ASSOCIATED CONTENT
Supporting Information

Experimental Procedures.

Materials. Rabbit polyclonal anti–FRS2α (H-91) and anti-flotillin (H-104) antibodies were from Santa Cruz Biotechnology. Mouse monoclonal anti-DDK antibody (TA50011) was from Origene. Fluorescently labeled secondary antibodies for Infrared Imaging System were from LI-COR. Reagents for electrophoresis and immunoblotting were from Bio-Rad. [9,10-^{3}H]palmitic acid was from PerkinElmer Life Sciences. Phosphatase inhibitors cocktail (PhosSTOP) was from Roche. Thiopropyl Sepharose and other reagents for analysis of palmitoylation were from Sigma-Aldrich. Transfection reagents were from Invitrogen (Lipofectamine 2000) and from Mirus Bio (TransitIT-LT1). Subcellular fractionation markers were prepared as described: mCherry-KDEL[1] (ER marker Lys-Asp-Glu-Leu) and mCherry-K-Ras tail[2] (PM marker), mCherry P4M[3] (Golgi marker; gift of Dr. Tamas Balla, NIH). Tissue culture reagents were from Sigma (for biochemical experiments) and HyClone (for microscopy). Primers were from Invitrogen. Cloning reagents were from Thermo Scientific.

Bioinformatic analyses of FRS2α lipidation sites. Palmitoylation sites were predicted using the CSS-PALM 4.0 program[4] (http://csspalm.biocuckoo.org). The effect of mutating palmitoylated cysteines on FRS2α myristoylation was predicted using the ExPASy Myristoylator tool[5] (https://web.expasy.org/myristoylator/) and GPS-Lipid[6] (http://lipid.biocuckoo.org).

Generation of mutant and fluorescently-tagged constructs. Human FRS2α cDNA (Myc-DDK-tagged, variant 1, NM-006654) was from Origene. This construct was used for transfections in all biochemical experiments. Site-directed mutagenesis was performed to generate point mutants of FRS2α using the FRS2-myc-DDK construct as a template. These constructs were then used as templates for generating fluorescently tagged FRS2α by subcloning into pEGFP-N1 vector (Contech).

Cells. HEK293, HeLa, and U2OS cells were purchased from American Type Culture Collection. They were cultured in DMEM or, for two-photon experiments, in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (HyClone) and antibiotics. For biochemical experiments the cells were transfected with Lipofectamine 2000 according to the manufacturer's instructions and were used 20-24 h after transfection.

Analysis of palmitoylation. Palmitoylation was detected using Acyl-Resin Assisted Capture (Acyl-RAC) method[7], described in detail in Barylko et al.[8]. Cells were solubilized with 2.5% SDS in 100 mM N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.5), 1 mM ethylene diamine tetraacetic acid (EDTA), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM dithiothreitol (DTT), and a protease inhibitor cocktail (consisting of 10 µg/ml each of N-p-tosyl-L-lysine chloromethyl ester, N-p-tosyl-L-arginine methyl ester, N-p-tosyl-L-lysine chloromethyl ketone, leupeptin, and pepstatin). They were then incubated at 40^{0}C for 0.5 h (to reduce potential S-S bonds), then for additional 4 h with methyl methanethiosulfonate (MMTS) to block free thiols. Proteins were then precipitated with cold acetone, and the pellet was extensively washed with 70% acetone to remove excess MMTS. Dried pellets were re-solubilized in 1% SDS

and mixed with thiopropyl-Sepharose resin. Half of the sample was incubated with hydroxylamine (NH_2OH) to cleave thioester bonds, the other half was incubated with 2 M NaCl as negative control, for 3 h at room temperature. Proteins with free thiols (i.e., from cysteines that were originally palmitoylated before NH_2OH treatment) are captured on the resin. NaCl treatment was control against false positives. After extensive washing, proteins released from the resin were analyzed by SDS-PAGE and identified by immunoblotting.

To detect palmitoylation directly, HeLa cells expressing FRS2α-myc-DDK were radiolabeled with [^{3}H]palmitate (2.5 mCi/ml) for 4 h, washed with cold PBS, then lysed with buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.05% SDS, 0.5% deoxycholate, and protease and phosphatase inhibitors for 15 min on ice. Lysates were centrifuged for 15 min at 20,000 x g, and FRS2α was immunoprecipitated with anti-DDK antibodies. The immunoprecipitates were subjected to SDS-PAGE, stained with Coomassie blue and [^{3}H] incorporation was detected by autoradiography.

Preparation of lipid rafts. HEK293 cells transfected with FRS2α^{WT} were treated with 1 mM 2-bromopalmitate (2-BP) or vehicle for 6 h. Cells were then resuspended in a solution containing 0.1 M MES buffer pH 6.5, 150 mM NaCl, 1 mM EDTA, phosphatase and protease inhibitor cocktails, and solubilized in 0.5% Triton X-100. The lysates were adjusted to 42% sucrose and overlaid with 35% and then 5% sucrose, prepared in the above solution but without detergent. Samples were centrifuged for 18 h at 210,000 x *g* in an SW41 rotor. Fractions (1 ml) were collected from the bottom of the tubes. Equal volumes of each fraction were analyzed for the presence of FRS2α by immunoblotting with anti- DDK antibodies and for endogenous flotillin (raft marker) using anti-flotillin antibodies.

Cell fractionation. Transfected cells were washed with PBS and scraped from plates in a solution containing 20 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM EDTA, 5 mM EGTA, 0.2 mM PMSF, and protease and phosphatase inhibitor cocktails. They were lysed by 5 min sonication at medium setting in cell disruptor then centrifuged at 1000 x *g* for 10 min to obtain post-nuclear supernatant (PNS). PNS was divided into 4 aliquots: No treatment (1) or resuspended in solution supplemented with 1 M NaCl (2), 0.2 M Na2CO3, pH 11 (3), or 1% Triton X-100 (4). The samples were centrifuged at 210, 000 x *g* for 15 min. Supernatants and pellets (resuspended in the initial sample volume) were used for immunoblotting.

Live-cell confocal imaging. To assess the subcellular distribution of WT and mutant forms of FRS2α (Figure 2C), HeLa cells transfected with WT and mutant forms of FRS2α with C-terminal EGFP tags were grown on coverslips and visualized with a Zeiss LSM 510 laser scanning confocal microscope. To identify organelles containing WT and mutant FRS2α (Figure 3), cells were plated on 8-well Lab-Tek chambered cover glass (Nunc) at a low density the day before transfection. 100ng plasmids were transfected into HeLa cells using TransitIT-LT1 (Mirus Bio). Approximately 12-14 h after transfection, cells were imaged at room temperature with a 60× objective on a spinning-disc confocal system built around a Ti-E Perfect Focus microscope (Nikon) with an EM camera (c9100-13; Hamamatsu) controlled by Micro-Manager software[9].

Two-photon fluorescence correlation spectroscopy (FCS) and Z-scan microscopy. U2OS cells were subcultured into 8-well coverglass chambered slides (Nunc, Rochester, NY) and transiently transfected using GenJet (SignaGen Laboratories, Rockville, MD) 12-24 h prior to measurement

according to the manufacturer's instructions. Immediately prior to measurements, growth medium was replaced with Dulbecco's phosphate-buffered saline containing calcium and magnesium (Biowhittaker, Walkerville, MD).

FCS experiments were conducted using a 63x C-Apochromat water immersion objective with numerical aperture (NA) = 1.2 (Zeiss, Oberkochen, Germany) and the two-photon focus centered in the cytoplasm of a U2OS cell. Data were collected at an excitation wavelength of 1000 nm and power of 0.3-0.4 mW for 60 s with a sampling rate of 20 kHz. The autocorrelation function of the data was calculated and fit using a 2D-Gaussian beam profile to recover the diffusion time and concentration of the EGFP-labeled protein[10]. Fits were performed using a single-species diffusion model for measurements of FRS2α^{C4,5S}-EGFP and FRS2α^{G2A}-EGFP, while a two-species diffusion model was required for the measurements of FRS2α^{WT}-EGFP.

Z-scan measurements on PM binding were carried out as described previously[11]. The objective, excitation wavelength and power were the same as used in the FCS experiments. Photon counts were detected by an avalanche photodiode (APD, SPCM-AQ-141, Perkin-Elmer, Dumberry, Quebec), recorded by a Flex04-12D card (correlator.com, Bridgewater, NJ) with a sampling frequency of 250 Hz, and analyzed with programs written in IDL 8.5 (Research Systems, Boulder, CO). An arbitrary waveform generator (Model No. 33522A, Agilent Technologies, Santa Clara, CA) moved a PZ2000 piezo stage (ASI, Eugene, OR) axially to perform z-scans. The driving signal from the arbitrary waveform generator was a linear ramp function with a peak-to-peak amplitude of 1.6 V and a period of 10 seconds. The peak-to-peak voltage corresponded to 24.1 μm of axial travel at a speed of v_z = 4.82 μm/s.

Z-scans were analyzed using a modified squared Gaussian-Lorentzian (mGL) point spread function (PSF)[12]. Z-scan calibration was performed as described in order to determine the radial and axial beam-waist (ω_0 and z_0) as well as the axial decay parameter *y*, resulting in values of $\omega_0 = 0.45 \pm 0.05$ μm, $z_0 = 1.1 \pm 0.2$ μm, and $y = 2.5 \pm 0.4$. The z-scan intensity profile (*F*) was fit by the delta-slab-delta model to identify the intensity contributions from the dorsal and ventral plasma membranes (F_M), as well as the cytoplasm (F_c)[11]. These intensity contributions were then converted to plasma membrane intensity fractions[13], f_M, by dividing the membrane intensity contribution by the total intensity at the plasma membrane using the equation $f_M = \frac{F_M(z = z_M)}{F(z = z_M)}$.

Other procedures. Protein concentrations were determined using a modified Lowry method[14,15] with BSA as a standard. SDS-PAGE was carried out according to Laemmli[16]. Proteins were transferred to nitrocellulose, and immunoblotted with the indicated antibodies. Bound primary antibodies were detected and quantified using fluorescently labeled secondary antibody in the LI-COR Odyssey system.

Supplemental Figures

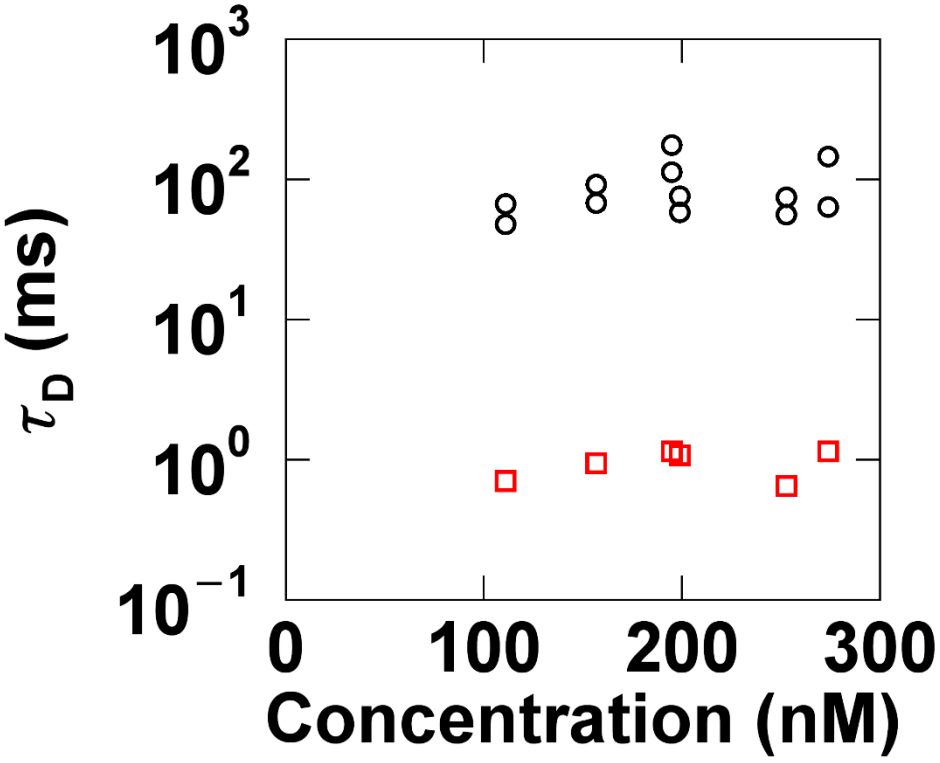

Figure S1. FCS characterization of the diffusion of FRS2α^{WT}-EGFP. Fits of the autocorrelation function to a two-species model identified the cytoplasmic diffusion time (red squares) and the PM-bound diffusion time (black circles). The cytoplasmic diffusion time is on the order of 1 ms, which is close to the observed diffusion data of the mutants at equivalent concentrations (Fig. 4A). PM binding results in a sharp reduction of mobility as evidence by a diffusion time of ~100 ms.

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