Supporting Information

Yudushkin and Vale 10.1073/pnas.1016388108

SI Text

Design of the Reporters. Binding of ZAP-70 to dually phosphorylated (but not mono- or unphosphorylated) immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 complex is mediated by its SH2 domains and results in allosteric activation of its kinase activity. Because of the low micromolar activity and high specificity of this interaction (1), we sought to use the ITAM-binding SH2 domains of ZAP-70 to construct fluorescence reporters of TcR/CD3 phosphorylation. Binding of SH2 domains to tyrosine-phosphorylated TcR/CD3 can be monitored either by recruitment of fluorescently tagged probe to the plasma membrane or by Förster's resonant energy transfer (FRET) between phosphorylated CD3 subunits and the probe, tagged with donor and acceptor fluorophores. Although intramolecular FRET in the conformation-sensitive reporter with fixed donor:acceptor stoichiometry (like ZIP) could be used to simultaneously monitor both the phosphorylation and trafficking of a specific receptor subunit, membrane translocation of the mCherry-$tSH2^{ZAP\text{-}70}$ allows probing of the rapid phosphorylation dynamics of the ITAM-containing subunits expressed endogenously. Similar strategies were used previously to monitor trafficking and phosphorylation dynamics of the epidermal growth factor receptor or focal adhesion kinase using PTB domain from Akt or two consecutive SH2 domains of Src, correspondingly (2, 3).

To monitor FRET, we used time-correlated single-photon counting fluorescence lifetime imaging microscopy (TCSPC-FLIM). FRET results in faster depopulation of the donor fluorophore (eGFP) and can be observed as a decreased fluorescence lifetime of eGFP. Fluorescence lifetime is an internal property of a fluorophore reflecting the net rates of the processes that depopulate the excited state. Because it does not depend on light path length, concentration of donor molecules, or unequal rates of photobleaching for donor and acceptor fluorophores, measuring fluorescence lifetime by FLIM provides a more robust and reproducible readout of FRET efficiency.

SI Materials and Methods

Antibodies. Purified mouse anti-human CD3ε HIT3 mAb and IgG2a, κ isotype control mAb were from BD Biosciences. Purified mouse anti-human CD3ζ (CD247) MCA1297T mAb was from AbD Serotec. Alexa Fluor 647-conjugated mouse anti-human $CD3\zeta^{pY142}$ clone K25-407.69 and mouse anti-human c-Src^{pY418} monoclonal antibodies were from BD PhosFlow. Purified mouse anti-human CD45 mAb was from BD Immunochemistry. Rabbit polyclonal antibodies against EEA1, LAMP1, CI-M6PR, GM130, TGN46, CHMP2B, and Rab11A as well as mouse anti-human c-Myc 9E10 mAb were from Abcam. Antibody against α-tubulin (DM1a) was from Sigma. Secondary Alexa Fluor 647-conjugated goat anti-rabbit or Alexa Fluor 633-conjugated goat anti-mouse antibodies were from Molecular Probes.

Plasmids. Mouse CD3ζ-eGFP construct was a gift from Dr. M. Krummel [University of California, San Francisco (UCSF)]. DsRed-tagged Rab5 and Rab11 constructs were a gift from Dr. R. Pagano (Mayo Clinic, Rochester, MN). Monomolecular reporter was generated by fusion of mouse CD3ζ-eGFP with mCherry-$tSH2^{ZAP\text{-}70}$ via 17-aa-long GPGSASGEGLPGSAGPG spacer and subcloned into eGFP/N1 plasmid (Invitrogen) at BsrGI and NotI restriction sites. For production of lentiviral particles, the reporter was subcloned into pHR' vector at MluI and NotI restriction sites, generated by PCR. Y→F and R37K/R190K point mutations were introduced using PCR. The identity of all constructs was verified by direct sequencing.

Cell Culture. Jurkat E6.1, J.CaM1, and P116 cell lines (gift of A. Weiss, UCSF) were maintained in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated FCS (Gibco), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. For microscopy, the cells were serum-starved for 2–4 h, rinsed with PBS and transferred into HBS. For immunofluorescence microscopy, the cells were fixed for 20 min at room temperature by 4% formaldehyde solution in PBS (pH 7.2), rinsed twice with PBS, formaldehyde quenched by incubation with 50 mM Tris·Cl (pH 7.5) in isotonic buffer, and incubated with the respective antibodies in 10% FCS/PBS containing 0.2% saponin.

Production of Lentiviral Particles and Cell Transformation. The vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped HIV1-derived viral particles were generated by FuGENE6-mediated transient cotransfection of HEK293T cells with second-generation packaging plasmid (pCMVΔ8.91), a plasmid encoding the VSV-G envelope proteins (pMD2.G) and a pHR'-derived transfer plasmid, containing the reporter. HEK293T cells were seeded in six-well plates at ≈0.5×10^6 cells/mL in 10% FCS-supplemented DMEM, containing 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The following day, the cells were co-transfected with equal amounts of the three plasmids (1.5 μg total DNA per well) and 4.5 μL/well FuGENE6 transfection reagent (Roche) according to the manufacturer's guidelines. Virus-containing supernatants were harvested 2 to 3 d after transfection, cellular debris was removed by centrifugation at $14{,}000 \times g$ for 10 min, and the supernatants were directly used for infection of the target Jurkat cells. For transient expression, the cells were transformed by electroporation using 10 μg DNA of the constructs used.

Cell Lysis and Immunoblot Analysis of the Lysates. Cells ($0.5–1 \times 10^6$) were lysed in 100 μL lysis buffer, containing 50 mM Tris·Cl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM PMSF, PhosStop, and EDTA-free CompleteMINI phosphatase and protease inhibitors mixtures (Roche) for 1 h at 4 °C. The cell debris was removed by centrifugation at $14{,}000 \times g$ for 10 min, and the cleared cell lysate fractions were used for SDS/PAGE using 4–12% Bis-Tris gradient precast gel (Invitrogen). Separated proteins were electroblotted onto nitrocellulose membrane, and immune complexes were visualized using HRP-conjugated secondary antibodies and an ECL immunoblotting detection system (GE Healthcare).

Image Acquisition and Analysis. eGFP-, mCherry-, and Alexa633/Alexa647 fluorescence was excited at 488, 543, and 633 nm lines of Ar- or HeNe lasers and detected using HFT488/HQ510-560, HFT543/BP565-615IR, or -LP560 and HFT UV/488/543/633/HQ650-700 dichroic beamsplitter/filter combinations, correspondingly. Contrast enhancement and confocal image overlays were performed using ImageJ v. 1.37 software, equally applied across the entire image. No nonlinear adjustments were used.

1. Isakov N, et al. (1995) ZAP-70 binding specificity to T cell receptor tyrosine-based activation motifs: The tandem SH2 domains of ZAP-70 bind distinct tyrosine-based activation motifs with varying affinity. *J Exp Med* 181:375–380.
2. Kirchner J, Kam Z, Tzur G, Bershadsky AD, Geiger B (2003) Live-cell monitoring of tyrosine phosphorylation in focal adhesions following microtubule disruption. *J Cell Sci* 116:975–986.
3. Offterdinger M, Georget V, Girod A, Bastiaens PI (2004) Imaging phosphorylation dynamics of the epidermal growth factor receptor. *J Biol Chem* 279: 36972–36981.

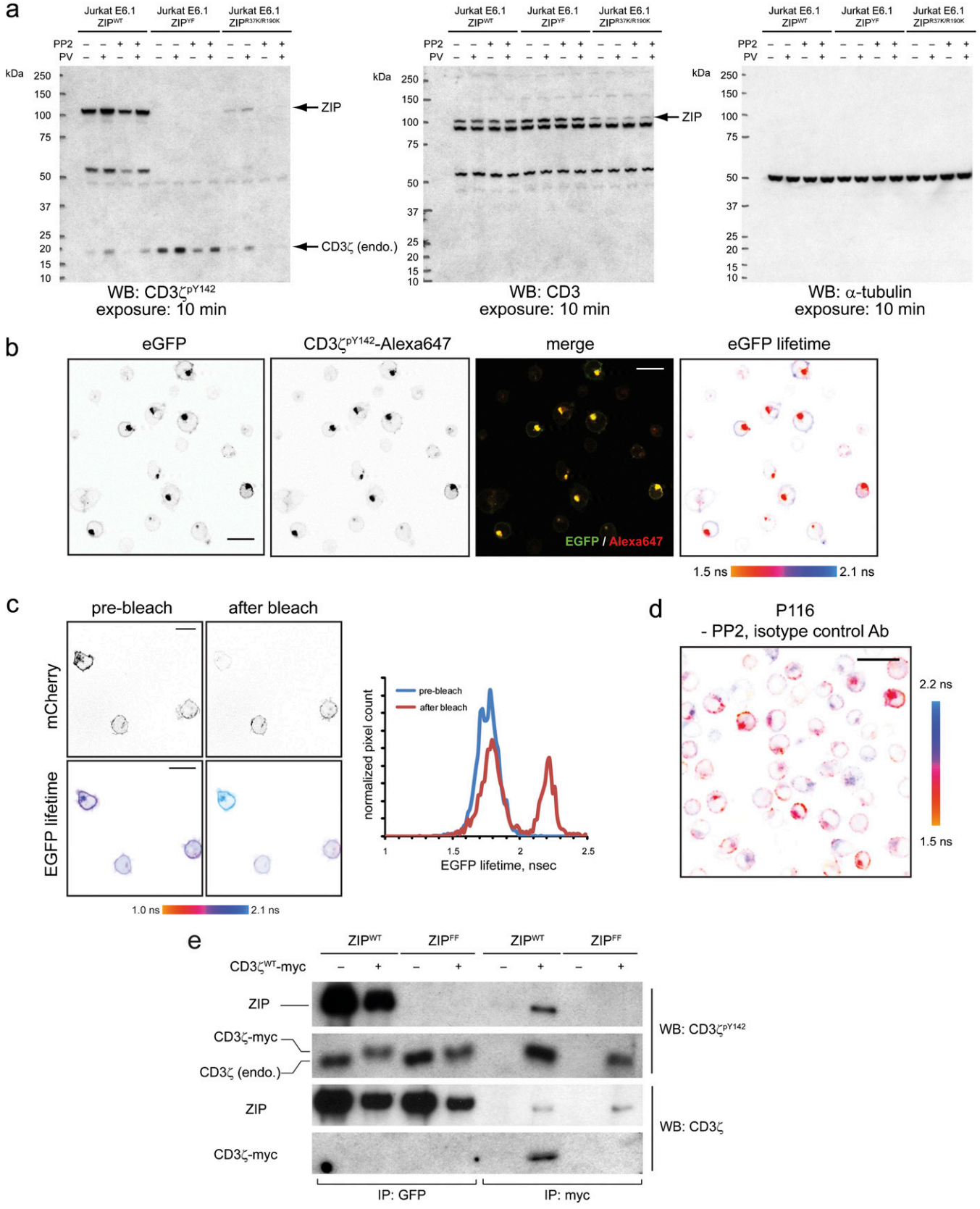

Fig. S1. (*A*) Full-length Western blots for Fig. 1*B* in the main text. (*B*) FRET signal corresponds to tyrosine-phosphorylated ZIP reporter. ZIP^{WT} reporter-expressing Jurkat E6.1 cells were incubated on a glass-bottom dish precoated with 20 μg/mL isotype control antibody for 20 min, fixed, permeabilized, and immunostained using Alexa647-conjugated CD3ζ^{pY142}-specific mouse antibody. Shown are confocal images of the ZIP^{WT} reporter in the eGFP channel, antibody staining in the far-red channel, and the confocal overlay of these channels. The right-most panel shows the corresponding eGFP lifetime image, pseudocolored between 1.5 and 2.1 ns. (Scale bar, 20 μm.) (*C*) Jurkat E6.1 cells transiently expressing ZIP^{YF} mutant of the reporter were allowed to adhere onto a glass-bottom

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dish coated with poly-D-lysine, and eGFP fluorescence lifetime before and after acceptor (mCherry) photobleaching in the top left cell was monitored using FLIM. Shown are the the confocal images in the mCherry channel and the corresponding pseudocolored eGFP fluorescence lifetime images (scaled between 1.0 and 2.1 ns) before and after acceptor photobleaching. (Scale bar, 10 μm.) *Right:* Corresponding cumulative eGFP lifetime histogram. (*D*) ZAP-70-deficient P116 Jurkat cells stably expressing the ZIP^{WT} reporter were adhered onto a glass-bottom dish precoated with 20 μg/mL isotype control antibody, fixed, and eGFP lifetime monitored using TCSPC-FLIM. Shown are the pseudocolored images of the eGFP fluorescence lifetime, scaled between 1.5 and 2.2 ns. (Scale bar, 20 μm.) (*E*) Jurkat E6.1 cells stably expressing ZIP^{WT} or ZIP^{YF} were infected with lentiviral stocks containing CD3ζ-myc construct, grown for several days, and lysed. The lysates were used for coimmunoprecipitation using either anti-GFP or anti-myc antibodies coupled to agarose beads. The beads were boiled for 5 min in sample buffer, and the bound proteins were separated by SDS/PAGE using 4–12% Bis-Tris gradient precast gel. The membranes were probed using antibodies against $CD3ζ^{pY142}$ and pan-CD3ζ. Although we could detect phosphorylation of the endogenous CD3ζ in the immunoprecipitates, it constituted only a minor fraction of the total protein. Conversely, only a minute fraction of ZIP reporter coimmunoprecipitated with CD3ζ-myc. The results suggest that although in cells ZIP could in principle form heterodimers with endogenous CD3ζ, upon overexpression the reporter is more likely to form a (disulfide-linked) homodimer.

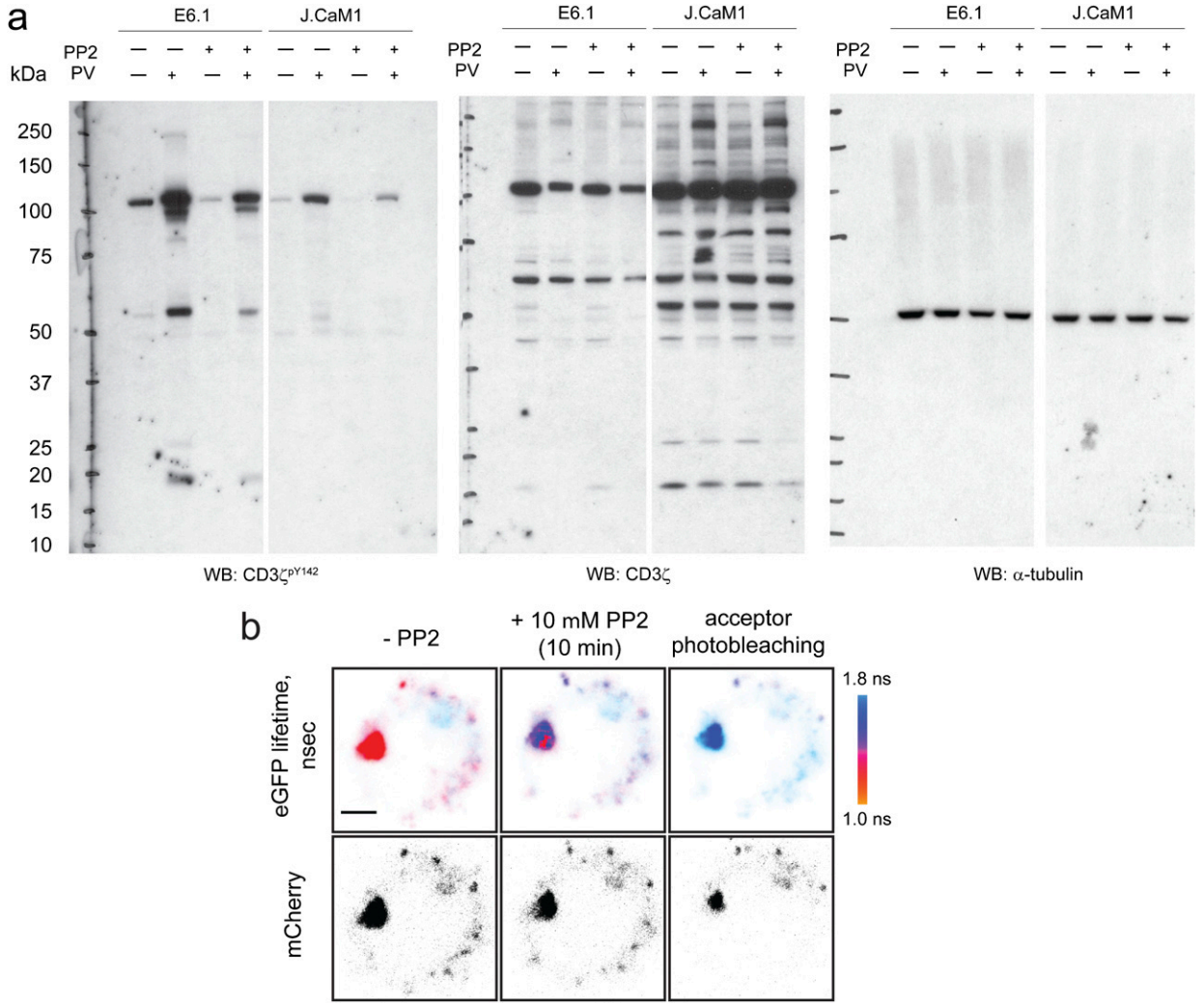

Fig. S2. (*A*) Full-length Western blot for Fig. 3*C* in the main text. (*B*) Jurkat E6.1 cell transiently expressing the ZIP^{WT} reporter was allowed to adhere onto a glass-bottom dish coated with poly-D-lysine, and eGFP lifetime before and after addition of 10 μM PP2 was monitored using TCSPC-FLIM. Shown are pseudocolored eGFP lifetime images scaled between 1.5 and 2.2 ns. (Scale bar, 2 μm.)

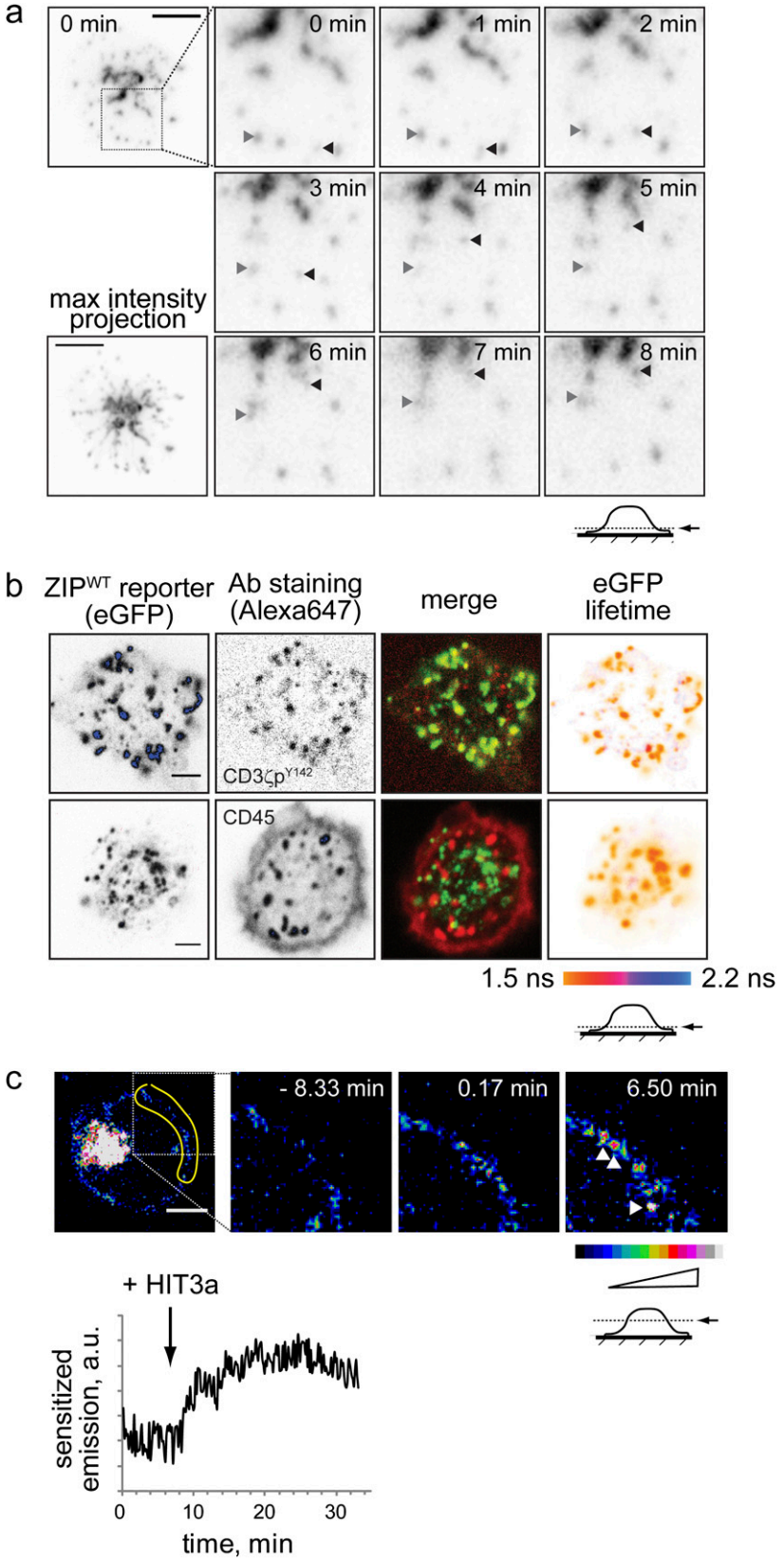

Fig. S3. (*A*) Wild-type Jurkat E6.1 cell transiently expressing mCherry-tSH2$^{ZAP\text{-}70}$ was allowed to adhere onto a supported lipid bilayer, functionalized with adhesion molecules ICAM-1 and anti-CD3 mAb HIT3a. Reporter-containing membrane microclusters were imaged using TIRF microscopy (excitation 561 nm; 100×/NA 1.45 oil immersion objective; NF01-405/488/561/633 triple dichroic mirror) at 1-s intervals for ≈15 min. Microclusters coalesced into a central supramolecular activation cluster-like structure within 5–10 min after contact with the bilayer. Movement of the reporter-containing microclusters (arrowheads) and the maximum-intensity projection over ≈15 min are shown. Full time-lapse sequence is presented in Movie S2. (Scale bars, 5 μm.) (*B*) Wild-type Jurkat E6.1 cells expressing the ZIP^{WT} reporter were allowed to adhere for 5 min onto glass-bottom dishes precoated with 10 μg/mL HIT3 mAb, fixed, and immunostained using

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CD3ζ^{pY142}-Alexa647- or mouse anti-CD45/goat anti-mouse Alexa633-conjugated antibodies. eGFP fluorescence was monitored using TCSPC-FLIM and presented as pseudocolored images, scaled between 1.5 and 2.2 ns. Confocal and FLIM images were acquired at the basal cell membrane, close to the bottom of the dish. (Scale bars, 2 μm.) (*C*) Wild-type Jurkat E6.1 cells expressing the ZIP^{WT} reporter were allowed to adhere onto a glass-bottom dish, and sensitized emission from the acceptor was imaged as described in *Materials and Methods* (main text) before and after addition of 50 μg/mL mAb HIT3a. Note high signal in the perinuclear endosomal compartment and increased signal at the membrane and membrane-proximal endosomes (*arrowheads*) upon stimulation. The plot shows increased sensitized emission from the membrane region (*yellow*). Full time-lapse sequence is presented in Movie S7. (Scale bar, 2 μm.)

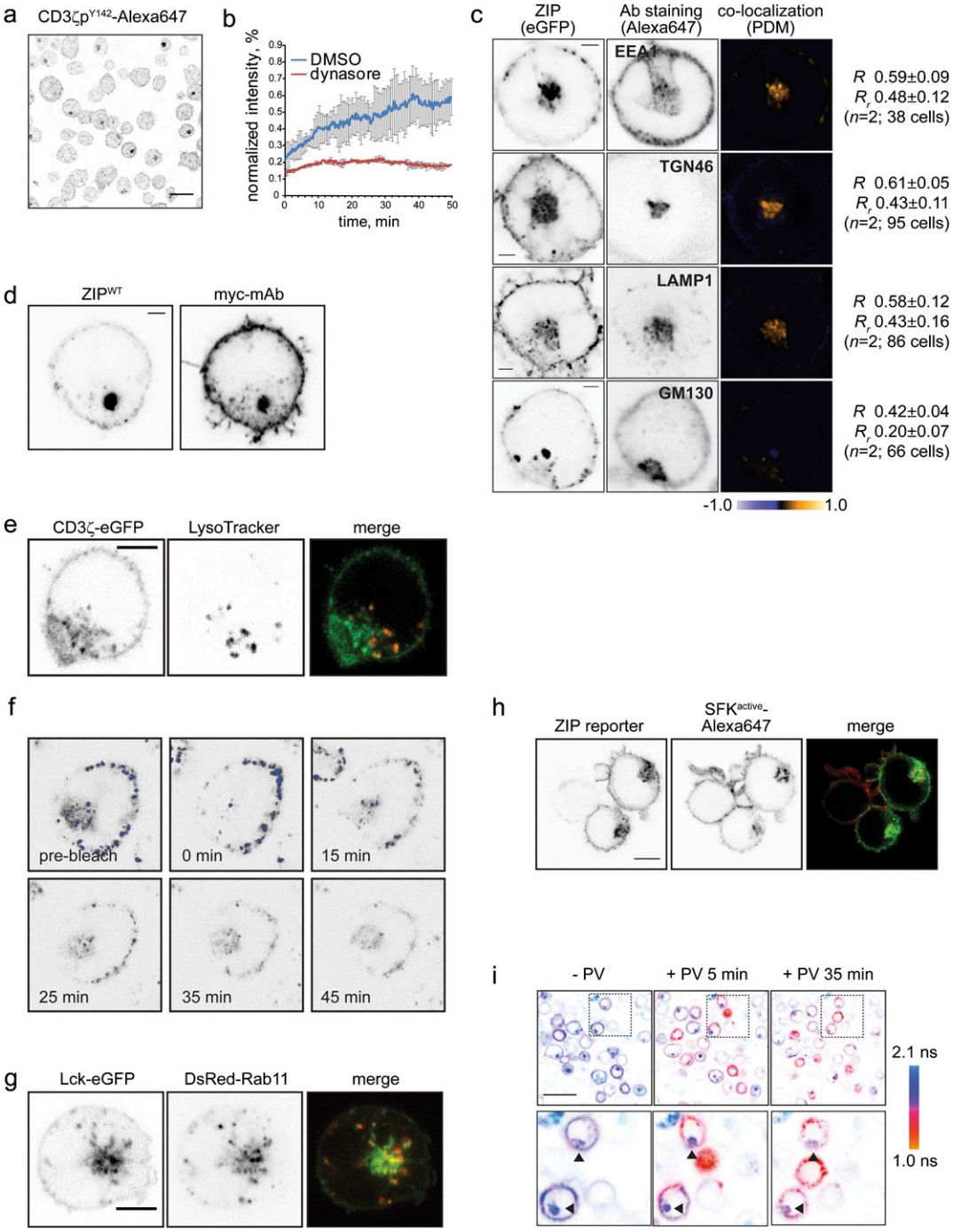

Fig. S4. (*A*) Untransformed wild-type Jurkat E6.1 cells were allowed to adhere onto a glass-bottom dish coated with poly-D-lysine, fixed, immunostained using Alexa647-conjugated mouse CD3ζ^{pY142} antibody, and imaged by confocal microscopy. (Scale bar, 20 μm.) (*B*) Wild-type Jurkat E6.1 cells stably expressing the ZIP^{WT} reporter were preincubated for 10 min with either 80 μM dynasore or the vehicle (DMSO) and adhered onto a poly-D-lysine–coated glass-bottom dish for 10 min. mCherry in the perinuclear endosomal compartment was photobleached, and the rate of mCherry fluorescence recovery was observed. Shown is the mean ± SD of the mCherry fluorescence in the bleached region, normalized to the prebleach intensity. n = 3 (5 cells). (*C*) Jurkat E6.1 cells expressing the ZIP^{WT} reporter were allowed to adhere onto poly-D-lysine–coated glass-bottom dishes, fixed, and immunostained with antibodies against EEA1, TGN46, LAMP1, and GM130. Colocalization of the reporter with the antibody staining is shown as the pseudocolored PDM (product of differences of the mean) images, scaled between −1 and 1, as well as overlap coefficient R and Mander's R_r coefficients averaged over several regions of interest. (Scale bars, 2 μm.) (*D*) Jurkat E6.1 cells

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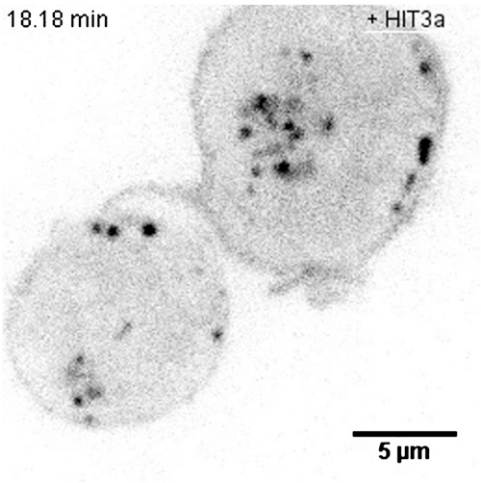
transiently coexpressing the ZIP^{WT} reporter and myc-tagged human CD3ζ were allowed to adhere onto a glass coverslip coated with poly-L-lysine, fixed, and immunostained using mouse anti-human c-myc mAb and Alexa633-conjugated goat anti-mouse secondary antibody. Shown are the confocal images of the reporter in the eGFP channel and anti-myc antibody staining in the far-red channel. (Scale bar, 2 μm.) (*E*) Wild-type Jurkat E6.1 cell transiently expressing CD3ζ-eGFP was preloaded with 0.1 μM LysoTracker Red for 30 min and adhered onto a poly-D-lysine–coated glass-bottom dish for 10 min. Shown are the confocal images and an overlay in the eGFP and DsRed channels at the midcell level. Time-lapse sequence before and after cell activation with 1 mM sodium pervanadate is presented in Movie S5. (Scale bar, 2 μm.) (*F*) Supplementary panel for Fig. 4*C* (main text) demonstrating mCherry fluorescence recovery after photobleaching in the perinuclear endosomal compartment. Saturated pixels shown in blue. (*G*) Wild-type Jurkat E6.1 cells transiently coexpressing Lck-eGFP and DsRed-Rab11 were adhered onto a poly-D-lysine–coated glass-bottom dish. Shown are the confocal images and an overlay in the eGFP or DsRed channels at the midcell level, so that intracellular vesicles are clearly seen. (Scale bar, 5 μm.) (*H*) Wild-type Jurkat E6.1 cells stably expressing the ZIP^{WT} reporter were allowed to adhere onto a glass-bottom dish, precoated with mAb HIT3a, fixed, and immunostained using Alexa647-conjugated mAb against Src^{pY418}. Shown are the confocal images and an overlay of the reporter and the mAb staining. (Scale bar, 5 μm.) (*I*) Supplementary panel for Fig. 5*F* demonstrating internalization of the tyrosine-phosphorylated ZIP^{WT} from the plasma membrane 35 min after stimulation with pervanadate in Lck-deficient J.CaM1 cells. *Lower:* Zoomed-in view of the frames above.

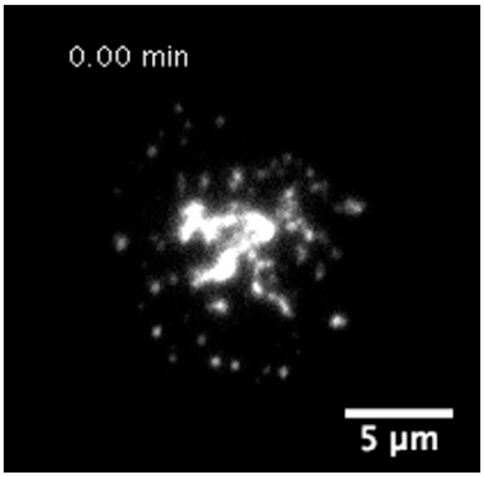
Table S1. Equipment and settings

Fig. ID	Acquisition settings	Image analysis and enhancement settings	Comments
Fig. 1			
1*C* (eGFP)	Ex: 488 nm; 5.1% HFT488/HQ510-560 512 × 512 (21.8 × 21.8 μm) 8-bit	Min..Max: 0–255 (both prebleach and postbleach)	Inverted LUT
1*C* (mCherry)	Ex: 543 nm; 10% HFT543/BP565-615 512 × 512 (21.8 × 21.8 μm) 8-bit	Min..Max: 0–255 (both prebleach and postbleach)	Inverted LUT
1*C* (FLIM)	Ex: 476 nm; ND 0% HFT488; BP525/50 256 × 256 (21.8 × 21.8 μm) 8-bit ADC	Model: incomplete double exponential decay; constrained for 300–7000 ps lifetime range; binning 4; threshold 15 Min..Max: 0–255 (both prebleach and postbleach)	Scaled 1.5–2.2 ns; pseudocolored
Fig. 2			
2*B*	Ex: 488 nm; exp: 250 ms 16-bit	Min..Max: 100–250 8 bit	Changed bit depth; inverted LUT EM-CCD camera C9100-13 (Hamamatsu Photonics) and a spinning disk confocal scan head CSU10 (Yokogawa)
2*C* (eGFP), all panels	Ex: 488 nm; 1.1% HFT488/HQ510-560 512 × 512 (17.0 × 17.0 μm) 8-bit	Min..Max: 0–255	Inverted LUT; saturated pixels highlighted blue
2*C* (mCherry), all panels	Ex: 543 nm; 5.1% HFT543/LP560 512 × 512 (17.0 × 17.0 μm) 8-bit	Min..Max: 145–245	Inverted LUT; saturated pixels highlighted blue
2*C* (FLIM), all panels	Ex: 476 nm; ND 10% HFT488; BP525/50 256 × 256 (17.0 × 17.0 μm) 8-bit ADC	Model: incomplete double exponential decay; constrained for 300–7,000 ps lifetime range; binning 4; threshold 15 Min..Max: 0–255	Scaled 1.8–2.7 ns; pseudocolored
Fig. 3			
3*A* (FLIM), all panels	Ex: 476 nm; ND 0% HFT488; BP525/50 256 × 256 (142.9 × 142.9 μm) 8-bit ADC	Model: incomplete double exponential decay; constrained for 300–7,000 ps lifetime range; binning 4; threshold 25 Min..Max: 100–255	Scaled 1.5–2.2 ns; pseudocolored
Fig. 4			
4a (eGFP), *Upper*	Ex: 488 nm; exp: 250 ms 16-bit	Min..Max: 70–251 8-bit	Changed bit depth; inverted LUT EM-CCD camera C9100-13 (Hamamatsu Photonics) and a spinning disk confocal scan head CSU10 (Yokogawa)
4*A* (DsRed), *Upper*	Ex: 568 nm; exp: 500 ms 16-bit	Min..Max: 68–252 8-bit	Same
4*A* (eGFP), *Lower*	Ex: 488 nm; exp: 250 ms 16-bit	Min..Max: 110–253 8-bit	Same
4*A* (DsRed), *Lower*	Ex: 568 nm; exp: 500 ms 16-bit	Min..Max: 66–253 8-bit	Same
4*B* (eGFP)	Ex: 488 nm; 8.1% HFT488/HQ510-560 512 × 512 (14.4 × 14.4 μm) 8-bit	Min..Max: 50–235	Inverted LUT
4*B* (Alexa647)	Ex: 633 nm; 1.1% HFT UV/488/543/633 NFT545/HQ655-697 512 × 512 (14.4 × 14.4 μm) 8-bit	Min..Max: 0–245	Inverted LUT; saturated pixels highlighted blue
4*B* (FLIM)	Ex: 476 nm; ND 0% HFT488; BP525/50 256 × 256 (14.4 × 14.4 μm) 8-bit ADC	Model: incomplete double exponential decay; constrained for 300–7,000 ps lifetime range; binning 4; threshold 15 Min..Max: 155–255	Scaled 1.0–2.1 ns; pseudocolored
4*C* (FLIM), all panels	Ex: 476 nm; ND 50% HFT488; BP525/50 256 × 256 (20.6 × 20.6 μm) 8-bit ADC	Model: incomplete double exponential decay; constrained for 300–7,000 ps lifetime range; binning 4; threshold 15 Min..Max: 100–255	Scaled 1.3–2.3 ns; pseudocolored
4*D* (FLIM), all panels	Ex: 476 nm; ND 33% HFT488; BP525/50 256 × 256 (90.0 × 90.0μm) 8-bit ADC	Model: incomplete double exponential decay; constrained for 300–7,000 ps lifetime range; binning 4; threshold 15 Min..Max: 100–255	Scaled 1.0–2.1 ns; pseudocolored



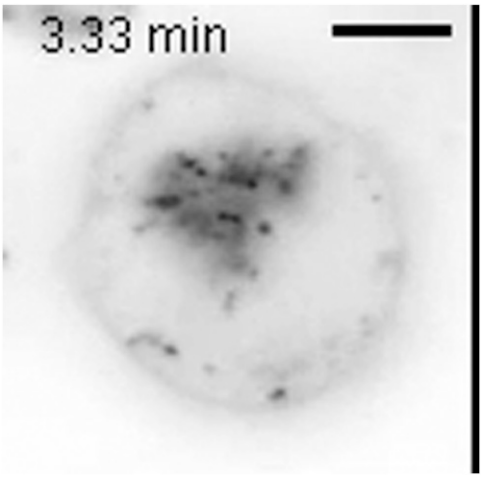
Movie S1. Jurkat E6.1 cells stably expressing mCherry-tSH2$^{ZAP\text{-}70}$ were allowed to adhere onto a glass-bottom dish coated with poly-D-lysine. Dynamics of the mCherry-tSH2$^{ZAP\text{-}70}$ before and after addition of 20 μg/mL HIT3a anti-CD3 mAb was monitored using a spinning disk confocal microscope (excitation 488 and 568 nm; 100×/NA1.4 oil immersion objective) at 5-s intervals for ≈20 min. Note the reporter-containing endosomal vesicles and antibody-induced translocation of the reporter to the plasma membrane. Movie playback: 25 fps. (Scale bar, 5 μm.)

Movie S1



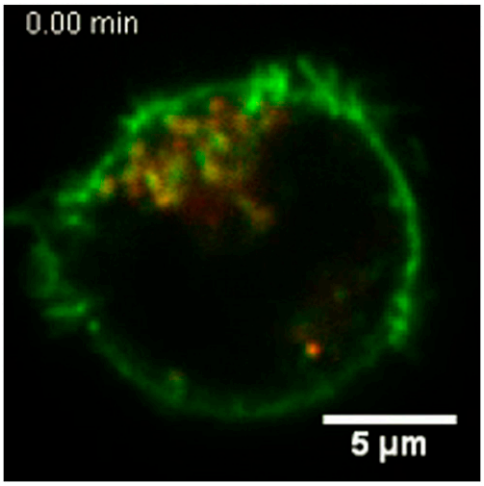
Movie S2. Jurkat E6.1 cell transiently expressing mCherry-tSH2$^{ZAP\text{-}70}$ was allowed to adhere onto a supporter lipid bilayer, functionalized with adhesion molecule ICAM-1 and anti-CD3ε mAb HIT3a. Reporter-containing plasma membrane microclusters were imaged using TIRF microscopy (excitation 561 nm; 100×/NA1.45 oil immersion objective; NF01-405/488/561/633 triple dichroic mirror) at 1-s intervals for ≈15 min. Microclusters coalesced into a central supramolecular activation cluster-like structure within 5–10 min after contact with the bilayer. Movie playback: 30 frames per second. (Scale bar, 5 μm.)

Movie S2



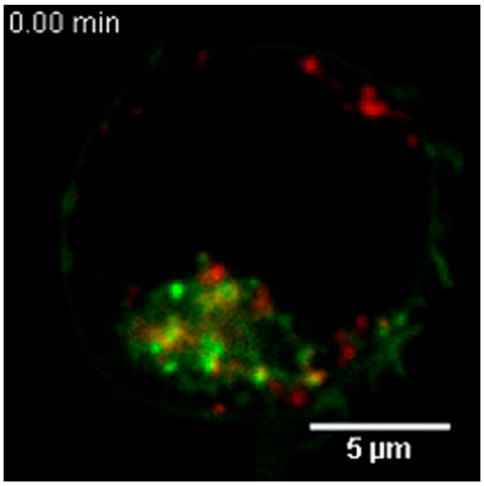
Movie S3. Jurkat E6.1 cells stably expressing the ZIP^{WT} reporter were allowed to adhere onto a glass-bottom dish coated with poly-D-lysine. Dynamics of the reporter was monitored in the mCherry channel using a wide-field fluorescence microscopy (excitation 488 nm, 40×/NA1.2 oil-immersion objective) at 5-s internals for ≈23 min. Movie playback: 25 frames per second. (Scale bar, 2 μm.)

Movie S3



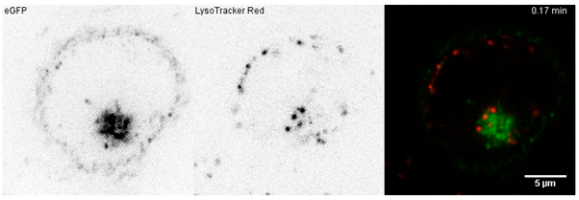
Movie S4. Jurkat E6.1 cells transiently coexpressing mouse CD3ζ-eGFP (green) and DsRed-Rab5 (red) were allowed to adhere onto a glass-bottom dish coated with poly-D-lysine. Dynamics of the CD3ζ- and Rab5-containing vesicles was monitored using spinning-disk confocal microscope (excitation 488 and 568 nm, respectively; 100×/NA1.4 oil-immersion objective) at 5-s intervals for ≈16 min. A subpopulation of internalized CD3ζ is concentrated on Rab5-containing early endosomes. Movie playback: 20 frames per second. (Scale bar, 5 μm.)

Movie S4



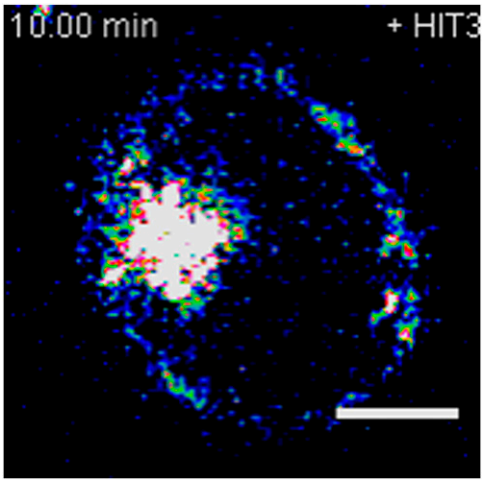
Movie S5. Jurkat E6.1 cells transiently coexpressing mouse CD3ζ-eGFP (green) and DsRed-Rab11 (red) were allowed to adhere onto a glass coverslip coated with poly-L-lysine. Dynamics of the CD3ζ- and Rab11-containing vesicles was monitored using spinning-disk confocal microscope (excitation 488 and 568 nm, respectively; 100×/NA1.4 oil-immersion objective) at 5-s intervals for ≈6 min. A subpopulation of internalized CD3ζ is concentrated on Rab11-containing recycling endosomes. Movie playback: 20 frames per second. (Scale bar, 5 μm.)

Movie S5



Movie S6. Jurkat E6.1 cells transiently expressing CD3ζ-eGFP (green) and preloaded for 1 h at 37 °C with 0.1 μM LysoTracker Red (Molecular Probes) were allowed to adhere onto a glass coversip precoated with poly-L-lysine. Dynamics of the CD3ζ- and LysoTracker-containing vesicles was monitored using spinning-disk confocal microscope (excitation 488 and 568 nm, respectively; 100×/NA1.4 oil-immersion objective) at 5-s intervals for ≈20 min. Sodium pervanadate (1 mM) increased colocalization of internalized CD3ζ with lysosomes and increased apparent internalization of CD3ζ. Movie playback: 25 frames per second. (Scale bar, 5 μm.)

Movie S6



Movie S7. Jurkat E6.1 cell stably expressing ZIP^{WT} was allowed to adhere onto a poly-D-lysine–coated glass-bottom dish. The dynamics of sensitized emission from the acceptor was monitored using a spinning-disk confocal microscope (excitation 488 and 568 nm, emission filters HQ530/60M and HQ605/75M; 100×/NA1.4 oil-immersion objective) at 10-s intervals for ≈30 min. Shown is pseudocolored apparent sensitized emission calculated as described in *Materials and Methods* (main text). Anti-CD3 mAb HIT3a (50 μg/mL) increased sensitized emission from the plasma membrane and induced formation of membrane-proximal vesicles containing ZIP^{WT}. Movie playback: 25 frames per second. (Scale bar, 2 μm.)

Movie S7