Supporting Information

A Highly Sensitive Fluorescent Akt Biosensor Reveals Lysosome-Selective Regulation of Lipid Second Messengers and Kinase Activity

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Materials and Methods

Reagents and constructs
PDGF (P3201) and IBMX (I5879) were purchased from Sigma. Forskolin (F9929) and PMA (P1680) were purchased from LC Labs. GDC0068 (RG7440) was purchased from APEXBio. Dyno-4a (S7163) and PIK-75 (S1205) were purchased from SelleckChem. To optimize ExRai based AktAR, the seven best candidates with different linker sequences next to cpGFP for ExRai–AKAR were selected: AI-SM, FC-LL, LQ-LL, SY-IS, HF-CR, YY-IT, TA-LL. The SacI/EcoRI-digested fragment containing the cpEGFP, FHA1, and linkers from pRSETb (Invitrogen) was subcloned into a SacI/EcoRI-digested fragment containing the cpEGFP, FHA1, and linkers from pRSETb (Invitrogen) was subcloned into a pcDNA3′ backbone containing the Akt substrate. ExRai-AktAR2 (T/A) was generated by subcloning a SacI/EcoRI-digested fragment containing the cpEGFP, FHA1, linkers from ExRai-AktAR2 into a SacI/EcoRI-digested ExRai-AktAR1 (T/A) backbone. Cyto, Nuc, PM, Golgi- and Lyso- ExRai-AktAR2 variants were prepared by subcloning a BamHI/EcoRI-digested fragment containing full-length ExRai-AktAR2 to BamHI/EcoRI-digested backbones containing a C-terminal 11 amino acid sequence from NES (EFLPPLERLTL), a C-terminal 10 amino acid sequence from NLS (PKKKRKVEDA), an N-terminal 12 amino acid sequence from Lyn (GCIKSKRKDLP), eNOS, and LAMP1-derived sequence, respectively. Lysosome targeted InPAkt reporter was prepared by subcloning a BamHI/EcoRI-digested fragment containing full-length InPAkt to BamHI/EcoRI-digested pcDNA3′ backbone containing an N-terminal lysosome targeting motif derived from LAMP1 protein. Lyso-PTEN A4-mCherry was generated by inserting BamHI/EcoRI-digested PTEN A4-mCherry fragment into BamHI/EcoRI-digested pcDNA3′ backbone with an N-terminal lysosome targeting motif. To assemble Lyso-INP4B-mCherry, standard cloning procedures were performed, including PCR with specific primers using Phusion polymerase (Catalog No. F530S, Thermo Fisher Scientific), and Gibson assembly. The PCR fragment of INP4B with an mCherry tag was assembled to BamHI/EcoRI digested pcDNA3′ backbone through Gibson assembly. All constructs were verified by sequencing. All plasmids will be made available by direct request from the investigators, or through Addgene.

Cell culture, transfection, and starvation
NIH3T3 cells (CRL-1658, ATCC) were cultured in DMEM (11885, Gibco) supplemented with 10% calf serum (30-2030, ATCC) and 1% penicillin-streptomycin (Sigma-Aldrich) and were routinely tested for mycoplasma contamination and found negative. HCT116 wild-type and Akt1/2 double knockout cells were cultured in McCoy’s 5A (16600, Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin (Sigma-Aldrich) at 37°C and 5% CO2. For live-cell imaging, cells were plated onto sterile 35-mm glass-bottomed dishes (D35-14-1.5N, CellVis) and grown to ~40% confluence at 37°C with 5% CO2. Transfection was conducted with lipofectamine 2000 (Invitrogen) and incubated for 24 hr. For expansion microscopy, cells were plated onto 18x18 microscope cover glass (48280-046, VWR) in a 12-well plate (Corning), and cultured and transfected as described above. For serum starvation, cells were starved in serum-free DMEM for 24 hr. For serum- and amino acid-starvation, cells were serum-starved for 24 hr (DMEM without serum) followed by a 2 hr amino acid starvation in modified Hank's balanced salt solution (1 × HBSS with 2 g/l glucose, pH 7.4, made from 10 × HBSS (14065, GIBCO)) at 37°C.

Biosensor localization
NIH3T3 cells expressing Lyso-ExRai-AktAR2 were stained with LysoTracker Red DND-99 (L7528, ThermoFisher) at a final concentration of 50 nM in DMEM (11885, Gibco). NIH3T3 cells expressing Nuc- and Golgi-ExRai-AktAR2 were stained with DAPI and anti-Golgin97 antibody (13192, Cell Signaling Technology), respectively. These cells, as well as cells expressing Cyto-ExRai-AktAR2 and PM-ExRai-AktAR2, were imaged on Zeiss Axio Observer Z7 microscope equipped with a 40x/1.4NA oil objective and Photometrics Prime95B sCMOS camera (Photometrics) controlled by METAFLUOR 7.7 software (Molecular Devices) or Leica SP8 confocal microscope equipped with a 63x/1.40 oil objective and PMT detector controlled by LAS X software (Leica) in the Microscopy Core at UC San Diego.

Live-cell imaging
NIH3T3 cells were washed once with modified Hank’s balanced salt solution (1 × HBSS with 2 g/l glucose, pH 7.4, made from 10 × HBSS (14065, GIBCO)) and imaged in the dark at room temperature. Images were acquired on Zeiss Axio
Observer Z7 microscope (Carl Zeiss) equipped with a 40x/1.4NA oil objective, a Definite Focus 2 system (Carl Zeiss), and Photometrics Prime95B sCMOS camera (Photometrics) controlled by METAFLUOR 7.7 software (Molecular Devices). For InPAkt and TORCAR imaging, dual-emission ratio imaging was performed with a 420DF20 excitation filter, a 455DRLP dichroic mirror, and two emission filters, 473DF24 and 535DF25 for CFP and YFP, respectively. For ExRai-AktAR2 imaging, dual-excitation ratio imaging was performed using 480DF20 and 405DF20 excitation filters, a 505DRLP dichroic mirror and a 535DF50 emission filter. For pHRed imaging, dual-excitation ratio imaging was performed using a 420DF20 and 572DF35 excitation filters, a 594DRLP dichroic mirror, and a 645DF75 emission filter. For RFP, a 572DF35 excitation filter, a 594DRLP dichroic mirror, and a 645DF75 emission filter were used. Filter sets were alternated by an LEP MAC6000 control module (Ludl Electronic Products Ltd). Exposure times were 50–500 ms, and images were taken every 30 s. Imaging data was analyzed with Metafluor 7.7 software (Molecular Device). Raw fluorescence images were background-corrected by deducting the background (regions with no cells) from the emission intensities of cells expressing biosensors. Excitation ratios (Ex480/405) for ExRai-AktAR2 related imaging, yellow-to-cyan (Y/C) emission ratios for InPAkt related imaging, and cyan-to-yellow (C/Y) emission ratios for TORCAR related imaging were then calculated at different time points. The resulting time courses were normalized by dividing the ratios at each time point by the average ratio of the time points before the addition of drugs. For biosensor responses, the maximum ratio changes (∆R/R₀) were calculated as (∱max – R₀)/R₀, where ∱max is the maximum ratio value recorded after stimulation, and R₀ is the ratio value at time 0 min. For analysis of untargeted reporters, such as in Fig. 1c, whole-cell regions of interest (ROIs) were used. Separate analyses of cytosolic and nuclear ROIs in cells expressing untargeted ExRai-AktAR or ExRai-AktAR2 are shown in Fig. S1a-b. ROIs at given subcellular regions, such as the plasma membrane, lysosome, golgi, cytosol, or nucleus, were used to analyze each respective subcellularly targeted reporter. For characterizing the kinetics, Tlag was calculated as the time needed to reach 50% of the maximum amplitude, and the lag time was calculated as the time needed to reach 5% of the maximum amplitude.

**Sustained activity metric analysis**

For calculating the Sustained Activity Metric at 15 min post-treatment (SAM15), we used the following equation where R15 represents the normalized emission ratio at 15 min post-treatment, and Rmax represents the maximal signal response after treatment and R₀ is the signal at time 0 min.

\[
\frac{R_{15} - R_0}{R_{max} - R_0}
\]

**Characterization of pH sensitivity of ExRai-AktAR2**

For pH sensitivity characterization in living cells, buffers contain 1 × HBSS (made from 10 × HBSS (14065, Gibco) with 2 g/l glucose, 10 mM HEPES (H3375, Sigma-Aldrich), 10 mM MES (M2933, Sigma-Aldrich)), with pH values adjusted to 5.0, 6.0, and 8.0 either with NaOH or with HCl. To permeabilize cells, 10 µM monensin (16488, Cayman Chemical) was added to the pH buffers. To validate the intracellular pH changes, NIH3T3 cells expressing pHRed (Addgene plasmid # 31473) were imaged upon treatment with sequential buffer exchange from modified HBSS buffer (1 × HBSS with 2 g/l glucose, pH 7.4) to the pH buffers with monensin at different pH values (5.0, 6.0, and 8.0). NIH3T3 cells expressing ExRai-AktAR2 were imaged upon the same treatment, followed by stimulation with 50 ng/ml PDGF.

**Immunoblotting**

Cells were washed with ice-cold PBS and then lysed in RIPA lysis buffer containing protease inhibitor cocktail, 1mM PMSF, 1mM Na₃VO₄, 1mMNaF, and 25 mM calyculin A. Total cell lysates were incubated on ice for 30 min and then centrifuged at 4 °C for 20 min. Total protein was separated via 4–15% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with TBS containing 0.1% Tween-20 and 5% bovine serum albumin and then incubated with primary antibodies overnight at 4 °C. After incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies, the membranes were developed using horseradish peroxidase-based chemiluminescent substrate (34579 and 34076, Thermoscientific). The intensity of the bands was quantified with ImageJ 1.52s software. The following primary antibodies used for immunoblotting were purchased from Cell Signaling Technology: p-TSC2 (T1462) (#3617), TSC2 (#3612), p-FoxO1/3 (T24/32) (#9464), FoxO1 (#2880), and p-GSK3β (S9) (#9322). GFP (sc9996) and
GAPDH (sc365062) were purchased from Santa Cruz. GSK3β (#610201) antibody was purchased from BD Bioscience. The horseradish peroxidase-labeled goat anti-rabbit (PI31460) or anti-mouse (PI31430) secondary antibodies were purchased from Pierce.

Immunofluorescence

Cells were washed 3 times with PBS and fixed with 4% paraformaldehyde in PBS (15710 S, Electron Microscopy Sciences) for 30 min at room temperature. Cells were then washed 3 times with PBS and permeabilized with PBS containing 0.1% Triton X-100 for 15 min at room temperature. Following 1h incubation in blocking buffer (PBS containing 0.1% Triton X-100 and 5% BSA) at room temperature, coverslips were incubated for 12 h at 4 °C in Akt antibody diluted in blocking buffer (#9272, Cell Signaling Technology, 1:100). Following three 5-min washes in PBS, coverslips were incubated for 1 h at room temperature in the dark in secondary antibody (anti-rabbit Alexa Fluor 488, A11006 (1:1000), Life Technologies/Molecular Probes)diluted in blocking buffer containing 0.1% Triton X-100. Following three 5-min washes with PBS, coverslips were mounted in Prolong Glass anti-fade Mountant with NucBlue (P36981, Invitrogen). For validation of Akt antibody, the mean intensity of entire cell was quantified using CellProfiler 3.0.0 software (Broad Institute).

Proximity ligation assay

PLA experiments were performed using the Duolink in situ red starter kit for proximity ligation assays (Sigma Aldrich, DUO92101) following the manufacturer’s protocol. Briefly, cells grown on coverslips were fixed and permeabilized as in the immunofluorescence experiments then blocked with blocking buffer supplied with the kit at 37 °C for 1 h. Cells were incubated with the primary antibody (rabbit anti-Akt, #9272, Cell Signaling Technology, 1:100, and mouse anti-LAMP1, sc20011, Santa Cruz, 1:100) overnight at 4 °C, and then with the provided secondary antibody (conjugated with nucleotides) for 1 h at 37 °C with washes after each step. Ligation of the nucleotides and amplification of the strand occurred sequentially by incubating cells with first ligase and then polymerase and detection solution. Negative control includes PLA experiments with mouse anti-LAMP1 with normal rabbit IgG (#2729, Cell Signaling Technology). Images were acquired on a Zeiss Axio Observer Z7 microscope (Carl Zeiss). The number of dots were counted using ImageJ software after proper thresholding.

Expansion Microscopy

After transfection for 24 hr, cells were washed three times with PBS. To preserve microtubule structure, cells were fixed in fresh 4% formaldehyde and 0.25% glutaraldehyde for 30 minutes. Following three washes with PBS, the cells were permeabilized with 3% bovine serum albumin (BSA) and 0.3% Triton X-100 for 30 min at room temperature. Cells were incubated with primary antibody against pan-Akt (#9272, Cell Signaling Technology, 1:100) in PBS buffer for 48h at 4°C. Cells were then washed and incubated with 0.01% acrylic acid N-hydroxysuccinimide ester (Santa Cruz) in PBS for 2 hr. The monomer solution contained 1x PBS, 2 M NaCl, 25% sodium acrylate (w/w), 4% acrylamide (w/w), 0.04% (w/w) N,N'-methylenebisacrylamide. Before gel polymerization, the monomer was mixed with 0.2% (w/w) ammonium persulfate (APS) and 0.2% (w/w) tetramethylethylenediamine (TEMED) to promote polymerization. 70 µL of the mixture was added to each sample and incubated for 3 hr at 37°C.

Following polymerization, the gel was digested with 10 excess gel volumes of 0.2 mg/ml Proteinase K (Roche, Proteinase K recombinant PCR grade) in digestion buffer (0.8 M guanidine HCl, 50 mM Tris, 2mM CaCl2, 3M NaCl, 0.5% Triton X-100, pH 8) at 37°C for 3 hr. The digested gels were washed in 100 excess volumes of PBS to dialyze proteinase K. This process was repeated with four washes of 30 min each to completely remove proteinase K. Then the sample was stained with Alexa 568 conjugated secondary antibodies (A11011, Life Technologies/Molecular Probes) for 24 hr at room temperature. Before imaging, the sample was washed in PBS four times for 30 min each to remove free dye. For imaging, the samples were placed in 35-mm glass-bottomed dish. The sample was imaged with Leica SP8 with lightning mode (Leica, 100x oil objective, 70% laser power).

Co-localization analysis
The quantitative analysis of co-localization of two different fluorescence channels usually contains two meanings: the co-occurrence of both fluorophores in the same pixels, and the correlation between the intensities of the two channels. The Pearson correlation coefficient (PCC) is calculated as follows:

\[ r = \frac{\sum (R_i - \bar{R}) \cdot (G_i - \bar{G})}{\sqrt{\sum (R_i - \bar{R})^2 \cdot \sum (G_i - \bar{G})^2}} \]

The Mander’s overlap coefficient (MOC) is given as follows:

\[ r = \frac{\sum R_i \cdot G_i}{\sqrt{\sum R_i^2 \cdot \sum G_i^2}} \]

Here, \( R_i \) is the intensity of the first channel in pixel \( i \), and \( \bar{R} \) is the arithmetic mean intensity; \( G_i \) and \( \bar{G} \) are the corresponding intensities for the second channel in the same pixel. Our quantitative co-localization analysis was conducted with the JACOP plugin on ImageJ software.

**Statistics and reproducibility**

All experiments were independently repeated as noted in the figure legends. All replication attempts were successful. The data were analyzed using GraphPad Prism 6. For Gaussian data, pairwise comparisons were performed using two-sided Student’s t-test or Welch’s unequal variance t-test, and comparisons among three or more groups were performed using ordinary one-way ANOVA followed by Dunnett’s test or Tukey’s test for multiple comparisons. Statistical significance was set at \( P < 0.05 \). **** indicates a \( P \)-value < 0.0001; *** indicates a \( P \)-value between 0.0001 to 0.001; ** indicates a \( P \)-value between 0.001 to 0.01; * indicates a \( P \)-value between 0.01 to 0.05; ns (not significant) indicates a \( P \)-value > 0.05. \( n \) numbers, as indicated in the figure legends and the main text, represent the numbers of cells. All data are presented as mean ± SEM, as indicated in the figure legends.

**Safety statement**

No unexpected or unusually high safety hazards were encountered.
**Figure S1**

ExRai-AktAR2 enables selective detection of Akt kinase activity. (a-b) Average time courses of normalized excitation ratio (Ex480/405) in serum-starved NIH3T3 cells expressing ExRai-AktAR (a) \( (n = 14) \) or ExRai-AktAR2 (b) \( (n = 24) \) treated with 50 ng/ml of PDGF. Separate analyses of cytosolic and nuclear regions of interest (ROIs) are shown. (c) Immunoblots showing that ExRai-AktAR2 is phosphorylated following the addition of PDGF. Serum-starved NIH3T3 cells were treated with 50 ng/ml PDGF for the indicated times. Data are representative of two independent experiments. (d) Average time courses of normalized excitation ratio (Ex480/405) in serum-starved NIH3T3 cells expressing ExRai-AktAR2 treated with 50 ng/ml of PDGF (green, \( n = 24 \) ), 100 ng/ml of PMA (blue, \( n = 8 \) ), or Fsk (50 µM) /IBMX (100 µM) (pink, \( n = 12 \) ). (e) Responses of ExRai-AktAR2, ExRai-AKAR2, and FRET-based CKAR2. From left to right: serum-starved NIH3T3 cells expressing ExRai-AktAR2 (green) were treated with PDGF (\( n = 13 \) ), PMA (\( n = 8 \)) or Fsk/IBMX (\( n = 12 \)); serum-starved NIH3T3 cells expressing ExRai-AKAR2 (pink) were treated with Fsk/IBMX (\( n = 19 \)); serum-starved NIH3T3 cells expressing FRET-based CKAR2 (blue) were treated with PMA (\( n = 7 \)). ****, \( P < 0.0001 \); ordinary one-way ANOVA followed by Dunnett's multiple comparison test. (f) Summary of responses of subcellularly targeted ExRai-AktAR2 to PDGF. From top to bottom, the responses of ExRai-AktAR2 to PDGF are 335 ± 22% (Cyto-ExRai-AktAR2, \( n = 20 \)), 130 ± 11% (PM-ExRai-AktAR2, \( n = 23 \)), 110 ± 15% (Nuc-ExRai-AktAR2, \( n = 13 \)), 173 ± 21% (Golgi-ExRai-AktAR2, \( n = 12 \)), and 73 ± 9.0% (Lyso-ExRai-AktAR2, \( n = 22 \)). Solid lines in a, b, and d indicate mean responses; shaded areas, SEM. Bars denote mean ± SEM (e, f).
Figure S2

ExRai-AktAR2 is insensitive to pH change. (a) pHRed reports intracellular pH in NIH3T3 cells. Average time courses of normalized excitation ratio (Ex572/420) in serum-starved NIH3T3 cells expressing pHRed permeabilized with 10 µM monensin treated with buffers at different pH values (n = 10 cells). Data are representative of three independent experiments. (b) ExRai-AktAR2 is insensitive to pH. Average time courses of normalized excitation ratio (Ex480/405) in serum-starved NIH3T3 cells expressing ExRai-AktAR2 permeabilized with 10 µM of monensin treated with buffers at different pH values, followed by stimulation with 50 ng/ml PDGF (n = 18 cells). Data are representative of three independent experiments. Solid lines in a, b indicate mean responses; shaded areas, SEM.
Figure S3. Single-cell traces of PM-ExRai-AktAR2 demonstrate cell heterogeneity, and ExRai-AktAR2 outperforms FRET-based AktAR2 in detecting Akt activities at various subcellular compartments. (a) Single-cell traces of PM-ExRai-AktAR2 revealed that there are distinct populations of cells showing different activity kinetics. This cell-to-cell heterogeneity\textsuperscript{6,7} was previously observed with other Akt activity reporters.\textsuperscript{8,9} (b) Average time courses of normalized emission ratio (Y/C) in serum-starved NIH3T3 cells expressing untargeted AktAR2 stimulated with PDGF (50 ng/ml) (n = 41). Curves are representative of and pooled from four experiments. (c) Average time courses of normalized emission ratio (Y/C) in serum-starved NIH3T3 cells expressing cytosolic AktAR2 (Cyto-AktAR2) stimulated with PDGF (50 ng/ml) (n = 21). Curves are representative of and pooled from five experiments. (d) Average time courses of normalized emission ratio (Y/C) in serum-starved NIH3T3 cells expressing plasma membrane-targeted AktAR2 (PM-AktAR2) stimulated with PDGF (50 ng/ml) (n = 20). Curves are representative of and pooled from three experiments. (e) Average time courses of normalized emission ratio (Y/C) in serum-starved NIH3T3 cells expressing nuclear AktAR2 (Nuc-AktAR2) stimulated with PDGF (50 ng/ml) (n = 18). Curves are representative of and pooled from four experiments. (f) Average time courses of normalized emission ratio (Y/C) in serum-starved NIH3T3 cells expressing Golgi-targeted AktAR2 (Golgi-AktAR2) stimulated with PDGF (50 ng/ml) (n =
20). Curves are representative of and pooled from four experiments. (g) Average time courses of normalized emission ratio (Y/C) in serum-starved NIH3T3 cells expressing lysosome-targeted AktAR2 (Lyso-AktAR2) stimulated with PDGF (50 ng/ml) (n = 25). Curves are representative of and pooled from four experiments. (h) Summary of responses of ExRai-AktAR2 and FRET-based AktAR2 to PDGF treatment in serum-starved NIH3T3 cells. From left to right, the responses of cytosolic AktAR2 (n = 21) and Cyto-ExRai-AktAR2 (n = 20) are 16 ± 1.1% and 334 ± 22%, respectively; the responses of plasma membrane-targeted AktAR2 (n = 20) and PM-ExRai-AktAR2 (n = 23) are 18 ± 1.1% and 130 ± 11%, respectively; the responses of nuclear AktAR2 (n = 18) and Nuc-ExRai-AktAR2 (n = 13) are 6.3 ± 0.6% and 110 ± 15%, respectively; the responses of Golgi-targeted AktAR2 (n = 20) and Golgi-ExRai-AktAR2 (n = 12) are 16 ± 1.7% and 173 ± 21%, respectively; and the responses of lysosome-targeted AktAR2 (n = 25) and Lyso-ExRai-AktAR2 (n = 22) are 7.3 ± 0.8% and 73 ± 9.0%, respectively. ****, P < 0.0001; unpaired two-tailed Student’s t-test. Solid lines in b-g indicate mean responses; shaded areas, SEM. Bars denote mean ± SEM (h).
Figure S4

(a) Measurement of macroscopic expansion ratio. Cells on cover glass (pre-expansion, diameter 18 mm) were expanded on a hydrogel (post-expansion, diameter 46 mm) showing the expansion ratio of 2.55 (46 mm/18 mm = 2.55).

(b) and (c) Measurement of microscopic expansion ratio. Images from the same region for pre-expanded (b) and post-expanded (c) NIH3T3 cells expressing a mitochondrial marker protein (DAKAP-GFP) are shown. Scale bars = 10 µm (yellow scale bar indicates post-expansion image with a physical size of 26.69 µm). The microscopic expansion ratio was calculated as 26.69/10 = 2.69.

(d) and (e) Line scans through mitochondria staining in pre-expanded regions (d) and in the corresponding post-expanded regions (e) reveal that the mitochondria structure separated in the post-expanded samples.
**Figure S5.** Validation of the Akt antibody. (a) Western blot analysis showing the knockout of Akt1/2 in HCT116 cells. Representative of four independent experiments. (b) Representative images of Akt immunostaining in wild-type (WT) or Akt1/2 double knockout (Akt KO) HCT116 cells. Representative of two independent experiments. Scale bars = 10 μm. (c) Quantification of mean intensity of Akt immunostaining per cell in WT and Akt KO cells. n = 157 and 323 cells for WT and Akt KO from two independent experiments, respectively. Bars denote mean ± SEM. ****, P < 0.0001; unpaired two-tailed Student’s t-test with Welch’s correction.
Figure S6. Non-expanded NIH3T3 cells fail to show the spatial correlation information between Akt and the lysosomes. (a) Confocal images of non-expanded, serum-starved NIH3T3 cells expressing LAMP1-GFP (green) and stained with total Akt (magenta). Bottom: Line scan showing spatial pattern between Akt (magenta) and the lysosome (green). (b) Confocal image of non-expanded, PDGF-treated (50 ng/ml of PDGF for 30 min) NIH3T3 cells expressing LAMP1-GFP (green) and stained with total Akt (magenta). Bottom: Line scan showing spatial pattern between Akt (magenta) and the lysosomes (green). (c) Quantification of Akt co-localization with the lysosomes without (gray) and with (red) PDGF stimulation by Pearson correlation coefficient (PCC, triangles) and the Mander's overlap coefficient (MOC, dots). *P*-values were determined by unpaired two-tailed Student’s t-test. PCC: - PDGF vs. + PDGF, ns, not significant, *P* = 0.4855; MOC: - PDGF vs. + PDGF, ns, not significant, *P* = 0.5114. Scale bars = 10 µm (a, b).
Figure S7. Proximity ligation assay (PLA) confirms that Akt is localized to the lysosome. (a) Representative epifluorescence images of in situ PLA in non-starved NIH3T3 cells. Left: mouse anti-LAMP1 and normal Rabbit IgG; Right: rabbit anti-Akt antibody and mouse anti-LAMP1 antibody. PLA puncta (red) were detected with epifluorescence imaging. Nuclei were stained in blue. Data are representative of three independent experiments. Scale bars = 10 µm. (b) Quantification of average puncta per cell from three randomly chosen regions with n = 40 and 36 cells for LAMP1/IgG and LAMP1/Akt, respectively. Data are representative of three independent experiments. *P*-value was determined by unpaired two-tailed Student’s t-test. Bars denote mean ± SEM.
Figure S8. Accumulation of 3-phosphoinositides at the plasma membrane and the lysosome upon PDGF stimulation. (a) Comparison of apparent half-time ($T_{1/2}$) of PM-InPAkt (n = 22) and Lyso-InPAkt (n = 29) from three independent experiments. **, $P = 0.0024$; unpaired two-tailed Student’s t-test with Welch's correction. (b) Analysis of Sustained Activity Metric at 15 min post-treatment (SAM15) in PDGF-treated serum-starved NIH3T3 cells expressing PM-InPAkt (n = 22) or Lyso-InPAkt (n = 29). The SAM15 for PM-InPAkt and Lyso-InPAkt are 0.63 ± 0.04 and 0.74 ± 0.03, respectively. *, $P = 0.0379$; unpaired two-tailed Student’s t-test. (c) Analysis of SAM15 in PDGF-treated serum-starved NIH3T3 cells expressing PM-InPAkt without (n = 22) or with pretreatment of 50 µM Dyngo-4a (n = 13), or with overexpression of DNM2/K44A (n = 21). The SAM15 for PM-InPAkt in cells without treatment, with expression of DNM2/K44A, or with Dyngo-4a pre-treatment are 0.68 ± 0.04, 0.88 ± 0.02, and 0.86 ± 0.04, respectively. ***, $P = 0.0008$, Control vs. Dyngo-4a; ****, $P < 0.0001$, Control vs. DNM2/K44A; ordinary one-way ANOVA followed by Tukey’s multiple comparison test. (d) Responses of Lyso-InPAkt in PDGF-treated serum-starved NIH3T3 cells without (5.0 ± 0.5%, n = 17) or with pretreatment of PIK-75 (1.1 ± 0.2%, n = 15). ****, $P < 0.0001$; unpaired two-tailed Student’s t-test with Welch’s correction. Bars denote mean ± SEM (a-d).
**Figure S9.** Endocytosis blockade suppresses the activities of Akt and mTORC1 at the lysosome. (a) Immunoblots showing that phosphorylation of T1462 in TSC2 by Akt was reduced by pretreating cells with Dyngo-4a. Serum-starved NIH3T3 cells were treated with PDGF (50 ng/ml) for 30 min without or with pretreatment of 50 µM of Dyngo-4a for 10 min. Data are representative of three independent experiments. (b) Quantification of reduction in phosphorylation of TSC2 (*, P = 0.037) and GSK3β (ns, not significant, P = 0.3387) by Dyngo-4a treatment in western blot experiments described in Figure S8a after normalization to the corresponding PDGF-induced phosphorylation in conditions without Dyngo-4a treatment. Data were representative of three independent experiments. P-values were determined by paired two-tailed student’s t-test. (c) Response of Lyso-ExRai-AktAR2 in PDGF-treated serum-starved NIH3T3 cells without (n = 18) or with expression of DNM2/K44A (n = 14). ***, P = 0.0004; unpaired two-tailed Student’s t-test. (d) Response of PM-ExRai-AktAR2 in PDGF-treated serum-starved NIH3T3 cells without (n = 5) or with expression of DNM2/K44A (n = 9). ns, not significant, P = 0.6481; unpaired two-tailed Student’s t-test. (e) Response of Lyso-TORCAR in PDGF-treated serum- and amino acid-starved NIH3T3 cells without (n = 19) or with expression of DNM2/K44A (n = 17). ***, P = 0.0003; unpaired two-tailed Student’s t-test. Bars denote mean ± SEM (b-e).
Figure S10. Lysosome-targeted lipid phosphatases perturb lysosomal Akt/mTORC1 signaling activities. (a) Representative images showing serum-starved NIH3T3 cells co-expressing Lyso-InPAkt (left) and Lyso-PTEN A4-mChe, Lyso-INPP4B-mChe, or Lyso-mChe (middle). (b) Representative images showing serum-starved NIH3T3 cells co-expressing Lyso-ExRai-AktAR2 (left) and Lyso-PTEN A4-mChe, Lyso-INPP4B-mChe, or Lyso-mChe (middle). (c) Domain structure of FRET-based lysosome-targeted AktAR2 (Lyso-AktAR2). (d) Responses of Lyso-AktAR2 in PDGF-treated serum-starved NIH3T3 cells without (10.3 ± 1.0%, n = 16) or with expression of Lyso-PTEN A4-mChe (PTEN, 5.8 ± 0.8%, n = 20), Lyso-INPP4B-mChe (INPP4B, 6.3 ± 0.7%, n = 14), and Lyso-mChe (mChe, 10.9 ± 0.8%, n = 17). Error bar represents mean ± SEM. **, P = 0.0015, Control vs. Lyso-PTEN A4-mChe; ***, P = 0.0002, Lyso-mChe vs. Lyso-PTEN A4-mChe; *, P = 0.0142, Control vs. Lyso-INPP4B-mChe; **, P = 0.0030, Lyso-mChe vs. Lyso-INPP4B-mChe; ns, not significant, P = 0.9600, Control vs. Lyso-mChe; Ordinary one-way ANOVA followed by Tukey’s multiple comparison test. (e) Representative images showing serum-starved NIH3T3 cells co-expressing Lyso-AktAR2 (left) and Lyso-PTEN A4-mChe, Lyso-INPP4B-mChe, or Lyso-mChe (middle). (f) Representative images showing serum-starved NIH3T3 cells co-expressing Lyso-TORCAR (left) and Lyso-PTEN A4-mChe, Lyso-INPP4B-mChe, or Lyso-mChe (middle). Bars denote mean ± SEM (d). Scale bars = 10 μm (a, b, e, f).
Figure S11. A model showing that lysosomal Akt/mTOR signaling activities are regulated by lysosomal 3-PIs. Upon growth factor stimulation, active PI3K leads to production of PI(3,4)P₂ and PIP₃, resulting in plasma membrane recruitment and activation of Akt. Throughdynamin-mediated endocytosis, PI(3,4)P₂ and PIP₃ accumulate at the lysosomes, which positively regulate Akt activity resulting in an increase in the lysosomal activity of mTORC1 via the TSC/Rheb axis. Inhibiting dynamin by either Dyngo-4a or dominant negative mutant (DNM2/K44A) suppresses the lysosomal accumulation of 3-PIs, inhibiting local Akt and mTORC1 activities.
**Figure S12.** Uncropped Western blots. Uncropped images of Western blots from Fig. S1c, S5a and S9a are shown, as well as the approximate extent of the cropped regions.
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