Aoki et al., http://www.jcb.org/cgi/content/full/jcb.200609017/DC1

Supplemental materials and methods

Calculation of the raw number of translocated proteins

First, the average intensities of background-subtracted EGFP and RFP images were obtained from the whole area of the cell, and the ratio of EGFP/RFP (named as α value) was calculated in each time point. Second, the normalized RFP image was created by multiplying α value to each pixel; the resulting image was names as αRFP. Third, we created two images by subtracting EGFP image from αRFP image (EGFP – αRFP) and αRFP image from EGFP image (αRFP − EGFP), respectively. In this process, all negative values were replaced with zero by the MetaMorph software. Fourth, the average intensities of the two images, i.e., EGFP – αRFP and αRFP – EGFP, were obtained and averaged, and this intensity was then converted to the number of EGFP proteins by calibrating with fluorescent microspheres (Sugiyama et al., 2005). This value was "the raw number of translocated proteins" (Fig. S4).

Dynamic range of the raw number of translocated proteins

To examine the dynamic range, the raw numbers of translocated proteins were obtained for positive and negative controls. As a positive control, the cells expressing EGFP-pm (plasma membrane marker; EGFP fused with CAAX box of Ki-Ras4B (amino acids 169–188), and RFP-NES were used. As a negative control, the cells expressing Vav2-EGFP (cytosolic marker) and RFP-NES were used. Fig. S4 B shows the plots of the raw number of translocated proteins against the number of total proteins for positive (red) and negative (blue) controls. The raw number of translocated proteins increased linearly against the number of total EGFP-tagged proteins. Slopes of positive and negative controls were 0.146 and 0.0251, respectively.

Calculation of the corrected number of translocated proteins

Next, we imaged cells expressing GRP-EGFP (PIP_3 indicator) and RFP-NES during NGF stimulation, and the raw number of translocated proteins was calculated as described

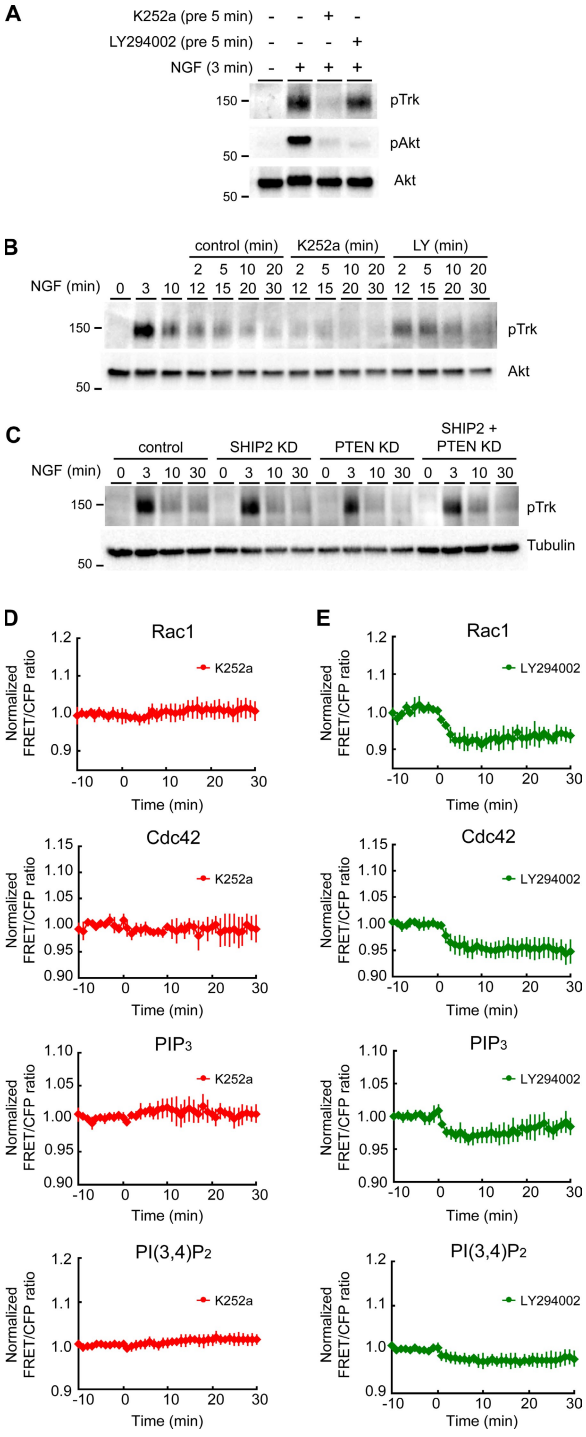

Figure S1. **TrkA phosphorylation, Rac1/Cdc42 activity, and PIP_3 levels in various conditions.** (A) PC12 cells were pretreated with or without 10 nM K252a or 20 μM LY294002 for 5 min and then stimulated with 50 ng/ml NGF for 3 min. Cell lysates were immunoblotted with anti–phospho-TrkA (Tyr490), anti–phospho-Akt (Thr308), and anti-Akt antibodies. (B) Starved PC12 cells were stimulated with NGF and then mock-treated or treated with K252a or LY294002 for the indicated periods. Cell lysates were probed with anti–phospho-TrkA (Tyr490) and anti-Akt antibodies. (C) PC12 cells transfected with pSUPER, pSUPER-SHIP2, pSUPER-PTEN, or both pSUPER-SHIP2 and pSUPER-PTEN were serum starved and stimulated with NGF for the indicated periods. Cell lysates were immunoblotted with anti–phospho-TrkA (Tyr490) and anti-tubulin antibodies. (D and E) PC12 cells expressing Raichu-Rac1, Raichu-Cdc42, Pippi-PIP_3, or Pippi-$PI(3,4)P_2$ were starved and treated with 10 nM K252a (D) or 20 μM LY294002 (E). The average FRET/CFP ratios over the whole cell are expressed by measuring the relative increase as compared to the reference value, which was averaged over 10 min before K252a or LY294002 addition. The number of experiments is as follows: (D) $n = 10$, 6, 5, and 10 for Rac1, Cdc42, PIP_3, and $PI(3,4)P_2$, respectively; (E) $n = 6$, 6, 5, and 9 for Rac1, Cdc42, PIP_3, and $PI(3,4)P_2$, respectively.

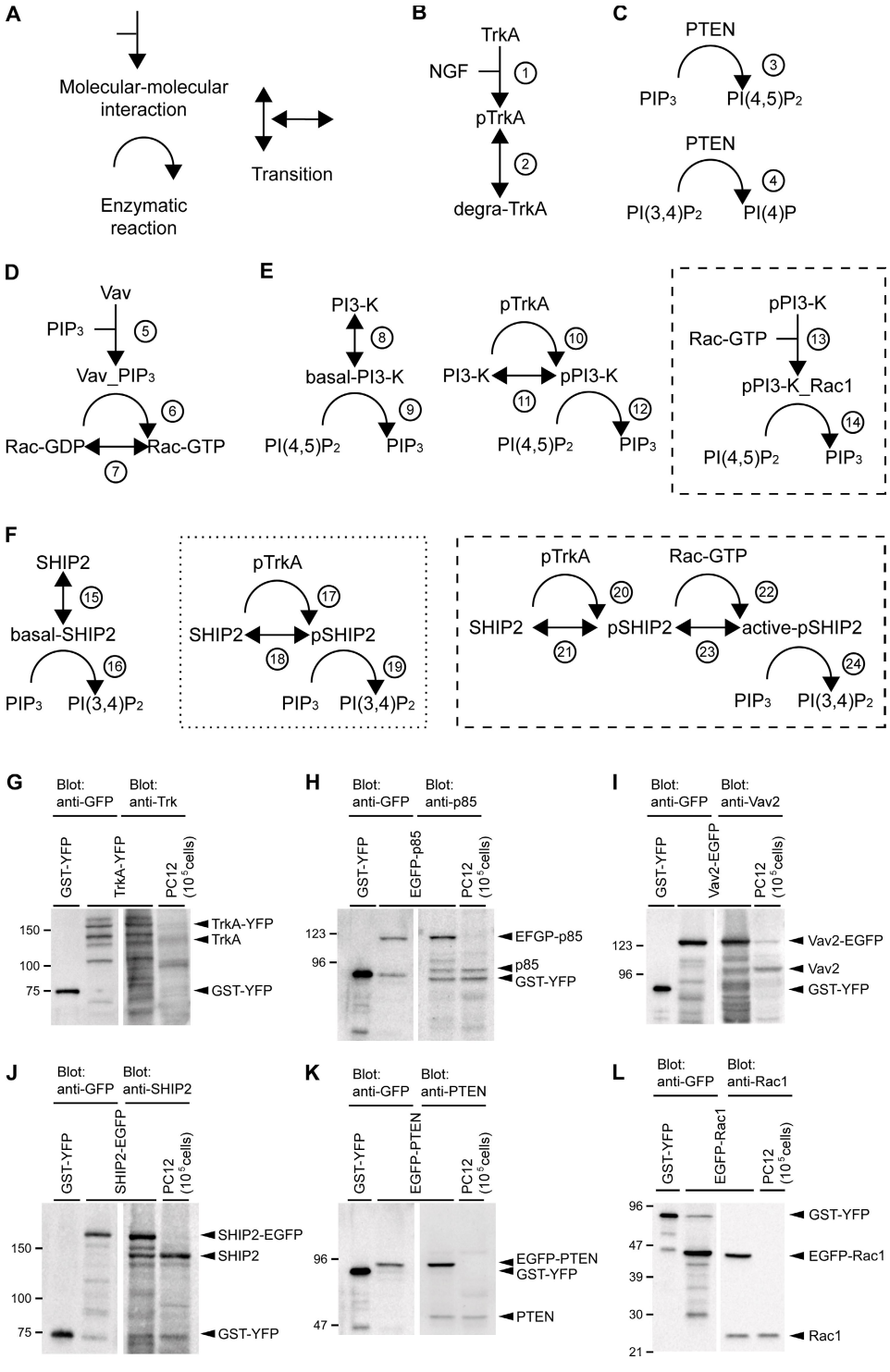

Figure S2. **All reactions in models of the NGF–PIP_3–Rac1 signaling pathway and quantification of endogenous proteins.** (A) Three types of reaction, i.e., molecular–molecular interaction, enzymatic reaction, and transition, are used in B–F. (B) NGF binds to TrkA, followed by phosphorylation of TrkA (pTrkA). pTrkA is degradated and recycled. (C) PTEN dephosphorylates PIP_3 and $PI(3,4)P_2$ and generates $PI(4,5)P_2$ and PI(4)P, respectively. (D) PIP_3 binds to Vav (Vav_PIP_3). Vav–PIP_3 complex activates Rac1 (Rac-GTP). Rac1-GTP is hydrolyzed and inactivated (Rac-GDP) constantly. (E) Spontaneous shuttling between PI3-kinase (PI3-K) and basal-PI3-K determines the basal activity of PI3-K. Basal-PI3-K phosphorylates $PI(4,5)P_2$ to maintain basal PIP_3 level. pTrkA phosphorylates and activates PI3-K (pPI3-K), which also stimulates the generation of PIP_3 from $PI(4,5)P_2$. pPI3-K is continuously dephosphorylated. In the improved model (Fig. 5 A), we introduced NGF-dependent and Rac1-mediated positive feedback (dashed line). In this model, activated Rac1 (Rac-GTP) directly binds to pPI3-K (pPI3-K_Rac1), which is also able to generate PIP_3 like pPI3-K. We assume that the binding between Rac1 and pPI3-K delays the dephosphorylation (inactivation) of pPI3-K. (F) Spontaneous shuttling between SHIP2 and basal-SHIP2 determines the basal activity of SHIP2. In the initial model (dotted line), pTrkA phosphorylates and activates SHIP2 (pSHIP2), which dephosphorylates PIP_3 and generates $PI(3,4)P_2$. The activated SHIP2 is constantly inactivated. In the improved model (dashed line), we introduced NGF-dependent and Rac1-mediated negative feedback. In this model, Rac1-GTP activates the phosphorylated SHIP2 (active-pSHIP2), which hydrolyzes PIP_3 and produces $PI(3,4)P_2$. All kinetic parameters are listed in Tables S1–S4. (G–L) The protein concentrations of the endogenous TrkA (G), p85 (H), Vav2 (I), SHIP2 (J), PTEN (K), and Rac1 (L) in PC12 cells were determined using a recombinant protein as a standard. The recombinant GST-tagged YFP protein was purified from *Escherichia coli*. The lysate of the cells expressing TrkA-YFP (G), EGFP-p85 (H), Vav2-EGFP (I), SHIP2-EGFP (J), EGFP-PTEN (K), or EGFP-Rac1 (L) was used as a reference. Standard (10 ng of GST-YFP), reference (the lysate of the cells expressing fluorescent protein-tagged protein), and the total cell lysate containing 10^5 cells were separated by SDS-PAGE, followed by immunoblotting with anti-GFP (standard and reference) or specific antibodies (reference and total cell lysate). The bound antibodies were detected with an ECL chemiluminescence detection system (GE Healthcare) and quantified with an LAS-1000 image analyzer (Fujifilm). The amount of reference protein was obtained from the relative intensity of the reference to GST-YFP, and the amount of endogenous protein was calculated by comparison with the reference.

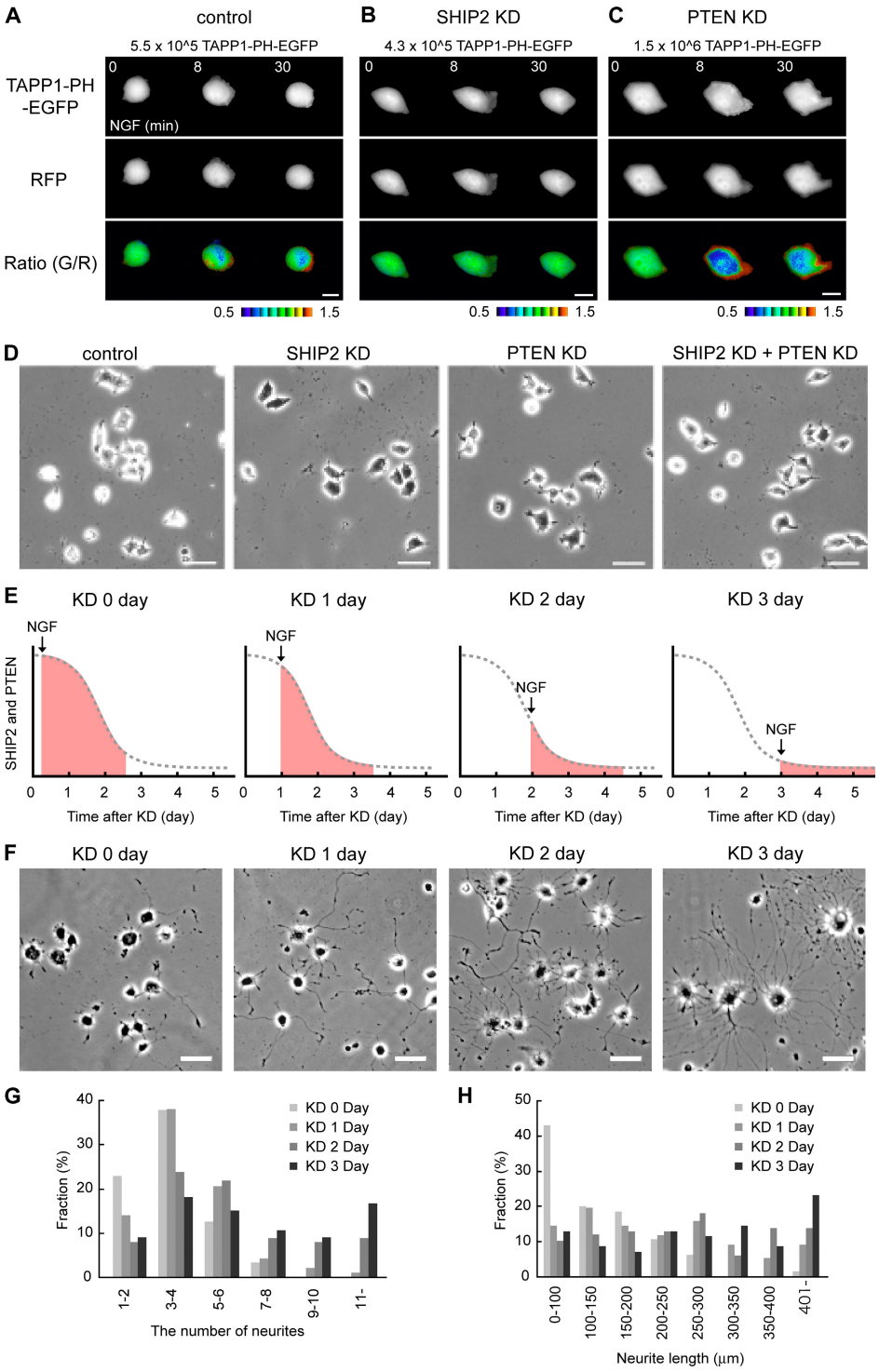

Figure S3. **Effect of depletion of SHIP2 and PTEN on NGF-induced PI(3,4)P_2 production and neurite outgrowth of PC12 cells.** (A–C) PC12 cells were transfected with pSUPER (A), pSUEPR-SHIP2 (B), or pSUPER-PTEN (C). After selection with puromycin, the cells were further transfected with the plasmids encoding TAPP1-PH-EGFP used as the PI(3,4)P_2 indicator and RFP used as the cytosolic marker. Cells were stimulated with NGF and time-lapse imaged as described. Shown here is the localization of TAPP1-PH-EGFP (top) and RFP (middle) at the indicated time points after NGF stimulation. The numbers of EGFPs were calculated by calibrating with fluorescent microspheres (Sugiyama et al., 2005) and are indicated on the top of each EGFP image. Ratio images of GFP/normalized RFP (bottom) were created as described in the legend to Fig. 4. Bars, 10 μm. (D) PC12 cells were transfected with pSUPER, pSUPER-SHIP2, pSUPER-PTEN, or both pSUPER-SHIP2 and pSUPER-PTEN. After puromycin selection for 2 d, the cells were serum starved for 48 h and fixed for microscopy. Representative phase-contrast images of control, SHIP2 knockdown (SHIP2 KD), PTEN knockdown (PTEN KD), and SHIP2 and PTEN double-knockdown cells (SHIP2 KD and PTEN KD) are shown. Bars, 50 μm. (E–H) PC12 cells were treated with NGF at 0, 1, 2, or 3 d after the transfection of both pSUPER-SHIP2 and pSUPER-PTEN. (E) Schematic diagrams of relative amounts of SHIP2 and PTEN during NGF-induced neurite outgrowth in each experimental condition. Dashed lines indicate the time-dependent change of the amounts of SHIP2 and PTEN after the transfection of shRNA vectors. The difference of relative amounts of SHIP2 and PTEN during 2.5-d NGF treatment is shown by pink areas. (F) Representative phase-contrast images of the cells treated with NGF at 0 d (KD 0 day), 1 d (KD 1 day), 2 d (KD 2 day), and 3 d (KD 3 day) after the transfection of shRNA vectors. The cells were incubated with NGF for 2.5 d and fixed for microscopy. Bars, 50 μm. (G) The number of neurites in neurite-bearing cells was divided into six segments as indicated under the horizontal axis. A histogram plotting the percentage of cell number in each segment is presented for the KD 0 day (n = 87), KD 1 day (n = 92), KD 2 day (n = 101), and KD 3 day (n = 66). (H) The length of neurites in neurite-bearing cells was divided into eight segments as indicated under the horizontal axis. A histogram plotting the percentage of cell number in each segment is presented as in G.

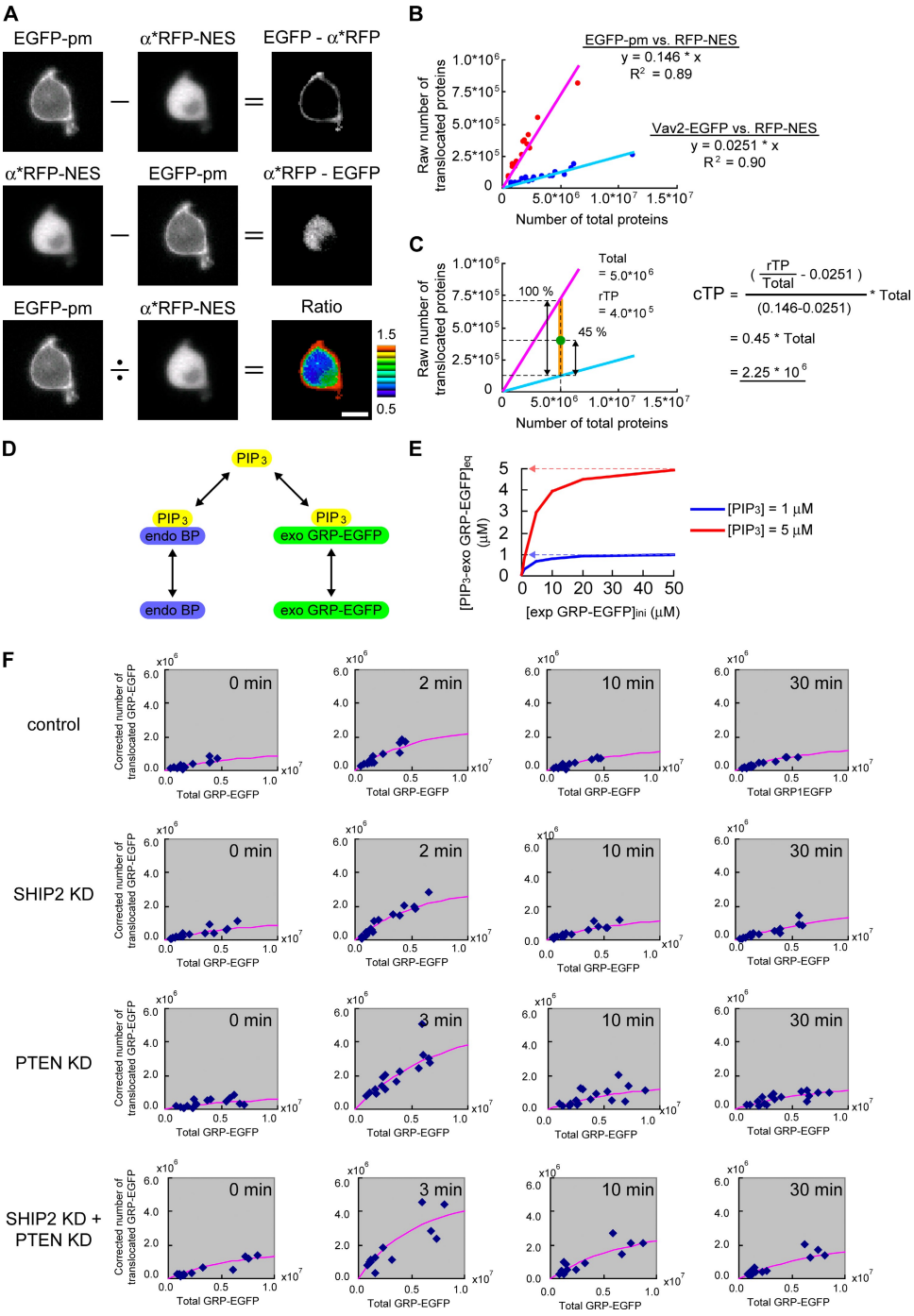

Figure S4. **Semiquantitative measurement of intracellular PIP_3 level.** PIP_3-dependent translocation of GRP-EGFP from the cytosol to the plasma membrane was measured semiquantitatively by using RFP-NES as a reference. For imaging, we used the following filters: two excitation filters (FX0480 [ASAHI SPECTRA, Tokyo, Japan] for EGFP and XF1017 [540AF30, Omega Optical] for RFP), an XF2052 (450-520-590TBDR, Omega Optical) dichroic mirror, and two emission filters (XF3079 [535AF26, Omega Optical] for GFP and XF3094 [600ALP, Omega Optical] for RFP). The cell was time-lapse imaged with exposure times 200–400 ms for GFP images and 100 ms for RFP images. See supplemental Materials and methods for details of image data processing.

(see Calculation of the raw number of translocated proteins). As a PIP_3 indicator, we used the full-length GRP protein fused with EGFP, which was mainly localized in cytoplasm in unstimulated cells because the PH domain of GRP fused with EGFP was preferentially localized to the nucleus and did not efficiently translocate to the plasma membrane upon NGF stimulation. By using the relationship obtained as described (see Dynamic range of the raw number of translocated proteins), the corrected number of translocated proteins (cTP) is obtained as follows: cTP = (rTP/total – 0.0251)/(0.146 – 0.0251) × total, where rTP and total represent the raw number of translocated GRP-EGFP and the total number of GRP-EGFP, respectively (Fig. S4 C).

Calculation of the amount of PIP_3 as an asymptotic value

The quantitative relationship (Fig. S4 D) among the concentrations of PIP_3 ([PIP_3]), endogenous PIP_3 binding proteins ([endo BP]), and exogenous GRP-EGFP ([exo GRP-EGFP]) is as follows: [PIP_3] + [endo BP] ⟷ [PIP_3-endo BP] and [PIP_3] +

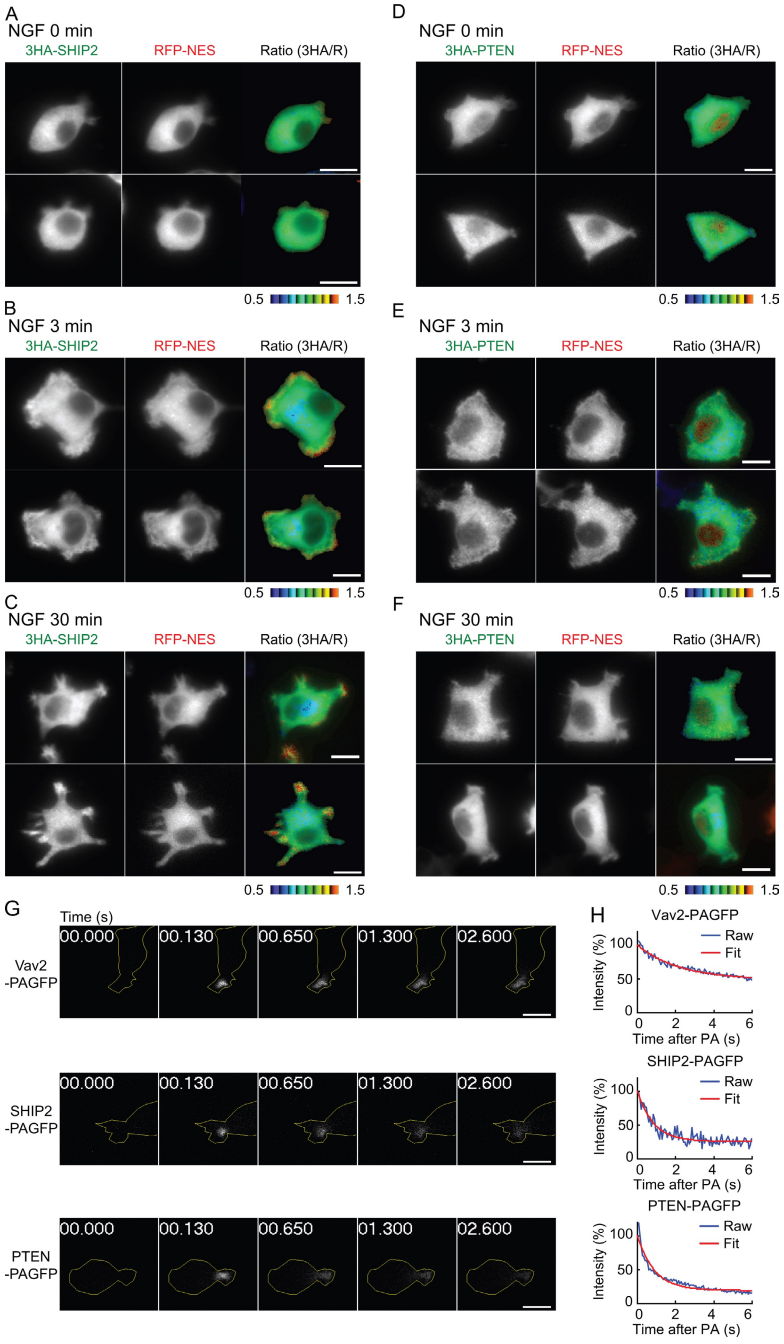

Figure S5. **Subcellular localization of SHIP2 and PTEN,and different diffusion rate among Vav2, SHIP2, and PTEN in PC12 cells stimulated with NGF.** (A–F) PC12 cells expressing both 3HA-SHIP2 and RFP-NES (A–C) or both 3HA-PTEN and RFP-NES (D–F) were treated with 50 ng/ml NGF for 0 min (A and D), 3 min (B and E), or 30 min (C and F). Then, the cells were fixed and stained with anti-HA antibody. The left and middle panels show the localization of 3HA-SHIP2 (A–C) or 3HA-PTEN (D–F) and RFP-NES, respectively. Ratio images of 3HA/normalized RFP (bottom) were created as described in the legend to Fig. 4. Bars, 10 μm. (G and H) PC12 cells expressing Vav2-PAGFP, SHIP2-PAGFP, or PTEN-PAGFP were treated with NGF for 3-30 min. At time point zero, PA-GFP proteins in a region of interest in NGF-induced protrusions were photoactivated by UV irradiation. (G) Representative images of Vav2-PAGFP (top), SHIP2-PAGFP (middle), and PTEN-PAGFP (bottom) are shown at the indicated time points after photoactivation. Bars, 10 μm. Yellow lines indicate cell contours. (H) Representative time courses of relative fluorescent intensities of PA-GFP proteins after photoactivation (PA). Blue and red lines indicate raw data and fitting curves, respectively.

[exo GRP-EGFP] ⟷ [PIP_3-exo GRP-EGFP]. We assume that the initial concentration of exogenous GRP-EGFP ($[\text{exo GRP-EGFP}]_{ini}$) is variable and obtained the equilibrium values using GENESIS/Kinetikit. Fig. S4 E plots the equilibrium value of the concentration of the complex between PIP_3 and exogenous GRP-EGFP ($[\text{PIP3-exo GRP-EGFP}]_{eq}$) against $[\text{exo GRP-EGFP}]_{ini}$. $[\text{exo GRP-EGFP}]_{ini}$ equals to the concentration of total GRP-EGFP. As shown in Fig. S4 E, the asymptotic value means the concentration of PIP_3.

Next, we plotted the experimental values of the corrected number of translocated GRP-EGFP against the number of total GRP-EGFP (Fig. S4 F, blue dots). Then, we fitted the data with the following single exponential function using a solver of Microsoft Excel software: cTP = amplitude × [1 − exp (− total/k)]

Amplitude and k are parameters that are changed during the fitting process (Fig. S4 F, red line). As shown in Fig. S4 E, the amplitude after fitting indicates the amount of PIP_3. We obtained the amount of PIP_3 in each time point, and plotted it against the time after NGF stimulation (Fig. 4 G).

Table S1. **Parameters of molecular–molecular interactions**

Reaction number	Fig. 2		Figs. 6 and 7	
	kf (/s/μM)	kb (/s)	kf (/s/μM)	kb (/s)
1	0.1	0	0.1	0
5	0.1	1	0.1	1
13			1	1

Table S2. **Parameters of enzymatic reactions**

Reaction number	Fig. 2			Figs. 6 and7		
	kf (/s/μM)	kb (/s)	kcat (/s)	kf (/s/μM)	kb (/s)	kcat (/s)
3	2	8	2	2	8	2
4	2	8	2	2	2	2
6	1	4	1	1	4	1
9	2	8	2	2	8	2
10	2.5	10	2	4	4	4
12	2	8	2	2	8	2
14				2	8	2
16	0.5	4	1	1	4	1
17	0.2	0.8	0.2			
19	1	4	1			
20				0.05	0.2	0.05
22				0.5	0.5	0.5
24				1	1	1

Table S3. **Parameters of transitions**

Reaction number	Fig. 2		Figs. 6 and 7	
	kf (/s)	kb (/s)	kf (/s)	kb (/s)
2	0.01	0.001	0.01	0.001
7	0	0.0083[a]	0	0.0083 [a]
8	0.02	2.0	0.02	2.0
11	0	0.4	0	2
15	0.05	1	0.005	0.1
18	0	0.004		
21			0	0.005
23			0	0.01

[a]This value was obtained from the rate of decrease in FRET/CFP ratio of Raichu-Rac1 in response to LY294002.

Table S4. **Initial concentration**

Molecule	Concentration
	μM
NGF	1[a]
$PI(4,5)P_2$	10

[a]NGF concentration was rapidly elevated from 0 to 1 μM at 10 min after starting simulation.