Supplementary Methods

Synthesis of C16-(*S*)-3-Methylindole rapamycin (iRap)

Rapamycin (50.3 mg, 55.0 mol), 3-methylindole (14.4 mg, 110.0 mol), and CH_2Cl_2 (5.0 mL) was added to a flame dried and argon cooled 25-mL round bottom flask. This solution was cooled to –40 ˚C and trifluoroacetic acid (17.0 L, 220.0 mol) was added. After 5 h at –40 ˚C, 8 mL ethyl acetate and 8 mL brine were added. The solution was warmed to room temperature, the layers separated, and the organic layer dried with sodium sulfate. After concentration under reduced pressure, the crude material was chromatographed using a mobile phase gradient of 1:1 hexanes/ethyl actetate to 2:3 hexanes/ethyl acetate to yield 55.1 mg (99%) of the desired product. Purity was demonstrated by HPLC using a methanol/water/acetonitrile (36:13:1) mobile phase, a Waters XTerra phenyl 5- m column at 50 ˚C, and monitoring at 278 nm. The C16-(*S*) stereochemistry was assigned by comparison of the C22 chemical shift in the ^{1}H NMR spectra of the product with rapamycin and C16-(*R*)-trimethoxyphenyl rapamycin [1,2]. Only the *trans*-conformer is described in the ^{1}H NMR characterization. **IR** (film) 3418 s, 2931 m, 1717 s, 1652 s, 1456 m, 1386 w, 990 w, 737m. **^{1}H NMR** (400 MHz, DMSO-d_6, 25 ˚C) 10.03 (s, 1H), 7.36 (d, 1H, J=8 Hz), 7.25 (d, 1H, J=8 Hz), 6.98 (t, 1H, J=7 Hz), 6.91 (t, 1H, J=7 Hz), 6.44-6.08 (m, 5H), 5.43 (dd, 1H, J=10, 15 Hz), 5.25 (d, 1H, J=4 Hz), 5.20 (d, 1H, J=10 Hz), 5.05 (m, 1H), 4.91 (d, 1H, J=6 Hz), 4.59 (d, 1H, J=4 Hz), 4.09 (m, 1H), 3.98 (d, 1H, J=4 Hz), 3.66 (m, 1H), 3.32-3.29 (m, 7H), 3.18-3.16 (m, 3H), 2.92-2.78 (m, 2H), 2.46-2.38 (m, 2H), 2.26-2.06 (m, 3H), 2.14 (s, 3H), 2.04-1.50 (m, 13H), 1.46-1.06 (m, 12H), 1.04-0.80 (m, 15H), 0.74-0.56 (m, 4H). **^{13}C NMR** (125 MHz, DMSO-d_6, 25 ˚C) δ 210.4, 208.1, 199.0, 169.2, 167.1, 140.3, 138.1, 137.2, 135.4, 135.2, 130.8, 129.0, 128.4, 125.1, 122. 7, 120.1, 117.9, 117.5, 110.5, 106.6, 98.9, 85.6, 83.8, 75.6, 73.3, 73.3, 66.9, 57.0, 56.8, 51.1, 44.8, 43.6, 38.5, 38.1, 35.3, 34.9, 33.5, 32.9, 32.6, 31.5, 31.4, 29.0, 26.6, 26.1, 24.5, 21.8, 20.4, 17.4, 16.0, 15.5, 14.7, 13.7, 13.2, 8.6. **HRMS** calculated for $C_{59}H_{85}N_2O_{12}$ (M+H): 1013.6103 amu, found (ESI) 1013.6096 amu.

DNA construction

DNA construction was performed with a Site-Directed Mutagenesis kit (Stratagene) and/or conventional restriction and ligation technique. Sequences of all DNA constructs were verified. The constitutively active form of the Rho GTPases tagged with either CFP or YFP were described in a previous paper [3]. The dominant negative form of Rac1 was generated from wild type Rac1 using mutagenesis. The CAAX box of these mutant Rho GTPases were removed by inserting a stop codon right before the CAAX sequence.

Src_{13}-YFP

The Src N-terminus sequence (ggg agt agc aag agc aag cct aag gac ccc agc cag cgc) was inserted at the N terminus of the YFP (N1) vector (Clontech).

Lyn_{11}-FKBP-FKBP-CFP (LF_2C)
The Lyn N-terminus sequence (gga tgt ata aaa tca aaa ggg aaa gac agc gcg gga gca) was inserted at the N terminus of tandem FKBP of the $pC_4M\text{-}F_2E$ (provided by ARIAD Phamceuticals), and the resulting construct flanked with EcoRI and BamHI sites was then inserted into the multiple cloning site of the CFP (N1) vector (Clontech).
Lyn_{11}-FKBP (LF)
A stop codon (taa) was introduced between the two FKBPs of LF_2C.
Lyn_{11}-linker-FKBP (LDF)
A flexible linker sequence (gat agt gct ggt agt gct ggt) was inserted between the Lyn N-terminus and FKBP of LF.
Lyn_{11}-FRB (LR)
The Lyn N-terminus sequence was inserted at the N terminus of FRB in the $pC_4\text{-}R_HE$ vector (provided by ARIAD Pharmaceuticals)
Lyn_{11}-linker-FRB (LDR)
A flexible linker sequence (gat agt gct ggt agt gct ggt agt gct ggt) was inserted between the Lyn N-terminus and FRB of LR.
YFP-FRB (YR)
FRB was taken out from the $pC_4\text{-}R_HE$ using SpeI and XbaI, and then inserted into the multiple cloning site of the YFP (C1) vector (Clontech).
FRB-YFP (RY)
FRB was taken out from $pC_4\text{-}R_HE$ with flanking sequence encoding NheI and AgeI, and then inserted into the YFP (C1) vector.
RY-Rac2
Rac2 was taken out from the vector encoding CFP-Rac2 using EcoRI and BamHI, and this fragment was inserted into the multiple cloning site of RY.
Rac2-GKKKK (Rac2++)
The GKKKK corresponding sequence (ggt aaa aag aag aaa) was inserted at the C terminus of RY-Rac2.
Rac2-YR
Rac2 was taken out from the CFP-Rac2 with a flanking sequence encoding NheI and AgeI site along with a linker sequence (agt gct ggt agt gct ggt agt gct ggt agt gct ggt agt gct ggt agt gct ggt) located at the N terminus of Rac2, and inserted at the N-terminus of YR.
Y-Rac2-R
Rac2 was taken out from the vector encoding CFP-Rac2 using EcoRI and BamHI, and this fragment was inserted into the multiple cloning site of YR.
FKBP-YFP (FY)
FKBP was taken out from the $pC_4M\text{-}F_2E$ with a flanking sequence encoding NheI and AgeI along with flexible linker sequence (agt gct ggt ggt agt gct ggt ggt agt gct ggt ggt agt gct ggt ggt agt gct ggt ggt) at the C terminus of FKBP, and inserted into the YFP (C1) vector.
FY-Rac2, FY-Rac1

Rac2 and Rac1 were taken out from the vector encoding CFP-Rac2 and CFP-Rac1, respectively, using EcoRI and BamHI, and these fragments were inserted into the multiple cloning site of FY.

YFP-FKBP (YF)

FKBP was taken out from pC_4M-F_2E with a flanking sequence encoding BsrGI and XhoI, along with a flexible linker sequence (agt gct ggt ggt agt gct ggt ggt agt gct ggt ggt agt gct ggt ggt agt gct ggt ggt) at the C terminus of FKBP, and then inserted into the YFP (C1) vector.

YF-Rac1, YF-RhoA, YF-Rac1(DN)

Rac1, Cdc42, RhoA and Rac1(DN) were inserted into the multiple cloning site of YF using EcoRI and BamHI.

YF-Cdc42

Cdc42H was inserted into the multiple cloning site of YF using EcoRI and BamHI, and the resulting construct was then sequentially mutated to form Cdc42.

YF-Tiam1

580 amino acids locating at C terminus of Tiam1 was taken out from pCS-Tiam1 (provided by Dr. Haruhiko Sugimura) with a flanking sequence encoding XhoI and SacII, and then inserted into the multiple cloning site of YF.

Cell Culture and Transfection

Cell culture and transfection of NIH3T3 cells were performed as described previously[14] with the exception that cells were plated directly in a transfection solution containing DNA plasmids and Lipofectamine (Invitrogen). Briefly, RBL cells were cultured in DMEM (GIBCO) supplemented with 20% FCS. For transfection of RBL cells, $1x10^6$ cells were suspended with 10- g YFP construct DNA and 20- g CFP construct DNA in 300- l transfection buffer (120 mM KCl, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 2 mM EGTA and 25 mM HEPES [pH 7.6]) containing 1.9 mM ATP (Sigma) and 4.7 mM glutathione (Sigma), followed by electroporation using an ECM830 electroporater (Genetronics) at 200 V for 50 ms.

Live Cell Confocal Microscopy

Live cell dual color measurements were performed on a spinning-disc confocal microscope. CFP and YFP excitations used a helium-cadmium laser (Kimmon Electrics) and an argon laser (Melles Griot), respectively. The two lasers were aligned to focus into the confocal scan head (UltraView, Perkin Elmer), mounted with a dual CFP/YFP dichroic mirrors. The lasers were processed with appropriate filter sets for CFP and YFP to capture fluorescent cellular images with a CCD camera (Hamamatsu Photonics), driven by Metamorph 4.6 imaging software (Universal Imaging). Images were taken using a 60x (NA 1.40) objective mounted on an inverted IX-70 microscope (Olympus).

Assay for membrane translocation and morphological changes

The cell culture medium on the RBL cells was changed to extracellular buffer (150 mM NaCl, 1.3 mM $CaCl_2$, 1.3 mM $MgCl_2$, 5 mM KCl, 5.6 mM glucose and 25 mM Hepes [pH 7.4]) and fluorescence images were taken every 3 or 5 s during the translocation assay. Membrane translocation induced by iRap dissolved in DMSO (0.1%) was evaluated by fitting the initial part of the normalized time course of the decrease in fluorescent signal intensity of cytoplasm to exponential function, e^{-rt}, where r is the rate constant used as an index of membrane translocation. The morphology assay of fixed NIH3T3 cells was performed as described previously[3] in the presence of 5 M iRap at 37°C. For the live cell morphology assay, the culture medium of transfected NIH3T3 cells was changed to Dulbecco's phosphate-buffered saline (GIBCO) after 6-hr serum starvation. Fluorescence images were taken every 30 s with minimal laser power and exposure time to reduce photo-induced damage of the actin cytoskeleton. Live cell imaging was performed at room temperature.

Immunohistochemistry and pull-down assay
After staining with polyclonal antibody against phospho (Ser63) c-Jun (Cell Signaling), the fluorescence intensity of Alexa Fluor 594 attached to goat anti-rabit IgG (Molecular Probes) within a nucleus of the cells was measured using confocal microscopy. Pull-down assays were performed using a Rac1 activation assay kit according to the manufacturer's protocol (Upstate).Supplementary References

1. Luengo, J. I. *et al. Chemistry & Biology* **2**, 471-481 (1995).
2. Kessler, H. *et al. Helvetica Chimica Acta* **76**, 117-130 (1993).
3. Do Heo, W. *et al. Cell* **113**, 315-328 (2003).