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Regulation of CRAC channels by protein interactions and post-translational modification

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Store-operated Ca^{2+} entry (SOCE) is a widespread mechanism to elevate the intracellular Ca^{2+} concentrations and stimulate downstream signaling pathways affecting proliferation, secretion, differentiation and death in different cell types. In immune cells, immune receptor stimulation induces intracellular Ca^{2+} store depletion that subsequently activates Ca^{2+}-release-activated-Ca^{2+} (CRAC) channels, a prototype of store-operated Ca^{2+} (SOC) channels. CRAC channel opening leads to activation of diverse downstream signaling pathways affecting proliferation, differentiation, cytokine production and cell death. Recent identification of STIM1 as the endoplasmic reticulum Ca^{2+} sensor and Orai1 as the pore subunit of CRAC channels has provided the much-needed molecular tools to dissect the mechanism of activation and regulation of CRAC channels. In this review, we discuss the recent advances in understanding the associating partners and posttranslational modifications of Orai1 and STIM1 proteins that regulate diverse aspects of CRAC channel function.

Introduction

In non-excitable cells such as T cells, Ca^{2+} entry via store-operated Ca^{2+} (SOC) channels is a primary mechanism to increase intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$).[1-4] Under resting conditions, cytoplasmic $[Ca^{2+}]$ is ~100 nM while that in the endoplasmic reticulum, which serves as an intracellular Ca^{2+} store, is much higher (~0.4–1 mM). Extracellular $[Ca^{2+}]$ reaches almost 2 mM establishing a huge $[Ca^{2+}]$ gradient between the extracellular milieu, Ca^{2+} store, and the cytoplasm. SOC channels were so named because they are activated by depletion of intracellular Ca^{2+} stores.[2] Upon pathogen infection, specialized antigen-presenting cells (APCs) (e.g., dendritic cells, macrophages, B cells) present foreign antigens on their surface to activate T cells. Antigen engagement of T cell receptor triggers a cascade of tyrosine phosphorylation events that results in stimulation of phospholipase C (PLC) γ, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) into inositol trisphosphate (Ins(1,4,5)P_3) and diacyl glycerol (DAG). Ins(1,4,5)P_3 binds to the Ins(1,4,5)P_3 receptor (Ins(1,4,5)P_3R) on the ER membrane and releases Ca^{2+} from the ER into the cytoplasm and this store depletion leads to activation of CRAC channels. The CRAC channel is a prototype and specialized class of SOC channel, very well characterized in immune cells. Because ER Ca^{2+} store especially in T cells is limited, SOCE via CRAC channels is important to maintain elevated levels of $[Ca^{2+}]_i$ for long time durations required for activation of downstream signaling pathways. In the short term, this Ca^{2+} increase induces a decrease in motility to provide stable interactions between T cells and APCs, and promotes granule secretion important for cytolytic activity of T cells. In the long term, the increased Ca^{2+} induces activation of downstream signaling pathways such as protein kinase C (PKC), extracellular-signal-regulated kinases (ERKs) or nuclear factor of T cell (NFAT) to affect the transcriptional programs necessary for generating a productive immune response. Defective function or lack of expression of the CRAC channel components cause severe combined immune deficiency in humans.[5] Hence, an in depth understanding of CRAC channel mediated Ca^{2+} signaling in T cells is crucial for developing drug therapies for immune deficiency or inflammatory disorders.

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Current Understanding of CRAC Channel Activation

Genome-wide RNAi screens identified Orai1 (heaven's gatekeeper in Greek myth, also known as CRACM1 or TMEM142A) as a pore subunit of the CRAC channels.[6-9] Prior to identification of Orai1, limited RNAi screens in Drosophila and HeLa cells identified STIM1, a Ca^{2+}-binding protein localized predominantly in the endoplasmic reticulum (ER) as an important regulator of SOCE.[10-12] STIM1 plays a pivotal role in sensing ER $[Ca^{2+}]$ via its N-terminal EF-hands and CRAC channel opening by direct interaction with Orai1. The EF-hand of STIM1 has low affinity of Ca^{2+}, between 0.2–0.6 mM[13] and hence remains bound to Ca^{2+} under resting conditions. Upon ER Ca^{2+} depletion, STIM1 loses Ca^{2+}-binding, multimerizes, translocates to PM-proximal ER, mediates clustering of Orai proteins on the PM and stimulates Ca^{2+} entry (**Fig. 1**).[10-12] Under resting conditions, Orai1 and STIM1 are homogenously distributed at the PM and the ER membrane, respectively. Detailed studies have identified a minimal domain of STIM1 necessary for activation of Orai1 as the CRAC activation domain (CAD)/STIM1 Orai1 activating region (SOAR)/Orai1-activating small fragment (OASF)/Ccb9 domain

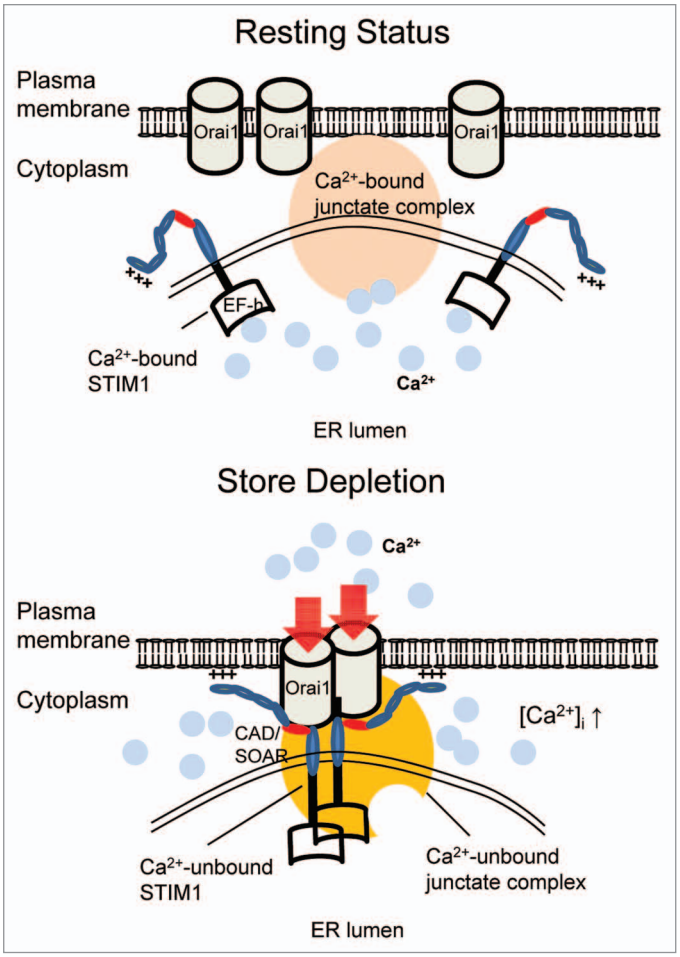

Figure 1. Molecular mechanism of CRAC channel regulation. Schematic showing a mechanism of CRAC channel regulation by intracellular Ca^{2+} sensors. Under resting conditions, Orai1 and STIM1 are distributed at the PM and the ER membranes. Upon store depletion, STIM1 oligomerizes by sensing ER Ca^{2+} depletion with its ER-luminal EF-hand domain, and translocates to form clusters at the ER-PM junctions marked by junctate. By physical interactions with Orai1 through the CAD/SOAR domain, clustered STIM1 recruits and activates Orai1 in the ER-PM junctions. Junctate also contains Ca^{2+}-sensing motif in the ER lumen and ER Ca^{2+} depletion increases protein interactions between STIM and junctate.

that directly binds to the cytosolic N and C terminus of Orai1.[14-17] Further studies showed that Ca^{2+} bound STIM1 in resting conditions exhibits a folded structure mediated by intramolecular protein interactions between the positively charged residues within the CAD/SOAR domain and the negatively charged, autoinhibitory region preceding the CAD/SOAR domain.[18] STIM1 activation requires unfolding of this intramolecular interaction to allow the basic residues within the CAD/SOAR domain to interact with the acidic residues in the C-terminus of Orai1.[18] Tetrameric assembly of Orai1 after store depletion has been observed under different experimental conditions.[19-23] However, this stoichiometry is currently in question, given the recent identification of a hexameric assembly of Drosophila Orai in crystal structure studies.[24] The emerging change in the paradigm of Orai protein stoichiometry will be an interesting aspect for future studies.

While Orai and STIM are the principal components, multiple auxiliary proteins as well as posttranslational mechanisms have been shown to regulate CRAC channel function. Some of these including golli myelin basic proteins, partner of STIM1 (POST), plasma membrane Ca^{2+} ATPase (PMCA), calmodulin, CRAC channel regulator 2A (CRACR2A), secretary pathway Ca^{2+}-ATPase 2 (SPCA2) have been reviewed in detail[25] and are only briefly mentioned here and summarized in **Table 1**. In the current review, we discuss regulation of CRAC channel gating and focus on recently identified auxiliary molecules as well as posttranslational modification mechanisms that modulate the activity of Orai1 and STIM1.

The Gating Mechanism of CRAC Channels

In recent years, extensive structure function studies have been conducted to understand the gating and regulation of CRAC channels. This information is highly relevant for our understanding not only of the pore structure and channel gating, but also of the way other molecules may interact with and regulate channel gating. Studies from several independent laboratories suggested that CRAC channels are tetramers of four independent Orai subunits each comprising four transmembrane segments (TM1–TM4) with the N and C terminus facing the cytoplasm (**Fig. 2A**).[19-23] The TM1 of Orai1 has been shown to line the pore, and residues R91, G98, V102, E106, $D^{110}xD^{112}xD^{114}$ in TM1 are important for Ca^{2+} selectivity and gating.[1-5,51] It has been suggested that the pore has a constriction around residue E106 which forms the selectivity filter.[1-4] Prakriya and colleagues have also suggested that V102 in TM1 faces the pore, possibly forming a gate within the ion permeation pathway just below the putative selectivity filter.[51] A model has been proposed for channel gating whereby the channel gate and the ion selectivity filter are closely associated. Other studies have suggested alternate gating mechanism for CRAC channels. First, Romanin and colleagues demonstrated that addition of a "bulky" hydrophobic residue at position R91, which is on TM1 near the cytoplasmic interface, blocks ion permeation.[52] Then, more recently, Cahalan and colleagues suggested that the R91 residues from the different subunits come close together to form a cytoplasmic gate near the bottom of TM1.[50]

The recently published crystal structure of the Drosophila Orai (dOrai) protein by Long and colleagues conforms to some evidences; however, it also challenges many of the prior experimental observations.[24] Hou et al. show that instead of tetramers, CRAC channels are formed of hexamers of Orai subunits each comprising four transmembrane segments (TM1–TM4) with the N and C terminus facing the cytoplasm.[24] With regards to the gating of the channels, the structural studies suggest that the selectivity filter (E106) and the lower hydrophobic residues (V102) form a rigid structure, which may be permeable to Ca^{2+} even in the absence of STIM, i.e., the selectivity filter may allow permeation of Ca^{2+} even in closed state. Below the hydrophobic region and near the intracellular face of the channel, the pore is lined by three basic residues (Arg/Lys) from each subunit that are likely to bind intracellular anion(s) like sulfate or phosphate species. The narrow width and extreme positive charge of this region

Table 1. Summary of associating partners of Orai1 and STIM1

	Interacting partners	Binding region on Orai1/STIM1	Function	Refs.
Orai1	SPCA2	N and C terminus	Store-independent gating	26
	CRACR2A	N terminus	Stabilization of Orai1-STIM1 protein complex	27
	Calmodulin	N terminus	Ca^{2+}-dependent inactivation	28
	Caveolin	N terminus	Orai1 recycling	29, 30
	Adenylyl cyclase 8 (AC8)	N terminus	cAMP signaling	31
	TrpC channel	N.D.	Store/agonist-operated Ca^{2+} entry	32–36 but also see 37
STIM1	ERp57	ER luminal domain	Negative regulation of STIM1	38
	Junctate	ER luminal domain	Marking the ER-PM junctions and recruiting STIM1 to these junctions	39
	CRACR2A	Cytoplasmic domain	Stabilization of Orai1-STIM1 protein complex	27
	EB1	Cytoplasmic domain	ER tubulation	40, 41
	Golli MBP	Cytoplasmic domain	Negative regulation of STIM1	42
	SARAF (TMEM66)	N.D.	Slow inactivation of CRAC channels	43
	Polycystin1-P100	N.D.	Inhibition of STIM1 translocation	44
	Stanniocalcin 2 (STC2)	N.D.	Negative regulation of STIM1	45
	POST (TMEM20)	N.D.	Indirect interaction via PMCA	46
	TrpC channel	Cytoplasmic	Activated by STIM1	32–36 but also see 37
	PMCA	Cytoplasmic	Inhibited by STIM1	47, 48
	ARC channels	N.D.	Store-independent activation by STIM1	49
	$Ca_v1.2$	Cytoplasmic	Inactivated by STIM1	73, 74

The interacting partners of Orai1 and STIM1 and their binding regions involved are summarized. N.D., not determined.

may preclude cation permeation and form a closed gate. Channel opening would then involve dilation of the pore's basic region at R91 by outward bending of the TM1 helices at around Ser89 and Gly98. Such pore widening would reduce the affinity of the basic region for anions and allow for Ca^{2+} flux.

The crystal structure data suggests the presence of a hydrogen bond between W176 in TM3 and S89 in TM1, where the pore expands to open the ion permeation pathway as suggested by the authors.[24] Based on KirBac channel data where change in conformation of the outer helices may affect the inner or pore-forming helix to open and close the channel gate,[53] it is tempting to speculate that TM2 and TM3, which form a ring of α helices surrounding the pore-forming TM1, may apply pressure on TM1 to regulate the channel gate and this process is regulated by the interaction of S89 (in TM1) with W176 (in TM3) and/or S93 (in TM1) with H134 (in TM2). A study supporting this hypothesis showed that mutation of W176 in TM3 of Orai1 confers STIM1-independent channel opening.[54] Exaggerated widening of the pore due to the mutation of W176 to cysteine may dissipate the ring of positive charges near the intracellular mouth of the channel and allow for outward flow of cations, as observed with W176C mutant of Orai1.[54] Future studies examining gating properties of Orai1 channels using mutation of specific residues based on the crystal structure should provide the much needed functional validation of this novel gating mechanism proposed by Huo et al.[24]

Besides STIM-dependent gating, CRAC channels exhibit very characteristic Ca^{2+}-dependent inactivation (CDI) properties, including fast and slow inactivation.[55-57] Mutational studies have shown that the cytoplasmic regions of Orai1, including the N terminus, the intracellular loop, and C terminus are all involved in CDI.[28,58,59] It was shown that a short peptide comprising the residues $N^{153}VHNL^{157}$ from the intracellular loop blocked CRAC currents generated by wild-type Orai1, supporting the hypothesis that the intracellular loop plays an important role in channel inactivation (**Fig. 2A**).[58] Therefore, the intracellular domains of Orai1 are not only important for STIM-dependent channel gating but are also essential for inactivation and cross talk with other signaling pathways as discussed below.

Molecules that Associate with STIM1

Several proteins localized to the ER membrane have been shown to interact with STIM1. Srikanth et al. recently identified junctate as an interactor of STIM1.[39] Previous electron microscopy studies showed that Orai1 and STIM1 clustered into pre-existing junctional areas, a space of 10–25 nM, between the PM and ER membranes (**Fig. 1**).[60-62] In excitable cells (e.g., neurons and muscle cells), proteins localized to the junctions between the PM and ER/sarcoplasmic reticulum (SR) membranes form a structural foundation for regulating the intracellular Ca^{2+} stores and Ca^{2+} entry.[63,64] Various biochemical screening approaches have identified junctophilins, mitsugumins, sarcalumenin, junctin and junctate as important components of these ER/SR-PM junctions.[63-66] Using biochemical methods of affinity protein purification and pulldown analyses, Srikanth et al. showed a

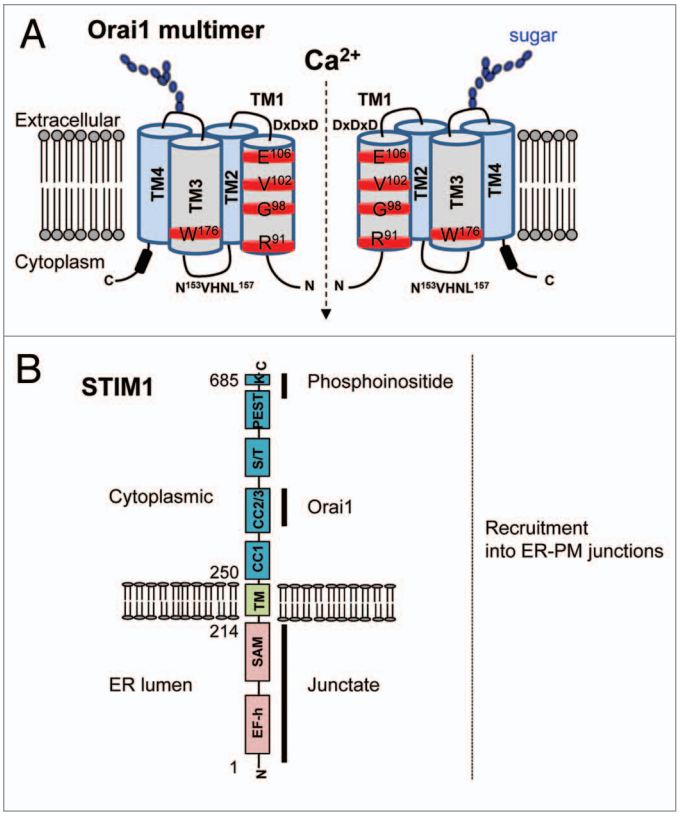

Figure 2. Structural architecture of Orai1 and STIM1. (**A**) Schematic of Orai1. Orai1 has four transmembrane segments (TM1–TM4). It has two extracellular domains and the second extracellular domain between TM3 and TM4 contains the asparagine (N^{223}) residue involved in glycosylation. The TM1 lines the pore and the residues in TM1 involved in Ca^{2+} selectivity and gating are indicated. Orai1 contains three intracellular domains, including the N terminus, intracellular loop and C-terminal coiled-coil domain, that are important for protein interactions and channel activation/inactivation. The stoichiometry of CRAC channels is currently in question, but the schematic indicates a dimeric form for convenience of representation. Arginine at position 91 was mutated in patients (R91W) with non-functional CRAC channels suffering from severe combined immunodeficiency. Orai1 channels with a mutation of tryptophan in TM3 (W176C) show STIM1-independent channel gating. (**B**) STIM1 contains the ER-luminal region, a single transmembrane segment and a cytoplasmic region. The cytoplasmic region has three coiled-coil domains, serine/threonine-rich domain, proline/glutamate/serine/threonine-rich (PEST) domain and a polybasic region at the C terminus that interacts with phosphoinositides. The fragment of STIM1 (340–450) involved in Orai1 interaction, the ER-luminal region of STIM1 involved in multimerization and interaction with junctate are indicated.

direct interaction between the ER luminal regions of junctate and STIM1.[39] The authors suggested that junctate localization defined the sites of accumulation of Orai1 and STIM1 since after store depletion, Orai1 and STIM1 accumulated at junctions that were already marked by junctate. Furthermore, after uncovering a Ca^{2+}-binding EF-hand motif in the C terminus of junctate, the authors generated an EF-hand mutant that loses its Ca^{2+} binding and described a functional association between junctate and STIM1 which is $[Ca^{2+}]_{ER}$-dependent. STIM1 contains a polybasic stretch of amino acids in its C terminus that binds to PM phosphoinositides, and is important for STIM1 clustering at the ER-PM junctions.[67-69] Truncation of this polybasic segment (STIM1-ΔK) abolished STIM1 accumulation at ER-PM junctions, and overexpression of Orai1 recovered clustering of STIM1-ΔK mutant,[16,67] suggesting Orai1 binding as alternate mechanism for STIM1 accumulation into the ER-PM junctions. Co-expression of the EF-hand mutant of junctate with STIM1-ΔK mutant in Orai1-depleted cells also recovered STIM1-ΔK accumulation, identifying another mechanism facilitating STIM1 accumulation into the ER-PM junctions.[39] Thus, the authors concluded that there are multiple pathways of STIM1 accumulation at the ER-PM junctions: 1) a pathway mediated by direct binding of the polybasic amino acids on the C terminus of STIM1 to the phosphoinositides on the PM, 2) an Orai1-dependent mechanism mediated by direct interaction and 3) a junctate-dependent mechanism mediated by protein interaction between STIM1 and junctate (**Fig. 2B**). In a physiological condition when the concentration of Orai1 and STIM1 is low, multiple mechanisms of STIM1 accumulation could become predominant for efficient and timely assembly of a functional CRAC channel complex. This study identifies a conserved function of the components of the ER-PM junctions in excitable and non-excitable cells.

Another ER protein SARAF (SOCE-associated regulatory factor; TMEM66) was identified as an interacting partner of STIM1 that facilitates the Ca^{2+}-dependent slow inactivation of CRAC channels.[43] Using cDNA library overexpression screen, Palty et al. identified SARAF as a candidate which reduces mitochondrial Ca^{2+} levels. SARAF is a single-pass transmembrane containing ER resident protein, with its N terminus facing the ER lumen and its C terminus facing the cytoplasm, similar to STIM1. The C-terminal portion of SARAF contains a serine-proline rich domain and polybasic region, which may interact with the PM phospholipids. SARAF was shown to interact with STIM1 via its cytoplasmic C terminus under resting conditions. After store depletion, SARAF translocates to the ER-PM junctions with STIM1 and facilitates the inactivation/dissociation of STIM proteins from Orai1 to regulate ER Ca^{2+} refilling and cytoplasmic Ca^{2+} homeostasis. Accordingly, knockdown of SARAF induced increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and increased SOCE after store depletion, whereas overexpression of SARAF showed an opposite effect. Considering the positive role of junctate, ehich enhances STIM1 accumulation into the ER-PM junctions,[39] SARAF plays an exact opposite, negative role by enhancing dissociation of STIM1 clusters at the ER-PM junctions. Further studies are required to examine whether there is any crosstalk between junctate and SARAF in associating with STIM1 for regulation of CRAC channel function.

Non-CRAC Channel-Related Interacting Partners of STIM1

A novel function of STIM1 was discovered in ER tubulation. STIM1 was identified as binding partner of EB1, a microtubule tip-binding protein.[40] When a growing microtubule tip came across the ER membrane, STIM1 bound to EB1 and pulled out a new ER tubule through the "tip attachment complex"

mechanism.[40,41] EB1 interacts with the residues ^{642}TRIP^{645} of STIM1 (human) and a STIM1 mutant defective in EB1-binding did not localize to the microtubule tips.[41] Interestingly, this interaction of STIM1 with EB1 does not affect SOCE, since siRNA-mediated depletion of EB1 did not alter STIM1 accumulation at ER-PM junctions and Ca^{2+} entry.[40] Therefore, it is possible that STIM1-mediated ER tubulation and STIM1 translocation upon store depletion are separate events. Involvement of microtubules in regulating SOCE is controversial. It was shown that CRAC channel-mediated SOCE was mildly reduced in mast cells treated with nocodazole, an antineoplastic agent that depolymerizes microtubules, whereas an earlier study reported reduced SOCE in HEK293 cells treated with nocodazole.[70,71] However, fluorescence recovery after photobleaching (FRAP) studies have suggested that STIM1 moves passively by diffusion in the ER membrane after store depletion supporting the observation that intact microtubules are not necessary for STIM1 translocation into The ER-PM junctions.[67,72] Hajkova et al. showed that STIM1 induced Ca^{2+} entry plays a very important role in microtubule reorganization to facilitate movement of secretory granules in bone marrow derived mast cells.[70] Thus, while CRAC channel mediated-Ca^{2+} entry is very important for microtubule reorganization for downstream functions in mast cells, microtubules are unlikely to be necessary for STIM1 translocation to the ER-PM junctions.

Two independent groups have shown an interaction between STIM proteins and $Ca_v1.2$, a subtype of voltage-gated Ca^{2+} channels (VGCCs) channels.[73,74] In mammals Ca_V family members have been categorized into 5 groups (L, N, P/Q, R and T) based on their electrophysiological and pharmacological properties. The Ca_v1 family belongs to the L-type VGCC, and is primarily studied in excitable cells where it is involved in initiating contraction, secretion and regulation of gene expression.[75] These channels comprise of multiple subunits including a pore-forming α1 and other auxiliary subunits including α2, β, δ and γ. Wang et al. and Park et al. independently showed that STIM1 interacted with Cav1.2 and inhibited the channels in a short-term and blocked its surface expression in a long-term.[73,74] Both the studies showed a direct interaction between the CAD/SOAR domain of STIM1 (required for Orai1 activation) and the C terminus of the $Ca_v1.2$ channel. These results provide an interesting scenario where the same region of STIM1 plays exactly opposite roles in regulation of CRAC channels and $Ca_v1.2$ channels. In non-excitable cells such as T cells, the function of L-type Ca^{2+} channel family, Ca_V1 was examined using mice genetically deleted for expression of the regulatory β3 or β4 subunits.[76] These mice showed normal development of T cells, however, upon TCR stimulation, naïve $CD4^+$ T cells lacking expression of either the β3 or β4 subunits showed reduced Ca^{2+} influx, an impairment in NFAT nuclear translocation and consequently reduced cytokine production. A recent study from the same group showed a prominent defect in $CD8^+$ T cells lacking the regulatory β3 subunit.[77] $CD8^+$ T cell numbers were reduced in β3-deficient mice and the strength of the Ca^{2+}/NFAT signaling pathway was also significantly reduced. The authors showed an association of the β3 subunit with $Ca_v1.4$ and β3 deficiency abrogated the cellular expression of $Ca_v1.4$, suggesting that β3 subunit maybe required for functional $Ca_v1.4$ channel in T cells and may protect $Ca_v1.4$ from degradation.[77] Another study examined mice genetically deleted for expression of $Ca_v1.4$ and observed reduction in numbers of both $CD4^+$ and $CD8^+$ T cells, as well as reduced TCR stimulation dependent downstream signal activation.[78] All the studies using mice deficient in $Ca_v1.4$ or the regulatory β subunits identified a positive role of $Ca_v1.4$ in promoting cell survival and naïve T cell homeostasis.[76-78] Considering the negative role of STIM1 in regulation of $Ca_V1.2$ channels, it would be interesting to examine the activity of these channels in excitable as well as non-excitable cells from mice lacking expression of STIM proteins.

An interaction between STIM1 and transient receptor potential type C (TrpC) channels has been studied by several groups.[32-34] Zeng et al. showed that the Ezrin, Radixin, Moesin (ERM) domain and the C-terminal polybasic tail of STIM1 interact with and activate TrpC channels via an electrostatic interaction between the di-lysine motif in STIM1's polybasic tail and a conserved di-aspartate motif in TrpC1 (and other TrpC channels).[35] Conversely, using overexpression and siRNA-mediated knockdown approaches, DeHaven et al. showed no effect of STIM1 on various TrpC channel activities including TrpC1, TrpC3, TrpC5 and TrpC7.[37] Since TrpC channel function or insertion into the PM is known to be enhanced by Ca^{2+},[79,80] it is also possible that Ca^{2+} ions entering the cell via the CRAC channels indirectly regulate TrpC channels. Future studies of measurement of endogenous TrpC channel activities in STIM1-deficient cells in the presence of known amounts of cytosolic free Ca^{2+} can shed some light on STIM1 dependence of TrpC channel-mediated SOCE.

STIM1 has also been shown to regulate opening of a closely related ARC family of ion channels. The ARC channel is a class of highly Ca^{2+}-selective ion channel which is activated specifically by low concentrations of arachodinic acid.[81] They function in a store-independent manner and share biophysical properties with the CRAC channels. While the CRAC channels are formed of homomultimers of Orai1, the ARC channels are heteropentamers of three Orai1 and two Orai3 monomers and are opened by the pool of STIM1 that constitutively resides in the PM.[49,82]

Interacting Partners of Orai1

Several interactors of Orai1 involved in channel gating, inactivation, recycling, translocation, and association have been identified. Recent studies have shown calmodulin (CaM) to bind the N terminus of Orai1 at elevated $[Ca^{2+}]_i$ and potentiate Ca^{2+} dependent inactivation (CDI).[28] In addition to STIM1 and CaM, other proteins interacting with the N terminus of Orai1 include a novel cytoplasmic EF-hand-containing protein, CRAC channel regulator 2A (CRACR2A).[27] It was shown that CRACR2A forms a ternary complex by direct interaction with Orai1 and STIM1 in a $[Ca^{2+}]_i$-sensitive manner, with low $[Ca^{2+}]_i$ favoring association of CRACR2A with Orai1 and STIM1 and high $[Ca^{2+}]_i$ favoring its dissociation. Although CRACR2A plays a positive role in CRAC channel activation by enhancing association of Orai1 and STIM1, it is not directly involved in channel

gating because expression of CRACR2A alone or in combination with STIM1 did not influence the CRAC currents when measured by electrophysiological methods (Srikanth S, Ribalet B and Gwack Y, unpublished observations). The human genome shows expression of three alternatively spliced isoforms of CRACR2A gene (isoform a, b and c encoding proteins with predicted MW of ~80 KDa, 65 KDa and 45 KDa respectively), while the mouse genome shows only one, corresponding to isoform c, studied by Srikanth et al.[27] The transcripts encoding isoforms a and b are longer than isoform c in the 3' end. While the regions interacting with Orai and STIM proteins are conserved in all the isoforms, the longest isoform a, has an additional Rab GTPase domain in its C-terminus. It would be interesting to identify which of the three isoforms is predominant in T cells and understand its physiological role in CRAC channel regulation.

Machaca and colleagues used xenopus oocyte as a model and identified a role for caveolin in internalization of Orai1.[29] It was shown that SOCE is inhibited due to internalization of Orai1 and impairment of STIM1 clustering during meiosis.[30] The authors showed that Orai1 internalization occurred via a caveolin and dynamin-dependent endocytic pathway. The authors mapped a caveolin-binding site in the N terminus of Orai1 and demonstrated protein interaction between Orai1 and caveolin.[29] Recent studies have also shown that inhibition of Orai1 and STIM1 activity greatly diminishes cervical and mammary tumor cell migration and metastasis in vitro and in vivo.[26,83,84] Interestingly, an isoform of the secretary pathway Ca^{2+}-ATPase, SPCA2 was shown to enhance mammary tumor cell growth by raising $[Ca^{2+}]_i$ via a direct interaction with Orai1.[26] Overexpression of SPCA2 increased $[Ca^{2+}]_i$ by directly binding to the N and C terminus of Orai1 in a STIM1- and store-independent manner. In addition to cancer cell migration, the role of SOCE via Orai1 has been identified in wound healing.[85] Lysophosphatidic acid (LPA) was known to promote cell migration and would healing, but the detailed signaling pathway was unknown. Reynolds and colleagues showed that LPA treatment stimulates SOCE via Orai1 and STIM1 leading to activation of calcineurin NFAT signaling pathway in keratinocytes.[85] Together, these studies strongly indicate the important role of Orai1 and STIM1 in a variety of other cell types in addition to immune cells.

Recently, a cross talk between the CRAC channel pathway and cyclic adenosine monophosphate (cAMP) signaling pathway has been identified. cAMP, another important intracellular signaling molecule, is generated by adenylyl cyclase (AC) mediated cleavage of adenosine triphosphate, and in turn activates downstream protein kinase A (PKA) pathway. Among the nine membrane-bound ACs, four of them (AC1, AC8, AC5 and AC6) are known to be regulated by Ca^{2+}. Of those four, AC1 and AC8 are stimulated through an interaction with Ca^{2+}-calmodulin (CaM). A role of STIM1 in activation of AC activity has been demonstrated.[86] It was shown that intracellular store depletion, independent of cytosolic Ca^{2+} concentration, led to recruitment of ACs in a STIM1 dependent mechanism referred by the authors as "store-operated cAMP signalling." Another study demonstrated a direct protein interaction between Ca^{2+}-CaM activated AC8 and Orai1 N terminus.[31] Using Forster resonance energy transfer (FRET) technique the authors detected a constitutive association between Orai1 and AC8, which was not affected by store depletion and Orai1 activation. Using FRET, GST pulldown and immunoprecipitation analyses, the authors show a direct interaction between the N termini of Orai1 and AC8.[31] Both these studies together show that STIM1 itself or Ca^{2+} entry via Orai1 plays an integral role in regulating cross talk between SOCE and cAMP signaling pathways.

Posttranslational Modification of Orai1 and STIM1

Several groups have described posttranslational modifications of Orai1 and STIM1. Putney and colleagues demonstrated the first example of phosphorylation mediated regulation of STIM1 during mitosis.[87] It was known that SOCE was suppressed during cell division, however, the molecular mechanism behind this event was not known. Smyth et al. showed that S486 and S668 located within the serine/threonine-rich domain of STIM1 were phosphorylated during mitosis by cyclin dependent kinase 1 (CDK1) and these phosphorylations suppressed SOCE (**Fig. 3A**). Expression of STIM molecules that were truncated at position 482 or with mutations of S486 and S668 significantly rescued SOCE in mitotic cells.[87] In a different study, Machaca and colleagues showed that SOCE was suppressed even in meiotic cells. The authors observed trapping of Orai1 in intracellular vesicles (as discussed above) and impaired STIM1 clustering in xenopus oocytes during meiosis.[30] Overexpression of $STIM1^{D76A}$, a constitutive active mutant also showed impaired cluster formation, suggesting that STIM1 multimerization was blocked during meiosis. STIM1 was found to be hyperphosphorylated in these cells; however, overexpression of STIM1 with alanine substitutions in all the conserved serine residues did not rescue the clustering defect. The authors concluded that hyperphosphorylation did not cause clustering defect in STIM1 in xenopus eggs. Independently, to examine the importance of blocking SOCE during cell division, constitutively active mutants of Orai1 may provide a useful tool. STIM1 phosphorylation may have other functions during cell division, in addition to regulating SOCE. Another study by Smyth et al. identified a function of phosphorylated STIM1 in ER exclusion from the mitotic spindle.[88] ER escapes from the mitotic spindle during cell division; however, the underlining mechanism was not known. STIM1 was shown to interact with EB1; however, in mitotic cells, STIM1 segregated from EB1. A STIM1 mutant harboring ten serine/threonine substitutions was not phosphorylated during cell division, colocalized extensively with EB1 and blocked ER exclusion from the mitotic spindle. These studies identify multiple functions of phosphorylated STIM1 during cell division, in regulating SOCE, as well as in exclusion of ER from the mitotic spindle.

A study by Pozo-Guisado et al. identified a positive role of phosphorylation of STIM1 on SOCE.[89] The authors showed that extracellular-signal-regulated kinases 1 and 2 (ERK1 and ERK2) phosphorylate S575, S608 and S621 residues located within the C-terminal S/T-rich and PEST domains of STIM1 (**Fig. 3A**).[89] These phosphorylation events were induced by store depletion with thapsigargin as well as ERK agonist, 12-O-tetradecanoylphorbol-13-acetate (TPA). FRET

experiments measuring interaction between Orai1 and STIM1 showed that phosphorylation enhanced STIM1 association with Oria1 when compared with a mutant STIM1 that cannot be phosphorylated by ERK1/2.[89] Another study reported a positive role of tyrosine phosphorylation of STIM1 in human platelets.[90] STIM1 was shown to be phosphorylated at tyrosine residues by immunoblotting with a classical anti-phospho-tyrosine antibody, 4G10. This event is possibly mediated by Bruton's tyrosine kinase (Btk) because a specific blocker of Btk, LFM-A13 suppressed tyrosine phosphorylation of STIM1 and protein interactions between Orai1 and STIM1.[90]

Phosphorylation of Orai1 has not been extensively investigated. Kawasaki et al. showed that Orai1 was phosphorylated at S27 and S30 in the cytoplasmic N terminus and this phosphorylation suppressed SOCE.[91] The PKC subfamilies include conventional PKC (cPKC; α, βI, βII and γ), novel PKC (nPKC; δ, ε, η and θ), and atypical PKC (aPKC; ζ, ι and λ). The authors suggested that cPKC family member, PKCβ phosphorylated Orai1 N terminus based on in vitro kinase assays, inhibitors of PKCβ, and knockdown experiments. Cells expressing Orai1 mutated at S27 and S30 showed a marginal enhancement of SOCE and CRAC currents.[91] Interestingly, Orai proteins contain several conserved serine residues, some in the N terminus close to the pore-forming TM1 (S75, S82, S89 and S90; amino acid numbering based on human Orai1), in the intracellular loop (S159 and S163), and C terminus (S260, **Fig. 3B**). Considering their positions, it is possible that phosphorylation of these residues may influence the regulation of channel gating, inactivation, or interaction with STIM1. Future studies examining the mutations of these residues and their impact on SOCE and CRAC currents would be important to understand phosphorylation-mediated regulation of Orai proteins.

Another post-translational mechanism regulating STIM1 and SOCE is ubiquitination. Keil et al. showed that STIM1 is ubiquitinated in hippocampal neurons, which reduces its surface expression.[92] In the presence of proteasome inhibitors, surface expression of STIM1 as well as SOCE was enhanced in HEK293 cells. Conversely, overexpression of POSH (Plenty of SH3's), an E3 ubiquitin ligase, reduced surface expression of STIM1.[92] Unlike STIM1, so far there is no biochemical data depicting ubiquitinated Orai1. Eylenstein et al. showed that overexpression of the ubiquitin ligase Nedd4–2 reduced surface expression of Orai1.[93] Further studies aimed at biochemical detection of ubiquitinated Orai1 is required to carefully examine ubiquitination-mediated regulation of Orai1.

Conclusions and Perspectives

A tremendous amount of progress in understanding CRAC channel gating and regulation has been made since the discovery of STIM and Orai proteins. Using these molecular tools, several groups have uncovered a ubiquitous role of SOCE in multiple cell types including immune cells, platelets, keratinocytes, osteoblasts, cardiac myocytes, skeletal muscle cells and even neuronal cells. Structure function studies using mutants of both Orai1 and STIM1 has helped in identifying the minimal domains necessary

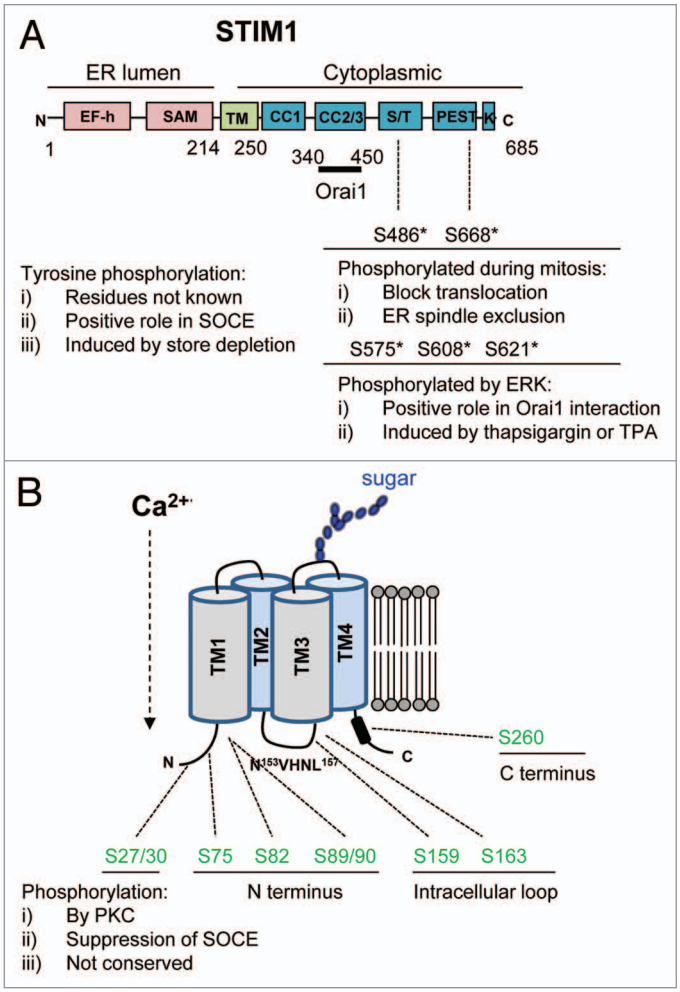

Figure 3. Posttranslational modifications of STIM1 and Orai1. (**A**) Identified phosphorylated residues within STIM1 are indicated. Tyrosine phosphorylation of STIM1 was identified, but the specific residues involved remain unknown. TPA, 12-O-tetradecanoylphorbol-13-acetate. (**B**) Potential phosphorylated serine residues within Orai1. Phosphorylation of Orai1 N terminus by PKCβ at is S27 and S30 indicated. Potential serine residues for phosphorylation that are conserved among Orai1, Orai2, Orai3 and the Drosophila Orai are indicated.

for channel activity and in understanding the mechanism of gating, inactivation and regulation of CRAC channels. Several studies examining the Ca^{2+} affinity of the EF hand motifs of STIM proteins and the structure of the ER-luminal region of STIM1 in the absence and presence of Ca^{2+} provides important insights into the mechanism involved in detecting ER Ca^{2+} depletion and STIM1 activation. The recent crystallization of the Drosophila Orai protein challenges the long held consensus about tetrameric assembly of Orai proteins.[24] While this study does not include data about any of the extra- or intra- cellular loops, it does have the atomic model of the pore-forming TM1 and the other TM segments of Drosophila Orai. An in depth examination of the currently available structure function data in the light of the crystal structure data and further mutational analyses will provide a giant leap forward in understanding the gating mechanism of CRAC channels.

In addition to structural studies, numerous interacting partners and posttranslational mechanisms regulating Orai1 and/or STIM1 have been identified. Some of the interacting molecules play a direct role by regulating the gating properties of CRAC channels and/or translocation of Orai1 and STIM1, while some others like caveolin are indirectly involved in regulating surface expression of Orai1. Furthermore, these studies have shed important insights into crosstalk of the SOCE pathway with other pathways including the cAMP signaling pathway. These studies have also uncovered novel functions of STIM1 in regulating ER segregation from the mitotic spindle during cell division or in regulating functions of other Ca^{2+} channels including the voltage-gated $Ca_v1.2$ channels. Identification of molecules or posttranslational mechanisms that affect SOCE will advance our understanding about the physiologically diverse mechanisms involved in regulation of CRAC channels.

In earlier studies, pharmacological agents (e.g., 2-aminoethoxydiphenyl borate, 2-APB) have advanced the field by providing useful information about the unique properties of CRAC channels.[94] Identification of small molecule blockers of CRAC channels can be an excellent direction for future studies because human patients with non-functional CRAC channels suffer from fatal SCID, which can be rescued by bone marrow transplantation.[95] These results suggest a predominant role for CRAC channels in immune cells that stems from the idea that CRAC channels in the immune cells consist of homomultimers of Orai1 while heteromultimers of Orai1 (with Orai2 or Orai3 or other channels like TrpC family members) may from SOC channels in other cell types. Hence, specific blockers of CRAC channels are likely to have fewer side effects than widely used immunosuppressive drugs such as cyclosporin A and tacrolimus that block the ubiquitously expressing calcineurin phosphatase. Identification of specific blockers of CRAC channels will also provide a molecular probe for examination of the role this signaling pathway in the immune system.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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