Published in final edited form as:

Methods Mol Biol. 2017 ; 1487: 113–126. doi:10.1007/978-1-4939-6424-6_8.

Single-Step Affinity Purification of ERK Signaling Complexes Using the Streptavidin-Binding Peptide (SBP) Tag

Liu Yang[1] and **Alexey Veraksa**[2]

[1]Department of Biology, University of Massachusetts Boston, 100 Morrissey Blvd., Boston, MA, 02125, USA.

[2]Department of Biology, University of Massachusetts Boston, 100 Morrissey Blvd., Boston, MA, 02125, USA. Alexey.veraksa@umb.edu.

Abstract

Elucidation of biological functions of signaling proteins is facilitated by studying their protein–protein interaction networks. Affinity purification combined with mass spectrometry (AP-MS) has become a favorite method to study protein complexes. Here we describe a procedure for single-step purification of ERK (Rolled) and associated proteins from *Drosophila* cultured cells. The use of the streptavidin-binding peptide (SBP) tag allows for a highly efficient isolation of native ERK signaling complexes, which are suitable for subsequent analysis by mass spectrometry. Our analysis of the ERK interactome has identified both known and novel signaling components. This method can be easily adapted for SBP-based purification of protein complexes in any expression system.

Keywords

Streptavidin-binding peptide; SBP; Affinity purification; Mass spectrometry; ERK; *Drosophila*

1 Introduction

Receptor tyrosine kinase (RTK)/extracellular signal-regulated kinase (ERK) signaling controls many cellular processes, including proliferation, differentiation, and apoptosis [1, 2]. Dysregulation of this pathway has been implicated in multiple human diseases, including cancer [1, 3, 4]. The development of relevant therapies depends on the knowledge of the structure and dynamics of the ERK signaling network. Genetic analysis of the RTK/ERK pathway in *Drosophila* and other model systems identified its major components, and subsequent biochemical studies revealed key protein–protein associations, such as those involving the core Raf-MEK-ERK kinase cascade, as well as scaffolding and adaptor proteins [5–7]. However, emerging evidence suggests that the ERK signaling network is complex and includes dozens of substrates and additional regulatory molecules [8, 9].

Affinity purification of protein complexes followed by mass spectrometry-based identification of interacting components has emerged as a powerful method to study signaling networks [10, 11]. Previously, we applied tandem affinity (TAP) purification to isolate signaling complexes in *Drosophila*, first using the original TAP tag [12, 13], and

more recently using an improved version, the GS-TAP tag [14, 15]. Both of these methods are rather lengthy, as they require two affinity binding steps separated by tobacco etch virus (TEV) protease cleavage after the first affinity column. Due to inevitable sample loss at every step, they require a large amount of starting material. In order to overcome these limitations, we have developed and present here an efficient single-step purification approach for isolating signaling complexes from *Drosophila* cultured cells, based on the use of streptavidin-binding peptide (SBP).

SBP is a 38-amino acid peptide that was artificially selected for high-affinity binding to native streptavidin [16]. Compared to other affinity purification methods, the SBP tag offers several advantages. First, the SBP tag is relatively compact and therefore less likely to impair protein function (4 kDa vs. 20 kDa for the TAP or GS-TAP tags). Second, SBP interacts with native streptavidin with high affinity (K_d = 2.5 nM), which results in an efficient association of the tagged protein with the matrix [16]. Third, we have found that the SBP tag works well when placed either at the amino or carboxy terminus of the protein, or even in the middle. Fourth, SBP-tagged proteins can be eluted with a heterologous compound, biotin, which results in a lower carryover of contaminants compared to other elution methods, such as competition with excess tag peptide. Fifth, streptavidin matrices are less costly than many other affinity resins. Finally, single-step purification is faster and less labor-intensive than TAP. These properties make SBP tagging an attractive option for affinity purification studies. Usability of this approach has been validated in our studies involving various signaling proteins [17–19], and this method has also been used to purify complexes from vertebrate cultured cells [20].

We describe a procedure to generate *Drosophila* cultured cells stably expressing SBP-tagged ERK, followed by purification of ERK protein complexes. The mass spectrometry step is not presented; however, we give suggestions for preparing samples for this analysis and for analyzing mass spectrometry data using the SAINT (significance analysis of interactome) program [21]. Using this workflow, we were able to identify most of the known core components of ERK signaling, including two major ERK phosphatases. In addition, our list of ERK interactors includes several proteins that have not been previously associated with ERK signaling. Though the method we describe is for *Drosophila* cultured cells, it can be easily adapted for mammalian cells or any other cell culture system.

2 Materials

A tissue culture facility and a general molecular biology lab with −20 and −80 °C freezers and a 4 °C cold room or a refrigeration chamber are needed for carrying out this protocol. Prepare all solutions using ultrapure (18.2 MΩ cm) water.

2.1 Tissue Culture and Cell Transfection Materials and Reagents

1. Tissue culture hood with vacuum connection. Cell transfections are performed in the hood.
2. Cell culture incubator at 25 °C. Use a dish with ultrapure water at the bottom shelf to maintain humidity in the incubator.

3. pMK33-SBP-N and pMK33-SBP-C expression vectors.
4. Qiagen Maxiprep plasmid purification kit.
5. Qiagen Effectene transfection reagent.
6. *Drosophila* S2 cells (*see* **Note 1**).
7. Tissue culture treated sterile 6-well plates, e.g., Corning catalog number 3516.
8. Tissue culture treated vented 25- and 75-cm^2 flasks, e.g., Corning catalog number 353109.
9. 15- and 50-mL Falcon tubes, sterile and regular Eppendorf tubes.
10. 250- and 500-mL sterile disposable filter units for preparing S2 cell media, 0.2 μm pore size.
11. Clinical centrifuge that accommodates 15- and 50-mL Falcon tubes, at 4 °C.
12. Microcentrifuge, at 4 °C.
13. Vortex mixer in or next to the tissue culture hood.
14. Gibco Schneider's *Drosophila* Medium (1×) with l-glutamine (Life Technologies catalog number 21720024). Store at 4 °C.
15. Gibco Fetal Bovine Serum (FBS), heat-inactivated (Life Technologies catalog number 10082147). Aliquot by 50 mL in 50-mL Falcon tubes, store at −20 °C.
16. Gibco Penicillin-Streptomycin, "Pen/Strep" (5000 U/mL) (Life Technologies catalog number 15070063). Aliquot by 5 mL in 15-mL Falcon tubes, store at −20 °C.
17. Complete S2 cell medium: in the tissue culture hood, combine in a 500-mL filter unit: 500 mL Schneider's Drosophila Medium, 50 mL FBS, and 5 mL Pen/Strep, filter using vacuum connection, cap and swirl to mix. Store at 4 °C. Warm to room temperature before use.
18. Hygromycin: 300 mg/mL stock solution. Dissolve 250 mg hygromycin (e.g., Sigma catalog number H3274) in 800 μL sterile PBS or ultrapure water, store at 4 °C in the dark.
19. Complete S2 cell medium with 300 μg/mL hygromycin: co-filter 250 mL complete S2 cell medium (**item 17**) with 250 μL hygromycin stock solution (1:1000 dilution). Wrap medium with hygromycin with aluminum foil to protect from light and store at 4 °C for up to 1 month. A larger (500 mL) batch can be made as needed.
20. 20. Anti-SBP antibody: Santa Cruz Biotechnology sc-101595, anti-SBP Tag (clone SB19-C4). Use 1:1000 for western blotting to check the expression of SBP-tagged proteins.

2.2 Affinity Purification Reagents

1. 0.07 M $CuSO_4$: dissolve 8.74 g $CuSO_4$ $5H_2O$ in 500 mL water, filter-sterilize, store at room temperature.
2. 1× phosphate buffer saline (PBS). Store at 4 °C.
3. 5× lysis buffer: 250 mM Tris pH 7.5, 25 % glycerol, 1 % IGEPAL, 7.5 mM $MgCl_2$, 625 mM NaCl, 125 mM NaF, 5 mM Na_3VO_4. To make 200 mL: completely dissolve 1 g NaF powder and 184 mg Na_3VO_4 powder in 71 mL water with constant stirring. Add 50 mL 1 M Tris pH 7.5, 2 mL 100 % IGEPAL (Sigma catalog number I8896), 1.5 mL 1 M $MgCl_2$, 25 mL 5 M NaCl, then add 50 mL glycerol (add last) and continue stirring for 1 h. Filter-sterilize using 250-mL filter unit (this step can be slow). Aliquot by 10 mL in 50-mL Falcon tubes and store at −80 °C.
4. 1 M dithiothreitol (DTT) solution. Store at −20 °C.
5. cOmplete™ protease inhibitor cocktail tablets, with EDTA (Roche catalog number 11697498001; 20 tablets). Store at 4 °C.
6. Streptavidin beads: Pierce™ Streptavidin Plus UltraLink™ Resin (Pierce catalog number 53117).
7. 10-mL Luer-Lok disposable syringes.
8. Syringe filters, 26 mm diameter, SFCA membrane, 0.45 μm pore size (e.g., Corning catalog number 431220).
9. Dry heating block at 95 °C.
10. 4× SDS sample buffer: 8 % SDS, 160 mM Tris pH 6.8, 30 % glycerol, 1 mg/mL bromophenol blue. To make 100 mL; combine 50 mL water, 8 g SDS powder, 16 mL 1 M Tris pH 6.8, 30 mL glycerol, 100 mg bromophenol blue powder. Dissolve well with constant stirring, filter-sterilize and store at room temperature. Before use, mix 950 μL buffer with 50 μL 1 M DTT (to get 50 mM final DTT concentration), vortex to mix. Buffer with DTT is stored at −20 °C.
11. 2× SDS sample buffer: mix equal volumes of 4× SDS sample buffer (with DTT) and water.
12. Rotating wheel with clamps for Falcon tubes and Eppendorf tubes, at 4 °C.
13. 200 mM biotin stock solution: in the chemical hood, mix 875 μL water with 125 μL NH_4 OH (ammonium hydroxide solution, 28 % NH_3 in water), this will make 2 M NH_4OH solution. To this solution, add 50 mg biotin (e.g., Sigma catalog number B4501) and vortex well. Store at −20 °C.
14. 2 mM biotin working solution: dilute 200 mM biotin stock solution 1:100 with lysis buffer with DTT and cOmplete™ protease inhibitor, *see* protocol Subheading 3.2, **step 2** (e.g., 990 μL Lysis buffer and 10 μL of 200 mM biotin stock). Make 2 mM biotin working solution right before use and discard unused portion.

15. 100 % (w/v) trichloroacetic acid (TCA) solution: combine 500 g TCA crystals with 350 mL water, mix and store at room temperature.
16. 10 % (w/v) TCA solution: dilute 100 % TCA 1:10 with water. Store at 4 °C.
17. Acetone at −20 °C.

2.3 Reagents for Silver-Staining Gels

1. Molecular weight marker: any unstained marker can be used; we prefer Bio-Rad Precision Plus unstained standards. Dilute 1:10 with 2× SDS sample buffer. Store at −20 °C.
2. Gel for silver staining: a regular SDS-PAGE can be used; however, we find that commercial gradient gels provide a better coverage of the complete molecular weight range. We use Novex NuPAGE 4–12 % Bis-Tris gels, 1.5 mm thick, with 10 wells, Life Technologies catalog number NP0335. They require a corresponding gel apparatus, such as the XCell SureLock Mini system.
3. Electrophoresis buffer for NuPage Bis-Tris gels: MOPS SDS running buffer, with antioxidant, Life Technologies catalog number NP0001.
4. Silver staining kit: Life Technologies SilverQuest Staining Kit, catalog number LC6070.
5. Dish for silver staining.

3 Methods

3.1 Transfection and Establishment of Stable S2 Cell Lines

1. Clone your protein of interest into the pMK33-SBP-N or pMK33-SBP-C vector (Fig. 1a) (*see* **Note 2**). For this procedure, we used full-length *Drosophila* ERK (Rolled) cloned into pMK33-SBP-C. Prepare DNA for transfection using Qiagen Maxiprep protocol following manufacturer's recommendations (*see* **Note 3**).
2. Dispense 1.5 mL per well of complete S2 cell medium in a 6-well plate. Add 0.5 mL of *Drosophila* S2 cells from dense cultures (4–5 days). Incubate at 25 °C overnight (optional) or at least 3 h to allow cell attachment to the bottom of the well.
3. Use Qiagen Effectene transfection reagent to prepare DNA for transfection into cells (*see* **Note 4**). Perform all transfection steps in the hood. Mix in a sterile Eppendorf tube: 150 μL buffer EC, 2 μg DNA, 16 μL Enhancer, vortex for 5 s. Incubate at room temperature for 5 min, briefly spin down. Add 21 μL Effectene reagent, immediately vortex for exactly 10 s, do not spin down. Incubate at room temperature for 15 min.
4. Remove old medium from cells using 1-mL tip. Add 1 mL of fresh complete medium to cells, then add 1 mL of fresh complete medium to the tube with DNA from **step 3**, gently but thoroughly pipet up and down 4–5 times, add DNA/

medium solution dropwise to cells, swirl to mix. Total volume will be 2 mL per well. Incubate plates with transfected cells for 48 h at 25 °C.

5. Resuspend cells in the well, transfer to 15-mL Falcon tube, spin down for 3 min at 500 × *g* in a clinical centrifuge, resuspend in 10 mL of complete medium with 300 μg/mL hygromycin, and seed the cells in a 25 cm^2 vented flask.

6. Carry out selection of stable cell lines. Watch the number of cells in the flasks and allow them to reach good density before splitting. During the first 2–3 weeks, significant cell death will be visible, and splitting can be done more rarely and retaining a higher volume of cells (e.g., 1:1 split once a week instead of a normal 1:5 split every 4–5 days). Medium should contain hygromycin during all passages. Cells in a control well (e.g., transfected with actin-GFP) will completely die out after 3–4 weeks, and cells in the experimental well should grow normally after about a month of continuous selection (*see* **Note 5**).

3.2 Cell Lysis and Affinity Purification

A general workflow for affinity purification steps is shown in Fig. 1b. Unless indicated otherwise, all cell collection, lysis and affinity purification steps should be performed on ice (*see* **Note 6**).

1. Amplify cells in two 75-cm^2 vented flasks, 25 mL in each flask, for a total of 50 mL (*see* **Note 7**). Use untransfected S2 cells grown in parallel as a negative control sample for purifications, and follow all of the same steps with that sample. Allow the cells to grow to medium-high density (3–4 days) and induce overnight with 0.07 mM $CuSO_4$ by adding 25 μL of 0.07 M $CuSO_4$ stock solution to 25 mL of cells in a flask (1:1000 dilution). Mix well by swirling and rocking the flask (*see* **Note 8**).

2. Prepare lysis buffer: add 40 mL water to 10 mL of 5× concentrated lysis buffer (stored at −80 °C) in a 50-mL Falcon tube. Add 50 μL of 1 M DTT to a final concentration of 1 mM, mix well and separate into two 50-mL Falcon tubes, 25 mL in each. To one of the tubes, add one cOmplete™ protease inhibitor tablet and rotate at 4 °C for 30 min (*see* **Note 9**). At the end, check to make sure the cOmplete™ tablet has fully dissolved. The second tube can be stored at −80 °C and will only require addition of the cOmplete™ tablet before the next experiment. 25 mL of lysis buffer is sufficient for up to 3 purification samples.

3. While cOmplete™ tablet is dissolving, resuspend cells in flasks with a 10-mL pipette and collect into a 50-mL Falcon tube on ice.

4. Spin in a clinical centrifuge at 500 × *g* for 3 min at 4 °C.

5. Remove supernatant by aspiration and wash cells with 50 mL of cold PBS. Mix by inversion.

6. Spin in a clinical centrifuge at 500 × *g* for 5 min at 4 °C. Remove as much supernatant as possible.

7. Lysis: add 1 mL of cold lysis buffer with cOmplete™ protease inhibitor (from **step 2**) to cells and pipet up and down 4–5 times to lyse the cells. For more efficient lysis, press the tip against the bottom of the tube to create shearing force. Transfer lysate to a chilled Eppendorf tube and incubate on ice for 15–20 min.

8. Prepare streptavidin beads, aiming for 50 μL of packed beads per sample. Take the appropriate amount of 50 % bead slurry and add to an Eppendorf tube. Wash beads three times with 1 mL of lysis buffer (*see* **step 2**), mixing by inversion. After each wash, centrifuge the tube for 1 min at 500 × *g* at 4 °C. After the last wash, remove supernatant, leaving the volume that is equal to the volume of packed beads (to obtain 50 % slurry after subsequent mixing). Keep washed beads on ice.

9. Centrifuge cell lysates from **step 7** at maximum speed (e.g., 14,000 × *g*) at 4 °C for 15 min.

10. This step is best performed in the cold room. Aspirate supernatants with 1-mL tip and load into chilled 10-mL syringes with 0.45 μm filters attached. Push all of the solution into fresh Eppendorf tubes on ice. Optional: save Before Binding (BB) analytical sample by mixing 50 μL of lysate with 25 μL of 4× SDS sample buffer (*see* **Note 10**). Vortex and heat at 95 °C for 5 min. Store at −20 °C.

11. Binding: resuspend washed streptavidin beads by pipetting and add 100 μL of slurry (corresponding to 50 μL of packed beads) to each sample (*see* **Note 11**). Rotate for 2–3 h at 4 °C on a rotating wheel.

12. Remove the tubes from the rotating wheel and centrifuge at 500 × *g* for 1 min at 4 °C. Optional: save Flow-through (FT) analytical sample by mixing 50 μL of supernatant with 25 μL of 4× SDS sample buffer. Vortex and heat at 95 °C for 5 min. Store at −20 °C. Aspirate and discard the rest of the supernatants, keeping the bead pellets.

13. Washes: add 1 mL of lysis buffer (*see* **step 2**) to the beads, mix by inversion 4–5 times and spin down at 500 × *g* for 1 min at 4 °C. Aspirate and discard the supernatant.

14. Repeat **step 13** four more times for a total of five washes. After the last wash, take care to remove as much supernatant as possible, without dislodging the beads.

15. Elution: prepare 2 mM biotin working solution, 300 μL per sample, right before use, by diluting 200 mM biotin stock solution 1:100 with lysis buffer. Resuspend bead samples in 100 μL of 2 mM biotin working solution. Use a pipet tip with a large orifice to resuspend the beads gently but completely by pipetting. Incubate on ice for 5 min. Centrifuge at 500 × *g* for 1 min at 4 °C. Carefully collect 100 μL of supernatant, avoiding the beads, and place in a new Eppendorf tube on ice.

16. Repeat **step 15** two more times, each time adding 100 μL of the supernatant to the same Eppendorf tube on ice, for a total of 300 μL of eluate after three elution

steps. Optional: collect Retentate (RT) sample: place the tubes with the beads into a rack at room temperature and let stand for 2 min. Add 50 μL of 4× SDS sample buffer to the beads, mix by swirling with a tip without pipetting, heat at 95 °C for 5 min and store at −20 °C (*see* **Note 12**).

17. Spin down pooled eluate at full speed for 30 s at 4 °C and transfer supernatant into a fresh Eppendorf tube, avoiding any beads that may remain at the bottom. Optional: collect Eluate (EL) sample by combining 10 μL of eluate with 10 μL of 4× SDS sample buffer. Mix by flicking, heat at 95 °C for 5 min and store at −20 °C.

18. TCA precipitation: the final eluate will be divided into two unequal parts to prepare samples for mass spectrometry and silver-stained gel analysis (*see* Fig. 1b). For the mass spectrometry sample, combine 180 μL of eluate with 20 μL of 100 % TCA in a fresh Eppendorf tube labeled “MS.” For the silver-stained gel sample, combine 90 μL of eluate with 10 μL of 100 % TCA in a fresh Eppendorf tube labeled “SSG.” Mix well and incubate on ice for 1 h.

19. Centrifuge MS and SSG samples at maximum speed for 15 min at 4 °C.

20. TCA wash: remove supernatant, add 500 μL of 10 % TCA, mix by inversion, and centrifuge samples at maximum speed for 15 min at 4 °C.

21. Acetone washes: remove TCA wash and add 500 μL of cold acetone. Mix by inversion. Centrifuge samples at maximum speed for 5 min at 4 °C.

22. Remove supernatant and repeat acetone washes (**step 21**) three more times for a total of four washes. After the last wash, remove supernatant as completely as possible and allow the pellets to dry overnight in open tubes at room temperature. Loosely cover the rack with the tubes with aluminum foil to prevent dust from falling into tubes. Store dried samples at −20 °C until ready to analyze by gel electrophoresis or mass spectrometry.

3.3 Silver-Stained Gel

1. Prepare samples for analysis on a silver-stained gel. To the SSG dried pellet from Subheading 3.2, **step 22**, add 20 μL of 2× SDS sample buffer and let stand at room temperature for 15 min, flicking the tube periodically. Heat at 95 °C for 5 min.

2. Run the samples using your choice of SDS-PAGE setup. We have successfully used Novex NuPAGE 4–12 % Bis-Tris gradient gels run with MOPS running buffer, as they offer excellent separation of most molecular weights. Other brands can be used for this purpose.

3. After the dye front reaches the bottom of the gel, open the cassette and place the gel in a staining dish. Perform silver staining of the gel using the SilverQuest Staining Kit following the Basic Staining Protocol, per manufacturer’s instructions.

4. Capture the image of the stained gel using a scanner or another imaging device. An example of a silver-stained gel after ERK-SBP purification is shown in Fig. 2a (*see* **Note 13**).

3.4 Suggestions for Preparation of Samples for Mass Spectrometry and Analysis of Interacting Proteins

After obtaining the final dried pellets of purified protein complexes, samples can be analyzed by a variety of methods, including mass spectrometry (MS). Generally, the user is advised to follow recommendations of their MS facility of choice. Dried pellets can be used directly for trypsin digestion and liquid chromatography/tandem mass spectrometry (nanoLC-MS/MS) analysis. In that case, care should be taken to purify the peptides away from biotin and detergent. We found that separating the samples on a short SDS-PAGE gel prior to submission for MS analysis improves protein identification. The dye front is allowed to migrate in the separating gel up to a distance of 1 cm, then the gel is stained using a standard Coomassie dye/methanol/acetic acid procedure, destained with 25 % methanol/5 % acetic acid and extensively washed in water, after which the lane is cut into two square 5 mm × 5 mm pieces which are submitted for MS analysis.

MS results can be analyzed in various ways. When applied to studying protein–protein interactions, often a goal is to identify genuine interacting components and eliminate contaminants. We found that one reliable and unbiased way to do it is using the program SAINT (significance analysis of interactome) [21]. We and others have validated SAINT in studies of various signaling complexes [18, 22]. The user is advised to follow the procedure described in the original SAINT publication [21]. We use the number of unique peptides identified for each protein in a given purification dataset as input values, and run SAINT using defaults; however, using total peptide numbers is also possible, as long as the user consistently applies the same approach for both the control and experimental samples. The most reliable results are obtained when two or more experimental purifications are compared to two or more controls, preferably (but not critically) run in parallel with experimental samples. A higher number of controls will further increase the reliability of identifying genuine interactions.

Using this approach, we analyzed data from two independent ERK-SBP purifications from *Drosophila* S2 cells and compared them to 5 control samples obtained from untransfected S2 cells. The top portion of the SAINT output for these experiments is shown in Fig. 2b, and a complete dataset is available at [23]. A key value to consider for evaluating protein interactions returned by SAINT is AvgP, which is an average probability of interaction for every identified protein (Fig. 2b). AvgP values above 0.8 are considered significant [21]; however, relevant proteins may be identified even with lower values. Using SAINT cutoff of 0.6, we were able to identify most of the known components of ERK signaling in our studies (such as the core kinases Phl (Raf) and Dsor1 (MEK) [6], scaffolds and adaptors Sos, Ksr, and Cnk [5], phosphatases Mkp3 and PTP-ER [24], and a transcriptional repressor Cic [25]), as well as several novel putative ERK interactors (Fig. 2c).

4 Notes

1. There are different “flavors” of S2 cells grown in various laboratories. We obtained our S2 cells from S. Artavanis-Tsakonas. Once a given line is selected for work, it should be consistently used for both control and experimental samples.
2. We have developed these vectors based on our previously published pMK33-NTAP(GS) and pMK33-CTAP(SG) vectors [15]. pMK33-based vectors allow for inducible expression of a gene of interest using induction with $CuSO_4$, and facilitate establishment of stable cell lines using hygromycin selection, because hygromycin resistance gene is carried in the same construct. These vectors are medium-copy plasmids.
3. We find that a final phenol/chloroform extraction of plasmid DNA after Qiagen Maxiprep followed by precipitation with sodium acetate/ethanol further improves transfection efficiency.
4. Qiagen Effectene transfection reagent does not require incubation of either cells or DNA in serum-free media. It is always good to include a separate transfection control performed in parallel to the main experiment, e.g., a plasmid encoding actin-GFP. This way general success of transfection can be verified by assessing the number of GFP-positive cells in a small aliquot from that well, and also the same well can be used to verify successful selection using hygromycin, as control cells that do not have the pMK33 plasmid should all die during selection.
5. After establishment of a stable cell line, expression of the tagged protein can be verified after an overnight induction with 0.35 mM $CuSO_4$ followed by cell lysis and western blotting using anti-SBP antibody.
6. For holding Eppendorf tubes during the washes and solution changes, we use an aluminum block that is embedded in ice (e.g., from a dry bath). This makes it convenient to perform tube inversions by removing the whole block with the tubes, rather than doing it one by one for each tube, and keeps the samples at 0 °C at all times. Care should be taken to make sure that the ice is well packed around the block and there is little water in the ice bucket, otherwise the block may sink.
7. While hygromycin-containing medium should be used for maintaining the main cell line at all times, hygromycin can be omitted from the medium for final cell amplification step, which can reduce the cost.
8. This concentration of $CuSO_4$ will result in a medium level of protein induction. This is recommended to avoid possible artifacts resulting from extreme overexpression of the tagged protein.
9. We are using 2× recommended concentration of cOmplete™ protease inhibitor for a stronger inhibition of proteases. Remaining buffer with cOmplete™ protease inhibitor can be stored in 1-mL single-use aliquots at −80 °C.

10. Analytical samples can be collected throughout the procedure where indicated. These fractions can be analyzed by western blotting with anti-SBP antibody using approximately 10 μL from each of the collected samples.

11. Cut off the tip to obtain a larger orifice for bead resuspension.

12. The Retentate (RT) sample may contain a lot of protein, so further dilution (e.g., 1:5 with 2× SDS buffer) is recommended for western blot analysis.

13. Silver staining is used for a sensitive quality assessment of the sample. Some proteins may be visible in a control sample from untransfected S2 cells. The major band in the experimental lane will be the SBP-tagged bait protein, which should migrate at the expected molecular weight (taking into account 4 kDa added by the SBP tag). Additional bands of interacting proteins should be visible in the experimental lane.

Acknowledgements

This protocol was developed with participation of Manuel Valdes, Marla Tipping, and Wenjian Xu. The authors thank Heya Zhao for helpful comments on the manuscript. A.V. was supported by the NIH grant GM105813. L.Y. was supported by the UMass Boston Sanofi Genzyme Doctoral Fellowship. Mass spectrometry was performed at the Taplin Mass Spectrometry Facility at Harvard Medical School.

References

1. Lemmon MA, Schlessinger J (2010) Cell signaling by receptor tyrosine kinases. Cell 141:1117–1134 [PubMed: 20602996]
2. Futran AS, Link AJ, Seger R et al. (2013) ERK as a model for systems biology of enzyme kinetics in cells. Curr Biol 23:R972–R979 [PubMed: 24200329]
3. Newbern J, Zhong J, Wickramasinghe RS et al. (2008) Mouse and human phenotypes indicate a critical conserved role for ERK2 signaling in neural crest development. Proc Natl Acad Sci U S A 105:17115–17120 [PubMed: 18952847]
4. Rauen KA (2013) The RASopathies. Annu Rev Genomics Hum Genet 14:355–369 [PubMed: 23875798]
5. Li WX (2005) Functions and mechanisms of receptor tyrosine kinase Torso signaling: lessons from Drosophila embryonic terminal development. Dev Dyn 232:656–672 [PubMed: 15704136]
6. Sopko R, Perrimon N (2013) Receptor tyrosine kinases in Drosophila development. Cold Spring Harb Perspect Biol 5(6):a009050
7. Shilo BZ (2014) The regulation and functions of MAPK pathways in Drosophila. Methods 68:151–159 [PubMed: 24530508]
8. Friedman AA, Tucker G, Singh R et al. (2011) Proteomic and functional genomic landscape of receptor tyrosine kinase and ras to extracellular signal-regulated kinase signaling. Sci Signal 4(196):rs10
9. von Kriegsheim A, Baiocchi D, Birtwistle M et al. (2009) Cell fate decisions are specified by the dynamic ERK interactome. Nat Cell Biol 11:1458–1464 [PubMed: 19935650]
10. Gavin AC, Maeda K, Kühner S (2011) Recent advances in charting protein-protein interaction: mass spectrometry-based approaches. Curr Opin Biotechnol 22:42–49 [PubMed: 20934865]
11. Veraksa A (2013) Regulation of developmental processes: insights from mass spectrometry-based proteomics. Wiley Interdiscip Rev Dev Biol 2:723–734 [PubMed: 24014456]
12. Rigaut G, Shevchenko A, Rutz B et al. (1999) A generic protein purification method for protein complex characterization and proteome exploration. Nat Biotechnol 17:1030–1032 [PubMed: 10504710]

13. Veraksa A, Bauer A, Artavanis-Tsakonas S (2005) Analyzing protein complexes in Drosophila with tandem affinity purification-mass spectrometry. Dev Dyn 232:827–834 [PubMed: 15704125]
14. Burckstummer T, Bennett KL, Preradovic A et al. (2006) An efficient tandem affinity purification procedure for interaction proteomics in mammalian cells. Nat Methods 3:1013–1019 [PubMed: 17060908]
15. Kyriakakis P, Tipping M, Abed L et al. (2008) Tandem affinity purification in Drosophila: the advantages of the GS-TAP system. Fly (Austin) 2:229–235 [PubMed: 18719405]
16. Keefe AD, Wilson DS, Seelig B et al. (2001) One-step purification of recombinant proteins using a nanomolar-affinity streptavidin-binding peptide, the SBP-Tag. Protein Expr Purif 23:440–446 [PubMed: 11722181]
17. Gilbert MM, Tipping M, Veraksa A et al. (2011) A screen for conditional growth suppressor genes identifies the Drosophila homolog of HD-PTP as a regulator of the oncoprotein Yorkie. Dev Cell 20:700–712 [PubMed: 21571226]
18. Dent LG, Poon CL, Zhang X et al. (2014) The GTPase regulatory proteins pix and git control tissue growth via the Hippo pathway. Curr Biol 25(1):124–130 [PubMed: 25484297]
19. Zhang C, Robinson BS, Xu W et al. (2015) The ecdysone receptor coactivator Taiman links Yorkie to transcriptional control of germline stem cell factors in somatic tissue. Dev Cell 34:168–180 [PubMed: 26143992]
20. Kim JH, Chang TM, Graham AN et al. (2010) Streptavidin-binding peptide (SBP)-tagged SMC2 allows single-step affinity fluorescence, blotting or purification of the condensin complex. BMC Biochem 11:50 [PubMed: 21194474]
21. Choi H, Larsen B, Lin ZY et al. (2011) SAINT: probabilistic scoring of affinity purification-mass spectrometry data. Nat Methods 8:70–73 [PubMed: 21131968]
22. Kwon Y, Vinayagam A, Sun X et al. (2013) The Hippo signaling pathway interactome. Science 342:737–740 [PubMed: 24114784]
23. Yang L, Veraksa A (2015) SAINT output for a complete ERK-SBP purification dataset. https://xythos.umb.edu/xythoswfs/webui/_xy-e3032387_1-t_4JUTz7Bi. Accessed 10 Dec 2015
24. Rintelen F, Hafen E, Nairz K (2003) The Drosophila dual-specificity ERK phosphatase DMKP3 cooperates with the ERK tyrosine phosphatase PTP-ER. Development 130:3479–3490 [PubMed: 12810595]
25. Jimenez G, Shvartsman SY, Paroush Z (2012) The Capicua repressor—a general sensor of RTK signaling in development and disease. J Cell Sci 125:1383–1391 [PubMed: 22526417]

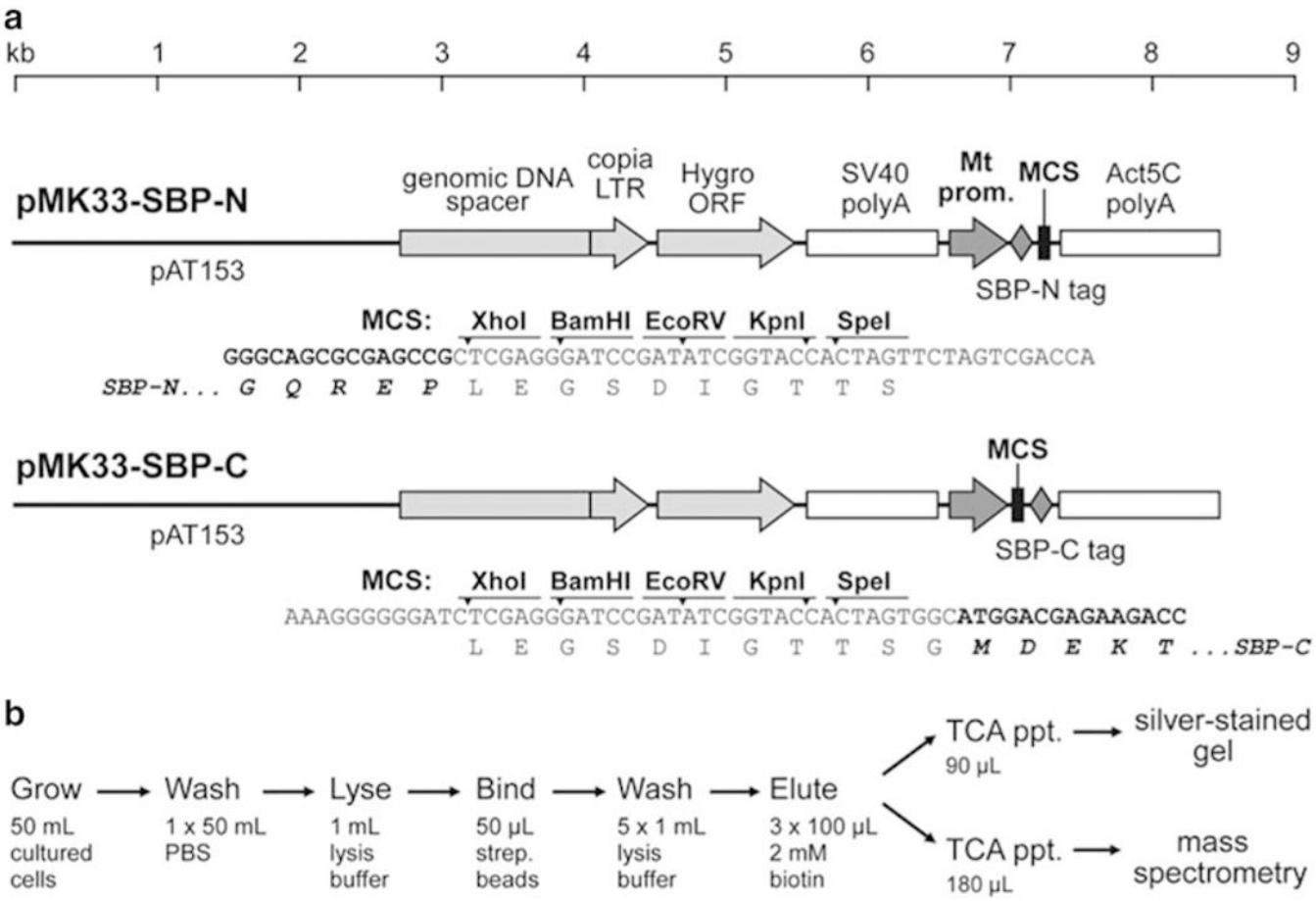
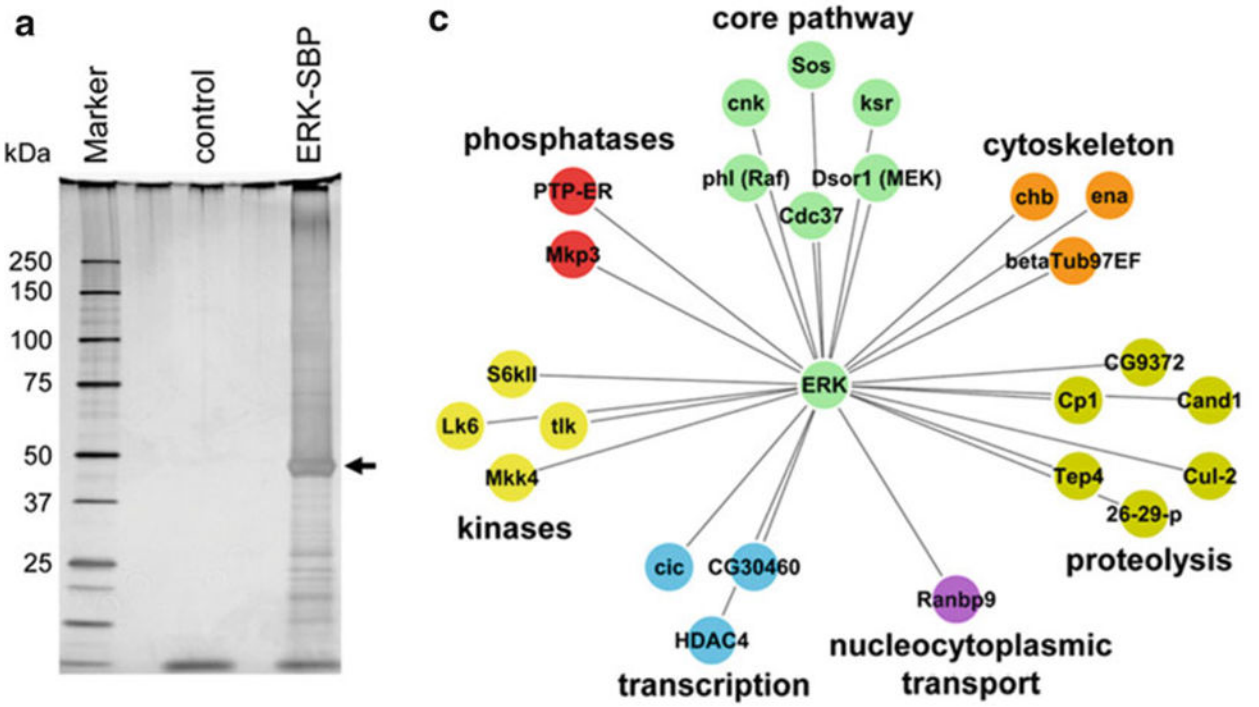


Fig. 1.

Diagram of the pMK33-SBP vectors and workflow for single-step SBP-based purification. (**a**) Diagram of the pMK33-SBP vectors. *Mt prom.* metallothionein promoter for $CuSO_4$-inducible expression, *MCS* multiple cloning site (polylinker). Unique cloning sites in the MCS are shown in *bold* and *underlined,* (**b**) Workflow for single-step SBP-based purification, as described in the protocol



b

	A	B	C	D	E	F	G	H	I	J	K	L
1	Bait	Prey	PreyGene	IP	Spec	SpecSum	NumRep	Prob	iProb	ctrlCounts	AvgP	MaxP
2	ERKWT	FBgn0003256	rl (ERK)	ERKWT1|ERKWT2	51|46	97	2	1	1.00|1.00	3|0|0|0|0	1	1
3	ERKWT	FBgn0041180	Tep4	ERKWT1|ERKWT2	40|32	72	2	1	1.00|1.00	2|1|0|0|0	1	1
4	ERKWT	FBgn0260748	CG5004	ERKWT1|ERKWT2	46|34	80	2	1	1.00|1.00	0|0|0|0|0	1	1
5	ERKWT	FBgn0001225	Hsp26	ERKWT1|ERKWT2	7|7	14	2	1	1.00|1.00	0|0|0|0|0	1	1
6	ERKWT	FBgn0262582	cic	ERKWT1|ERKWT2	8|7	15	2	1	1.00|1.00	0|0|0|0|0	1	1
7	ERKWT	FBgn0016081	fry	ERKWT1|ERKWT2	75|21	96	2	1	1.00|1.00	0|0|0|0|0	1	1
8	ERKWT	FBgn0016641	PTP-ER	ERKWT1|ERKWT2	53|50	103	2	1	1.00|1.00	0|0|0|0|0	1	1
9	ERKWT	FBgn0017581	Lk6	ERKWT1|ERKWT2	48|44	92	2	1	1.00|1.00	0|0|0|0|0	1	1
10	ERKWT	FBgn0262866	S6kII	ERKWT1|ERKWT2	38|27	65	2	1	1.00|1.00	0|0|0|0|0	1	1
11	ERKWT	FBgn0036844	Mkp3	ERKWT1|ERKWT2	16|8	24	2	1	1.00|1.00	0|0|0|0|0	1	1
12	ERKWT	FBgn0013275	Hsp70Aa	ERKWT1|ERKWT2	15|9	24	2	1	1.00|1.00	4|1|1|0|0	0.9995	1
13	ERKWT	FBgn0000578	ena	ERKWT1|ERKWT2	6|3	9	2	1	1.00|1.00	0|0|0|0|0	0.9965	0.997
14	ERKWT	FBgn0250848	26-29-p	ERKWT1|ERKWT2	3|3	6	2	0.99	0.99|0.99	0|0|0|0|0	0.993	0.994
15	ERKWT	FBgn0013770	Cp1	ERKWT1|ERKWT2	5|3	8	2	0.99	1.00|0.98	1|0|0|0|0	0.9895	0.998
16	ERKWT	FBgn0052016	CG32016	ERKWT1|ERKWT2	4|38	42	2	0.98	0.96|1.00	0|0|0|0|0	0.9825	1
17	ERKWT	FBgn0001226	Hsp27	ERKWT1|ERKWT2	3|3	6	2	0.98	0.98|0.98	0|0|0|0|0	0.979	0.981
18	ERKWT	FBgn0003079	phl	ERKWT1|ERKWT2	6|2	8	2	0.97	0.99|0.95	0|0|0|0|0	0.972	0.993

Fig. 2.

Results of ERK-SBP purification from *Drosophila* S2 cells. (**a**) Silver-stained gel showing a typical result from SBP-based purifications. Control sample was obtained using untransfected S2 cells processed in parallel with the stable ERK-SBP-expressing cell line. *Arrow* indicates position of the bait ERK-SBP protein. Multiple additional bands are visible in the ERK-SBP lane. (**b**) Top portion of SAINT output. Proteins are sorted according to the average probability of interaction, AvgP (column K). The Spec column shows the numbers of unique peptides identified for each protein in the experimental samples, whereas

ctrlCounts reports peptides for the same protein observed in control samples. A complete dataset is available at [23]. (**c**) The ERK protein interactome in *Drosophila* S2 cells. The proteins shown were identified with the SAINT score of >0.6, and were grouped into functional classes. In (**b**) and (**c**), proteins are listed by their standard FlyBase gene identifiers