes ⁴²
80	IP100136618	toll interacting protein	5.7	1.1		1.1	no
81	IP100272559	Vav2 protein	5.6	1.1			yes ⁴¹
82	IP100381394	filamin C, gamma	5.5	1.9	2.4	0.5	no
83	IP100133591	vacuolar protein sorting 28	5.5	0.8			no
84	IP100308222	drebrin-like	5.5	1.0	2.0	0.1	no
85	IP100227149	YTH domain family protein 3	5.4	1.7	1.3	0.2	no
86	IP100272148	Cytchesin-3	5.4	1.0	1.2	0.4	no
87	IP100132604	unnamed protein product	5.2	2.1	0.6	3.0	no
88	IP100153207	unnamed protein product	5.2	0.1	2.8	2.2	no
89	IP100325146	annexin A2	5.2		3.2		no
90	IP100130883	Putative RNA-binding protein 3 (RNA-binding motif protein 3)	5.1		0.9		no
91	IP100323483	programmed cell death 6 interacting protein	4.9	1.8	0.6	0.6	no
92	IP100454019	unnamed protein product	4.9	1.5			no
93	IP100458001	ataxin 2-like, isoform CRA_g	4.8	0.9	1.3	0.3	no
94	IP100231715	protein phosphatase 1 gamma2	4.8		2.0		no
95	IP100130115	Vesicle transport through interaction with t-SNAREs homolog 1B	4.8	2.4	0.6	1.6	no
96	IP100626620	SEC24 related gene family, member C (S. cerevisiae), isoform CRA_b	4.8	1.3			no
97	IP100230035	ATP-dependent RNA helicase DDX3X	4.4	0.7	1.9	1.4	no
98	IP100553792	Isoform 2 of Caskin-1	4.4		2.0		no
99	IP100114332	ribosomal protein S6 kinase polypeptide 1	4.3	1.2			no
100	IP100132322	Trafficking protein particle complex subunit 5	4.1	1.0			no
101	IP100402900	Isoform 1 of Engulfment and cell motility protein 2	4.1	0.5			no

No.	accession	protein name	mean ratio	Rep. 1 CV	Rep. 2 CV	CV (reps 1,2)	Known EphB effector?
102	IP100222107	FERM domain containing 4A	4.0	0.5	0.4	0.3	no
103	IP100264501	phosphatidylinositol-binding clathrin assembly protein	3.9	0.9			no
104	IP100114613	Cdc42 binding protein kinase beta	3.9	1.2	0.3	0.8	no
105	IP100331016	SEC24 related gene family, member B	3.8	0.6	1.0	1.1	no
106	IP100420553	Serine/threonine-protein kinase TAO2	3.8				no
107	IP100330862	Ezrin	3.7	1.0	1.1	0.7	no
108	IP100221494	Lipoma-preferred partner homolog	3.7	0.1			no
109	IP100313841	ATPase, H+ transporting, V0 subunit D isoform 1	3.7		0.6	0.9	no
110	IP100118899	actinin alpha 4	3.7	0.6	1.0	0.3	no
111	IP100380817	breakpoint cluster region homolog	3.6	0.0			no
112	IP100223070	dedicator of cytokinesis 4	3.6	1.0	0.9	0.1	no
113	IP100458995	polyubiquitin C	3.5	0.1	1.7	0.6	no
114	IP100313275	Sorting nexin-9	3.5	0.7	0.4	0.3	no
115	IP100132462	cytotoxic granule-associated RNA binding protein 1	3.3	0.0	0.8	0.6	no
116	IP100206710	pleckstrin homology, Sec7 and coiled/coil domains 2	3.2		0.9		no
117	IP100154057	protocadherin 1	3.2	0.7	0.8	0.2	no
118	IP100380436	actinin, alpha 1	3.2	0.6	0.6	0.3	no
119	IP100110247	TBC1 domain family member 15	3.0	0.5			no
120	IP100464282	Hbs1-like (S. cerevisiae), isoform CRA_b	2.9	1.0			no
121	IP100368041	similar to DNA-directed RNA polymerase II largest subunit	2.8		0.1		no
122	IP100109334	Proto-oncogene tyrosine-protein kinase FER (p94-FER) (c-FER)	2.8	0.2		0.0	no
123	IP100226727	Isoform 2 of Discs large homolog 2	2.8	0.6	0.9	0.0	no
124	IP100189519	Histone H3.3	2.7				no
125	IP100108150	Rho-associated protein kinase 2 (p164 ROCK-2)	2.7	0.6			no
126	IP100117159	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide	2.7	0.8	0.4	0.3	yes ³⁹
127	IP100120923	Vacuolar protein sorting 16	2.6				no

No.	accession	protein name	mean ratio	Rep. 1 CV	Rep. 2 CV	CV (reps 1,2)	Known EphB effector?
128	IP100380814	target of myb1 homolog	2.6	0.4			no
129	IP100124753	misshapen-like kinase 1 isoform 2	2.6	0.5	0.8	0.0	no
130	IP100380108	transmembrane protein 1	2.6	0.0			no
131	IP100136498	lin 7 homolog c	2.6	0.3			no
132	IP100558156	61 kDa protein	2.6	0.3	0.4	0.0	no
133	IP100204923	ubiquitin specific peptidase 9, X chromosome	2.5		1.0		no
134	IP100109932	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6	2.5	0.4			no
135	IP100123313	ubiquitin-activating enzyme E1, Chr X	2.5	0.6			no
136	IP100123349	SEC23A	2.4	0.3	0.3	0.2	no
137	IP100130423	Growth factor receptor bound protein 2-associated protein 2	2.4	0.3			no
138	IP100462445	E3 ubiquitin-protein ligase NEDD4	2.3	0.9	0.1	0.2	no
139	IP100133024	I110059P08Rik protein	2.2		0.6		no
140	IP100114948	interferon induced transmembrane protein 2	2.2	0.3	0.4	0.3	no
141	IP100457533	ubiquitin-associated protein 2	2.2	0.2	0.1	0.2	no
142	IP100229895	dispatched homolog 2	2.2				no
143	IP100331579	synaptogyrin 3	2.2		0.1		no
144	IP100123292	Isoform 2 of Far upstream element-binding protein 1	2.2	0.3			no
145	IP100226563	twenty homolog 3 (Drosophila), isoform CRA_a	2.1	0.5	0.3	0.2	no
146	IP100329998	Histone cluster 1, H4h	2.1	0.2	0.2	0.0	no
147	IP100116697	RAB6A, member RAS oncogene family	2.1	0.7	0.7	0.2	no
148	IP100676192	latrophilin 2	2.1				no
149	IP100111416	syntaxin 12	2.1	0.3	0.4	0.5	no
150	IP100223253	heterogeneous nuclear ribonucleoprotein K	2.1	0.2	1.0	0.6	no
151	IP100380824	MKIAA0144 protein	2.0	0.1	0.4	0.1	no
152	IP100408378	Isoform 2 of 14-3-3 protein theta	2.0	0.3	0.4	0.0	no
153	IP100762547	Isoform 1 of Intersectin-2	2.0	0.2	0.6	0.2	yes ³¹
154	IP100322492	Ewing sarcoma homolog	2.0	0.1	0.3	0.5	no

No.	accession	protein name	mean ratio	Rep. 1 CV	Rep. 2 CV	CV (reps 1,2)	Known EphB effector?
155	IP100360064	PREDICTED: similar to Son of sevenless homolog 1	2.0			0.5	yes ²⁰
156	IP100133428	protease (prosome, macropain) 26S subunit, ATPase 1	2.0				no
157	IP100312527	Crmp1 protein	1.9	0.5	0.4	0.1	no
158	IP100109375	Poliovirus receptor-related protein 2 precursor	1.9	0.2			no
159	IP100125778	Transgelin-2	1.9		0.2		no
160	IP100117039	Tyrosine-protein kinase ABL2	1.9	0.2	0.1	0.0	no
161	IP100116112	Dynactin subunit 2	1.9	0.2			no
162	IP100515195	eukaryotic translation initiation factor 4, gamma 1 isoform b	1.9		0.4		no
163	IP100309413	non-catalytic region of tyrosine kinase adaptor protein 2	1.9	0.4	0.3	0.1	no
164	IP100622847	Heterogeneous nuclear ribonucleoproteins A2/B1	1.9	0.2	0.2	0.4	no
165	IP100221796	poly(rC) binding protein 2, isoform CRA_a	1.9		0.5		no
166	IP100372054	PEF protein with a long N-terminal hydrophobic domain	1.9				no
167	IP100135887	transmembrane protein 106B, isoform CRA_b	1.8	0.2			no
168	IP100136917	Tyrosine-protein kinase-protein kinase Sgk269	1.8		0.3		no
169	IP100365284	eukaryotic translation initiation factor 4 gamma, 3	1.8				no
170	IP100125298	SHC-transforming protein 1	1.8	0.3	0.2	0.0	yes ⁴⁰
171	IP100420185	Epidermal growth factor receptor substrate 15-like 1	1.8	0.4	0.1	0.2	no
172	IP100415402	Syntaxin-binding protein 1	1.8	0.4			no
173	IP100463573	eukaryotic translation initiation factor 3 subunit 6 interacting protein	1.8	0.2			no
174	IP100116966	Asparagine synthetase	1.8	0.3			no
175	IP100226275	WD repeat domain 26	1.7	0.1			no
176	IP100406118	NS1-associated protein 1 isoform 2	1.7	0.3	0.0	0.1	no
177	IP100553633	cullin 7	1.7	0.3			no

No.	accession	protein name	mean ratio	Rep. 1 CV	Rep. 2 CV	CV (reps 1,2)	Known EphB effector?
178	IP100129417	heterogeneous nuclear ribonucleoprotein D-like	1.7	0.1		0.2	no
179	IP100227392	14-3-3 protein eta	1.7	0.2	0.5	0.3	no
180	IP100470095	G protein-coupled receptor kinase-interactor 1	1.7	0.3	0.1	0.1	no
181	IP100114401	emerin	1.6		0.3	0.5	no
182	IP100128202	eukaryotic translation initiation factor 3, subunit 3 (gamma)	1.6		0.1		no
183	IP100308162	solute carrier family 25 (mitochondrial carrier, Aralar), member 12	1.6	0.4			no
184	IP100280250	SH3 and PX domains 2A	1.6				no
185	IP100321647	eukaryotic translation initiation factor 3, subunit 8	1.6	0.1	0.1	0.1	no
186	IP100118384	14-3-3 protein epsilon (14-3-3E)	1.5	0.1	0.2	0.1	no
187	IP100230704	betaPix-c	1.5	0.1			no
188	IP100229548	Solute carrier family 1 (neural amino acid transporter), member 5	1.5	0.1			no
189	IP100331334	Bcl-2-binding protein Bis	1.5	0.0	0.0	0.1	no
190	IP100230707	14-3-3 protein gamma	1.5	0.2	0.1	0.0	no
191	IP100114560	Ras-related protein Rab-1A	1.5	0.0	0.1	0.2	no
192	IP100131329	Sorting nexin-18	1.5	0.1			no
193	IP100116498	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	1.5	0.1	0.1	0.0	no
194	IP100362014	Tln1 protein	1.5				no
195	IP100230445	peripherin	0.68	0.04	0.09	0.13	no
196	IP100331738	52 kDa Ro protein	0.67	0.03	0.07	0.08	no
197	IP100222801	Neuronal proto-oncogene tyrosine-protein kinase Src	0.67	0.12			no
198	IP100117821	Breast cancer anti-estrogen resistance protein 1 (p130cas)	0.66	0.05	0.07	0.02	yes ⁴⁷
199	IP100113563	Focal adhesion kinase 1 (FADK 1) (pp125FAK)	0.64	0.06	0.05	0.02	yes ^{47, 48}
200	IP100135965	Alpha-internexin	0.57	0.04			no
201	IP100137970	SH2 domain containing 3C	0.55	0.05	0.04	0.01	yes ⁴⁹

No.	accession	protein name	mean ratio	Rep. 1 CV	Rep. 2 CV	CV (reps 1,2)	Known EphB effector?
202	IP100223751	Rho GTPase activating protein 12 isoform 1	0.52	0.05	0.05	0.01	no
203	IP100330231	Rap guanine nucleotide exchange factor (GEF) 1 isoform 3	0.52	0.05			no
204	IP100111258	unnamed protein product	0.46	0.04	0.04	0.01	no

^afor proteins identified based on the same set of peptides, only one protein is shown in this table for each protein group. More detailed information about each protein group is included in Supporting Information Table 1.