Supplemental Methods

Materials.

Acetyl-methacholine was purchased from Sigma (St. Louis, MO), thapsigargin from Alexis (San Diego, CA), and fura-5F/AM from Molecular Probes (Eugene, OR).

Plasmids.

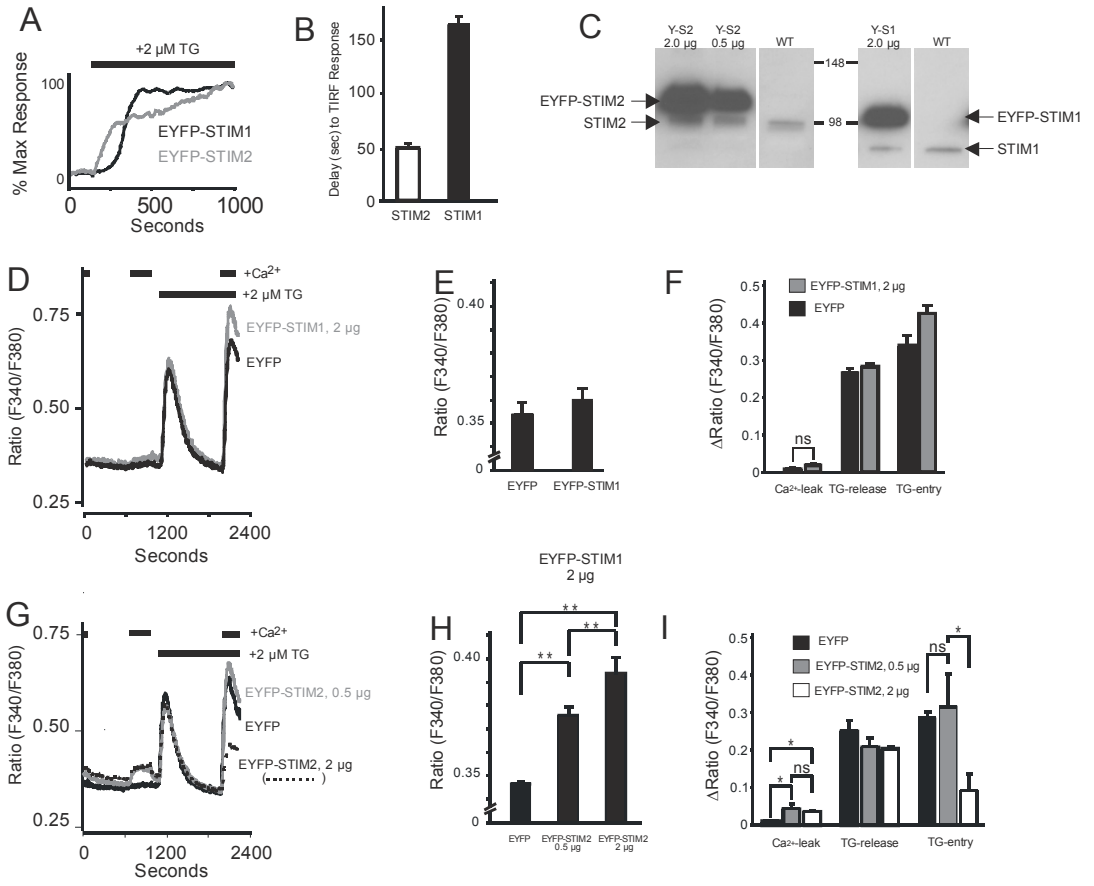
EYFP-C1 (EYFP: Enhanced Yellow Fluorescent Protein) was obtained from Clontech (Mountain View, CA). STIM1 and STIM2 with the enhanced yellow fluorescent protein fused to the N terminus (EYFP-STIM1 and EYFP-STIM2 respectively) were obtained from Tobias Meyer, Stanford University. D76N/D78N-EYFP-STIM1 was described previously [11]. An EF-hand mutant of EYFP-STIM2, EYFP-STIM2-(D80A), was made by site-directed mutagenesis with the QuikChange® II XL kit (Stratagene).

Western Blots

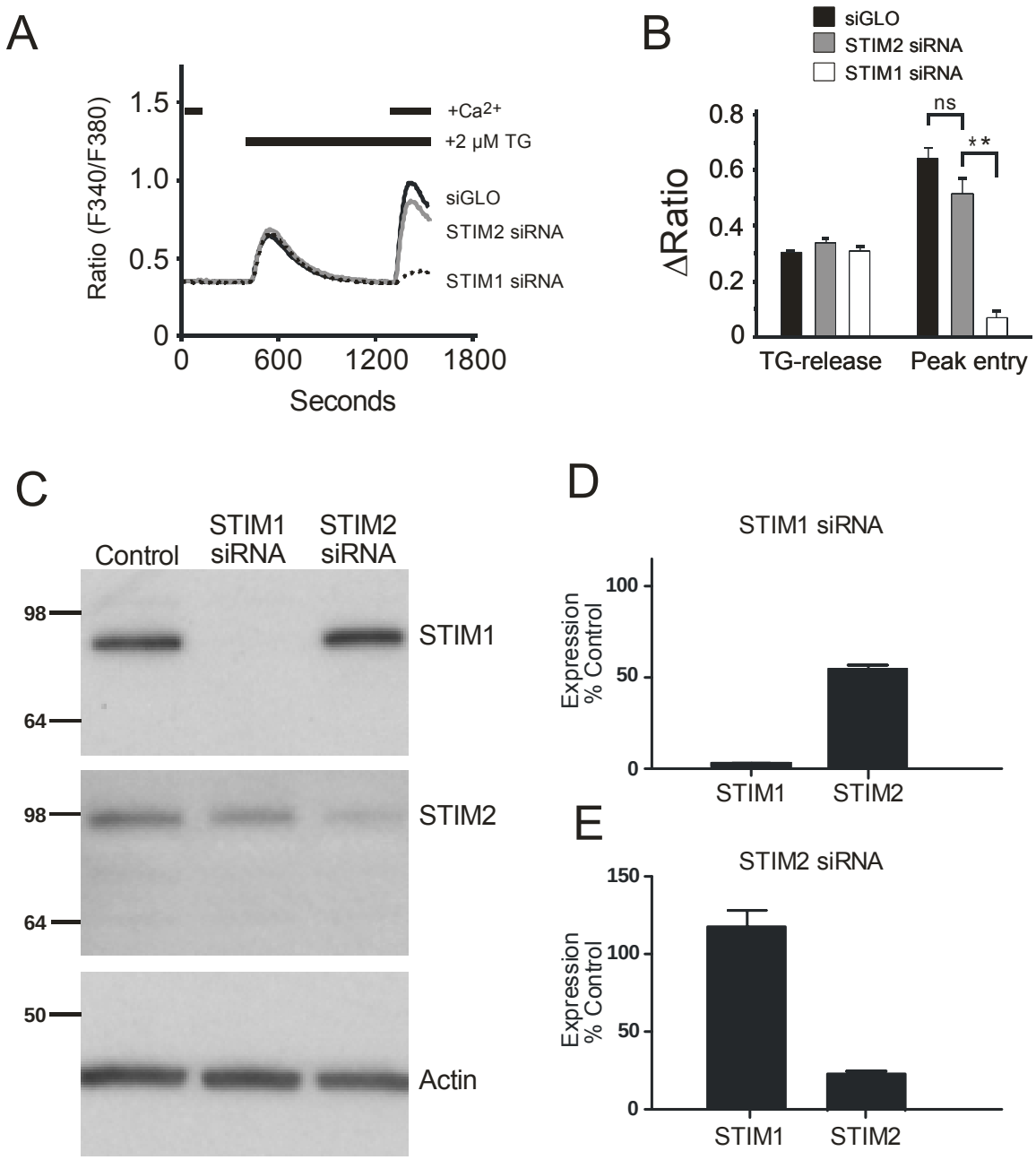
Cells were lysed in RIPA buffer (50 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% w/v sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, pH 7.5) containing one Complete Mini Protease Inhibitor Tablet (Roche Applied Sciences) per 10 ml, lysates were cleared by centrifugation, and protein concentrations were determined using the DC Protein Assay Kit (BioRad). For analysis of native Orai1 expression, lysates were treated with N-glycosidase F (Boehringer Mannheim; 1 Unit enzyme per 30 μl lysate) for 16 hr at 37°C to reduce effects of multiple glycosylation patterns of Orai1 protein. Lysates were normalized by protein content and analyzed by Western blot as previously described [24]. Primary antibodies used were anti-STIM1 (1:6,000; BD Biosciences), anti-STIM2 (1:7,000; Alomone), and anti-Orai1 (1:10,000; Sigma). When necessary, blots were stripped with Restore Stripping Buffer (Thermo Scientific) and reprobed.

Statistical analyses.

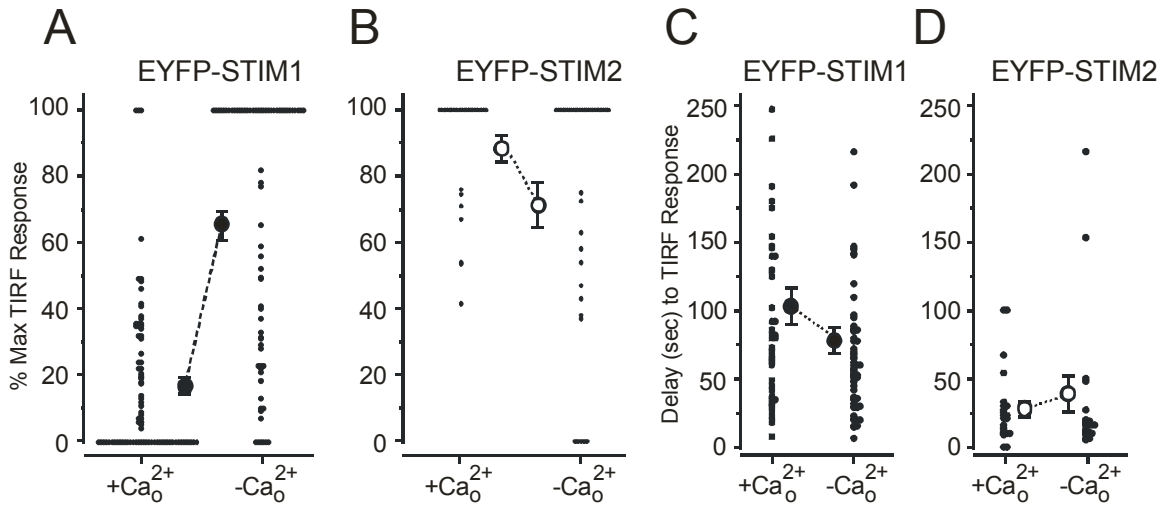
Statistical analyses were carried out using GraphPad Prism Software.



Supplemental Figure 1. *STIM2, but not STIM1, is partially active under basal conditions.* A. TIRFM imaging of EYFP-STIM1 and EYFP-STIM2 demonstrates that STIM2 responds faster than STIM1 following store depletion by thapsigargin. Data are from a single experiment representative of n= 17 cells for EYFP-STIM1 and n= 20 for EYFP-Stim2. B. Means ± SEM for the delays to onset of the TIRFM response for all experiments. C. Western blots showing expression of endogenous STIM2 and overexpressed EYFP-STIM2 at 0.5 μg and 2.0 μg (left panel) and expression of endogenous STIM1 and overexpressed EYFP-STIM1 at 2.0 μg (right panel). D-F. Overexpression of EYFP-STIM1 does not affect basal Ca^{2+}, Ca^{2+} leak, thapsigargin-induced release and increases Ca^{2+} entry only slightly (Data are mean ± SEM for 3 experiments). D. Results of one experiment examining thapsigargin-activated entry in cells expressing EYFP or EYFP-STIM1. E. Summary of basal Ca^{2+} at the beginning of the experiment. F. Summary of the magnitude of Ca^{2+} leak (elevation of $[Ca^{2+}]_i$ upon restoration of extracellular Ca^{2+}, prior to thapsigargin addition), the magnitude of the Ca^{2+} release upon thapsigargin addition, and the magnitude of the thapsigargin-induced Ca^{2+} entry (peak elevation of Ca^{2+} following restoration of Ca^{2+} after thapsigargin addition). For STIM1, none of these parameters were significantly affected. G-I. Effects of overexpression of EYFP-STIM2 with two different plasmid concentrations, 0.5 and 2.0 μg/well. The panels each show experiments carried out and analyzed as in D-F, including the two EYFP-STIM2 plasmid concentrations. Overexpression of EYFP-STIM2 at either plasmid concentration has no significant effect on thapsigargin-induced Ca^{2+} release and significantly increases basal Ca^{2+} and Ca^{2+} leak. EYFP-STIM2 at 2.0 but not at 0.5 μg/well significantly inhibits thapsigargin-activated Ca^{2+} entry (data are mean ± SEM for 3 experiments). Data were analyzed by ANOVA where * $p< 0.05$, ** $P< 0.01$ and ns is not significant.



Supplemental Figure 2. *Effects of STIM1 and STIM2 siRNA on thapsigargin-induced Ca^{2+} entry*. HEK293 cells were transfected with control siRNA (siGLO), or siRNA directed against STIM1 or STIM2. A. Traces of $[Ca^{2+}]_i$ from a single experiment showing thapsigargin-activated Ca^{2+} entry has a strong dependence on STIM1 and a minimal dependence on STIM2. B. Summary of 5 experiments showing effects of knocking down STIM1 or STIM2 on thapsigargin-induced release of Ca^{2+}, and thapsigargin-induced Ca^{2+} entry (data were analyzed by ANOVA where ** $P< 0.01$ and ns is not significant). C: Cells were transfected with control siRNA or siRNAs directed against STIM1 or STIM2, and blots were probed with antibody against STIM1 (upper), STIM2 (middle), and β-actin (lower). D: For three independent experiments carried out as described in panel C, band intensities were quantified by densitometry and normalized against respective β-actin band intensities. Data are shown as percent of control, mean ± S.E.M.



Supplemental Figure 3. *Summary of effects of Methacholine (MeCh) on EYFP-STIM1 and EYFP-STIM2 TIRFM response parameters in the presence and absence of extracellular Ca^{2+}.* HEK293 cells were activated with low concentration of MeCh (5 μM) in the presence or absence of extracellular calcium and the TIRFM responses for EYFP-STIM1 and EYFP-STIM2 were analyzed for both the magnitude (A,B) and the delay to initiation (C,D) of the TIRFM signal, The magnitude of the TIRFM response is expressed as a percentage (%) of the maximal response elicited by thapsigargin or ionomycin-treatment in the same cell. Data were captured in experiments reported in Figs. 3 and 4. Within each panel, the magnitudes (A, EYFP-STIM1; B, EYFP-STIM2) and delays (C, EYFP-STIM1; D, EYFP-STIM2) of the TIRFM responses to low [MeCh] in the presence or absence of extracellular Ca^{2+} are compared. The result for each cell is represented in a scatter plot, with the mean ± SEM for each condition also plotted and connected by the dashed line. The panels summarize data from (#cells/#expt); (A) 83/24, 69/12, (B) 23/6, 32/8, (C) 49/24, 63/12, (D) 23/6, 26/6 respectively.