

Chapter 2

Dissecting Kinase Effector Signaling Using the RapRTAP Methodology

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Abstract

Kinases are involved in a broad spectrum of cell behaviors. A single kinase can interact with different ligands each eliciting a specific cellular response. Dissecting downstream signaling pathways of kinases is a key step to understanding physiological and pathological cell process. However, directing kinase activity to specific substrates remains challenging. Here, we present a new tool to selectively activate a kinase in a specific protein complex in living cells. This technology uses a rapamycin-inducible kinase activation coupled to interaction with FKBP12-binding domain (FRB) tagged protein. Here, we demonstrate application of this method by targeting Src to either p130Cas or FAK and discriminating cell morphodynamic changes downstream each of these signaling complexes.

Key words Kinase, Phosphorylation, FKBP12, Targeted signaling pathway, Rapamycin

1 Introduction

Protein kinase signaling is critical for the regulation of many pathways contributing to normal cellular function [1]. Because kinase signaling activates many pathways in parallel, it can be difficult to identify the immediate consequences of this activation and dissect the individual roles of each substrate. Traditionally, these signaling pathways have been studied utilizing pharmacological inhibitors and genetic manipulations of the cell, but there are many limitations to these approaches [2]. The use of small molecule inhibitors can elicit many off target effects [3]. Additionally, genetic manipulations are achieved with poor temporal resolution and are therefore vulnerable to cellular compensatory mechanisms. Here, we describe a new method called “rapamycin-regulated targeted activation of pathways” (RapRTAP) which gives us the ability to temporally regulate the activation of a specific kinase as well as to couple its activation to a specific protein complex or a subcellular location. As an example, we will discuss manipulation of signaling

downstream of Src, a ubiquitously expressed protein kinase known to regulate signaling processes responsible for normal cellular motility and cancer cell metastasis [4–6]. Furthermore, Src signaling in complex with specific proteins or at particular subcellular locations has been shown to elicit distinct effects, making it an ideal candidate for testing this new methodology [7].

To develop RapRTAP technology, we built upon our previously described RapR-kinase approach. This method involves insertion of a modified FK506-binding protein (iFKBP) into a highly conserved region of the catalytic domain of a kinase, rendering it catalytically inactive. Addition of rapamycin mediates an interaction of iFKBP with a co-expressed FKBP12-rapamycin-binding domain (FRB) leading to activation of the kinase (Fig. 1a) [8, 9]. With RapRTAP technology, we add a level of regulatory specificity over the previous RapR-kinase methodology, enabling activation of the kinase in a complex with a specific binding partner or at a specific subcellular location. This increased specificity is accomplished by attaching FRB to a known binding partner of the kinase or to a subcellular localization signal (Fig. 1b). Previous studies of RapR-kinases indicate that this approach will be broadly applicable to many different kinase signaling networks in living cells. Here, we discuss application of RapRTAP for targeted activation of Src kinase in complexes with Crk-associated substrate (p130Cas) and focal adhesion kinase (FAK). Both proteins interact with the SH2 domain of Src, and this interaction is proposed to activate Src. Thus, by using RapRTAP, we aim to mimic Src activation and signaling through these two complexes. To prevent Src SH2 domain from interacting with other endogenous binding partners, we utilize Arg175 to Leu mutant of Src (R175L), which abolishes SH2 domain binding without affecting Src catalytic domain [10]. To investigate Src signaling through interactions with p130Cas and FAK specifically, we generated chimeras where the FRB domain was inserted, replacing the Src binding site (FAK Tyr397 [11] and p130Cas Tyr668 [12]) (Fig. 2). Mutation of SH2 domain of Src (R175L) and insertion of FRB into the SH2 binding domain in FAK and p130Cas target the activation of Src only to either FAK or p130Cas-FRB chimera (Fig. 2) [10].

We had previously shown that Src activation stimulates cell spreading that is accompanied by formation of lamellipodia and filopodia, as well as changes in focal adhesion number and organization [5]. Inactivation of SH2 domain of Src abolishes these morphological changes, suggesting that interactions mediated by this domain are critical. Dissecting Src signaling through p130Cas and FAK using RapRTAP method revealed that Src-p130Cas complex stimulates formation of lamellipodia, filopodia, and new focal adhesions, whereas targeted activation of Src-FAK pathway

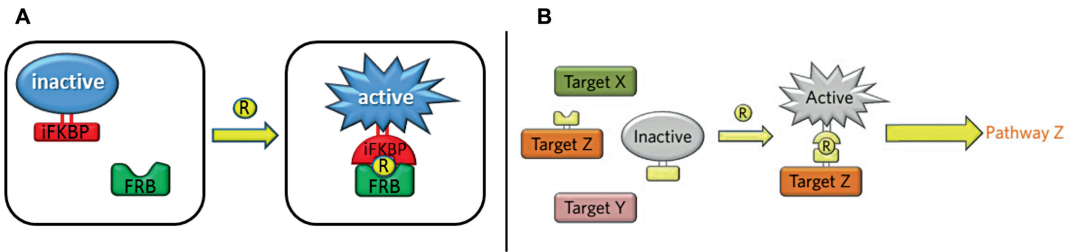


Fig. 1 RapR-kinase and RapRTAP design. (a) RapR-kinases allow for temporal control of enzyme activation in cells. Insertion of an engineered iFKBP domain into the catalytic domain of the kinase renders it inactive. Rapamycin-induced heterodimerization with a co-expressed FRB domain restores catalytic activity. However, this activation is global throughout the cell. (b) RapRTAP technology allows for the activation of a specific kinase in a specific protein complex and/or at a particular subcellular location when FRB is fused with a known binding partner of the kinase or a localization signal

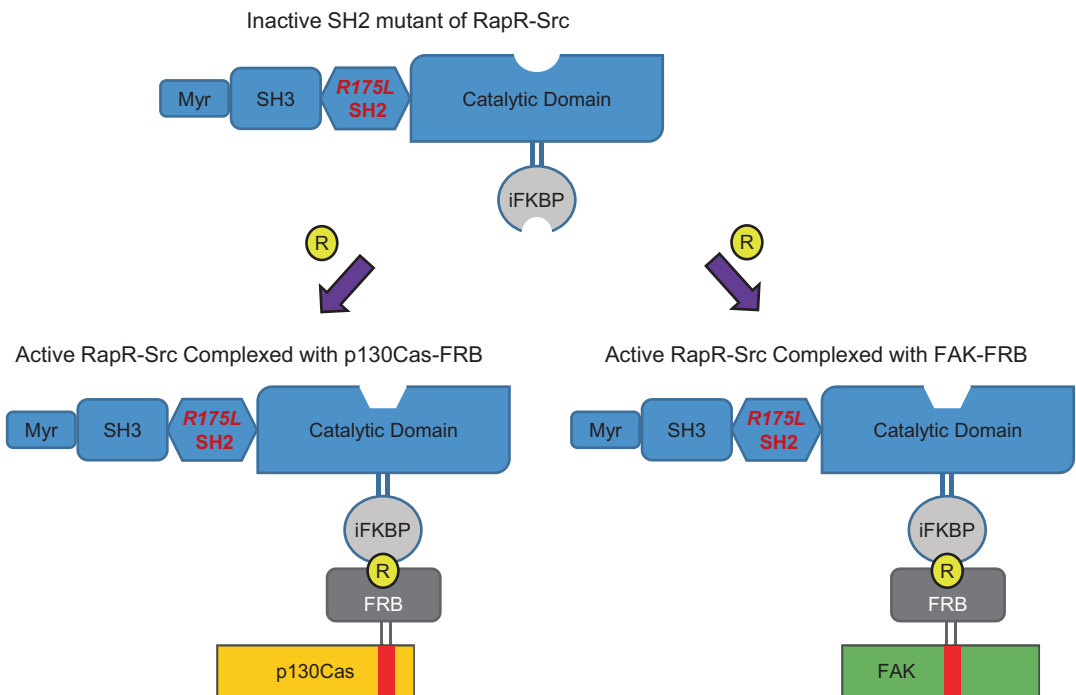


Fig. 2 Schematic of RapRTAP technology applied to Src signaling. Targeted activation of RapR-Src in the complex with p130Cas or FAK. A mutant of RapR-Src (*R175L*) was used to ensure that observed physiological changes were only triggered by addition of rapamycin (R) and subsequent interaction with the target substrate. FRB is inserted into FAK and p130Cas replacing known binding site for Src SH2 domain

predominantly stimulates rearrangement of existing focal adhesions [10]. RapR-TAP technology provides the ability to differentiate individual roles of p130Cas and FAK in the induction of early Src-induced morphodynamic changes in living cells.

2 Materials

2.1 Generation of FRB-Tagged Binding Partners

Insertion of FRB into FAK and p130Cas to replace the binding site for Src SH2 domain is achieved using the previously described modification of site-directed mutagenesis method (the “megaprimer” technique) [9] (Fig. 3). FRB can also be inserted before or after the target protein using alternative cloning techniques not described here.

1. Primers used for “megaprimer” synthesis are composed of three parts (Fig. 3a):
 - (a) A target sequence, about 30 nucleotides long and anneals to the target sequence (FAK or p130Cas) before or after insertion site (Fig. 3c)

A. Primer design

forward primer

Part	a. 28-32nt annealing to the target	b. linker	c. 20-25nt annealing to FRB
FAK	CATGCAGTCTCTGTGTCAGAGACAGATGAC	GGCCCCGGA	TGGCATGAGATGTGGCATGAAGG
p130Cas	GAGAACAGTGAAGGGGTTGGATGGAGGAC	GGCCCCGGA	TGGCATGAGATGTGGCATGAAGG

reverse primer

Part	a. 28-32nt annealing to the target	b. linker	c. 20-25nt annealing to FRB
FAK	GTATGTGCTTCCTCATCGATGATCTCTGC	ACCGGGTCC	ACTAGTCTTTGAGATTCGTCGGAA
p130Cas	CAAATTCCTCCTCCCTGCAGATGAACGTAGTC	ACCGGGTCC	ACTAGTCTTTGAGATTCGTCGGAA

B. Megaprimer synthesis



C. FRB insertion

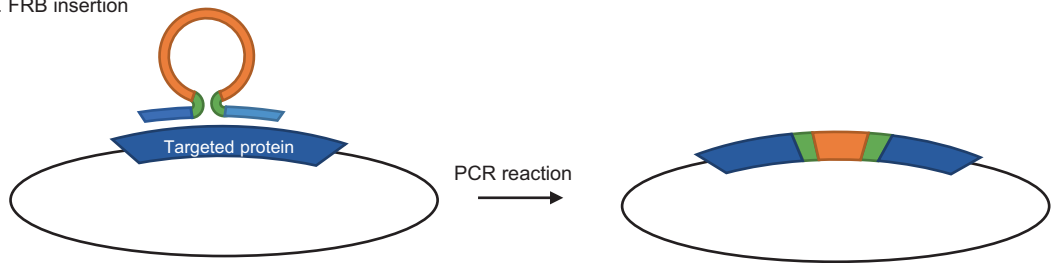


Fig. 3 Different steps required for the cloning strategy via a megaprimer method. (a) Example primers. The primers are composed of three parts: (a) a portion which anneals to the targeted protein (FAK and p130Cas, blue), (b) a linker (green), and (c) a portion which anneals to the insert fragment (FRB, yellow). (b) First step, generation of the “megaprimer” is achieved through a PCR reaction using FRB gene as a template. (c) FRB is inserted into the target protein (FAK and p130Cas) via a PCR reaction with the megaprimer from part (b) and a plasmid containing the target protein

- (b) A linker sequence, variable in size, that encodes a peptide linker connecting the target protein (FAK or p130Cas) to the FRB domain.
 - (c) An insertion sequence, about 20–25 nucleotides long and anneals to the insert sequence (FRB).
2. DNA constructs: pmCherry-FRB (Addgene 25920), pmVenus-p130Cas, pmVenus-FAK [9], dissolved in water or TE buffer.
 3. *PfuTurbo* DNA polymerase (Agilent Technology) sold with *PfuTurbo* 10× reaction buffer.
 4. Apex 2.0× Taq RED Master Mix Kit (Genesee Scientific).
 5. 10 mM dNTP mix.
 6. H₂O molecular grade.
 7. PCR tubes.
 8. PCR thermal cycler.
 9. DNA gel purification kit (Qiagen) or equivalent.
 10. GeneJET Gel Extraction Kit (Thermo Fisher Scientific) or equivalent.
 11. Transformation-competent DH5α bacterial cells.
 12. LB medium and agar plates with the appropriate antibiotic for selection (50 µg/ml of kanamycin in this example).

2.2 Evaluation of Kinase Activity

1. DNA constructs: RapR-*Src*-myc R175L mutant [10], mVenus-FAK-FRB, and mVenus-p130Cas-FRB (both generated as described in Subheading 3.1), dissolved in water or TE buffer.
2. HEK293 cells (ATCC).
3. DMEM media (Gibco or equivalent) supplemented with 4.5 g/L glucose, 4 mM L-glutamine, and 110 mg/L sodium pyruvate.
4. Complete DMEM: DMEM media supplemented with 4.5 g/L glucose, 4 mM L-glutamine, and 110 mg/L sodium pyruvate and 10% (v/v) of fetal bovine serum (FBS).
5. 1 mM rapamycin stock solution in ethanol.
6. FuGene6 transfection reagent (Promega).
7. Ethanol 200 proof.
8. IP buffer: 20 mM HEPES-KOH, pH 7.8, 50 mM KCl, 1 mM EGTA, 1% NP40.
9. Lysis buffer: IP buffer containing 1 mM NaF, 0.2 mM Na₃VO₄.
10. Wash buffer: IP buffer containing 100 mM NaCl.

11. Protein G agarose beads (Millipore).
12. Bovine serum albumin.
13. Sample buffer: 2× Laemmli Sample Buffer (BioRad) complemented with 5% 2-mercaptoethanol or equivalent.
14. Antibodies:
 - (a) Anti-GFP (Thermo Fisher Scientific, A-6455).
 - (b) Anti-myc tag (clone 4A6, Millipore).
 - (c) Anti-phospho-tyrosine (clone 4G10, Millipore).
15. Cell culture plate, 6-well format.

2.3 Live Cell Imaging

1. Epifluorescence microscope with a motorized and heated stage (the imaging system used for these experiments: Olympus IX-81 microscope equipped with objective-based TIRF system and a PlanApo N 60× TIRFM objective (NA 1.45) and an open heated chamber (Warner Instruments)). Images are collected via Photometrics CoolSnap ES2 CCD camera controlled by MetaMorph software. For TIRF imaging, the 488 nm line from an omnichrome series 43 Ar/Kr laser and the 594 nm line from a Cobolt Mambo continuous-wave diode-pumped solid-state laser are used. A high-pressure mercury arc light source is used for illumination of epifluorescence images. Other TIRF microscopes suitable for live cell imaging can be used; however, verify that the instrument will allow the addition of reagents during imaging.
2. DNA constructs (dissolved in water or TE buffer):
 - (a) mVenus-FAK-FRB.
 - (b) Venus-p130Cas-FRB.
 - (c) RapR-Src-cerulean R175L.
 - (d) mCherry-Paxillin.
 - (e) Stargazin-mCherry [10].
3. HeLa cells (ATCC).
4. FuGene6 transfection reagent (Promega).
5. Cell culture medium: DMEM media (Gibco or equivalent) supplemented with 4.5 g/L glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate, and 10% (v/v) FBS.
6. Live cell imaging medium: L15 Leibovitz medium (Invitrogen) containing 5% FBS.
7. Mineral oil, sterile filtered, suitable for mouse embryo cell culture (Sigma-Aldrich).
8. 25 mm round glass coverslips, 0.17 mm thick (Fisher Scientific), store in 70% ethanol solution.
9. 1 mg/ml fibronectin stock solution dissolved in TBS (Sigma).
10. Phosphate-buffered saline (PBS).

11. 1 mM rapamycin (LC Laboratories) stock solution in ethanol.
12. 35 mm tissue culture plates.
13. Attotfluor cell chamber (Invitrogen).

3 Methods

3.1 Generation of FRB Tag Targeted Protein

1. To generate the megaprimer, prepare a PCR reaction as follows:
 - 2.5 U of *PfuTurbo* DNA polymerase.
 - 50 ng pmCherry-FRB.
 - 125 ng of each primer designed as described above.
 - 1 µl of 10 mM dNTP mix.
 - 5 µl of 10× *PfuTurbo* reaction Buffer.
 - H₂O to 50 µl.
2. Perform 25 cycles of PCR with the following cycle conditions:

Denaturation	30 s	95 °C
Annealing	30 s	60 °C
Extension	30 s	72 °C

3. Purify the resulting PCR product (megaprimer, expected size for FRB insert is around 360 nt) product using GeneJET Gel Extraction Kit following the manufacturer's recommendations or an analogous technique. Dissolve resulting DNA in molecular grade water.
4. To insert the FRB fragment into the target sequence, prepare a PCR reaction as follows, one for each target protein:
 - 2.5 U of *PfuTurbo* DNA polymerase.
 - 50 ng of template (pmVenus-p130Cas or pmVenus-FAK).
 - 200–400 ng purified previously generated megaprimer.
 - 2.5 µl of 10× *PfuTurbo* reaction buffer.
 - 1 µl of 10 mM dNTPs mix.
 - H₂O to 25 µl.
5. Perform 18 cycles of PCR with the following cycle parameters:

Denaturation	30 s	95 °C
Annealing	30 s	55 °C
Extension	16 min	72 °C

6. Add 1 μ l of *DpnI* enzyme to the PCR mix and incubate for 1 h at 37 °C.
7. Transform 1–2 μ l of obtained product into DH5 α competent cells following the manufacturer's protocol.
8. Plate cells on LB agar plates, supplemented with the appropriate selection antibiotics (50 μ g/ml of kanamycin for both pmVenus-p130Cas and pmVenus-FAK). Incubate plates overnight at 37 °C.
9. Perform a PCR screen to identify positive colonies [13] (*see Note 1*).
10. Inoculate with a positive colony 5 ml of LB liquid medium with appropriate selection antibiotics. Incubate the culture overnight at 37 °C. The following day, prepare plasmid DNA, and confirm the insertion of FRB via DNA sequencing.

3.2 Evaluation of Kinase Activity

1. Seed 1×10^6 HEK293 cells per well into four wells of a 6-well plate in 2 ml complete DMEM media (two wells will be used for mVenus-FAK-FRB and two wells will be used for mVenus-p130Cas-FRB). Incubate for 24 h in a 37 °C, 5% CO₂ incubator.
2. Transfect cells with 1:1 ratio of RapR-Src-myc R175L mutant construct with mVenus-FAK-FRB or mVenus-p130Cas-FRB, two wells each. Use 2 μ g of total DNA and 6 μ l of FuGene6 per well, as recommended by the manufacturer (1:3 w/v ratio). Other transfection techniques also can be used. Incubate cells overnight in a 37 °C, 5% CO₂ incubator.
3. Next day, prepare 60 μ l of protein G-coupled agarose beads (10 μ l per sample). Wash the beads two times with 1 ml of IP buffer (*see Notes 2 and 3*).
4. Resuspend beads in 400 μ l IP buffer containing 1 μ g/ μ l of BSA and 3 μ l the anti-GFP antibody (0.5 μ l per sample). Incubate the beads for 2 h at 4 °C.
5. Wash beads two times with 1 ml of IP buffer; resuspend the beads in 300 μ l IP buffer (50 μ l per sample). Distribute 50 μ l of beads into each fresh tubes.
6. Treat one well of FAK-FRB transfected cells and one well of p130Cas-FRB transfected cells with 500 nM of rapamycin (1 μ l per well of 1 mM stock solution). The remaining two wells are treated with the same volume of solvent (ethanol). Incubate 1 h in a 37 °C, 5% CO₂ incubator.
7. Stop incubation by placing the plate on ice, aspirate media, and wash with 2 ml of PBS (*see Note 4*). Add 300 μ l of lysis buffer to each well, scrape cells into the buffer, and transfer lysate to

fresh tube. Spin samples for 10 min at $3000 \times g$ and 4°C (*see Note 5*).

8. Mix 20 μl of each sample supernatant with 20 μl of sample buffer; boil for 5 min (*see Note 6*).
9. Transfer the leftover supernatant into the tubes containing the beads from the **step 5**. Incubate 2 h at 4°C under agitation.
10. Wash beads three times with 500 μl of cold wash buffer (*see Note 3*). Add 60 μl of sample buffer to the beads, and boil for 5 min.
11. Run two SDS-polyacrylamide gels (one for the kinase assay samples, one for total cell lysates). To detect the efficiency of the transfection, perform a Western blot analysis of lysates using anti-myc antibody (clone 4A6) to detect RapR-Src-myc and anti-GFP antibody to detect FRB-tagged constructs. To analyze the kinase activation and the interaction of RapR-Src with the targets, perform a Western blot analysis of immunoprecipitated sample using anti-phospho-tyrosine (clone 4G10) to detect the phosphorylation of the targets (FAK-FRB and p130Cas-FRB) and anti-myc tag antibody to detect RapR-Src (Fig. 4). The presence of RapR-Src in the immunoprecipitated samples will indicate interaction of RapR-Src with the target. Increased phosphorylation of FAK-FRB and p130Cas-FRB will demonstrate activation of RapR-Src (Fig. 4).

3.3 Live Cell Imaging

1. Plate 2×10^5 HeLa cells per 35 mm tissue culture dish with cell culture media, and incubate for 2–4 h at 37°C , 5% CO_2 (two plates total).

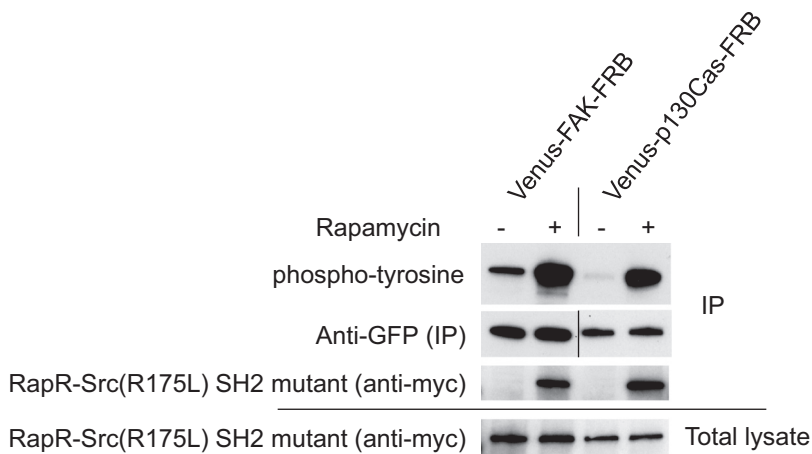


Fig. 4 Evaluation of kinase activity. Changes in tyrosine phosphorylation of FAK-FRB and p130Cas-FRB induced by targeted activation of RapR-Src R175L mutant

2. Make sure that the cells are attached and the confluency is 60–70%. Co-transfect HeLa cells with the following four mixes (one mix per plate):
 - (a) Mix 1: 0.5 μ g mVenus-FAK-FRB/1.2 μ g RapR-Src-cerulean R175L/0.3 μ g Stargazin-mCherry.
 - (b) Mix 2: 0.5 μ g mVenus-FAK-FRB/1.2 μ g RapR-Src-cerulean R175L/0.3 μ g mCherry-Paxillin.
 - (c) Mix 3: 0.5 μ g mVenus-p130Cas-FRB/1.2 μ g RapR-Src-cerulean R175L/0.3 μ g Stargazin-mCherry.
 - (d) Mix 4: 0.5 μ g mVenus-p130Cas-FRB/1.2 μ g RapR-Src-cerulean R175L/mCherry-Paxillin (*see Note 6*).Use 4 μ l of FuGene6 following manufacturer recommendation (1:2 w/v ratio) (*see Note 7*), and incubate overnight at 37 °C, 5% CO₂.
3. On the day of transfection, place one coverslip per well in a 6-well plate. Prepare 2–3 coverslips per transfection experiment. Rinse coverslips with PBS, and then add 1.5 ml of 5 mg/ml fibronectin solution in PBS (*see Note 8*). Incubate at 37 °C overnight.
4. Wash fibronectin-coated coverslips with PBS, and then plate transfected HeLa cells onto them, about 1×10^5 per coverslips. Incubate in cell culture medium for 2 h at 37 °C, 5% CO₂ (*see Note 9*). Cells should be plated at low density so most cells are not touching each other.
5. Carefully wash coverslips with transfected cells using PBS, place the coverslip into an Attotfluor cell chamber, and add 0.9 ml of L15 imaging media (*see Note 10*). Cover with 1 ml of mineral oil (*see Note 11*).
6. Place chamber onto a microscope stage pre-heated to 37 °C, and select cells co-expressing mVenus-FAK-FRB or mVenus-p130Cas-FRB, RapR-Src-cerulean R175L, and Stargazin-mCherry or mCherry-Paxillin (*see Note 12*).
7. Image selected cells every minute for a total of 120 min. Mix 0.5 μ l of 1 mM rapamycin with 100 μ l of imaging media (*see Note 13*), and add to the chamber after 30 min of imaging (*see Notes 13 and 14*). Epifluorescence can be used for monitoring the fluorescently labeled co-transfected proteins. Use Stargazin-mCherry images to analyze cell spreading, protrusive activity, and filopodia utilizing “CellGeo” [14] image analysis software or using other alternative methods. Use mCherry-Paxillin images to analyze focal adhesions. Changes in focal adhesion number and morphology can be determined using a computational image analysis method described by Berginski et al. [15] or other alternative methods. Activation of RapR-Src in complex with p130Cas stimulates cell spreading (Fig. 5a, b),

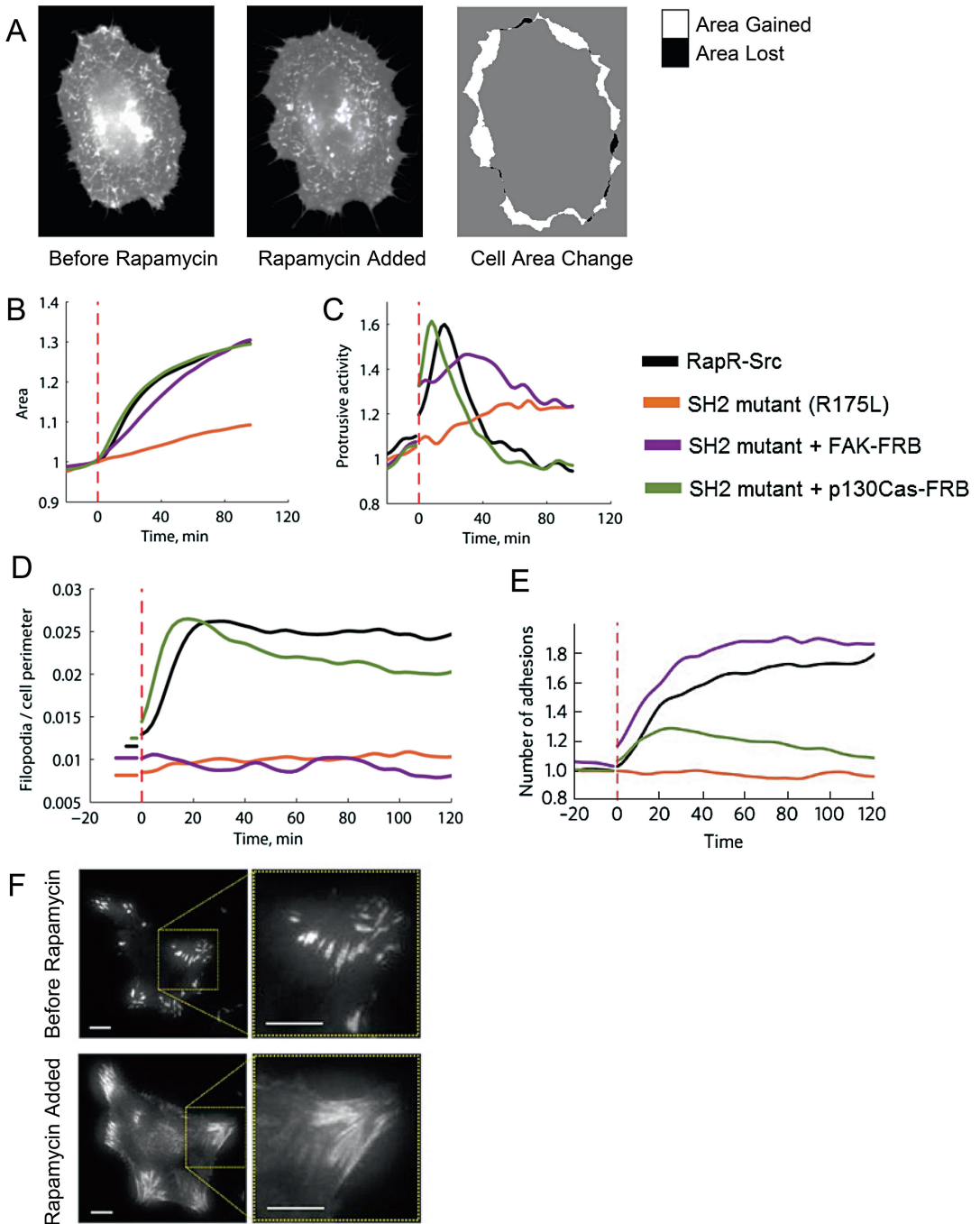


Fig. 5 Morphological changes induced by activation of RapR-Src SH2 mutant in complexes with FAK-FRB and p130Cas-FRB. (a) Cell area before and after activation of Src-p130Cas signaling complex. Changes in cell area (b), protrusive activity (c), filopodia (d), and the number of focal adhesions (e) induced by targeted activation of RapR-Src in complexes with FAK-FRB and p130Cas-FRB. Data are smoothed as a running average using a Gaussian filter. (f) Rearrangement of focal adhesions induced by activation of Src-FAK signaling complex

protrusive activity (Fig. 5c), filopodia (Fig. 5d), and formation of new focal adhesions (Fig. 5e). Stimulation of Src-FAK signaling induces slower cell spreading (Fig. 5b) and protrusive activity (Fig. 5c) but causes rearrangement of focal adhesions (Fig. 5f).

4 Notes

1. To perform the colony screen, use a forward primer that anneals to the p130Cas (or FAK) sequence upstream of FRB insertion site and the reverse primer that anneals to the FRB sequence. Apex 2.0× Taq RED Master Mix or equivalent reagents can be used to perform this screen.
2. For pipetting agarose beads, cut the pipette tip, and then resuspend the agarose beads vigorously to homogenize the solution.
3. To wash agarose beads, add IP/wash buffer to the tube, vortex, and centrifuge 1 min at $1500 \times g$; carefully remove the buffer without disturbing beads.
4. Wash carefully with PBS to avoid detachment of cells.
5. After this step, keep the samples on ice, unless otherwise indicated.
6. These samples are used for protein gel electrophoresis. They are used to test phosphorylation of endogenous proteins and transfection efficiency.
7. Alternative transfection reagents can be used and optimized.
8. Prepare the fibronectin mixture in PBS at the time of coverslip coating.
9. This is the time required for HeLa cells to attach and spread on the fibronectin coated coverslips.
10. L15 medium and FBS should be stored separately at 4 and -20°C , respectively, until the day of imaging. On the day of imaging, prepare a fresh mixture of L15 medium and FBS; a minimum of 1 ml will be needed per experiment. Preincubate imaging media at 37°C incubator for at least 1 h prior to imaging.
11. Preincubate mineral oil at 37°C for at least 1 h prior to imaging. Addition of oil on top of the media prevents evaporation but still allows for the ability to add reagents.
12. Using a microscope which is equipped with a motorized stage enables imaging of multiple cells in the same experiment. The number of positions that will be imaged depends on the

amount of time it takes to capture all images at one position and move to the next position.

13. Mix rapamycin and media immediately before adding it to the cells. Make sure rapamycin is added below the layer of oil.
14. Imaging for the first 30 min prior to the addition of rapamycin is necessary in order to establish a baseline. A baseline is required to determine whether the activation of the kinase via rapamycin causes any changes in cell behavior and/or changes in protein localization.

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