

# A Basic Sequence in STIM1 Promotes $\text{Ca}^{2+}$ Influx by Interacting with the C-Terminal Acidic Coiled Coil of Orai1<sup>†</sup>

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**ABSTRACT:** Store-operated  $\text{Ca}^{2+}$  entry (SOCE) is a ubiquitous signaling process in eukaryotic cells in which the endoplasmic reticulum (ER)-localized  $\text{Ca}^{2+}$  sensor, STIM1, activates the plasma membrane-localized  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  (CRAC) channel, Orai1, in response to emptying of ER  $\text{Ca}^{2+}$  stores. In efforts to understand this activation mechanism, we recently identified an acidic coiled-coil region in the C-terminus of Orai1 that contributes to physical association between these two proteins, as measured by fluorescence resonance energy transfer, and is necessary for  $\text{Ca}^{2+}$  influx, as measured by an intracellular  $\text{Ca}^{2+}$  indicator. Here, we present evidence that a positively charged sequence of STIM1 in its CRAC channel activating domain, human residues 384–386, is necessary for activation of SOCE, most likely because this sequence interacts directly with the acidic coiled coil of Orai1 to gate  $\text{Ca}^{2+}$  influx. We find that mutation to remove positive charges in these residues in STIM1 prevents its stimulated association with wild-type Orai1. However, association does occur between this mutant STIM1 and Orai1 that is mutated to remove negative charges in its C-terminal coiled coil, indicating that other structural features are sufficient for this interaction. Despite this physical association, we find that thapsigargin fails to activate SOCE following coexpression of mutant STIM1 with either wild type or mutant Orai1, implicating STIM1 residues 384–386 in transmission of the  $\text{Ca}^{2+}$  gating signal to Orai1 following store depletion.

$\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  (CRAC) channels play a key role in  $\text{Ca}^{2+}$  influx and mobilization in numerous cellular responses. These channels are activated by coupling of the ER-store  $\text{Ca}^{2+}$  sensor STIM1 (1, 2) to the plasma membrane channel protein Orai1, also known as CRACM1 (3, 4). Recently, we showed that six acidic residues in a putative coiled coil at the C-terminus of Orai1 contribute to STIM1–Orai1 interactions in response to  $\text{Ca}^{2+}$  store depletion (5). The mutant lacking these acidic residues, Orai1 $\Delta$ DE, exhibits a clustered distribution at the plasma membrane in the absence of and following stimulation with thapsigargin. Similar clustering of wild-type (wt) Orai1 in unstimulated cells is caused by sphingosine derivatives that flip to the inner leaflet of the plasma membrane (5), suggesting that electrostatic repulsion prevents homo-oligomerization of unliganded Orai1. Stimulated oligomerization of Orai1 that is induced by clustered STIM1 is known to play a role in channel activation (6, 7).

On the basis of these results, we hypothesized that electrostatic attraction and the resulting neutralization of the acidic coiled coil in Orai1 by basic residues in STIM1 facilitate oligomerization of Orai1 necessary for activation of the complex in response to store depletion. We therefore sought to identify a sequence on STIM1 that could mediate this charge neutralization. We first considered that the C-terminal polybasic sequence of STIM1 could be

responsible for this coupling. However, our findings and those of others (8–10) demonstrated that this region is not necessary for robust SOCE or for formation of the STIM1–Orai1 complex. Furthermore, STIM1 residues 342–448 (human numbering), named the CRAC activating domain (CAD), were recently identified as a minimal region of STIM1 sufficient to activate  $\text{Ca}^{2+}$  mobilization (ref 9; see also refs 8 and 10). Motivated by this information, we identified a short, highly conserved, basic sequence within CAD, STIM1(382–387), as a potential region for interaction with the Orai1 acidic coiled coil. We used fluorescence resonance energy transfer (FRET) and  $\text{Ca}^{2+}$  mobilization measurements of mutants to show that these residues are key for functional coupling of STIM1 and Orai1.

## MATERIALS AND METHODS

**Constructs and Cloning.** STIM1 K(384–6)Q-mRFP cDNA was generated using the Stratagene Quickchange site-directed mutagenesis kit on our previously constructed STIM1-mRFP vector (5). The following primers were used: 5'-GCCAAGGAGGGGGCTGAGAAGATACAACAGCAGAGAAACACACTCTTTGGCACCTTCCAC-3' and its reverse complement. Preparation of the AcGFP-Orai1 and AcGFP-Orai1 $\Delta$ DE constructs was previously described (5).

**Cell Culture.** RBL-2H3 mast cells were cultured in minimal essential medium supplemented with 1  $\mu\text{g}/\text{mL}$  gentamicin and 20% (v/v) fetal bovine serum. In preparation for transfection and imaging, cells were plated at 25% confluence in 35 mm MatTek wells. After approximately 20 h, cells were transfected with either mutant or wild-type versions of STIM1-mRFP and AcGFP-Orai1. These constructs were transfected using either Geneporter (Genlantis) or Fugene HD (Roche) per the manufacturer's

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instructions, with modifications to enhance transfection efficiency in the RBL cells previously described (11). Cells were imaged 24 h after transfection.

COS7 cells were cultured as monolayers in Dulbecco's modified Eagle's medium supplemented with 1  $\mu$ g/mL gentamicin and 10% (v/v) fetal bovine serum. In preparation for imaging, cells were harvested and transfected with wt or mutant STIM1-mRFP and Orai1 using Fugene HD according to the manufacturer's instructions. Cells were transfected with nonfluorescent derivatives of Orai1 and mRFP-labeled derivatives of STIM1 as a marker for positive transfectants. Cells were imaged 24 h after transfection.

**Confocal Microscopy.** Immediately prior to being imaged, RBL-2H3 cells were washed and incubated for 5 min at 37 °C in 2.5 mL of buffered salt solution (BSS) [135 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 5.6 mM glucose, 1 mg/mL BSA, and 20 mM HEPES (pH 7.4)]. Cells were then imaged on a Leica TCS SP2 confocal microscope with a Leica APO 63 $\times$  dipping objective. Cells were excited at 488 and 543 nm, with laser intensity and phototube sensitivity adjusted to maximize the signal-to-noise ratio. Fluorescence emission was monitored at 495–540 and 555–675 nm. All live cell imaging was conducted at 37 °C. After observing the resting state of the cells, we stimulated them for the time specified by the addition of 0.5 mL of BSS containing thapsigargin (final concentration of 150 nM). Leica Confocal was used during the experiment to acquire images, and ImageJ was used postacquisition to prepare composite micrographs by uniform contrast adjustment.

**Ca<sup>2+</sup> Measurements.** Immediately prior to being imaged, COS7 cells were incubated with 0.9  $\mu$ M fluo-4-AM (Molecular Probes) for 10 min in BSS containing 0.5 mM sulfinpyrazone. Cells were then washed and resuspended in BSS with sulfinpyrazone. Fields of cells were imaged before and during thapsigargin stimulation under the same conditions and settings that were described for multicolor imaging. For single-cell time courses, images were collected every 15 s for 2.5 min prior to stimulation and for 10 min after thapsigargin stimulation. For quantitation of pre- and poststimulation Ca<sup>2+</sup> levels, several fields of cells were imaged before stimulation and 10 min after thapsigargin stimulation. Cytoplasmic Fluo-4 fluorescence was quantified using ImageJ. For each dish of cells, the average prestimulation fluorescence in untransfected cells was normalized to 1.0 to account for small differences in dye loading between multiple samples.

**FRET Measurements.** RBL-2H3 cells were imaged for FRET as previously described (5). Briefly, cells expressing a combination of mutant or wild-type STIM1-mRFP and AcGFP-Orai1 were imaged at 10 s intervals with excitation at 476 nm to minimize spectral bleedthrough. An automated mask-drawing algorithm in Matlab was used to select the pixels of interest at the plasma membrane for every time point using fluorescence from AcGFP as the template. The integrated red and green intensities under the mask were adjusted by background subtraction and correction for spectral bleedthrough. We report the ratio of the corrected red fluorescence to corrected green fluorescence as FRET (5).

## RESULTS AND DISCUSSION

The majority of the STIM1 CAD sequence shows homology to coiled-coil regions from a wide array of proteins [based on a homology search using the Position-Specific Iterated Basic Local Alignment Search Tool, PSI-BLAST, provided by the National

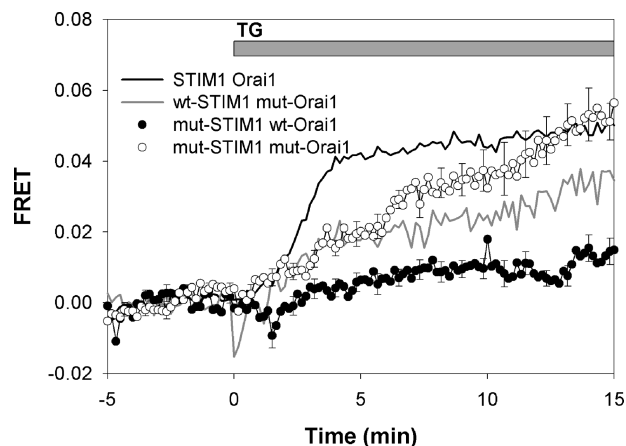


FIGURE 1: Thapsigargin-mediated FRET. Thapsigargin (150 nM)-stimulated FRET between STIM1-mRFP and AcGFP-Orai1 constructs in RBL mast cells: wtSTIM1-mRFP and AcGFP-wtOrai1 (black line), wtSTIM1-mRFP and AcGFP-Orai1 $\Delta$ DE (mut-Orai1; gray line), STIM1 K(384–6)Q-mRFP (mut-STIM1) and AcGFP-Orai1 $\Delta$ DE (○), and STIM1 K(384–6)Q-mRFP and AcGFP-wtOrai1 (●). Error bars show the standard error of the mean for 12–18 cells from four or five different experiments, and every fifth error bar is shown for the sake of clarity.

Institutes of Health (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), but residues 382–395 have a lower degree of sequence homology to other proteins, suggesting a specialized function of this segment. We therefore mutated the three central lysines in this sequence (384–386) to glutamines to eliminate their positive charge while maintaining the hydrophilic and steric character of the endogenous sequence [STIM1 K(384–6)Q]. Our modeling predicts that the flanking basic residues (K382 and R387) in this sequence are involved in intramolecular salt bridges with neighboring acidic amino acids and therefore might be structurally important. The sequence we selected is similar to a slightly longer sequence of homologous, positively charged amino acids in the CAD region of the *Bombyx mori* (silkworm) homologue of STIM1 that was recently shown to be important for translocation of STIM1 to plasma membrane punctae (12). It is very close to residue L373 that was identified by Fischau et al. as being critical both for the interaction of STIM1 and Orai1 and for Ca<sup>2+</sup> mobilization (13). We speculate that the STIM1 L373S mutation may disrupt the coiled-coil structure in the segment of STIM1 containing the basic residues we identified, thereby preventing proper engagement with Orai1.

We previously developed a microscopy-based FRET assay to monitor the physical association of wtSTIM1-mRFP with AcGFP-wtOrai1 and AcGFP-Orai1 $\Delta$ DE (5). Using this method, we measured FRET between STIM1 K(384–6)Q-mRFP and AcGFP-wtOrai1 transfected into RBL mast cells. We found that these mutations in STIM1 almost completely prevent its association with wtOrai1 that is normally observed following stimulation by thapsigargin (Figure 1). In unstimulated cells, STIM1 K(384–6)Q-mRFP is expressed like wtSTIM1-mRFP throughout the ER (Figure 2A,C) (5). However, unlike wtSTIM1-mRFP, this mutant fails to undergo thapsigargin-stimulated coclustering with AcGFP-wtOrai1 in plasma membrane punctae (Figure 2D), consistent with the FRET results. Also shown in Figure 2A is the punctate distribution of AcGFP-Orai1 $\Delta$ DE at the plasma membrane of this unstimulated RBL cell as previously described (5).

To evaluate the functional capacity of this STIM1 mutant, we monitored Ca<sup>2+</sup> mobilization in COS7 cells using Fluo-4 as

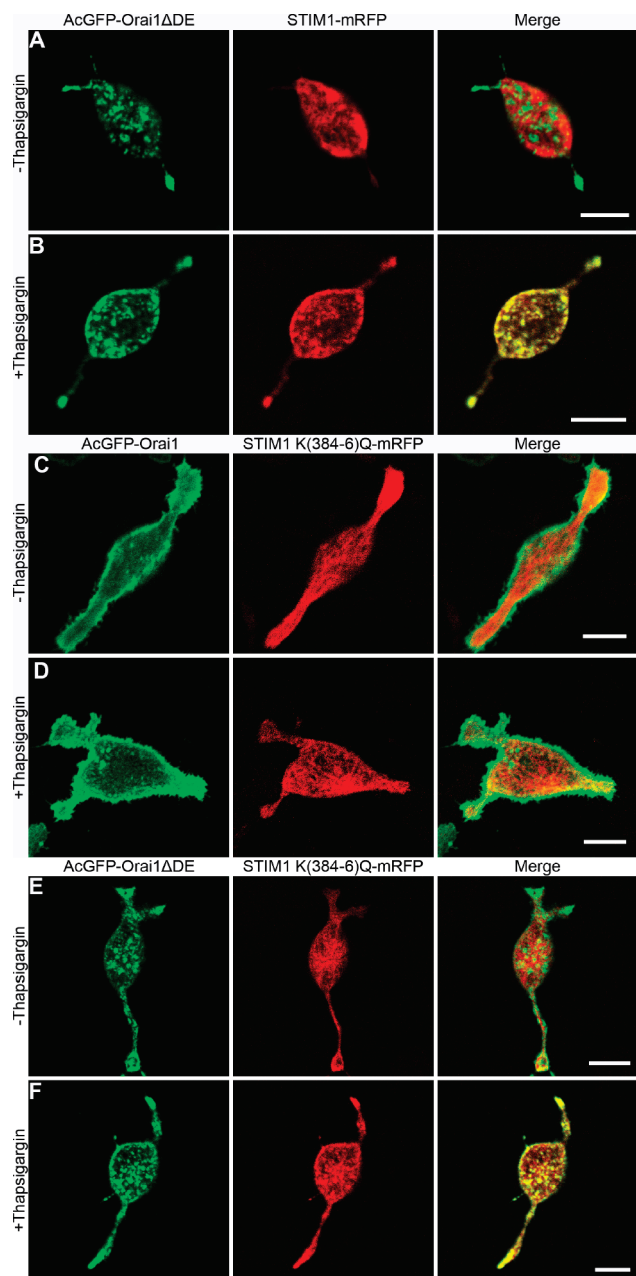


FIGURE 2: Cellular distributions of Orail and STIM1. Ventral surface confocal microscopy images of transfected RBL cells showing distributions of (A and B) AcGFP-OrailΔDE (green) and wtSTIM1-mRFP (red), (C and D) AcGFP-wtOrail (green) and STIM1 K(384-6)Q-mRFP (red), or (E and F) AcGFP-OrailΔDE and STIM1 K(384-6)Q-mRFP in the absence (A, C, and E) or presence (B, D, and F) of 150 nM thapsigargin for 30 min at 37 °C. Scale bars represent 10  $\mu$ m.

previously described (5). COS7 cells exhibit little or no endogenous SOCE, but they exhibit a robust response to thapsigargin when wtSTIM1-mRFP and wtOrail are coexpressed (Figure 3A, blue circles). In contrast, when STIM1 K(384-6)Q-mRFP is coexpressed with wtOrail at similar levels, no thapsigargin-stimulated SOCE is observed (Figure 3A, green triangles). Results in Figures 1 and 3 indicate that STIM1 lysine residues 384-386 are necessary for coupling to Orail and consequent activation of SOCE.

These observations, together with our previous results, led us to hypothesize that basic residues 384-386 of STIM1 directly engage the acidic coiled coil at the C-terminus of Orail, involving

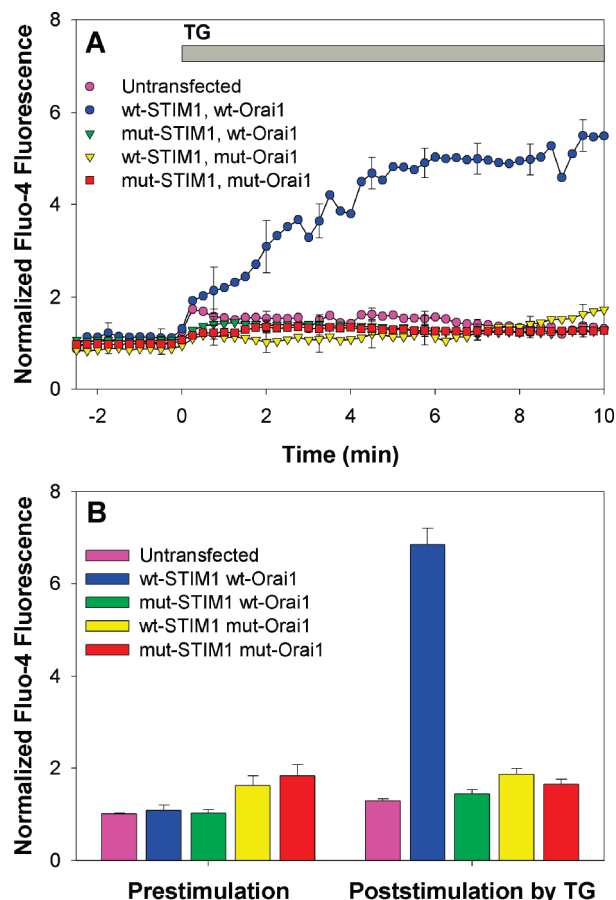
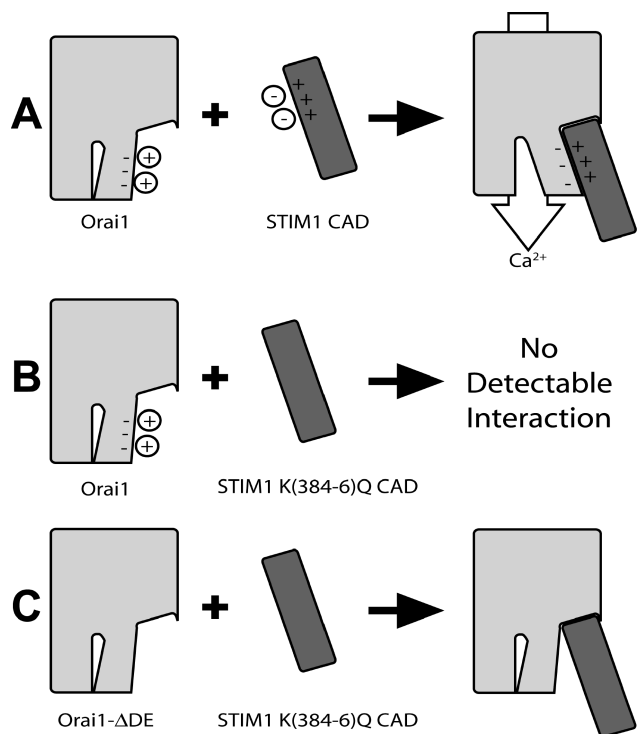


FIGURE 3: Stimulated  $\text{Ca}^{2+}$  responses. (A)  $\text{Ca}^{2+}$  responses to 150 nM thapsigargin in COS7 cells expressing wtSTIM1-mRFP and wtOrail (blue circles), STIM1 K(384-6)Q-mRFP and wtOrail (green triangles), wtSTIM1-mRFP and OrailΔDE (yellow triangles), or STIM1 K(384-6)Q-mRFP and OrailΔDE (red squares) and untransfected cells (pink circles). Fluo-4 fluorescence was normalized relative to the average prestimulation basal  $\text{Ca}^{2+}$  level in untransfected cells in the same field. Each time course represents the average of 10-18 cells, and error bars show the standard of the mean. Cells with basal  $\text{Ca}^{2+}$  levels  $>3$  times higher than the average for untransfected cells were excluded. (B) Mean  $\text{Ca}^{2+}$  levels before and 10 min after addition of 150 nM thapsigargin for averaged individual COS7 cells expressing a combination of wtSTIM1-mRFP or mutant STIM1 K(384-6)Q-mRFP with wtOrail or mutant OrailΔDE. Each bar represents the mean for 20-60 cells from three or more different experiments, and error bars show the standard error of the mean.

electrostatic attraction between the oppositely charged residues. If this is the case, wtOrail might not couple with STIM1 K(384-6)Q without this electrostatic attraction. Alternatively, electrostatics may contribute but may not be the dominant energetic contribution to the interaction between STIM1 and Orail. In this latter case, the stimulated association of the wild-type proteins might be restored if the charged sequences on both of these proteins are mutated to neutral amino acids. To distinguish these possibilities, we measured FRET between STIM1 K(384-6)Q-mRFP and AcGFP-OrailΔDE, and we found that thapsigargin stimulates their physical association to an extent similar to that for the wild-type proteins, but with substantially slower kinetics (Figure 1). This finding is similar to the slow FRET-detected association between wtSTIM1-mRFP and AcGFP-OrailΔDE that we previously reported (5) (Figure 1). Consistent with the FRET observed, STIM1 K(384-6)Q-mRFP forms copatches with AcGFP-OrailΔDE in





**FIGURE 4:** Cartoon depicting basic features of the proposed model for wild-type and mutant STIM1 and Orai1 interactions. (A) In unstimulated cells, the negatively charged acidic amino acids on wtOrai1 and the positively charged lysine sequence (K384–K386) in the STIM1 CAD domain (dark gray) defined by our mutations are shown associated with solvated counterions. Upon stimulation, these ions are displaced from the binding cleft between the two proteins, allowing these charged regions to interact with each other and causing the  $\text{Ca}^{2+}$ -selective channel to open in an allosteric manner. (B) When K384–K386 in STIM1 are mutated to glutamines, STIM1 no longer binds and activates wtOrai1 because of the loss of electrostatic attraction and resulting repulsion at this interaction subsite. (C) When these charged sequences on Orai1 and STIM1 are both mutated, STIM1 CAD regains the capacity to bind Orai1 via interactions at other sites because the electrostatic repulsion has been eliminated. However, this association does not cause allosteric activation of the  $\text{Ca}^{2+}$  gate. The oligomerization state of these proteins has been omitted for the sake of clarity. Orai1 is oligomerized in the active wtSTIM1–wtOrai1 complex and in all cases involving Orai1ΔDE.

plasma membrane punctae in response to thapsigargin, similar to the behavior seen with wtSTIM1-mRFP and AcGFP-Orai1ΔDE (Figure 2B,F). As with FRET-detected association, this visible copatching of STIM1 K(384–6)Q-mRFP with AcGFP-Orai1ΔDE requires longer stimulation times with thapsigargin than for the corresponding wt constructs.

This finding that STIM1 K(384–6)Q undergoes stimulated association with Orai1ΔDE but does not associate with its wild-type partner suggests a model for the interaction between these two proteins and the role of the charged sequences in this interaction (Figure 4). Although the charged sequence on STIM1 and its resulting electrostatic attraction to the acidic sequence on Orai1 appears to contribute little to the overall binding energy of the active complex, mutation to eliminate the charged residues on STIM1 clearly reduces the interaction energy with its wild-type partner. Thus, the simplest model to account for these observations is one in which this acidic sequence on Orai1 normally interacts with the basic sequence on STIM1 in an attractive electrostatic interaction.

To determine whether stimulated association between STIM1 K(384–6)Q and Orai1ΔDE results in SOCE, we monitored  $\text{Ca}^{2+}$

mobilization in COS7 cells expressing these mutants. As shown in Figure 3A, we did not detect a significant  $\text{Ca}^{2+}$  response to thapsigargin (red squares), compared to untransfected COS7 cells (pink circles). The absence of SOCE with these constructs, which clearly undergo thapsigargin-stimulated association detected by FRET (Figure 1) and copatching (Figure 2F), implicates these sequences in the gating of  $\text{Ca}^{2+}$  influx. Our proposed model accounts for these results:  $\text{Ca}^{2+}$  gating is controlled by the C-terminal acidic coiled coil of Orai1, and engagement of this sequence with basic residues 383–387 in the CAD region of STIM1 induces the formation of the active CRAC channel (Figure 4A).

Our previous results provided evidence that the acidic coiled coil of Orai1 controls its oligomerization state, possibly on the basis of mutual electrostatic repulsion (5). In our current model, STIM1 facilitates  $\text{Ca}^{2+}$  mobilization in part by inducing the oligomerization of Orai1. Modulation of electrostatics on the faces of coiled coils has been implicated previously in the control of the oligomerization state of different proteins (14, 15), consistent with our view that, by neutralizing the acidic coiled coil on Orai1, STIM1 383–387 weakens the charge–charge repulsion between individual Orai1 monomers and allows them to oligomerize into an active state. Our model is also consistent with our observations that neutralization of the acidic residues on the C-terminal coiled coil of Orai1 induces plasma membrane patching of this protein in the absence of store depletion or STIM1 translocation (Figure 2A) (5).

We investigated possible  $\text{Ca}^{2+}$  influx through spontaneous oligomerization of Orai1ΔDE. Figure 3B summarizes average  $\text{Ca}^{2+}$  levels before and 10 min after addition of 150 nM thapsigargin for a large number of individual cells. For unstimulated cells, there is a small but significant increase in the average  $\text{Ca}^{2+}$  levels of cells expressing Orai1ΔDE compared to untransfected cells or cells transfected with wtOrai1. We find that a small fraction (~10–20%) of these cells has a basal state of cytoplasmic  $\text{Ca}^{2+}$  that is >3-fold greater than that of untransfected cells, suggesting they have spontaneously activated CRAC channels (Figure S1A of the Supporting Information). However, cells expressing Orai1ΔDE with both high and low resting  $\text{Ca}^{2+}$  levels are similarly unresponsive to thapsigargin, consistent with the incapacity of Orai1ΔDE to be activated by STIM1 (Figure S1B of the Supporting Information). This variation in resting  $\text{Ca}^{2+}$  levels we observe for cells expressing clustered Orai1ΔDE indicates that there must be additional factor(s) controlling the gating of  $\text{Ca}^{2+}$  entry through Orai1 that varies among cells; one possibility is population differences in polyphosphoinositide levels (16).

To account for the activation of SOCE, we propose that, in addition to inducing Orai1 oligomerization, binding of the STIM1 K(384–386) sequence to the acidic residues in the coiled coil of Orai1 causes an allosteric change that directly opens the  $\text{Ca}^{2+}$  selective pore in the STIM1–Orai1 complex. Park et al. showed that STIM1 CAD interacts with both the N- and C-termini of Orai1 (9). A possible scenario is that in which the C-terminal acidic coiled coil of Orai1 interacts with its own N-terminus in the resting state, and STIM1 (346–348) disengages this interaction in activated cells, thereby opening the channel in an allosteric manner (Figure 4A). The N-terminus of Orai1 contains a polyarginine sequence (Orai1 28–33) that could serve as a potential binding partner for its acidic coiled coil in unstimulated cells. Yuan et al. found that the N-terminus of Orai1 is important for activation of CRAC channels with

full-length STIM1, but not with a minimal interaction domain that is similar to CAD (10), suggesting additional changes in Orail structure may be involved in this activation process.

In summary, we find that the basic sequence in the C-terminus of STIM1, K384–K386, is important for functional coupling of STIM1 with Orail that is driven by thapsigargin-mediated  $\text{Ca}^{2+}$  store depletion (Figure 4A,B). Furthermore, we demonstrate that mutation to neutralize acidic residues in the C-terminal coiled coil of Orail restores its stimulated association with STIM1 K-(384–6)Q but does not restore SOCE (Figure 4C). Our results support a model in which both oligomerization of Orail and  $\text{Ca}^{2+}$  gating are dependent on electrostatic interaction between these sequences in Orail and STIM1. In addition, our results reveal that these two processes can be uncoupled from each other in this exquisitely regulated mechanism of SOCE activation.

## SUPPORTING INFORMATION AVAILABLE

Population distribution of  $\text{Ca}^{2+}$  levels in cells expressing mutant and wild-type STIM1 and Orail (Figure S1). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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