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Use of Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) for Phosphotyrosine Protein Identification and Quantitation

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Summary

In recent years, stable isotope labeling by amino acids in cell culture (SILAC) has become increasingly popular as a quantitative proteomic method. In SILAC experiments, proteins are metabolically labeled by culturing cells in media containing normal and heavy isotope amino acids. This makes proteins from the light and heavy cells distinguishable by mass spectrometry (MS) after the cell lysates are mixed and the proteins separated and/or enriched. SILAC is a powerful tool for the study of intracellular signal transduction. In particular, it has been very popular and successful in quantitative analysis of phosphotyrosine (pTyr) proteomes to characterize pTyr-dependent signaling pathways. In this chapter, we describe the SILAC procedure and use EphB signaling pathway as an example to illustrate the use of SILAC to investigate such pathways.

Keywords

SILAC; Tyrosine phosphorylation; Quantitation; Identification; Mass spectrometry; HPLC; Immunoprecipitation; RTK; Phosphoproteome; Signal transduction

1. Introduction

Phosphorylation is one of the most common posttranslational modifications of proteins. In eukaryotic cells, phosphorylation mainly occurs on serine, threonine, and tyrosine residues (1). Despite its infrequent occurrence compared with serine and threonine phosphorylation (pSer: pThr: pTyr=1,800:200:1), tyrosine phosphorylation plays critical roles in regulating intracellular signal transduction (1). In a typical receptor tyrosine kinase (RTK) pathway, upon stimulation by binding with ligand the receptor becomes tyrosine phosphorylated and activated. The activated receptor then recruits and tyrosine phosphorylates other effectors, which triggers further signaling events down the pathway. Traditional approaches to study these pathways involve looking at one or a few effectors at a time, and it is difficult to get a complete picture of the signaling pathway in this way. Mass spectrometry (MS)-based quantitative proteomics has become a major tool for high throughput investigation of cell signal transduction, owing to rapid development of both quantitative methods and instrumentation in recent years (2, 3). Quantitative proteomics allows the comparison of different cellular conditions to obtain a global view of changes in protein phosphorylation.

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¹⁰The SILAC ratio of a protein is essentially a measure of the differences in the protein's affinity (directly or indirectly through a binding partner) towards anti-pTyr antibody before and after RTK activation. It generally reflects the activation/deactivation of the protein upon the RTK stimulation, but there are exceptions in rare occasions. The overall tyrosine phosphorylation level of a protein does not always reflect its level of activation. For example, Src family kinases are tyrosine phosphorylated at different sites both when inhibited and activated (26).

These experiments provide valuable data to help us to screen for new signaling effector molecules and interpret the dynamic regulation of the pathway under study.

Stable isotope labeling by amino acids in cell culture (SILAC) is an excellent approach for high-accuracy quantitative proteomics (4, 5). It involves culturing cells in a medium supplemented with amino acids containing either normal or heavy stable isotopes. The amino acids are metabolically incorporated into the proteins of the cells through protein synthesis. When the mixed light and heavy isotope labeled proteins are analyzed by MS, the source of the sample can be easily distinguished by the mass difference caused by the differential labeling. The relative abundance of the proteins is measured based on the intensities of the light and heavy peptides. Compared with other quantitative methods, SILAC has the following advantages:

- a. It allows combining the differentially labeled samples early on during sample preparation (usually directly after cell lysis and before any purification or fractionation steps). This feature is very important as it minimizes the possible quantitative error caused by handling different samples in parallel.
- b. It does not require chemical reactions to modify proteins or peptides.
- c. It is easy to obtain a high level of incorporation. (d) It is simple to perform.

SILAC is not limited to mammalian cells. It can be applied to organisms that can be metabolically labeled with amino acids, which include bacteria, yeasts, and plants (6–9).

Particularly in combination with anti-phosphotyrosine (pTyr) immunoprecipitation (IP), SILAC has proven to be a very powerful method to study RTK signaling (10). It has been successfully used to study EGF, PDGF, FGF, EphB, insulin, and T-cell receptors (11–19). The general workflow for such studies is shown in Fig. 1. Cells are first differentially labeled with SILAC. 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-pTyr IP to pull down pTyr proteins together with their tight binding partners. The precipitated proteins are then identified and quantified by MS, and the SILAC ratio of a protein (the abundance of proteins in the IP of stimulated cells/the abundance of proteins from the IP of unstimulated cells) is used to indicate whether the protein participates in the RTK pathway or not.

In this chapter, we describe the protocols to use SILAC to screen for downstream effector proteins in the EphB signaling pathway. These protocols can be easily adapted to study other similar RTK pathways.

2. Material

2.1. Cell Culture and SILAC Labeling

1. Cell line: NG108-15 cell line (mouse neuroblastoma and rat glioma hybrid) stably over-expressing EphB2 (20).
2. Culture medium: Dulbecco's Modified Eagle Medium (DMEM) deficient in lysine and arginine (Specialty Media, Millipore).
3. Amino acids for labeling: Normal (light) L-lysine and L-arginine hydrochloride; Stable isotope-labeled (heavy) L-lysine $^{13}\text{C}_6$ and L-arginine $^{13}\text{C}_6$ hydrochloride (Cambridge Isotope Labs, Andover, MA). Heavy isotope amino acids (usually contain ^2H , ^{13}C , ^{15}N or ^{18}O) used in SILAC are not radioactive and therefore do not need special handling precautions.

4. Supplements: Dialyzed fetal bovine serum; post-fusion selective medium HAT (liquid mixture of sodium hypoxanthine, aminopterin and thymidine); penicillin/streptomycin; plasmid selecting antibiotic G418.
5. Other: Sterile phosphate buffered saline (PBS); trypsin-EDTA solution; 0.22- μ m filter flasks.
6. Ligand stimulation: EphrinB1-Fc (Sigma-Aldrich, St. Louis, MO), recombinant human Fc and goat anti-human Fc IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

2.2. Cell Lysis, Immunoprecipitation, and SDS-PAGE

1. Lysis buffer: Contains 1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 2 mM tyrosine phosphatase inhibitor sodium orthovanadate, 2 mM serine/threonine phosphatase inhibitor sodium fluoride, and protease inhibitors (Complete tablet; Roche, Mannheim, Germany). Fresh phosphatase and protease inhibitors should be added just before use.
2. Agarose conjugated anti-pTyr antibody PY99 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).
3. Elution buffer: 0.2% trifluoroacetic acid (TFA)/0.5% SDS.
4. Precast 7.5% Tris-HCl polyacrylamide gel.
5. Coomassie brilliant blue R250 (CBB) staining solution: 0.1% CBB/7% acetic acid/40% ethanol.

2.3. In-gel Digestion

1. Destaining buffer: 25 mM ammonium bicarbonate/50% ace-tonitrile.
2. Acetonitrile (HPLC grade).
3. Trypsin solution: 12.5 ng/ μ L sequencing grade trypsin (Promega Corporation, Madison, WI) in 25 mM ammonia bicarbonate. The trypsin stock solution (200 ng/ μ L in water) can be stored at -70°C for 6 months.
4. Extraction buffer: 5% formic acid (FA)/50% acetonitrile.

2.4. High Performance Liquid Chromatography–Tandem Mass Spectrometry (HPLC-MS/MS)

1. Nano-Aquity HPLC (Waters, Milford, MA) equipped with a Symmetry C18 trap column (180 $\mu\text{m} \times 2$ cm, 5- μm particle diameter, Waters) and a Symmetry C18 analytical column (75 $\mu\text{m} \times 15$ cm, 3.5- μm particle diameter, Waters).
2. LTQ-Orbitrap (Thermo Scientific, San Jose, CA), connected to the Nano-Aquity LC via a nanoelectrospray ion source.
3. Mobile phase A: 0.1% FA in water (HPLC grade).
4. Mobile phase B: 0.1% FA in acetonitrile (HPLC grade).

3. Methods

3.1. SILAC Medium Preparation

SILAC cell medium is the same as regular medium except for the following two components: (a) The amino acids that are used for labeling should be left out of the original formulation. Later the light and heavy amino acids are added to the base medium to make

the light and heavy labeling media. (b) Dialyzed serum is used instead of regular serum because the latter contains free amino acids that can cause problems for labeling (4).

1. Prepare 1,000× stock solutions for the light and heavy labeling amino acids by dissolving the amino acids in PBS. The use of stock solutions is generally recommended for ease of use. However this does not apply to some amino acids because of their poor solubility in water (*see* Note 1). In our case based on the DMEM formulation, the working concentrations of light arginine and lysine are 84 and 146 mg/L respectively. Note that because the molecular weights of heavy amino acids are different from their light counterparts, their concentrations (in mg/L) are different. In this case the concentrations of heavy arginine and lysine are 87.2 and 152.8 mg/L respectively. The use of lower concentrations of amino acids has been reported (*see* Notes 2 and ³) (10). In addition to arginine and lysine, other amino acids can be used for labeling (*see* Notes 4 and ⁵) (21).
2. Dilute the light and heavy amino acids 1,000-fold with DMEM deficient in arginine and lysine.
3. Add supplements to both media to their working concentrations: HAT (1×), penicillin/streptomycin (1×), and G418 (0.4mg/mL).
4. Filter both media with 0.22-μm filter flasks.
5. Save some of the serum free media for the purpose of cell starvation prior to stimulation.
6. Add 10% dialyzed fetal bovine serum to light and heavy media. These media are now ready for cell culture.

3.2. SILAC Cell Culture

SILAC cell culture is nearly the same as regular cell culture.

¹Some hydrophobic amino acids, such as tyrosine, do not dissolve well in water and are therefore prepared directly at their working concentrations in medium.

²Labeling amino acids can be used at concentrations lower than those specified in our standard formulations. For example, four times less arginine in the DMEM has been used for HeLa cell cultures (10). This significantly decreases the cost of SILAC experiments.

³A known issue when using arginine labeling is that some cell lines can convert arginine metabolically into proline (5, 24). In MS, this will cause the signal of a proline containing peptide to split into multiple signals, depending on the number of prolines in the sequence. Decreasing the concentration of arginine in medium may prevent this conversion from happening (10). For cell lines in which the conversion cannot be inhibited, the options are: (a) exclude all proline containing peptides from quantitation; (b) for each heavy proline-containing peptide, add up all its split signals for quantitation; (c) use specially designed labeling strategies to cancel out the error (24).

⁴In addition to arginine and lysine, some other amino acids can be used in SILAC (21). When choosing labeling amino acids, several important factors should be considered: (a) The mass difference between the light and heavy versions should be at least 4 Da to ensure sufficient separation of the naturally occurring isotopic envelopes of peptide doublets in MS. Mass differences smaller than 4 Da will cause overlaps of isotope peak clusters from light and heavy peptides (especially for large peptides) and complicate quantitation. (b) Peptides labeled with amino acids deuterated on side chains can elute earlier than their light counterparts in reverse-phase LC (25). The retention time difference depends on the number of ²H and their positions within the molecule. This may affect the accuracy of quantitation. In contrast C¹³ and N¹⁵ labelings do not cause LC retention time shift and are considered better than ²H labeling in this regard. (c) Arginine and lysine are the most commonly used labeling amino acids in SILAC. The reason is because trypsin, the most commonly used pro-tease in proteomics, specifically cleaves at these amino acids. Therefore after digestion all peptides (except the one at the C-terminus of the protein) contain single labeling amino acids (more if trypsin misses one or more cleavages) and are thus quantifiable in MS.

⁵In this chapter we describe a SILAC protocol to compare two cell conditions. Comparison of more than two conditions can be achieved by employing more labeling amino acids. For example, triple SILAC labeling with Arg0-Arg6 (¹³C6)-Arg10 (¹³C6¹⁵N4) can be used for a three-condition experiment. Usually to conduct a time course study, two triple-labeling experiments can be conducted with one common control condition in both experiments. Then the results of the two experiments are combined to obtain a five time point result (12).

1. Split one dish of NG108-EphB2 cells into two dishes: One with light medium and the other with heavy medium. When the cells are >80% confluent, rinse the cells with PBS, detach the cells with trypsin/EDTA and split the cells to new dishes.
2. To ensure complete labeling, cells should undergo at least five doublings in the SILAC media. Protein labeling is achieved through: (a) dilution of the non-labeled protein by newly synthesized (labeled) protein and (b) degradation of the non-labeled protein. Five cell doublings corresponds to a labeling incorporation of at least 96.9% when only the dilution effect is considered. Considering natural protein turnover, the actual incorporation should be even higher.
3. Prepare about 10^8 cells (10 cm I.D. dishes) for each labeling condition (*see* Note 6).

3.3. Cell Stimulation and Lysis

1. After SILAC labeling is complete, culture the cells in serum-free SILAC media (containing heavy or light amino acids consistent with previous labeling) for 24 h. The purposes of starvation are (a) to synchronize the cells and (b) to lower the basal tyrosine phosphorylation level to facilitate identification of pTyr proteins specific to the signaling pathway under study. The optimal starvation time is generally 12–24 h depending on cell lines.
2. Cluster the ligand by incubating ephrinB1-Fc (250 $\mu\text{g/mL}$) and anti-human Fc (65 $\mu\text{g/mL}$) at 4°C for 1.5 h. Non-clustered ephrin ligands are unable to activate the receptors efficiently (20).
3. Add clustered ligand to one set of cells (either light or heavy) with a final ligand concentration of 2 $\mu\text{g/mL}$. To the other set of cells add 1 $\mu\text{g/mL}$ anti-human Fc. These cells are used as a control. Incubate for 45 min or other desired time at 37°C.
4. Remove media from the dishes, wash twice with ice cold PBS. Tilt the dishes to facilitate complete removal of PBS. To each dish add 1 mL of ice cold lysis buffer. Scrape the cells and incubate the lysates on ice for 20 min. To remove cell debris, centrifuge the lysates at $14,000 \times g$ for 20 min. Carefully aspirate and save the supernatant fluids. Save 20 μL of the heavy lysate. This may be used to double check the labeling incorporation if necessary.
5. Mix equal amount of light and heavy lysates. The Bradford assay can be used to determine the lysate protein concentrations to ensure equal mixing. Save 20 μL of the mixed lysates. This will be analyzed by MS and used as a loading control.

3.4. Anti-pTyr IP and SDS-PAGE

The use of anti-pTyr IP is to isolate the pTyr proteome, which is critical because signaling proteins are usually of low abundance and difficult to detect by MS without enrichment. Care should be taken to minimize nonspecific binding in IP. Although SILAC is able to discriminate nonspecific binding proteins based on their ratios, if nonspecific binding proteins are too abundant they will dominate the analytical space of LC-MS/MS, leading to poor identification of target proteins.

1. Pre-clear the SILAC lysate: Add agarose beads to the lysate (10 μL of agarose beads per mL lysate) and incubate with rotation at 4°C for 1 h. Spin at $14,000 \times g$ for 20 min. Carefully aspirate and save the supernatant.

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⁶The number of cells needed for a SILAC study depends heavily on the cell line used, the expression level and tyrosine phosphorylation level of the proteins of interest, and the sensitivity of the LC-MS technology used. Based on our experiments, it seems that 50 mg total protein is a good starting point (This may translate into significantly different cell numbers depending on the cell lines used).

2. Add the agarose-conjugated anti-phosphotyrosine antibody PY99 to the lysate (10 μg in 20 μL of PY99 beads per mL lysate) and incubate with rotation at 4°C for 4 h. Remove the lysate and transfer the beads into 1.5-mL Handee™ spin cups with paper filters (Pierce, Rockford, IL).
3. Wash the beads four times with lysis buffer and once with water. Elute precipitated proteins by incubating the beads in equal volume of 0.2% TFA per 0.5% SDS for 10 min at room temperature. Repeat the elution once and combine eluates. Alternatively the proteins can be eluted using other approaches (*see* Note 7).
4. Neutralize the eluate with 1 M ammonium bicarbonate (1 μL ammonium bicarbonate per 100 μL elution buffer). Concentrate the eluate up to tenfold using a vacuum centrifuge (SpeedVac) to reduce the volume. Add Laemmli sample loading buffer. At this point the color of the sample should be blue. If the color is yellow it indicates the pH is acidic and should be further adjusted with ammonium bicarbonate. Heat the sample at 95°C for 5 min before loading the sample onto a Bio-rad 7.5% precast Tris–HCl PAGE gel (*see* Note 8).
5. After electrophoresis stain the gel with Coomassie blue, which can detect as little as 50–100 ng of a single protein in a gel. With sample loading of protein immunoprecipitated from five dishes of cells per PAGE lane, there should be hardly any bands visualized. Presence of dark bands often suggests considerable nonspecific binding in the IP.

3.5. In-Gel Digestion

Before digestion, horizontally cut each gel lane into 10–20 gel bands. This serves as a fractionation step to reduce sample complexity and improve the dynamic range of protein identification by LC-MS/MS. Wear gloves to prevent keratin contamination. Do the digestion in a laminar flow tissue culture hood if possible to minimize contamination by dust or other atmospheric particulates.

1. Cut each gel band into small pieces (~1-mm³ cubes) and destain in 25 mM ammonium bicarbonate in 50% acetonitrile. Repeat the destaining step until the gels are completely clear. Add acetonitrile to shrink the gels. Now the gels should be white in color. Any blue color indicates insufficient destaining, which may cause decreased sensitivity in LC-MS/MS analysis. Remove the acetonitrile by aspiration and dry the gels with a SpeedVac.
2. Rehydrate the gel pieces with ice-cold 12.5 ng/ μL trypsin solution in 25 mM ammonium bicarbonate and incubate on ice for 20 min or till the gels are fully swollen to allow trypsin to enter into the gel pieces. Remove surplus trypsin solution. Add 25 mM ammonium bicarbonate (about 25 μL for a 0.6-mL tube or 50 μL for a 1.5-mL tube) to cover the gels to prevent the gels from drying during digestion. Incubate overnight at 37°C.
3. Peptide extraction: Add the extraction buffer to the gels, mix for 10 min and sonicate for 10 min. Spin briefly and save the supernatant. Repeat the extraction once with the extraction buffer and once with acetonitrile. Concentrate the digests to almost dryness in a SpeedVac and reconstitute with 0.1% FA in 2% acetonitrile to about 5 μL . Now the digests are ready for LC-MS analysis.

⁷Another commonly used elution approach is to boil beads in Laemmli buffer. However, the use of DTT and heat will cause leaching of the crosslinked antibody from agarose beads. As a result, the light chain and heavy chain of the antibody will show up on the SDS-PAGE gel and affect identification of proteins with molecular weights near 25 kDa (IgG light chain) and 50 kDa (IgG heavy chain).

⁸Load the sample in as few lanes as possible to maximize the protein concentration in each lane. This will facilitate subsequent in-gel digestion and LC-MS/MS.

3.6. HPLC-MS/MS

In this step the peptides from in-gel digestion are separated by HPLC and introduced into MS via online nanoelectrospray. In MS the peptide ions are analyzed in a data-dependent manner: First the m/z values of the intact peptides are measured. Then the most intense ions are selected for MS/MS through collision induced fragmentation (22), in which a peptide is broken into fragment ions. The pattern of the fragment ions contains sequence information and is used to search protein databases for peptide sequence identification.

1. Load the peptides onto a trap column ($180\ \mu\text{m} \times 2\ \text{cm}$ Symmetry C18, Waters) with 100% mobile phase A for 4 min at $5\ \mu\text{L}/\text{min}$.
2. After sample loading, the peptides are eluted using a gradient of 6–40% mobile phase B over 120 min at $0.25\ \mu\text{L}/\text{min}$.
3. Mass spectra are 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 most intense five peaks selected from each survey scan. Automatic gain control is set to 500,000 for Orbitrap survey scans and 10,000 for LTQ MS/MS scans. Survey scans are acquired in profile mode and MS/MS scans are acquired in centroid mode.

3.7. Data Processing

Peak list files containing peptide masses and their corresponding fragment ion masses and intensities are extracted from raw MS files. Then these peak lists are used by protein search tools to match the MS information to sequences in protein databases for protein identification. In this example Mascot is used for protein identification and MSQuant is used for quantitation. Alternatively there are other tools with similar functions available (23).

1. Mascot generic format files are generated from the raw data using DTASuperCharge (version 1.01) and Bioworks (version 3.2, Thermo Fisher Scientific) for database searching.
2. Mascot software (version 2.1.0, Matrix Science, London, UK) is used for database searching. An IPI database containing mouse and rat protein sequences is used. Peptide mass tolerance is 10 ppm, fragment mass tolerance is 0.6 Da, trypsin specificity is 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.
3. Proteins that are introduced during sample preparation should be excluded from the reported protein list. These proteins include: Keratins and trypsin (from in-gel digestion); immu-noglobins (from the PY99 antibody and Fc fusion protein); ephrinB1 (the stimulating ligand); ferritin and serum albumin (from cell culture).
4. SILAC quantitation is 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 SILAC ratio of a protein is calculated by comparing the summed MS intensities of all matched light peptides with those of the heavy peptides.
5. As a loading control, a small volume of the combined lysate 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.

6. The SILAC protein ratios are used to determine whether the proteins are involved in EphB signaling (*see* Note 9–¹¹). If after cell stimulation the SILAC ratio of a protein is “up regulated,” i.e., more abundant in the pTyr IP, it indicates that the protein is very likely to be tyrosine phosphorylated after activation of EphB2 receptor. Another possibility is that the protein binds to another “up regulated” protein through non-pTyr dependent interactions (*see* Note 9). Conversely, the most likely explanations for a “down regulated” SILAC ratio are that the protein is dephosphorylated or binds to another “down regulated” effector. An unchanged SILAC ratio suggests either the tyrosine phosphorylation level of the protein is not affected by the stimulation or the protein is pulled down by pTyr IP through nonspecific binding. Figure 2 shows an example of an MS spectrum corresponding to each scenario.

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⁹In general the anti-pTyr IP should pull down pTyr proteins together with their interacting proteins and thus these interacting proteins should also be detected by SILAC. However, it should be noted that the interactions that rely on phosphorylated tyrosines are generally not present in the IP due to competitive binding from the antibody.

¹¹The cutoff SILAC ratio to define significant changes depends on the accuracy of quantitation, which in turn can be affected by the type of MS instrument used, the quantitation software, the signal to noise ratio of the peptide in MS and the biological reproducibility. 1.5-fold is a commonly used cutoff when QTOF and Orbitrap instruments are used (11, 12, 17). Ideally one should perform a control experiment using SILAC samples with predefined ratios to test the accuracy of the quantitation.

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Fig. 1.

Strategy for using SILAC to study RTK signaling. Two sets of cells are cultured in light and heavy SILAC medium, respectively. After complete SILAC labeling, one set of cells is treated to activate the RTK while the other is used as a control. The two cell lysates are combined at an equal ratio for anti-pTyr IP to pull down phosphotyrosine proteins and their binding partners. The immunoprecipitated proteins are fractionated by SDS-PAGE, digested in-gel and analyzed by LC-MS/MS. In MS spectra, light and heavy labeled peptides appear as peak doublets (light: *dashed line*; heavy: *solid line*). Their ratios can be used to indicate if the proteins participate in the RTK signaling.

Fig. 2.

Protein quantitation using SILAC. The lower-mass peak clusters (*open circles*) are from light Arg/Lys peptides from the stimulated cells while the higher-mass peak clusters (*black circles*) are from heavy Arg/Lys peptides from the control cells. All peptide ions shown are doubly charged and the m/z difference between the light and heavy peaks is three (mass difference is 6 Da). Ratios were determined by comparing the heights of the peaks from light and heavy peptides. **(a)** A SILAC peptide pair (LQLSVTEVGTEK) from Afadin, a downstream effector of EphB2 receptor, which was highly enriched in the anti-pTyr IP after ephrinB1 stimulation. **(b)** A peptide pair (YLEASYGLSQGSSK) from Shep1, a downstream effector of EphB2 receptor, which was less abundant in the anti-pTyr IP after ephrinB1 stimulation. **(c)** A peptide pair (GYSFTTTAER) from actin with a ratio of 1:1, indicating this protein does not participate in the signaling pathway.