Ebner et al., https://doi.org/10.1083/jcb.201610060

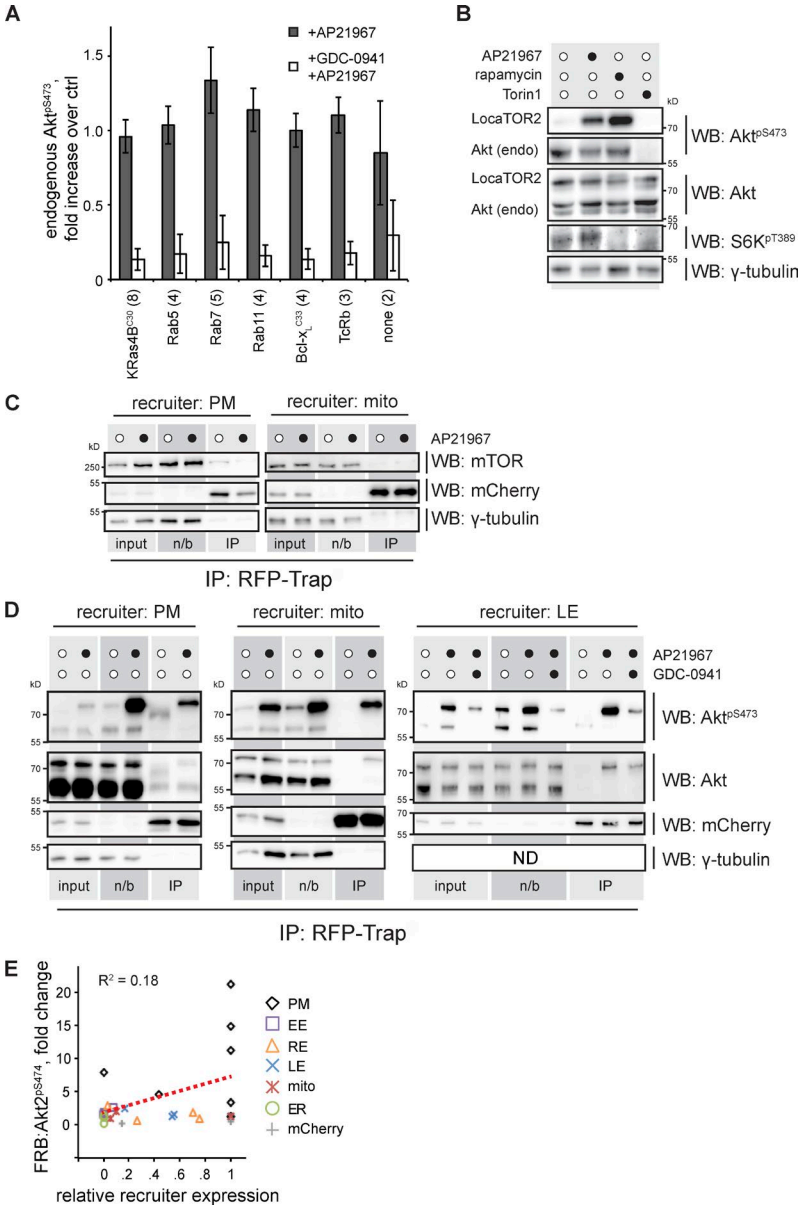


Figure S1. **WBs demonstrating no effect of AP21967 on endogenous Akt, S6K, and mTOR, and the independence of LocaTOR2 phosphorylation on the expression level of the recruiter constructs is shown.** (A) Phosphorylation of endogenous Akt is insensitive to AP21967 but depends on PI3K activity. HEK293T cells were cotransfected with LocaTOR2 and the corresponding recruiter constructs for 18 h and then preincubated for 30 min with 500 nM GDC-0941 or the vehicle and treated with 250 nM AP21967 for 40 min. The cells were lysed, and the level of pSer473-phosphorylated endogenous Akt was analyzed by quantitative WBs and normalized against total endogenous Akt. The data are presented as fold increase over untreated vehicle control averaged over several independent experiments (shown in parentheses); data are presented as means ± SEM. ctrl, control. (B) Phosphorylation of S6K is insensitive to AP21967. HEK293T cells were transiently cotransfected with LocaTOR2 and plasma membrane (PM) recruiter construct and then treated with 250 nM AP21967, 250 nM rapamycin, 250 nM Torin1, or the vehicle for 40 min. The cells were lysed, and phosphorylation of LocaTOR2, endogenous Akt, and S6K was analyzed using PAGE/WBs. endo, endogenously expressed Akt. (C) AP21967 does not recruit endogenous mTOR. HEK293T cells were transiently transfected with the indicated mCherry-tagged recruiter constructs for 18 h and then treated with 250 nM AP21967 for 40 min. The cells were lysed, and mCherry-tagged proteins were pulled down using RFP-Trap beads. Total, unbound, and immunoprecipitated (IP) fractions were analyzed using PAGE/WBs. (D) LocaTOR2 phosphorylation is induced by AP21967-induced recruitment to the plasma membrane, outer mitochondrial membrane (mito), and late endosomal membrane (LE). HEK293T cells were cotransfected with LocaTOR2 and the indicated mCherry-tagged recruiter constructs for 18 h, serum starved overnight, and then treated with 250 nM AP21967 for 40 min. The cells were lysed, and mCherry-tagged proteins were pulled down using RFP-Trap beads. Phosphorylation of LocaTOR2 in the total, unbound, and immunoprecipitate fractions was determined using PAGE/WBs with the indicated antibodies. n/b, nonbound. (E) LocaTOR2 phosphorylation does not depend on the relative expression of the recruiter construct. Shown are the cumulative data from seven experiments using various recruiter constructs. The relative expression of the recruiter was determined by scaling (between 0 and 1) the cumulative mCherry antibody intensity from various recruiter constructs loaded onto the same gel. EE, early endosome; RE, recycling endosome.

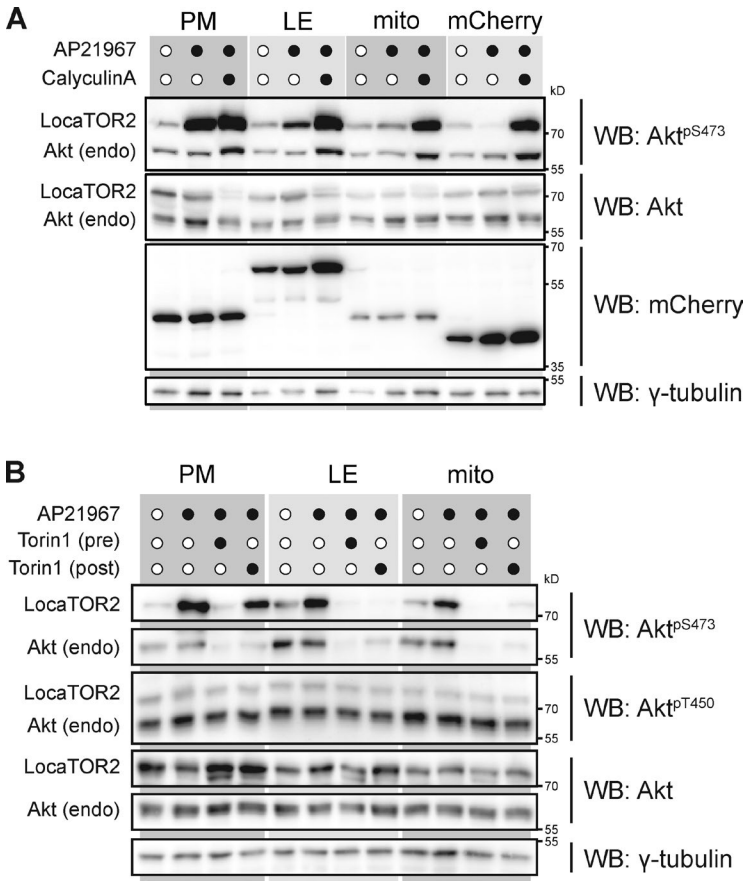


Figure S2. **WBs demonstrating that membrane recruitment does not protect LocaTOR2 from dephosphorylation and has no effect on Thr450 phosphorylation.** (A) Membrane recruitment does not protect LocaTOR2 from dephosphorylation by cytosolic phosphatases. HEK293T cells were cotransfected with LocaTOR2 and the indicated recruiter constructs for 18 h and then treated with the vehicle or with 250 nM AP21967 for 40 min. After AP21967, the cells were treated with 50 nM calyculin A for 15 min. (B) Phosphorylation of Akt turn motif (Thr450) is insensitive to AP21967. HEK293T cells were transiently cotransfected with LocaTOR2 and the indicated recruiter constructs, serum starved for 18 h, and incubated with 250 nM Torin1 for 5 min before or after treatment with 250 nM AP21967. (A and B) The cells were lysed, and the level of LocaTOR2 phosphorylation was determined by PAGE/WBs. endo, endogenously expressed Akt; LE, late endosome; mito, outer mitochondrial membrane; PM, plasma membrane.

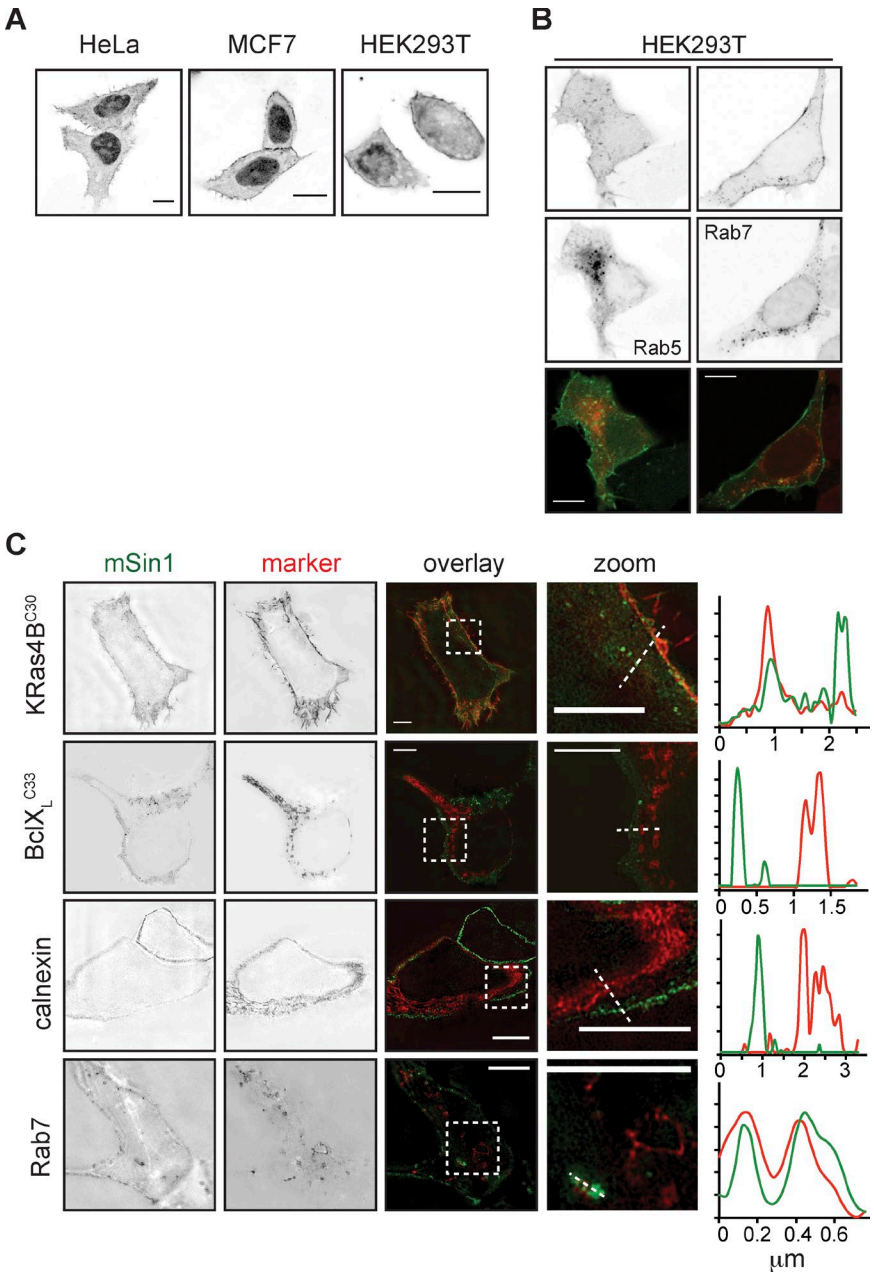
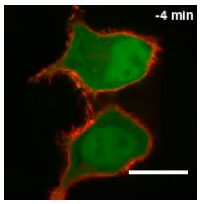
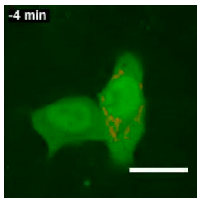


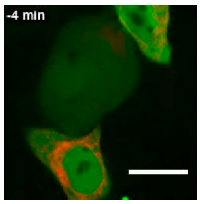
Figure S3. **Colocalization analysis of mSin1-GFP and various membrane markers using SIM.** (A) mSin1.1-GFP was transiently expressed in HeLa, MCF7, and HEK293T cells, and the cells were fixed and imaged using an LSM700 confocal microscope. (B) HEK293T cells were transiently cotransfected with mSin1.2^{WT}-GFP and mCherry-tagged Rab5 and Rab7. The cells were fixed and imaged using an LSM710 confocal microscope. (A and B) Bars, 10 µm. (C) HEK293T cells were transiently cotransfected with mSin1.2^{WT}-GFP and the indicated mCherry-tagged compartment markers. The cells were fixed, permeabilized, stained with Atto488-conjugated anti-GFP scAb, washed, and mounted using Mowiol mounting medium. 3D SIM images were acquired using an OMX system as described in the Image acquisition and analysis section of Materials and methods. Intensity profiles across the white dotted lines in both channels are indicated. Bars, 5 µm for both full images and insets.



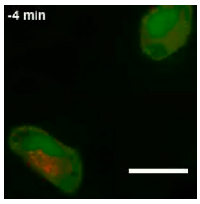
Video 1. **AP21967-induced recruitment of the LocaTOR2 probe to the plasma membrane.** HEK293T cells were transiently cotransfected with FRB:Akt2-citrine (Akt; green channel in the overlay) and the FKBP:mCherry-KRas4B^{C30} plasma membrane recruiter construct (red channel in the overlay). The cells were treated with 250 nM AP21967 for the time indicated. Shown are individual channels and channel overlay. The playback rate is 15 frames per second. Bar, 10 µm.



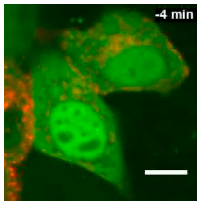
Video 2. **AP21967-induced recruitment of the LocaTOR2 probe to the outer mitochondrial membranes.** HEK293T cells were transiently cotransfected with FRB:Akt2-citrine (Akt; green channel in the overlay) and the FKBP:mCherry-Bcl-X_L^{C33} mitochondrial recruiter construct (red channel in the overlay). The cells were treated with 250 nM AP21967 for the time indicated. Shown are individual channels and channel overlay. The playback rate is 15 frames per second. Bar, 10 µm.



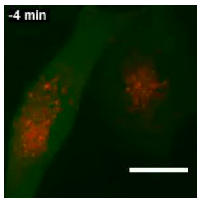
Video 3. **AP21967-induced recruitment of the LocaTOR2 probe to the ER membranes.** HEK293T cells were transiently cotransfected with FRB:Akt2-citrine (Akt; green channel in the overlay) and the TcR-β–mCherry-FKBP ER recruiter construct (red channel in the overlay). The cells were treated with 250 nM AP21967 for the time indicated. Shown are individual channels and channel overlay. The playback rate is 15 frames per second. Bar, 10 µm.



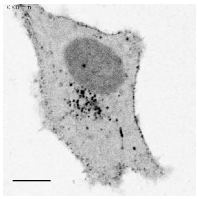
Video 4. **AP21967-induced recruitment of the LocaTOR2 probe to the early endosomes.** HEK293T cells were transiently cotransfected with FRB:Akt2-citrine (Akt; green channel in the overlay) and the FKBP:mCherry-Rab5a early endosome recruiter construct (red channel in the overlay). The cells were treated with 250 nM AP21967 for the time indicated. Shown are individual channels and channel overlay. The playback rate is 15 frames per second. Bar, 10 µm.



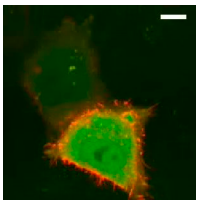
Video 5. **AP21967-induced recruitment of the LocaTOR2 probe to the recycling endosomes.** HEK293T cells were transiently cotransfected with FRB:Akt2-citrine (Akt; green channel in the overlay) and the FKBP:mCherry-Rab11 recycling endosome recruiter construct (red channel in the overlay). The cells were treated with 250 nM AP21967 for the time indicated. Shown are individual channels and channel overlay. The playback rate is 15 frames per second. Bar, 10 µm.



Video 6. **AP21967-induced recruitment of the LocaTOR2 probe to the late endosomes.** HEK293T cells were transiently cotransfected with FRB:Akt2-citrine (Akt; green channel in the overlay) and the FKBP:mCherry-Rab7 late endosome recruiter construct (red channel in the overlay). The cells were treated with 250 nM AP21967 for the time indicated. Shown are individual channels and channel overlay. The playback rate is 15 frames per second. Bar, 10 µm.



Video 7. **Time-lapse video showing intracellular dynamics of mSin1.2^{WT}-GFP in a live cell.** HeLa cells were transiently transfected with mSin1-GFP and imaged using a laser-scanning confocal microscopy system at 0.1 Hz. The playback rate is 15 frames per second. Bar, 10 µm.



Video 8. **SLF'-TMP–induced recruitment and TMP-induced dissociation of FKBP^{F36V}:mCherry-Akt2^{K14A}.** HEK293T cells were transiently cotransfected with FKBP^{F36V}:mCherry-Akt2^{K14A} (left; green channel in the overlay) and TagBFP:2×DHFR-CAAX membrane recruiter construct (middle; red channel). The cells were grown in 10% serum and treated with 1 µM SLF'-TMP and then 10 µM TMP, as indicated. The playback rate is 15 frames per second. Bar, 10 µm.