STIM1/Orai1 coiled-coil interplay in the regulation of store-operated calcium entry

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Orai1 calcium channels in the plasma membrane are activated by stromal interaction molecule-1 (STIM1), an endoplasmic reticulum calcium sensor, to mediate store-operated calcium entry (SOCE). The cytosolic region of STIM1 contains a long putative coiled-coil (CC)1 segment and shorter CC2 and CC3 domains. Here we present solution nuclear magnetic resonance structures of a trypsin-resistant CC1–CC2 fragment in the apo and Orai1-bound states. Each CC1–CC2 subunit forms a U-shaped structure that homodimerizes through antiparallel interactions between equivalent α-helices. The CC2:CC2′ helix pair clamps two identical acidic Orai1 C-terminal helices at opposite ends of a hydrophobic/basic STIM–Orai association pocket. STIM1 mutants disrupting CC1:CC1′ interactions attenuate, while variants promoting CC1 stability spontaneously activate Orai1 currents. CC2 mutations cause remarkable variability in Orai1 activation because of a dual function in binding Orai1 and autoinhibiting STIM1 oligomerization via interactions with CC3. We conclude that SOCE is activated through dynamic interplay between STIM1 and Orai1 helices.
store-operated calcium (Ca\(^{2+}\)) entry (SOCE) is activated in response to cellular stimuli that deplete the sarco/endo-plasmic reticulum (ER) lumen of Ca\(^{2+}\), a prerequisite event that opens exquisitely Ca\(^{2+}\) selective plasma membrane (PM) channels, augmenting cytosolic Ca\(^{2+}\) levels and refilling ER stores (reviewed in Lewis\(^{1}\) and Putney\(^{2}\)). The principal molecular components of SOCE in many cell types include the ER-inserted stromal interaction molecule-1 (STIM1)\(^{3,4}\) and the Orai1 PM channel subunits\(^{5-8}\), which physically interact at ER-PM junctions in a functional Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channel complex (reviewed in Muik et al.\(^{9}\)). CRAC channels have an extremely high selectivity for Ca\(^{2+}\) over monovalent ions (that is, >1,000-fold more selective for Ca\(^{2+}\) than Na\(^{+}\)), a low unitary conductance, a low permeability to large cations (that is, Cs\(^{+}\)) and are regulated by intra- and extracellular Ca\(^{2+}\), conferring a unique functional fingerprint among the Ca\(^{2+}\) channel protein family (reviewed in Prakriya and Lewis\(^{10}\)). SOCE through CRAC channels is integral to myriad signalling pathways in electrically excitable and non-excitable cells, the most obvious coupling to SOCE is integral to myriad signalling pathways in electrically excitable and non-excitable cells, the most obvious role in STIM1 multimerization and/or STIM1:Orai1 interactions was recognized. One symmetric antiparallel CC forms along residues E320 to A331 of each subunit with V324 and A327 side chains occupying the buried ‘a’ and ‘d’ knob positions in a canonical heptad repeat, respectively, that pack into holes made up of four residues that include reciprocating ‘a’ and ‘d’ knobs from the partner helix (Fig. 1d). More extensive left-handed supercoiling is observed in the second symmetric antiparallel CC along with in-line multi-angle light scattering, pull-down experiments, live-cell electrophysiology and fluorometric colocalization studies in the absence of TFE to further substantiate our high-resolution structures and the structure-derived functional models.

We used SOCKET\(^{26}\) to locate the CC interaction network and heptad positions within the CC1\(_{[TM-distal]}-CC2\) structure. Consistent with primary sequence prediction, two key CC interactions were recognized. One symmetric antiparallel CC forms along residues E320 to A331 of each subunit with V324 and A327 side chains occupying the buried ‘a’ and ‘d’ knob positions in a canonical heptad repeat, respectively, that pack into holes made up of four residues that include reciprocating ‘a’ and ‘d’ knobs from the partner helix (Fig. 1d). More extensive left-handed supercoiling is observed in the second symmetric antiparallel CC along with in-line multi-angle light scattering, pull-down experiments, live-cell electrophysiology and fluorescent colocalization studies in the absence of TFE to further substantiate our high-resolution structures and the structure-derived functional models.

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one another, not all four residues making up the hole; see Supplementary Fig. S5a, S5b and S5d for the proximity and orientation of the ‘a’ and ‘d’ side chains.

The side chains (sticks) of the Orai1 C272–292 peptide, which pack into the pocket, are coloured salmon. (f) Cartoon view of the CC(1(TM-distal))-CC2:Orai1 C272-292 structure. α1 Helix (α1); Loop 1 (L1); α2 helix (α2). Comprehensive structural validation was performed (Supplementary Figs S2c–e, S3 and S4). (g) Zoomed view of the SOAP shown in f (broken black boxes). The N-terminal α2 and C-terminal α2’ side chains (sticks) forming one Orai1-binding site are coloured teal. The side chains (sticks) of the Orai1 C272-292 peptide, which pack into the pocket, are coloured salmon. (h) Supercoiling within the α2:Orai1 C272-292 interface (defg/abcdefg/a = 4/7/1). In d,e,h, the helical wheels show the heptad positions with only reciprocating ‘a’ (purple) and ‘d’ (magenta) packing residues adjacent to one another, not all four residues making up the hole; see Supplementary Fig. S5a, S5b and S5d for the proximity and orientation of the ‘a’ and ‘d’ side chains.
Table 1 | NMR statistics for the apo CC1\textsubscript{TM-distal}-CC2 structure ensemble.

| NMR distance and dihedral constraints |  |
|----------------------------------------|---|
| Total NOE distance limits | 2,199 |
| Intraresidue | 578 |
| Sequential: | 504 |
| Medium-range: | 643 |
| 1<|i−j|<5 | 102 |
| Long-range: | 186 ± 2 |
| Hydrogen bonds | 118 ± 2 |
| Total dihedral angle restraints |  |
| | 67 ± 2 |
| | 67 ± 2 |

| Violations (mean ± s.d.$^{\dagger}$) |  |
|----------------------------------------|---|
| Number of distance violations > 0.3 Å | 0.10 ± 0.45 |
| Number of dihedral violations > 5° | 0.00 ± 0.00 |
| Max. distance constraint violation (Å) | 0.24 ± 0.03 |
| Max. dihedral angle violation (°) | 3.94 ± 0.41 |
| Distance constraint rmsd (Å) | 0.02 ± 0.04 |
| Dihedral angle constraint rmsd (°) | 0.55 ± 0.04 |

| Idealized geometry deviations (mean ± s.d.$^{\ddagger}$) |  |
|----------------------------------------|---|
| Bond length rmsd (Å) | 0.01 ± 0.00 |
| Bond angle rmsd (°) | 1.35 ± 0.03 |
| Improper rmsd (°) | 1.42 ± 0.08 |

| Ramachandran statistics (% of residues) |  |
|----------------------------------------|---|
| Most favourable regions | 94.7 |
| Allowed regions | 98.5 |
| Generously allowed regions | 1.5 |
| Disallowed regions | 0.0 |

| Average pairwise rmsd (Å)$^{\S}$ |  |
|----------------------------------------|---|
| Heavy (to lowest energy) | 1.53 ± 0.25 |
| Backbone (to lowest energy) | 1.14 ± 0.29 |

| CC, coiled-coil, Max., maximum; NMR, nuclear magnetic resonance; NOE, Nuclear Overhauser effect; rmsd, root mean square deviation. |
|---|
| $^{\dagger}$Based on Cα-Cβ chemical-shift index and amide-exchange data. |
| $^{\ddagger}$Calculated from the chemical shifts in TALOS.$^{49}$ |
| $^{\S}$Calculated for the ensemble using PROCHECK-NMR.$^{57}$ |

The Y342 and A343 residues of L1 are positioned to interact with the C-terminal end of the α2$'$ helix, and although A376', A380' and I383' residues are in close proximity to L1 (Supplementary Fig. S5c), the L1:α2$'$ contacts appear only marginally stable as the α2 C-terminal region shows high internal dynamics (Supplementary Fig. S6). Consistent with the increased structural variability (that is, relatively higher backbone root mean square deviation (rmsd)) at the N- and C-termini of the ensemble (Supplementary Fig. S6a), $^{15}$N-$^{1}$$H$ heteronuclear NOE measurements show decreased saturated/ reference peak intensity ratios, indicating greater mobility on the ~ns timescales compared with the central α1 and α2 regions, which exhibit the highest ratios (Supplementary Fig. S6b,c). The most rigid backbone regions within apo CC1\textsubscript{TM-distal}-CC2 are congruent with the α1:α1' and α2:α2' CC configurations observed in dimer stabilization. The increased dynamics at the termini reflect regions of conformational instability that are apt to undergo or initiate structural changes related to regulatory mechanisms.

STIM1 CC1\textsubscript{TM-distal}-CC2:Orai C272–292 complex structure. STIM1 binding to both the Orai N- and C-terminal domains are required for recruitment and gating at ER–PM junctions; however, the interaction with the Orai C-terminal domain occurs with higher apparent affinity than the N-terminal domain, as deletion of the C-terminal domain completely abolishes channel activity but not the ability to co-cluster with STIM1, whereas deletion of the C-terminal domain eliminates the interaction.$^{9,16,19,27–29}$ We engineered a glutathione-S-transferase (GST)-Orai C272–292 fusion (that is, Orai residues 272–292) (Fig. 1b), which exhibited resistance to degradation and was soluble to >3 mM after GST cleavage. NMR chemical-shift perturbation data demonstrated Orai C272–292 binding to STIM1 CC1\textsubscript{TM-distal}-CC2. Specifically, the CC1\textsubscript{TM-distal}-CC2 $^{1}$$H$–$^{15}$N-HSQC (heteronuclear single quantum coherence) spectrum exhibited residue-specific chemical-shift perturbations upon addition of unlabelled Orai C272–292 up to 2 mM (Supplementary Fig. S7a). Similarly, we observed chemical-shift changes in a titration of unlabelled CC1\textsubscript{TM-distal}-CC2 into uniformly $^{15}$N-Orai C272–292 (Supplementary Fig. S7b). The NMR spectra of the mixed samples exhibited no peak doubling, indicating that this dimeric STIM1 fragment maintains two equivalent Orai C272–292-binding sites with single magnetic environments at all residue positions. The greatest chemical-shift changes observed in the Orai C272–292 $^{1}$$H$–$^{15}$N-HSQC spectrum occurred on both the N- and C-terminal halves of the peptide and included residues E272, L273, N274, A277, E278, A280, R281, H288 and R289 (Supplementary Fig. S7c). The ability of CC1\textsubscript{TM-distal}-CC2 to bind Orai C272–292 in the absence of TFE was confirmed using pull-down experiments (Supplementary Fig. S7d; Supplementary Discussion).

We used the perturbation data to estimate the concentration of protein or peptide required to saturate our NMR samples (Supplementary Fig. S7e) and proceeded to determine the solution structure of STIM1 CC1\textsubscript{TM-distal}-CC2 in complex with Orai C272–292 using NOE-derived distance restraints (Table 2). The dimeric STIM1 protein equivalently binds two Orai C272–292 molecules (Fig. 1f); furthermore, the Orai C272–292-binding pocket (that is, SOAP) within the STIM1 CC1\textsubscript{TM-distal}-CC2 complex is formed primarily by side chains of the α2 helices; specifically, P344, L347, L351, H355 and V359 from the N-terminal side of α2 on one subunit and Y362', K366', A369', L373', A376', A380' and I383' from the C-terminal side of α2' on the second subunit form a predominantly hydrophobic groove that accommodates one Orai C272–292 molecule (Fig. 1g). Side chains from two sides of one Orai α-helix pack against opposite faces of the SOAP. The N274, A277, R281, Q285 and R289 Orai residues interact with the N-terminal α2 surface, while L273, L276, A280, Q283 and L286 residues interact with the C-terminal side of the second α2' subunit within the SOAP (Fig. 1g).

Analysis of the complex structure using SOCKET$^{26}$ revealed the absence of supercoiling at the α1:α1' and α2:α2' interfaces. Instead, two new parallel CC interactions are formed along L276 to D287 within each Orai C272–292 domain and residues A369 to A380 within each of the STIM1 α2 helices (Fig. 1h). The α' and 'd' positions of the α2 helices are occupied by L373 and A376, respectively, while A280 and Q283 residues of Orai are in the α' and 'd' positions, respectively (Fig. 1h). The supercoiling occurs through uninterrupted heptad repeats in 12 residue stretches of the STIM1 α2 and Orai C-terminal helices.

STIM1 conformational changes upon Orai C272–292 binding. Along with the α' and 'd' interactions, the α1:α1' interface exhibits central V324:L328' hydrophobic side-chain packing in the absence of Orai C272–292 binding (Fig. 2a). In complex with Orai C272–292, the α1 helices undergo a registry shift, facilitating...
The surface of apo CCl1(TM-distal)-CC2 structure is markedly basic in amino-acid composition; however, the α1:α1' supercoiling facilitates the formation of a negative patch by orienting E318, E319, E320 and E322 residues of the two subunits close to one another (Fig. 2g). This acidic region is not interfered with the α2 C-terminal basic stretch of amino acids (that is, K382, K384, K386 and R387) previously suggested to keep STIM1 in a quiescent state through an electrostatic clamp10; however, longer-range charge attractions between these structural features may help position the α2 C-terminal region close to L1'. The acidic stretch of residues on each α1 helix (that is, E318, E319, E320 and E322) is closer to the identical stretch on the second subunit in the Orai C272–292-bound versus the apo state, creating a large contiguous negative patch on the α1:α1' face (Fig. 2h). The surface of the SOAP is primarily positive created by H355, K363', K366', K377', K382', K384', K385', K386' and R387' of the α2 helices (Fig. 2h). This SOAP basicity is complementary to the predominantly negative surface of each Orai C272–292 helix created by acidic residues including E272, E275, E278, D284, D287 and D291 and likely contributes to the stability of the complex (Fig. 2h).

Several aspects of the CCl1(TM-distal)-CC2 dimer backbone dynamics are consistent with the structural changes accompanying the binding of two Orai C272–292 peptides in an antiparallel manner (Supplementary Fig. S8a). First, the increased variability in the 15N-{1H} NOE ratios compared with the apo state can be attributed to relatively weak peptide binding, promoting conformational exchange (Supplementary Fig. S8b). Second, the decrease in the backbone dynamics of the N-terminal region (that is, residues 312–316) is consistent with the registry shift moving the α1:α1' interaction site closer to the termini, thereby decreasing mobility of the termini (Supplementary Fig. S8b). Most importantly, the attenuated backbone mobility compared with the apo state, observed at the N- and C-terminal regions of α2 that create the SOAP, correlates with the Orai C272–292 binding sites (Supplementary Fig. S8b,c).

### STIM1 CCl1(TM-distal)-CC2 structural integrity in SOCE.

What is the correlation between CCl1(TM-distal)-CC2 structural integrity and the ability of STIM1 to activate Orai channels? We answered this question using patch-clamp electrophysiology experiments of HEK-293 cells co-overexpressing full-length monomeric Cherry fluorescent protein (mCh)-STIM1 and YFP-Orai (Fig. 3a) after in vitro assessments of the effect of mutations (Supplementary Fig. S9a) on the folding, stability and dimerization propensity of CCl1(TM-distal)-CC2 (Supplementary Table S3; Supplementary Discussion). Cells co-overexpressing the STIM1 E318Q/E319Q/E320Q/E322Q (that is, 4EQ) charge-neutralizing quadruple mutant, which stabilized the α1:α1' interface and promoted dimerization of the CCl1(TM-distal)-CC2 region (Supplementary Figs S1h, S10a and S11a), exhibited spontaneous inward-rectifying currents at the time of patch pipette break-in (Fig. 3b,i). The inward currents were blocked by La3+, confirming a CRAC channel-dependent entry. Disruption of the α1:α1' interaction and destabilization of the CCl1(TM-distal)-CC2 dimer via the 324P mutation (Supplementary Figs S1h, S10b and S11b) significantly attenuated the maximal inward-rectifying current compared with the wild type after passive ER Ca2+ store depletion with EGTA (Fig. 3c,i), underscoring the importance of an intact α1:α1' interface to transduce a conformation that maximally activates CRAC entry. These data suggest that the α1 structural integrity plays an important role in the quiescent-to-active STIM1 transduction efficiency; furthermore, the meta-stability of α1 conferred by the acidic cluster ensures that the region is readily susceptible to allosteric changes.
Consistent with this notion, the 4EQ mutant that increased the CC1\textsubscript{TM-distal}-CC2 dimer propensity enhanced the CC1\textsubscript{TM-distal}-CC2 interaction with Orai1 C\textsubscript{272–292}, while the V324P mutation that disrupted CC1\textsubscript{TM-distal}-CC2 dimerization diminished the CC1\textsubscript{TM-distal}-CC2:Orai1 C\textsubscript{272–292} interaction (Supplementary Fig. S7d).

Our structures revealed centrally located, double Tyr residues within the ε2 helices; hence, the Y361K/Y362K double Tyr mutation was engineered to fully eliminate the thermodynamically favourable propensity for Tyr-Tyr stacking\textsuperscript{30,31} at this crossover point. The Y361K/Y362K double mutation completely precluded the ability of full-length STIM1 to induce an activation-competent STIM1 conformation. The possibility that the Y361K/Y362K double mutant causes gross unfolding of the resting SOAR dimer cannot be discounted. Additionally, the Y361K mutation could potentially disrupt contacts with ε1; however, since STIM1-Y361K is capable of eliciting maximal Orai currents (Fig. 3d,i), this scenario is unlikely.

Our NMR structures revealed that the C-terminal region of ε2 hinges away from L1' by >30° upon Orai1 C\textsubscript{272–292} binding compared with the apo state (Fig. 2f). Interestingly, C-terminal ε2 mutations that may affect CC2 homomerization as well as heteromerization with the Orai1 C-terminal domain produce somewhat disparate CRAC current profiles. Cells co-overexpressing Orai1 and STIM1 A380R show constitutive CRAC entry characterized by maximal inward-current density at the time of break-in and La\textsuperscript{3+} inhibition (Fig. 3e,i). The preservation of the CC1\textsubscript{TM-distal}-CC2:Orai1 C\textsubscript{272–292} interaction observed with the A380R and I383R mutants (Supplementary Fig. S7d) is congruent particularly with the A380R-induced constitutive CRAC activation, and since both the mutations only marginally affect the stability, folding and dimer propensity of CC1\textsubscript{TM-distal}-CC2 (Supplementary Figs S1h,S10d,e and S11d,e), these residues must be involved in other aspects of the signalling process, such as an intramolecular transition in STIM1 prerequisite to adopting an Orai1 activation-competent state\textsuperscript{21,32} and/or Orai1 N-terminal interactions. It should be noted that A380 and I383 residues are relatively distant (that is, ~20–25 Å) from the Orai N-terminus in our assembled channel-coupling model (see below); however, additional high-resolution data are required to completely rule out interactions with the Orai1 N-terminus.

**Figure 2** | CC1\textsubscript{TM-distal}-CC2 structural changes associated with Orai1 C\textsubscript{272–292} binding. (a) V324 and L328' side-chain (green sticks) proximity in the apo ε1:ε1' interface. The distance between the V324-C\textsubscript{g} and L328'-C\textsubscript{g} atoms is indicated (broken black line). (b) V324 and L328' side-chain (green sticks) proximity in the ε1:ε1' interface of the CC1\textsubscript{TM-distal}-CC2:Orai1 C\textsubscript{272–292} structure. The distance between the V324-C\textsubscript{g} and L328'-C\textsubscript{g} atoms is indicated (red broken line). (c) Central pivot point of the apo ε2:ε2' interface. The intermolecular Y362-OH (green sticks) distance (broken black line) is shown. (d) Central ε2:ε2' pivot point in the CC1\textsubscript{TM-distal}-CC2:Orai1 C\textsubscript{272–292} structure. The intermolecular Y362-OH (green sticks) distance (broken red line) is shown. The Orai L273-C\textsubscript{g} (brown sticks) to Y362-OH and intermolecular Y361-OH (green sticks) distances (broken black lines) are also shown. (e) Distance between the ε2 helical axes in the apo (blue cartoons; broken black line) versus Orai C\textsubscript{272–292} bound (white cartoons; broken red line) states. (f) Angular opening (broken curved line) of the C-terminal ε2 region upon Orai C\textsubscript{272–292} binding. The apo ε2 helices (blue cartoons) are shown relative to Orai-bound ε2 helices (white cartoons). (g) Surface electrostatics of the CC1\textsubscript{TM-distal}-CC2 structure. The distinct ε1:ε1' acidic and the C-terminal basic residues are labelled. (h) Electrostatic complementarity between CC1\textsubscript{TM-distal}-CC2 and Orai C\textsubscript{272–292} derived from the complex structure. The basic rim residues of the SOAP (broken black circle) and acidic patches are labelled. The Orai C\textsubscript{272–292} peptides (yellow cartoons) and the acidic side chains are shown (red space fill). The electrostatic gradient in g,h is from −2 (red) to +2 (blue) kT/e.
Disruption of the surface basicity at the C-terminal α2 region via the K382E/K384E/K385E/K386E (that is, 4KE) quadruple mutation, which only marginally alters the stability, folding and dimerization of CCI[TM-distal]-CC2 compared with the wild type (Supplementary Figs S1h,S10f and S11f), completely abrogates CCI[TM-distal]-CC2-Orai1 C272–292 interactions (Supplementary Fig. S7d) as well as inward CRAC current (Fig. 3e,i), advocating a role for electrostatic complementarity in STIM1-Orai1 coupling and the activation of CRAC entry. Taken together, these data demonstrate that the α2L1' interface minimally affects the CCI[TM-distal]-CC2 dimer structure, consistent with the high backbone mobility of the C-terminal α2 region (Supplementary Fig. S6); however, residues of the C-terminal α2 region are involved in multiple facets of CRAC channel activation, including coupling to Orai1 and aspects currently unresolved in available high-resolution structures (Supplementary Discussion).

**STIM1 CCI[TM-distal]-CC2-Orai1 C272–292 interface in SOCE.** We employed live-cell patch-clamp experiments in conjunction with rationalized mutagenesis (Supplementary Fig. S9b) to validate the structurally elucidated STIM1-Orai1 interactions. First, we separately introduced L347R and L351R mutations into full-length STIM1, aimed at disrupting the N-terminal α2 hydrophobic face of the SOAP, while maintaining the electrostatic complementarity to Orai1. Cells co-overexpressing Orai1 and STIM1 with these substitutions exhibited no inward-rectifying current upon passive Ca²⁺ store depletion (Fig. 3f,i); furthermore, OASF-L347R and L351R mutants had no ability to colocalize with Orai1 at the PM (Supplementary Fig. S9c,d). We separately engineered R281A, L286S and R289A mutations in full-length Orai1, as these amino acids were identified to make contacts within the SOAP and have not been previously studied (Supplementary Discussion). Co-overexpression of each of these full-length Orai1 mutants with wild-type STIM1 resulted in significantly attenuated maximal Orai1 currents after store depletion (Fig. 3g,i).

Three anionic Glu amino acids (that is, E272, E275 and E278) are located on the N-terminal half and three Asp residues (that is, D284, D287 and D291) are present on the C-terminal half of Orai1 C272–292. Cells co-overexpressing an Orai1 E272A/E275A/E278A triple mutant with wild-type STIM1 did not appreciably affect the current–density profiles compared with the wild-type Orai1 (Fig. 3h,i). However, the Orai1 D284A/D287A/D291A triple mutant significantly attenuated the maximum inward currents versus wild type (Fig. 3h,i). Hence, the acidic D284, D287, D291 triplet within Orai1 C272–292 plays a dominant role in complementing the electropositive surface of the SOAP and is consistent with a close proximity to the basic C-terminal α2 stretch (that is, K382', K384', K385' and K386') elucidated in the complex structure. All Orai1 mutations that inhibited CRAC currents also attenuated colocalization with STIM1-OASF at the PM (Supplementary Fig. S9c,e), suggesting reduced STIM1-Orai1 coupling. Hence, both non-polar and polar forces promote Orai1 C272–292 binding to CCI[TM-distal]-CC2.

**Discussion.** The present structures of apo and Orai1 C272–292-complexed STIM1 CCI[TM-distal]-CC2 complemented by *in vitro* biophysical and live-cell functional analyses have illuminated several mechanistic features of STIM1-Orai1 signalling. First, the nature of the CCI:CCI' interaction is coupled to the efficiency of Orai1 activation by STIM1. CCI[TM-distal]-CCI[TM-distal] interactions that favour a dimer conformation promote coupling to and activation of Orai1, as evidenced by the 4EQ mutant, which increases dimer propensity and stability of CCI[TM-distal]-CC2, resulting in constitutive Orai1 currents in the full-length STIM1.
context (Supplementary Table S3). A STIM1 E318A/E319A/E320A/E322A (that is, 4EA) mutant, previously shown to constitutively activate CRAC channels in Orai1 co-overexpressing COS-7 cells, likely has a similar mode of action as 4EQ. Of the 4E residues in the 4EQ and 4EA mutants (that is, E318, E319, E320 and E322), only E322 (that is, E264 in Caenorhabditis elegans) contacts the CAD/SOAR region based on the C. elegans STIM crystal structure (3TER.pdb); furthermore, deletion of residues 318–322 containing the 4E stretch does not alter the Ca$^{2+}$-store-dependent wild-type behaviour of STIM1 in terms of puncta formation and Orai1 activation. Taken together, these data suggest that the mechanism by which the 4EQ and 4EA mutations constitutively activate Orai1 is distinct from the effects of the 310–337 deletion mutant16, which probably eliminates all folding constraints on the CAD/SOAR region, resulting in constitutively Orai1 activation. Disruption of CC1[TM-distal] via a helix-breaking Y324P mutation destabilizes CC1[TM-distal]–CC2 and promotes monomer, resulting in less efficient Orai1 activation (Supplementary Table S3; Supplementary Discussion).

Second, each STIM1 dimer forms two identical Orai1 C272–292 binding sites through an antiparallel configuration of the β2 helices, positioning N- and C-terminal β2 residues on opposite faces of each binding site within the SOAP (Fig. 1g). All previously identified STIM1 cytosolic fragments, which activate Orai channels, preserve the SOAP (that is, CAD19, SOAR18, OASF17 and CC boundary 9 (ref. 20)). This structural organization is corroborated by several previous studies demonstrating that the efficacy of CRAC channel activation by CAD/SOAR in live cells is sensitive to truncations and mutations at the N-terminus of β2. For example, the L347A/Q348A mutation abolishes SOAR colocalization and co-immuno-precipitation with Orai1 (refs 18,24). Moreover, L273S and L276D mutations in the Orai1 C-terminal domain strongly inhibit CRAC entry16,18,34, and our data reveal that these non-polar residues contact opposite sides within one hydrophobic cleft of the SOAP (Fig. 1g). Further, STIM1 fragments that exclude ~6–8 residues from the N-terminal β2 helix (that is, fragments encompassing residues 350–450 or 350–448) inefficiently induce CRAC entry compared with CAD and SOAR fragments, which retain these residues18,19. Mutations on the β2 C-terminal face of the SOAP can also disrupt Orai1 activation. The OASF L373S mutant fails to induce constitutive Orai1 currents concomitant with a decrease in STIM1–Orai1 colocalization27, STIM1 A376K constitutively forms puncta at resting ER Ca$^{2+}$, with no ability to recruit Orai1 to these ER–PM sites35. The non-polar interactions within the SOAP are reinforced by charge complementarity (that is, STIM1 K382, K384, K385, K386 with Orai1 D284, D287, D291), an interaction mechanism first proposed by Baird and co-workers36,37 who showed that K384Q/K385Q/K386Q or Orai1 D291Q/D294Q/D297Q/D298Q/D301Q/D304Q/D307Q/D310Q mutations can abolish STIM1–Orai1 colocalization and SOCE.

Foremost, our structural data reveal that the supercoiling changes from the apo CC1[TM-distal]–CC2 to the Orai1 C272–292 complexed state, suggesting a dynamic CC interplay is involved in the activation of Orai1 channels. In the apo STIM1 state, CC1[TM-distal]–CC2 exhibits intersubunit (that is, β1:β1’ and β2:β2’) CC interactions, constricting the SOAP within the β2 CC interface. Complexed with Orai1 C272–292, the homotypic intersubunit supercoiling is alleviated and, instead, STIM1 engages in heterotypic β2:Orai1 C272–292 supercoiling. Structural alignment of CC2 from the SOAP structure24 with CC2 of the apo CC1[TM-distal]–CC2 and CC1[TM-distal]–CC2–Orai1 C272–292 structures shows that CC3 of SOAR occupies the same position as CC2 (that is, α2) of the apo structure (Supplementary Fig. S12a) and Orai1 C272–292 within the complex structure (Supplementary Fig. S12b). The analogous positions allude to an elegant CC-switching mechanism involved in CRAC activation (see below). The STIM1 CC3 region drives the oligomerization of the cytosolic domains necessary to activate Orai1 channels, as previous work showed that STIM1 residues 420–450 are required for the enhancement of STIM1 homomerization as well as CRAC activation17, and residues 392–448 are essential for stabilizing higher-order STIM1 oligomers after ER Ca$^{2+}$ store depletion35. Indispensable CC3-mediated oligomerization is also congruent with reports showing that C-terminal truncations decrease the intramolecular FRET between CAD molecules35 and markedly disrupt the ability of SOAR fragments to activate Orai1 (ref. 18). We speculate that the SOAP crystalline state represents a quiescent, non-Orai1-coupled structure, which may undergo a conformational change such that the CC2:CC2’ orientation mimics that observed in the apo CC1[TM-distal]–CC2 solution structure prior to coupling with and gating of the Orai channels. The basis for this speculation includes the facts that SOAR CC2:CC3 interface mutations can cause an OASF extension, which has been linked with Orai1 activation32, and the apo CC1[TM-distal]–CC2 structure is more analogous to the Orai1 C272–292 complexed state and appears better primed for the Orai C-terminal interaction.

Recently, Drosophila melanogaster Orai was crystallized in a hexameric conformation with individual dimers stabilized through antiparallel CC interactions between the cytosolic C-terminal helices25. Remarkably, the antiparallel configuration of the Orai C-terminal helices in D. melanogaster appears primed for an interaction with STIM, highly analogous to the one elucidated in our human STIM1 CC1[TM-distal]–CC2–Orai1 C272–292 complex structure (Fig. 4a). The interhelix angle between the two interacting D. melanogaster Orai C-terminal helices (that is, 152°) is very similar to the angle observed in our human complex structure (that is, 136°) (Fig. 4a). Docking of three dimer structures of CC1[TM-distal]–CC2–Orai1 C272–292 on the Orai hexamer by structurally aligning the common Orai C-terminal regions (Fig. 4b,c) confirms the noteworthy structural compatibility. Specifically, the N-termini of CC1[TM-distal]–CC2, indicating the positions of CC1[TM-proximal], are directed away from the cytosolic channel face towards the ER membrane; further, the C-termini of CC1[TM-distal]–CC2, marking the locations of CC3, are adjacent to the C-termini from neighbouring CC1[TM-distal]–CC2 dimers and are compatible with homotypic oligomerization of CC1[TM-distal]–CC2 via CC3 interactions into a required functional stoichiometry (Fig. 4b).

Our solution NMR structures and functional data in conjunction with recent CRAC component crystal structures convey a CC-switching mechanism in the transition of the cytosolic STIM1 region from a quiescent to an Orai1 activation-competent state. We propose that intramolecular CC2:CC3 supercoiling (Supplementary Fig. S12c; Supplementary Discussion) contributes to the suppression of CC3-mediated STIM1 assembly and internal autoinhibition of intermolecular CC2:CC2’ as well as CC2–Orai1 C272–292 interactions, since CC3 occupies the same space as CC2’ and Orai1 C272–292 after structural alignment of CC2 (Fig. 5a; Supplementary Fig. S12a,b). Store depletion drives self-association of CC1[TM-proximal], which induces a cytosolic domain rearrangement28 that we believe favours the β1:β1’ CC interaction elucidated in our apo structure. Accessing this conformation releases the intramolecular CC2:CC3 supercoiling, permitting the structurally elucidated intermolecular CC2:CC2’ supercoiling (Fig. 5a). Critically, the antiparallel β2:β2’ arrangement forms the SOAP, which ultimately facilitates β2:Orai1 C272–292 supercoiling upon complexation. The released CC3 module promotes the assembly of STIM1 dimers into higher-order oligomers such as hexamers (Fig. 5b) or tetramers.
Recent studies show that concatenated hexameric Orai1 is permeable to Na\(^+\) and Ca\(^2+\) ions in addition to Ca\(^2+\), in contrast to the highly Ca\(^2+\) selective concatenated Orai1 tetramer and native Orai1 channels. Importantly, our STIM1 oligomerization model predicts that CC1 and CC2 mediate intradimer supercoiling essential to maintain the dimer unit building blocks of the STIM1-activation apparatus, while CC3 coordinates the assembly of these units to present a tetramer, hexamer or any other even-numbered multimeric structure to the cytoplasmic face of the Orai1 proteins for coupling and activation.

We suggest that the binding-induced arrangement of the Orai1 C-termini within each STIM1 dimer is a key step in eliciting an Orai1 subunit reorientation necessary for channel-gating. At ER–PM junctions, two Orai1 C-termini couple to the \(\alpha_2\alpha_2\) SOAP formed by each STIM1 dimer, stabilized by CC2/Orai1 C272–292 supercoiling (Fig. 5a,b; Supplementary Fig. S13a). The \(D.\ melanogaster\) Orai C-terminal helices are inwardly twisted compared with the human Orai1 C272–292 following structural alignment of the homologous residues (Supplementary Fig. S13b). Hence, we speculate that the interaction not only recruits Orai1 to the ER–PM junctions but also causes an allosteric movement in the Orai1 TM helices, an outward angular rotation of both TM4 helices within the CC1\(_{\text{TM-distal}}\)-CC2 complex were structurally aligned through sequentially similar regions in each \(D.\ melanogaster\) Orai dimer. CC3 locations are inferred from the position of the \(\alpha_2\) C-termini. The Orai dimer unit is indicated (broken black box).

**Methods**

**Protein expression and purification.** The 6 \times His-tagged STIM1 constructs (that is, residues 234–474/491 (OASF and OASF\_ext, respectively), residues 312–387 (renamed CC1\(_{\text{TM-distal}}\)-CC2; CC1\(_{\text{TM-distal}}\)-CC2-CC3) (Fig. 1a)) were cloned into PET-28a using the Ndel/XhoI sites. STIM proteins were expressed in BL21-AE3 Escherichia coli at 24°C overnight and pullout of crude lysate solubilized with 6 M guanidine HCl, 20 mM TRIS, 7.5 mM \(\beta\)-mercaptoethanol (BME), pH 8 using Ni-NTA agarose (Qiagen Inc.). Subsequently, the agarose resin was washed in 6 M urea, 20 mM TRIS, 1 mM dithiothreitol, pH 8, and protein was eluted using wash buffer supplemented with 350 mM imidazole. After dialysis (that is, 6,000–8,000 molecular weight cutoff; Spectra/Por membrane) and overnight digestion with bovine thrombin at 4°C (12.5 units mg\(^{-1}\) protein), gel filtration chromatography (that is, Superdex S200 10/300 GL; GE Healthcare Corp.) in 20 mM TRIS, 300 mM NaCl, 1 mM DTT, pH 7.3 and 8 (that is, pH 8 for OASF, OASF\_ext and CC1\(_{\text{TM-distal}}\)-CC2-CC3; pH 7.3 for CC1\(_{\text{TM-distal}}\)-CC2) was employed as a final purification step. Protein homogeneity to > 95% was confirmed with Coomassie blue-stained SDS–PAGE. The commercially available QuikChange kit (Stratagene Inc.) was used for the introduction of point mutations, and all mutagenesis was confirmed with the help of DNA sequencing (AOGT Corp.).

The Orai C272–292 fragment (Fig. 1b) cloned in pGEX-4T1 using the BamHI/EcoRI sites was expressed in BL21-AE3 E. coli at 37°C for 4 h. Cell lysate was collected after resuspension in phosphate-buffered saline, probe-sonication on ice and incubation in the presence of 1% (v/v) Triton X100 and 2 mM BME at 4°C. The GST fusions were pulled out of the lysate using GST Sepharose 4B beads (GE Healthcare Inc.), washed with phosphate-buffered saline in the presence of 2 mM BME and eluted with 20 mM TRIS, 150 mM NaCl, 2 mM DTT and 10 mM reduced glutathione, pH 7.5. Subsequently, the GST was cleaved by overnight incubation with bovine thrombin (that is, 15 units mg\(^{-1}\) fusion protein, 20 mM TRIS, 150 mM NaCl, pH 7.5, 4°C). Free Orai C272–292 was collected and concentrated using ultrafiltration, sequentially through 10,000 Da and 2,000 Da molecular weight cutoff membranes. The mass of intact Orai C272–292 was confirmed using mass spectrometry.
CC1[TM-distal]-CC2:Orai1 C272–292 supercoiling (elucidated herein) and via Orai1 N-terminal domain (currently unresolved).

Yellow cylinders represent the Orai1 channel with each dimer unit separated by a broken line. Channels are closed in the absence of STIM1 binding; open the channel pore. CC1 refers to the CC1[TM-distal] region; CC1[TM-proximal] interactions known to have a vital role in the quiescent-to-active shift. CC interactions are indicated (solid black lines). The assembly of STIM1 dimer units occurs through CC3:CC3 indicated (broken black circle). STIM1 domains from individual subunits are cyan and light blue, while the Orai1 C-terminal domains are yellow.

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Solution NMR spectroscopy. NMR experiments were performed on 600 and 800 MHz Avance (Bruker Biospin Ltd.) spectrometers equipped with cryogenic, triple-resonance probes (1.7 and 5 mm, respectively). Unless otherwise stated, the NMR buffer was 20 mM TRIS, 17.5% (v/v) TFE, pH 5.5. Backbone data were acquired using standard 1H-15N-HSQC, 1H-13C-HNCO40, CBCA(CO)NH41 and HNCA42 experiments, while side-chain data were obtained using 1H-13C-HSQC, 1H(CO)NH-TOCSY43 and (H)C(CO)NH-TOCSY43,44 experiments.

Solution small angle X-ray and DLS. To verify that the overall conformation of CC1[TM-distal]-CC2 is not influenced by TFE, we collected solution SAXS and DLS data. X-Ray scattering measurements were carried out at the 12-ID-C beamline of the Advanced Photon Source, Argonne National Laboratory (Argonne, IL). The energy of the X-ray beam was 18 keV (wavelength, \( \lambda = 0.6888 \) Å). The sample to charge-coupled device detector (MAR research, Hamburg) distances were adjusted to achieve scattering q values of 0.006 < q < 0.28 Å\(^{-1}\), where q = (4\pi/\(\lambda\))sin(θ/2), and θ is the scattering angle. Twenty-two dimensional images were recorded for each buffer or sample using a flow cell at 4°C, with an accumulated exposure time of 1-2 s to reduce radiation damage. No radiation damage was observed as confirmed by the absence of systematic signal changes in sequentially collected X-ray scattering images. DLS measurements were made on a temperature-controlled setup.
Dynapro Titan (Wyatt Technology) using a 12 µl, 8.5 mm centre height quartz cuvette. The incident laser light was 827.4 nm, the scattering angle was 90° and the temperature was 20°C. Analysis was performed using the accompanying Dynamics software version 6.7.7.9 (Wyatt Technology).

**Solution NMR-binding assays.** Equilibrium dissociation constants ($K_d$) were calculated from plots of chemical-shift perturbation versus $CC1_{[3D-danala]}$-CC2 concentration in monomers. All $^{1}H$-$^{15}$N- and $^{1}H$-$^{13}$C NMR titration experiments were performed on a Varian Unity Plus 500 at ambient temperature. Data were acquired in 90 min increments. NMR titration data were fitted to a 1:1 binding model using the program NMRPipe (Molecular Simulations Inc.)

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Author contributions
P.B.S., G.M.G.-S., M.L. R.S. and C.R. determined and analysed the structures. P.B.S. and L.Z. performed the in vitro biophysical studies and related molecular biology. R.S., M.F. and M.M. performed the live-cell electrophysiology studies and related molecular biology. P.B.S. and M.I. wrote the manuscript with input from all other authors. M.I. and C.R. supervised work in their respective laboratories and jointly coordinated the project.

Additional information
Accession codes: The structure coordinates are deposited in the RCSB protein data bank (pdb codes: 2MAJ.pdb and 2MAK.pdb). NMR chemical shifts and structure constraints are deposited in the BMRB (accession codes: 19362 and 19363).

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