Translocation between PI(4,5)P_2-poor and PI(4,5)P_2-rich microdomains during store depletion determines STIM1 conformation and Orai1 gating

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The Orai1–STIM1 current undergoes slow Ca^{2+}-dependent inactivation (SCDI) mediated by the binding of SARAF to STIM1. Here we report the use of SCDI by SARAF as a probe of the conformation and microdomain localization of the Orai1–STIM1 complex. We find that the interaction of STIM1 with Orai1 carboxyl terminus (C terminus) and the STIM1 K-domain are required for the interaction of SARAF with STIM1 and SCDI. STIM1–Orai1 must be in a PM/ER microdomain tethered by E-Syt1, stabilized by septin4 and enriched in PI(4,5)P_2 for STIM1–SARAF interaction. Targeting STIM1 to PI(4,5)P_2-rich and -poor microdomains reveals that SARAF-dependent SCDI is observed only when STIM1–Orai1 are within the PI(4,5)P_2-rich microdomain. Notably, store depletion results in transient localization of STIM1–Orai1 in the PI(4,5)P_2-poor microdomain, which then translocates to the PI(4,5)P_2-rich domain. These findings reveal the role of PM/ER tethers in the regulation of Orai1 function and a mode of regulation by PI(4,5)P_2 involving translocation between PI(4,5)P_2 microdomains.
Ca$^{2+}$ is a unique second messenger whose cytoplasmic concentration is determined by Ca$^{2+}$ pumps and channels. Physiological receptor-evoked Ca$^{2+}$ signals regulate virtually all cell functions on timescales from ms to days. At the same time, excess cytoplasmic Ca$^{2+}$ ([Ca$^{2+}]_{i}$) is highly toxic. Most often, toxic [Ca$^{2+}]_{i}$ is due to excessive Ca$^{2+}$ influx through the plasma membrane (PM) Ca$^{2+}$ channels. Store-operated TRPC and Orai channels are key Ca$^{2+}$ influx channels. While TRPC channels are mostly cell specific, all cells express the major isoform Orai1, which mediates the Ca$^{2+}$ release-activated Ca$^{2+}$ (CRAC) current. Shortly after Orai1 is activated, it is partially inhibited by the rise in [Ca$^{2+}]_{i}$: this limits Ca$^{2+}$ influx and prevents Ca$^{2+}$ toxicity.

Orai1 is activated in response to Ca$^{2+}$ release from the ER and is gated by the ER Ca$^{2+}$ sensor STIM1 (ref. 5). STIM1 has several domains including an ER-resident EF hand that mediates Ca$^{2+}$ sensing; a cytoplasmic coiled-coil domain 1 comprising an inhibitory helix that occludes STIM1 in the resting state; a SOAR domain that activates Orai1 (refs 9,10); a CTID domain that mediates the interaction of the STIM1 inhibits SARAF$^{11}$, with SOAR$^{12}$; a C terminus linker and a polybasic domain with multiple lysines (K-domain) that may mediate the interaction of STIM1 with PI(4,5)P$_2$ (ref. 13).

Store depletion results in Orai1–STIM1 clustering in ER/PM microdomains. The nature of these domains and their role in Ca$^{2+}$ signalling is not well understood. Several recent studies have begun to define these domains. A search for proteins that tether the ER/PM in yeast identified ER-resident homologues of the phosphatidylinositol transfer protein Nir2; this restores high [Ca$^{2+}]_{i}$ when both STIM1–Orai1 complex formation and the STIM1 Ca$^{2+}$ release channel are inhibited. This is in turn is followed by the translocation of the STIM1–Orai complex to the PI(4,5)P$_2$-rich domain, recruitment of SARAF and SCDI. These findings identify a role for tethered ER/PM microdomains in regulating Ca$^{2+}$ influx and directing STIM1–Orai1 conformational changes, and report on a new mode of regulation by PI(4,5)P$_2$.

**Results**

Orai1 C terminus facilitates interaction of SARAF with STIM1. Previous work reported that SARAF mediates the SCDI of Orai1 (ref. 11). Supplementary Fig. 1a shows that SARAF also affects FCDI. FCDI is affected by the STIM1–Orai1 ratio$^{22,23}$. At a STIM1–Orai1 expression ratio of 1:1 and 20 mM EGTA in the pipette, (to minimize FCDI and better resolve the effect of SARAF), FCDI has mainly one component with a single time constant t of 7.0 ± 0.5 ms (n = 3; in all results, the ± indicates s.e.m. and n indicates the number of experiments). In the presence of SARAF, FCDI is described best by two exponentials with time constants t of 15.4 ± 0.2 and t of 241 ± 26 ms (n = 3). The effect of SARAF on FCDI was not examined further in this study, as here we were interested mainly in using SCaDi as readout of STIM1 conformation and localization in the PM/ER microdomain.

Orai1 was reported to facilitate the interaction of SARAF with STIM1 (ref. 11). We extended these findings in Fig. 1a,b, which compare the time course of STIM1–STIM1, STIM1–Orai1 and STIM1–SARAF interaction using FRET (Fig. 1a) and co-immunoprecipitation (Co-IP; Fig. 1b) assays. The basal FRET efficiency of STIM1–Orai1 and STIM1–SARAF (see Supplementary Fig. 2a,b) are similar. To better illustrate the time course of FRET increase, Fig. 1a,c shows the normalized FRET ratio. The results show that the STIM1–SARAF interaction is increased minimally in the absence of Orai1. STIM1–STIM1 and STIM1–Orai1 FRET start shortly after the initiation of store depletion. Notably, FRET (Fig. 1a) and Co-IP (Fig. 1b) show that the STIM1–SARAF interaction is delayed by about 20–45 s. It then progresses more slowly than the STIM1–STIM1 and STIM1–Orai1 interactions. Several Orai mutants were used to identify the Orai1 domain that facilitates the STIM1–SARAF interaction (Fig. 1c). Orai1(D1–73), Orai1(ΔN) lacking the entire Orai1 amino terminus (N terminus), constitutively active Orai1(V102C) and channel-dead Orai1(R91W) facilitated the STIM1–SARAF interaction. Only Orai1(L273S) with a disrupted C terminus CCD$^{24}$ failed to support the STIM1–SARAF interaction. Figure 1e shows the Orai1-mediated CRAC current. The current is activated by depleting ER Ca$^{2+}$ by including relatively low concentration (1.5 mM) of the slow Ca$^{2+}$ buffer EGTA in the pipette solution so that SCaDi can be reliably measured. Three minutes after the initiation of store depletion, the current was measured by including 10 mM Ca$^{2+}$ in the bath solution. SARAF overexpression enhanced and its knockdown (see Fig. 1d) markedly reduced SCaDi.

These findings suggest that the STIM1–SARAF interaction is independent of the Orai1 channel function and can take place at basal [Ca$^{2+}]_{i}$, and that the interaction of STIM1 with the Orai C terminus CCD mediates recruitment of SARAF to the STIM1–Orai1 complex. Recruitment of SARAF to the complex starts only after initiation of the interaction between STIM1 and Orai1.

The STIM1 K-domain in STIM1–SARAF interaction. To determine if STIM1 domains other than SOAR are required for its interaction with SARAF, we tested the role of the STIM1 K-domain. Deletion of the K-domain prevented STIM1–SARAF interaction. This was the case even before store depletion as...
Figure 1 | STIM1–Orai1 interaction is required for SARAF interaction with STIM1. (a) FRET was measured in HEK293 cells transfected with CFP-STIM1 and YFP-STIM1, CFP-STIM1 and YFP-Orai1, CFP-STIM1 and YFP-SARAF and with (red) or without (green) HA-Orai1. Store depletion by CPA in Ca²⁺-free solution was initiated where indicated. The delay in STIM1–SARAF interaction is marked by grey rectangle. The averaged FRET efficiencies before and after 5 min treatment with CPA are shown in Supplementary Fig. 2. The images are example FRET images at the indicated conditions before (basal) and after 5 min store depletion. Scale bars, 5 μm. (b) Co-IP of STIM1 and SARAF. HEK cells were transfected with STIM1 and SARAF alone (left blots) or with Orai1 (right blots) and were incubated in Ca²⁺-free solution containing 25 μM CPA for the indicated times before preparation of lysates. The lysates were used to IP SARAF and blotted for STIM1. (c) CFP-STIM1-YFP-SARAF FRET was measured in cells expressing the indicated HA-Orai1 mutants. Scale bars in images are 5 μm. (d) HEK cells were treated with siSARAF for 48 h and analysed for expression of SARAF. (e) HEK cells were treated with scrambled or siSARAF were transfected with Orai1, STIM1 and with or without SARAF and Orai1 current was measured by dialyzing cells with pipette solution containing 1.5mM EGTA for 3 min in Ca²⁺-free bath. CRAC current was initiated by superfusing with a solution containing 10 mM Ca²⁺. The residual current was inhibited with 10 μM La³⁺. The same protocol was used in all current measurements unless otherwise stated. The currents were leak subtracted and normalized to the zero current to facilitate demonstrating the SCDI. Pick current density in pA/pF is given in the text. For all FRET experiments, of the two numbers listed in parenthesis next to the traces, the first indicates the number of experiments and the second indicates the number of cell analysed. All results (FRET and current) are given as the means ± s.e.m.

Tethered PM/ER microdomain in STIM1–SARAF interaction. It has been suggested that the K-domain interacts with PM PI(4,5)P₂ (ref. 13). Therefore, we tested the effect of depleting PM PI(4,5)P₂ with the FRB/FKBP12 rapamycin-activated system²¹. The scheme in Fig. 3a illustrates the experimental system for Figs 3 and 4 and Fig. 3b shows that the FRB/FKBP12 system efficiently depletes cellular PI(4,5)P₂. Supplementary Fig. 2c shows that the depletion of PI(4,5)P₂ reduces the basal STIM1–SARAF interaction (reduced basal FRET efficiency). More importantly, Fig. 3c shows that the depletion of PI(4,5)P₂ strongly inhibits the STIM1–SARAF interaction in response to store depletion. Significantly, as reported previously²¹, PI(4,5)P₂ depletion had no effect on Orai1 peak current (19.8 ± 0.04 and 20.0 ± 0.6 pA/pF without and with PI(4,5)P₂ depletion; n = 8 and 6), but prevented the effect of SARAF (Fig. 3d).

It has recently been reported that filamentous septin4 participates in the formation of a PI(4,5)P₂ microdomain around Orai1 and it is required for STIM1–Orai1 clustering and Orai1 activation²⁰. We confirmed that the knockdown of septin4 (Fig. 4a) reduced Orai1 current by 36 ± 3%. It is unlikely that this can be attributed solely to the disruption of a PI(4,5)P₂ microdomain²⁰, since depletion of PI(4,5)P₂ did not reduce current density. Nevertheless, Fig. 4b,c and Supplementary Fig. 2d show that the knockdown of septin4 prevented STIM1–SARAF interaction and the effect of SARAF on SCDI (the currents were normalized to better demonstrate the effect of septin4 knockdown).
Recent studies have shown that all three E-Syts participate in tethering the PM to the ER\(^{18}\) and that E-Syt1 in particular has a key role\(^{14,18,19}\). Here we tested the effects of knockdown and overexpression of E-Syt1 on STIM1–SARAF interaction and SCDI. Preliminary experiments showed that HEK cells express predominantly E-Syt1 and E-Syt2 (Supplementary Fig. 4a) and

**Figure 2 | SCDI by SARAF required the STIM1 K-domain.** (a) HEK293 cells were transfected with Orai1, CFP-STIM1 or CFP-STIM1\(^{ΔK}\) and YFP-SARAF, and FRET was measured in response to store depletion by 25 \(\mu\)M CPA in \(Ca^{2+}\)-free solution. The columns show FRET efficiency before (basal) and 5 min after treatment with CPA. FRET efficiencies of the same experiments were normalized to show the time course of FRET changes in response to CPA. The images are examples of FRET images measured in cells expressing STIM1\(^{ΔK}\), SARAF and Orai1 before and after 5 min treatment with CPA, as indicated. Scale bars, 5 \(\mu\)m. (b) The Orai1 current and SCDI were measured with STIM1 or STIM1\(^{ΔK}\) and in the presence and absence of SARAF. Results are given as mean \(±\) s.e.m of the number of experiments.

**Figure 3 | SCDI by SARAF requires PM PI(4,5)P\(_2\).** (a) A model illustrating a microdomain formed by PI(4,5)P\(_2\), tethered by E-Syt1 and stabilized by septins. (b) Cells transfected with EGFP-PH domain (green, PI(4,5)P\(_2\) probe) and the FRB/FKBP12 (red, PI(4,5)P\(_2\) depletion systems) were imaged before (left) and after exposure to 0.1 \(\mu\)M rapanycin for 2 min to deplete the PI(4,5)P\(_2\). Scale bars, 5 \(\mu\)m. (c) HEK293 cells were transfected with Orai1, STIM1 and SARAF and the FRB/FKB12 or FRB/FKB12-stop (no phosphatase control) and were either untreated (control) or treated with 0.1 \(\mu\)M rapanycin for 5 min to deplete PM PI(4,5)P\(_2\) (red trace) and CFP-STIM1, YFP-SARAF FRET was measured in response to store depletion by 25 \(\mu\)M CPA in \(Ca^{2+}\)-free solution. Basal and 5-min store depletion FRET efficiencies are shown in Supplementary Fig. 2. (d) Orai1 current was measured in cells transfected with Orai1, STIM1 and with or without SARAF and the FRB/FKB12 system and SCDI by SARAF measured. Shown are rapanycin control (grey), FRB/FKB12, no SARAF, no rapamycin control (blue) and FRB/FKB12, SARAF, no rapamycin control (red). Depletion of PI(4,5)P\(_2\) inhibited the effect of SARAF on SCDI (green). Results are given as mean \(±\) s.e.m of the number of experiments indicated in brackets.
that the knockdown of E-Syt1 increased E-Syt2 mRNA levels (Supplementary Fig. 4b). Although E-Syt2 and E-Syt3 were shown to localize to the peripheral ER and participate in PM/ER tethering, the knockdown of E-Syt2 and E-Syt3 had no effect on SARAF-mediated SCDI (Supplementary Figs. 4c,d). In contrast, Fig. 4d shows that the knockdown of E-Syt1 (see Supplementary Fig. 4c) reduced basal STIM1–SARAF FRET efficiency. Conversely, the overexpression of E-Syt1 maximally increased STIM1–SARAF FRET efficiency and FRET efficiency did not increase further by store depletion. The overexpression of E-Syt1 was sufficient to significantly increase STIM1–SARAF FRET efficiency in the absence of Orai1. These findings, together with the findings that the overexpression of E-Syt1 markedly increases the number and size of the PM/ER microdomains, indicate that E-Syt1 overexpression preassembles the Orai1–STIM1–SARAF complex and thus no further recruitment of SARAF is observed on store depletion. Accordingly, Fig. 4e shows that knockdown of E-Syt1 prevented the SARAF-mediated SCDI, while the overexpression of E-Syt1 maximized SCDI to the level observed by the overexpression of SARAF, without affecting current density (Control 18.1 ± 0.5, siE-Syt1 18.5 ± 0.2, E-Syt1 18.0 ± 0.7 pA/pF; n = 4, 8, 6, respectively).

Together the findings in Figs 3 and 4 suggest that tethering of the ER to the PM by E-Syt1 forms a microdomain that requires filamentous septins, is rich in PI(4,5)P2 and to which the STIM1–Orai1 complex is targeted to recruit SARAF. In addition, the findings with the knockdown of the various E-Syts suggest some specificity in PM/ER tethers. The tethers formed by E-Syt1 recruit and regulate the function of the Orai1–STIM1 complex, while the extensive tethers formed by E-Syt2 and E-Syt3 (ref. 18) do not.

Targeting STIM1 to defined PM/ER sites and SCDI by SARAF.

To support the hypothesis that the STIM1–Orai1 complex is targeted to the PI(4,5)P2-rich microdomain for interaction with SARAF, we sought means to target STIM1 to different PM microdomains. We achieved this using the Lyn- and Hras-targeting motifs. These motifs were shown to target proteins to different PM microdomains. This is illustrated in Supplementary Fig. 5d,e showing that STIM1-Kras and STIM1-Lyn are likely not localized in the same microdomain. First, the puncta formed by STIM1-Kras and STIM1-Lyn are clustered in PM puncta in the absence of Ca2+ store depletion.

Importantly, the puncta formed by STIM1-Kras and STIM1-Lyn are likely not localized in the same microdomain. First, PM localization of STIM1-Lyn was enhanced by deletion of the STIM1 K-domain, while STIM1-Kras was retained in the PM puncta in spite of deletion of the K-domain (Supplementary Fig. 6c,e). Second, STIM1-Kras strongly co-IP`ed with SARAF, while STIM1-Lyn did not. It was not possible to show the differential localization of STIM1-Kras and STIM1-Lyn in the same cells since the strong targeting motifs of STIM1-Kras and STIM1-Lyn recruited the respective STIM1s to the same puncta. This is illustrated in Supplementary Fig. 5d,e showing that STIM1-Kras and STIM1-Lyn recruited wild-type STIM1 to the...
Figure 5 | Targeting STIM1 to PI(4,5)P₂-poor and PI(4,5)P₂-rich PM microdomains. (a) Scheme illustrating the microdomain targeting of STIM1 by the Lyn/Hras- and Kras-targeting motifs. (b) Examples of FRET images before and after treatment with CPA for 5 min in cells transfected with CFP-STIM1-Lyn + SARAF + Orai1 or STIM1-Kras + SARAF + Orai1 related to the experiments in c. Scale bars, 5 μm. (c) HEK293 cells were transfected with CFP-STIM1 and YFP-SARAF in the absence (grey) and presence of Orai1 (black); YFP-SARAF and CFP-STIM1-Kras (blue) or CFP-STIM1-Lyn (green) in the absence or presence of Orai1 (red and purple, respectively) and FRET efficiency was measured in response to store depletion by 25 μM CPA in Ca²⁺-free solution. (d) For the Co-IP, HEK cells were transfected with SARAF and either STIM1-Kras (upper blots) or STIM1-Lyn (lower blots) and with (right blots) or without Orai1 (left blots). The cells in Ca²⁺-free solution were treated with 25 μM CPA for the indicated times and were used to measure the Co-IP of STIM1 and SARAF. (e) Orai1 current was measured in pipette solution containing 10 mM BAPTA and bath containing 10 mM Ca²⁺ to evaluate the spontaneous channel activity with STIM1 (black), STIM1-Kras (red) and STIM1-Lyn (green). In several experiments at each condition, where indicated, the residual currents were inhibited by 10 μM La³⁺. (f) SCDI was measured in pipette solution containing 1.5 mM EGTA and cells transfected to measure the Co-IP of STIM1 and SARAF. (g) SCDI was measured in pipette solution containing 1.5 mM EGTA and cells transfected with Orai1 related to the experiments in Fig. 5e-g. Figure 5e shows the CRAC current measured in the presence of the strong and fast Ca²⁺-buffer 10 mM BAPTA to minimize SCDI and Supplementary Fig. 3d shows the Ca²⁺ permeability of cells expressing STIM1-Kras alone. Notably, STIM1-Kras/Orai1 was largely constitutively active independent of store depletion, while STIM1-Lyn/Orai1 was not, although both generated puncta in the absence of store depletion (Supplementary Fig. 5). However, the store depletion fully activated STIM1-Lyn/Orai1 and STIM1-Lyn/Hras with similar current density to STIM1-Kras/Orai1 (19.7 ± 0.9, 17.8 ± 0.4 and 18.7 ± 0.4 pA/pF, respectively; n = 7, 5, 13, respectively). Most significantly, Fig. 5f,g show that the Orai1 current activated by STIM1-Kras undergoes rapid and strong SCDI with no further effect of SARAF, whereas the currents activated by STIM1-Lyn and STIM1-Hras are not
affect by SARAF. The effects of targeting STIM1 by the Kras, Hras and Lyn motifs were not non-specific effects of extension of the STIM1 C terminus. Extending STIM1 C terminus by a similar size α helix had no effect on current amplitude (19.1 ± 0.5 pA/pF; n = 4) or SCDI by SARAF (Supplementary Fig. 8).

The results in Supplementary Figs 5–7 and Fig. 5 suggest that the Kras, Hras and Lyn motifs target STIM1 to different PM microdomains. STIM1-Kras is likely targeted to the domain dependent on caveolin/PI(4,5)P2/E-Syt1/septins, while STIM1-Lyn and STIM1-Hras are targeted to a different domain. STIM1-Kras and STIM1-Lyn/Hras then recruit Orai1 to the two respective domains. STIM1-Kras/Orai1, but not STIM1-Lyn-Hras/Orai1, then recruits SARAF to mediate SCDI. In support of this conclusion, the fast and maximal SCDI observed with STIM1-Lyn/Hras is not affected by SARAF. The effects of targeting STIM1 by the Kras, Hras and Lyn motifs to the PI(4,5)P2-poor domain and YFP with C terminus Lyn motif or YFP with C terminus Hras motif to the PI(4,5)P2-poor domain and YFP with C terminus Kras motif to the PI(4,5)P2-rich domain (Fig. 7a and model in Fig. 7b). The probes were co-expressed with CFP-STIM1, and FRET in response to store depletion was measured. Most notably, FRET between Lyn-EGFP (Fig. 7c) and YFP-Hras (Fig. 7d) and CFP-STIM1 started upon start of store depletion but was transient and returned to basal levels within 1–2 min. Conversely, FRET between YFP-Kras and CFP-STIM1 started after a delay of about 1 min and then remained stable. Most notably, the depletion of PI(4,5)P2 resulted in retention of STIM1 in the Lyn/Hras PI(4,5)P2-poor domain and its exclusion from the Kras PI(4,5)P2-rich domain.

**Discussion**

Excess Ca\(^{2+}\) influx is associated with, and in many cases is the initiator of, cellular and tissue pathologies. Hence, Ca\(^{2+}\) influx channels are extensively regulated, most prominently by Ca\(^{2+}\) itself, to restrict Ca\(^{2+}\) influx. This is very well described for the Orai1 channel, which undergoes both FCDI and SCDI, with STIM1 (refs 12,27–29) and SARAF (Fig. 1 and Supplementary Fig. 1a) participating in both through the STIM1 CTID domain. The present work indicates that the regulation of Orai1 by Ca\(^{2+}\) depends on the localization of the Orai1–STIM1 complex to specific PM microdomains and dynamic translocation of the complex between the microdomains; this sets the STIM1 conformation that recognizes SARAF. The microdomain is assembled by the E-Syt1-mediated tethering of the ER to the PM and appears to require several proteins known to demarcate PM microdomains, such as caveolin and septins. Caveolin is a scaffolding protein that participates in the formation of caveolae and assembly of signalling complexes. Septins are a family of small GTPases that polymerize into non-polarized filaments and interact with both microtubules and the actin cytoskeleton to restrict distribution of proteins and lipids in the PM31. We note that the depletion of caveolin, E-Syt1 and PI(4,5)P2 did not reduce the Orai1 current but only eliminated the effect of SARAF on SCDI. However, the depletion of septin4 while eliminated the

**Translocation of STIM1 between PI(4,5)P2 microdomains.** The above findings indicate that the STIM1–Orai1 complex must localize to the PI(4,5)P2-rich domain to assume a conformation that allows it to recruit SARAF and mediate SCDI. Conversely, when the STIM1–Orai1 complex is in a PI(4,5)P2-poor domain it is in a conformation that cannot access SARAF, and thus the Orai1 current is more sustained. A critical question is whether STIM1 translocates between the two domains during physiological activation of the current. The first evidence that this is the case can be deduced from the delayed interaction between STIM1 and SARAF (Fig. 1a,b). Direct evidence for the shift of STIM1–Orai1 between microdomains is given in Fig. 7. We targeted EGFP with N terminus Lyn motif or YFP with C terminus Hras motif to the PI(4,5)P2-poor domain and YFP with C terminus Kras motif to the PI(4,5)P2-rich domain (Fig. 7a and model in Fig. 7b). The probes were co-expressed with CFP-STIM1, and FRET in response to store depletion was measured. Most notably, FRET between Lyn-EGFP (Fig. 7c) and YFP-Hras (Fig. 7d) and CFP-STIM1 started upon start of store depletion but was transient and returned to basal levels within 1–2 min. Conversely, FRET between YFP-Kras and CFP-STIM1 started after a delay of about 1 min and then remained stable. Most notably, the depletion of PI(4,5)P2 resulted in retention of STIM1 in the Lyn/Hras PI(4,5)P2-poor domain and its exclusion from the Kras PI(4,5)P2-rich domain.

**Figure 6 | The rate and maximal SCDI with STIM1-Kras requires native SARAF and intact PI(4,5)P2-rich microdomain.** (a) Cells were treated with scrambled (black, red) or siSARAF (blue, green), and SCDI by STIM1 (black, blue) or STIM1-Kras (red, green) was measured. (b) SCDI by STIM1-Kras was measured in cells transfected with Orai1 and FRB/FKBP12 (red, control) and treated with rapamycin to deplete PI(4,5)P2 (green). (c) SCDI by STIM1-Kras was measured in cells transfected with Orai1 and E-Syt1 (red) or treated with si-E-Syt1 (green). The control orange trace is reproduced from Fig. 4e. (d) Depletion of PI(4,5)P2 has no effect on the current measured with STIM1-Lyn (blue). Results are given as mean ± s.e.m. of the number of experiments indicated in brackets.
The present studies reveal dynamic translocation of the STIM1–Orai complex between PI(4,5)P2-poor and PI(4,5)P2-rich microdomains to control Ca\(^{2+}\) influx. This is a new form of regulation by PI(4,5)P2 that has not been previously described. Prior modes of regulation by PI(4,5)P2, including the regulation of ion transporters, has been attributed to a reduction or increase in PM PI(4,5)P2 levels or to targeting of proteins to PI(4,5)P2-rich microdomains (reviewed in ref. 32). Dynamic translocation between PM microdomains with different PI(4,5)P2 levels allows efficient temporal and spatial regulation independent of PI(4,5)P2 synthesis and breakdown. Translocation back and forth between PI(4,5)P2 microdomains should result in oscillatory activity of the regulated proteins. In the case of Orai1–STIM1, this will result in oscillations in Ca\(^{2+}\) influx to support receptor-evoked Ca\(^{2+}\) oscillations.

STIM1 and SARAF are localized in the ER and interact to some extent in the basal state with filled Ca\(^{2+}\) stores, as indicated by their basal Co-IPs (Fig. 1b) and FRET efficiency (Figs 4d and 5c and Supplementary Fig. 2). Moreover, SARAF stabilizes STIM1 in the basal conformation since the knockdown of SARAF resulted in the store-independent clustering of STIM1 and Ca\(^{2+}\) influx\(^{11,12}\). Activation of Ca\(^{2+}\) influx by store depletion needs to relieve the basal inhibition of STIM1 by SARAF leading to the formation of the STIM1–Orai1 complex. Ca\(^{2+}\) influx should be sustained for some time before its reinitiation by SARAF. The present studies provide evidence to suggest that the STIM1–Orai1 complex is first targeted to a PI(4,5)P2-poor microdomain, where STIM1 is in a conformation that fully activates Orai1 but minimally interacts with SARAF to increase Orai activity. Subsequently, the STIM1–Orai complex translocates to a PI(4,5)P2-rich microdomain (Fig. 7) formed by tethering the ER and PM by E-Syt1 (Fig. 4). In this microdomain, STIM1 is now in a conformation that fully activates Orai1 but minimally interacts with SARAF, which mediates the SCDI to limit Orai1 activity. This mechanism allows the precise and timed regulation of Ca\(^{2+}\) influx between fully activated and partially inhibited Ca\(^{2+}\) influx to guard against cell toxicity.

**Figure 7 | Dynamic translocation of STIM1 between PI(4,5)P2-poor and PI(4,5)P2-rich domains in response to Ca\(^{2+}\) store depletion.** (a) PM localization of Lyn-GFP, mCherry-Kras and YFP-Hras. YFP-Kras shows similar PM localization. Scale bars, 5 μm. (b) The scheme illustrates the localization of Lyn-GFP/YFP-Hras and YFP-Kras. (c) HEK293 cells were transfected with CFP-STIM1, Lyn-GFP (orange, grey, green) or YFP-Kras (red, blue) and the PI(4,5)P2-depletion system (grey, green, blue) and were treated with 0.2 μM rapamycin (green, blue) or left untreated (grey, control). (d) The cells were transfected with CFP-STIM1 and YFP-Hras (purple) and the PI(4,5)P2-depletion system (dark yellow), and FRET was measured in response to store depletion by 25 μM CPA in Ca\(^{2+}\)-free solution. Note the early but transient FRET with Lyn-GFP and YFP-Hras, the delayed but sustained FRET with YFP-Kras and the effect of PI (4,5)P2 depletion on the two responses. Results are given as mean ± s.e.m. of the number of experiments and cells indicated in brackets.
SARAF-YFP was made by digesting out SARAF at MluI and HindIII mutagenesis kit from Agilent Technologies. Primers used for cloning are listed in Supplementary Table 1. All mutations were generated using the QuikChange Lightning site-directed mutagenesis kit from Agilent Technologies. Primers used for cloning are listed in Supplementary Table 1. The cells were harvested after 48 h. The cells were transfected using Lipofectamine 2000 with siRNA duplexes after 12 h (20–40 nM each as pA/pF).

siRNA probes and RT-PCR. The scrambled control and short interfering RNA (siRNA) duplex sequences for SARAF, SEPTIN4, E-syt1 and Caveolin-1 are listed in Supplementary Table 1. The primers used for the RT-PCR were designed using the primer design tool available at the life technologies website.

FRET measurements. HEK293 cells were transfected with STIM1, SARAF and with or without Orai1. After 24 h, the cells were washed once in Ca2+-free solution and treated with 25 mM CPA for 0, 15, 30 or 45 s and the reactions were stopped and lysates prepared by a rapid removal of Ca2+ and ATP by centrifugation. The cleared lysates were used to IP STIM1 with anti-myc (SARAF) antibodies by standard IP protocols. The complexes were collected by addition of 50 μl of Protein G Sepharose beads and incubation for 4 h at 4°C. The precipitates were analysed by SDS-PAGE and blots were stained with Ponceau S.

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