Supplementary Methods:

Cells, Solutions, and Reagents

Jurkat E6-1 human leukemic T cells (American Type Culture Collection) and Jurkat YC4.2er cell lines were maintained as described previously[30]. Unless indicated otherwise, experiments were performed at 22-25°C after cells were attached to poly-D-lysine-coated coverslip chambers and were bathed in Ringer's solution containing (in mM): 155 NaCl, 4.5 KCl, 2 or 20 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 5 Na-Hepes (pH 7.4). For Ca^{2+}-free Ringer's, $CaCl_2$ was replaced with 1 mM EGTA + 2 mM $MgCl_2$. To deplete Ca^{2+} stores, cells were exposed to Ca^{2+} -free Ringer's supplemented with CPA (0.5-20 μM) or TG (1μM). All salts and chemicals were from Sigma (St. Louis, MO) unless otherwise stated. Thapsigargin was purchased from LC Laboratories (Woburn, MA), ionomycin and digitonin were from EMD Biosciences, Inc. (La Jolla, CA), OKT3 was from eBioscience (San Diego, CA) and fura-2/AM and Mitotracker Red were purchased from Invitrogen (Carlsbad, CA). AP21967 (rapalog) was generously provided by Ariad Pharmaceuticals (Cambridge, MA, www.ariad.com/regulationkits).

Plasmids and Transfection

Yellow cameleon YC4.2er. To reduce the environmental sensitivity of EYFP, point mutations V68L and Q69M were introduced into the original YC4er (kindly provided by Dr. R. Tsien, UC San Diego, CA) to generate YC4.2er (refs 25-27). Jurkat E6-1 cells

were transfected by electroporation and selected for stable growth with G418. A monoclonal line with the highest cameleon expression and normal SOCE was used. Construction of Cherry-STIM1 and GFP-myc-Orai1 were described previously[11].

F-STIM1. Mutant FRB (pC_4-R_HE) and tandem FKBP (pC_4M-F2E) plasmids were provided by Ariad Pharmaceuticals. The provided variant of FRB binds an analog of rapamycin (AP21976, or rapalog) that fails to bind native mTOR, enabling heterodimerization of FRB and FKBP while avoiding potential side effects from inhibition of mTOR kinase activity[17]. FRB- and tandem FKBP-containing STIM1 plasmids were made by engineering two unique restriction sites, a Nhe1 site after Cherry and a Mlu1 site after the SAM domain, into Cherry-STIM1 using site-directed mutagenesis (Quikchange XL, Stratagene, La Jolla, CA). The plasmid was then digested with Nhe1 and Mlu1 to remove the EF-hand and SAM domains (aa 35-207 in the wtSTIM1 sequence). FRB was amplified from pC_4-R_HE using the primers (5'-3')

TGATTAGCTAGCGGTGCTGGTGCTGGTGCTGGTGCTGGTGCTGGTATCCTCTGGCATG
AG

and (5'-3')

CGACGAATCTCAAAGGGAGCAGGAGCAGGAGCAGGAGCAGGAGCAGGAACGCGTG
TAATT

to append 11 amino acid linkers (GAGAGAGAGAG) flanked by Nhe1 or Mlu1 restriction sites, respectively. Tandem FKBP (2XFKBP) was amplified from pC_4M-F2E using the primers (5'-3')

TGATTAGCTAGCGGTGCTGGTGCTGGTGCTGGTGCTGGTGCTGGTGGAGTGCAGGTG
GAA

and (5'-3)'

CTGCTGAAGCTGGAGGGAGCAGGAGCAGGAGCAGGAGCAGGAGCAGGAACGCGTG TAATT. Amplified, the FRB and 2XFKBP were separately ligated into Nhe1- and Mlu1-digested Cherry-STIM1. The introduction of the NheI site introduced a premature STOP codon which was removed by site-directed mutagenesis.

FRB-STIM1 and FKBP-STIM1 were transiently transfected into Jurkat E6-1 or Jurkat YC4.2er cells by electroporation as described[8], or into HEK293 cells by lipofection following the manufacturer's protocol (Invitrogen) using 0.5 μg of each construct. Cells were studied 48-72 h after transfection.

Fluorescence Microscopy

Widefield epifluorescence and TIRF microscopy was performed essentially as described using a Zeiss Axiovert 200M microscope[8]. For widefield epifluorescence microscopy of YC4.2er, cells were excited at 420 nm and dual emission ratios were collected using a 455 DCLP filter cube (Chroma, Rockingham, VT) and by rapidly alternating D485/40 and D535/30 emission filters with a filter changer (Lambda 10-2, Sutter Instruments, San Rafael, CA) positioned at the exit port of the microscope. All images were acquired with a cooled CCD camera (ORCA-ER, Hamamatsu, Bridgewater, NJ) using 2x2 binning (GFP, Cherry, YC4.2er) or 4x4 binning (YC4.2er when co-expressed with Cherry).

FRET Measurements of $[Ca^{2+}]_{ER}$

Background-corrected emission intensities at 535 nm and 485 nm were averaged across the cell to yield a raw F535/F485 emission ratio. At the end of every experiment, *in situ*

calibration of YC4.2er cells was performed by adding digitonin (50-75 µg/ml) and ionomycin (10 µM) to permeabilize the plasma membrane while leaving intracellular organelles intact and to equilibrate Ca^{2+} across the ER membrane (Supp. Fig. 2). In the presence of ionomycin and digitonin, two standard F535/F485 ratios (R_1 and R_2) were obtained after exposing the cells to (in mM): 75 K aspartate, 60 KCl, 1 $MgCl_2$, and either 10 EGTA (for R_1) or 20 $CaCl_2$ (for R_2). For every cell, raw F535/F485 ratios (R) were normalized using these standard solutions:

$$R_{norm}=(R-R_1)/(R_2- R_1)$$

where R_{norm} is the normalized YC4.2er ratio[26]. R_{norm} was converted to $[Ca^{2+}]_{ER}$ using a calibration curve determined separately.

A complete calibration curve for YC4.2er (Supp. Fig. 2) was generated by exposing cells to solutions with various Ca^{2+} concentrations (listed in Supp. Table 1) after permeabilization with digitonin and ionomycin as described above. The concentrations of K aspartate and KCl were adjusted to maintain constant $[Cl^-]$ and osmolarity. Free $[Ca^{2+}]$ was calculated with MaxChelator software (http://maxchelator.stanford.edu) and subsequently adjusted using a calibrated Ca^{2+}-sensitive electrode (Orion Research Inc.). Normalized F535/F485 ratios were calculated as described above and plotted against free $[Ca^{2+}]$, and this relation was fitted with the following equation using IgorPro (Wavemetrics, Lake Oswego, OR):

$$[Ca^{2+}]_{ER} = K_d\,[(R_{norm}-R_{min})/(R_{max}-R_{norm})]^{1/n}$$

where K_d (819 µM) is the apparent dissociation constant, n is 0.54, R_{min} is 0.193, and R_{max} is 1.134. YC4.2er is reported to an additional, high-affinity binding site for $[Ca^{2+}]_{ER}$ (K_d =83 nM; ref 26); the calibration curve was fitted only to the lower affinity site which

reported $[Ca^{2+}]_{ER}$ from ~400 nM to > 1 mM (Supp. Fig. 2b). Thus, in the few cells where R_{norm} fell below the R_{min} for this low affinity binding site, $[Ca^{2+}]_{ER}$ was assigned a value of 400 nM.

Immunocytochemistry

Jurkat YC4.2er cells were attached to poly-D-lysine-coated glass coverslips and fixed in 4% fresh paraformaldehyde at 22-25°C for 15 min. For staining with polyclonal anti-calnexin antibody (Stressgen Biotechnologies Corp, Victoria, BC, Canada), cells were permeabilized for 5 min in cold methanol at –20°C. For staining with monoclonal anti-golgin-97 (Molecular Probes, Eugene, OR), cells were permeabilized in 0.5% Triton X-100 in phosphate-buffered saline containing 0.2% bovine serum albumin (PBS/BSA) for 5 min at 22-25°C. After permeabilization, cells were rinsed three times with PBS and incubated in blocking buffer containing 20 mM glycine, 75 mM NH_4Cl, 0.2% BSA, and 1% goat serum in PBS for 1 h. Cells were then incubated with primary antibodies (1:1000 dilution in blocking buffer) for an additional 1 h and rinsed three times with blocking buffer to remove unbound antibody. Alexa 594-conjugated secondary antibody (Molecular Probes; 1:1000 dilution in PBS/BSA) was applied for 45 min, followed by three rinses with PBS/BSA. Coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and viewed with a Zeiss Axiovert 200M microscope (40x NA1.3 oil), or a Molecular Dynamics Multiprobes 2010 confocal microscope (40x NA1.3 oil).

Quantification of STIM1 Redistribution

A binary mask of the cell periphery was applied to each background-corrected Cherry image by drawing a polygonal region with a width of 0.5 μm around the edge of the cell. The extent of Cherry redistribution was assessed as the mean intensity within the masked region (F_P) divided by the intensity averaged across the whole cell (F_{TOT}). In TIRF experiments, background-corrected Cherry images were thresholded to exclude remaining background fluorescence, and intensity was measured within an outline drawn around the cell footprint in the first frame of each time-lapse series. To compensate for any cell-to-cell differences in the abundance of peripheral ER, changes in redistribution measured by TIRF or widefield epifluorescence microscopy were normalized to the maximal response obtained in each cell after store depletion with TG. Mean values of F_P/F_{TOT} or normalized TIRF intensities were calculated from 3-4 images for each data point.

Heterodimerizer Experiments

All experiments were performed using 1 μM AP21967. For timelapse imaging of F-STIM1 redistribution in response to AP21967, cells were imaged using widefield epifluorescence at 37°C in full medium. TIRF measurements of F-STIM1 were made at 22-25°C in 2 mM Ca^{2+} Ringer's solution, before and after treatment with AP21967 for 30 min in full medium at 37°C on the microscope stage; untreated cells were incubated in full medium alone under the same conditions. Otherwise, cells were pretreated for 30 min in full medium with (treated cells) or without (untreated cells) AP21967 at 37°C in a CO_2 incubator before imaging at 22-25°C. Cells overexpressing STIM1 or F-STIM1 at high levels often displayed puncta in the resting state, and correspondingly elevated

resting $[Ca^{2+}]_i$. This effect of overexpression has also been reported by others for STIM1 (ref 7). To avoid these effects of overexpression, we restricted analysis to cells with ~3-10% of the fluorescence of the brightest cells in each experiment.

BN-PAGE and Western Blot Analysis

BN-PAGE was performed using the NativePAGE Novex Bis-Tris gel system (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Briefly, 10^7 HEK293 cells expressing F-STIM1 were solubilized using NativePAGE sample buffer supplemented with 1% n-dodecyl-β-D-maltoside. For the rapalog condition, cells were incubated with 1 μM AP21967 for 30 min at 37°C prior to solubilization, and 1 μM AP21967 was included in the sample buffer. Coomassie G-250 was added to samples, and 0.5-1% of the sample was loaded onto the NativePAGE Novex 4-16% Bis-Tris gel. Proteins bound to Coomassie G-250 were transferred electrophoretically to Hybond-P membrane (GE Healthcare, Piscataway, NJ) in Tris/Glycine buffer (BioRad, Hercules, CA) for 14 h at 60 mA, then fixed with 10% acetic acid and de-stained with methanol. Membranes were blocked with 5% skim milk powder in Tris/NaCl buffer (0.05 M Tris pH 7.4, 0.15 M NaCl, 0.05% Tween 20) and incubated for 8 h at 4°C with a monoclonal antibody against the STIM1 C-terminus (1:250, Abnova , Taipei City, Taiwan). The membrane was washed with Tris/NaCl buffer and then incubated with alkaline phosphatase-conjugated 2° antibody (1:30,000; Sigma) for 1 h at room temperature. After subsequent washing, alkaline phosphatase was detected using Lumi-Phos substrate (Pierce, Rockford, IL).

Cytosolic $[Ca^{2+}]_i$ Measurements

Video microscopic measurements of $[Ca^{2+}]_i$ were performed as described previously[28].

Electrophysiology

Patch-clamp experiments were conducted in the standard whole-cell recording and perforated-patch configurations as previously described[28, 29]. The internal solution for whole-cell recording contained (in mM): 140 Cs aspartate, 5 $MgCl_2$, 0.5 $CaCl_2$, 1.2 EGTA, and 10 HEPES (pH 7.2 with CsOH). The free $[Ca^{2+}]$ of this solution was calculated to be 146 nM using MaxChelator. For perforated-patch experiments, the internal solution contained (in mM): 115 Cs aspartate, 1 $CaCl_2$, 5 $MgCl_2$, 10 NaCl, 10 HEPES, and 100 μg/ml amphotericin B (pH 7.2 with CsOH). All data were leak-subtracted using currents collected in Ca^{2+}-free Ringer's. For whole-cell recording of I_{CRAC} in cells expressing F-STIM1, resting cells were perfused with 20 mM Ca^{2+} Ringer's in the cell-attached configuration, and voltage stimuli consisting of a 100-ms step to –112 mV followed by a 100-ms voltage ramp from –112 to +88 mV were applied from the holding potential of +38 mV every 2-5 s, beginning within 5 s of break-in. I_{CRAC} was measured as a 10-ms average at the end of 100-ms pulses to -112 mV, and spontaneous I_{CRAC} was measured from the first voltage step to -112 mV after break-in.

Measuring I_{CRAC} and $[Ca^{2+}]_{ER}$

After 8-15 min pretreatment with CPA (0.5-20 μM) in Ca^{2+}-free Ringer's solution, the perforated-patch configuration was established with YC4.2er Jurkat cells. I_{CRAC} was measured after perfusing the cells with a 20 mM Ca^{2+} Ringer's solution, using a

step/ramp stimulus consisting of a 50-ms step to -100 mV followed by a voltage ramp from –100 to +100 mV delivered every 2.5 s from the holding potential of +30 or +50 mV. $[Ca^{2+}]_{ER}$ was measured simultaneously with I_{CRAC} by exciting at 440 ± 10 nm (Chroma) for 40 ms every 2.5 s through a Nikon Fluor 40X objective (NA 1.3). The emissions from YC4.2er at 485 ± 12.5 nm and 535 ± 12.5 nm were collected simultaneously from an area slightly larger than the cell using two photomultipliers (HC120 05-MOD; Hamamatsu, Japan). YC4.2er ratios were calibrated *in situ* as described above.

Image and Data Analysis

Image analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD). The dependence of STIM1 redistribution and I_{CRAC} (y) on $[Ca^{2+}]_{ER}$ was described by the Hill equation:

$$y = y_{min} + (y_{max} - y_{min}) / (1 + (K_{1/2} / [Ca^{2+}]_{ER})^{n_H})$$

where y_{max} and y_{min} are the maximal and minimal values of STIM1 redistribution or I_{CRAC}, $K_{1/2}$ is the $[Ca^{2+}]_{ER}$ at which these values are half-maximal, and the Hill coefficient, n_H, is a measure of the steepness of the relation. A curve was fitted by non-linear least squares in Igor Pro to the entire collection of single-cell data using this equation. For display purposes in Figures 1 and 2, the single-cell responses within 25-μM bins of $[Ca^{2+}]_{ER}$ were averaged and plotted as mean values ± s.e.m. Because of the difficulty of obtaining large numbers of cells in the perforated-patch experiments, some $[Ca^{2+}]_{ER}$ bins contained fewer than three cells; for these bins, I_{CRAC} in single cells is plotted instead (Fig. 1).

Supplementary Tables:

Supplementary Table 1. Composition of YC4.2er calibration solutions (in mM).

$[Ca^{2+}]_{free}$	$CaCl_2$	$MgCl_2$	K asp	KCl	HEPES	Buffer
0		2	70	40	10	20 EGTA
0.0004	4.9571	2.2835	87.6203	29.5188	10	10 EGTA
0.003	0.2335	11.18	89.71	21.173	10	10 HEDTA
0.01	0.7392	10.7	89.72	21.12	10	10 HEDTA
0.02	1.2088	10.4332	89.79	20.74	10	10 HEDTA
0.06	1.3	6.03	87.635	29.34	10	10 NTA
0.1	2.109	6.33	88.2	27.14	10	10 NTA
0.2	0.2	2	100.1	39.6	10	
0.3	0.3	2	100.15	39.4	10	
0.5	0.5	2	100.25	39	10	
1	1	2	100.5	38	10	
5	5	2	102.5	30	10	
20	20	2	110		10	

Supplementary Figures:

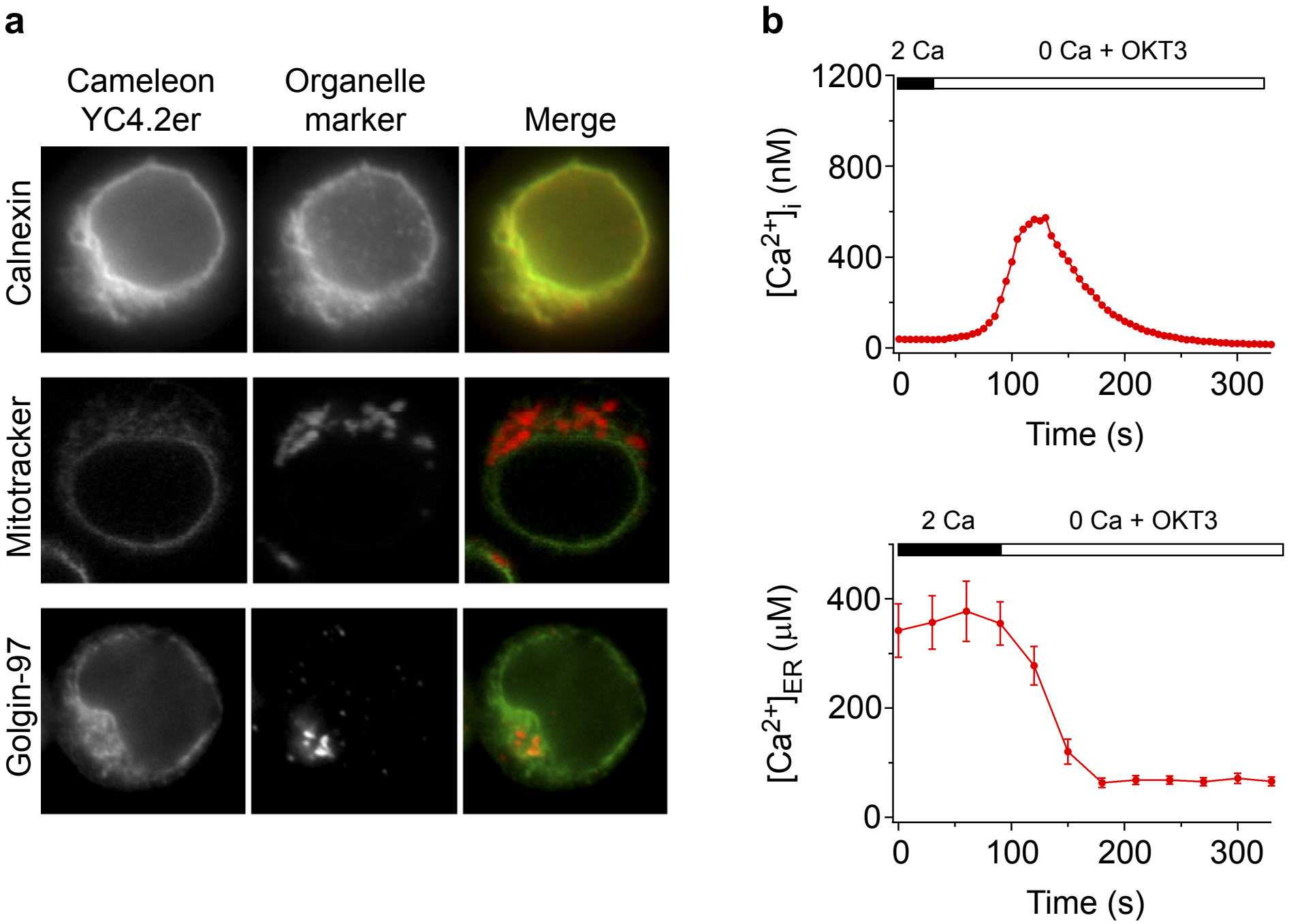
Supplementary Figure 1. Targeted localization of YC4.2er to the ER in a stably transfected Jurkat cell line. a, To verify ER-specific targeting of the indicator, YC4.2er fluorescence (left panels) was compared with fluorescent staining of specific organelle markers (middle panels). The ER was stained with a polyclonal antibody against the ER luminal protein calnexin (top); the mitochondria were labeled with MitoTracker Red (middle); and the Golgi was labeled with a monoclonal antibody against the Golgi resident protein golgin-97 (bottom). **b,** Cytosolic and $[Ca^{2+}]_{ER}$ responses to 1:100 dilution of α-CD3 mAb OKT3, applied in Ca^{2+}-free Ringer's as indicated by the bars. (Top) Ca^{2+} release causes a transient rise of $[Ca^{2+}]_i$ monitored with fura-2. Mean response of 119 cells. (Bottom) The resulting depletion of the ER store monitored with YC4.2er. Mean response of 97 cells.

Supplementary Figure 2. *In situ* calibration of YC4.2er Jurkat cells. a, Normalized F535/F485 emission ratios for YC4.2er cells are shown (n = 67-134). Cells were permeabilized with digitonin in 20 mM Ca^{2+} and subsequently washed with calibration solutions containing the indicated concentrations of Ca^{2+} (in mM) in the presence of 10 μM ionomycin, to facilitate equilibration of $[Ca^{2+}]$ between ER and the calibration solutions. Emission ratio was normalized to the range produced by 20 mM Ca^{2+} and 0 Ca^{2+}. **b,** *In situ* calibration curve for YC4.2er, fitted to the data as described in Supplementary Methods.

Supplementary Figure 3. Validation of widefield epifluorescence assay for STIM1 redistribution. a, TIRF (left column) and widefield epifluorescence (right column) images of Cherry-STIM1 in the same cell. As stores are depleted with 3 μM CPA (middle row) followed by TG (bottom row), TIRF images show the accumulation of Cherry-STIM1 in puncta within ~200 nm of the plasma membrane, and widefield images at the cell equator show the redistribution of Cherry-STIM1 to the cell periphery. Scale bar = 2 μm. **b,** F_P/F_{TOT} (blue) and TIRF intensities (red) for the cell shown in **a**. Both data sets are normalized to the maximal response obtained with TG treatment. Individual data points (open circles) and the average response (bars) are plotted for each extracellular solution. **c,** F_P/F_{TOT} versus TIRF intensities for individual cells (n=11) treated with 0-3 μM CPA. Means ± s.e.m. are shown. The linear relation validates the widefield method for measuring STIM1 redistribution.

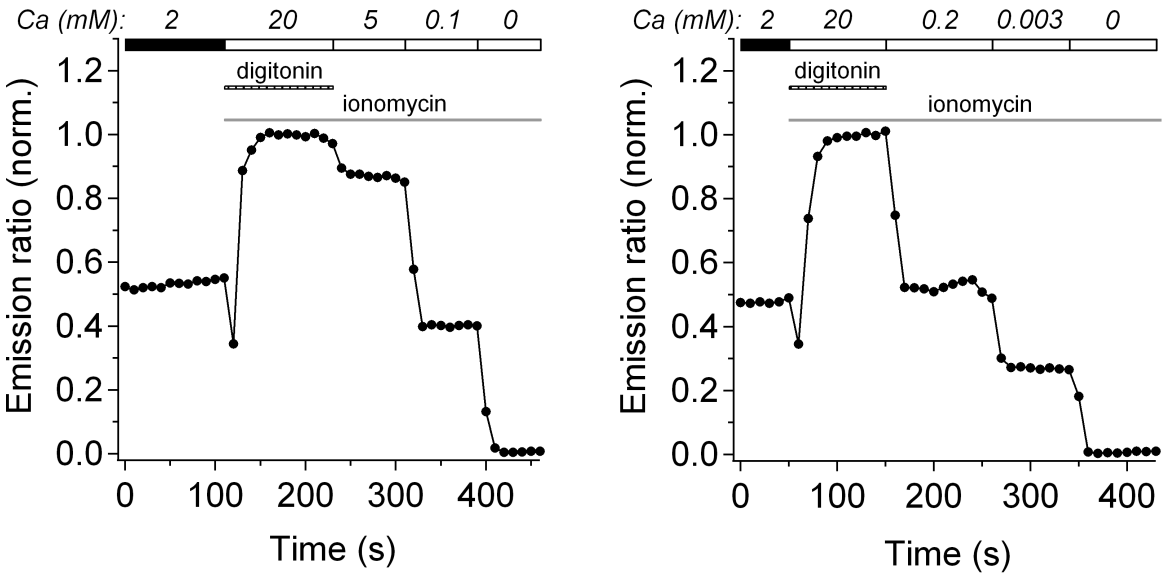
Supplementary Figure 4. Ca^{2+} entry triggered by F-STIM1 oligomerization occurs through CRAC channels. a, La^{3+} block of constitutive Ca^{2+} influx was monitored in Jurkat cells loaded with fura-2. Resting $[Ca^{2+}]_i$ was elevated in cells expressing F-STIM1 after treatment with rapalog (black trace, n=35), and declined rapidly in response to Ca^{2+}-free Ringer's or 1 μM La^{3+}. Resting $[Ca^{2+}]_i$ was not elevated in untreated F-STIM1-expressing cells (red, n=25), or in untransfected Jurkat cells with (green, n=369) or without (blue, n=313) rapalog treatment. **b,** Inhibition of I_{CRAC} by 50 μM 2-APB in rapalog-treated (black, n=4) and untreated (red, n=4) Jurkat cells expressing F-STIM1. I_{CRAC} was measured in 20 mM Ca^{2+} Ringer's during voltage steps to -112 mV beginning within 5 s of break-in.

Supplementary Figure 1



Supplementary Figure 2

a



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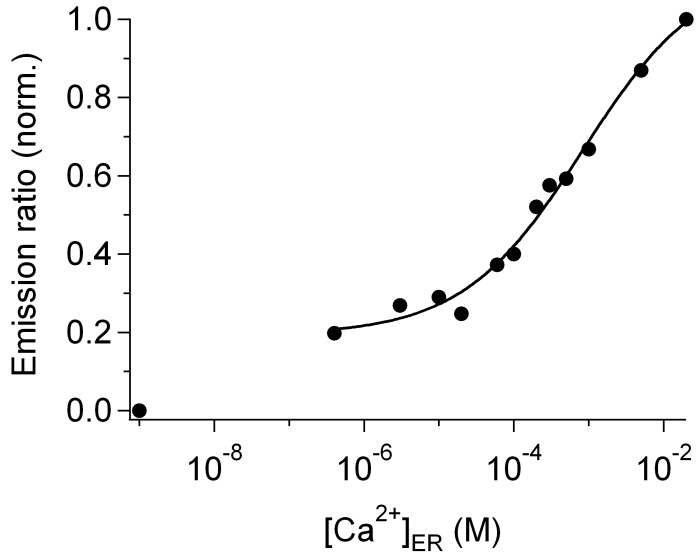
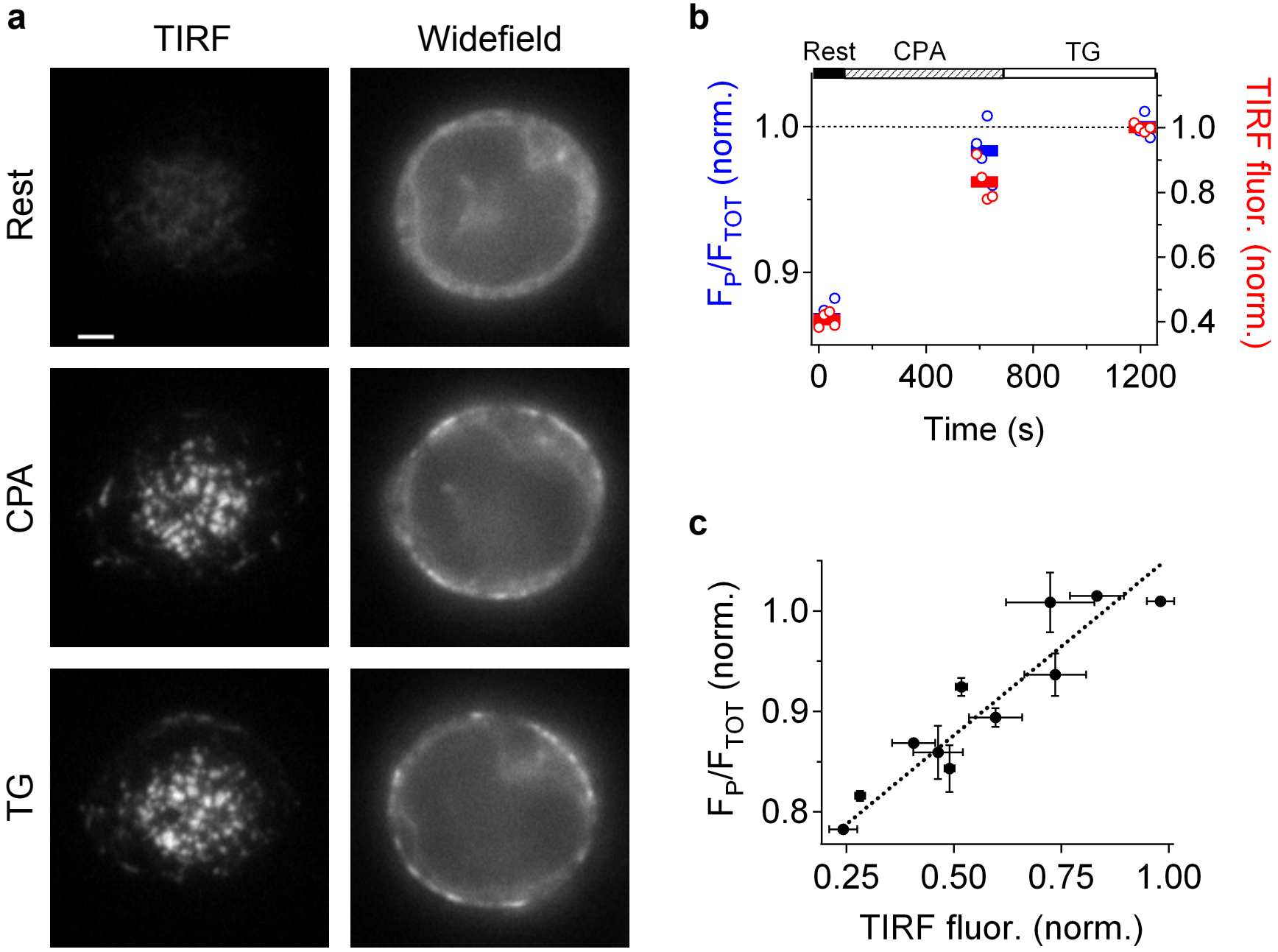


Supplementary Figure 3



Supplementary Figure 4