Chapter 12

Characterization of an Engineered Src Kinase to Study Src Signaling and Biology

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Abstract

Pharmacologic inhibitors of protein kinases comprise the vast majority of approved signal transduction inhibitors for cancer treatment. An important facet of their clinical development is the identification of the key substrates critical for their driver role in cancer. One approach for substrate identification involves evaluating the phosphorylation events associated with stable expression of an activated protein kinase. Another involves genetic or pharmacologic inhibition of protein kinase expression or activity. However, both approaches are limited by the dynamic nature of signaling, complicating whether phosphorylation changes are primary or secondary activities of kinase function. We have developed rapamycin-regulated (RapR) protein kinases as molecular tools that allow for the study of spatiotemporal regulation of signaling. Here we describe the application of this technology to the Src tyrosine kinase and oncoprotein (RapR-Src). We describe how to achieve stable expression of this tool in cell lines and how to subsequently activate the tool and determine its function in signaling and morphology.

Key words FK506-binding protein, KRAS, mTOR, Oncogene, Pancreatic cancer, Rapamycin, Src, Tyrosine kinase

1 Introduction

Since Food and Drug Administration (FDA) approval of the first kinase inhibitor just over a decade ago [1], many small-molecule inhibitors of protein kinases have been developed successfully for cancer treatment. However, although the aberrant overexpression or activation of protein kinases have well-validated driver roles in cancer, the precise substrates critical for their cancer causing functions remain unclear. Establishing the role these kinase-dependent signaling events have in promoting cell transformation has been limited by the conventional methods utilized to study their function. A majority of the data implicating kinase activity in various disease phenotypes was collected using depletion of the kinase by RNA interference or pharmacologic inhibition, often resulting in

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off-target effects. Furthermore, these approaches provide an assessment of the loss of activity rather than the consequences of activation itself. For such studies, a common strategy is the expression of a constitutively active kinase. However, this approach is not applicable to many protein kinases. Furthermore, this approach allows for evaluation of cells stably overexpressing the protein kinase. All of these approaches make it difficult to identify transitional, directly kinase-activity-dependent steps leading to transformation.

A recently-developed tool circumvents many of these issues in the form of an inducible kinase [2]. This tool utilizes the well-characterized rapamycin-mediated heterodimerization of small FK506-binding protein (FKBP12) and an FKBP12-rapamycin-binding (FRB) domain of the mammalian target of rapamycin (mTOR). Rapamycin (MW 914.2 g/mol) is an FDA-approved drug used as an immunosuppressive and anticancer drug. This engineered protein contains a modified FKBP12 protein suitable for insertion into the middle of other proteins (insertable FKBP; iFKBP) (Fig. 1). The insertion of iFKBP into a structurally conserved portion of the kinase catalytic domain increases mobility

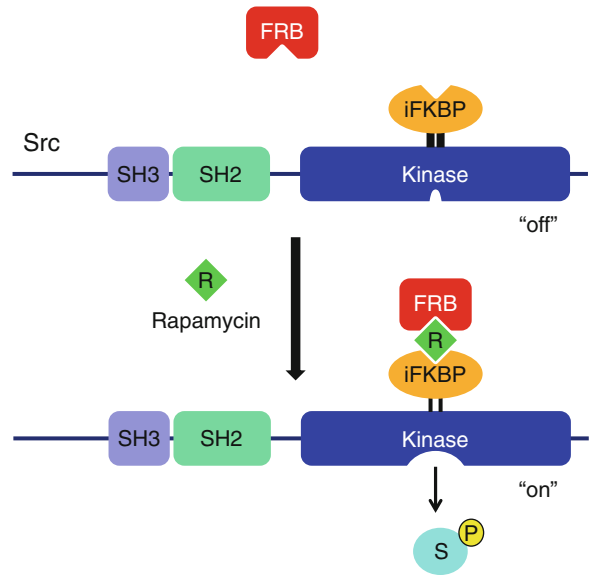

Fig. 1 RapR-Src construct: The Src tyrosine kinase is comprised of an N-terminal Srch homology domain 2 (SH2) followed by an SH3 domain, and a C-terminal kinase catalytic domain. A fragment of human FKBP12 (iFKBP12; residues 22–108) is inserted at a position in the kinase catalytic domain where it abrogates catalytic activity. A retrovirus vector encoding a Cerulean fusion with RapR-Src was generated. The FRB (FKBP12-rapamycin binding) domain of human mTOR (residues 2015–2114) was expressed as an mCherry-fusion protein. Binding to rapamycin (R) and FRB restores activity, leading to phosphorylation (P) of substrates (S)

of the region and thus decreases its ability to catalyze phosphate transfer. This construct is expressed in cells simultaneously with an exogenous FRB construct, where it remains inactive. Upon addition of rapamycin, the kinase domain is stabilized, restoring kinase activity and beginning downstream signaling. By controlling kinase activity through the addition of rapamycin or its non-immunosuppressive analogs, one can monitor events immediately after activation. Here, we discuss the application of this tool using the example of modified Src tyrosine kinase, termed RapR-Src (rapamycin-regulated Src). Src has long been studied due to its known contribution to progression of many cancers including colorectal, pancreatic, and prostate [3–5], and its early signaling events were of extreme interest when developing this tool. RapR-Src gives temporal control of Src activation. We recently demonstrated the usefulness of RapR constructs encoding different Src family members [6, 7]. Here we describe the implementation of RapR-Src to study Src signaling and biology in pancreatic cancer.

2 Materials

2.1 Expression Constructs and Plasmids

1. pBabe-Cerulean-RapR-Src: Retroviral expression vector (puromycin resistant) encodes an N-terminal cerulean fluorescent tag followed by full-length Src with the iFKBP in the kinase domain.
2. pLHCX-mCh-FRB retroviral expression vector (hygromycin resistant) encodes an N-terminal fluorescent tag fused to the FRB domain.
3. PCL10A-1 vector was used for retroviral packaging.
4. pBabe-tet-CMV-RapR-Src-GFP-Myc: Retroviral expression vector (puromycin resistant) encodes green fluorescent protein (GFP)-tagged RapR-Src under the control of a Tet-off expression system (Clontech).
5. pBabe-CMV-mCherry-FRB (puromycin resistant) retroviral expression vector encodes an mCherry-tagged FRB domain.

2.2 Cell Lines

1. HEK293T cells were obtained from the ATCC and maintained in Dulbecco's modified minimum essential medium (DMEM) supplemented with 10 % fetal calf serum (FCS).
2. Two immortalized human epithelial cell lines, HPDE and HPNE, and their matched mutant *KRAS* oncogene-transformed counterparts (HPDE-KRAS and HPNE-KRAS) cells were obtained from Michele Ouelette (University of Nebraska) and Ming Tsao (University of Toronto) [8, 9], respectively, and maintained in DMEM supplemented with 10 % FCS.

3. Mouse embryonic fibroblasts (Clontech Laboratories MEFs; Tet-off cell system).

2.3 Chemicals, Antibodies

1. Rapamycin: 1 mM stock solution made with ethanol (LC Laboratories). Store at –20 °C.
2. Polybrene: 8 mg/mL stock solution in water. Store at –20 °C.
3. Hygromycin: 50 mg/mL stock solution in sterile ddH_2O. Store at 4 °C.
4. Puromycin: 2 mg/mL stock solution in sterile ddH_2O. Store at –20 °C.
5. Anti-GFP mouse monoclonal antibody (clone JL-8; Clontech), 1 mg/mL.
6. Anti-Src rabbit monoclonal antibody (36D10; Cell Signaling).
7. Anti-FAK rabbit polyclonal antibody (Cell Signaling).
8. Anti-phospho-FAK (Tyr925) rabbit polyclonal antibody (Cell Signaling).
9. NP-40 lysis buffer: 1 % NP-40, 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 5 % glycerol, 0.25 % Na-deoxycholate.
10. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4.
11. HEPES-buffered saline (HBS): 140 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4. Adjust to pH 7.1 with 1 M NaOH, filter sterilize, and store at room temperature.
12. Calcium chloride (1.25 M): Dissolve $CaCl_2$ in distilled water and filter sterilize. Store at room temperature.
13. Poly-l-lysine: 0.01 % stock solution (Sigma), stored at 4 °C.

2.4 Imaging Components

1. #1.5 Glass cover slips (0.13–0.17 mm thick), 25 mm diameter. Store in 70 % ethanol.
2. Live cell imaging medium: L15 Leibovitz medium (Invitrogen) supplemented with 5 % FCS (*see* **Note 1**).
3. Mineral oil, sterile filtered, suitable for mouse embryo cell culture (Sigma-Aldrich).
4. Fibronectin: 1 mg/mL stock solution (BD Bioscience) dissolved in 0.5 M NaCl, 0.05 M Tris, pH 7.5 (*see* **Note 2**).
5. Collagen: Collagen Type I (rat tail) (BD Bioscience) suspended in 0.2 % acetic acid to 5 mg/mL.
6. Attofluor® cell chamber (Invitrogen) (*see* **Note 3**).
7. Cell culture plates; 35 mm.
8. Inverted microscope equipped with a 40× objective, CCD camera, a high-pressure mercury arc light source, and an open heated chamber (*see* **Note 4**).

3 Methods

3.1 Sequential Retroviral Infections of Pancreatic Epithelial Cells

1. Plate HEK293T cells at a density of 2.5×10^6 cells in a T25 flask 24 h prior to transfection.
2. To transfect cells, first add 5 μg of pLHCX-mCh-FRB DNA and 5 μg of pCL10A-1 DNA to 400 μL of HBS in a 1.5 mL microcentrifuge tube. Then add 100 μL of $CaCl_2$, briefly vortex the mixture, and add dropwise to the cells.
3. Five hours after transfection, change the growth medium.
4. The following day, change media 16 h prior to harvesting virus. On this day, split HPDE and HPDE-KRAS cells to a density of 20–30 % in T25 flasks.
5. To harvest virus, filter the conditioned medium from transfected 293T cell cultures using a syringe and a 0.45 μm filter. Add polybrene to the filtered medium to a concentration of 8 μg/mL.
6. Add 4 mL of growth medium back to flask containing to 293Ts to allow for a second transduction.
7. Add 2 mL of virus to target cells and incubate for 5 h, then replenish with fresh growth medium.
8. After 48 h, begin selecting for cells containing mCh-FRB using 50 μg/mL hygromycin. Selection should take 3–4 days (*see* **Note 5**).
9. After hygromycin selection, follow **steps 1–2** to infect cells containing mCh-FRB with pBabe-Cerulean-RapR-Src.
10. After 48 h, select cells using 1 μg/mL puromycin while maintaining a 25 μg/mL dose of hygromycin.
11. Continue to grow cells in the presence of 25 μg/mL of hygromycin and 0.5 μg/mL puromycin until you achieve 9×10^6 cells.
12. Sort cells positive for both Cerulean (excitation 433 nm, emission 475 nm) and mCherry (excitation 590 nm, emission 610 nm) fluorescence using cell sorter to enrich population of cells co-expressing RapR-Src-GFP-myc and mCherry-FRB (*see* **Note 6**).
13. Use untransduced cells and cells transduced with only one virus as controls for cell sorting. Continue growing sorted cells in the presence of 25 μg/mL of hygromycin and 0.5 μg/mL puromycin. If expression of RapR-Src is not desired while propagating the cells, then cells should be grown in the presence of doxycycline. Cell samples with different concentration of doxycycline should be tested to establish optimal concentration.

3.2 Simultaneous Retroviral Infection of Mouse Embryonic Fibroblasts

1. Prepare retroviruses as described in Subheading 3.1 using pBabe-tet-CMV-RapR-Src-GFP-myc and pBabe-CMV-mCherry-FRB constructs.
2. Plate MEFs to 2×10^5 cells onto 3 cm tissue culture plate the day before transduction (three plates total).

3. On the day of transduction remove media and add 1 mL of fresh media containing 16 μg/mL polybrene.
4. Add 2 mL 1:1 mixture of the two viruses to one plate and 1 mL of each virus to the remaining two dishes separately. Cells transduced with only one virus will be used as controls. Incubate overnight.
5. Change media and incubate for 48 h.
6. Re-plate cells into 6 cm plate and next day add puromycin at concentration 8 μg/mL to start selection.
7. Continue growing cells in the presence of puromycin until you achieve 3×10^6 viable cells.
8. Sort cells positive for both GFP (excitation 488 nm, emission 510 nm) and mCherry fluorescence using cell sorter to enrich population of cells co-expressing RapR-Src-GFP-myc and mCherry-FRB (*see* **Note 6**). Continue as described in Subheading 3.1, **steps 12** and **13**.

3.3 Activation of RapR System

1. The day before activation, plate HPDE or HPDE-KRas cells in 6-well plate.
2. To activate RapR-Src, add 0.5 μM rapamycin to growth medium, using 0.5 μM EtOH in a control well.
3. Three hours after activation, immediately wash cells with cold PBS and lyse cells on ice in ice-cold NP-40 lysis buffer. Incubate lysed cells in lysis buffer for 20 min on ice.
4. Centrifuge lysate for 15 min at $16{,}000 \times g$ to remove cell debris.
5. Take protein concentration by Bradford Assay and prepare 20 μg samples.
6. Run samples on SDS-PAGE and perform a western blot.
7. Use anti-Src, anti-FAK, anti-Y925 FAK, and anti-JL8 antibodies as directed by the manufacturer (Fig. 2).

3.4 Imaging

HPDE and HPDE-KRAS cells must be plated on collagen-coated cover slips for imaging.

1. Place one cover slip per well in a 6-well plate.
2. Wash cover slips with 70 % EtOH and aspirate.
3. Once cover slips are dried, coat with poly-l-lysine for 30 min in a sterile hood.
4. Aspirate and wash with sterile ddH_2O.
5. Next, cover cover slips with collagen at a concentration of 5 μg/mL in 0.2 % acetic acid and let sit overnight at 4 °C.
6. Aspirate solution and seed cells directly on collagen.
7. Plate HPDE or HPDE-KRAS cells at a density of 50,000 cells/well 24 h prior to imaging.

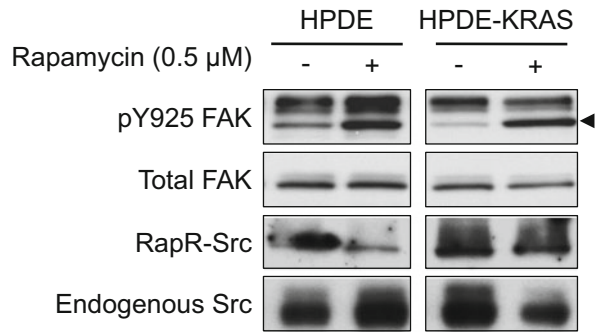

Fig. 2 RapR-Src activation induces phosphorylation of FAK tyrosine residue 925. HPDE and HPDE K-Ras cells co-expressing Cerulean-RapR-Src and mCherry-FRB were treated with 0.5 μM rapamycin to activate RapR-Src. Lysates were collected 3 h after treatment. Lysates were resolved by SDS-PAGE followed by blot analyses for phosphorylated Y925 FAK (indicated by *triangle*), total FAK, ectopically expressed RapR-Src (anti-JL8), and endogenous Src

8. On the day of imaging, aspirate media and wash cells one time with PBS. Place cover slip in an Attofluor® cell chamber and add 1 mL imaging media, ensuring that there are no leaks. Add 1 mL of mineral oil to top of media to reduce evaporation during imaging.
9. Place chamber on heated stage of microscope. Select cells that are expressing RapR-Src and FRB.
10. Image cells co-expressing Cerulean-RapR-Src and mCherry-FRB, taking images every min for 120 min. Mix 0.5 μL of 1 mM rapamycin solution with 100 μL of L15 Leibovitz Media and add to the cells (final concentration of 0.5 μM) 30 min after imaging has begun (*see* **Note 7**).
11. Continue imaging for the remaining 90 min. DIC imaging can be used to monitor cell movement and overall changes in cell morphology (i.e., protrusion formation and cell shape) (Fig. 3). Epifluorescence can be used to monitor RapR-Src, FRB, or any other fluorescently labeled co-transfected protein.

3.5 Imaging MEF Cells Expressing RapR-Src and FRB

1. Place a glass cover slip in 35 mm tissue culture plates or 6-well plates. Wash with 2–4 mL of PBS.
2. Incubate the cover slip in 2 mL of 5 μg/mL fibronectin solution in PBS at 37 °C overnight.
3. Wash the cover slip with PBS and add 2 mL of DMEM supplemented with 10 % FCS.
4. Plate transduced 50,000 MEF cells onto fibronectin-coated cover slip. Incubate in DMEM/10 % FCS medium for 2 h at 37 °C, 5 % CO_2 (*see* **Note 8**).
5. Preincubate mineral oil and imaging medium (L15 supplemented with 5 % FCS) at 37 °C for at least 30 min before imaging.

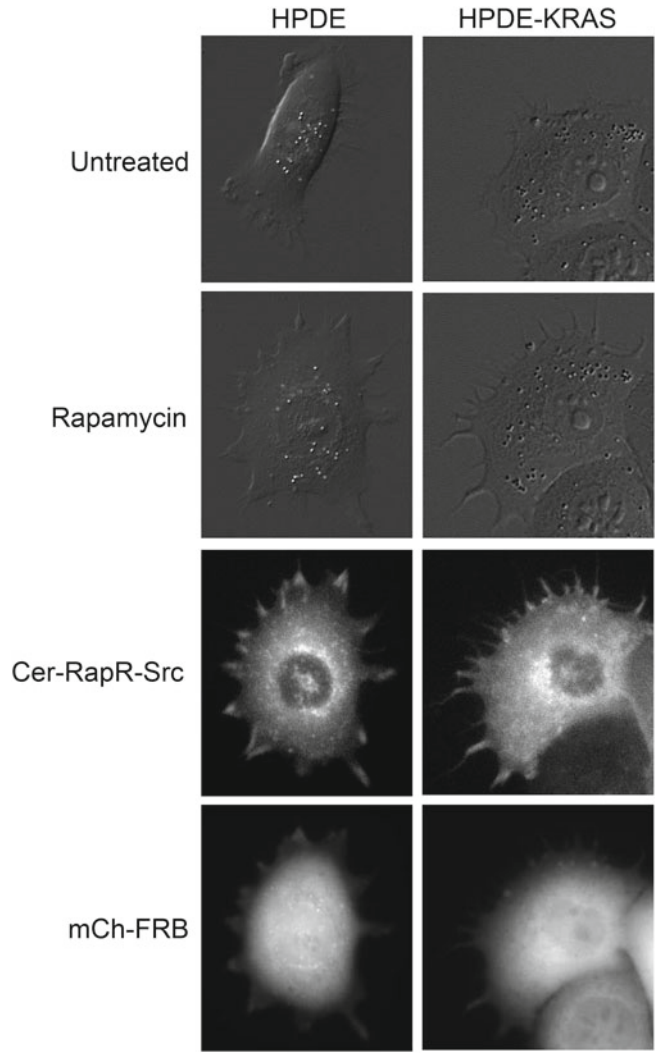

Fig. 3 RapR-Src activation induces cell spreading and protrusion formation. HPDE and HPDE-KRAS cells co-expressing Cerulean-RapR-Src and mCherry-FRB were treated with 0.5 μM rapamycin to activate RapR-Src. Image of rapamycin-treated cells was taken 1 h after treatment

6. Aspirate media and wash cells one time with PBS.
7. Place cover slip in an Attofluor® cell chamber and add 0.9 mL imaging media, ensuring that there are no leaks.
8. Add 1 mL of mineral oil to top of growth medium to reduce evaporation during imaging.
9. Place cell chamber onto heated stage of the microscope and select cells co-expressing RapR-Src-GFP and mCherry-FRB (*see* **Note 9**).

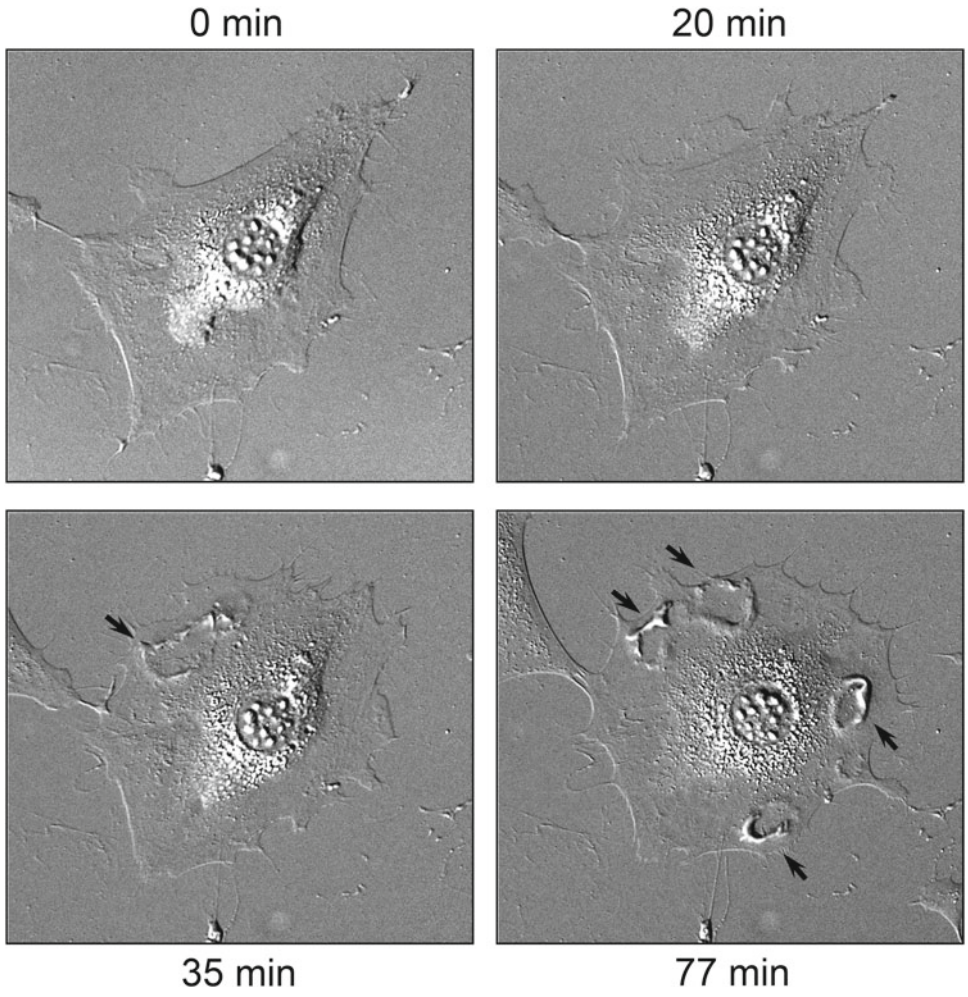

Fig. 4 RapR-Src activation induces cell spreading and dorsal wave formation (marked by arrows). MEFs co-expressing RapR-Src-GFP and mCherry-FRB were treated with 0.5 μM rapamycin to activate RapR-Src. Time after treatment is indicated

10. Image cells co-expressing RapR-Src-GFP and mCherry-FRB, taking images every min for 120 min. Mix 0.5 μL of 1 mM rapamycin solution with 100 μL of L15 Leibovitz Media and add to the cells (final concentration of 0.5 μM) 30 min after imaging has begun (*see* **Note 7**).
11. Continue imaging for the remaining 90 min. DIC imaging can be used to monitor cell movement and overall changes in cell morphology (i.e., protrusion formation and cell shape) (Fig. 4). Epifluorescence can be used to monitor RapR-Src, FRB, or any other fluorescently labeled co-transfected protein.

4 Notes

1. L15 medium is stored at 4 °C. FBS is stored separately at –20 °C. Fresh mix of L15 medium supplemented with FCS should be prepared on the day of the experiment. The amount depends on the number of imaging experiments performed; a minimum of 1 mL will be needed per experiment.
2. Fibronectin stock solution should be stored at 4 °C. Final solution used for coating cover slips should be prepared right before application.
3. Other devices suitable for live cell imaging using inverted epifluorescence microscopes can be used. The instrument should allow for addition of reagents during cell imaging.
4. We routinely use an Olympus IX-81 microscope equipped with a UPlanFLN 40× (Oil, NA 1.30) objective. All images are collected using a Photometrix CoolSnap ES2 CCD camera controlled by Metamorph software. Illumination for epifluorescence was provided from a high-pressure mercury arc light source.
5. Before selection, if expression of fluorescently tagged construct is not visible or is dim, perform a second round of viral transduction following **steps 2–4**.
6. We use Beckman Coulter MoFlo cell sorter equipped with tunable 355/405/568 nm laser and two fixed wavelength lasers (488 and 633 nm) provided by the UNC-Chapel Hill cell sorting facility.
7. Mix rapamycin with the media right before adding it to the cells. Make sure that you penetrate the oil layer when adding rapamycin solution to the cells.
8. It takes 1–2 h for MEFs to attach to the cover slips and spread.
9. A microscope equipped with a mechanized motorized stage enables consecutive imaging of several cells by selecting and logging the positions of cells expressing RapR-Src-GFP and mCherry-FRB. The number of positions depends on the time it takes all the images at one position and move to the next one, and on the desired imaging time intervals.

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