Accepted Manuscript

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Author: Rajesh Bhardwaj Matthias A. Hediger Nicolas Demaurex

PII: S0143-4160(16)30025-2
DOI: http://dx.doi.org/doi:10.1016/j.ceca.2016.03.006
Reference: YCECA 1748

To appear in: Cell Calcium

Received date: 1-3-2016
Accepted date: 8-3-2016

Please cite this article as: [http://dx.doi.org/]

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Title: Redox modulation of STIM-ORAI signaling

Authors: Rajesh Bhardwaj¹, Matthias A. Hediger¹ and Nicolas Demaurex²

Affiliations: ¹Institute of Biochemistry and Molecular Medicine, University of Bern, Bühlstrasse 28, CH-3012 Bern, Switzerland. ²Department of Cell Physiology and Metabolism, University of Geneva, 1 rue Michel-SERVET CH-1211 Geneva 4, Switzerland.

Corresponding Authors:
Rajesh Bhardwaj
Institute of Biochemistry and Molecular Medicine
University of Bern
Bühlstrasse 28
CH-3012 Bern, Switzerland
Email: rajesh.rajesh@ibmm.unibe.ch

Nicolas Demaurex
Department of Cell Physiology and Metabolism
University of Geneva
1 rue Michel-Servet
CH-1211 Geneva 4, Switzerland
Ph: +41 (0) 22 379 53 99
Fax: +41 (0) 22 379 54 02
Email: Nicolas.Demaurex@unige.ch
HIGHLIGHTS

- Oxidative modifications of STIM and ORAI proteins modulate store-operated Ca\(^{2+}\) entry.
- Redox modification of the luminal Cys-49 and Cys-56 residues alters STIM1 Ca\(^{2+}\) sensing.
- Modification of ORAI1 Cys-195 by extracellular oxidants reduce channel function.
- Cytosolic cysteine residues in STIM and ORAI isoforms might also undergo redox modifications.

Abstract

STIM1 and ORAI1 constitute the core machinery of the ubiquitous store-operated calcium entry pathway and loss of function in these proteins is associated with severe immune and muscular disorders. Other isoforms - STIM1L, STIM2, ORAI2 and ORAI3 exhibit varied expression levels in different cell types along with several other interaction partners and thereby play different roles to facilitate, regulate and fine-tune the calcium entry. STIM proteins convey the Ca\(^{2+}\) store-depletion message to the PM and thereby participate in refilling of the ER by physically interacting with the Ca\(^{2+}\)-selective ORAI channels at the PM. STIM and ORAI are exposed to oxidative modifications in the ER, the cytosol, and at the cell surface, and redox-mediated alterations in STIM/ORAI coupling might contribute to autoimmune disorders and cancer progression. This review discusses the redox reactivity of cysteine residues in STIM and ORAI isoforms, focusing on the oxidative modifications of STIM and ORAI proteins by which STIM-ORAI signaling can be modulated.

Keywords:

STIM, ORAI, CRAC, store-operated calcium entry, redox, hypoxia, ROS

Introduction

All living organisms require calcium (Ca\(^{2+}\)) which must be taken up from the environment. In higher organisms, such as humans, Ca\(^{2+}\) is a major component of the mineral phase of bone and teeth and the plasma levels of Ca\(^{2+}\) are kept constant within narrow limits. At the cellular
level, the cytosolic Ca\textsuperscript{2+} concentrations are kept low, as calcium is central to a ubiquitous intracellular messenger system that controls vital functions such as cell differentiation and proliferation, muscle contraction, hormone secretion and neurotransmitter release. Intracellular Ca\textsuperscript{2+} is a uniquely versatile signalling molecule because just about any Ca\textsuperscript{2+} spatiotemporal pattern can be generated by the coordinated action of channels, pumps and exchangers, regulating Ca\textsuperscript{2+} fluxes at the plasma membrane (PM) and across intracellular organelles. Ca\textsuperscript{2+}-storage organelles include the endoplasmic reticulum (ER) and its sarcoplasmic reticulum (SR) equivalent in muscle cells, acidic organelles such as lysosomes and mitochondria which can passively take up Ca\textsuperscript{2+} by a regulated uniporter system [1, 2]. Active Ca\textsuperscript{2+} extrusion by plasma membrane Ca\textsuperscript{2+} ATPases (PMCA) and sequestration by SR/ER Ca\textsuperscript{2+} ATPases (SERCA) maintain the free cytosolic Ca\textsuperscript{2+} concentration at nanomolar levels. The large gradient generated by the pumps enables transient elevations in the local Ca\textsuperscript{2+} concentration via controlled opening of Ca\textsuperscript{2+} release and influx channels, while Ca\textsuperscript{2+} binding proteins prevent the rapid diffusion of Ca\textsuperscript{2+} ions within the cytosol and favour their accumulation into Ca\textsuperscript{2+} stores. The Ca\textsuperscript{2+} depletion of the ER, in turn, activates Ca\textsuperscript{2+} entry channels at the PM to sustain the refilling of stores and the maintenance of long-lasting Ca\textsuperscript{2+} signalling processes. This store-operated Ca\textsuperscript{2+} entry (SOCE) process is particularly important for the maintenance of long-lasting Ca\textsuperscript{2+} signals that drive transcriptional response in immune cells.

The two major families of proteins that mediate the SOCE process, STIM and ORAI, were discovered nearly 20 years after Jim Putney formulated the concept that the content of Ca\textsuperscript{2+} stores controls the opening of plasma membrane channels [3]. This concept stems from early observations that Ca\textsuperscript{2+} entry persists following agonist or second messenger withdrawal and gained strong support through the electrophysiological characterization of a Ca\textsuperscript{2+} release activated Ca\textsuperscript{2+} current (CRAC) in mast cells and T cells [4]. This prototypical current has a characteristic inward rectification, an extreme selectivity for Ca\textsuperscript{2+}, and a very small unitary conductance. Although in 2001, Clapham and colleagues proposed that the calcium channel
TRPV6 (also known as CaT1) assumes this role [5], genome-wide RNAi screens in Drosophila and human cell lines in 2005 and 2006 (deploying NFAT translocation reporter assays among other approaches) revealed STIM1 and ORAI1 as the essential components for Ca\(^{2+}\) entry and CRAC [6-9]. There are two STIM isoforms (STIM1 and STIM2) and three ORAI isoforms (ORAI1, ORAI2, ORAI3) in humans, and genetic linkage analysis pinpointed a mutation in ORAI1 (R91W) in a patient suffering from severe combined immunodeficiency (SCID) associated with impaired SOCE in his T cells [10]. T cells from the SCID patient homozygous for the ORAI1-R91W mutation completely lacked CRAC currents that were restored by the expression of wild-type (wt) ORAI1, firmly linking the defect in CRAC channel function to ORAI1. Subsequent genetic studies revealed the full spectrum of diseases associated with loss or gain of functions in STIM or ORAI, a phenotype now termed “CRAC channelopathy”. Patients deficient in STIM1 or ORAI1 function suffer from SCID-like immunodeficiency associated with recurrent and chronic bacterial, viral, and fungal infections, and from blood, muscle and skin disorders that can comprise autoimmune hemolytic anemia and thrombocytopenia, muscular hypotonia and atrophy and ectodermal dysplasia with hypocalcified dental enamel and anhidrosis. Gain-of-function mutations in STIM1 and ORAI1 have been associated with bleeding disorders and tubular aggregate myopathy (TAM), a skeletal muscle disorder causing muscular pain, weakness and cramping with exercise.

STIM1 and its paralog STIM2 are single-pass ER Ca\(^{2+}\) sensing proteins that act as intracellular ligands to control the gating of ORAI channels (Fig. 1). Their N-terminus extending into the ER lumen contains EF-hand calcium sensing domains while the C-terminal cytosolic tail contains self-associating coiled-coiled motifs, a channel activating domain (CAD, also known as STIM-ORAI activating region, SOAR) and a polybasic lysine(K)-rich C-terminal domain that binds phosphoinositides [11-18]. A decrease in the free Ca\(^{2+}\) concentration within the ER lumen promotes an inside-out conformational change that extends STIM1 cytosolic tail and promotes its oligomerization as well as the exposure of the
CAD/SOAR and the lysine-rich tail. The resulting increased affinity of STIM1 for lipids promotes its accumulation within cortical ER structures, located near phosphoinositide-rich domains of the PM or of organelles, while the exposure of the SOAR allows STIM1 to trap ORAI1 channels via interactions with their C-terminal cytosolic domain. This is followed by the gating of the trapped channel complex via productive interactions of STIM1 with the N-terminal region of ORAI1 [11, 19, 20], reviewed in [21]. As a result of these trafficking events and the conformational changes, both STIM1 and ORAI1 accumulate within membrane clusters visible in the TIRF microscope as fluorescent “puncta”. Store depletion as well as STIM1-enforced expression induces the extension of junctional ER structures which can be visualized in the electron microscope as extended thin sheets of cortical ER [22-25]. The STIM-ORAI coupling process occurs at membrane contacts sites between the ER and the target membranes. This structural arrangement, sustaining lipid transfer between membranes, is conserved from yeast to human. SOCE thus shares molecular and cellular components that are essential for lipid homeostasis and proper assembly of the STIM1 and ORAI complex. Activation of this complex requires a range of associated proteins including CRACR2A, SARAF, STIMATE, junctate, POST, and septins (reviewed in [26]). In addition certain TRP channels located at the plasma membrane can also contribute to SOCE [27-30]. The regulation of SOCE by the associated proteins, by phosphorylation, and by Ca$^{2+}$ itself, has been extensively reviewed [31, 32]. In this present review, we focus on recent studies reporting that the SOCE process can be modulated by changes in the redox status of STIM and ORAI proteins, the core components of the SOCE machinery. The sequential steps linking store depletion to the activation of CRAC channels are summarized below [26] (Fig. 1).

**Redox regulation of STIM1**

Human STIM1 contains five highly conserved cysteine residues (Fig. 2A), one in the signal peptide, two just upstream of the canonical EF-hand motif in the N-terminal luminal domain,
one in the transmembrane domain and another one in the conserved cytosolic C-terminal STIM-ORAI activating region (SOAR). Two studies have implicated the ER luminal cysteines (Cys-49 and Cys-56) in the redox regulation of STIM1 [45, 46].

**Effect of oxidant challenges on STIM1-mediated SOCE:** Hawkins et al. (2010) demonstrated that exposure of COS7 cells to H$_2$O$_2$ or buthionine sulfoximine (BSO) redistributes exogenous STIM1 from the perinuclear ER to PM puncta, indicating that oxidative stress promotes oligomerization of STIM1 and activation of SOCE [45]. A similar oxidant insult caused store-independent Ca$^{2+}$ entry in mouse embryonic fibroblasts (MEFs) but was ineffective in MEFs lacking STIM1, establishing STIM1 as an important oxidative stress sensor [45]. In agreement with this, STIM1 knockout DT40 chicken B-lymphocytes were resistant toward oxidative stress-induced SOCE, triggered by BSO or H$_2$O$_2$, and regained oxidant sensitivity upon re-expression of STIM1 [45].

**Redox reactivity of STIM1 luminal cysteines:** H$_2$O$_2$ targets the reactive thiol groups of cysteines in proteins and forms sulfenic acid (R-SOH), which undergoes nucleophilic attack by another thiol (RSH), resulting in disulfide bond (RSSR) formation. The direct redox reactivity of the ER-luminal cysteines in STIM1 was investigated by exposing the recombinant human STIM1 N-terminal fragment to 100 $\mu$M H$_2$O$_2$ for 30 min. This treatment failed to alter the protein mobility on non-reducing SDS-PAGE devoid of DTT or any other reducing agent [45]. Thus, the authors concluded that oxidant stress does not facilitate the formation of luminal disulfide bonds in STIM1. However the presence of a preformed disulfide bridge in STIM1 was not assessed since the protein was not exposed to reducing conditions and the presence of cross-linked cysteines might account for the failure of H$_2$O$_2$ to trigger further modifications.

Under oxidative stress conditions, reactive thiol groups of proteins can undergo S-glutathionylation, a reversible reaction between cysteine residues of a protein and glutathione (GSH) that plays important regulatory roles in redox signalling and oxidative
stress responses [47]. This was explored by Hawkins et al. (2010) who treated full length recombinant human STIM1 with 100 µM H₂O₂ in presence of 10 mM GSH in a cell-free system and detected a strong S-glutathionylation of STIM1 by Western blotting [45]. S-glutathionylation was associated with the formation of higher order oligomers of STIM1, as indicated by a dramatic shift in the molecular mass upon H₂O₂ treatment that exceeded by far the effect expected through addition of reduced GSH (the molecular mass of which is only 0.307 kDa). Furthermore, upon DTT addition, the recombinant human STIM1 ran at its calculated monomeric molecular weight, regardless of the presence of H₂O₂, indicating that the S-glutathionylation-mediated effect on STIM1 mobility is reversible [45]. Importantly, the authors further showed that the S-glutathionylation of purified recombinant human STIM1 decreases its Ca²⁺-binding affinity by at least 5-fold [45]. These findings indicate that S-glutathionylation of STIM1 mimics the effects of luminal Ca²⁺ depletion and promotes the oligomerization of STIM1.

Subsequently, Hawkins et al. (2010) showed that Cys-56 in STIM1 is the target of S-glutathionylation under H₂O₂-induced oxidative stress, based on studies using lysates from COS7 cells expressing wt STIM1 or the C56A, C49A and C49/56A mutants [45]. The decreased affinity of S-glutathionylated STIM1 for Ca²⁺-binding triggers STIM1 oligomerization, thereby facilitating constitutive Ca²⁺ entry and increasing basal cytosolic Ca²⁺ levels [45]. Somewhat unexpectedly, alanine substitution at Cys56 alone or at Cys49 and Cys56 led to constitutive Ca²⁺ entry when the mutated STIM1 proteins were expressed in STIM1⁻⁻ DT40 cells, possibly due to allosteric modulation of the proximal EF-hand domain, thereby impairing Ca²⁺-binding [45]. Consistent with this hypothesis, when HEK293T cells were co-transfected with ORAI1 and either C56A mutant or the C49/56A double mutant STIM1, a substantial redistribution of STIM1 towards the PM and colocalization with ORAI1 was observed. Furthermore, coexpression of ORAI1 and STIM1 C49/56A in HEK293 resulted in a constitutive CRAC current [45].
In a separate study, Prins et al. (2011) reported that YFP-STIM1 protein derived from MEFs migrated slightly faster on PAGE under non-reducing conditions than under reducing conditions, suggesting the presence of an intramolecular disulfide bond in STIM1 [46]. The redox-dependent migratory shift was absent in the YFP-STIM1-C49/56A double cysteine mutant, indicating a possible involvement of these residues in creating an intramolecular disulfide bridge [46]. This observation is at odds with the findings of Hawkins et al. (2010) who failed to provide evidence for disulfide bridge formation between C49 and C56. The double STIM1 cysteine mutant had altered mobility when expressed in MEFs lacking the ER-resident oxidoreductase ERp57 [46], suggesting that ERp57 is dispensable for the formation of the luminal disulfide bridge in STIM1. Biochemical and functional evidence for an association between Erp57 and STIM1 was obtained: Erp57 was shown to interact with the ER luminal N-terminal domain of STIM1 by surface plasmon resonance, and overexpression of YFP-STIM1 and ERp57-CFP generated a robust FRET signal in resting and store-depleted STIM1<sup>−/−</sup> MEFs, indicating that these two proteins interact [46]. Coexpression of YFP-STIM1 C49/56A and ERp57-CFP in STIM1<sup>−/−</sup> or ERp57<sup>−/−</sup> MEFs resulted in a 33% or 28% lower FRET signal, respectively compared to wt STIM1 indicating that the luminal C49 and C56 contribute to the binding of STIM1 to ERp57 [46]. Finally, deletion of ERp57 in MEFs caused partial pre-clustering of STIM1 and potentiated SOCE whereas overexpression of ERp57 had the opposite effect [46]. These data indicate that ERp57 binding to STIM1 has inhibitory effect on SOCE by hindering the recruitment of STIM1 to puncta. However, robust evidence for disulfide bridge formation between C49 and C56 of STIM1 and its involvement in SOCE regulation still remains to be provided.

Although the two studies support the notion that STIM1 is regulated by redox state, highlighting the importance of the luminal cysteine residues in this regulation, they diverge on the functional consequences. When expressed in STIM1<sup>−/−</sup> MEFs, the YFP-STIM1-C49/56A double mutant showed ER-like reticular localization and failed to form puncta even in store-depleted conditions [46]. In contrast, the same mutant was constitutively recruited to puncta
when expressed in HEK-293 cells [45]. In keeping with this diverging localization, the YFP-STIM1 C49/56A double mutant did not mediate SOCE when co-expressed with ORAI1-dsRed in STIM1−/− MEFs [46] but was constitutively active when co-expressed with ORAI1 in HEK-293 cells [45]. The simplest explanation for this discrepancy is that endogenously expressed STIM1 of HEK-293 cells participate in plasma membrane recruitment of the STIM1 mutants. Other possible explanations might be related to different amounts of ROS and antioxidants and/or the presence of ancillary proteins in the two different cell lines used for STIM1 expression. Nevertheless, in spite of all the discrepancies, both studies confirm that Cys49 and Cys56 are both critical for SOCE regulation.

The most parsimonious explanation of these diverging observations is that a disulfide bond forms between C49 and C56 in the oxidative environment of the ER, stabilizing the STIM1 resting conformation by maintaining its luminal EF-hand domains in a high-affinity state. Disruption of this bond would destabilize STIM1 but does not promote the extension of its cytosolic domain at resting luminal Ca\(^{2+}\), unless it oligomerizes with endogenous STIM1 (HEK cells), while S-glutathionylation at these sites poises STIM1 for activation by decreasing luminal calcium sensing and promoting the extension of its cytosolic domain and thus the formation of active STIM1 oligomers. Further experimental work will be required to clarify the precise role of these luminal cysteine residues in STIM1 function.

**Cytosolic and TMD-cysteines in regulation of STIM1 function:** Formation of disulfide bridges has been thought to be limited to the oxidative milieu of the ER, as the reducing environment of the cytosol was considered to prevent the creation of disulfide bonds [48]. However, in recent years, it has been recognized that thiols in cytosolic proteins may actually form disulfide bridges under oxidative stress conditions, due to saturation of the major cytosolic thiol-reducing systems, rendering cytosolic cysteines prone to oxidation [49]. Under such conditions, for example, mitochondrial ROS were shown to cause the redox-modulation of three proximal cysteine residues present in the cytosolic tail of the human cardiac L-type Ca\(^{2+}\) channel, leading to inhibition of this channel [50]. STIM1 possess a cytosolic cysteine at
position Cys-437 that could mediate reversible intermolecular disulfide bridge formation between STIM1 molecules during oligomerization but the redox reactivity of this residue has not yet been studied.

Cys-437 of STIM1 is the last C-terminal residue of the Sø4 helix (Fig. 2A) in the highly conserved CAD/SOAR [51]. Several investigators performed mutagenesis at this site. Park et al. (2009) demonstrated that the mCherry-STIM1 C437G mutant trafficked normally to PM puncta upon store-depletion and co-localized with eGFP-ORAI1, but was ineffective at inducing SOCE in HEK293 cells [11]. The initial rate of SOCE was 4-fold lower for the C437G mutant compared to wt STIM1, while the fraction of STIM1 or ORAI1 in puncta under resting and store-depleted conditions were similar for both mutant and wt STIM1 [11]. These data indicate that Cys-437 is not needed for STIM/ORAI clustering but plays a direct or indirect role in channel opening.

The involvement of Cys-437 of SOAR in SOCE regulation was further addressed in two biochemical studies. Yang et al. (2012) crystallized SOAR from human STIM1 and mutated the Cys-437 residue to Thr to improve the crystallization process. They confirmed that this substitution, along with the L347M and V419A mutations, did not affect the PM targeting of SOAR-eGFP and STIM1-eGFP before and after store depletion. Both the WT and mutant SOAR coimmunoprecipitated with the exogenously expressed ORAI1 channel and mediated SOCE when expressed in HeLa cells [51]. Analytical ultracentrifugation revealed that human wt SOAR is partially tetrameric in solution, whereas SOAR bearing the C437T, L347M and V419A mutations exclusively forms dimers [51], hinting towards a possible role of this cysteine residue in regulating the oligomeric state of STIM1. In another study, Zhou et al. (2013) employed a lanthanide-based resonance energy transfer approach to investigate the conformational changes in STIM1 C-terminus during activation of STIM1 [52]. For this, a cysteine was added to the C-terminus of the purified recombinant full-length C-terminal fragment (res. 233-685) of mouse STIM1 (STIM1\textsuperscript{CT}) for labelling with an acceptor fluorophore, and C437 was substituted by serine. They found that STIM1\textsuperscript{CT} is in a locked
conformation with its C-terminal K-rich domain in close proximity to the N-terminus of CC1. Further, their data suggests that CC1 dimerize during activation of STIM1 which releases SOAR from association with monomeric CC1, thereby exposing SOAR and the K-rich domain to interact with ORAI1 and PM lipids, respectively. Size exclusion chromatography revealed that the STIM C-terminus with this cysteine mutation still formed dimers, excluding its role in dimer formation, and the similar CD spectra of mutant and wt STIM1 C-termini showed that this residue does not define the conformational state of STIM1 [52].

Together, these findings indicate that Cys-437 of STIM1 might not be critical for producing conformational changes in STIM1. However, single point mutations of this residue appear to adversely interfere with CAD/SOAR-mediated channel gating. Thus, whether the cysteine 437 is still involved in intermolecular disulfide bridge formation and/or whether it contributes toward the redox regulation of STIM1 remain open questions.

Fuchs et al. (2012) reported two immunodeficiency syndrome patients with a homozygous mutation of Arg-429 located at the distal end of CC3 of STIM1 to cysteine. This mutation completely abolished SOCE in T-cells of these patients [53]. Fibroblasts from a patient carrying the homozygous R429C mutation were incapable to undergo SOCE, while transduction with wt STIM1 rescued SOCE [54]. It was shown that R429C destabilizes the structure of this CC3 coiled-coil and impairs intra- and intermolecular STIM1 protein interactions. This mutation also leads to a conformational change causing extension of STIM1 C-terminus exposing the K-rich domain thereby promoting constitutive translocation of STIM1 to ER-PM contact sites [54]. R429C also impaired the oligomerization of STIM1 C-terminus and binding of STIM1 to ORAI1, thereby abolishing SOCE. However, STIM1-R429E or R429L also abolished SOCE in HEK293 cells (coexpressed with ORAI1), as did STIM1-R429C, suggesting that the effect does not depend on the substitution to cysteine [54]. Nevertheless, it is of interest to consider whether genetic mutations leading to cysteine substitutions in SOCE components are evolutionary favoured to enhance the sensitivity toward oxidative stress as an additional regulatory factor.
Does ER-transmembrane cysteine 227 of STIM1 regulate STIM1 activity? The redox reactivity of C227 present in the transmembrane domain (TMD) of STIM1 has not been investigated. Ma et al. (2015) showed that a mutation of this residue to tryptophan reorients the TM helices and causes elongation of the cytoplasmic domain of STIM1, leading to constitutive STIM1 activity and calcium entry [55]. These data suggest that the TMDs of the STIM1 dimer in the resting state are associated with each other in the membrane and that a local rearrangement within the TMDs initiate conformational changes in the C-terminal domain of STIM1, without alteration of the oligomeric state, to activate STIM1 and subsequently expose SOAR to gate ORAI channels.

**STIM1 and Hypoxia/ROS:** Hypoxia, a condition marked by inadequate supply of oxygen to cells, increases reactive oxygen species (ROS) generation through mitochondrial complex III, causing nuclear accumulation of the transcription factor subunit HIF-1α, the key cellular oxygen sensor [56, 57]. HIF-1 regulates the expression of numerous target genes involved in maintaining oxygen homeostasis and thereby increases vascularization in hypoxic areas found at sites of focal ischemia and within tumors [57-59]. The hepatocarcinoma cell lines, HepG2 and Huh-7 exhibited, upon hypoxic treatment (cultured in 1% O2 for 24h), enhanced expression levels of HIF-1α and STIM1 but not of STIM2 [60]. In agreement with this, Li et al. (2015) reported that hypoxic conditions enhanced SOCE and I_{CRAC} in HepG2 cells. Also, downregulation or pharmacological inhibition of HIF-1α in hypoxic HepG2 cells led to decreased expression levels of STIM1 and concomitantly reduced SOCE. In addition to the drop in STIM1 expression, pharmacological inhibition of HIF-1 also suppressed xenograft hepatic tumorigenesis [60]. Altogether, these findings reveal a role of STIM1 in hypoxia and hypoxia-driven tumorigenesis.

Several studies showed that induction of hypoxia/oxidative stress with either the O2 scavenger sodium dithionite (Na-Dit), low (1.5%) O2-containing medium or tert-butyl hydroperoxide (tBHP) promotes accumulation of endogenous or overexpressed STIM1 at ER-PM junctions in various cell lines [61-63]. In two back-to-back publications, Gutarova et.
al and Mungai et al. reported that hypoxia-stimulated ROS production (1.5% O\textsubscript{2}) and oxidant signalling cause store-depletion and trigger CRAC channel activation, as assessed by pharmacological and RNA-silencing approaches in human A549 adenocarcinomic alveolar basal epithelial cells and 143B osteosarcoma cells, respectively [61, 63]. Another group, however, observed that hypoxic treatment (1 mM Na-Dit) of HEK293 or human airway smooth muscle cells (hASMC) inhibited Tg-induced SOCE and \(I_{\text{CRAC}}\), but this inhibition was attributed to the cytosolic acidification evoked by Na-Dit, which prevented functional coupling between STIM1-SOAR and the C-terminal tail of ORAI1 despite clustering of STIM1 at ER-PM junctions [62]. The authors further showed by FRET analysis that Na-Dit addition after store-depletion impaired the STIM2-ORAI1 interaction and that cytoplasmic alkalization with ammonium chloride reversed the inhibition of STIM1-ORAI1 interaction and \(I_{\text{CRAC}}\) [62]. Consistent with these findings, others also reported internal and external acidosis-induced inhibition of SOCE and \(I_{\text{CRAC}}\), and several residues were identified that confer pH sensitivity to ORAI1 [64-67].

Different mechanisms were proposed to account for the hypoxia-mediated store-depletion. Mancarella et al. (2011) demonstrated that hypoxia leads to a reduction in ATP levels which decreases SERCA-mediated Ca\textsuperscript{2+} pumping in the ER, resulting in Ca\textsuperscript{2+}-store depletion [62]. In contrast, Gusarova et al. (2011) and Mungai et al. (2011) reported no changes in ATP levels upon hypoxia induction with 1.5% O\textsubscript{2} [61, 63]. A direct role of ROS in STIM1 activation was shown by trapping ROS using the oxidative species scavenger EUK-134 [61]. Scavenging ROS prevented the clustering of endogenous STIM1 in hypoxia-treated ATII alveolar epithelial cells. Also, hypoxia-triggered intracellular Ca\textsuperscript{2+} release and SOCE were absent in A549 adenocarcinomic human alveolar basal epithelial cells either devoid of mitochondria or of a component of mitochondrial complex III which is crucial for ROS generation. Further research is required to clarify the mechanism of the reported effects of hypoxia/ROS on CRAC channel activation or inhibition. Gusarova et al. (2011) and Mungai et al. (2011) did not measure pH in their experiments and given that acidosis/alkalosis leads to
inhibition/potentiation of SOCE [64-67], pH differences could explain the discrepancies reported by Mancarella et al. (2011) [61-63]. Alternatively, a distinct expression ratio of STIM isoforms (STIM1L, STIM2, inhibitory isoform of STIM2) [41, 43, 44] might account for the different sensitivities toward hypoxia/ROS of the cell lines used. It will also be of interest to determine whether ROS generated by mitochondria have a different effect on STIM1 function than externally added ROS.

**Redox-regulation of the actin-binding isoform of STIM1**: STIM1L is a splice variant of STIM1 with an additional actin-binding domain downstream of SOAR. This enables STIM1L to bind cortical actin in muscle cells in order to form permanent clusters that colocalize with ORAI1, even under resting ER Ca^{2+} levels [41]. This renders STIM1L able to rapidly activate ORAI1 upon store-depletion without undergoing redistribution of the preformed clusters [41]. This prerecruitment of STIM1L to cortical actin is required for very rapid activation of SOCE during repetitive muscle contraction. STIM1L encompasses an additional cysteine residue in its actin-binding domain (Fig. 2A). Interestingly, STIM1L expression is detected in all skeletal muscles and many different tissues such as brain, lungs, liver, spleen, and heart but not in the kidney, suggesting that the constitutive pre-clustering of STIM1L-ORAI1 under resting conditions may be a common feature of the SOCE pathway in different cell types. However STIM1L did not form constitutive membrane clusters when expressed in fibroblasts [68] indicating that accessory proteins are likely required to recruit STIM1L to cortical structures. Whether hypoxia/ROS regulate the activity of this STIM1 isoform is not known but the presence of an additional cysteine residue within its actin-binding domain suggests that the pre-recruitment of STIM1L to cortical actin might be regulated in a redox-dependent manner.

During acute hypoxia, STIM1 associates with both ORAI1 and TRPC1 to mediate SOCE in mouse PASMCs [69]. The association of STIM1L with TRPC1 has not yet been tested, but STIM1L binds to ORAI1 with a 4-fold higher affinity than STIM1 and associates with TRPC3 and TRPC6, leading to stronger inhibition of receptor-operated Ca^{2+} entry via these TRPC channels [70]. STIM1 was also shown to interact with and inhibits voltage-operated calcium
channel CaV1.2 [71], but whether STIM1L similarly regulates CaV1.2 has not yet been demonstrated. The specific interactions involving STIM1 or STIM1L with STIM-regulated PM calcium channels other than ORAI (TRPC or L-type calcium channels) might lead to different redox sensitivities in different cell-types, depending on their expression levels. The redox regulation of these channels has recently been reviewed [72, 73].

In mouse models of muscular dystrophy, increased SOCE was associated with the upregulation of ORAI1, STIM1, and STIM1L [74]. The ratios of STIM1L to STIM1S were similar in wt and dystrophic mdx muscle and PMCA expression was increased, balancing the inhibitory effect of STIM1 on PMCA and preserving the Ca\(^{2+}\) extrusion capacity of muscle fibers [75]. The causal role of SOCE in the pathology of dystrophic muscle was directly tested in a mouse model of muscle-specific STIM1 overexpression [76]. Enhanced muscle-specific expression of STIM1 was associated with increased Ca\(^{2+}\) influx and with typical histological and biochemical features of muscular dystrophy, including myofiber necrosis, swollen mitochondria, increased inflammation, interstitial fibrosis, and elevated creatine kinase levels [76]. Most of these pathological features were reverted by expressing a dominant-negative ORAI1 mutant, which also significantly reduced the severity of muscular dystrophy in two mouse models of the disease [76]. This firmly links increased STIM1-ORAI1 activity to muscular dystrophy, but whether an altered redox regulation of STIM1, STIM1L, and/or ORAI proteins participate in the increased SOCE of dystrophic myofibers is not known.

**Redox regulation of STIM2**

**Lower Ca\(^{2+}\) activation threshold of STIM2 compared to STIM1:** The affinity of the STIM2 EF-hand domain for Ca\(^{2+}\) is about 400 \(\mu\)M whereas that of STIM1 is 200 \(\mu\)M [77]. This difference in the EF-hand Ca\(^{2+}\)-binding affinity renders STIM2 active at basal ER Ca\(^{2+}\) concentrations (~400 \(\mu\)M) and sensitive to small reductions in ER Ca\(^{2+}\), whereas STIM1 needs a higher drop in ER Ca\(^{2+}\) concentration for activation [78]. Thus, STIM2 may function as a feedback regulator that stabilizes basal cytosolic and ER Ca\(^{2+}\) levels. STIM2, in addition to lower Ca\(^{2+}\) affinity of
the EF-hand, has higher affinity of its C-terminal K-rich domain for PI(4,5)P$_2$. The increased lipid-binding avidity enables STIM2 to cluster at ER-PM junctions in response to minimal depletion of ER Ca$^{2+}$ stores [12, 79].

**Differential expression of STIM isoforms:** While most tissues express higher levels of STIM1 than STIM2 [80-82], the levels of STIM2 protein in brain are higher than those of STIM1 [83]. STIM2 is the predominant STIM isoform in mouse primary hippocampal and cortical neurons and is essential to induce SOCE [83-86]. STIM2, like STIM1 can also activate all the three ORAI isoforms upon store-depletion [87]. However, different STIM isoforms can contribute to Ca$^{2+}$ entry in different cell types. For example, Bandyopadhyay et al. (2011) demonstrated that STIM2 regulates SOCE by coupling to ORAI2 in mouse dendritic cells [88].

**Role of STIM2 in ER-PM coupling and constitutive calcium entry:** The expression of a chimeric ER membrane protein, *i.e.* Kir6.2, fused to the C-terminus of STIM2, led to constitutive ER-PM contact formation, whereas fusion of Kir6.2 with the C-terminus of STIM1 remained reticular, indicating that STIM2 leads to constitutive coupling of ER and PM [79]. STIM2 also has a higher lipid-binding affinity than STIM1 in PI(4,5)P$_2$-containing liposome-binding assays [12]. The higher affinity of the C-terminal K-rich domain of STIM2 for PI(4,5)P$_2$ at the PM thus favors the recruitment of STIM2-containing ER to the PM, even in resting conditions [12, 79]. Overexpression of STIM2, but not STIM1 had been reported to trigger constitutive Ca$^{2+}$ entry [89] and cortical neurons co-transfected with YFP-STIM2 and ORAI1 had much higher resting Ca$^{2+}$ levels than neurons expressing YFP-STIM1 and ORAI1 [84]. Even though STIM2 is a poor activator of ORAI1 [90], altogether these findings suggest that the higher lipid-binding affinity of STIM2 leads to constitutive recruitment of STIM2 in ER-PM contacts, resulting in constitutive Ca$^{2+}$ entry by activation of PM Ca$^{2+}$ channels. Recent findings from Ambudkar and colleagues suggest that, under mild depletion of ER Ca$^{2+}$ stores, STIM1 is corecruited to ER-PM contact sites with STIM2. In this recruitment of STIM1-STIM2 heterooligomers, the K-rich domain of STIM2 is indispensable [91]. Therefore,
in the light of the formation of mixed STIM1 and STIM2 complexes, the function of STIM2 isoform and its redox-regulation is of great interest.

**Redox-regulation of STIM2:** The luminal cysteine residues present in STIM1 are also conserved in STIM2. Moreover, there is a higher number of conserved cysteine residues present in the C-terminal domain of STIM2 compared to STIM1 (eleven versus one) which may indicate the possibility of redox-mediated STIM2 activation (Fig. 2B). While it is established that 1) STIM1 forms homodimers at resting state via coiled-coil domains [33-36, 92] and 2) unlocking of the STIM1 protein exposes the K-rich domain during activation-driven conformational changes [52], the resting assembly state of STIM2 and the exposure state of its K-rich domain is unknown. Given the high degree of sequence homology between the STIM1 and STIM2 coiled-coil domains, it is likely that STIM2 also forms dimers at resting state. Therefore, it is tempting to hypothesize that the redox environment in the cytosol can regulate the intermolecular reversible thiol bridge formation involving the cysteines present in the cytosolic tail of STIM2, triggering the extension and activation of the STIM2 molecule, thus allowing it to increase the ER-PM coupling.

Enhanced ER-PM contact formation may stimulate STIM2-dependent activation of Ca^{2+}-channels which may lead to chronic Ca^{2+} overload and finally to necrosis and cell death by apoptosis [93]. Consistent with a redox-related modulation of STIM2 function, it had been reported that neuronal cells of STIM2^{-/-} mice fail to undergo apoptosis under oxidative stress conditions [83]. However, there is no direct evidence for redox-related control of STIM2 activity.

Upregulated protein levels of STIM2 were reported under chronic hypoxic conditions in rat pulmonary arterial smooth muscle cells (PASMCs) [94]. Also, increased levels of STIM2 protein but not STIM1 were found in PASMCs of idiopathic pulmonary arterial hypertension patients [94]. It had been reported that STIM2 knockdown decreased SOCE in response to
acute hypoxia in PASMC but to a lesser extent when compared to STIM1 knockdown [95]. Altogether these data indicate a redox-regulated role of STIM2 in mediating SOCE.

Reactivity of STIM2 cytosolic cysteines: Bhardwaj and colleagues showed that the oxidation of the last C-terminal cysteine of STIM2 at position 725 (located just upstream of the lipid-binding K-rich domain) led to the dimerization of the purified recombinant eGFP-tagged polybasic domain of STIM2. This dimer exhibited high affinity binding to PI(4,5)P₂-containing PM-like liposomes in vitro [12]. Under reducing conditions this recombinant protein, however, is a monomer and failed to bind PM lipids [12]. It remains to be determined whether, in vivo, oxidation of cys-725 in STIM2 (or by any other cytosolic cysteine residues) may place in close proximity the two STIM2 monomeric units of the lipid-binding domains and whether this dimerization is sufficient for ER-PM contact formation and activation of PM Ca²⁺ channels.

Redox regulation of other STIM2 isoforms: STIM2 preprotein (Fig. 2C) generated by inefficient cleavage of the signal peptide, is localized in the cytosol and has been implicated in a store-independent mode of SOCE activation [42]. Whether this isoform, now having all the cysteines of STIM2 exposed to the redox environment of the cytosol, can be modified upon variation of the intracellular redox conditions, thereby modulating SOCE, would be the subject of additional investigations. Recently a new STIM2 inhibitory isoform containing an eight-residue insert in its SOAR (Fig. 2B) that disrupts its binding to ORAI1 has been identified and studied [43, 44]. We also noticed that the STIM2 preprotein isoform [42] also exists as a variant with this 8-residue insertion (NCBI accession number: AAJ36450 or AAJ71766). These inhibitory isoforms of STIM2 are interesting modulatory players of SOCE and need to be investigated in the context of redox regulation, as all the cysteines residues present in STIM2 are also conserved in these isoforms. Moreover, the finding that the inhibitory isoform of STIM2 which is retained in the ER can oligomerize with other STIM isoforms and can be recruited to CRAC channels, thereby inhibiting the Ca²⁺ influx, suggests that the redox modulation of STIM isoforms can have either a potentiating or an inhibiting effect on SOCE [44].
Redox regulation of ORAIs

The topology and domain architecture of the tetra-spanning PM ORAI calcium channel subunits are presented in Fig. 3. The function of ORAI proteins has been shown to be differentially regulated by oxidative stress [96]. Three cysteine residues are present in ORAI1, Cys-126 in the second TMD, Cys-143 in the cytosolic loop between TMD 2 and TMD 3 and Cys-195 at the extracellular end of the third TMD (Fig. 3). C126 and C143 are conserved in all the three ORAI isoforms whereas Cys-195 is missing in ORAI3 and is substituted by a glycine (Fig. 3). ORAI3 has a 34 residue longer extracellular loop between the third and fourth TMD and this loop contains two additional cysteine residues located five residues apart, which are not present in ORAI1 or ORAI2.

Bogeski and colleagues studied the redox reactivity and sensitivity of the ORAI isoforms in naive and effector T cells and in HEK293 cells coexpressing each ORAI isoform along with STIM1 [96]. They reported that a pre-treatment with H$_2$O$_2$ before store depletion inhibited the ORAI1- and ORAI2-mediated calcium influx, whereas the ORAI3-mediated calcium influx remained resistant to H$_2$O$_2$-mediated block. Lack of Cys-195 in ORAI3, which was identified as major redox sensor in ORAI1, was reported to be the cause of this differential redox sensitivity of ORAI1 and ORAI3. Calcium imaging and electrophysiological recordings revealed that substitution of Cys-195 of ORAI1 to the non-oxidizable serine residue conferred partial resistance to H$_2$O$_2$-block, without changing the ion selectivity of the channel, whereas mutation of all three cysteines to serines completely abolished the redox sensitivity of ORAI1 [96]. Supporting this finding, a mutation of Gly-170 in ORAI3 to cysteine (at a homologous position to Cys-195 of ORAI1, see Fig. 3) conferred redox-sensitivity to ORAI3, confirming that Cys-195 is a critical redox sensor in ORAI1 [96].

Bogeski and colleagues also showed that the CRAC channels in effector T$_H$ cells are less vulnerable toward H$_2$O$_2$ than those in naive T$_H$ cells [96]. Mild H$_2$O$_2$ treatment (10 µM) led to a 50% decrease in Ca$^{2+}$ influx upon store-depletion in naive T cells and a marginal decrease
in effector T<sub>H</sub> cells. Similarly, acute H<sub>2</sub>O<sub>2</sub> treatment (500 µM) almost completely inhibited the CRAC current in Jurkat T cells and had only a partial inhibitory effect in effector T<sub>H</sub> cells (87% vs. 40% inhibition of I<sub>CRAC</sub> [96]). The decreased redox sensitivity of effector T<sub>H</sub> cells compared to undifferentiated naive T cells correlated with upregulation of ORAI3 in T<sub>H</sub> cells as assessed by qRT-PCR. Moreover, silencing ORAI3 mRNA in human effector T<sub>H</sub> cells rendered store-operated Ca<sup>2+</sup> influx more redox sensitive [96]. This showed that effector T<sub>H</sub> cells, by upregulating ORAI3 expression and thus decreasing the ORAI1:ORAI3 ratio, undergo a remarkable adaptation to protect the SOCE mechanism while operating in the harsh oxidative environment during inflammation. Consistently, expressing STIM1 together with ORAI1 and ORAI3 at a five to one ratio in HEK293 led to 45% block of I<sub>CRAC</sub> by 1 mM H<sub>2</sub>O<sub>2</sub>, whereas expressing STIM1 and ORAI1 without ORAI3 led to 80% block [96]. These findings demonstrate that ORAI1 is redox-sensitive and mediates the H<sub>2</sub>O<sub>2</sub> inhibition of I<sub>CRAC</sub> and that upregulation of ORAI3 can tune this I<sub>CRAC</sub> inhibition by generating redox-resistant ORAI3 homomers or by participating in heteromeric complexes (ORAI1/ORAI3). In support for this, a recent study using heteromeric concatenated channel subunits (one ORAI3 and three ORAI1) showed that a single subunit of ORAI3 can confer redox resistance to ORAI1-ORAI3 heteromeric channels [97]. The authors also showed that ORAI1 facilitates the recruitment of ORAI3 to STIM1 clusters at ER-PM junctions in HEK293 cells and also that ORAI1 in Jurkat T cells is required for escorting ORAI3 to an immunological synapse mimic. Altogether these findings further support the notion that ORAI3 might work together with ORAI1 in a heteromeric complex to bring about resistance to oxidative stress.

Interestingly, active STIM/ORAI complexes formed after store-depletion in T cells and in STIM1-ORAI1 overexpressing HEK293 cells were found to be resistant to H<sub>2</sub>O<sub>2</sub>-mediated inhibition of calcium entry, ruling out a possible open channel block by H<sub>2</sub>O<sub>2</sub> [96]. This suggests that ORAI1 undergoes a conformational change when docked with STIM1 that alters the accessibility of the extracellular Cys-195 to oxidants, thus imparting redox resistance to ORAI1-mediated Ca<sup>2+</sup> influx after store-depletion. In such a scenario, the
STIM1L isoform, which is pre-coupled to ORAI1, could maintain the channel in a redox-insensitive state. Further investigation is required to understand the molecular mechanism by which H$_2$O$_2$ inhibits ORAI1 function and the impact of the redox changes in channel state on the STIM-ORAI1 interaction.

Altered ORAI1/ORAI3 expression ratios have been reported to play a role in abnormal calcium signaling in prostate cancer [98, 99]. Since prostate cancer cells are exposed to elevated levels of ROS [100], it would be of great pathophysiological interest to study the mechanism of redox-modulation of ORAI1-ORAI3 heteromeric channels, functioning in calcium store-dependent manner or in a store-independent manner regulated by stimuli such as arachidonic acid [101] or leukotriene C4 [102].

**Concluding remarks**

STIM and ORAI proteins are the core components of the store-operated Ca$^{2+}$ entry process that sustain long-lasting Ca$^{2+}$ signals required for the proliferation and differentiation of T lymphocytes and for the development of skeletal muscle cells. Loss of function mutations in STIM and ORAI are associated with severe immunodeficiency [10, 103] and with muscular hypotonia and gain-of-function mutations with tubular aggregate myopathy [104-106] (reviewed in [107]). Reactive oxygen species modulate SOCE activity in immune cells and in STIM/ORAI expression systems but the molecular basis and functional significance of this redox regulation are unclear. The SOCE machinery is exposed to local redox alterations occurring within the ER lumen and at the plasma membrane, as STIM proteins sense a drop in the luminal ER Ca$^{2+}$ concentration and redistribute to the cell periphery to trap and gate ORAI channels at ER-PM contact sites. Two cysteine residues in STIM1 luminal domain (Cys-49 and Cys-56) have been implicated in redox sensing within the lumen of the ER and a cysteine within ORAI1 extracellular loop (Cys-195) has been implicated in redox sensing of extracellular hydrogen peroxide. Interactions with the ORAI3 isoform lacking Cys-195 renders ORAI1 redox resistant and might represent an adaptive mechanism to preserve SOCE function in oxidative conditions. Whether interactions with STIM proteins also
modulate the redox sensitivity of ORAI1 remains to be determined. Future research should
explore the role of the additional cytosolic cysteines present in the STIM2 isoform, which has
distinct Ca\(^{2+}\) sensing, lipid binding, and ORAI gating efficiencies, and in the STIM1L isoform,
which is pre-recruited to cortical actin structures. More work is also needed to clarify whether
cytoplasmic cysteine residues within STIM and ORAI proteins are subject to redox modifications
and establish the consequence of these redox changes for ER Ca\(^{2+}\) sensing, membrane
trafficking, and channel gating.

Acknowledgements

This work was funded by the Swiss National Foundation grant numbers 31003A_149566 (to N.D.) and
CRSII3_160782/1 (to M.H.)

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Figure 1. Schematic step-wise representation of SOCE pathway:

1) At resting ER Ca\(^{2+}\) levels both STIM1 and ORAI1 form homodimers [33-37] and have diffused localization in the ER membrane and the PM, respectively [10, 25]. STIM1 forms a dimer via intermolecular interactions involving cytosolic coiled-coil domains, as described in the text (also see Fig. 2).

2) The agonist-induced stimulation of G-protein coupled receptors (GPCRs) or tyrosine kinase receptors (RTKs) activates phospholipase C\(\beta\) or phospholipase C\(\gamma\) (PLC), respectively resulting in hydrolysis of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P\(_2\)] on the inner leaflet of PM and release of Inositol 1,4,5-trisphosphate (IP\(_3\)).

3) IP\(_3\) diffuses into the cytosol and binds to the IP\(_3\) receptor (IP\(_3\)R) on the ER membrane causing release of ER Ca\(^{2+}\) from the IP\(_3\)R.

4a) STIM1 or STIM2, upon significant or minor drop in ER Ca\(^{2+}\) concentration, respectively, lose Ca\(^{2+}\)-bound to their EF-hand domains. Ca\(^{2+}\)-dissociation activates the STIM dimer by aggregation and interaction of EF-SAM domains in the ER lumen.

4b) Aggregation and interaction of EF-SAM induces an extended conformation of the cytoplasmic coiled-coil domains, leading to exposure of SOAR and the K-rich domain (also see Fig. 2).

5a) Activated STIM1 dimer further oligomerizes via coiled-coils and translocates to ER-PM contact sites.

5b) Oligomerized STIM1 binds PI(4,5)P\(_2\) at the inner leaflet of the PM avidly via their K-rich domains leading to extension and stabilization of ER-PM contact sites.

6) Activated STIM1 oligomers and ORAI1 accumulate at ER-PM junctions.

7) The exposed SOAR domains of activated STIM1 oligomers activate the ORAI1 channel, leading to influx of extracellular Ca\(^{2+}\) into the cytosol. Each exposed SOAR dimer interacts with and activates one ORAI1 protein [38]. While the Drosophila structure reports a hexameric ORAI1 channel [39], human ORAI1 in its active state is suggested to be tetrameric [36, 37]. Eight STIM1 molecules forming an active complex with one tetrameric ORAI1 channel are shown as per current stoichiometric model of STIM1-ORAI1 interaction [26, 38].

8) Consequently, ER stores are refilled with Ca\(^{2+}\) by SERCA, which is co-recruited at these ER-PM contacts [40]. Figure was redrawn, using information from Soboloff et al. (2012) [26] and others.
Figure 2. Schematic outline of the domain architecture of human STIM1 and STIM2 isoforms drawn to scale with conserved cysteine residues shown as yellow circles. (A) Human STIM1 and STIM1L [41] isoforms, comprising 685 and 791 amino acid residues, respectively (B) STIM2, comprising 746 amino acids and (C) STIM2 preprotein [42], comprising 833 residues are shown. Shown are the signal sequence (SS), the canonical EF-hand domain (cEF) with amino acid sequence of Ca$^{2+}$-binding loop, the hidden EF-hand domain (hEF), the sterile alpha motif (SAM), the transmembrane domain (TMD), the coiled-coil domains 1-3 (CC), the three predicted $\alpha$-helices of CC1 (Ca1-3) [26] STIM-ORAI activating region (SOAR), four $\alpha$-helices of SOAR (So1-4), the inactivation domain (ID), the actin-binding domain (ABD) in STIM1L, the serine/proline-rich region (S/P), the proline/histidine-rich region (P/H), the microtubule plus end-tracking protein (EB1)-binding motif - TRIP motif in STIM1 and the analogous SGIP/SSIP motifs in STIM2 (Ser/Thr-x-Ile-Pro), the lysine (K)-rich domains with their amino acid sequences (all with residue numbers) and the orientations of N- and C-termini. Asn-linked glycosylation sites are shown as hexagons. Arginine retention signals (RXR) are depicted as blue circles with residue numbers indicated on STIM1 and STIM2. The 8 amino acid insertion in the SOAR in STIM2 inhibitory splice variant [43, 44] is shown on both (B) STIM2 and (C) STIM2 preprotein.
Figure 3. ORAI proteins share common topology. Cysteine residues in ORAI1 are indicated as yellow circles with residue numbers and sequence alignments of segments from each ORAI isoform spanning the corresponding cysteine residues. Cysteines in the alignments of ORAI isoforms are highlighted in bold and square boxes. The glycosylation site of ORAI1 (N223) is shown in blue circle with a hexagon. Putative STIM1-binding site at the N-terminus of ORAI1 (residues 73-85) [19] and the coiled-coil domain in ORAI1 at the C-terminus (residues 268-291) [9] spanning the STIM1-binding site are depicted as black thick lines. Glu-106 (selectivity filter), Val-102 (hydrophobic gate), Gly-98 (gating hinge) on the TMD 1 of ORAI1 are indicated as yellow lines. Arg-91 on TMD1 of ORAI1 that is mutated to Trp in SCID patients [10] is indicated with red line.