Initial activation of STIM1, the regulator of store-operated calcium entry

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Physiological Ca\(^{2+}\) signaling in T lymphocytes and other cells depends on the STIM-ORAI pathway of store-operated Ca\(^{2+}\) entry. STIM1 and STIM2 are Ca\(^{2+}\) sensors in the endoplasmic reticulum (ER) membrane, with ER-luminal domains that monitor cellular Ca\(^{2+}\) stores and cytoplasmic domains that gate ORAI channels in the plasma membrane. The STIM ER-luminal domain dimerizes or oligomerizes upon dissociation of Ca\(^{2+}\), but the mechanism transmitting activation to the STIM cytoplasmic domain was previously undefined. Using Tb\(^{3+}\)-acceptor energy transfer, we show that dimerization of STIM1 ER-luminal domains causes an extensive conformational change in mouse STIM1 cytoplasmic domains. The conformational change, triggered by apposition of the predicted coiled-coil 1 (CC1) regions, releases the ORAI-activating domains from their interaction with the CC1 regions and allows physical extension of the STIM1 cytoplasmic domain across the gap between ER and plasma membrane and communication with ORAI channels.

The Ca\(^{2+}\) release–activated Ca\(^{2+}\) (CRAC) current of T cells and mast cells has been a classical example of store-operated Ca\(^{2+}\) entry (reviewed in ref. 4). The classical CRAC current is triggered by interaction of the ER Ca\(^{2+}\) sensor STIM1 with the plasma membrane Ca\(^{2+}\) channel ORAI1 (refs. 5–9). Inherited deficits in this pathway in humans or oligomerization of the STIM luminal domain and movement of STIM within the ER to sites where the ER is apposed to the plasma membrane (reviewed in refs. 10–11). There is increasing evidence that the STIM1-ORAI1 pathway and CRAC current also contribute to Ca\(^{2+}\) signaling in other cells.

Store-operated Ca\(^{2+}\) entry is controlled by the ER-resident Ca\(^{2+}\) sensors STIM1 and STIM2 (refs. 5,6,12–16; reviewed in refs. 17–19). Physiological stimulation—through the T-cell receptor, the Fcε receptor or mast cells or various G protein–coupled receptors in other cells—initiates a sequence of ER Ca\(^{2+}\) depletion, dimerization or oligomerization of the STIM luminal domain and movement of STIM within the ER to sites where the ER is apposed to the plasma membrane (reviewed in refs. 10–11). The STIM cytoplasmic domain, through its SOAR(CAD) region, then recruits and directly activates the ORAI channel20–25.

STIM1 at ER–plasma membrane junctions interacts with plasma membrane phosphoinositides, ORAI channels and other plasma membrane proteins20–33. Several of these interactions involve direct physical contact, in which STIM1 and its partner must bridge an ER–plasma membrane separation estimated at 10–25 nm (refs. 34–36). The interaction of a polybasic segment at the extreme C terminus of STIM1 with the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP\(_2\))26–28,37 imposes a crucial geometric constraint. The PIP\(_2\) head group is at the cytoplasmic face of the plasma membrane, and hence the cytoplasmic domain of STIM1 must itself bridge the distance from ER to plasma membrane.

To gain insight into the conformational changes in STIM1 that lead to store-operated Ca\(^{2+}\) entry, we mapped distances within STIM1 by Tb\(^{3+}\)-acceptor energy transfer38,39. The lanthanides Tb\(^{3+}\) and Eu\(^{3+}\) have favorable properties as donor fluorophores for distance measurements, as exemplified in studies of the protein conformations of myosin, the Shaker K\(^+\) channel and RNA polymerase40–43. Moreover, the Tb\(^{3+}\) donor and its acceptor can be incorporated into STIM1 as small probes that are unlikely to cause appreciable changes in STIM1 conformations. Experiments described below indicate that the resting STIM1 cytoplasmic domain (STIM1\(_{\text{CT}}\)) in its preferred conformation does not span the >10 nm separating the ER and plasma membrane, thus implying that STIM1\(_{\text{CT}}\) undergoes a substantial conformational change upon activation. Here we characterize this conformational change in biophysical and functional assays and delineate its mechanism.

RESULTS

‘Activating’ mutations increase STIM1\(_{\text{CT}}\) binding to PIP\(_2\)

Initial targeting of STIM1 to the plasma membrane has been attributed to an interaction of the C-terminal polybasic segment of STIM1 with plasma membrane PIP\(_2\) (refs. 26–28,37). We confirmed that STIM1\(_{\text{CT}}\) binds PIP\(_2\) and phosphatidylinositol 3,4,5-trisphosphate (PIP\(_3\)) in a commercially available array of seven phosphoinositides, phosphatidylinositol and certain other common membrane lipids (Fig. 1a) and binds PIP\(_2\) in nanodiscs (Fig. 1b and Supplementary Fig. 1a).
lipoprotein particles in which a belt of engineered apolipoprotein A1 scaffold protein surrounds a small circular lipid bilayer.

To dissect the targeting of activated STIM1, we examined the ‘activated’ STIM1 variant L251S, which has been shown to interact more effectively with ORAI1 than does wild-type STIM1 (ref. 45). We asked whether it also interacts more strongly with PIP2. We quantified GFP-STIM1CT binding to liposomes, using an equilibrium dialysis assay, in which binding to PIP2-containing liposomes is reflected in excess fluorescence in the PIP2 chamber over that in the control chamber (Fig. 1c and Supplementary Fig. 1b–d). Binding is dependent on the STIM1 polybasic segment and is modestly increased by the L251S replacement (Fig. 1c and Supplementary Fig. 1d). The increased binding correlates with an increased exposure of the C-terminal polybasic segment of STIM1, as reported by the environment-sensitive fluorescence probe IAEDANS introduced directly adjacent to the indicated STIM1 proteins at introduced residue Cys668. Bottom, wavelengths of peak fluorescence emission. The shift of the peak to a shorter wavelength in the wild-type protein is due to partial burial of the fluorophore. Representative of two experiments.

**Figure 1** STIM1-PIP2 interaction. (a) GFP-STIM1CT binding to PIP2 and other lipids arrayed on a lipid strip. GFP-STIM1CT lacking the C-terminal polybasic segment (GFP-STIM1CT-∆K) and GFP alone are controls. LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; S1P, sphingosine-1-phosphate; PA, phosphatidic acid; PS, phosphatidylserine. (b) GFP-STIM1CT binding to nanodiscs.

Left, schematic of the experiment. A fluorescent phospholipid analog (1,2-dioleoyl-sn-glycero-3-phosphaethanolamine-N-(carboxyfluorescein)) serves as donor, and tetramethylrhodamine (TMR) attached to STIM1 CT serves as acceptor. Right, binding measurements on control and PIP2-containing nanodiscs, respectively. In the subtracted spectra, donor fluorescence was removed from the nanodisc + STIM1 CT spectra by subtraction of an appropriately scaled nanodisc-alone spectrum. (c) GFP-STIM1CT binding to liposomes. Top, schematic of the equilibrium dialysis experiment. One chamber was loaded with PIP2-containing liposomes, the other with control liposomes. The initial concentration of STIM1CT in the two chambers was identical. Bottom, excess GFP-STIM1CT recovered from the PIP2 chamber after equilibration, as a fraction of total GFP-STIM1CT in both chambers. WT, wild type. Error bars, s.e.m. for three independent experiments. (d) Exposure of the STIM1 CT C terminus, assessed with an environment-sensitive probe. Top, fluorescence spectra of IAEDANS and of AEDANS covalently attached to the indicated STIM1 CT proteins at introduced residue Cys668. Bottom, wavelengths of peak fluorescence emission. The shift of the peak to a shorter wavelength in the wild-type protein is due to partial burial of the fluorophore. Representative of two experiments.

**Activating mutations cause extension of STIM1CT**

Wild-type STIM1CT binds PIP2 (Fig. 1a–c)28 and ORAI1 (ref. 25) in vitro, and the soluble STIM1CT fragment expressed in cells binds and gates ORAI1 channels20,25,47, results prompting the question of why STIM1 is inactive in resting cells. One hypothesis is that the cytoplasmic domain of STIM1 is retained near the ER until STIM1 is activated.

We used Tb3+-acceptor energy transfer18,39 to measure the distance between STIM1 residue 233—the site at which the cytoplasmic portion of STIM1 emerges from the ER membrane—and the STIM1 C-terminal polybasic tail that interacts with the plasma membrane (Fig. 2a). In the initial experiments, we compared unlabeled wild-type and L251S STIM1CT proteins. The wild-type protein is an elongated dimer that migrates ahead of compact globular proteins of comparable molecular weight in size-exclusion chromatography.25 The L251S variant migrates slightly ahead of wild-type STIM1CT (Fig. 2b and Supplementary Fig. 3), reflecting a more extended conformation rather than increased oligomerization, on the basis of the finding that STIM1CT234–491 with the L251S replacement is a dimer45 and the direct measurements below. The CD spectrum of L251S variant STIM1CT reports no gross change in secondary structure (Supplementary Fig. 3b), but there is a subtle increase in thermal melting at temperatures below 40 °C that may reflect loss of an intramolecular interaction that stabilizes a portion of the α-helical secondary structure in the wild-type protein (Fig. 2c). On the basis of data presented below, it is likely that this is the intramolecular CC1-SOAR(CAD) interaction.

Tb3+ was excited through the lanthanide-binding-tag (LBT) tryptophan antenna by illumination at 280 nm, and energy transfer from Tb3+ to BODIPY FL was assessed in emission spectra gated at 200 μs to eliminate scattered light and fluorescence from direct excitation of acceptor. Protein labeled with Tb3+ donor alone displays the characteristic narrow Tb3+ emission peaks at 490 and 545 nm (Fig. 2d). Protein labeled with both donor and acceptor has, in addition, a broad BODIPY FL emission peak centered at 515 nm superimposed on the Tb3+ peaks (Fig. 2d). Control experiments established that the fluorescence peak at 515 nm is due to Tb3+-BODIPY FL energy transfer within a STIM1CT dimer (Supplementary Note).
Figure 2 Distance measurements in STIM1 cytoplasmic domain. (a) Schematic of STIM1CT donor-acceptor labeling. STIM1CT C437S was engineered with an LBT58 at its N terminus for labeling with donor Tb3+ and an added cysteine residue at its C terminus for labeling with acceptor fluorophore (Supplementary Fig. 6). SOAR, STIM1 Orai activating region; K, C-terminal polybasic tail. (b) Size-exclusion chromatography of wild-type and L251S variant STIM1CT proteins. (c) Thermal melting monitored as change in CD at 222 nm, showing a difference in stability between wild-type STIM1CT and the activated variant L251S at temperatures below 40 °C. (d) Gated luminescence spectra of labeled STIM1CT proteins. Arrow indicates BODIPY FL acceptor emission from the labeled wild-type protein. (e) Luminescence decay of the indicated STIM1CT proteins, followed at the donor wavelength in the absence and presence of acceptor (τ0, τD) and at the acceptor wavelength (τAD). Residuals indicate no systematic deviation of the data from the fitted curves. (f) Cartoon interpreting the results in d and e. The distance measured between residues 233 and 686 implies that the wild-type protein is folded back, whereas the activated L251S protein is extended.

Decay of Tb3+ luminescence in the presence of acceptor is a sensitive test of Tb3+-acceptor energy transfer. LBT-STIM1CT with Tb3+ donor alone exhibits a lifetime (τ) of ~2.32 ms (Fig. 2e), as expected for Tb3+ completely shielded from water by coordination to its LBT ligand48–50. In wild-type STIM1CT labeled with BODIPY FL acceptor, Tb3+ emission exhibits a major quenched component having τ of ~1.21 ms and a second component having τ of ~0.28 ms (Fig. 2e, Table 1 and Supplementary Table 1). A third component with τ of ~2.3 ms represents STIM1 not labeled with acceptor. The sensitized acceptor emission displays components with τ of ~0.98 ms and τ of ~0.21 ms (Fig. 2e, Table 1 and Supplementary Table 1), in agreement with the quenched donor lifetimes. A straightforward interpretation is that there are two populations of wild-type STIM1CT with different donor-acceptor distances of ~4.2 nm and ~3.0 nm (Table 1 and Supplementary Table 1). An alternative dynamic interpretation (Supplementary Note) is that there is a single folded-back conformation with a donor-acceptor distance of ~3.1 nm and that the second time constant reflects conformational changes occurring during the excited-state lifetime of Tb3+ donor (Table 2). In either interpretation, extrapolation to STIM1 in cells suggests that wild-type STIM1CT has a preferred conformation in which the polybasic segment at its C terminus is near the site of STIM1 anchoring in the ER and distant from the plasma membrane.

The gated spectrum of STIM1CT variant L251S labeled with BODIPY FL shows very little sensitized acceptor signal (Fig. 2d) despite efficient labeling with acceptor (Supplementary Fig. 3c). Correspondingly, the donor lifetime in the presence of acceptor is only marginally less than the lifetime of donor alone (Fig. 2e, Table 1 and Supplementary Table 1). To exclude bias introduced by the specific labeling strategy, we made independent measurements on LBT-STIM1CT proteins labeled at the C terminus with a different acceptor fluorophore, BODIPY TMR (Supplementary Fig. 4a–d, Table 1 Luminescence resonance energy transfer (LRET) distance estimates

| Protein | Donor-acceptor pair | R0 (Å) | τDA (µs (%)) | E | R (Å) | τAD (µs (%)) | E | R (Å) |
|---------|---------------------|-------|-------------|---|------|-------------|---|------|
| Wild type | Tb3+-BODIPY FL | 44.0 | 277 (32) | 0.88 | 31.5 | 266 (40) | 0.91 | 29.8 |
| | Tb3+-BODIPY TMR | 54.3 | 1,214 (41) | 0.48 | 44.7 | 977 (60) | 0.58 | 41.7 |
| | Tb3+-GFP | 44.1 | 1,214 (41) | 0.48 | 44.7 | 977 (60) | 0.58 | 41.7 |
| L251S | Tb3+-BODIPY FL | 44.0 | 2,167 (100) | [0.066] | [68.4] | Negligible acceptor emission |
| | Tb3+-BODIPY TMR | 54.3 | 2,208 (100) | [0.048] | [89.2] | Negligible acceptor emission |
| | Tb3+-GFP | 44.1 | 2,164 (100) | [0.067] | [68.4] | Negligible acceptor emission |

Data have been fitted to a model in which energy transfer reports on two populations of STIM1CT with distinct Tb3+-acceptor distances. Tabulated values of τDA and τAD are averages from three experiments. Data from the individual experiments are listed in Supplementary Table 1. Amplitudes (%) of τDA and τAD have been corrected to zero time, and τAD has been corrected for energy-transfer rate as in ref. 59. All energy-transfer efficiency and distance calculations are based on τD of 2.32 ms. E is the efficiency of Tb3+-acceptor energy transfer. R is the corresponding Tb3+-acceptor distance. Bracketed values [] are outside the range for accurate estimation.
Table 2 LRET distance and conformational-change parameters

| Protein       | Donor-acceptor pair | \( R_0 \) (Å) | \( t_1 \) (µs) | \( E \) | \( R \) (Å) | \( \tau_2 \) (µs) | \( F \) |
|---------------|---------------------|----------------|--------------|-------|----------|----------------|------|
| Wild type     | Tb\(^{3+}\)-BODIPY FL | 44.0          | 309          | 0.87   | 32.3     | 1,309         | 0.58 |
|               | Tb\(^{3+}\)-BODIPY TMR | 54.3          | 215          | 0.91   | 37.2     | 716           | 0.63 |

The same data shown in Table 1 have been fitted to a model in which STIM1\(^{CT}\) has two states, a folded-back state in which energy transfer occurs and an extended state in which there is no energy transfer. The folded-back state extends with rate constant \( \alpha \), and the extended state folds back with rate constant \( \beta \). Calculations are based on \( \tau_2 \) of 2.3 ms. \( t_1 \) is the lifetime \( \left( k_{\text{on,exc}} + k_{\text{off}} \right) \) of excited Tb\(^{3+}\) in the folded-back state of STIM1\(^{CT}\). \( E \) is the efficiency of Tb\(^{3+}\)-acceptor energy transfer in the folded-back state. \( R \) is the corresponding Tb\(^{3+}\)-acceptor distance. \( \tau_2 \) is the characteristic time \( (\alpha + \beta)^{-1} \) for exchange between the folded-back and extended states of STIM1\(^{CT}\). \( F \) is the fraction of STIM1\(^{CT}\) in the folded-back state.

Table 1 and Supplementary Table 1) and on STIM1\(^{CT}\) proteins with a C-terminal Tb\(^{3+}\) donor and an N-terminal GFP acceptor (Supplementary Fig. 4e–g, Table 1 and Supplementary Table 1). In each case, the wild-type donor-acceptor distance is 3–4 nm, and the L251S donor-acceptor distance falls beyond the range that can be estimated confidently with these donor-acceptor pairs, results indicating that the distance is increased in the L251S protein by at least 4–5 nm (Fig. 2f).

The other activating STIM1\(^{CT}\) variant, 4EA, also has negligible energy transfer and negligible change in donor lifetime and thus has a large estimated donor-acceptor separation (Supplementary Fig. 4h.i and Supplementary Table 1). Three nonactivating variants of STIM1\(^{CT}\) are similar to wild-type STIM1\(^{CT}\) in their size-exclusion chromatography profiles (Supplementary Fig. 3a) and CD spectra (Supplementary Fig. 3b), and they do not differ appreciably from wild-type protein in the estimated distances between residue 233 and the polybasic tail (Supplementary Table 1).

**CC1-CC1 association induces a conformational change**

The CC1 region, STIM1\(_{233-343}\), is positioned to take part both in bridging the distance between ER and plasma membrane and in propagating an activating conformational change in wild-type STIM1\(^{CT}\). There has been a tacit belief that the basal state of CC1 is a coiled coil\(^{21,53}\), though the primary STIM1 dimerization is through its SOAR(CAD) domain\(^{23,53}\). In fact, CC1 in isolation does not assemble as the predicted coiled coil. The molecular mass of the isolated recombinant CC1 fragment, estimated from size-exclusion chromatography coupled with multichannel light scattering (SEC-MALS), is 12.9 ± 1.1 kDa, comparable to the calculated monomer mass, 14.3 kDa (Fig. 3a). Notably, the CC1 regions are not closely associated in the context of the dimeric STIM1\(^{CT}\) protein either, as established by a lack of intradimer energy transfer between donor and acceptor probes placed at the N termini of individual monomers within a STIM1\(^{CT}\) dimer (Fig. 3b–f).

For these experiments, we introduced a HAP2-peptide docking site for fluorescent \( \alpha \)-bungarotoxin\(^{54}\) at the...
N terminus of STIM1<sup>CT</sup>, coexpressed LBT-STIM1<sup>CT</sup> and HAP-STIM1<sup>CT</sup> and purified heterodimers bearing both a donor and an acceptor tag (Supplementary Note). Control experiments in which the N termini of the heterodimer were artificially brought together by a disulfide link verified that the assay detects CC1-CC1 proximity.

This finding led us to explore the hypothesis that bringing CC1 N termini together might trigger a conformational change. We first investigated the secondary structure of the recombinant CC1 monomer and of a CC1 dimer produced by engineering a disulfide bond between the N termini of CC1 monomers (Fig. 4a and Supplementary Fig. 5a). CD spectroscopy shows that the CC1 monomer has a modest content of α-helix at 4 °C, with a broad melting transition indicative of independent unfolding of parts of CC1 rather than concerted unfolding of a single compact domain (Fig. 4b,c). In contrast, the disulfide-linked dimer possesses increased α-helical secondary structure and, tellingly, exhibits a cooperative melting transition at ~60 °C, when the monomer is already fully denatured (Fig. 4b,c). Thus, physical apposition of the N termini of two CC1 monomers leads to increased CC1-CC1 interactions and a marked stabilization of a portion of CC1 secondary structure.

The effect of the L251S replacement casts light on the probable nature of the conformational change. The substitution does not, by itself, affect the thermal melting curve of the CC1 monomer (Fig. 4d–f). However, it prevents the stabilization of α-helical structure by forced dimerization. Given that L251 is in the core of the predicted coiled coil, and taking into account the fractional helix content stabilized, it is likely that the conformational change is the formation of a partial coiled coil (Fig. 4g).

**CC1-CC1 association reduces interaction with SOAR(CAD)**

A further key observation is that CC1 dimerization reduces the interaction between CC1 and SOAR(CAD), the minimal ORAI-activating domain within STIM1<sup>CT</sup>. We purified monomeric CC1 and dimeric disulfide-linked CC1 and measured the interaction between these proteins and malto-binding protein (MBP)-SOAR<sup>33</sup> immobilized on amyllose resin. Comparison shows less binding of dimeric CC1 to SOAR(CAD) when equal amounts of CC1 protein are incubated (Fig. 4h, lanes 4 and 6). There is no binding to the MBP negative control (Fig. 4h). These findings are consistent with the hypothesis that bringing the N termini of CC1 into apposition within a STIM1<sup>CT</sup> dimer triggers a conformational change in CC1 and releases SOAR(CAD) from its interaction with CC1, thus resulting in extension of STIM1<sup>CT</sup>. The replacement L251S in CC1 likewise weakens the interaction between CC1 and MBP-SOAR (Fig. 4h). However, the activating mutation L251S and the activating conformational change triggered by CC1-CC1 interaction have a similar effect by different detailed mechanisms. On the basis of data shown above (Fig. 4d–f), the L251S replacement releases SOAR(CAD) without triggering the conformational change in CC1, thus suggesting that L251S is not part of the CC1-SOAR(CAD) interface in resting STIM1<sup>CT</sup> or participates in interactions that allosterically stabilize the interface. We propose that the key to STIM1 activation is the release of SOAR(CAD) and the polybasic tail from interactions that tether them near the ER. Active wild-type STIM1 achieves this by sequestering L251 in a CC1-CC1 coiled coil, thereby weakening the interaction with SOAR(CAD). The L251S replacement directly weakens the interaction with SOAR(CAD).

**Intradimer CC1-CC1 association causes extension of STIM1<sup>CT</sup>**

Finally, we tested whether apposition of the N termini in STIM1<sup>CT</sup> can initiate a conformational change that propagates throughout STIM1<sup>CT</sup>, using STIM1<sup>CT</sup> engineered with an LBT at the N terminus and a HAP2 docking site for fluorescent α-bungarotoxin at the C terminus of each monomer (Fig. 5a). An introduced cysteine at the extreme N terminus was either blocked by reaction with
Fig. 5 c
domain is clearly dimeric 25, with its primary dimerization through
extension of STIM1 CT (Fig. 5 c). We conclude that apposition of the N termini of CC1 within a STIM1 CT
dimer triggers a propagating conformational change that results in
extension of STIM1 CT (Fig. 5 d).

DISCUSSION
The initial step in STIM1 activation
Our findings have direct implications for the process of STIM1 activ-
ation and plasma membrane targeting in cells (Fig. 6). We have
established here that CC1 regions do not associate detectably, either
in isolation or when attached to SOAR(CAD). The isolated STIM1 ER-luminal domains also do not interact at Ca2+ concen-
trations typical of the ER in resting cells 13,16. Nevertheless, the STIM1 cytoplasmic domain is clearly dimeric 25, with its primary dimerization through
SOAR(CAD) 23. Therefore we propose that the dimerized SOAR(CAD)
regions are connected to the ER in resting cells by two physically
independent CC1 tethers. Owing to the low density of native STIM1
in the ER, a decrease in luminal Ca2+ will elicit, preferentially, the
association of the paired luminal domains within a STIM1 dimer.
Association of the luminal domains will bring the initial portions of
the CC1 regions into apposition, favor an otherwise weak CC1-CC1
interaction and trigger the physical extension of STIM1 CT and the
enhanced exposure of the polybasic segment that interacts with PIP2.
A similar mechanism is likely to describe activation of STIM2.

The mechanism is roughly the converse of integrin inside-out sig-
signaling 35–37. For integrin β dimers, protein-protein interactions at
the cytoplasmic face of the plasma membrane, exemplified by talin
binding to the integrin β subunit, allow the transmembrane segments
of the α and β subunits to move apart, thus triggering an extension
of the integrin extracellular domains and an increase in affinity for
physiological integrin ligands. The detailed mechanisms of the STIM
and integrin conformational changes are different, and for STIM it
remains to be seen whether the transmembrane segments have a
specific role other than to connect the luminal and cytoplasmic
domains. However, a feature common to STIM1 and integrins is that
the conformational change and clustering steps are distinct. In con-
trast, all previous models for STIM1 activation 15,19,26,45,46,52,53 have
posited a single activation step tied to STIM oligomerization.

STIM-STIM FRET changes detected in cells
An increase in STIM-STIM FRET after store depletion was taken to
indicate that oligomerization beyond the level of resting STIM1 is the
first step in activation 26. This view was supported by evidence that
artificial oligomerization activates STIM1 15 and by later STIM-STIM
FRET experiments 47,52. We have defined an activation mechanism
initiated by the association of paired luminal domains within a pre-
existing STIM dimer, an association that parallels the dimerization
of recombinant STIM1 luminal domains observed in the absence of
Ca2+ in vitro 13,16. It is reasonable to ask whether there is a correlate
in the cellular experiments. Is luminal dimer formation temporally
separable in the cellular assays from higher-order oligomerization?
Is it even detectable?

It can be argued that luminal-domain dimerization has gone
undetected in cellular studies of full-length STIM1. A previous
study showed, using a STIM protein (STIM1-ΔC) consisting of only
the luminal domain and transmembrane segment, that the luminal
domain alone cannot drive oligomerization to the extent observed
with full-length STIM1 (ref. 52). The very small FRET signal from
STIM1-ΔC at rest in those experiments could plausibly come from
luminal-domain monomers, with the larger signal after store deple-
tion reflecting the formation of luminal-domain dimers. In this view,
because the FRET signal from CFP- and YFP-labeled full-length STIM
at rest is already comparable to that from STIM1-ΔC luminal-domain
dimers after store depletion, the initial step of luminal-domain asso-
ciation could be silent. Then the first step detected by FRET would
be SOAR(CAD)-dependent oligomerization.

The high resting FRET signal in the case of full-length STIM1
deserves comment because it has two interpretations leading to

Figure 5 Intradimer CC1-CC1 association triggers extension of the STIM1
cytoplasmic domain. (a) Schematic illustrating the placement of labels,
with an LBT binding Tb3+ at the N terminus of STIM1 CT and a HAP tag
binding Alexa Fluor 488–labeled α-bungarotoxin at the C terminus. To
allow cross-linking in this experiment, we introduced a cysteine residue
at the extreme N terminus of STIM1 CT. (b) Reducing SDS-PAGE analysis
documenting the effectiveness of cross-linking after a 16-h reaction.
IAM, iodoacetamide. (c) CC1-CC1 cross-linking abolishes energy
transfer between N-terminal donor and C-terminal acceptor. Left, gated
fluorescence spectra of the iodoacetamide-blocked and cross-linked
samples. Right, corresponding luminescence decay curves. Tb3+ donor
emission was monitored except in the trace labeled td2 (magenta), for
which acceptor emission was monitored. (d) Cartoon interpreting the
results in e. CC1-CC1 cross-linking, like the L251S mutation, leads to
an extended conformation of STIM1 CT.
different conclusions about the configuration of unlabeled STIM1. In the first possibility, FRET between CFP- and YFP-labeled STIM proteins in resting cells accurately reflects the proximity of the transmembrane segments of the unlabeled STIM dimer in resting cells. This possibility must be taken seriously because the very pronounced conservation of the STIM1 transmembrane region across vertebrates could well indicate preservation of a sequence that allows a specific local rearrangement during activation. However, because we find that the two N termini of STIM1CT dimers in solution are well separated, this interpretation implies that there is an unidentified geometric constraint on full-length STIM1 in cells that does not apply to the isolated STIM1CT dimers. The second possibility is that the CFP and YFP labels in labeled STIM1 are appreciably closer together than are the transmembrane segments of the unlabeled STIM dimer. In that case, store depletion would induce both a substantial relative movement of the luminal domains and SOAR(CAD)-dependent oligomerization in unmodified wild-type STIM1. Both possibilities are represented in Figure 6.

CC1-CC1 interaction
A previous study also documented a CC1-CC1 interaction in an engineered STIM1 protein truncated after CC1 (STIM1-CC1)52. The protein-protein interaction was weak, in agreement with our observations on isolated CC1, stabilizing luminal-domain dimers for immunoprecipitation only in conditions of low Ca2+ and low-ionic-strength buffer. Yet, somewhat unexpectedly, FRET measurements indicated that the interaction resulted in dimerization of the luminal domains in resting cells52. Whereas the previous study52 took this as evidence that CC1 supports the formation of inactive STIM1 oligomers in resting cells, we attribute the CC1-based dimerization in cells to the heightened CC1-CC1 interactions of overexpressed STIM1-CC1 confined to the ER membrane and the absence of competition from SOAR(CAD). Indeed, because loss of Ca2+ favors CC1-CC1 interaction, the converse is true as well, and the Ca2+ dependence of monomer-dimer equilibrium may shift so that the luminal domains of STIM1-CC1 are present as the Ca2+-free dimer even before store depletion.

Earlier views of the activating conformational change
A pioneering study of activating mutations in the STIM1 cytoplasmic domain demonstrated that the 4EA variant of STIM1 is constitutively active46. It found further that artificial recruitment of a long CC1 fragment containing the EEEELE sequence, STIM1238–343, to the plasma membrane inhibited STIM-ORAI communication in a fraction of cells. On the basis of these data, the authors suggested that the acidic sequence interacts under resting conditions with a basic segment of STIM1CT, thus masking the ORAI-interacting SOAR(CAD), and that STIM oligomerization unmasks SOAR(CAD). The specific interaction proposed is at variance with subsequently published structural evidence53 and with the failure of the 382KIKKK386→QIQQQ mutant to cause extension of STIM1CT in the current study, but the notion that STIM oligomerization unmasks SOAR(CAD) has continued to resonate.

Another study characterized further mutations in CC1 and SOAR(CAD), assessed in the context of STIM1233–474 termed ORAI1 activating small fragment or OASF45. Several of these mutations caused a reduction in the FRET signal from a CFP-OASF-YFP protein, thus indicating that the mutated OASF proteins assume a more extended conformation than does wild-type OASF. The conformational change correlated with activation of STIM1 in cases selected for examination because the mutant OASF proteins displayed increased binding to ORAI1 and because introduction of the substitutions into full-length STIM1 led to constitutive activation of ORAI1. On the basis of these findings, the authors proposed that physiological activation of STIM1 triggers oligomerization and conversion of the STIM1 cytoplasmic domain to an extended form45, but they did not address the mechanism linking oligomerization and conformational change. The most potent single-residue changes identified in the FRET assay were substitutions at Leu251 or at Leu248, a neighboring residue predicted to be in the CC1 core, thus providing the first evidence that the initial region of CC1 is a determinant of the STIM1 resting conformation.

The recently published structure of a portion of Caenorhabditis elegans STIM-1 cytoplasmic domain (PDB 3TER) shows an α-helix spanning C. elegans STIM-1 residues 260–276 folded back against the SOAR domain35. This short segment, which corresponds to human STIM1 residues 318–334, was designated the inhibitory helix, though experimental support for the designation is limited to the activated phenotype of the human 4EA variant46 and that of a variant produced by aggressive deletion of human STIM1 residues 310–337 (ref. 53). This interpretation of the structure led to a model in which store depletion causes the STIM1 luminal domain to dimerize or oligomerize, thus bringing about conformational changes in the inhibitory helices and release of SOAR(CAD). This proposal is more specific than the previous model45, but, in focusing on the inhibitory helix, it is likely to reflect only one part of the CC1-SOAR interaction. First, C. elegans CC1 is considerably shorter than mammalian CC1, and the part of C. elegans CC1 resolved in the crystal structure comprises
only the 27 residues immediately N terminal to SOAR. Thus, a stretch of more than 80 residues of mammalian STIM1 CC1 is not represented, including an extensive region flanking Leu251. Second, the human 318E3E322 segment is present in the structure as C. elegans 260HTEME264, but, of these residues, only Gnu264 is seen to make contact with SOAR(CAD). Most tellingly, the presence of the ‘inhibitory helix’ is not sufficient to maintain mammalian STIM1 in its inactive state because STIM1315–346 is fully active46, as is full-length STIM1 with the L251S substitution45.

With our study, these proposals can be updated to incorporate a concrete mechanism in which luminal-domain association leads to CC1–CC1 association and the release of SOAR(CAD) and the STIM1 polybasic tail. Although the precise configuration of CC1 in the inactive STIM1 cytoplasmic domain remains to be defined, it seems likely that CC1 is folded against SOAR(CAD), with several regions including the segment around residues Leu248 and Leu251 contributing to maintenance of the inactive conformation. Our data suggest that CC1 redeployed upon activation as an extended α-helical structure with at least its N-terminal portion forming a coiled coil.

Conclusion
We have shown here that a key step in physiological activation of STIM1 is a conformational change that enables STIM1 to bridge the distance from ER to plasma membrane where it can engage PIP2 and ORAI. The conformational change arises from the association of STIM1 ER-luminal domains and is independent of subsequent STIM oligomerization. Because this conformational rearrangement of the STIM1 dimer involves only two STIM1 Ca2+–binding sites, the steep cooperativity of physiological STIM1 activation14,15 must depend on further oligomerization following the initial activation step. It remains to be determined how higher-order STIM oligomerization is facilitated by the high local density of STIM1 at ER–plasma membrane junctions, by partner proteins and by binding to plasma membrane lipids or ORAI.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
Y.Z. and P.G.H. designed the study. Y.Z. designed engineered proteins, developed the assays and carried out the experiments with assistance from S.R. and S.S. P.S. made a detailed study of STIM1 binding to liposomes. P.M. prepared and characterized liposomes for the binding assays. A.G. contributed recombinant proteins and technical suggestions. P.B.S. and M.I. contributed the SEC-MALS characterization of recombinant CC1. Y.Z., A.R. and P.G.H. analyzed data, with input from the other authors. Y.Z. and P.G.H. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available in the online version of the paper.
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ONLINE METHODS

Engineered proteins. STIM1CT, its fragments CC1, SOAR(CAD) (as the MBP-SOAR fusion protein33) and STIM1CT-AK, STIM1 variants and GFP-STIM1CT variants were expressed in E. coli and purified with standard techniques. Short LBT34 or HAP-tag34 sequences were inserted into the proteins as indicated, for labeling, respectively, with Tb3+ as donor fluorophore or with α-bungarotoxin carrying an acceptor fluorophore. Cysteine residues for covalent labeling or for cross-linking were engineered into cysteine-less STIM1CT C437S or into the CC1 carrying an acceptor fluorophore. Cysteine residues for covalent labeling or for the two conformations in equilibrium.

Distance estimates. Emission-decay curves were fitted to a sum of one, two or three exponentials with Felix GX software supplied with the spectrofluorometer. Energy-transfer efficiency E and donor-acceptor distance R were calculated with the equations for Förster energy transfer39. Because each STIM1CT protein for corresponding to Tb3+-acceptor energy transfer, this conventional treatment resulted in two distance estimates for each case and an estimate of the relative occupancies during the lifetime of excited Tb3+. Details are provided in Supplementary Note. This fitting procedure results in estimates of a single donor-acceptor distance, a rate of exchange between the conformations and the relative occupancies of the two conformations in equilibrium.

STIM1-PIP2 binding. For the nanodisc binding assay, membrane scaffold protein 1D1 (MSP1D1) was purified and assembled into nanodiscs with 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)60. The fluorescent lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein) (PE-CF) was incorporated into the nanodiscs as a donor fluorophore. In control nanodiscs, the DMPC/PE-CF ratio was 79:1. In nanodiscs containing PIP2, the DMPC/PE-CF/PIP2 ratio was 79:1:2. STIM1-CT-MTR was titrated into a nanodisc sample at concentrations up to 2 μM, and STIM1-nanodisc binding was monitored by measurements of PE-CF-sensitized TMR fluorescence emission, with excitation at 450 nm to minimize direct excitation of acceptor.

Binding of GFP-STIM1CT to PIP2-containing liposomes was assessed quantitatively in a two-chamber microdialysis assay. Control liposomes consisted of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) in a 4:1:2 molar ratio. PIP2 liposomes contained additionally 2 mol% PIP2 unless a different PIP2 content was specified. For the assay, a microdialysis cell of 200-μL total capacity was divided into two equal chambers by a cellulose acetate membrane with a nominal molecular-weight cutoff of 300 kDa, permeable to GFP-STIM1CT but not to the substantially larger liposomes. The cell was loaded initially with PIP2 liposomes and GFP-STIM1CT or the indicated GFP-STIM1CT variant (10 nM) in one dialysis chamber, and with control liposomes and the same protein at the same concentration in the other chamber. After equilibration, GFP fluorescence was determined in samples recovered from each chamber and corrected for the scattering signal due to the liposomes, and the fraction of GFP-STIM1CT bound was calculated as (FPIP2 – Fcontrol) / (Fcontrol + FPIP2), where FPIP2 is the fluorescence intensity in the chamber with PIP2 liposomes and Fcontrol is the fluorescence intensity in the control chamber.

CC1-SOAR(CAD) interaction. MBP or MBP-SOAR was immobilized on amyllose resin, incubated for 4 h at 4 °C with 400 μg of the recombinant CC1 protein indicated and washed extensively. Bound protein was analyzed by nonreducing SDS-PAGE. Protein bands stained with Coomassie Brilliant Blue R-250 were quantified with ImageJ (http://rsweb.nih.gov/ij/).

Data analysis and statistics. Error bars indicate mean ± s.e.m., except in the case of CC1 L251S (Fig. 4h), where they indicate the range. Because fitted lifetimes need not be distributed according to a Gaussian function, mean values are reported in Table 1 without an attempt to estimate statistical variability, and the actual fitted values from all experiments are reported in Supplementary Table 1.

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