The CCT chaperonin is a novel regulator of Ca\(^{2+}\) signaling through modulation of Orai1 trafficking

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Store-operated Ca\(^{2+}\) entry (SOCE) encodes a range of cellular responses downstream of Ca\(^{2+}\) influx through the SOCE channel Orai1. Orai1 recycles at the plasma membrane (PM), with ~40% of the total Orai1 pool residing at the PM at steady state. The mechanisms regulating Orai1 recycling remain poorly understood. We map the domains in Orai1 that are required for its trafficking to and recycling at the PM. We further identify, using biochemical and proteome approaches, the CCT [chaperonin-containing TCP-1 (T-complex protein 1)] chaperonin complex as a novel regulator of Orai1 recycling by primarily regulating Orai1 endocytosis. We show that Orai1 interacts with CCT through its intracellular loop and that inhibition of CCT-Orai1 interaction increases Orai1 PM residence. This increased residence is functionally significant as it results in prolonged Ca\(^{2+}\) signaling, early formation of STIM1-Orai1 puncta, and more rapid activation of NFAT (nuclear factor of activated T cells) downstream of SOCE. Therefore, the CCT chaperonin is a novel regulator of Orai1 trafficking and, as such, a modulator of Ca\(^{2+}\) signaling and effector activation kinetics.

INTRODUCTION
Ca\(^{2+}\) is a ubiquitous intracellular messenger that is central to many cell physiological processes ranging from cellular proliferation to cell death (1, 2). Specificity in Ca\(^{2+}\) signaling is encoded in the spatial and temporal features of cytosolic Ca\(^{2+}\) signals, which are mediated by various Ca\(^{2+}\) release and influx pathways. A ubiquitous Ca\(^{2+}\) influx pathway in both excitable and nonexcitable cells is store-operated calcium entry (SOCE). SOCE is activated in response to depletion of intracellular Ca\(^{2+}\) stores, mainly the endoplasmic reticulum (ER), in response to activation of phospholipase C (PLC)-coupled receptors. SOCE is central in shaping the dynamics of Ca\(^{2+}\) signals and, as such, in specifying downstream cellular responses. For example, it has been shown to be critical for the activation of immune cells through transcriptional induction of cytokines and to be down-regulated during M phase of the cell cycle (3, 4). The main molecular players in SOCE are members of the stromal interaction molecule (STIM) family (STIM1 and STIM2), which are ER Ca\(^{2+}\) sensors with luminal EF-hands (5–7), and members of the Orai family (Orai1, 2, and 3), which are Ca\(^{2+}\)-selective channels at the plasma membrane (PM) (8–10). Depletion of intracellular calcium stores results in clustering of STIM1 and stabilization of STIM1 clusters at ER-PM junctions, where STIM1 physically recruits Orai1 and gates it open, thus mediating Ca\(^{2+}\) influx (11). In addition to the STIM and Orai family, members of the transient receptor potential channel family have also been implicated in SOCE in some cell types (12).

Most of the known functions of Orai1 are centered around its Ca\(^{2+}\) conducting properties at the PM, despite the fact that the majority of the Orai1 protein pool (~60%) is intracellular at rest (13). Nonetheless, Orai1 trafficking has been implicated in various physiological functions.

Store depletion is coupled to an increase of Orai1 at the PM from ~40 to ~65% of the cellular Orai1 pool (13, 14). This is due to trapping of Orai1 by STIM1 at ER-PM junctions, thus removing Orai1 from the recycling pool at the PM (13). Orai1 PM enrichment in response to store depletion is dependent on the exocytotic machinery, as it was abrogated by interfering with SNAP-25 function (14). In mammary epithelial cells, the Ca\(^{2+}\)-ATPase (adenosine triphosphatase) (SPCA2) has been identified as important for cell surface localization of Orai1 (15). Furthermore, Orai1 PM insertion is increased in cystic fibrosis airways, resulting in increased cytokine production (16). Orai1 exhibits rapid PM recycling kinetics at rest, with an exocytosis rate constant of ~0.1 min\(^{-1}\) (13), which is high compared to the Glut4 transporter, for example, which has a K\(_{ex}\) of 0.003 min\(^{-1}\) at rest (17).

Orai1 trafficking has been studied in significant detail in Xenopus oocytes at steady state and during meiosis. At rest, Orai1 exhibits a similar distribution between the intracellular pool and PM as observed in mammalian cells and recycles through a Rho- and Rab5-dependent but dynamin-independent endocytic pathway (18). Store depletion is associated with enrichment of Orai1 at the plasma by recruiting the intracellular Orai1 pool (18). During meiosis, Orai1 is removed from the PM and internalized into an endocytic compartment through a Cav-, Rab5-, and dynamin-dependent endocytic pathway and, as such, is no longer able to mediate Ca\(^{2+}\) influx (18, 19). Orai1 internalization during meiosis is dependent on its N-terminal cytoplasmic domain, which contains an inverted caveolin binding site FXXXXΦΨXXΦ (Φ indicates an aromatic amino) (20) between residues 52 and 60, YPDWIGQSY (18). Mutating the first two aromatic residues (Y52 and W55) to Ala does not affect Orai1 trafficking or recycling at rest but blocks Orai1 internalization during meiosis (18).

Barring the studies in oocytes, the trafficking of Orai1 remains poorly understood, with little known about the molecular machinery or even domains within the Orai1 protein required for its trafficking and membrane residence at steady state. These are important issues to resolve as they would modulate SOCE signaling and cellular responses. Here, we systematically dissect the role of Orai1 cytoplasmic regions in its trafficking and show that neither the N terminus nor the C terminus of Orai1 modulates its recycling at the PM, although deletion of the C-terminal domain leads to trapping of Orai1 intracellularly. Using

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biochemical fractionation and antibody-dependent trapping of Orai1 intracellularly coupled to quantitative proteomics, we identify the chaperonin-containing TCP-1 (T-complex protein 1) (CCT, also known as TCP-1 Ring Complex or TRiC) complex as a modulator of Orai1 recycling at the PM. CCT affects Orai1 membrane residence by primarily regulating Orai1 endocytosis. We map the CCT binding domain to the Orai1 intracellular loop (ICL) between residues 157 and 167. A mutant Orai1 with residues 157 to 167 scrambled (ICL-S) is no longer able to bind to CCT and exhibits increased residence at the PM at steady state. This has significant consequences on signaling downstream of store depletion, resulting in prolonged Ca\(^{2+}\) signaling in response to agonist stimulation, faster STIM1-Orai1 puncta formation, and a more rapid activation of nuclear factor of activated T cells (NFAT). This validates a novel role for the CCT chaperonin complex as a regulator of Orai1 trafficking, in addition to its well-established role in supporting protein folding. Furthermore, these results show that Orai1 membrane residence is important in modulating the dynamics of the Ca\(^{2+}\) signal and the activation of effectors downstream of SOCE.

RESULTS

The C terminus of Orai1 is required for its biogenesis

At steady state, 40% of the total Orai1 protein pool distributes to the PM, with a portion of the intracellular Orai1 pool localizing to an ill-defined sub-PM intracellular compartment (13). We were interested in better defining the domains in Orai1 that are important for its PM residence and subcellular distribution. Orai1 is a four-pass transmembrane protein, with most of the cytoplasmic domains localizing to the N and C termini (Fig. 1A, cartoon). We therefore deleted the cytoplasmic N-terminal (residues 1 to 90, Orai1-ΔN) or C-terminal (residues 257 to 301, Orai1-ΔC) domain in the context of a yellow fluorescent protein (YFP)-tagged Orai1 construct with a hemagglutinin (HA) tag embedded within the second extracellular loop to allow quantification of Orai1 at the PM as previously described (13). Both the Orai1-ΔN and Orai1-ΔC are expressed at similar levels to full-length Orai1 (wild-type (WT)) when transiently transfected (Fig. 1B). While Orai1-ΔN is enriched at the PM and exhibits a similar distribution to WT Orai1, Orai1-ΔC has a diffuse intracellular distribution, arguing that the C-terminal domain of Orai1 is required for its trafficking to and residence at the PM (Fig. 1C). Staining for the HA tag in nonpermeabilized cells shows nonetheless that Orai1-ΔC does traffic to the PM (Fig. 1C).

We previously described detailed approaches to quantifying Orai1 at the PM, as well as its trafficking (exocytosis and endocytosis) kinetics, using the YFP-HA-Orai1 construct (13). Assessing the surface/total (S/T) ratio of Orai1 provides a sensitive measure of Orai1 trafficking to and residence at the PM. Orai1-ΔN exhibits a similar S/T to full-length Orai1, arguing that the N terminus is not important in regulating Orai1 subcellular distribution (Fig. 1D). In contrast, deletion of the C terminus significantly reduces the Orai1 S/T ratio (Fig. 1D). This confirms the intracellular trapping of Orai1-ΔC observed by imaging (Fig. 1C). The C-terminal domain of Orai1 has been shown to be critical for its interactions with STIM1 in response to store depletion to gate Orai1 (21, 22). Consistently, measuring SOCE following coexpression of Orai1-ΔC with STIM1 shows that it is unable to induce SOCE despite its PM localization (fig. S1). Collectively, these results argue that Orai1-ΔC either does not fold properly or lacks essential targeting domains to reach the steady-state distribution of the WT protein, resulting in intracellular trapping of a significant proportion of the protein.

To further map the region in the C terminus of Orai1 required for its PM residence, we generated a series of deletions of 10-residue increments starting from the C-terminal end of the protein. We expressed WT and truncated Orai1 mutants [Orai1-ΔC (1-256), 1-266, 1-275, and 1-285] (Fig. 1A) and tested their S/T distribution. When normalized to S/T levels for WT Orai1, Orai1-ΔC shows the most pronounced defect with the lowest enrichment at the PM at steady state (Fig. 1E). Similarly, 1-266 and 1-275 show significantly decreased PM localization compared to WT, but a less pronounced defect than Orai1-ΔC (Fig. 1E). In contrast, 1-285 behaves like WT Orai1, arguing that residues 286 to 301 are not required for Orai1 PM residence (Fig. 1E). We further show that the various C-terminal deletions, except for 1-285, are unable to mediate SOCE when coexpressed with STIM1 (fig. S1).

The C-terminal deletions can either lead to misfolding of the protein, preventing it from effectively reaching the PM, or be associated with increased internalization, resulting in decreased PM steady-state levels. To differentiate between these two possibilities, we measured the percentage of the Orai1 protein pool that recycles at the PM by measuring the exocytosis rate of the different deletions (Fig. 1F), as previously described (13). WT and 1-285 exhibit similar exocytosis rates and maximal labeled Orai1 (Fig. 1F). In contrast, the other C-terminal deletions plateaued at significantly lower levels, consistent with the S/T results, with no major differences in the rate of exocytosis (Fig. 1F). Using these measurements, we calculated the percentage of Orai1 pool that recycles at the cell membrane compared to WT Orai1 as follows

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\% \text{ Recycling Orai1} = \frac{(\text{cell-associated } \alpha\text{HA})_{\text{Max}} - (\text{cell-associated } \alpha\text{HA})_{T0}}{(\text{cell-associated } \alpha\text{HA})_{\text{WT Orai1 Max}}}.
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where (cell-associated \(\alpha\text{-HA}\))_{\text{Max}} is the plateau level reached for each mutant, (cell-associated \(\alpha\text{-HA}\))_{\text{T0}} is the level at the beginning of the uptake experiment, and (cell-associated \(\alpha\text{-HA}\))_{\text{WT Orai1 Max}} is the plateau level reached for the full-length Orai1. Consistent with the exocytosis rates, about 60% of the WT and 1-285 Orai1 populations recycle, whereas only ~18% of the Orai1-ΔC pool and 22 to 26% of the 1-266 and 1-275 pools recycle. This shows that the defect in C-terminal Orai1 deletions is the inability of the protein to reach the PM, as it is not engaged in recycling and cannot be accessed following incubation with \(\alpha\)-HA antibodies. Therefore, the Orai1 C-terminal cytoplasmic domain is important for its biogenesis and PM targeting.

Our deletion analysis further shows that residues 256 to 285 are important for Orai1 biogenesis, which would be consistent with protein quality control check before allowing it to transit to the cell membrane. The diffuse broad cytoplasmic distribution of Orai1-ΔC (Fig. 1C) is consistent with this conclusion. The region required for Orai1 trafficking to the cell membrane defined by our deletion analyses matches well with the predicted coiled-coil domain in the Orai1 C terminus (263 to 285) (21), arguing that proper folding of the C-terminal coiled coil are requisite for Orai1 PM targeting, as for its interactions with STIM1. Consistent with this conclusion, the COILS algorithm (23) does not predict a coiled-coil domain in the Orai1 deletions 1-256, 1-266, and 1-275; in contrast, in the full-length Orai1 and 1-285, a strong coiled coil is predicted between residues 263 and 285.

To better define the intracellular distribution of Orai1, we stained cells transfected with YFP-HA-Orai1 with markers for the Golgi
(GM130), ER [inositol 1,4,5-trisphosphate receptor (IP3R)], and the early endosome (EEA1). Orai1 shows faint localization to the Golgi, potentially through its biogenesis to the PM (fig. S2A). No colocalization of Orai1 to the ER was observed, but a subset of the Orai1 protein pool localized to the early endosome consistent with its recycling (fig. S2A). Feeding cells expressing YFP-HA-Orai1 with labeled transferrin further shows localization to the late endosome (fig. S2B).

We further show that the cellular trafficking machinery does not saturate at higher Orai1 expression levels (fig. S3A), as the levels of Orai1 at the PM remain fairly constant over a wide range of YFP expression. We finally tested the effects of glycosylation on Orai1 PM residence by quantifying the S/T ratio of two previously described mutants (N223A and 223NVS225 to IVI) in the second extracellular loop that disrupt Orai1 glycosylation (24). Neither glycosylation mutant affected Orai1 PM residence (fig. S3B), although the NVS mutant showed a trend toward higher PM levels but the data were not significant.

**Recycling Orai1 interacts with CCT**

The C-terminal deletion analysis argues that proper folding in that region is important for Orai1 transport to the PM and, as such, its steady-state distribution. However, it does not offer clues about the mechanisms regulating Orai1 recycling at the PM. Store depletion was shown to increase the levels of Orai1 at the PM by sequestering recycling Orai1 (13), which is likely to modulate SOCE dynamics and, as such, the downstream cellular responses. Furthermore, a significant population of the intracellular Orai1 pool localizes to a sub-PM vesicular pool of unknown identity (13). This sub-PM Orai1 pool is enriched when cells stably expressing YFP-HA-Orai1 are fed with anti-HA antibodies (Fig. 2A). Short incubation times (2 min) label resident PM Orai1 (Fig. 2A), whereas longer incubation times (45 min) allow recycling Orai1 to take up the anti-HA antibody, thus labeling the intracellular sub-PM compartment (Fig. 2A, arrows). We were interested in taking an unbiased approach to identifying the machinery required for Orai1 recycling at the PM, with a focus on endogenous Orai1 to rule out any potential artifacts from Orai1 overexpression or tagging. We therefore used sucrose gradients to isolate fractions enriched in sub-PM Orai1 (Fig. 2B). Chinese hamster ovary (CHO) cells were lysed under detergent-free conditions to maintain the integrity of vesicular membranes, and the homogenate was centrifuged at 1000g to remove nuclei and unbroken cells (Fig. 2B, p1000), followed by centrifugation at 8000g to pull down the PM (Fig. 2B, p8000). The post-8000g supernatant (Fig. 2B, Sup) was devoid of PM contamination, as confirmed by staining for the resident PM protein in the Na-K pump. This fraction was further separated on a continuous 10 to 40% (w/v) sucrose gradient overnight.
and 13 fractions were collected and probed for Orai1, which localized to fractions 9 to 11 (Fig. 2B). We then identified, using mass spectrometry (MS), proteins that coenrich with Orai1 in fractions 9, 10, and 11 but are absent in fractions 12 and 13 as candidate regulators of Orai1 trafficking. Given the high noise and potential nonspecific candidates that would comigrate with Orai1 vesicles on the sucrose gradient, we combined the above approach with a pulldown and proteomics approach to improve the specificity of identifying proteins that interact preferentially with intracellular Orai1. We used the stable YFP-HA-Orai1 CHO cell line and pulled down YFP-HA-Orai1 in control cells and cells fed with anti-HA antibodies to label Orai1 in the sub-PM vesicles (Fig. 2A), as outlined in Fig. 2C. Immunoprecipitated complexes were digested with trypsin and differentially labeled using reductive dimethylation with light (control) and heavy (HA-fed) dimethyl isotopes, followed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) (25). The heavy/light ratio provides a relative quantitative measure of the enrichment of a particular protein under the anti–HA-fed conditions as compared to control.

To identify candidates that regulate Orai1 trafficking, data from both proteomics approaches were analyzed using the following criteria: The HA-feeding experiment was repeated three times, and only proteins that were identified in all three experiments were kept. Furthermore, any candidate protein had to be present in fractions 9, 10, and 11 but not in fractions 12 and 13 to be considered. We further prioritized protein candidates that were enriched with endogenous Orai1 and kept only proteins that were identified by at least three independent peptides. Combined analysis of both datasets shows a striking enrichment of seven subunits of the TCP-1 chaperonin complex (Fig. 2D).

**Orai1 binds to CCT**

To verify the interaction between Orai1 and the CCT complex, we immunoprecipitated YFP-tagged Orai1 from CHO stables (S) or transients (T) (Fig. 3A). CCT1, CCT2, and CCT5 coimmunoprecipitated with Orai1 (Fig. 3A), thus supporting the proteomics data and showing that the CCT complex interacts with Orai1. However, given that the CCT complex is quite abundant in cells, the overexpression of YFP-HA-Orai1 may lead...
to erroneous interactions between the two proteins. We were therefore interested in confirming that endogenous Orai1 at physiological expression levels interacts with CCT. Endogenous Orai1 immunoprecipitates with the endogenous CCT complex, but not following immunoprecipitation using control immunoglobulin G (IgG) (Fig. 3B), although some nonspecific background is observed with the control IgG, presumably due to the abundance and chaperone role of CCT. We also found that immunoprecipitation of endogenous Orai1 by siRNA. Cell lysates were separated on SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and probed with CCT2 and actin antibodies, followed by IRDye 800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG secondary antibodies. Quantitative Western blot analysis was performed using LI-COR. (E) Stable YFP-HA-Orai1 CHO cells treated with CCT2 siRNA (50 nM) or nontargeting siRNA (control) for 48 hours and stained with anti-HA antibodies followed by a Cy5-labeled secondary. The surface Orai1–to–total Orai1 ratio [(Cy5/YFP in nonpermeabilized cells)/(Cy5/YFP in permeabilized cells)] was determined by quantitative immunofluorescence as indicated in Materials and Methods. Data are the means ± SEM from three independent experiments. **P < 0.01.

**Fig. 3. CCT complex binds to expressed and endogenous Orai1 and regulates Orai1 levels at the PM.** (A) YFP-HA-Orai1 complexes were immunoprecipitated from stably (S) or transiently (T) transfected YFP-HA-Orai1 CHO cells using GFP microbeads, separated on SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and probed with specific antibodies against CCT2, CCT1, CCT5, and HA. (B) Coimmunoprecipitation (IP) of endogenous Orai1-CCT complexes, CHO cell lysates incubated with antibodies against CCT2, CCT1, and CCT5 as the immunoprecipitating antibody or control IgG followed by Western blotting using Orai1 antibody. The middle blot shows efficient CCT2 pulldown using the CCT2 antibody. The blot on the right shows that the CCT1, CCT2, and CCT5 coimmunoprecipitate. (C) Reciprocal immunoprecipitation with antibody against Orai1 followed by Western blotting using CCT2, CCT1, and CCT5 antibodies. Some lysates were expressing CCT2-GFP. (D) Knockdown of endogenous CCT2 by siRNA. Cell lysates were separated on SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and probed with CCT2 and actin antibodies, followed by IRDye 800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG secondary antibodies. Quantitative Western blot analysis was performed using LI-COR. (E) Stable YFP-HA-Orai1 CHO cells treated with CCT2 siRNA (50 nM) or nontargeting siRNA (control) for 48 hours and stained with anti-HA antibodies followed by a Cy5-labeled secondary. The surface Orai1–to–total Orai1 ratio [(Cy5/YFP in nonpermeabilized cells)/(Cy5/YFP in permeabilized cells)] was determined by quantitative immunofluorescence as indicated in Materials and Methods. Data are the means ± SEM from three independent experiments. **P < 0.01.

**Knockdown of the CCT2 subunit increased cell surface levels of Orai1**

CCT is required for the folding of several newly synthesized proteins including cytoskeletal proteins, cell cycle regulators, and other cytosolic proteins (26). To investigate whether CCT modulates Orai1 trafficking, we initially tested the effect of CCT2 knockdown on Orai1 PM residence in YFP-HA-Orai1 stable CHO cells. Small interfering RNA (siRNA) against CCT2 effectively down-regulated CCT2 protein levels by 35 ± 3.8% as compared to a nontargeting control siRNA, assessed by quantitative LI-COR Western blotting (Fig. 3D). Actin levels, as a control, remained unchanged (Fig. 3D). Because CCT is known to affect folding of some proteins (27), we examined whether knocking down CCT2 expression affects Orai1 expression levels. In stable CHO cells treated with CCT2 siRNA, YFP-HA-Orai1 expression was unchanged compared to that in cells treated with control siRNA (Fig. 3D). Despite the fact that total Orai1 levels were not affected by CCT2 knockdown, the S/T ratio of Orai1 was significantly increased (Fig. 3E). Similarly, knocking down CCT1 increased Orai1 at PM, showing that the CCT complex regulates Orai1 trafficking and its PM residence.

**CCT interacts with Orai1 through its ICL**

Most of the cytoplasmic domains of Orai1 localize to the N- and C-terminal domains. However, results from Orai1-AN and Orai1-ΔC deletions do not support a role for these domains in regulating Orai1
trafficking at the PM (Fig. 1). Neither deletion phenocopies this increased S/T Orai1 observed following knockdown of CCT. This argues that CCT does not interact with Orai1 through either its N- or C-terminal domain. To determine whether this is the case, we immunoprecipitated Orai1 from cells expressing full-length Orai1 (Orai1), Orai1-ΔN, or Orai1-ΔC and tested interaction with CCT2. As shown in Fig. 4A, all three constructs pulled down similar levels of CCT2, arguing that Orai1 does not interact with CCT through either its N- or C-terminal domains.

CCT was previously shown to bind to the cytoplasmic domain of the LOX-1 [Lectin-like oxidized low-density lipoprotein (LDL) receptor-1] receptor and regulates its trafficking (28). Alignment of the LOX-1 receptor cytoplasmic domain sequence (residues 1 to 33) with Orai1 shows significant homology within the Orai1-ICL (residues 144 to 173) (Fig. 4B), particularly within residues 157 to 167 of Orai1 (Fig. 4B).

To directly test whether the Orai1-ICL is required for CCT interaction, we engineered an Orai1 construct with residues 157 to 167 scrambled. It was not possible to generate mutants with Orai1-ICL deleted, as this would result in fusion of the TM2 and TM3 and an unstable protein. We did, however, attempt several smaller deletions in the ICL, but these constructs did not express consistently. We further generated a mutant where residues 157 to 167 were replaced with alanines. Although this alanine mutant expressed, it resulted in a diffuse cytosolic distribution and did not traffic to the PM (fig. S4). Therefore, alterations in the ICL appear to affect Orai1 folding and trafficking. We therefore opted for scrambling residues 157 to 167, resulting in an Orai1 mutant, ICL-S (for scrambled ICL), with the following sequence: 157-NEKPHRSLVES-167 (Fig. 4B). ICL-S trafficked to and was enriched at the PM, suggesting that its folding and biogenesis were not affected (Fig. 4C). Strikingly, though, ICL-S showed significant reduction in its ability to interact with the CCT complex as compared to WT Orai1 (Fig. 4D). Consistently, ICL-S exhibited significantly higher enrichment at the PM (Fig. 4E), confirming that CCT-Orai1 interaction is required to maintain steady-state levels of PM Orai1. This further validates CCT as a bona fide regulator of Orai1 trafficking.

**CCT regulates Orai1 recycling kinetics, primarily endocytosis**

We next determined the recycling kinetics of ICL-S at the PM by measuring the rates of endocytosis and exocytosis as previously described (13). Exocytosis was measured using the anti-HA antibody uptake assay and showed a significant reduction in the exocytosis rate of ICL-S (0.0132 ± 0.0059 min⁻¹) as compared to WT Orai1 (0.048 ± 0.0067 min⁻¹) (Fig. 4F). Endocytosis was assessed by measuring the ratio of intracellular Orai1 to surface Orai1 over time (Fig. 4G). The endocytosis rate of ICL-S (0.0100 ± 0.0040 min⁻¹) was significantly reduced compared to WT Orai1 (0.0611 ± 0.0073 min⁻¹) (Fig. 4G), arguing that the CCT complex modulates Orai1 trafficking primarily by regulating its endocytosis at the PM through interactions that require Orai1-ICL.

**CCT modulates Ca²⁺ signaling downstream of SOCE**

The PM steady-state levels of Orai1 represent the readily available pool of Orai1 to interact with STIM1 following store depletion. SOCE develops over a time course of minutes following store depletion, a process that is coupled to enrichment of Orai1 at the PM as STIM1 clusters stabilize below the PM and recruit recycling Orai1 to form stable puncta

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**Fig. 4. Identifying CCT binding domain in Orai1.** (A) Coimmunoprecipitation of CCT from CHO cells expressing WT, Orai1-ΔC, and Orai1-ΔN, using GFP microbeads. Lysates from cells transfected with pEGFP-C1 (GFP) were used as control. (B) Sequence alignment of the LOX-1 receptor cytoplasmic domain shown to bind CCT with the Orai1-ICL. The scrambled ICL construct (ICL-S) is shown in red. (C) Representative epifluorescent images of a mutant Orai1 bearing the scrambled sequence compared to WT Orai1 (0.048 ± 0.0067 min⁻¹). (Fig. 4F). Endocytosis was assessed by measuring the ratio of intracellular Orai1 to surface Orai1 over time (Fig. 4G). The endocytosis rate of ICL-S (0.0100 ± 0.0040 min⁻¹) was significantly reduced compared to WT Orai1 (0.0611 ± 0.0073 min⁻¹) (Fig. 4G), arguing that the CCT complex modulates Orai1 trafficking primarily by regulating its endocytosis at the PM through interactions that require Orai1-ICL.
that underlie Ca\textsuperscript{2+} influx (13). This argues that increasing steady-state levels of Orai1 at the PM, as afforded by the ICL-S mutant, would modulate signaling downstream of SOCE. We first tested whether Ca\textsuperscript{2+} signaling in response to a physiological agonist is affected by Orai1 PM levels. Stimulating P2Y receptors using 50 μM adenosine triphosphate (ATP) in Ca\textsuperscript{2+}-containing medium results in Ca\textsuperscript{2+} mobilization from intracellular stores, followed by a plateau phase and recovery back to baseline during which time SOCE activates (Fig. 5A). Expressing WT Orai1 does not affect the kinetics of the Ca\textsuperscript{2+} response downstream of ATP as compared to control cells (Fig. 5A). In contrast, expressing ICL-S significantly extends the plateau phase and slows down the initial rate of decay of the Ca\textsuperscript{2+} signal, as measured by the time required to reach 75% plateau levels (Fig. 5A), where the time to 75% decay was 36.73 ± 2.18 s for ICL-S, which is significantly higher than that for WT (25.23 ± 1.94 s). These results suggest that inhibition of CCT-Orai1 interactions modulates the early phases of Ca\textsuperscript{2+} signaling downstream of SOCE.

The higher steady-state levels of the ICL-S mutant at the PM predict that it would form STIM1-Orai1 puncta at a faster rate compared to WT Orai1, as it provides a higher readily available Orai1 pool for STIM1 to interact with after store depletion without having to rely on the slower kinetics of Orai1 trafficking. We therefore measured the time course of puncta formation in both CHO and HeLa cells expressing either WT Orai1 or ICL-S using total internal reflection fluorescence (TIRF) microscopy (Fig. 5B). In response to store depletion with thapsigargin (TG), the Orai1 ICL-S mutant forms puncta with a significantly faster time course, tens of seconds earlier than WT Orai1 in both cell types (Fig. 5B). This argues that disruption of the ability of Orai1 to interact with CCT by scrambling the ICL regions increases its PM residence, thus allowing it to engage with STIM1 with a faster time course after store depletion.

To determine whether the faster rate of puncta formation (Fig. 5B) and the slower rate of Ca\textsuperscript{2+} decay (Fig. 5A) observed for the ICL-S mutant are functionally significant, we tested its ability to activate NFAT translocation to the nucleus in response to store depletion (Fig. 5C). NFAT is the canonical downstream effector of SOCE (29) and is known to activate specifically in response to SOCE and not Ca\textsuperscript{2+} release from stores (30). We measured the nuclear-to-cytoplasmic ratio of NFAT in cells transfected with GFP-NFAT in response to store depletion (Fig. 5C). Cells were transfected with STIM1 and either WT Orai1 or the ICL-S mutant. ICL-S expression significantly (P < 0.0012) enhanced the initial rate of NFAT translocation as compared to cells expressing WT Orai1 (Orai1) (Fig. 5C). The initial rate of NFAT translocation was measured as the rising slope within the first 200 s after TG addition (Fig. 5C, box). These data show that a higher membrane residence of Orai1, as afforded by the ICL-S mutant, allows Orai1 to engage with STIM1 following store depletion with faster kinetics in terms of both puncta formation and activation of downstream effectors.

**Fig. 5.** CCT-Orai1 interaction modulates Ca\textsuperscript{2+} signaling downstream of Orai1. (A) Ca\textsuperscript{2+} transients in response to 50 μM ATP stimulation in Ca\textsuperscript{2+}-containing (2 mM) Ringer solution measured using Fura-2 in CHO cells cotransfected with STIM1 and Orai1 (red) or STIM1 and ICL-S Orai1 (blue). Untransfected cells were used as control (black). Time to 75% decay was determined (means ± SEM from three independent experiments). (B) Accelerated STIM1-Orai1 puncta formation in CHO and HeLa cells transfected with ICL-S Orai1 after TG-dependent calcium store depletion. Live-cell images by TIRF microscopy and intensity recordings showing accumulation of the subplasmalemmal puncta in response to 1 μM TG in CHO cells cotransfected with STIM1 and Orai1 (red) or STIM1 and ICL-S (blue). (C) Time course for NFAT1 nuclear translocation in response to store depletion with TG (1 μM) (top). Cells were cotransfected with CFP (cyan fluorescent protein)--STIM1, YFP-Orai1, and GFP-NFAT1 or with CFP-STIM1, ICL-S, and GFP-NFAT1. Ratio of GFP-NFAT fluorescence in the nucleus/cytosol over time. The initial slope of N/C NFAT was calculated within 200 s after TG (bottom). * P < 0.05, ** P < 0.01.
**DISCUSSION**

Ca\(^{2+}\) is an ever-present messenger in eukaryotic cells that defines the cellular response to agonists under various physiological, pathological, and environmental conditions. Ca\(^{2+}\) signals are stimulated downstream of G-protein- and tyrosine kinase–coupled receptors through the activation of PLCs to encode specific cellular responses. The universal nature of Ca\(^{2+}\) signaling creates a conundrum in terms of the ability of cells to decode Ca\(^{2+}\) signals into specific cellular responses. Without having such specificity encoded in Ca\(^{2+}\) dynamics, this signaling module would not have survived the evolutionary pressure, leading to its omnipresence in eukaryotic cells. Ca\(^{2+}\) signals encode cellular responses as disparate as cell proliferation and cell death often in the same cell. Hence, the ability of the downstream Ca\(^{2+}\)-dependent machinery to detect and decode specific Ca\(^{2+}\) dynamics into a defined cellular response is vital. Ca\(^{2+}\) dynamics are molded by various Ca\(^{2+}\) influx and extrusion pathways at the PM, and Ca\(^{2+}\) release and reuptake pathways are the ER membrane (2, 31). SOCE is a ubiquitous Ca\(^{2+}\) signaling module that is active in both nonexcitable cells, where it represents one of the primary Ca\(^{2+}\) influx pathways, and excitable cells, where it modulates Ca\(^{2+}\) signaling dynamics and cellular development (32). SOCE modulates Ca\(^{2+}\) signaling dynamics and, as such, the ensuing cellular response. Therefore, elucidating the molecular mechanisms regulating the dynamics of SOCE activation becomes critical to understanding how it modulates the activation of downstream effectors and, as such, cellular responses.

One important factor in regulating SOCE is the trafficking and residence of the SOCE channel, Orai1, at the PM. Orai1 trafficking particularly at the PM is quite dynamic and is modulated during the cell cycle, leading to Orai1 internalization in oocyte meiosis, thus contributing to SOCE inhibition at this stage of the cell cycle (18, 19). Furthermore, in interphase at steady state, ~40% of the Orai1 protein pool resides at the PM, where it is continuously exchanging with the intracellular portion of the protein (13, 18). However, the molecular machinery and domains within Orai1 that regulate its trafficking are poorly understood. Here, we define the cytoplasmic Orai1 domains that regulate its trafficking and PM residence. We show that Orai1 recycling is not dependent on either the cytoplasmic N terminus (residues 1 to 89) or C terminus (residues 257 to 301), which encompasses the vast majority of the intracellular portion of the protein (Fig. 1). Orai1-ΔN with the entire N terminus deleted traffics normally to the PM and distributes at steady state in a similar fashion to the full-length protein (Fig. 1). This is consistent with previous studies that showed normal distribution of Orai1 N-terminal deletions (21, 33). However, in contrast to these studies, which argued that the Orai1 C-terminal deletion also traffics and distributes normally in HEK cells, here using quantitative measurements of Orai1 at the PM and confocal imaging, we show that the Orai1 C-terminal cytoplasmic domain is required for its biogenesis and PM targeting (Fig. 1). A significant percentage of Orai1-ΔC is retained intracellularly where only 25% of the protein pool resides at the PM, compared with 40% for the WT and Orai1-ΔN proteins (Fig. 1). This is due to the inability of Orai1-ΔC to reach the PM effectively, as only 18% of the protein pool recycles at the PM (Fig. 1, F and G). These data and confocal imaging of Orai1-ΔC distribution compared to WT or Orai1-ΔN argue that Orai1-ΔC is retained intracellularly. We further confirm, consistent with previous work, that Orai1-ΔC is unable to support SOCE (Fig. S1). Deletion analyses of the Orai 1 C terminus argue that residues 256 to 285 are critical for proper Orai1 folding and biogenesis (Fig. 1). This is consistent with results from Muik et al. who showed that the Orai1 C terminus is required for interaction with STIM1 and SOCE activation and that this interaction requires the proper folding of the coiled-coil domain in the Orai1 C terminus, since a mutation that disrupts the coiled coil, L273S, does not interact with STIM1 (21). Together, these results argue that proper folding of the coiled coil in the Orai1 C-terminal cytoplasmic region is important for its PM targeting. A potential explanation for the leaky trafficking of Orai1-ΔC is that it may form heterotetramers with endogenous Orai1, resulting in their transport to the PM. Regardless, these channels are not able to support SOCE (fig. S1), as shown previously (21, 33).

To further define the molecular machinery regulating Orai1 PM residence, we combined biochemical and proteomics approaches. We isolated the endogenous Orai1 intracellular pool on sucrose gradients and identified proteins that coenrich in the Orai1-positive fractions using MS. We further built on our previous finding that feeding cells expressing YFP-HA-Orai1 with anti-HA antibodies stabilizes Orai1 in an intracellular vesicular pool (13). Using quantitative proteomics, we searched for proteins enriched with Orai1 in the HA-fed cells as compared to controls in an effort to identify Orai1 interactors that regulate its trafficking. Combining all three approaches allowed a subtractive approach that identified practically all the subunits of the TCP-1 complex that form the CCT complex, also known as TCP-1 Ring Complex (TRiC) (Fig. 2). CCT is an essential and conserved chaperonin initially discovered as a folding machine for actin and tubulin (34, 35). It is a group II eukaryotic chaperonin that is homologous to the group I chaperonins (GroEL and GroES) found in prokaryotes and endosymbiotic organelles, including mitochondria (26, 27). TCP-1 is encoded on the T-Complex gene cluster on mouse chromosome 17, which, when mutated, produces tailless and lethality phenotypes (36). CCT is a ubiquitous chaperonin that facilitates the folding of 5 to 10% of newly synthesized cytosolic proteins (37–40).

We confirmed the proteomics findings by coimmunoprecipitation of either Orai1 or CCT subunits (Fig. 3, A to C). Furthermore, knockdown of CCT2 significantly increased Orai1 at the PM, without affecting Orai1 expression levels (Fig. 3, D and E). Orai1-ΔN and Orai1-ΔC interact with CCT with similar efficiency to the WT protein, arguing that CCT does not bind to either domain (Fig. 4A). This is consistent with the findings of the effects of the Orai1-ΔN and Orai1-ΔC deletions on Orai1 trafficking (Fig. 1). Furthermore, alignment of Orai1 with the Lox-1 receptor, whose trafficking has been shown to be regulated by the CCT complex, shows significant homology with the Orai1-ICL. To test whether CCT interacts with Orai1 through its ICL, we generated an Orai1 mutant where the residues in Orai1-ICL were scrambled while maintaining the same length of the loop and amino acid composition (ICL-S) (Fig. 4, B to E). ICL-S traffics normally to the PM but shows increased residence at the PM (Fig. 4, C to E), in a similar fashion to what was observed following CCT knockdown (Fig. 3E). Quantitative analyses of ICL-S exocytosis and endocytosis kinetics as compared to the WT Orai1 show that CCT interaction affects both exocytosis and endocytosis rate constants, with the most marked defect affecting Orai1 endocytosis. The decreased ability of Orai1-ICL to coimmunoprecipitate with CCT1 (Fig. 4D) correlated with the observed decrease in the endocytosis rate of Orai1-ICL (Fig. 4G). This argues that CCT modulates Orai1 endocytosis. Consistently, Orai1 is enriched at the PM either when CCT is knocked down or when the Orai1-ICL is mutated where Orai1 can no longer interact with CCT.

Collectively, these results show that CCT is crucial for maintaining Orai1 PM residence primarily through modulating Orai1 endocytosis and argue against a role for CCT in Orai1 biogenesis or folding. This is consistent with the known function of the CCT chaperonin in the
folding of soluble cytoplasmic proteins and not integral membrane proteins.

Our premise at the inception of these studies was that Orai1 PM residence at rest is important in modulating the dynamics of SOCE Ca\(^{2+}\) signals and the activation of downstream effectors. To assess whether this is the case, we used the ICL-S mutant, which is unable to bind CCT (Fig. 5). The results showing enrichment of Orai1-ICL at the PM compared to wild type suggest an accelerated activation of SOCE without necessarily affecting SOCE levels. To study this, ATP was used as a physiological agonist to induce Ca\(^{2+}\) mobilization from intracellular stores, followed by a plateau phase and recovery back to baseline during which time SOCE activates. ICL-S mediated Ca\(^{2+}\) signals are more sustained and result in a significantly more rapid activation of the SOCE prototypical effector NFAT (Fig. 5). These changes were associated with a more rapid formation of coincident STIM1-Orai1 clusters, which are required for SOCE activation (Fig. 5B). The more rapid puncta formation argues that PM Orai1 is readily available to interact with STIM1 at ER-PM junctions, avoiding the need to recruit Orai1 from the intracellular pool (I3). This is consistent with the data showing faster translocation of NFAT1 in cells expressing Orai1-ICL compared to wild type before reaching similar plateau levels where Orai1 is recruited from the intracellular pool.

The role of CCT in protein trafficking, in addition to its well-defined function as a folding chaperonin, is not limited to Orai1. In addition to its role in regulation trafficking of the Lox-1 receptor (28), a growing body of evidence suggests that CCT, through its association with the Bardet-Biedl syndrome (BBS) complex, plays a role in protein trafficking at the base of cilia (41–43). BBS is an autosomal recessive genetic disease in humans belonging to the cilipathy spectrum disorders (44). Furthermore, functional studies in Caenorhabditis elegans show that the BBS complex is involved in intraflagellar transport, and in zebrafish, knockdown of BBS proteins results in intracellular transport delay, specifically retrograde vesicle and organelle transport (45, 46). Similarly, knockdown of CCT1 and CCT2 results in a cilia trafficking defect (42). In another model, Trypanosoma brucei, the BBS complex was suggested to facilitate endocytic trafficking of specific cell surface proteins, mainly through interactions with clathrin and ubiquitin (47).

In summary, the present study identifies the CCT complex as a novel regulator of Orai1 trafficking and PM residence. Disruption of Orai1-CCT interaction enriches Orai1 at the PM, leading to more rapid and sustained Ca\(^{2+}\) signals and activation of NFAT as a downstream effector. Therefore, these results define CCT as a novel regulator of SOCE activation at 93°C, 35 cycles (15-s denaturation at 93°C, 30-s annealing at 62°C, and 10-min primer extension at 68°C).

MATERIALS AND METHODS

Reagents and antibodies

TG, Dulbecco’s modified Eagle’s medium:Nutrient Mixture F-12 (DMEM/F12), high-glucose DMEM with glucose (4500 mg/liter), Fura-2 AM, Cy3- and Cy5-conjugated secondary antibodies, and penicillin-streptomycin were from Invitrogen. Fetal bovine serum (FBS), rabbit polyclonal anti-Orai1 antibody, mouse anti-actin antibody, rabbit anti-CCT2 antibody, ATP, and protease inhibitor cocktails were from Sigma-Aldrich. Mouse monoclonal anti-CCT5 antibody (GT639) was from GeneTex. Rat monoclonal anti-CCT1 antibody (91A, sc-53454) was from Santa Cruz Biotecy Technology Inc. Purified monoclonal anti-HA.11 Epitope Tag antibody (#MMS-101P) was from Covance. Rabbit anti-Orai1 antibody (ab78471) was from Abcam. Mouse anti-GM130 was from BD Transduction Laboratories. Rabbit anti-EEA1 antibody and rabbit anti–Na,K-ATPase antibody (#3010) were from Cell Signaling. Rabbit anti-IP\(_3\)R antibody was from Affinity BioReagents. Horseradish peroxidase–conjugated goat anti-rabbit IgG and goat anti-mouse IgG antibodies were from Jackson ImmunoResearch Laboratories. Enhanced chemiluminescence detection reagents and immobilized streptavidin gel were from Pierce. IRDye 800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG antibodies were obtained from LI-COR Biosciences.

Cell culture

CHO-K1 cells obtained from the American Type Culture Collection (CCL-61) and TRvb1 CHO cell line lacking the endogenous transferrin receptor and stably expressing the human transferrin receptor (48) were maintained in high-glucose DMEM/F12. HeLa cells were maintained in high-glucose DMEM. Media for cell lines were supplemented with 10% heat-inactivated FBS (Invitrogen) and 5% (v/v) of a stock solution containing penicillin (5000 U/ml) and streptomycin (5 mg/ml) (Invitrogen) and cultured at 37°C and 5% CO\(_2\). For transient transfections, cells were grown to 50 to 70% confluency and transfected using Lipofectamine 2000 (Invitrogen). Generation of YFP-HA-Orai1 stable CHO cell lines was by pDS-YFP-HA-Orai1 DNA transfection and selected using selective media [DMEM/F12, 10% FBS, G418 (800 μg/ml), and penicillin and streptomycin (100 μg/ml)].

Molecular biology

Plasmids pDS-YFP-HA-Orai1, Orai1-AN, and Orai1-AC were a gift from R. Lewis (Stanford University, Palo Alto, CA) (8, 33). Plasmid expressing GFP-CCT2 was a gift from J. Granthem (The Wenner-Gren Institute, Stockholm University, Sweden) (49). HA-NFAT1(4-460)–GFP (Addgene plasmid #11107) and human STIM1-CFP (Addgene plasmid #19755) were from A. Rao’s Lab (La Jolla Institute for Allergy and Immunology, La Jolla, CA). To construct pDS-YFP-HA-Orai1(1-266), pDS-YFP-HA-Orai1(1-275), and pDS-YFP-HA-Orai1(1-285), nucleotides at respective positions were substituted to generate premature stop codon using a site-directed mutagenesis kit from Stratagene. All constructs were validated by sequencing. The primers used were as follows: for YFP-HA-Orai1(1-256), 5′-TCTTGGCCGTCACCAGTGCTCTGGTT-3′ (forward) and 5′-AACACGTAGGGGTATCGTGGACCACGAC-3′ (reverse); for YFP-HA-Orai1(1-266), 5′-GCTCAGCTGTAGCATAAGCCTAGGGCAGTTCCG-3′ (forward) and 5′-GCTCAGCTGTAGCATAAGCCTAGGGCAGTTCCG-3′ (reverse); for YFP-HA-Orai1(1-275), 5′-CAGAGCTCACTATAAGCCTAGGGCAGTTCCG-3′ (forward) and 5′-CAGAGCTCACTATAAGCCTAGGGCAGTTCCG-3′ (reverse); for YFP-HA-Orai1(1-285), 5′-CAGAGCTCACTATAAGCCTAGGGCAGTTCCG-3′ (forward) and 5′-CAGAGCTCACTATAAGCCTAGGGCAGTTCCG-3′ (reverse); for YFP-HA-Orai1(1-275), 5′-CAGAGCTCACTATAAGCCTAGGGCAGTTCCG-3′ (forward) and 5′-CAGAGCTCACTATAAGCCTAGGGCAGTTCCG-3′ (reverse); for YFP-HA-Orai1(1-285), 5′-CAGAGCTCACTATAAGCCTAGGGCAGTTCCG-3′ (forward) and 5′-CAGAGCTCACTATAAGCCTAGGGCAGTTCCG-3′ (reverse); for YFP-HA-Orai1(1-285), 5′-CAGAGCTCACTATAAGCCTAGGGCAGTTCCG-3′ (forward) and 5′-CAGAGCTCACTATAAGCCTAGGGCAGTTCCG-3′ (reverse). For generation of scrambled 157-167(ICL-S) Orai1 mutant, the QIAGEN LongRange PCR Kit (Qiagen) was used according to the manufacturer’s protocol. Briefly, polymerase chain reaction was done with 20 ng of template pDS-YFP-HA-Orai1 DNA using the primers 5′-PO4-AACGAGAAGGCCCCATCCTGCTGTGAGTGCTCCTCCTG-3′ (forward) and 5′-PO4-ATTGTGCACGTTGCTCCTCCTGCTGAGTGCTCCTCCTG-3′ (reverse) and 35 cycles of the following amplification protocol: 3-min initial activation at 93°C, 35 cycles (15-s denaturation at 93°C, 30-s annealing at 62°C, and 10-min primer extension at 68°C).
Transfections and immunofluorescence imaging
Cells grown on poly-D-lysine–coated glass-bottomed plates (MatTek Corporation) were transfected with desired constructs using Lipofectamine 2000. After 48 hours, cells were fixed in 4% (w/v) paraformaldehyde and permeabilized in phosphate-buffered saline (PBS) containing 0.2% (v/v) bovine serum albumin for 30 min. The primary antibodies used were GM130 (1:200) (BD Transduction Laboratories), IP3-R (1:100) (Affinity BioReagents), and EEA1 (1:200) (Cell Signaling). Labeling by primary antibodies was followed by incubation in a 1:200 dilution of the relevant Alexa Fluor 546–conjugated secondary antibody (Invitrogen) for 30 min. All antibody incubation steps were followed by extensive washes in PBS. Laser-scanning confocal microscopy of fixed cells was performed with a Leica SP inverted confocal imaging microscope (Leica; Lasertechnik). Images were processed with ImageJ (National Institutes of Health) and Adobe Photoshop CS3.

TIRF imaging and quantification
The kinetics of the formation of the Orai1 clusters at the PM plane was evaluated using TIRF microscopy on cells transiently transfected with equimolar amounts of Orai1-YFP and STIM1-CFP. Imaging was performed at room temperature on a Zeiss Cell observer TIRF microscope fitted with a 63×/1.46 lens using the following parameters: \(\lambda_{\text{ex}} = 488 \text{ nm}, \) filter for \(\lambda_{\text{em}} = 510 \text{ to } 555.\) The frame rate was 0.2 Hz and the typical exposure time was 50 ms. TIRF images in the PM plane were thresholded and normalized to the levels before TG to empty the stores. Puncta formation was assessed by increased fluorescence in the TIRF plane.

Orai1 S/T ratio assay
To measure surface Orai1, cells were washed once with PBS and immediately fixed using freshly prepared 4% (w/v) paraformaldehyde for 10 min at room temperature. In parallel, intracellular Orai1 was detected using cells fixed followed by permeabilization for 10 min in PBS containing 0.1% (w/v) Triton X-100. All subsequent antibody incubations were performed in PBS containing 5% (w/v) FBS. Cells were stained with saturating concentrations of monoclonal anti-HA antibodies (Covance) at 1:300 for 45 min at 37°C, followed by incubation in a 1:400 dilution of Cy5-conjugated goat anti-mouse IgG (Invitrogen) for 30 min at 37°C. The Cy5/YFP ratio was determined by quantitative fluorescence microscopy. In parallel, nonpermeabilized cells were fixed and stained. Then, Cy5/YFP was measured in both cell populations. The background signal of each of the YFP and Cy5 channels was set using two controls: YFP-HA-Orai1–transfected cells incubated only with Cy5 secondary antibody and cells that did not express the YFP-HA-Orai1 construct. The fraction of Orai1 at the PM was determined as the (Cy5/YFP in nonpermeabilized cells)/(Cy5/YFP in permeabilized cells) ratio.

Exocytosis assay
The exocytosis rate was determined as described previously (13). Briefly, cells were preincubated at 37°C with saturating concentration of anti-HA antibody to label YFP-HA-Orai1 and fixed after various incubation times. Initially, Orai1 at the PM was labeled, and an increase in total cell-associated anti-HA antibodies was observed over time as more Orai1 trafficked to the PM and interacted with anti-HA antibodies. Cells were then fixed, permeabilized, and stained with Cy5-labeled anti-mouse antibody. Therefore, the time course of the Cy5/YFP ratio reflects the exocytosis of YFP-HA-Orai1. Data were fit to a monoeponential growth curve as discussed previously to determine the exocytosis rate (17).

Endocytosis assay
Orai1 endocytosis rate was measured as the amount of Orai1 internalized as a function of time as described previously (13). Briefly, cells were incubated with saturating concentration of anti-HA antibody, and at various times, cells were fixed and stained with Cy5-labeled anti-mouse secondary antibody to label the Orai1 pool at the PM. Cells were fixed again, permeabilized, and stained with Cy3-labeled anti-mouse IgG to reveal anti-HA that has been internalized. The fluorescence intensities of the Cy3 and Cy5 were measured using quantitative fluorescence microscopy. Unlabeled cells were used for a background correction. The \(\frac{\text{Cy3/YFP}}{\text{Internal}}\)/(\(\text{Cy5/YFP}\) Surface ratio) was plotted over time and fitted with a linear regression function.

Immunoisolation of Orai1 for proteomics studies
Orai1 was immunoprecipitated under detergent-free conditions from CHO cells stably expressing YFP-HA-Orai1 using the μMACS GFP Isolation Kit (Miltenyi Biotec) according to the manufacturer’s protocol. Since the sub-PM Orai1–positive compartment becomes more evident after HA feeding, cells were incubated with saturating concentration of anti-HA antibody. In parallel, cells transfected with YFP-HA-Orai1 without HA incubation served as a control for the immunoprecipitation experiment. After treatment, cells were washed with ice-cold PBS, scraped in detergent-free HES buffer [20 mM Hepes (pH 7.4), 1 mM EDTA, and 0.25 M sucrose, with protease inhibitors], and lysed using syringe-based homogenization by passing them through 25G1/2 and 26G1/2 needles. Cell lysates were centrifuged at 10,000 g for 10 min at 4°C. The supernatant was incubated with GFP microbeads for 45 min on ice and then loaded on a microcolumn. Captured complexes were washed and then eluted by increasing the pH using 0.1 M trimethylamine and neutralized using 4-morpholineethanesulfonic acid. The efficiency of YFP-HA-Orai1 pulldown was checked by Western blotting before MS analyses.

Subcellular fractionation by discontinuous sucrose density gradient centrifugation
Three T-75 flasks grown to confluency were placed on ice, washed once with ice-cold PBS (pH 7.4) to remove growth medium, and followed by incubation in ice-cold 10 mM tris-HCl (pH 7.4) for 1 min to induce osmotic swelling as an aid to cell disruption. The buffer was rapidly removed, and cells were scraped into an ice-cold homogenization HES buffer composed of 20 mM Hepes (pH 7.4), 1 mM EDTA, 0.25 M sucrose, and protease inhibitors. Cells were mechanically lysed in the absence of detergent by passing them repeatedly through 25G1/2 and 26G1/2 needles. The procedure for subcellular fractionation was adapted from a previous protocol (50). Briefly, cell lysates were centrifuged for 10 min at 1000g at 4°C in a benchtop centrifuge to obtain a p1000 pellet containing cell nuclei and unbroken cells. The p1000 pellet was resuspended in 1 ml of homogenization buffer, sonicated 3× for 10 s each using a Transonic digital sonicator at an amplitude setting of 100, and stored as p1000. The post-p1000 supernatant was then centrifuged for 10 min at 8000g at 4°C to obtain a p8000 pellet containing PM, mitochondria, and ER. The p8000 pellet was resuspended in 1 ml of homogenization buffer, sonicated, and then stored as p8000. The post-8000g supernatant was loaded at the top of
and visualized by ECL using horseradish peroxidase (HRP)–conjugated secondary antibodies. For proteomics studies, lysozymes from GFP-CCT2–expressing cells were used. CCT2 and CCT5 were immunoprecipitated using CCT2 (Sigma–Aldrich) and anti-CCT5 (GeneTex) antibodies, respectively.

For Western blot analysis, protein extracts were obtained from cells lysed in cold lysis buffer containing 25 mM tris–HCl (pH 7.6), 150 mM NaCl, 1% IGE Plex CA–630, and protease inhibitors for 30 min, followed by centrifugation at 10,000 rpm for 10 min at 4°C. Protein concentrations were determined using a Bio–Rad protein assay. Equal amounts of proteins were loaded on 4 to 12% SDS–PAGE. Electrophoresed samples were transferred to a PVDF membrane and blocked with 5% milk in TBST (tris-buffered saline with Tween 20), and the membrane was incubated overnight at 4°C with primary antibody with constant shaking. After washing, horseradish peroxidase–conjugated secondary antibody was applied. Bound proteins to the secondary antibody were visualized using ECL (Amersham Biosciences). Quantitative Western blot analysis was performed using the secondary IRDye 800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG antibodies, and blots were scanned on a LI-COR Odyssey platform, followed by analysis using LI-COR Image Studio Lite v. 4.0.

For analysis of the subcellular fractions, an equal sample volume (30 µl) was mixed with an equal volume of 2× SDS loading buffer loaded onto 4 to 12% SDS polyacrylamide gels. Proteins were transferred onto PVDF membranes at 20 V for 1 hour, probed with primary antibodies, and visualized by ECL using horseradish peroxidase–conjugated secondary antibodies. For proteomics studies, lysozymes from fractions were resolved by electrophoresis on 4 to 12% SDS polyacrylamide gels and stained with Coomassie Brilliant Blue R-250.

Quantitative MS
Immunoprecipitated protein complexes using anti-GFP microbeads (described above) were washed [three iterations with triethylammonium bicarbonate (TEAB)] before reduction [200 mM tris(2-carboxyethyl)phosphine], alkylation (15 mM iodoacetamide), and precipitation (methanol/chloroform). The pellet is then dried down, resuspended in TEAB, and digested overnight using trypsin (Promega; weight ratio, 1:50). Stable isotope dimethyl labeling was performed according to Boersma et al. (25). Briefly, samples were incubated for 1 hour with either the light or the heavy form of formaldehyde and cyanoborohydride and then mixed in a 1:1 ratio. Combined samples were then loaded on R3 beads for cleaning and concentration before LC–MS analysis. Reconstituted sample (6 µl) was injected on C18 packed capillary emitter columns and eluted using either a 120-min or a 240-min gradient. High-resolution tandem MS was performed on a Q Exactive instrument (Thermo Fisher Scientific) using a data-dependent approach. Acquired data were then processed using the MaxQuant suite of programs (51), using a comprehensive Chinese hamster protein database from UniProt.org. For the proteomics studies on subcellular fractions, proteins for fractions positive for Orai1 (fractions 9 to 11) were separated on SDS–PAGE and stained with Coomassie Brilliant Blue R-250. Whole gel lanes were cut out from the gel. For in-gel digestion, the Shevchenko protocol was used (52).

After SDS–PAGE, the gel lanes were sliced, chopped in Eppendorf tubes, and washed in 25 mM ammonium bicarbonate/ethanol until completely destained. The reduction and alkylation were carried out by incubation with 10 mM dithiothreitol and 55 mM iodoacetamide. The gel pieces were dehydrated in ethanol, dried in a SpeedVac, and digested with trypsin at 37°C for a minimum of 4 hours. The tubes were then centrifuged for 1 min at 15,000g, and the supernatant was transferred to a fresh tube. After digestion, protein peptides were extracted by mixing with 30% acetonitrile (ACN)/3% trifluoroacetic acid for 10 min, followed by centrifugation to remove the supernatant. This step was repeated twice and then followed by two rounds of extraction in 100% ACN. The supernatants are combined and concentrated for 1 to 2 hours in a SpeedVac at 37°C before LC–MS/MS.

siRNA knockdown of CCT2
YFP–HA–Orai1 stable cells were transfected with the siRNA ON–TARGETplus SMARTpool (50 nM) designed against human CCT2 (NM_007636.2) or nontargeting siRNA as a control, using a DharmaFECT transfection reagent (Dharmacon). Four siRNA duplexes targeting human CCT2 were used: 5′-GCACAACAUUAUCCUAACAA-3′, 5′-AAAGUUAGCUGUAAGCA-3′, 5′-GAAGUUAAUCCGUCAAG-3′, and 5′-UGACACAGCUCCAAUAG-3′. These duplexes have 78 to 100% sequence identity to hamster CCT2 and effectively knocked down CCT2 in CHO cells (Fig. 3). Cells were incubated for 16 hours with the respective siRNA; the transfection medium was aspirated and replaced with Opti-MEM (Minimal Essential Medium) fresh medium without siRNA for 48 hours before imaging or Western blot analysis.

Statistical analysis
Results are expressed as means ± SEM. Statistical analysis was performed using analysis of variance and Student’s t test. Differences are considered significant at P < 0.05 and are indicated in the figures as follows: ∗P < 0.05, ∗∗P < 0.01, and ∗∗∗P < 0.001.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/9/eaau1935/DC1
Supplementary Methods
Fig. S1. Effects of Orai1 C-terminal truncations on SOCE.
Fig. S2. Subcellular localization of Orai.
Fig. S3. Orai1 PM targeting is independent of its expression levels and glycosylation.
Fig. S4. Replacing residues 157 to 167 in the Orai1–ICL with alanine prevents Orai1 trafficking to the PM.
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