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Translocation between PI(4,5)P₂-poor and PI(4,5)P₂-rich microdomains during store depletion determines STIM1 conformation and Orai1 gating

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Summary

The Orai1-STIM1 current undergoes slow Ca²⁺-dependent inactivation (SCDI) mediated by 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 interaction of STIM1 with Orai1 C terminus and the STIM1 K-domain are required for 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)P₂ for STIM1-SARAF interaction. Targeting STIM1 to PI(4,5)P₂ rich and poor microdomains reveals that SARAF-dependent SCDI is observed only when STIM1-Orai1 are within the PI(4,5)P₂-rich microdomain. Notably, store depletion results in transient localization of STIM1-Orai1 in the PI(4,5)P₂-poor microdomain, which then translocate to the PI(4,5)P₂-rich domain. These findings reveal the role of PM/ER tethers in the regulation of Orai1 function and a new mode of regulation by PI(4,5)P₂ involving translocation between PI(4,5)P₂ microdomains.

Introduction

Ca²⁺ is a unique second messenger whose cytoplasmic concentration is determined by Ca²⁺ pumps and channels. Physiological receptor-evoked Ca²⁺ signals regulate virtually all cell functions on time scales from msec to days¹. At the same time, excess cytoplasmic Ca²⁺ ([Ca²⁺]_i) is highly toxic^{2,3}. Most often, toxic [Ca²⁺]_i is due to excessive Ca²⁺ influx through the plasma membrane (PM) Ca²⁺ channels. Store operated (SOC) TRPC and Orai channels are key Ca²⁺ influx channels⁴. While TRPC channels are mostly cell specific, all cells

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express the major isoform Orai1, which mediates the Ca²⁺ release-activated Ca²⁺ (CRAC) current⁵. Shortly after Orai1 is activated, it is inhibited by the rise in [Ca²⁺]_i; this limits Ca²⁺ influx and prevents Ca²⁺ toxicity³.

Orai1 is activated in response to Ca²⁺ release from the ER and is gated by the ER Ca²⁺ sensor STIM1⁵. STIM1 has several domains, including: an ER-resident EF hand that mediates Ca²⁺ sensing⁶; a cytoplasmic coiled-coil domain 1 (CCD1) comprising an inhibitory helix that occludes STIM1 in the resting state^{7,8}; a SOAR domain that activates Orai1^{9,10}; a CTID domain that mediates interaction of the STIM1 inhibitor SARAF¹¹ with SOAR¹²; a C terminus linker, and a polybasic domain with multiple lysines (K-domain) that may mediate the interaction of STIM1 with PI(4,5)P₂¹³.

Store depletion results in Orai1-STIM1 clustering in ER/PM microdomains. The nature of these domains and their role in Ca²⁺ signaling is not well understood. Several recent studies have begun to define these domains. A search for proteins that tether the ER/PM in yeast¹⁴ identified ER resident homologues of mammalian extended synaptotagmins (E-Syts)¹⁵, VAP proteins, homologues of mammalian septins¹⁶, and TMEM16 proteins. The three mammalian E-Syts participate in lipid transfer between the ER and PM¹⁷. Their role in ER/PM tethering depends on PM PI(4,5)P₂ and, in the case of E-Syt1, also on [Ca²⁺]_i¹⁸. The E-Syts-formed tethers were suggested to be distinct from those formed by STIM1-Orai1¹⁸. However, the use of a PM/ER microdomain marker subsequently indicated that the receptor-evoked Ca²⁺ signal forms E-Syt1-dependent tethers that recruit the phosphatidylinositol transfer protein Nir2; this restores PM PI(4,5)P₂ levels and sustains the Ca²⁺ signal¹⁹. Finally, the filamentous septins 4 and 5 were reported to shape a PM PI(4,5)P₂ microdomain around clustered Orai1-STIM1 complexes²⁰. However, depletion of PI(4,5)P₂ does not inhibit activation of Orai1 by STIM1²¹. Thus, the exact roles of PI(4,5)P₂ and the ER/PM microdomain in regulation of Ca²⁺ signaling and particularly Ca²⁺ influx by Orai1 are not understood.

A major regulatory modality of Orai1 is inhibition by [Ca²⁺]_i to guard against excessive Ca²⁺ influx. [Ca²⁺]_i inhibits Orai1 in two ways, fast Ca²⁺-dependent inactivation (FCDI) that occurs within 10-20 msec, and slow Ca²⁺-dependent inactivation (SCDI) that develops in 1 min of channel activation³. SCDI (and possibly FCDI-see below) is mediated by the ER protein SARAF¹¹, which interacts with STIM1¹². In the present work, we use SCDI by SARAF as a reporter of Orai1-STIM1 complex conformation and microdomain localization. We report that both STIM1-Orai1 complex formation and the STIM1 K-domain are required for interaction of SARAF with STIM1. The STIM1-Orai1 complex must be present in a microdomain that is tethered by E-Syt1, that contains septin4, and that is enriched in PI(4,5)P₂. Furthermore, SCDI is observed only when the STIM1-Orai1 complex is in a PI(4,5)P₂-rich microdomain. Dynamics of STIM1-Orai1 complex localization were measured by following FRET between CFP-STIM1 and YFP targeted to the PI(4,5)P₂ rich and poor domains, revealing that store depletion is followed by STIM1-Orai1 complex formation in the PI(4,5)P₂-poor domain when the channel is fully active. This is in turn followed by translocation of the STIM1-Orai complex to the PI(4,5)P₂-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 $\text{PI}(4,5)\text{P}_2$.

Results

Orai1 C terminus facilitates interaction of SARAF with STIM1

Previous work reported that SARAF mediates the SCDI of Orai1¹¹. Supplementary Fig. 1a shows that SARAF also affect FCDI. FCDI is affected by the STIM1:Orai1 ratio^{22,23}. At the 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 τ_1 of 7.0 ± 0.5 msec ($n = ?$; in all results the \pm 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 τ_1 of 15.4 ± 0.2 and τ_2 of 241 ± 26 msec ($n=3$). The effect of SARAF on FCDI was not examined further in this study, as here we were interested mainly in using SCDI as readout of STIM1 conformation and localization in the PM/ER microdomain.

Orai1 was reported to facilitates the interaction of SARAF with STIM1¹¹. We extended these finding in Figs. 1a and 1b, which compare the time-course of STIM1-STIM1, STIM1-Orai1 and STIM1-SARAF interaction using FRET (Fig. 1a) and Co-IP (Fig. 1b) assays. The basal FRET efficiency of STIM1-SARAF was somewhat higher than that of the basal FRET efficiency of STIM1-Orai1 and STIM1-STIM1 (see Supplementary Figs. 2a, 2b). Therefore, to better illustrates the time course of FRET increase Fig. 1a and 1c shows the normalized FRET ratio. The results show that STIM1-SARAF interaction is increased minimally in the absence of Orai1. STIM1-STIM1 and STIM1-Orai1 FRET starts shortly after 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 sec. It then progresses more slowly than STIM1-STIM1 and STIM1-Orai1 interaction. Several Orai1 mutants were used to identify the Orai1 domain that facilitates the STIM1-SARAF interaction (Fig. 1c). Orai1(1-73), Orai1(N) lacking the entire Orai1 N terminus, constitutively active Orai1(V102C) and channel-dead Orai1(R91W) facilitated the STIM1-SARAF interaction. Only Orai1(L273S) with a disrupted C terminus CCD²⁴ failed to support STIM1-SARAF interaction. Fig. 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 SCDI can be reliably measured. Three minutes after initiation of store depletion, the current was measured by including 10 mM Ca^{2+} in the bath solution. SARAF over-expression enhanced and its knockdown (see Fig. 1d) markedly reduced SCDI.

These findings suggest that STIM1-SARAF interaction is independent of Orai1 channel function and can take place at basal $[\text{Ca}^{2+}]_i$ and that interaction of STIM1 with the Orai1 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. This was the case even before store depletion as evident from the reduced basal FRET efficiency. This was not due to interrupted STIM1-STIM1 or STIM1-Orai1 interaction (supplementary Fig. 1b). Importantly, while deletion of the K-domain had no effect on activation of Orai1 current by STIM1 (peak currents 19.1 ± 0.5 and 18.8 ± 0.5 pA/pF; $n=?$), the deletion eliminated the effect of SARAF on SCDI (Fig. 2b).

To define the PM microdomain that interacts with the K-domain, we first determined whether the scaffold protein caveolin was required for normal current density and for SARAF to mediate SCDI. Knockdown of caveolin (Supplementary Fig. 3a) had no effect on current density but eliminated both STIM1-SARAF interaction and the effect of SARAF on SCDI (Supplementary Figs. 3b and 3c). Hence, the STIM1-Orai1 complex has the same current whether in a caveolin-containing domain or outside this domain. However, the complex can only recruit SARAF to mediate SCDI when it is within the caveolin-containing domain. Therefore, SCDI by SARAF can function as a probe to report the microdomain localization of the STIM1-Orai1 complex

Tethered PM/ER microdomain in STIM1-SARAF interaction

It has been suggested that the K-domain interacts with PM $PI(4,5)P_2$ ¹³. Therefore, we tested the effect of depleting PM $PI(4,5)P_2$ 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 $PI(4,5)P_2$. Supplementary Fig. 2c shows that depletion of $PI(4,5)P_2$ reduces the basal STIM1-SARAF interaction (reduced basal FRET efficiency). More important, Fig. 3c shows that depletion of $PI(4,5)P_2$ strongly inhibits the STIM1-SARAF interaction in response to store depletion. Significantly, as reported previously²¹, $PI(4,5)P_2$ depletion had no effect on Orai1 peak current (19.8 ± 0.4 and 20.0 ± 0.6 pA/pF without and with $PI(4,5)P_2$ depletion; $n=?$), but prevented the effect of SARAF (Fig. 3d).

It has recently been reported that filamentous Septin4 participates in formation of a $PI(4,5)P_2$ microdomain around Orai1 and it is required for STIM1-Orai1 clustering and Orai1 activation²⁰. We confirmed that knockdown of Septin4 (Fig. 4a) reduced Orai1 current by $36 \pm 3\%$. It is unlikely that this can be attributed solely to disruption of a $PI(4,5)P_2$ microdomain²⁰, since depletion of $PI(4,5)P_2$ did not reduce current density. Nevertheless, Figs. 4b, c and Supplementary Fig. 2d show that 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 ER¹⁸, 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 that knockdown of E-Syt1 (increased *E-Syt1* mRNA levels

(Supplementary Fig. 4b). Although E-Syt2 and E-Syt3 were shown to localize to the peripheral ER and participate in PM/ER tethering¹⁸, knockdown of E-Syt2 and E-Syt3 had no effect on SARAF-mediated SCDI (Supplementary Figs. 4c,d). By contrast, Fig. 4d shows that knockdown of E-Syt1 (see Supplementary Fig. 4c) reduced basal STIM1-SARAF FRET efficiency. Conversely, overexpression of E-Syt1 maximally increased STIM1-SARAF FRET efficiency and FRET efficiency did not increase further by store depletion. 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 overexpression of E-Syt1 markedly increases the number and size of the PM/ER microdomains¹⁸ indicate that E-Syt1 over-expression 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 overexpression of E-Syt1 maximized SCDI to the level observed by 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=?).

Together the findings in Figs. 3, 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 in order to recruit SARAF. In addition, the findings with 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-Syt3¹⁸ 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 or Kras targeting motifs. These motifs were shown to target proteins to different PM microdomains^{25,26}. The Kras and Hras motifs are fused to the C terminus, but the Lyn motif is commonly attached to the N terminus of proteins. However, since the STIM1 N terminus resides in the ER lumen we fused all motifs to the C terminus of STIM1, which resulted in STIM1 targeting to the PM using all motifs, including STIM1-Lyn and STIM1-Hra. We determined their localization to test the specificity of the targeting. Supplementary Figs. 5a-c show that 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 eliminated 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-immunoprecipitates (co-IP'ed) with SARAF, the Co-IP is independent of store depletion and it is not further enhanced by Orai1 (Fig. 5d). On the other hand, STIM1-Lyn does not Co-IP with SARAF regardless of the presence or absence of Orai1 or of store depletion (Fig. 5d). It was not possible to show 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 Figs. 5d,e

showing that STIM1-Kras and STIM1-Lyn recruited wild-type STIM1 to the same puncta. Nevertheless, the differential role of the K-domain and interaction with SARAF indicate that STIM1-Kras and STIM1-Lyn are targeted to different PM microdomains.

The STIM1-Kras and STIM1-Lyn probes were then used to measure FRET efficiency with SARAF under various conditions. Figure 5a illustrates the experimental protocol. As expected from the Co-IP results (Figs. 5d) STIM1-Lyn failed to recruit SARAF (Fig. 5b, c) when the two were co-expressed. Co-expression of Orai1 slightly reduced the basal STIM1-Lyn/SARAF FRET efficiency before and after Ca^{2+} store depletion. Similarly, Supplementary Fig. 7 shows that the Hras motif also targets STIM1 to PM puncta in the absence of Ca^{2+} store depletion and that STIM1-Hras failed to interact with SARAF. By sharp contrast, STIM1-Kras/SARAF displayed high FRET efficiency, which was maximized by expression of Orai1 and did not increase by store depletion (Fig. 5c). Hence, the Kras motif targets STIM1 to a domain where it can recruit SARAF, whereas the Lyn and Hras motifs target STIM1 to a domain that prevents recruitment of SARAF, although STIM1 can access Orai1 in both domains.

The functional significance of targeting STIM1 to the different domains is shown in Figs. 5e-g. Fig. 5e shows the CRAC current measured in the presence of the strong and fast Ca^{2+} buffer 10 mM BAPTA to minimize SCDI and Supplementary Fig. 3d shows the Ca^{2+} 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, store depletion fully activated STIM1-Lyn/Orai1 and STIM1-Hras/Orai1, with similar current density to STIM1-Kras/Orai1 (19.7 ± 0.9 , 17.8 ± 0.4 and 18.7 ± 0.4 pA/pF, respectively; $n=?$). Most significantly, Figs. 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 affected 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; $n=?$) 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) P_2 /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-Kras is eliminated by knockdown of SARAF (Fig. 6a), knockdown of caveolin (Supplementary Fig. 3c), depletion of PI(4,5) P_2 (Fig. 6b) and knockdown of E-Syt1 (Fig. 6c), while the SCDI observed with STIM1-Lyn is not affected by depletion of PI(4,5) P_2 (Fig. 6d).

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 in order 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-SARAF (Figs. 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) or 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, 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 Ca²⁺ influx is associated with, and in many cases is the initiator of, cellular and tissue pathologies. Hence, Ca²⁺ influx channels are extensively regulated, most prominently by Ca²⁺ itself, to restrict Ca²⁺ influx. This is very well described for the Orai1 channel, which undergoes both FCDI and SCDI³, with STIM1^{12,27-29} and SARAF (Fig. 1 and Supplementary Fig. 1a) participating in both through the STIM1 CTID domain¹². The present work indicates that regulation of Orai1 by Ca²⁺ 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 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 signaling complexes³⁰. 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 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, depletion of Septin4 while eliminated the effect of SARAF, also reduced the Orai1 current by about 36%. This would suggest that Septins have a more global role in determining the composition and shape of the PM.

A puzzling observation in the present study is that STIM1-Lyn and STIM1-Hras clustered at the PM but did not cause store-independent activation of Orai1, although store depletion fully activated Orai1. Previous studies already concluded that STIM1 clustering is not sufficient to activate Orai1^{10,12}. However, in these cases the STIM1 mutants were able to cluster but not activate Orai1, whereas STIM1-Lyn and STIM1-Hras fully activate Orai1.

Moreover, when PI(4,5)P₂ is depleted STIM1-Lyn is retained in the PI(4,5)P₂-poor domain (Fig. 7) and remains fully active. Together, these findings suggest that store depletion does more than co-clustering STIM1-Orai1 to activate the channel. This may be due to dissociation of SARAF from STIM1, or due to stabilization of a particular STIM1-Orai1 conformation.

The present studies reveal dynamic translocation of the STIM1-Orai1 complex between PI(4,5)P₂-poor and PI(4,5)P₂-rich microdomains to control Ca²⁺ 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 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 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 Ca²⁺ influx to support receptor-evoked Ca²⁺ oscillations.

STIM1 and SARAF are localized in the ER and interact to some extent in the basal state with filled Ca²⁺ stores, as indicated by their basal Co-IP (Fig. 1b and ¹²) and FRET efficiency (Figs. 4d, 5c, Supplementary Fig. 2). Moreover, SARAF stabilizes STIM1 in the basal conformation since knockdown of SARAF resulted in store-independent clustering of STIM1 and Ca²⁺ influx^{11,12}. Activation of Ca²⁺ influx by store depletion needs to relieve the basal inhibition of STIM1 by SARAF, leading to formation of the STIM1-Orai1 complex. Ca²⁺ influx should be sustained for some time before its re-inhibition 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 Orai1 activity. Subsequently, the STIM1-Orai1 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 binds SARAF, which mediates the SCD1 to limit Orai1 activity. This mechanism allows precise and timed regulation of Ca²⁺ influx between fully activated and partially inhibited Ca²⁺ influx to guard against cell toxicity.

Methods

Constructs and antibodies

STIM1-YFP, HA-Orai1, mCherry-Orai1, the Orai1 mutants and SARAF clones have been previously described^{9,12}. The FKBP12 and FRB constructs are described in²¹. CFP-STIM1, CFP-STIM1 K and Orai1-CFP were a kind gift from Dr. Murali Prakriya³³. E-Syt1 was a gift from Dr. Jen Liou¹⁹ and E-Syt1, E-Syt2 and E-Syt3 were a kind gift from Dr. Pietro DeCamilli¹⁸. Primers were obtained from Integrated DNA Technologies. 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. Preparation of the SARAF-myc has been previously described¹². SARAF-YFP was made by digesting out SARAF at MluI and HindIII sites and ligating into pCMVYFP plasmid. STIM1-Kras, STIM1-Lyn, STIM1-Hras and Random α -helix constructs were made by mutating the bases

in frame 5 amino acids downstream of the STIM1 open reading frame in CFP-STIM1. Briefly CFPSTIM1 plasmid was amplified using the specified primers and the Quick change lightning kit from Agilent and then the backbone was digested using *dpnI* enzyme. The primer sequences are shown in Supplementary Table 1: Kras-Lys Lys Lys Lys Lys Ser Lys Thr Lys Cys Val Ile Met; Lyn-Arg Asn Met Gly Cys Ileu Ser Lys Ser Lys Arg Lys Asp; Hras-Gly Cys Met Ser Cys Lys Cys Val Leu Ser; α -helix - Ala Ala Leu Lys Leu Ile Glu Ala Leu Ala Trp Glu Leu Ala. pYFP-Kras was made by mutating the amino acids to the Kras motif 3 amino acids downstream from YFP open reading frame. YFP-Hras was made by mutating the Hras motif 5 amino acids downstream from YFP open reading frame. p3xflag-mCherry-Kras was made by mutating to the Kras sequence 5 amino acids downstream of the open reading frame of mCherry. Lyn-pEGFP (Cat # 35958) and PLC δ 1 PH-EGFP (Cat # 21179) were purchased from Addgene. Antibodies used at the present work are: polyclonal antiGFP (Life technologies, Cat # A11122) 1:1000; monoclonal anti-myc (Cell Signaling Inc., Cat # 2276) 1:1000; polyclonal anti-SARAF (Thermo Fisher Scientific, Cat # PIPA524237) 1:100; polyclonal anti-Cav-1 (Sigma, Cat # C449-200) 1:500; polyclonal anti-Esyt1 (Sigma, Cat # SAB1105095) 1:250 and polyclonal anti-Septin (Sigma, Cat # SAB2501449) 1:200.

siRNA probes and RT-PCR

The scrambled control and siRNA duplex sequences for SARAF, SEPTIN4, Esyt1 and Caveolin-1 are listed in Supplementary Table 1. The siRNA sequences for E-Syt2 and E-Syt3 were the same as those used before¹⁸. HEK293 cells were plated at 70–80% confluence and transfected using Lipofectamine 2000 with siRNA duplexes after 12 h (20-40 nM/well) in 6-well plates in serum free medium according to the manufacturer's protocol. The medium was changed to serum containing medium 6 hours after adding the duplexes to the cells. The cells were harvested after 48 h. The cells were harvested after 48 h. RNA was extracted using the TRIZOL reagent and the mRNA levels were determined by quantitative PCR as described³⁴. In brief, isolated mRNA was reverse-transcribed into cDNA by the iscript cDNA synthesis kit from Bio-Rad Laboratories. The primers for quantitative RT-PCR for *SARAF*, *Septin4*, *E-Syt1*, *Esyt2*, *Esyt3* and *GAPDH* were purchased from Applied Biosystems. The fold change in the transcript levels of the specified genes was calculated by normalizing the Ct values from control and siRNA-transfected cells (threshold values) to GAPDH.

FRET measurements

HEK293 cells were plated at low confluence on glass bottom dishes (MatTek Corporation) and transfected with ECFP (donor) and EYFP (acceptor) tagged constructs, for 12-16 h using Lipofectamine 2000 (Invitrogen) at 37°C. FRET imaging was performed at 37°C using a confocal system (FV1000; Olympus) equipped with UplanSApo 60X oil immersion objective (NA 1.35; Olympus) at 1X zoom. Images were acquired at 10s intervals using the simplified two-cube method for sensitized emission^{35,36}. To minimize photobleaching low laser power (1-3%) was used.

Image analysis was performed with NIH ImageJ software. Images were corrected for background fluorescence as necessary. FRET was determined on a pixel-by-pixel basis

using a two-step FRET efficiency calculation protocol³⁷. Briefly, bleed-through components were removed by generating a corrected FRET image (F_c) according to the equation $F_c = I_{DA} - dI_{DD}$ where I_{DA} and I_{DD} are the background-subtracted FRET and ECFP images, respectively. The microscope-specific bleed-through constants a and d were determined by measuring the bleed-through from cells expressing ECFP or EYFP alone. The derived values were $d = I_{DA}/I_{DD} = 0.061 \pm 0.0064$ ($n=52$ cells) and $a = I_{DA}/I_{AA} = 0.02 \pm 0.0015$ ($n=46$ cells). In the second step the apparent FRET efficiency (E_{app}) was calculated using the algorithm $E_{app} = F_c / (F_c + GI_{DD})$ where E_{app} is the fraction of ECFP exhibiting FRET and G is a microscope specific constant derived by measuring the increase in ECFP fluorescence following EYFP acceptor photobleaching with the intramolecular CFP–YFP construct YFP-OASF-CFP⁷, which was estimated to be 0.69 ± 0.12 ($n=18$ cells).

Confocal Imaging

For confocal imaging, HeLa cells plated at low confluence on glass bottom dishes were transfected with the indicated constructs for 8-12 h. The images were captured at room temperature with UplanSApo 60X oil immersion objective (NA 1.35; Olympus) at 4X zoom. Images were processed with Photoshop CS3 (Adobe).

PI(4,5)P₂ depletion

Plasma membrane PI(4,5)P₂ was depleted using the FRB/FKBP system provided by Dr. Tamas Balla (NIH) and described in^{38,39}. In brief, the plasma membrane Lyn targeting sequence was fused to the FRB domain of mTOR1. The wild-type or kinetic-dead 5'-phosphatase was fused to FKBP12 which was tagged with mRFP. The two constructs were co-expressed in the cells. Upon exposure of the cells to rapamycin, the FRB and the FKBP12 heterodimerize to recruit the phosphatase to the plasma membrane and hydrolyze PI(4,5)P₂.

Current measurements

HEK293T cells were cultured in Dulbecco's modified eagle's medium and 10% fetal bovine serum. Lipofectamine 2000 (Invitrogen) was used for cDNA or siRNA transfection. Treatment with siRNAs was with 20-40 nM for 48 hrs and transfection with cDNA was for 24-36 hrs before start of current recording. On the day of experiment, transfected cells were released and re-plated on square coverslips in 35-mm Petri dishes and incubated with culture media for at least 1 h to allow attachment to the coverslips. Expression pattern and levels of the various constructs were carefully monitored by fluorescence microscopy. Electrophysiological experiments were performed at 20-24 °C, using the patch clamp technique in the whole cell recording configuration. For current measurements, voltage ramps of -100 to 100 mV were applied every 4 sec from a holding potential of 0 mV. The current at -100 mV was used to calculate current density as pA/pF.

To measure slow Ca²⁺-dependent inactivation (SCDI) the pipette solution contained 135 mM CsCl, 6 mM MgCl₂, 2 mM MgATP, 1.2 mM EGTA, and 10 mM Hepes, pH 7.2 (with CsOH). During SCDI recording, after establishing the whole cell configuration, the cells were kept in Ca²⁺-free solution for 5 min to allow store depletion before exposing the cells to bath solution containing 10 mM Ca²⁺. The standard bath solution contained 130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 10 mM Hepes with or without 10 mM CaCl₂ (pH 7.4 with

NaOH). To measure the extent of spontaneous current, current was recorded as above, except that 10 mM BAPTA replaced the 1.5 mM EGTA. Membrane potentials were amplified with an Axopatch 200B (Axon Instruments, Foster, CA, USA). The currents were filtered at 5 kHz and displayed on computer monitor. Where indicated, the currents were normalized first by averaging multiple experiments and then normalizing to the peak current. Peak currents are given in the text. Results were analyzed using the Clampfit program (Axon Instruments) and all currents are given as mean±s.e.m.

Ca²⁺ measurement

Cells attached to coverslips were loaded with Fura2 by 30 min incubation at 37 °C with 5 μM Fura2/AM in culture media. After dye washing fluorescence was measured at 340 and 380 nm using a TILL system as detailed before¹². Collected images were analyzed using MetaFluor and the results are given as the 340/380 ratio.

Western blot and Co-IP measurements

HEK293T cells were transfected with STIM1, SARAF and with or without Orai1. After 24 hrs the cells were washed once in Ca²⁺-free solution and treated with 25 μM CPA for 0, 15, 30 or 45 seconds and reactions were stopped and lysates prepared by a rapid removal of media and attrition of 0.5 ml of lysis buffer. Lysates (100 μl) containing the same amount of protein were cleared by incubation with 50 μl of Protein G Sepharose beads. The cleared lysates were used to IP STIM1 with anti-myc (SARAF) antibodies by incubation with 1 μl anti-myc antibody overnight at 4°C. The complexes were collected by addition of 50 μl of Protein G Sepharose beads and incubation for 4 hrs at 4°C. The precipitates were analyzed for STIM1 (anti-GFP, 1:1000) and SARAF (anti-myc, 1:1000). Uncropped blot images are shown in Supplementary Figure 9.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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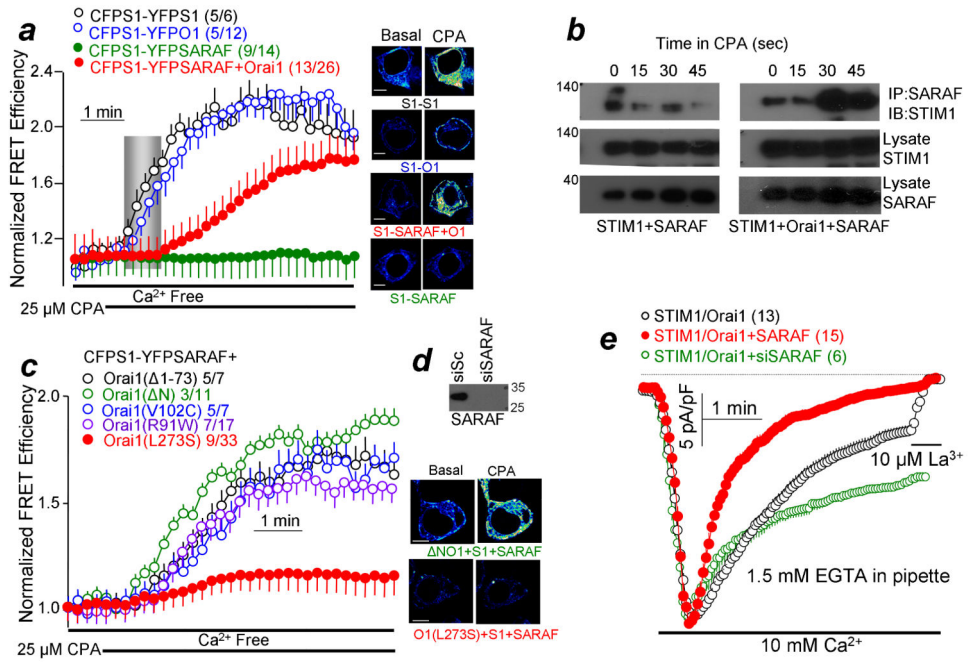


Figure 1. STIM1-Orai1 interaction is required for SARAF interaction with STIM1
 Panel (a): FRET was measured in HEK293 cells transfected with CFP-STIM1 and YFP-STIM1, CFP-STIM1 and YFP-Orai1, CFP-STIM1 and YFP-SARAF and with (red) or without (green) HA-Orai1. Store depletion by CPA in Ca^{2+} -free solution was initiated where indicated. The delay in STIM1-SARAF interaction is marked by gray 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 are 5 μ m. (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 Ca^{2+} -free solution containing 25 μ M 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 μ m. (d) HEK cells were treated with si-SARAF for 48 hrs and analyzed 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 dialyzing cells with pipette solution containing 1.5mM EGTA for 3 min in Ca^{2+} -free bath. CRAC current was initiated by superfusing with a solution containing 10 mM Ca^{2+} . The residual current was inhibited with 10 μ M La^{3+} . The same protocol was used in all current measurements unless otherwise stated. The currents were leak-subtracted and normalized to the 0 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 number of experiments and the second indicates the number of cell analyzed. All results (FRET and current) are given as the mean \pm s.e.m.

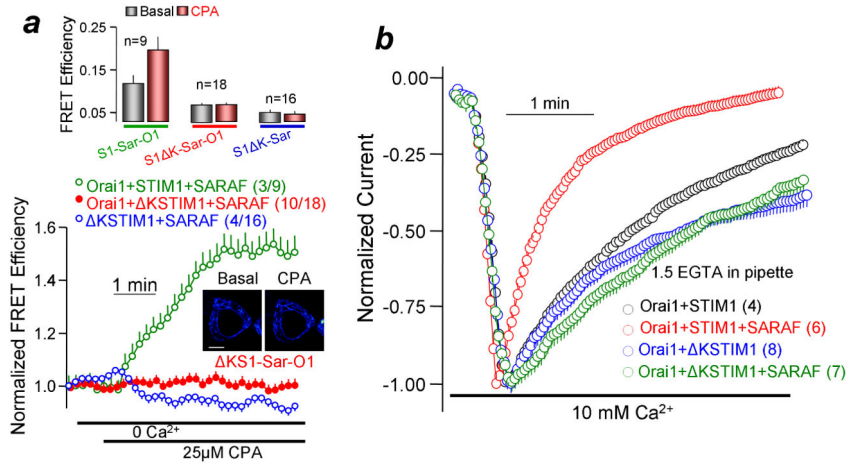


Figure 2. SCDI by SARAF required the STIM1 K-domain

(a): HEK293 cells were transfected with Orai1, CFP-STIM1 or CFP-STIM1 K and YFP-SARAF and FRET was measured in response to store depletion by 25 μ M CPA in Ca²⁺-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 K, SARAF and Orai1 before and after 5 min treatment with CPA, as indicated. Scale bars are 5 μ m. (b): The Orai1 current and SCDI were measured with STIM1 or STIM1 K and in the presence and absence of SARAF. Results are given as mean \pm s.e.m of the indicated number of experiments.

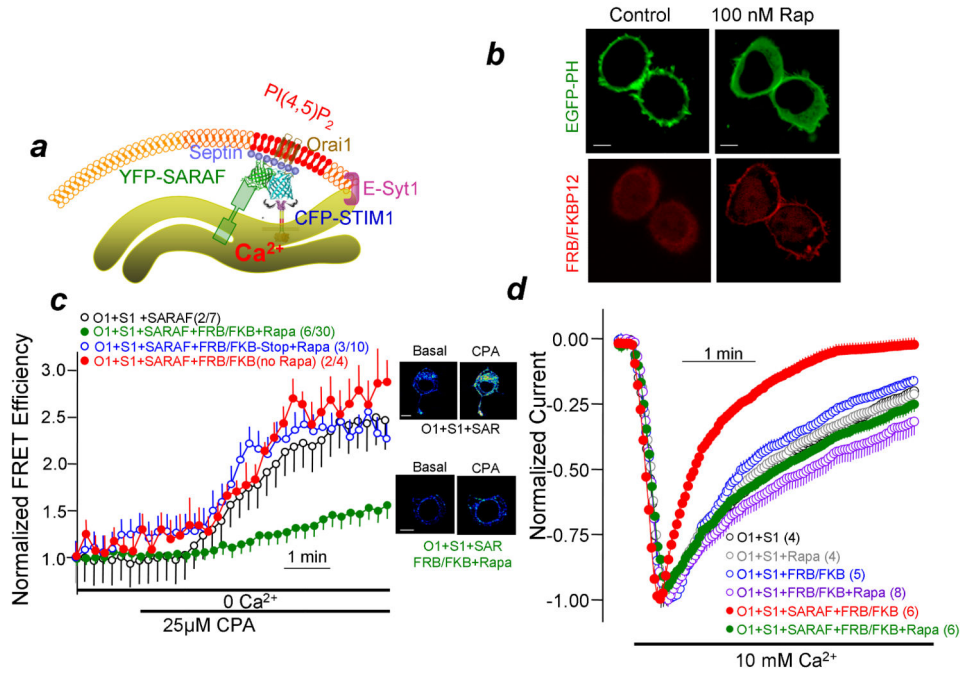


Figure 3. SCDI by SARAF requires plasma membrane PI(4,5)P₂

(a) A model illustrating a microdomain formed by PI(4,5)P₂, tethered by E-Syt1 and stabilized by Septins. (b) Cells transfected with EGFP-PH domain (green, PI(4,5)P₂ probe) and the FRB/FKBP12 (red, PI(4,5)P₂ depletion systems) were imaged before (left) and after exposure to 0.2 μM rapamycin for 2 min to deplete the PI(4,5)P₂. Scale bars are 5 μm. (c) HEK293 cells were transfected with Orai1, STIM1 and SARAF and the FRB/FKBP12 or FRB/FKBP12-stop (no phosphatase control) and were either untreated (control) or treated with 0.2 μM rapamycin for 5 min to deplete PM PI(4,5)P₂ (red trace) and CFP-STIM1, YFP-SARAF FRET was measured in response to store depletion by 25 μM CPA in Ca²⁺-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/FKBP12 system and SCDI by SARAF measured. Shown are rapamycin control (gray), FRB/FKBP12, no SARAF, no rapamycin control (blue) and FRB/FKBP12, SARAF, no rapamycin control (red). Depletion of PI(4,5)P₂ inhibited the effect of SARAF on SCDI (green). Results are given as mean±s.e.m of the number of experiments indicated in brackets.

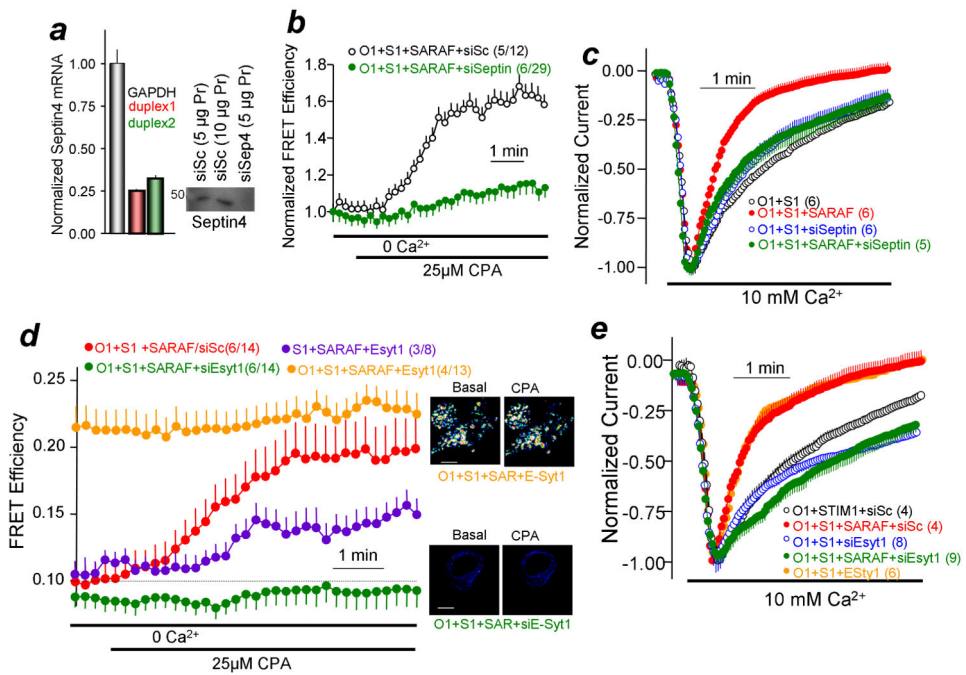


Figure 4. SCDI by SARAF requires Septin 4 and E-Syt1

(a): Effect of two siSeptin4 probes on *septin4* mRNA measured by qPCR and effect of duplex 1 on septin4 protein. Lanes 1 and 3 were loaded with 5 μ g protein and lane 2 with 10 μ M protein. (b) CFP-STIM1/YFP-SARAF in the presence of Orail was measured in cells treated with scrambled or siSeptin4 in response to store depletion by 25 μ M CPA in Ca^{2+} -free solution. Basal and 5 min store depletion FRET efficiencies are shown in supplementary Fig. 2. (c) Orail current was measured in cells expressing Orail, STIM1 and with or without SARAF and treated with scrambled or siSeptin4. (d) FRET efficiency was measured in response to store depletion by 25 μ M CPA in Ca^{2+} -free solution in cells expressing CFP-STIM1, YFP-SARAF and E-Syt1 in the absence (blue) or the presence of Orail (red) or cells treated with scrambled siRNA (black) or siE-Syt1 (green) and expressing Orail, CFP-STIM1 and YFP-SARAF. Scale bars for all images are 5 μ m. (e) the effect of E-Syt1 overexpression (orange) or knockdown (green) on SCDI by SARAF. Results are given as mean \pm s.e.m of the number of experiments indicated in brackets.

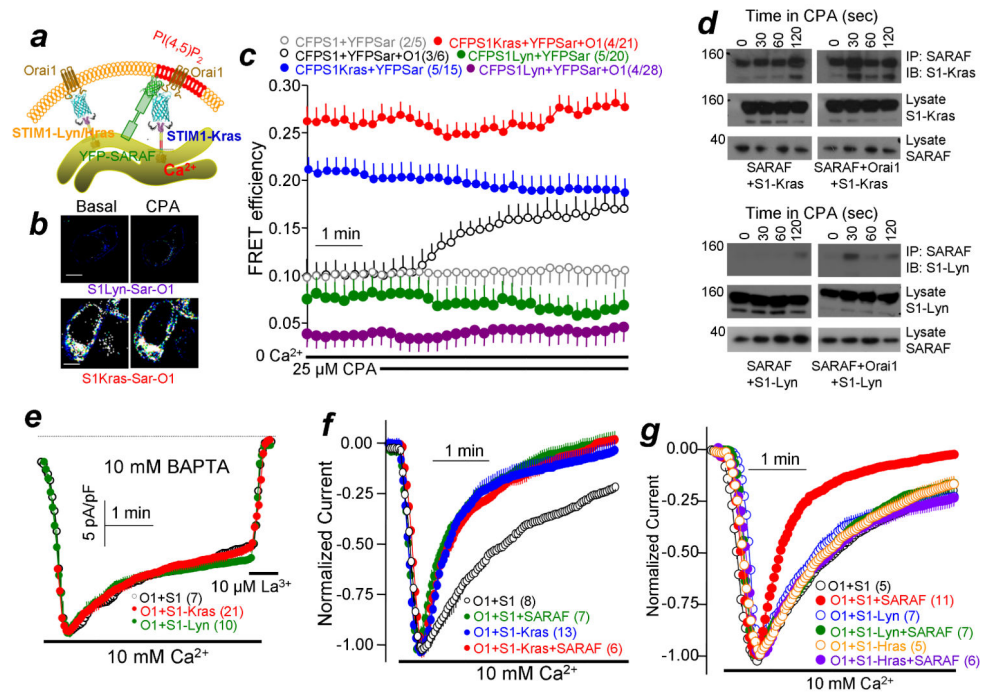


Figure 5. Targeting STIM1 to PI(4,5)P₂-poor and PI(4,5)P₂-rich PM microdomains
 (a) Scheme illustrating the microdomain targeting of STIM1 by the Lyn/Hras and Kras targeting motifs. (b) Examples of FRET images before and after treatment with CPA for 5 min in cells transfected CFP-STIM1-Lyn+SARAF+Orai1 or STIM1-Kras+SARAF+Orai1 related to the experiments in (c). Scale bars are 5 μm. (c) HEK293 cells were transfected with CFP-STIM1 and YFP-SARAF in the absence (gray) and presence of Orai1 (black); YFP-SARAF and CFP-STIM1-Kras (blue) or CFP-STIM1-Lyn (green) in the absence or presence of Orai1 (orange 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) Orail 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 with Orail and STIM1 (black, green), Orail and STIM1-Kras (blue, red) and in the presence of SARAF (red, green). (g) Experiments and conditions as in (f), except that STIM1-Lyn or STIM1-Hras replaced STIM1-Kras. All results are given as mean±s.e.m of the number of experiments indicated in brackets.

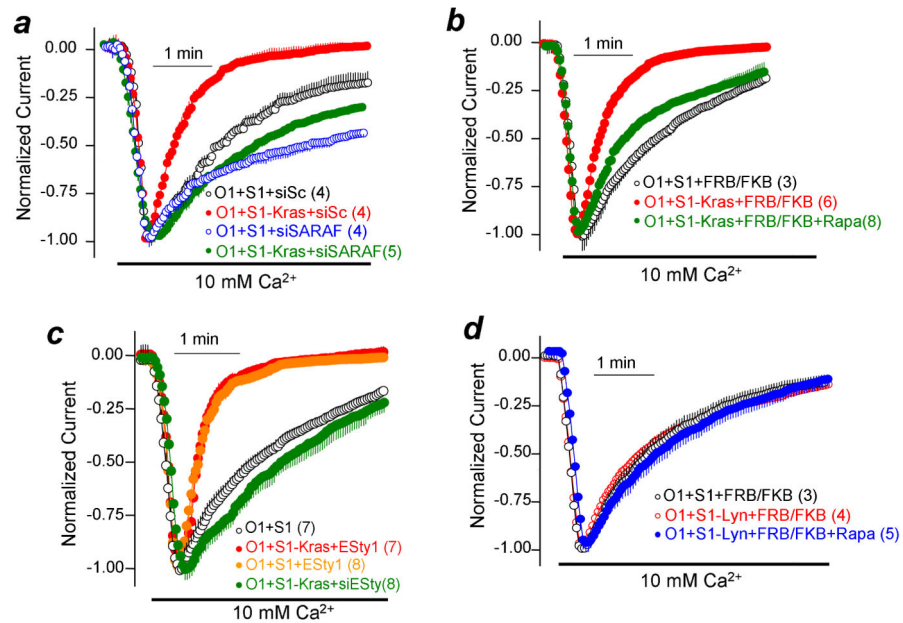


Figure 6. The rate and maximal SCDI with STIM1-Kras requires native SARAF and intact PI(4,5)P₂-rich microdomain

(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 siE-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.

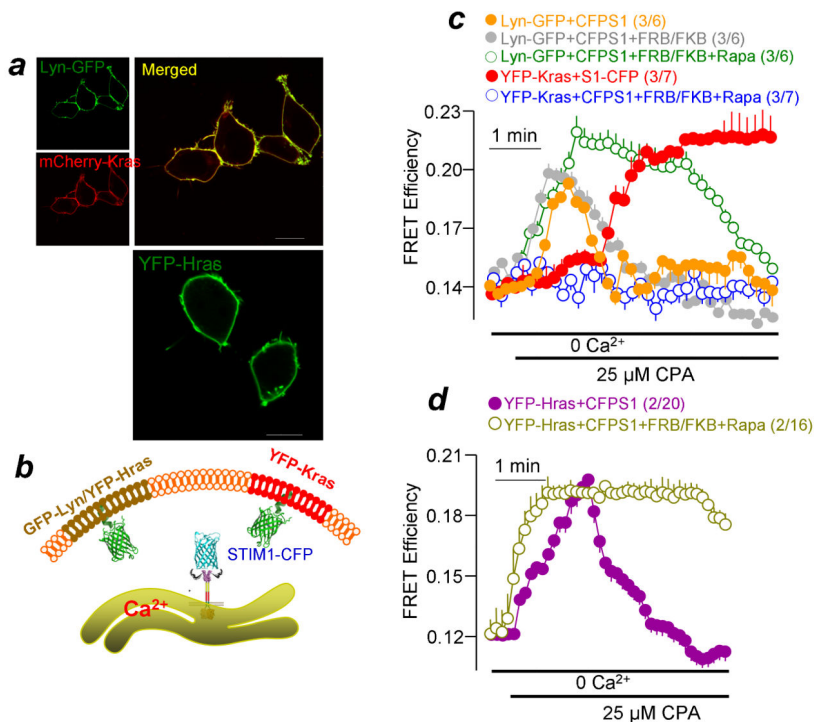


Figure 7. Dynamic translocation of STIM1 between PI(4,5)P₂-poor and PI(4,5)P₂-rich domains in response to Ca²⁺ store depletion

(a) Shows PM localization of Lyn-GFP, mCherry-Kras and YFP-Hras. YFP-Kras shows similar PM localization. Scale bars are 5 μ m. (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, gray, green), or YFP-Kras (red, blue) and the PI(4,5)P₂ depletion system (gray, green, blue) and were treated with 0.2 μ M rapamycin (green, blue) or left untreated (gray, control). In (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 μ M CPA in Ca²⁺-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 \pm s.e.m of the number of experiments and cells indicated in brackets.