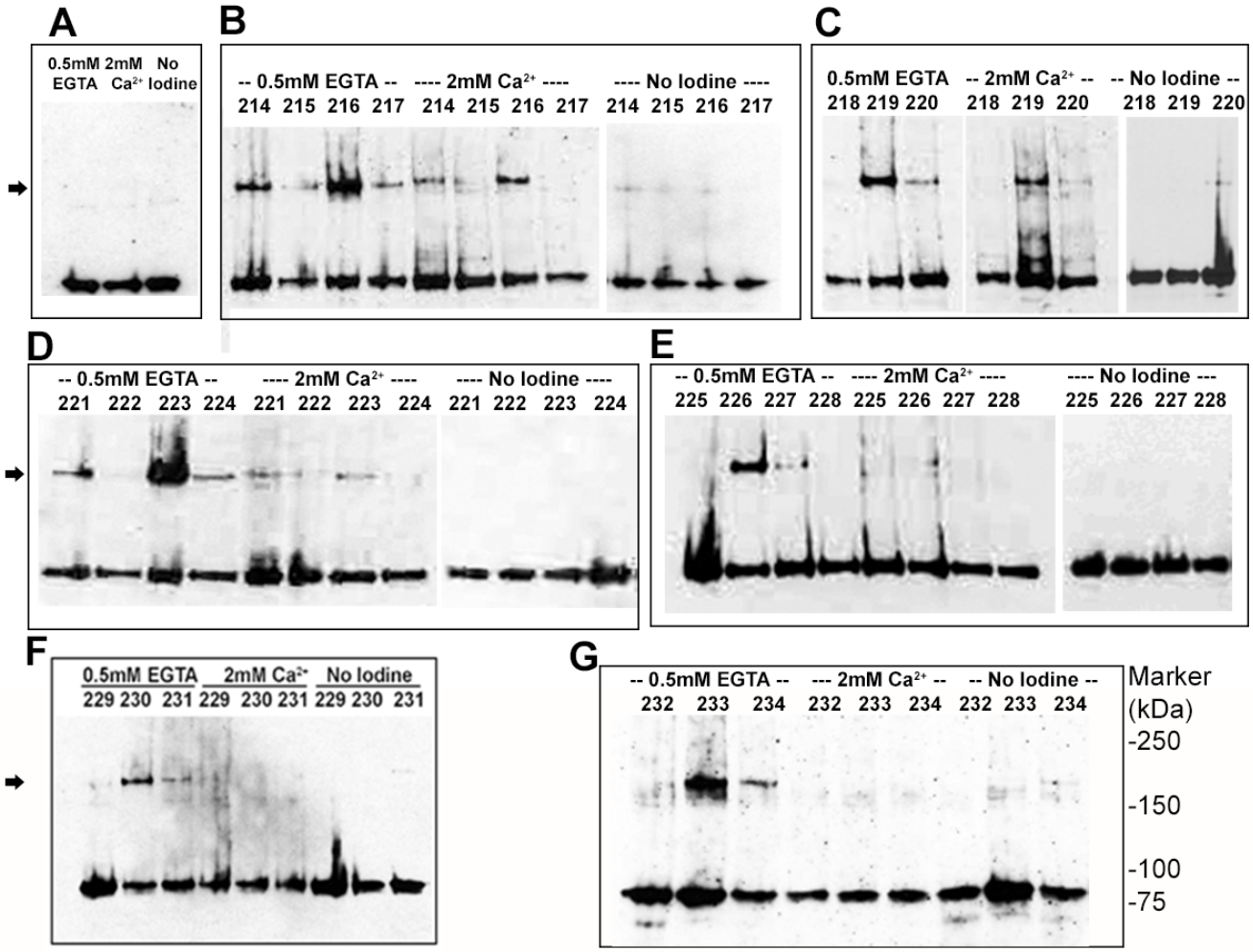

Figure S1. Crosslinking pattern of STIM1 transmembrane helix residues. Related to Figure 2. **A,** Representative western blots showing the crosslinking of cysteine-less STIM1 in EGTA and Ca^{2+} conditions, as well as the non-oxidized control (No Iodine). **B-G,** Representative western blots showing crosslinking of the specified single-cysteine mutants of STIM1 in isolated cellular membranes incubated in the presence of EGTA or Ca^{2+} and oxidized with iodine. Non-oxidized controls (No Iodine) are included in each western blot. The samples were subjected to nonreducing SDS-polyacrylamide gel electrophoresis, and the blots were probed with anti-STIM1 antibody. Representative of three biological replicates. Quantitative data from these experiments are summarized in Figure 2C. Positions of molecular weight markers are shown for the blot in panel G.

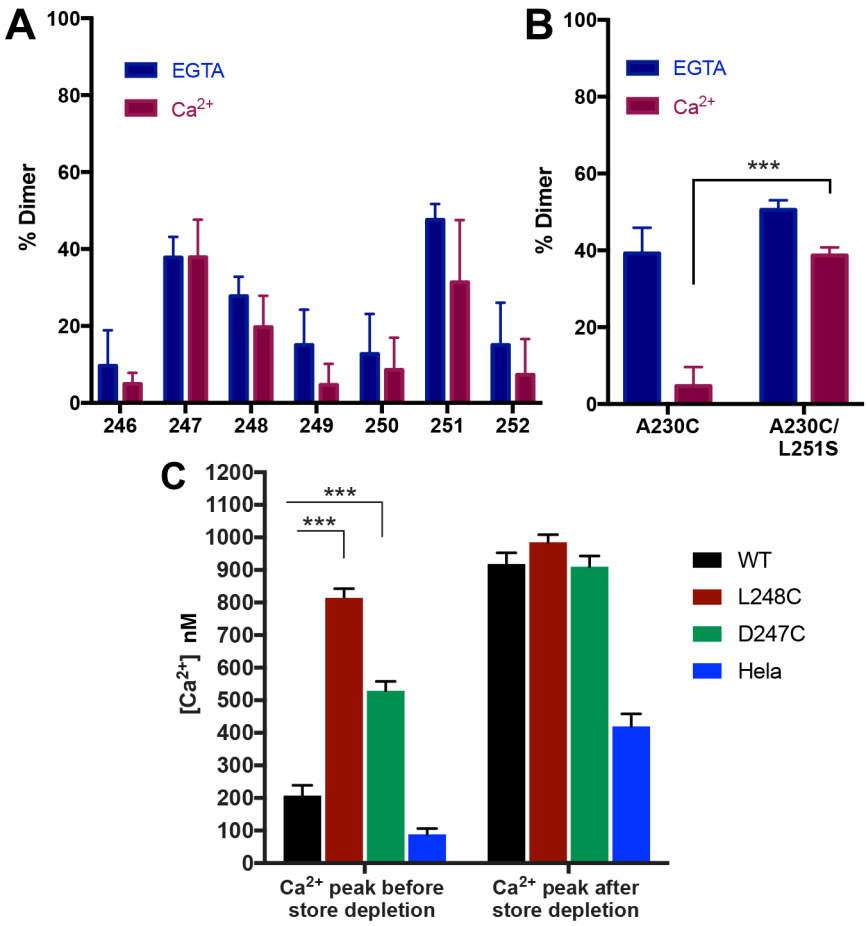

Figure S2. Analysis of mutants in CC1 residues 246–252. Related to Figure 4. **A,** Crosslinking efficiencies of STIM1 with single cysteine replacements at residues 246–252, in the presence of EGTA (blue) or Ca^{2+} (magenta). Data from three biological replicates. Error bars report SEM. **B,** The effect of an additional L251S mutation on the crosslinking efficiency of $STIM1^{A230C}$, in the presence of EGTA (blue) or Ca^{2+} (magenta). Data from three biological replicates. Error bars report SEM. The difference between STIM1(A230C/L251S) and STIM1(A230C) in the presence of Ca^{2+} is statistically significant ($p < 0.05$; marked with ***). **C**. Quantitation of peak $[Ca^{2+}]_i$ in Figure 4F in store-replete cells after addition of 1 mM $CaCl_2$, and in store-depleted cells after addition of 2 mM $CaCl_2$. For wildtype STIM1, n = 55; STIM1(L248C), n = 50; STIM1(D247C), n = 48; and non-transfected HeLa cells, n = 65. Error bars report SEM. The Ca^{2+} peaks for L248C and D247C mutants in store-replete cells were significantly different from the corresponding peak for wildtype STIM1 ($p < 0.05$; marked with ***).

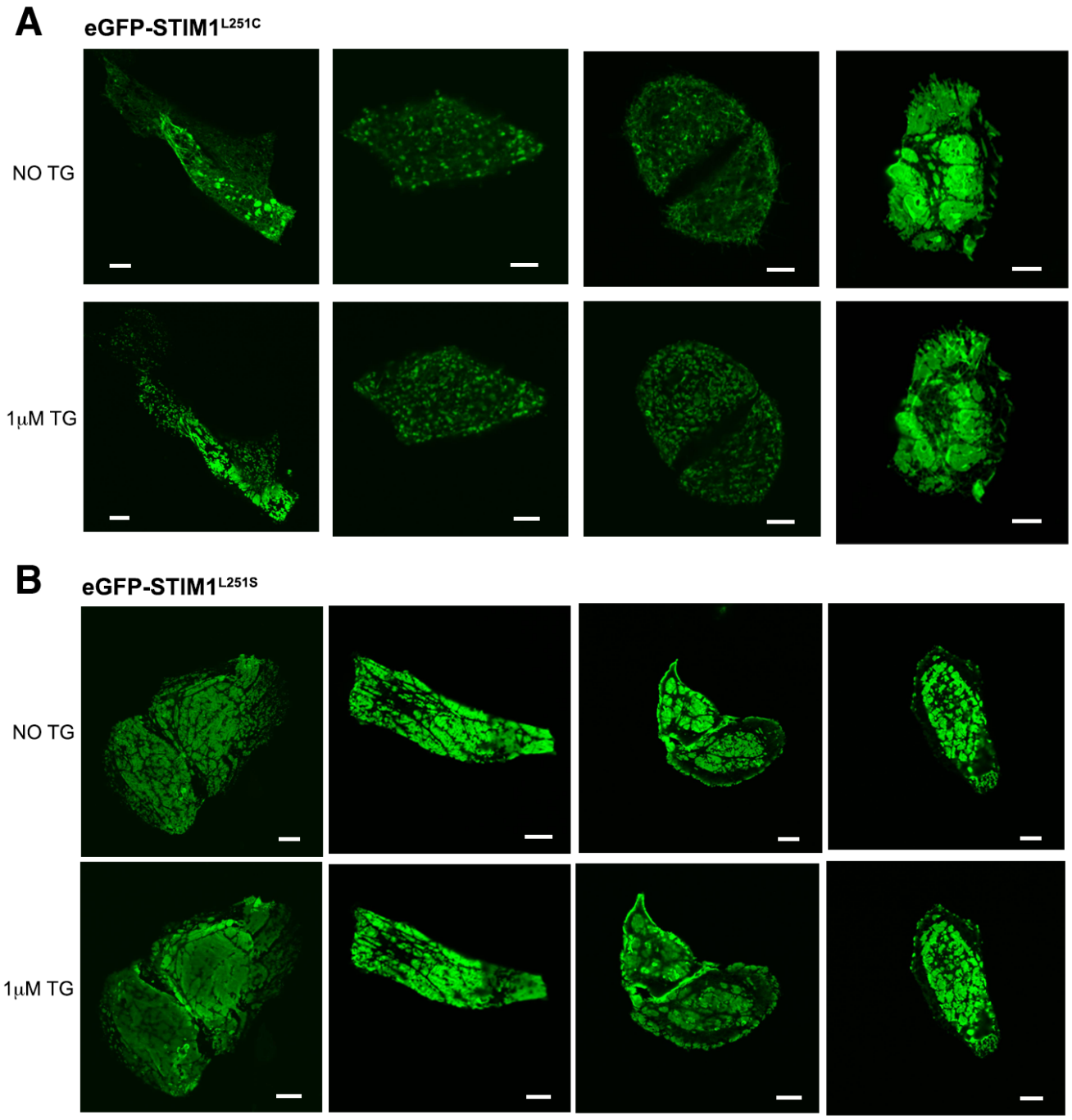

Figure S3. Additional confocal images of HeLa cells expressing the eGFP-STIM1 variants of Figure 4B and 4C. Related to Figure 4. **A,** eGFP-STIM1^{L251C}. **B,** eGFP-STIM1^{L251S}. The images illustrate the range of variation seen with each mutant. Scale bar, 10 μm.

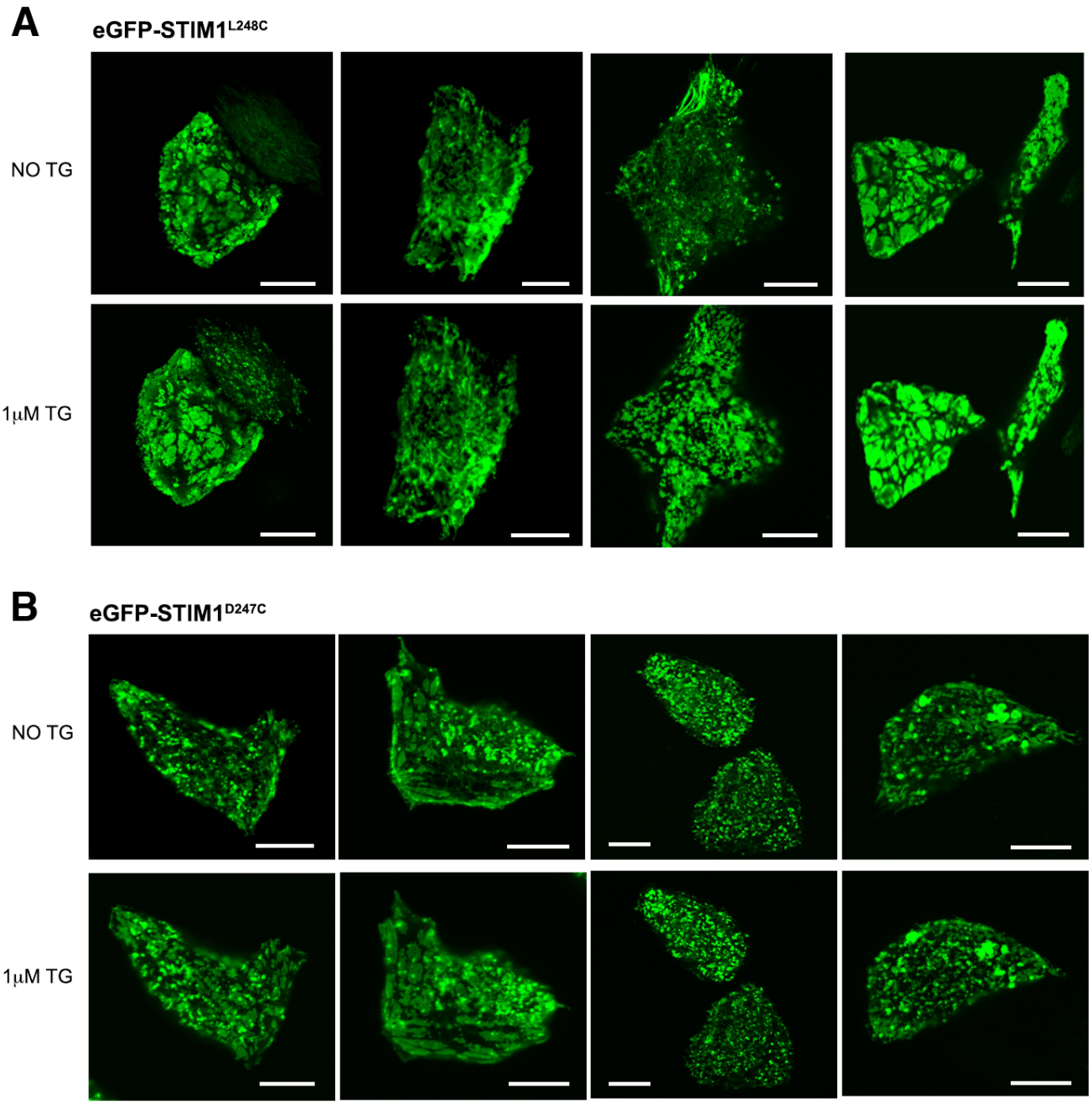

Figure S4. Additional confocal images of HeLa cells expressing the eGFP-STIM1 variants of Figure 4G and 4H. Related to Figure 4. **A,** eGFP-STIM1^{L248C}. **B,** eGFP-STIM1^{D247C}. The images illustrate the range of variation seen with each mutant. Scale bar, 10 μm.

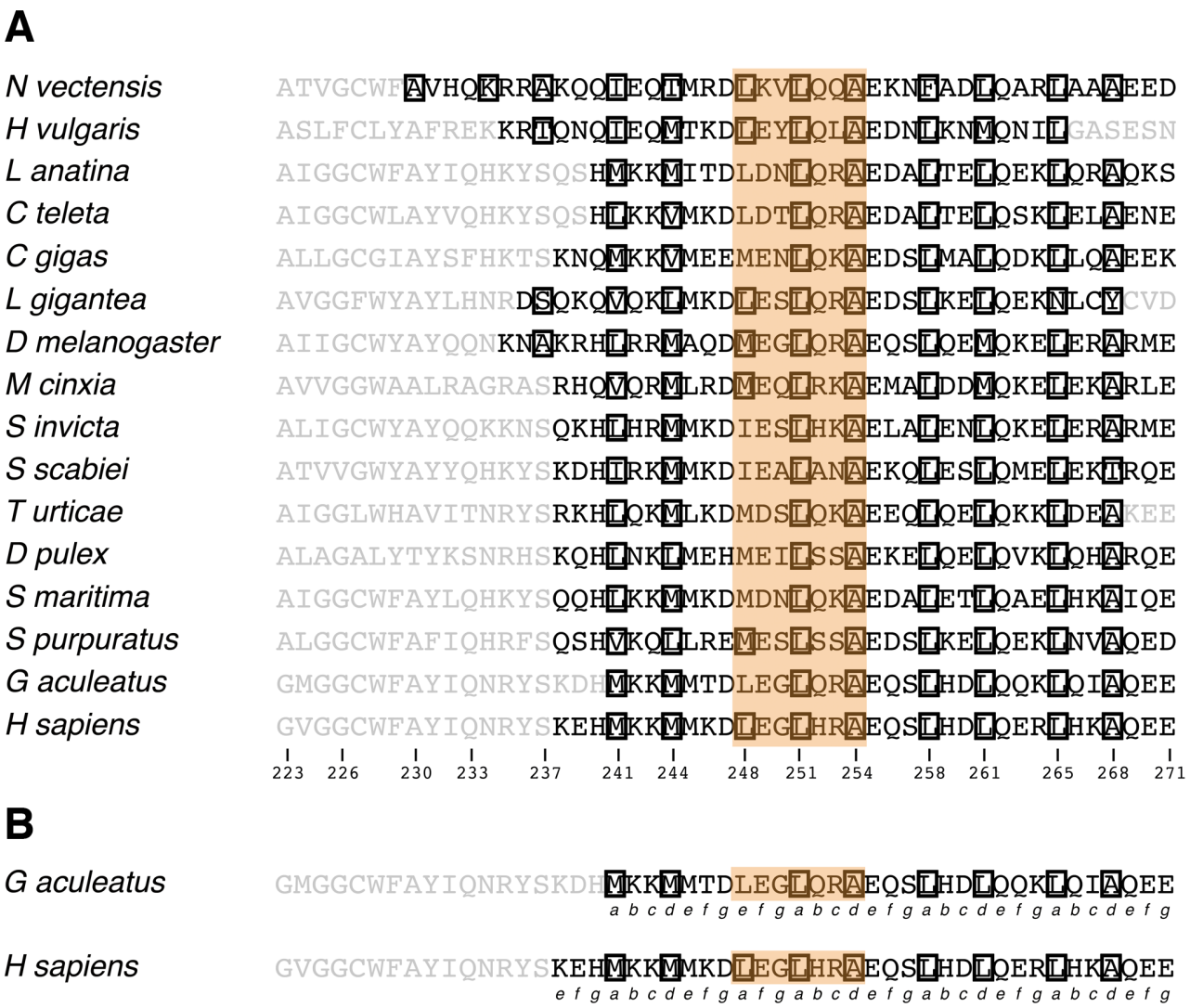

Figure S5. Conservation of predicted STIM coiled-coil organization. Related to Figure 5. **A**, Human STIM1(223–271) aligned with its orthologues from selected species. Residues are shown in black if COILS (input matrix MTIDK, using weights, window length 14) calculates a coiled-coil probability greater than 0.5. Other residues are shown in grey. Boxed residues are assigned to core *a* or *d* positions by COILS. The brown highlight indicates a break in the predicted heptad repeat that is conserved across these species. Transmembrane portions of the proteins, corresponding to human STIM1 residues 223–234, are shown only to anchor the alignment, since COILS is not designed to examine transmembrane helices. Given that a 14-residue window was applied, predictions at residues before human STIM1 residue 237 are not reliable. The species represented are *Nematostella vectensis*, starlet sea anemone; *Hydra vulgaris*, a freshwater polyp; *Lingula anatina*, a lingulid brachiopod; *Capitella teleta*, an annelid worm; *Crassostrea gigas*, pacific oyster; *Lottia gigantea*, owl limpet; *Drosophila melanogaster*, common fruit fly; *Melitaea cinxia*, Glanville fritillary; *Solenopsis invicta*, fire ant; *Sarcoptes scabiei*, itch mite; *Tetranychus urticae*, red spider mite; *Daphnia pulex*, a water flea; *Strigamia maritima*, a centipede; *Strongylocentrotus purpuratus*, purple sea urchin; *Gasterosteus aculeatus*, three-spined stickleback; *Homo sapiens*, human. **B**, Detail of the predictions for *G aculeatus* and *H sapiens* STIM1 with the assigned positions in the heptad register (*a*-*g*) indicated. Note that the respective assignments are *efgefg* and *efgafg* at the discontinuity flanking human STIM1 residue 248, so that the leucine residue in the human protein is judged to be a core residue and the leucine residue in the stickleback protein is not. The ambiguity in assignment extends to the other species represented, even though the corresponding residue is always nonpolar (leucine, isoleucine, or methionine). The commonality is the heptad discontinuity, and whether or not a core residue is predicted is incidental.

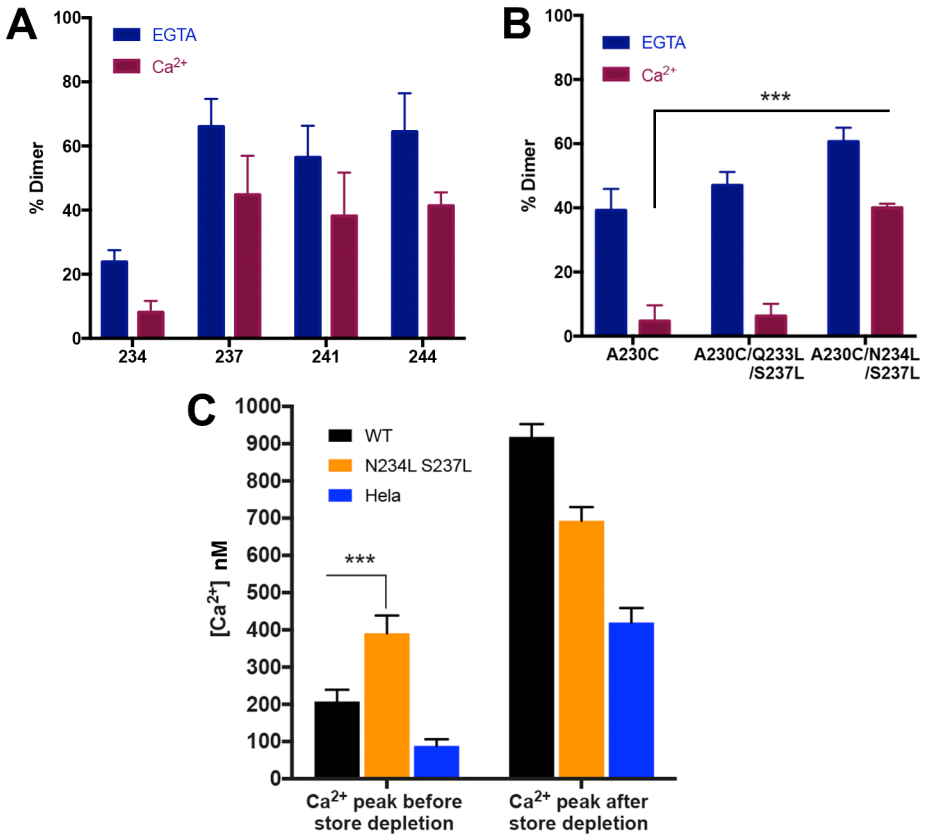

Figure S6. Analysis of mutants in the STIM1 juxtamembrane region. Related to Figures 5 and 6. **A,** Crosslinking efficiencies of STIM1 with single cysteine replacements at residues 234, 237, 241, and 244 in the presence of EGTA (blue) or Ca^{2+} (magenta). Data from three biological replicates. Error bars report SEM. **B,** The effect of pairwise mutations Q233L/S237L and N234L/S237L on the crosslinking efficiency of $STIM1^{A230C}$, in the presence of EGTA (blue) or Ca^{2+} (magenta). Data from three biological replicates. Error bars report SEM. The difference between STIM1(A230C/N234L/S237L) and STIM1(A230C) in the presence of Ca^{2+} is statistically significant ($p < 0.05$, marked with ***). **C**. Quantitation of peak $[Ca^{2+}]_i$ in Figure 6E in store-replete cells after addition of 1 mM $CaCl_2$, and in store-depleted cells after addition of 2 mM $CaCl_2$. For wildtype STIM1, n = 55; STIM1(N234L/S237L), n = 73; and non-transfected HeLa cells, n = 65. Error bars report SEM. Peak $[Ca^{2+}]_i$ for the N234L/S237L mutant in store-replete cells was significantly different from the peak for wildtype STIM1 ($p < 0.05$; marked with ***). Note that data for the control wildtype GFP-STIM1 and non-transfected HeLa cells are repeated from Figure S2C, since all the single-cell Ca^{2+} imaging experiments were performed in one series.

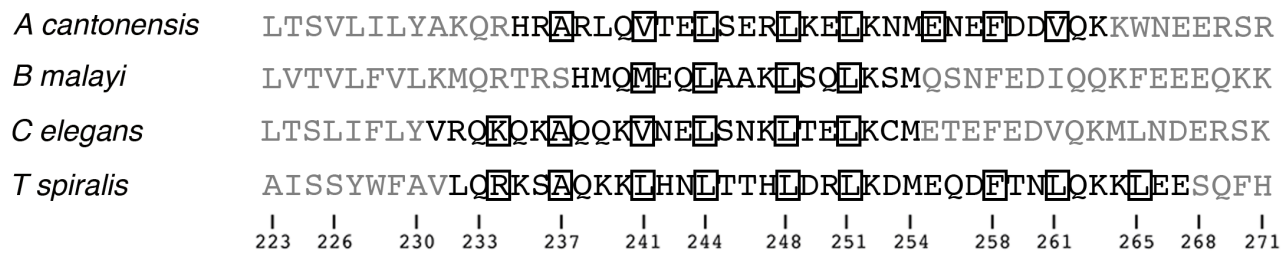

Figure S7. An alternative STIM coiled-coil pattern in nematodes. Related to Figure 5. Partial STIM sequences from four nematode species, displayed as in Figure S5. The species represented are *Angiostrongylus cantonensis*, *Brugia malayi*, *Caenorhabditis elegans*, and *Trichinella spiralis*. Residue numbering below the nematode sequences is that of human STIM1, aligned with the nematode STIM proteins at the transmembrane segment and continued through the region shown without introducing gaps.

Residue	Significant difference in dimer formation (EGTA vs Ca^{2+}) *(p < 0.05)*	Puncta in the resting state	Puncta after activation
F214C	No		
M215C	No		
L216C	Yes		
V217C	Yes		
V218C	No		
S219C	Yes		
I220C	No		
V221C	Yes		
I222C	No		
G223C	Yes		
V224C	No		
G225C	No		
G226C	Yes		
C227	No		
W228C	No		
F229C	No		
A230C	Yes	No	Yes
Y231C	No		
I232C	No		
Q233C	Yes		
N234C	Yes		
S237C	Yes		
A230C/Q233L/S237L	Yes		
A230C/N234L/S237L	Yes		
M241C	Yes		
M244C	Yes		
D247C	No	Yes	Yes
L248C	No	Yes	Yes
E249C	No		
G250C	No		
L251C	Yes	Yes	Yes
H252C	No		
Q233L/S237L		No	Yes
N234L/S237L		Yes	Yes

Table S1. Summary of STIM1 mutants examined and their phenotypes. For each single-cysteine mutant, the table indicates whether crosslinking in the presence of 0.5 mM EGTA differed significantly from crosslinking in the presence of 2 mM Ca^{2+}. Entries for selected mutants note whether GFP-STIM1 puncta were prominent in resting cells and after ER Ca^{2+} store depletion.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids

The expression vector to study STIM1 crosslinking in HeLa cell membranes was derived from a pIRES2-EGFPlHA-STIM1 construct (Wu et al., 2006) provided by Dr. Richard Lewis (Stanford University). The insert encoding HA-tagged human STIM1 was PCR-amplified and cloned between the HindIII and EcoRI sites of pcDNA3.1. The sequence encoding STIM1 residues between the 3xHA tag and residue 58 was deleted and cysteine codons 227 and 437 were replaced by serine codons to obtain the expression construct for cysteine-less human STIM1. The wild-type eGFP-STIM1 construct was made by cloning full-length human STIM1 into pCMV-XL5 vector with the STIM1 signal peptide preceding the eGFP tag. $STIM1^{CT}$ for Tb^{3+}-acceptor energy transfer experiments was expressed from the LBT-$STIM1^{CT}$-C437S/C686 construct in pProEX HTb vector (Zhou et al., 2013). In brief, this construct contains a cDNA encoding the mouse STIM1 cytoplasmic domain (residues 233-685 with the replacement C437S) cloned, with added sequences coding for a lanthanide-binding tag (GGFIDTNNDGWIEGDELLLEEG) at the N terminus and an engineered cysteine at the C-terminus. Mutations specified in the text were introduced into these expression plasmids using the Quikchange site-directed mutagenesis kit (Agilent).

STIM1 expression in mammalian cells

HeLa cells were transfected with the specified constructs using Lipofectamine 2000 and grown under 10% CO_2, in DMEM (HyClone media, Thermo) containing 10% heat-inactivated FBS for 24 hr before imaging or crosslinking experiments.

STIM1 crosslinking assay

HeLa cells (~$2x10^6$ cells) were scraped from the substrate and resuspended in 250 μl dilution buffer containing 25 mM Tris-Cl, pH 7.5, 25 mM NaCl, 12.5 U/ml DNase I (Thermo Fisher; catalog number 90083), protease inhibitors (Roche; catalog number 11873580001, 1 tablet per 50 ml buffer), 0.3 mM DTT, with either 0.5 mM EGTA (low Ca^{2+}) or 2 mM Ca^{2+} (high Ca^{2+}). The cells were lysed at 4°C by passing through a 25G syringe needle 30-35 times. The lysates were centrifuged at 167,000g for 20 min in an airfuge (Beckman Coulter) at 4°C. The pellet, predominantly composed of cellular membranes, was resuspended in 100 μl of resuspension buffer containing 25 mM Tris-Cl pH 7.5, 150 mM NaCl, and 0.3 mM DTT, with either 0.5 mM EGTA or 2 mM Ca^{2+}. DTT in the dilution and resuspension buffers was increased to 0.9 mM to maintain low background crosslinking when cytoplasmic cysteine substitutions were examined. (In some early experiments, the dilution and resuspension buffers were at pH 8.5.) The membrane pellets were resuspended by pipetting and dispensed into the wells of a round-bottom 96-well plate at 4°C. The membranes (in 20 μl) were oxidized by addition of 10 μl iodine working stock (Sigma; Lugol solution, catalog number L6146, diluted 1:10 into resuspension buffer) and incubation for 10 min at 4°C; quenched with 20 μl stop solution containing equal volumes of 60 mM Tris-HCl pH 6.8, 100 mM iodoacetamide, and of 5x nonreducing loading/dye buffer; heated at 55°C for 10 min; and analyzed by SDS-PAGE using 3-8% NuPAGE Bis-Tris gels (Invitrogen). Immunoblotting was performed using rabbit anti-STIM1 (Cell Signaling; catalog number 4916S) and HRP-labeled goat anti-rabbit secondary antibody (Sigma; catalog number A0545) and the blots were developed using ECL substrate (Perkin Elmer). The relative intensity corresponding to the entire area of each band was quantified on images of the blots using

ImageJ software (National Institutes of Health). Error bars report mean ± SEM. Statistical significance was determined using an unpaired, one-tailed Student's t-test.

Ca^{2+} concentration dependence of crosslinking

HeLa cells expressing STIM1(A230C) (~$12x10^6$ cells) were scraped from the substrate and resuspended in Chelex-treated (Chelex-100 resin, Bio-Rad) dilution buffer with no added EGTA or Ca^{2+}. Cellular membranes were prepared as described above and resuspended in 130 μL of Chelex-treated resuspension buffer containing 25 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.3 mM DTT. Equal volumes of membranes were apportioned to twelve wells containing resuspension buffer supplemented to give either a final EGTA concentration of 0.5 mM or final Ca^{2+} concentrations ranging from 0.3 μM to 2 mM. Iodine oxidation, SDS-PAGE analysis, immunoblotting, and quantitation were performed as described above.

Confocal microscopy

HeLa cells, transiently expressing wild-type eGFP-STIM1 or specified variants, were grown on 35-mm glass-bottom dishes (Mat-tek). Imaging was performed using an Olympus Fv10.1 confocal laser scanning microscope with a 60x 1.35 NA oil-immersion objective. Cells were imaged at room temperature, first in Ringer's buffer containing 20 mM HEPES (pH 7.4 with NaOH), 125 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 10 mM D-glucose, for the resting state images. This solution was replaced by a modified Ringer's buffer lacking $CaCl_2$ and containing 1μM thapsigargin (Life Tech) and incubated at least 5 min before obtaining the activated state images.

Coiled coil prediction

Sequences of STIM1 orthologues were retrieved from the Ensembl, Ensembl Metazoa, Wellcome Trust Sanger Institute, or KEGG (Kyoto Encyclopedia of Genes and Genomes) databases. Individual sequences were trimmed to the segments corresponding to *Homo sapiens* STIM1(201-300) or, in cases of less extensive alignment, an equivalent length of sequence flanking the transmembrane region and submitted to the COILS server (Max Planck Institute for Developmental Biology). COILS predictions (input matrix MTIDK, and using weights) are quoted for the narrowest window (window length 14) in order to maximize sensitivity to local sequence features.

Protein purification and fluorescein dye conjugation

Plasmids encoding LBT-STIM1^{CT}-C437S/C686 or its L251S or N234L/S237L variants were transformed into *E. coli* strain Rosetta2-DE3 (EMD Millipore) for efficient protein expression. *The* transformed cells were grown at 37ºC. Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when OD_{600} of the culture reached 0.6, followed by incubation for 12 hours at 16ºC. Harvested cells were resuspended in lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 5% (vol/vol) glycerol, 1 mM β-mecaptoethanol, and Roche protease inhibitors (1 tablet per 50 ml buffer), and sonicated. Cell debris was removed by centrifugation, and the lysate was applied to Ni^{2+}-nitrilotriacetic acid (Ni-NTA)-agarose resin (Qiagen). Bound recombinant proteins were eluted in lysis buffer containing 250 mM imidazole and further purified by gel filtration on a Superdex 200 column (GE Healthcare) in the presence of 2 mM TCEP (tris(2-

carboxyethyl)phosphine). Eluted proteins were concentrated using Amicon Ultra 30kDa concentration devices (EMD Millipore).

Single cysteine-containing LBT-STIM1^{CT}-C437S/C686 variants were labeled with fluorescein-5-maleimide (Thermo) for LRET measurements. Each protein sample, ~100 μM in 50 mM Tris pH 7.5, 150 mM NaCl, was first treated with 10 mM TCEP for at least 1 hr to reduce any disulfide bonds. Excess TCEP was removed by passing the fully reduced protein sample through an Amicon 30kDa device with a quick buffer exchange using 50 mM Tris pH 7.5, 150 mM NaCl buffer. Dye working stock (10-20 mM) was prepared in anhydrous DMF immediately prior to use. A 10-fold molar excess of fluorescein-5-maleimide was added slowly to the protein and incubated at 4°C for 1hr. The labeled protein was passed through a PD-10 desalting column (GE Healthcare) and further concentrated. Labeled proteins yielded a ~0.8-0.9:1 molar ratio of fluorophore to protein, based on measuring the absorbance at 495 nm (fluorescein-5-maleimide, ε_{495} = 68,000 M^{-1} cm^{-1}) and 280 nm (protein, ε_{280} = 34,950 M^{-1} cm^{-1}). Dye conjugation to each protein was further confirmed by resolving the labeled proteins on a 4-12% NuPAGE gel (Life Technologies) and imaging under a broad-range UV light using Gel Doc EZ (Bio-rad).

LRET assay

Luminescence resonance energy transfer (LRET) assays were performed according to the protocol in Zhou et al., 2013. Briefly, the measurements were made at 4 °C in buffer containing 80% (vol/vol) glycerol using a QuantaMaster 40 spectrofluorometer (PTI) equipped with a pulsed xenon excitation source for phosphorescence lifetime measurement. Protein concentration was 300 nM and Tb^{3+}:protein ratio was 0.9:1. The spectra were collected from 450 nm to 650 nm with the excitation set at 280 nm and the slit widths set at 12 nm. Luminescence spectra were collected with a 200-μs delay to eliminate background from scattering and direct excitation of fluorescein.

Single-cell Ca^{2+} influx assay

Single-cell Ca^{2+} imaging was performed using HeLa cells co-transfected with eGFP-STIM1 variants and mCherry-ORAI1 (Sharma, Quintana et al., 2013) and plated on 18-mm coverslips. The cells were loaded with 5 μM Fura-2-acetoxymethyl ester for 45-60 min at 37°C in DMEM containing 0.02% Pluronic F-127 and 10 mM HEPES pH 7.4, washed twice with fresh media, and analysed immediately. Modified Ringer's solution used in this assay consists of 125 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 10 mM D-glucose, and 20 mM HEPES (pH 7.4 with NaOH), with the addition of either 1 mM or 2 mM $CaCl_2$, or 1 μM TG, where indicated. Coverslips were assembled into a chamber on the stage of an Olympus IX 71 microscope equipped with an Olympus UPLSAPO 20×, NA 0.75, objective. Cells were alternately illuminated at 340 nm and 380 nm with the Polychrome V monochromator (TILL Photonics) using ET - Fura2 filter (Chroma Technology Corp, catalog number 79001). The fluorescence emission at $\lambda > 400$ nm (LP 400 nm, Emitter 510/80 nm) were captured with a CCD camera (SensiCam, TILL Imago). Ratio images were recorded at intervals of 4 s. Ca^{2+} concentration was estimated from the relation $[Ca^{2+}]_i = K*(R-R_{min})/(R_{max}-R)$, where the values of K, R_{min}, and R_{max} were determined from an *in situ* calibration of Fura-2 in HeLa cells. Data were analyzed using TILL Vision (TILL Photonics). 3–4 experiments were performed for each condition and error bars report mean ± SEM. Statistical

significance was determined using an unpaired, one-tailed Student's t-test, with Bonferroni adjustment for multiple testing in Figure S2C.