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Constitutive calcium entry and cancer : updated views and insights

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Abstract

Tight control of basal cytosolic Ca^{2+} concentration is essential for cell survival and to fine-tune Ca^{2+} -dependent cell functions. A way to control this basal cytosolic Ca^{2+} concentration is to regulate membrane Ca^{2+} channels including store-operated Ca^{2+} channels and secondary messenger-operated channels linked to G-protein-coupled or tyrosine kinase receptor activation. Orai, with or without its reticular STIM partner and Transient Receptor Potential (TRP) proteins, were considered to be the main Ca^{2+} channels involved. It is well accepted that, in response to cell stimulation, opening of these Ca^{2+} channels contributes to Ca^{2+} entry and the transient increase in cytosolic Ca^{2+} concentration involved in intracellular signaling. However, in various experimental conditions, Ca^{2+} entry and/or Ca^{2+} currents can be recorded at rest, without application of any experimental stimulation. This led to the proposition that some plasma membrane Ca^{2+} channels are already open/activated in basal condition, contributing therefore to constitutive Ca^{2+} entry. This article focuses on direct and indirect observations supporting constitutive activity of channels belonging to the Orai and TRP families and on the mechanisms underlying their basal/constitutive activities.

Keywords

STIM, Orai, TRP, SPCA, cancer, Constitutive/basal Ca^{2+} entry

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Abbreviations

ARC: Arachidonate Regulated Ca^{2+}

BKCa: Big conductance Ca^{2+} -activated K^+ channels

B-SOCE: Basal Store Operated Calcium Entry?

$\text{Ca}_v3.2$: voltage-gated Ca^{2+} channel 3.2

CRAC: Ca^{2+} Release-Activated Ca^{2+}

GPCR: G-Protein-Coupled Receptor

hEAG1: Human Ether-a-go-go K^+ Channel 1

IKCa: Intermediate conductance Ca^{2+} -activated K^+ channels

LPA: LysoPhosphatidic Acid

LPC: LysoPhosphatidylCholine

NVG- Ca^{2+} channel: Non-Voltage Gated Ca^{2+} channel

P2X: Purinergic ionotropic receptor

R-SOCE: Receptor-triggered Store Operated Ca^{2+} Entry influx

SPCA: The Secretory Pathway Ca^{2+} -ATPase.

SKCa: Small conductance Ca^{2+} -activated K^+ channels

SMOC: Secondary Messenger-Operated Channels

STIM: STromal Interaction Molecule

SAC: Stretch-Activated Channels

SOC: Store-Operated Channels

TRPC: Transient Receptor Potential Canonical

TRPM7: Transient Receptor Potential Melastatin-related 7

TRPV: Transient Receptor Potential Vanilloid

VOCC: Voltage-Operated Calcium Channels

1. Introduction.

A tight control of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) is essential for cell survival and normal cell function. Cells must maintain resting $[\text{Ca}^{2+}]_c$ at a low level (around 100 nM) in order to maintain the large dynamic range required for Ca^{2+} signals. Membrane ionic channels and transporters, cytosolic Ca^{2+} buffers and Ca^{2+} buffering organelles regulate Ca^{2+} influx, storage and extrusion to maintain this $[\text{Ca}^{2+}]_c$. This finely tuned control of $[\text{Ca}^{2+}]_c$ is essential for the differential modulation of various signaling pathways and intracellular Ca^{2+} -regulated proteins involved in specific cellular processes including regulation of metabolism, proliferation, death, gene transcription, cell migration, exocytosis, and contraction (Berridge et al. 2003).

Plasma membrane Ca^{2+} channels support Ca^{2+} entry into the cytosol along its electrochemical gradient across the plasma membrane leading to an increase of cytosolic free $[\text{Ca}^{2+}]_c$. Various Ca^{2+} channels are involved in such transmembrane Ca^{2+} influx including Voltage-Operated Ca^{2+} Channels (VOCC), ligand-gated channels (P2X purinergic ionotropic receptor families, for instance), Secondary messenger-operated channels (SMOC) linked to GPCR (G-protein-Coupled Receptor) or RTK (Receptor Tyrosine Kinase) activation and the production of secondary messengers, Store-Operated Channels (SOC) and Stretch-Activated Channels (SAC). Among these channels, STIM1 (stromal interacting molecules 1) and Orai1 proteins were firstly described as the molecular components of the ubiquitous store-operated Ca^{2+} entry (SOCE) pathway mediated by the archetypal Ca^{2+} release-activated Ca^{2+} channels (CRAC) in many cell types (Hogan and Rao 2015). Member of the Orai protein family (Orai1, Orai2, and Orai3) form highly selective Ca^{2+} channels (Feske et al. 2006) regulated by STIM1 and STIM2. These STIM proteins act as Ca^{2+} sensors mainly located in the membrane of the endoplasmic reticulum (ER) that can interact with and activate Orai channels upon ER-

Ca²⁺-store depletion. However, a pool of STIM1 located in the plasma membrane play a different role including the regulation of store-independent Ca²⁺ entry (SICE) pathways (Mignen et al. 2007). Along with Orai1, Orai3 contributes to store-independent Ca²⁺ channels, such as the arachidonate regulated Ca²⁺ (ARC) channels (Mignen et al. 2008) and Leukotriene C4-regulated Ca²⁺ (LRC) channels (Gonzalez-Cobos et al. 2013).

TRP channels form tetrameric assemblies (Hellmich and Gaudet 2014) and can be divided in seven main subfamilies based on sequence homology (Montell 2011): The TRPC ('Canonical') family; the TRPV ('Vanilloid') family; the TRPM ('Melastatin') family; the TRPP ('Polycystin') family; the TRPML ('Mucolipin') family; the TRPA ('Ankyrin') family; and the TRPN ('NOMPC') family. All functionally characterized TRP channels are cationic channels permeable to Ca²⁺ with the exceptions of TRPM4 and TRPM5, which are only permeable to monovalent cations. TRP channels also contribute to changes in [Ca²⁺]_c either directly by supporting Ca²⁺ entry through the plasma membrane, or indirectly through the modulation of membrane potential controlling the driving force for Ca²⁺ entry. These channels are activated by a wide range of stimuli that include the binding of intra- and extracellular messengers, changes in temperature, chemical agents, mechanical stimuli and osmotic stress (Zheng 2013). In some cellular models, TRPC channels also participate to Ca²⁺ entry activated by store depletion along with Orai1 (Liao et al. 2014). Like Orai proteins, members of the TRP family can be activated/mobilized by a variety of extracellular signals leading to changes in the Ca²⁺ concentration in spatially restricted micro/nanodomains underneath the plasma membrane that support various Ca²⁺ dependent intracellular pathways.

The activity of all these Ca^{2+} selective channels is tightly controlled by a number of different protein partners. The membrane environment defines micro/nanodomains that also regulate the spatio/temporal pattern of intracellular Ca^{2+} signal in response to extracellular signals.

It is well accepted that the opening of these Ca^{2+} channels contributes to Ca^{2+} entry in response to cell stimulation. However, in various experimental conditions, and in particular in pathological situations, Ca^{2+} entry and/or Ca^{2+} currents could be observed at rest in some experimental conditions. This led to the proposition that plasma membrane Ca^{2+} channels could be already be opened/activated at rest, at least in the chosen experimental conditions. As an example, an early study in dystrophic muscles cells showed that a class of channels, named leak channels, permeable to both barium and Ca^{2+} , are open more frequently in dystrophic mouse and human myotubes in resting conditions (Fong et al. 1990). It was suggested that this leak channel activity supports a higher basal Ca^{2+} entry, and in turn a higher resting $[\text{Ca}^{2+}]_c$ underneath the plasma membrane (Turner et al. 1991). These so called “leak” channels were not molecularly identified, and it was later suggested that they could be store-operated (Hopf et al. 1996).

The present review reports direct or indirect observations supporting a basal or a constitutive activity of identified channels belonging to the Orai or the TRP family. Many observations of a basal Ca^{2+} influx were realized in cancer cells and were suggested to contribute to the deregulation of intracellular Ca^{2+} signaling supporting the cancer phenotype. This also raised the question of the mechanisms underlying this constitutive or basal activity of Ca^{2+} channels. Gating of these TRP and Orai1 protein is most often dependent on specific mechanisms such as stimulation of the PLC pathway, or association with the STIM protein at junctions between the plasma membrane and the ER membrane. Changes in plasma membrane properties or in

intracellular signaling pathways but also post-translational modifications of channel proteins were suggested as explanations of their constitutive activation. The association of Ca^{2+} channels with protein partners such as scaffolding proteins, cytoskeletal proteins or even Ca^{2+} -activated potassium channels could also regulate their constitutive activity. The alternative hypothesis is an alteration of the intrinsic properties of Ca^{2+} channels due to some mutation or to their post-translational status.

2. Constitutive Ca^{2+} entries and regulation mechanisms

2.1. Orai1 complexes with potassium channels and SPCA

2.1.1. Orai1 complexes with SK3, BKCa and hEAG1 channels

Defective basal Ca^{2+} signaling could be linked to constitutive plasma membrane Ca^{2+} influx leading to higher $[\text{Ca}^{2+}]_c$ than usually observed in normal cells (excitable and non-excitable cells). $[\text{Ca}^{2+}]_c$ is controlled by the balance between Ca^{2+} pumped into the extracellular space by Ca^{2+} plasma membrane pumps and Ca^{2+} influx into the cytosol through Ca^{2+} channels such as Orai1.

Cancer cells are associated with major changes in the expression of Ca^{2+} channels and Ca^{2+} pumps (Monteith et al. 2012) and since these cells are generally found to be resistant to Ca^{2+} -induced apoptosis it is not surprising to observe high and non-harmful $[\text{Ca}^{2+}]_c$ in these cells.

A few years ago, it was observed that the highly metastatic cancer cell line, MDA-MB435s, has a high level of $[\text{Ca}^{2+}]_c$ close to 380 nM, a concentration that was reduced by SK3 channel inhibitors such as apamin and edelfosine (Potier et al. 2011). SK3 is a potassium channel that belongs to the SKCa family. The suppression of SK3 reduces cancer cell migration while rescue experiments with SK3 restore this capacity and enforced SK3 expression increases the

migrational capacity of cancer cells that do not express the channel (Chantome et al. 2009; Potier et al. 2006). When SK3 is expressed in a cancer cell, it increases twice the capacity of cells to migrate and invade a matrix similar to the physiological extracellular matrix (Chantome et al. 2009; Potier et al. 2006). The suppression of the SK3 channel, the addition of SK3 inhibitors or an increase in external K^+ solutions all depolarize SK3 expressing cells and reduce cell migration demonstrating that the SK3 channel regulates cell migration by polarized cells to values close to E_K (Chantome et al. 2009; Chantome et al. 2013; Potier et al. 2006). In addition, an elevation of external Ca^{2+} concentration increasing SK3 current and plasma membrane hyperpolarization, increases Ca^{2+} influx (Chantome et al. 2013).

It was established that Orai1 is involved in SK3-dependent Ca^{2+} entry (Chantome et al. 2013). Ca^{2+} entry through Orai1 channels is increased by plasma membrane hyperpolarization due to SK3 channels opening. Orai1 is referred as a SOC channel when activated by STIM1 located in the ER plasma membrane. However, in contrast to Orai1, STIM1 knockdown has no effect on SK3-dependent Ca^{2+} entry nor on SK3 dependent MDA-MB-435s cell migration. These findings revealed a novel signaling pathway in which Orai1 along with SK3 channels elicited a constitutive and STIM1 store independent Ca^{2+} influx that promoted MDA-MB-435s cell migration (Chantome et al. 2013). This Ca^{2+} entry is not regulated by STIM2 since the protein is not expressed in MDA-MB-435s. Figure 1 shows that basal $[Ca^{2+}]_c$ follows external Ca^{2+} concentration changes suggesting that Ca^{2+} enters the cell through a constitutive Ca^{2+} entry.

The working model is that high level of basal $[Ca^{2+}]_c$ results from basal Orai1 opening that in turns activates SK3 channels. Hyperpolarizing of plasma membrane due to SK3 activation increases the Ca^{2+} driving force and consequently favors Ca^{2+} entry through Orai1 that in turns increases basal $[Ca^{2+}]_c$ (Fig. 2). Thus, there isn't a need for much Orai1 and SK3

channel basal activity to increase basal $[Ca^{2+}]_c$ since a positive feedback loop exists between Orai1 and SK3 channels. Nevertheless, these channels need to be close to one another in the plasma membrane to support this positive feedback loop. SK3 and Orai1 channels were both found in caveolae-rich membrane fractions (Chantome et al. 2013). SK3 protein knockdown or the addition of the alkyl-lipid Ohmline (1-O-hexadecyl-2-O-methyl-sn-glycero-3-lactose) acting on lipid rafts, both reduce constitutive Ca^{2+} entry of MDA-MB-435s (Chantome et al. 2013). This novel function of SK3 allowed to hypothesize that the SK3 channel could participate in the formation of metastases. SK3 channels do not regulate primary tumour development in mouse models of metastatic breast cancers (orthotopic xenografts), but promote the development of metastases, especially in the bones. These observations may be linked to the activation of SK3 by Ca^{2+} and the high Ca^{2+} concentrations found in the bone environment (Chantome et al. 2013). In the absence of SK3, Orai1 is not embedded within lipid rafts and does not promote constitutive Ca^{2+} influx. The expression of SK3 in cancer cells triggers SK3-Orai1 to associate within lipid rafts, resulting in plasma membrane hyperpolarization and constitutive Ca^{2+} entry. Increased external Ca^{2+} concentration observed in osteolytic metastatic sites amplifies Ca^{2+} entry, leading to a positive feedback loop. Disrupting lipid rafts with the alkyl-lipid Ohmline allows Orai1-SK3 complex to dissociate and abolishes SK3-dependent constitutive Ca^{2+} entry. In mice treated with Ohmline, SK3-dependent cancer cell migration and bone metastasis is inhibited (Chantome et al. 2013).

The origins of the constitutive activity of Orai1 are unknown, this may be due to its physical interaction with SK3 channels. We found such a direct interaction between Orai1 and SK3 using co-immunoprecipitation and time-resolved FRET experiments (Gueguinou et al. 2017). Moreover, physical interaction between channels should be favored by the localization of Orai1 and SK3 in caveolae probably due to the high content of this sub-compartments in

specific lipids such as cholesterol and sphingomyelin. Another possibility is the alteration of the intrinsic properties of Orai1 channel in cancer cells due to mutations or posttranslational modifications. Recently the V102C mutation in the Orai1 gene was found to transform Orai1 into a non-selective cation channel constitutively active even in the absence of STIM1 (McNally et al. 2012). Future studies are needed to identify the mechanism of basal Orai1 activation in cancer cells.

The Orai1 channel associates with the SK3 channel in MDA-MB-435s cells, as previously mentioned, but also with other potassium channels including the Human Ether-a-go-go potassium Channel 1 (hEag1) and the big conductance Ca^{2+} activated K^+ channel, BKCa (Chen et al. 2016; Hammadi et al. 2013). The hEag1 channel regulates breast cancer cell migration through an Orai1-dependent Ca^{2+} entry (Hammadi et al. 2013). This complex has been also reported in invaded lymph nodes where a high hEAG1 level was associated with high Orai1 level expression (Hammadi et al. 2013). However, the nature of the Ca^{2+} entry and its role in regulating basal $[\text{Ca}^{2+}]_c$ remains to be explored in these cells. Indeed, and by analogy to observations with the SK3 channel, hEag1 would favor Ca^{2+} entry through hyperpolarization, but it is suspected that Ca^{2+} would in turn inhibit hEag1 (Ouadid-Ahidouch et al. 2016) and thus reduce Ca^{2+} entry. Thus, a role of this complex in a sustained constitutive Ca^{2+} entry is unlikely in this model.

BKCa activity is also increased by membrane depolarization and by intracellular Ca^{2+} concentration that amplifies Ca^{2+} dependent potassium channel activity at concentrations higher than 100 nM. In smooth muscle cells, BKCa regulates the resting membrane potential through its activation by spontaneous Ca^{2+} release from the peripheral sarcoplasmic reticulum (Benham and Bolton 1986; Lee and Earm 1994; Vandier et al. 1998). By hyperpolarizing the plasma membrane this channel plays a negative feedback controller role in excitation-

contraction coupling by limiting smooth muscle cell depolarization and contractions (Brayden and Nelson 1992). Recently, Orai1 channel was found to form a physical complex with BKCa channels in mesenteric smooth muscle cells. Store operated Ca^{2+} influx supported by Orai1 stimulates BKCa leading to membrane hyperpolarization (Chen et al. 2016). A similar role for the Orai1-SK3 complex has been also postulated by the same group in gallbladder smooth muscle (Song et al. 2015). However, a role of Orai1-SK3 and Orai1-BKCa complexes in a constitutive smooth muscle Ca^{2+} entry was not proposed.

BKCa also form a complex with the voltage-gated Ca^{2+} channel Cav3.2 (Gackiere et al. 2013). This complex controls the proliferation of LNCaP prostate cancer cells by regulating a constitutive Ca^{2+} entry (Gackiere et al. 2013). The BKCa-Cav3.2 complex maintains membrane potentials within a narrow window allowing a fraction of Cav3.2 to be activated but not inactivated. This voltage gated Ca^{2+} current window allows a constitutive Ca^{2+} influx in cells with activated BKCa channels that in turn regulate the membrane potential.

2.1.2. Orai1 complexes with SPCA

The Secretory Pathway Ca^{2+} -ATPase (SPCAs) not only carry Ca^{2+} , but also Mn^{2+} with submicromolar affinity into the secretory pathway, using the energy of ATP hydrolysis (Durr et al. 1998; Sorin et al. 1997). Two isoforms of SPCAs, SPCA1 and SPCA2, have been identified in humans (Vanoevelen et al. 2005; Xiang et al. 2005). Whereas SPCA1 is ubiquitously expressed, SPCA2 expression is limited to highly secretory or absorptive epithelia such as salivary and mammary glands, intestinal tract or lung (Vanoevelen et al. 2005) where SPCA2 is correlated with highly active Ca^{2+} absorbance and secretion. Thus, during mid-pregnancy, upon parturition and even through lactation, SPCA2 expression increased by 35-fold whereas SPCA1 expression showed a modest 2-fold induction (Faddy et

al. 2008). Orai1 expression is also up regulated in mammary gland tissue samples from mice at lactation (McAndrew et al. 2011). Orai1 appears to be, *in vitro*, a major contributor of the enhanced basal Ca^{2+} influx observed in mammary gland epithelial cells from a lactating host independently of STIM1. Silencing of STIM2 had a modest effect on this Ca^{2+} influx (Ross et al. 2013). In 2013, Cross *et al.*, showed that SPCA2 and Orai1 regulate together a store-independent Ca^{2+} entry, which mediates the massive basolateral Ca^{2+} influx into mammary epithelia needed to support the large Ca^{2+} transport requirements for milk secretion. Furthermore, SPCA2 is required for plasma membrane trafficking of Orai. A membrane-anchored C-terminal domain of SPCA2 is sufficient to address Orai to the plasma membrane (Cross et al. 2013). The SPCA2 N-terminus interacts directly with both the N- and C-terminal domains of Orai1. This interaction appears to induce the exposure of the SPCA2 C-terminal activating domain (Feng et al. 2010).

Feng *et al.*, also showed that SPCA2 is up-regulated in breast cancer tissues and elicits a constitutive Ca^{2+} entry mediated by Orai1, which correlates with oncogenic activities of mammary tumor cells. This Ca^{2+} influx is independent from endoplasmic reticulum Ca^{2+} stores release and downstream Ca^{2+} signaling pathway. Knockdown of STIM protein (STIM1 and STIM2) or mutational inactivation of the ATPase activity of SPCA2 does not affect constitutive Ca^{2+} influx (Feng et al. 2010).

Taken together, these reports showed a new function of SPCA2 as a regulator of a constitutive Orai1 dependent Ca^{2+} influx involved in physiological and pathological processes (Fig. 2). Nonetheless, many questions still remain to be answered. How is SPCA2 targeted to the plasma membrane? Is there a specific localization of SPCA2 and Orai1 in common nanodomain, which could explain the specific role of this complex in mammary tumors?

2.2. STIM regulation of basal Ca²⁺ signaling.

Long-term control of ER Ca²⁺ concentration is a function of cytosolic Ca²⁺ and is controlled by the balance between Ca²⁺ pumping into the ER and Ca²⁺ flux out of the ER. In order to prevent harmful changes in ER Ca²⁺ concentration, a link between ER loading level and a basal Ca²⁺ influx may be necessary to maintain the cytosolic and ER Ca²⁺ homeostasis. One intriguing question is the nature of this basal Ca²⁺ influx. Considering that basal Ca²⁺ influx may be dependent on the level of ER Ca²⁺ store content, this basal Ca²⁺ entry may consist in a so-called store operated entry. This basal store regulated entry (B-SOCE) would be therefore active in the absence of external receptor stimulation and so opposite to the well described receptor-triggered store operated Ca²⁺ influx (R-SOCE).

Meyer and colleagues identified first STIM2 as a key actor of basal intracellular Ca²⁺ levels maintenance (Brandman et al. 2007). This transmembrane protein STIM2 is ubiquitously expressed among human and murine tissues at different degrees and shares 47% homology with STIM1, the central player in the signaling pathway linking receptor-mediated release of ER Ca²⁺ to SOCE (Dziadek and Johnstone 2007; Liou et al. 2005; Lopez et al. 2012). STIM2 is located at membranes of the ER and acidic stores and, in contrast to STIM1, cannot be detected on the cell surface (Lopez et al. 2012; Soboloff et al. 2006).

Since Meyer's group work, a large body of evidence supporting the function of STIM2 as a major regulator of basal [Ca²⁺]_c and ER ([Ca²⁺]_{ER}) concentrations has been published. Consistent with this role, decreasing STIM2 expression reduces basal and ER Ca²⁺ levels while overexpressing STIM2 increases it in many different cellular models (HeLa, HUVEC, HEK293T cells, naive CD4⁺ T cells, breast cancer cells, myoblasts and murine neuronal cells) (Berna-Erro et al. 2009; Bird et al. 2009; Brandman et al. 2007; Darbellay et al. 2010;

McAndrew et al. 2011; Miederer et al. 2015; Rana et al. 2015). In contrast, STIM1 seems to play only a minor role in $[Ca^{2+}]_c$ and $[Ca^{2+}]_{ER}$ control.

Even with a lower ER Ca^{2+} sensitivity than STIM1, STIM2 can sense ER Ca^{2+} levels through its luminal EF-hand and trigger plasma membrane Ca^{2+} influx using a common regulatory mechanism with STIM1 that involves Ca^{2+} dissociation from their EF-Hand, oligomerization, and translocation to ER-PM junction sites (Brandman et al. 2007; Hoth and Niemeyer 2013; Lopez et al. 2012; Soboloff et al. 2012; Stathopoulos et al. 2009). However, unlike STIM1, STIM2 translocates to ER-PM junctions with only small decreases in ER Ca^{2+} concentration. STIM2 partial activation at basal ER Ca^{2+} levels would explain why STIM2 but not STIM1 serves as a regulator of basal Ca^{2+} homeostasis. Due to their different Ca^{2+} affinities, STIM1 requires much larger receptor-triggered reductions in ER Ca^{2+} to be activated than STIM2 (Parvez et al. 2008; Stathopoulos et al. 2009). The two-fold lower Ca^{2+} sensitivity of the STIM2 EF-Hand compared to STIM1 results in a weaker inhibition of STIM2 by resting ER Ca^{2+} levels (Stathopoulos et al. 2009). In resting conditions, most copies of STIM2 are proposed to be constitutively localized at ER/PM junctions and coupled to Orai1 in a store-independent manner, with a smaller fraction of STIM2 molecules remaining available to activate SOC channels after store depletion (remaining store coupled) (Brandman et al. 2007; Gruszczynska-Biegala and Kuznicki 2013; Parvez et al. 2008; Rana et al. 2015; Stathopoulos et al. 2009).

Up to now, among the different Orai isoforms, only Orai1 regulation by STIM2 has been clearly established (Brandman et al. 2007; Rana et al. 2015; Stanisiz et al. 2014). In order to activate Ca^{2+} channels, STIM proteins need to contact plasma membrane phosphoinositides via their cytosolic lysine (K)-rich domains. Consistent with a lower activation threshold and a function as a regulator of basal Ca^{2+} levels, the C-terminal domain of STIM2 has a higher affinity to PI(4,5)P₂-containing liposomes than STIM1. This higher affinity for PM lipids may

contribute to the constitutive localization of STIM2 at ER/PM junctions (Bhardwaj et al. 2013).

As mentioned above, the majority of the STIM2 population resides on the ER membrane. However, a second population (2 to 10%) of full-length pre-protein (preSTIM2) with its signal peptide intact has also been described (Graham et al. 2011). This preSTIM2 seems to escape ER targeting and to localize to the inner leaflet of the plasma membrane where it interacts with Orai1 in a store-independent manner to regulate basal Ca^{2+} influx, basal Ca^{2+} concentration and Ca^{2+} -dependent gene transcription (Graham et al. 2011). However, this hypothesis of a Ca^{2+} constitutive entry mediated by non-ER-localized pre-STIM2 as postulated by Graham *et al.* (2011) is somehow challenged by the increase in basal Ca^{2+} concentration observed after STIM2 expression in HEK cells independently of the nature of its signal peptide (short STIM1 or long STIM2) (Miederer et al. 2015). In some cells, a decrease in STIM2 expression only alters SOCE without affecting basal $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_{ER}$, suggesting that STIM2 might regulate or not the different aspects of Ca^{2+} homeostasis depending on the cell type (Berna-Erro et al. 2009; Schuhmann et al. 2010). The expression ratio between STIM isoforms seems also to determine whether STIM2 controls SOCE, basal $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_{ER}$, or just the latter two. However, regulation of basal $[\text{Ca}^{2+}]_c$ seems to be an exclusive function of STIM2.

Since STIM1 and STIM2 are able to form hetero-oligomers (Dziadek and Johnstone 2007; Soboloff et al. 2006; Stathopoulos et al. 2009; Williams et al. 2001), it is plausible that they function as a complex and that STIM2 requires STIM1 to signal to Orai1 channels for both B-SOC and R-SOC type Ca^{2+} influx. However, STIM1 and STIM2 may also act synergistically only if both are activated following Ca^{2+} stores are depleted by strong receptor stimuli whereas STIM2 may function independently of STIM1 for basal ER Ca^{2+} levels or weak receptor stimuli.

Conflicting results have also been published on a possible role of STIM2 in negatively (Soboloff et al. 2006) or positively regulating store-operated Ca^{2+} influx (R-SOCE) (Liou et al. 2005; Soboloff et al. 2006; Stanisiz et al. 2014); (Kar et al. 2012; Liou et al. 2005; Miederer et al. 2015; Oh-Hora et al. 2008) (Kraft 2015); (Kar et al. 2012; Miederer et al. 2015; Oh-Hora et al. 2008; Thiel et al. 2013). The description of STIM2 as a SOCE inhibitor may result from an artifact of STIM2 overexpression in the presence of a limiting amount of Orai1 (Bird et al. 2009; Parvez et al. 2008; Rana et al. 2015). These divergent results may also be attributed to the inhibition of STIM2-Orai1 coupling by cytoplasmic calmodulin depending on the level of calmodulin activity in resting conditions or following cell stimulation (Parvez et al. 2008). A study from Parekh and colleagues may somehow reconcile these contradictory findings by demonstrating that different agonists sustain cytoplasmic Ca^{2+} signals and gene expression through activation of different STIM proteins (Kar et al. 2012).

Important for the interpretation of STIM2 effects on B-SOCE or R-SOCE is the recent identification of STIM2 β (also name *STIM2.1*), a ubiquitous and highly conserved alternatively spliced isoform of STIM2 (STIM2 α , *STIM2.2*) (Miederer et al. 2015; Rana et al. 2015). In contrast to STIM2 α , STIM2 β negatively regulates the resting $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_{ER}$ in the absence of stimulation. However, as observed for STIM2 α , STIM2 β is constitutively activated and forms puncta in resting cells even without store depletion. Among all known STIM isoforms, STIM2 β displays a unique role as an inhibitor of SOCE through a sequence-specific allosteric interaction with Orai1. Further studies will be needed to decipher the involvement of STIM2 β in basal constitutive Ca^{2+} influx and homeostasis in normal and pathological conditions.

Dysregulation of STIM2 regulated Ca^{2+} entries will theoretically expose cells to the risk of Ca^{2+} overload or to significant decrease in basal $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_{ER}$ leading to potentially

harmful cell states and disruption of important Ca^{2+} dependent cellular functions as observed in cancer cells. Controversial roles for STIM2 as both a tumor suppressor and as a potential oncogene have been postulated. For example, STIM2 expression is enhanced in glioblastoma multiforme tumors (Ruano et al. 2006), in human melanoma (Stanisz et al. 2014), in colorectal cancer (Aytes et al. 2012), in prostate cancer (Ashida et al. 2012) and in esophageal squamous cell carcinoma (ESCC) (Zhu et al. 2014). High STIM2 expression decreases Ca^{2+} -dependent proliferation in colorectal cancer (Aytes et al. 2012) and in the melanoma cell line SK-Mel-5 (Stanisz et al. 2014). At the opposite, an increase in Orai1 and a decrease in STIM2 expression are critical for tumorigenesis in a model of colon cancer (Sobradillo et al. 2014). In melanoma cells, higher levels of both Orai1 and STIM2 lead to increased basal $[\text{Ca}^{2+}]_c$ and consequently to higher invasive potential, while reduction in their expression levels decreases basal $[\text{Ca}^{2+}]_c$ and causes enhanced melanoma growth.

Further studies are certainly needed to better identify the molecular nature of STIM2 regulated Ca^{2+} entries and the exact role of STIM2 in basal Ca^{2+} homeostasis and signaling dysregulations. Most published studies so far have concluded that STIM2-regulated Ca^{2+} channels consist of a unique molecular identify (Orai1) involved in SOCE supporting both B-SOCE and R-SOCE. However, many questions about STIM2 regulation of Orai2 or 3 and TRPCs channels are still waiting to be answered: for example, no one has yet considered the possible implication of STIM2 in the regulation of store independent Ca^{2+} channels like ARC channels (Shuttleworth et al. 2004, 2007).

Mechanisms controlling the specific participation of STIM2 in SOCE and in the regulation of basal $[\text{Ca}^{2+}]_c$ or $[\text{Ca}^{2+}]_{ER}$ have also to be better deciphered. A recent study raised the possibility that an ER-resident membrane protein, TMEM110, may cooperate with STIM2 to regulate the long-term maintenance of ER-plasma membrane junctions and the short-term physiological remodeling of the junctions during store-dependent Ca^{2+} signaling (Quintana et

al. 2015). Binding of Ca^{2+} /CaM to the K-rich domain of STIM2 contributes to the regulation of STIM2-mediated Ca^{2+} influx (Bauer et al. 2008).

Specific functions of a constitutive Ca^{2+} entry controlled by STIM2 still need to be clearly identified. Interestingly, in cells insensitive to STIM2 silencing for R-SOCE, the absence of STIM2 results in a significant reduction of interferon gamma and interleukin secretion suggesting the possible involvement in gene expression of a STIM2 regulated Ca^{2+} entry not dependent on STIM2-mediated SOCE or only dependent on STIM2-B-SOCE (Oh-Hora et al. 2008). Changes in basal Ca^{2+} concentration and Ca^{2+} influx consequent to changes in STIM2 expression may explain altered Ca^{2+} dependent transcription signaling observed in different pathological situations such as cancer (Muller and Rao 2010).

In conclusion, the working model is that STIM2 plays a housekeeping role serving as the primary positive regulator for basal Ca^{2+} influx (B-SOCE) that regulates $[\text{Ca}^{2+}]_c$ or $[\text{Ca}^{2+}]_{ER}$ at resting conditions or in response to the limited release of ER Ca^{2+} stores (Fig. 3). There is some consensus that STIM2-mediated regulation of Ca^{2+} entry may represent an important mechanism for the long-term Ca^{2+} homeostasis maintenance in cells. It is however quite clear that the molecular mechanisms underlying STIM2-regulated constitutive Ca^{2+} entry need further characterization. Up to now, STIM1 has not been associated with the regulation of constitutive or basal Ca^{2+} entry, but this is not surprising considering the Ca^{2+} sensitivity of STIM1.

2.3. TRPs: a large family of channels contributing to various Ca^{2+} entries

Transient receptor potential (TRP) channels are a large group of non-selective cation channels ubiquitously distributed in non-excitabile and excitable cells. The TRP proteins are sensitive to a remarkable range of stimuli through a large diversity of activation mechanisms. As a

consequence, TRP subunits have been shown to contribute to Receptor-Operated Ca^{2+} Entries (ROCE), SOCE but also to constitutive Ca^{2+} entries, which enable them to participate in various physiological and pathological conditions. Many studies have linked specific TRP channels to cancer progression (Nielsen et al. 2014).

2.3.1 TRPCs

All mammalian TRPCs build non-selective Ca^{2+} -permeable cation channels that demonstrate variable $\text{Ca}^{2+}/\text{Na}^+$ permeability ratio, and support relatively non-selective cation currents (Owsianik et al. 2006). Upon activation of transmembrane receptors by extracellular ligand, stimulation of PLC and production of Diacylglycerol (DAG) activates or potentiates TRPC-dependent current and Ca^{2+} entry, referred as receptor-operated Ca^{2+} entry (ROCE). Evidence is also accumulating that TRPC proteins are important components of SOCs in both excitable and non-excitable cells (Smani et al. 2015).

Nonetheless, recent reports showed that TRPC channels might play a role in basal constitutive Ca^{2+} entry. One first evidence of a basal constitutive activity of TRPC channels was provided by the study of Vandebrouck and collaborators in skeletal muscle (Vandebrouck et al. 2002). Using patch clamping in a cell-attached configuration, voltage-independent Ca^{2+} channels were recorded at the sarcolemma of mouse fibres. These channels were recorded at rest in normal fibres or in dystrophic fibres and displayed a low conductance of 8 pS (with 110 mM CaCl_2 in the pipette). However, the inward currents were recorded with a greater occurrence in fibers from *mdx* dystrophic mice. Using an antisense strategy directed against the TRP box, a motif conserved among TRPC members, this study showed that when TRPC1, TRPC4 and TRPC6 channels were decreased at the protein level, the occurrence of channel activity was drastically lowered in both normal and dystrophic fibres. This demonstrated that the sarcolemma of isolated muscle fibers displays TRPC-dependent basal Ca^{2+} currents. The

probability of the channels being open was increased by treatment with thapsigargin and caffeine, suggesting that these channels could also be store-operated.

Interestingly, the absence of Homer-1, a scaffolding protein, was also suggested to promote a constitutive activity of TRPC1 channels in skeletal muscle sarcolemma (Stiber et al. 2008). In accordance with the presence of a Homer-binding motif at the C-terminal and N-terminal domains of TRPC1, this channel could also be co-immunoprecipitated with endogenous Homer protein from mouse gastrocnemius muscle protein lysates. Homer-1 was found to be the predominant Homer isoform in skeletal muscle, and Homer-1 knock out (KO) mice exhibited a myopathy (Stiber et al. 2008). Interestingly, a drastic increase in a spontaneous barium influx was observed in Homer-1 KO myotubes, which could be blocked by forced expression of Homer-1b and also by transfection of a shRNA construct silencing TRPC1. This strongly supported the idea that dissociation of Homer-1 from TRPC1 channels induces an abnormal constitutive Ca^{2+} entry leading to Ca^{2+} mishandling in fibers. Because the outwardly rectifying current observed in Homer KO myotubes could be blocked by the GsMTx4 peptide, the authors suggested that changes in the stretch-activated TRPC1 channels might lead to its constitutive activity.

TRPC1 interacts with the scaffolding protein Homer, through two Homer-binding sites (PPXXF or PXXF) in a proline-rich motif (LPXPFX~~XXX~~PSPK), downstream of the TRP domain (EWKFAR). Homer expression was found to be crucial for mediating a TRPC1-IP₃R complex necessary for responses to G-protein-coupled receptor activation (Yuan et al. 2003). On the contrary, expression of TRPC1 with mutation of the proline-rich motif disrupted Homer binding and resulted interestingly in a constitutive activity of TRPC1 channels with reduced agonist regulation. Homer was thus proposed to permit the assembly of an agonist

responsive TRPC1-IP₃R complex. To further demonstrate the role of Homer in native cells, Ca²⁺ influx were measured in acini from Homer-1 KO mice (Yuan et al. 2003). Deletion of Homer-1 resulted in increased spontaneous Ca²⁺ entry into pancreatic acinar cells. This study also strongly supported the idea that the dissociation of Homer from TRPC1 induces TRPC1-dependent constitutive entry. These data imply that the right combination of TRPC proteins and accessory proteins must be assembled to form channels that are not constitutively active but are responsive to agonist. Interestingly the association of TRPC1 with other TRPC proteins also controls constitutive cation entry through TRPC1 in HEK-293 cells as shown by the effect of TRPC siRNA on Ba²⁺ leak influx (Zagranichnaya et al. 2005). Suppression of either TRPC3 or TRPC7 results in a high Ba²⁺ leak influx, and TRPC1 silencing dramatically reduces this Ba²⁺ leak influx, whereas the suppression of TRPC1 alone has no effect on the Ba²⁺ leak entry. This strongly suggested that TRPC1 homomeric channels were supporting a constitutive cation entry in HEK-293 whereas it is inhibited when TRPC1 was associated in heteromeric channels with TRPC3 and TRPC7.

TRPC proteins were also shown to support constitutive Ca²⁺ entry through the plasma membrane of cardiomyocytes. A so called background Ca²⁺ entry (BGCE) pathway was recorded in beating adult ventricular cardiomyocytes using the Mn²⁺ Fura2-quench assay (Camacho Londono et al. 2015). The analysis of multiple KO mice showed that this basal Ca²⁺ entry depends on TRPC1/C4 proteins but not on other TRPC proteins such as TRPC3/C6. The constitutively active TRPC1/C4-dependent BGCE affects both diastolic and systolic Ca²⁺ concentrations, in basal or stimulated conditions, as well as expression of genes regulated by Ca²⁺-dependent signaling.

In isolated synaptosomes, Nichols *et al.* (2007), demonstrated in presynaptic terminals the existence of a Ca^{2+} constitutive influx independent of voltage-gated Ca^{2+} channels and $\text{Na}^+/\text{Ca}^{2+}$ exchanger activities. The presynaptic voltage-independent Ca^{2+} influx could be revealed after re-addition of Ca^{2+} following depletion of extracellular Ca^{2+} and a divalent cation entry was also observed by using Mn^{2+} in a typical Fura-2 quench assay. This presynaptic constitutive Ca^{2+} influx (Nichols *et al.* 2007) was blocked by SKF9636 and attenuated by shRNA against TRPC5 or TRPC1 (but not TRPC3). The constitutive Ca^{2+} pathway could serve to sustain synaptic function under widely varying levels of synaptic activity by maintaining Ca^{2+} stores in mitochondria. A constitutive Ca^{2+} entry was also observed in human follicular thyroid ML-1 cancer cells, when Ca^{2+} was re-added to cells previously maintained in EGTA buffer solution. This constitutive influx was attenuated in TRPC1 knockdown (KD) thyroid cancer cells as well as a S1P-evoked Ca^{2+} entry after re-addition of external Ca^{2+} (Asghar *et al.* 2015). The secretion and activity of MMP2 and MMP9 were attenuated, and proliferation was decreased in TRPC1-KD cells. TRPC1 silencing prolonged G1 phase of the cell cycle, and led to a significant increase in the expression of the cyclin-dependent kinase inhibitors p21 and p27, and a decrease in the expression of cyclin D2, cyclin D3, and CDK6. Transfecting TRPC1 into TRPC1 KD cells rescued migration, and proliferation, suggesting a critical role of TRPC1-dependent basal Ca^{2+} influx (Asghar *et al.* 2015).

Constitutive activity has been also described for mouse TRPC5 (Yamada *et al.* 2000). TRPC5-transfected HEK293 cells showed single-channel activities at a holding potential (V_h) of -50 mV, whereas no control HEK293 cells had measurable single-channel activities indicating that spontaneous single-channel openings in the transfected HEK293 cells result from TRPC5 channel activity. In contrast, only constitutive activity was observed in HEK293

cells expressing the human TRPC5 (Zeng et al. 2004). Interestingly, the resting Ca^{2+} permeabilities of HSG cells line (human submandibular gland cell line) are similar in cell expressing or not the human TRPC1. This was determined by the re-addition of 1.0 mM Ca^{2+} to cells in a Ca^{2+} free medium (Singh et al. 2000).

TRPC2 mediates both receptor operated Ca^{2+} entry and SOCE (Chu et al. 2002; Jungnickel et al. 2001) in mammals, but is a pseudogene in humans (Wes et al. 1995). In rat thyroid cells, TRPC2 functions as a receptor-operated Ca^{2+} channel. In addition, Sukumaran *et al.* (2012), suggested that TRPC2 could negatively regulate basal Ca^{2+} entry. They proposed that TRPC2 could participate in the regulation of ER Ca^{2+} content by modulating PKC expression and SERCA (SarcoEndoplasmic Reticulum Ca^{2+} transport ATPase) activity (Sukumaran et al. 2012). Indeed, SERCA activity was decreased in shTRPC2 cells or following the down-regulation of PKC δ . This, in turn, induced STIM2 puncta formation and increased basal Ca^{2+} entry. As mentioned previously, recent investigation suggested that basal Ca^{2+} influx might be driven by the ER Ca^{2+} sensor whereas STIM1 is important for SOCE. Nonetheless silencing of either STIM1 or STIM2 potently attenuated Ca^{2+} entry in shTRPC2 cells but not in thyroid control cells suggesting a potential role of STIM1 in regulation of Ca^{2+} entry (Sukumaran et al. 2012). Consistent with the constitutive Ca^{2+} entry in HCG cells, STIM1D76A (mutation in the EF hand domain that renders it insensitive to ER- Ca^{2+}), showed relatively high association with TRPC1 even in unstimulated cells. Furthermore, a significant fraction of STIM1D76A was found to be raft-associated in unstimulated cells and showed a high level of constitutive Ca^{2+} entry, which was significantly reduced upon treatment with the methyl- β -cyclodextrin (M β CD) that reduces membrane cholesterol levels. Additionally, STIM1D76A-TRPC1 interaction was disrupted in cells treated with M β CD (Pani et al. 2008).

Taken together, these reports suggest a new function of TRPC channels in basal constitutive Ca^{2+} entry without excluding the possibility that several different processes regulate this pathway including phosphorylation of TRPC, activation of TRPC by lipids and/or STIM protein. Several studies also strongly supported the idea that the dissociation of the scaffolding protein Homer from TRPC1 induces TRPC1-dependent constitutive entry. These features imply that the right combination of TRPC proteins and accessory proteins must be assembled to form channels that are not constitutively active but are responsive to agonist.

2.3.2 TRPVs

Within the TRPV subfamily, only two channel subunits have been shown to be physiologically open at rest but with somehow different mechanisms, namely TRPV2 and TRPV6. Through this constitutive activity *per nature*, they regulate resting Ca^{2+} levels and control physiological cellular responses but can also promote cancer progression.

The TRPV6 (and TRPV5) subunit is highly selective for Ca^{2+} ions with multiple layers of Ca^{2+} -dependent inactivation mechanisms (Bodding and Flockerzi 2004; Wissenbach and Niemeyer 2007). As TRPV6 is a truly constitutively active channel, its function is directly correlated to its expression, which is tightly controlled. In normal tissues, TRPV6 is expressed with TRPV5 at the apical membrane of Ca^{2+} transporting epithelia where they play the role of Ca^{2+} entry channels in the first step of transcellular Ca^{2+} transport pathways (*e.g.* intestinal absorption, renal reabsorption of Ca^{2+} ...) (van Abel et al. 2005). As opposed to TRPV5, TRPV6 is found expressed in a broader variety of tissues. In skin, a natural Ca^{2+} gradient exists which spans from the stratum granulosum (high Ca^{2+}) to the basal layer (low Ca^{2+}) of the epidermis (Tsutsumi et al. 2009). This gradient is essential to prevent premature differentiation of keratinocytes (Hennings et al. 1980). When keratinocytes move from the

basal layer toward the stratum granulosum, increased extracellular Ca^{2+} concentration induces TRPV6 expression. The resulting TRPV6-dependent constitutive Ca^{2+} entry triggers in return the expression of differentiation markers allowing keratinocyte differentiation (Lehen'kyi et al. 2007).

Abnormal expression of TRPV6 has been linked to the progression of malignant diseases such as hormone-dependent breast cancers, colon carcinoma cells and prostate cancer (Liberati et al. 2013). For example, TRPV6 expression is not detected in the healthy prostate but appears in prostate cancer (PCa). TRPV6 expression level correlates with the grade of the tumor (Gleason grading), the highest TRPV6 transcripts levels being found in lymph node metastasis of prostate origin (Peng et al. 2001; Wissenbach et al. 2001). Further studies have revealed that this *de novo* expression of the TRPV6 Ca^{2+} channel increases PCa or other tumor cells survival by enhancing proliferation and conferring apoptosis resistance (Raphael et al. 2014). In PCa cells, TRPV6-dependent constitutive Ca^{2+} -entry promotes tumor progression at least in part by the transcription of NFAT-driven genes (Lehen'kyi et al. 2007). TRPV6 has thus been suggested to be an oncogene and has been proposed to be a potential diagnostic marker for tumor development, indicative of the degree of tumor aggression (Liberati et al. 2013).

The TRPV2 channel is not as selective for Ca^{2+} as TRPV6 but mediates cationic currents with higher divalent permeability (Peralvarez-Marin et al. 2013). In stably transfected cells, TRPV2 expression results in an outwardly-rectifying current that can be recorded at the resting membrane potential, and translate into an increased resting intracellular Ca^{2+} concentration (Penna et al. 2006). This indicates how TRPV2 has significant functional activity in resting cells and contributes to constitutive Ca^{2+} entry. Of note, TRPV2 activity

could be partly prevented by serum starvation in the same stably transfected cells (Penna et al. 2006). Thus, as opposed to the truly constitutively active TRPV6 subunit, TRPV2 “constitutive” activity seems rather more complex with different levels of regulation. TRPV2 was first identified (and named growth-factor-regulated channel (GRC)) based on the fact that it induces Ca^{2+} currents upon stimulation of cells by insulin-like growth factor 1 (IGF-1). Kanzaki and collaborators showed that IGF-1 regulates TRPV2 activity by promoting its dynamic translocation from the endosomal compartment to the PM through a phosphatidylinositol-3 kinase (PI3K)-dependent pathway (Kanzaki et al. 1999). Thus, TRPV2 activity was suggested to be modulated by regulated PM targeting of constitutively active channel rather than gating. TRPV2 dynamic trafficking was since described for a broader range of stimuli and seems to be the major regulatory mechanism accounting for TRPV2 activation in non-excitabile cells. Several studies have shown that growth factors like IGF-1 or platelet-derived growth factor (PDGF), hormones or chemokines such as the neuropeptide head activator or the chemotactic peptide fMLP, the exogenous agonist Cannabidiol or even mechanical stimulation, all promote membrane insertion of TRPV2 (Boels et al. 2001; Kojima and Nagasawa 2014; Nagasawa and Kojima 2015; Nagasawa et al. 2007). In addition, TRPV2 PM targeting seems also to be reinforced by Ca^{2+} entering the cells through the first channels inserted (Boels et al. 2001). But it is not clear yet whether the sole mechanism by which these stimuli up-regulate TRPV2 function is a dynamic and transient translocation of the TRPV2 channel from intracellular compartments to the PM as additional levels of regulation have been suggested and may also exist, including direct effects on TRPV2 gating. However these mechanisms are not well understood and still require further characterization (Cohen et al. 2013; Penna et al. 2006). Nevertheless, as some of the factors able to recruit/activate TRPV2 are present in serum, it is quite clear that in normal growth conditions, steady-state TRPV2-mediated Ca^{2+} influx regulates resting cytosolic Ca^{2+} levels. What is less

clear however are the precise physiological function(s) of such TRPV2-constitutive Ca^{2+} entry, TRPV2 being one of the least characterized members of the TRP family (Peralvarez-Marín et al. 2013). As suggested by IGF-1 activation, TRPV2-dependent intracellular increase of Ca^{2+} concentration occurs downstream of insulin/glucose signaling in pancreatic β -cells of the endocrine system, regulating in return insulin secretion and cell growth (Aoyagi et al. 2010; Hisanaga et al. 2009). TRPV2 is also highly expressed in immune-related tissues where it seems to play an important role in diverse aspects of the innate immune response such as mast cells degranulation or macrophage phagocytosis and cytokine secretion (for complete review see (Liberati et al. 2013)). It is however on the roles played by TRPV2 in carcinogenesis that the largest amount of data is currently available (for review see (Liberati et al. 2014)). Deregulation of TRPV2 expression has been reported in several solid cancers including prostate cancers, bladder cancers, hepatocellular carcinoma and esophageal squamous cell carcinoma (Santoni et al. 2011). TRPV2 was always found expressed at higher levels in samples from patients with metastatic disease than in solid primary tumors and associated with poor prognosis, consistent with a role in metastasis formation (Caprodossi et al. 2008; Monet et al. 2010; Zhou et al. 2014). TRPV2 characterization during prostate cancer progression best illustrates the tumorigenic potential of TRPV2. In prostate PC3 cells, translocation of TRPV2 to plasma membrane by serum, lysophospholipids or by adrenomedullin stimulation increases cell adhesion, migration and invasion (Monet et al. 2009; Oulidi et al. 2013). Knockdown experiments have demonstrated that TRPV2 affects prostate cancer cell aggressiveness and invasive capability by influencing basal intracellular Ca^{2+} levels and the induction of key proteases, namely matrix metalloproteinase-2 (MMP-2), MMP-9 and cathepsin B (Monet et al. 2010). The constitutive activity of TRPV2 is also critical for castration-resistant prostate cancer development and progression *in vivo*. Indeed, TRPV2 is required for the invasive properties of PC3 prostate tumors established in nude

mice xenografts. In patients, the progression of prostate cancer to the castration-resistant phenotype is characterized by a *de novo* expression of TRPV2, and higher levels of TRPV2 transcripts were found in metastatic cancers (stage M1) as compared to primary solid tumors (stage T2a and T2b) (Monet et al. 2010). Nevertheless, the mechanisms regulating TRPV2-dependent Ca^{2+} entries and the exact pathophysiological functions of this channel subunit require further studies to be fully elucidated.

Hence, TRPV6 and TRPV2 regulate basal cytosolic Ca^{2+} concentration through their constitutive activity, this without excluding the possibility of a modulation by several processes including phosphorylation or protein-protein interactions, like for other TRP. Of note, a constitutive activity has been described for some other TRPV members but only in pathological settings: Gain-of function mutation leading to constitutively open channels have been associated to human inherited diseases, including neuropathies and skeletal dysplasia for TRPV4 and Olmsted syndrome for TRPV3 (Landouere et al. 2010; Lin et al. 2012; Rock et al. 2008).

2.3.3. TRPM7 is involved in cancer cell fates through different mechanisms.

The transient receptor potential melastatin-related 7 (TRPM7) is an atypical cation channel fused with a serine/threonine kinase located at its C-terminus (Nadler et al. 2001; Runnels et al. 2001). Physiologically, TRPM7 is involved in cellular and systemic Mg^{2+} homeostasis (Ryazanova et al. 2010; Schmitz et al. 2003). Nevertheless, TRPM7 channels also conduct Ca^{2+} . For example, TRPM7 overexpression in HEK-293 cells induces Ca^{2+} -dependent m-calpain activation, cell rounding and detachment without Ca^{2+} overloading suggesting that TRPM7 regulates proteases through local Ca^{2+} influx (Su et al. 2006). Moreover, TRPM7 is coupled to $\text{Ins}(1,4,5)\text{P}_3\text{R}$ in human embryonic lung fibroblasts mediating high- Ca^{2+} microdomains and polarized migration induced by PDGF (Wei et al. 2009). However, recent

studies showed that TRPM7 also regulates polarized movements through cellular Mg^{2+} homeostasis (Liu et al. 2011; Su et al. 2011). These data suggest that TRPM7 is not involved in constitutive Ca^{2+} entry in non-cancer cells.

Aberrant expression of TRPM7 is found in numerous cancers including breast and pancreas (Dhennin-Duthille et al. 2014). Numerous studies showed that TRPM7 is involved in constitutive Ca^{2+} influx in cancer cell lines. Indeed, TRPM7 silencing induces manganese- quenching slope reduction as well as intracellular basal Ca^{2+} decrease in human breast cancer cell line MCF-7 that express estrogen receptor (Guilbert et al. 2009). Moreover, TRPM7 silencing also decreases MCF-7 proliferation in a Ca^{2+} -dependent manner. Similar results were found in retinoblastoma (Hanano et al. 2004), in head and neck squamous carcinoma (Jiang et al. 2007) and in neuroblastoma (Zhang et al. 2014) cell lines suggesting that TRPM7 channels promote constitutive Ca^{2+} uptake and regulate basal intracellular Ca^{2+} levels leading to sustained proliferation of cancer cells.

On the other hand, TRPM7 silencing has no effect on basal intracellular Ca^{2+} levels and in cell proliferation in the triple negative invasive breast cancer cell lines MDA-MB-231 and MDA-MB-435s (Guilbert et al. 2013). However, TRPM7 regulates invasive breast cancer cell lines migration through myosin heavy and light chain phosphorylation by the kinase domain. TRPM7 kinase domain is required for metastasis but not for breast cancer growth in mice xenografts (Middelbeek et al. 2012). Nevertheless, it has been shown that TRPM7 could regulate cell migration through Ca^{2+} entry in a model of stimulation or exogenous overexpression. Indeed, TRPM7 expression stimulation by bradykinin or low TRPM7 overexpression in NIE-115 mouse neuroblastoma cells allows Ca^{2+} influx and both Ca^{2+} - and kinase-dependent interactions with actomyosin cytoskeleton leading to cell spreading and migration (Clark et al. 2006). Moreover, TRPM7 stimulation by bradykinin also induces Ca^{2+} influx and human nasopharyngeal carcinoma cell migration (Chen et al. 2010). Importantly, it

has been shown recently that Ca^{2+} sparks through TRPM7 are dissociated from invadosome formation in neuroblastoma cells suggesting that TRPM7 regulates cancer cell invasion independently of Ca^{2+} influx (Visser et al. 2013). Interestingly, TRPM7 regulates pancreatic ductal adenocarcinoma cell migration by a Mg^{2+} - but not by a Ca^{2+} -dependent mechanism (Rybarczyk et al. 2012). Moreover, TRPM7 also regulates intracellular basal intracellular Mg^{2+} levels and constitutive cation influx in pancreatic ductal adenocarcinoma cells (Rybarczyk et al. 2012).

Taken together, these data indicate that TRPM7 regulates constitutive Ca^{2+} influx in non-invasive cancer cells leading to sustained proliferation (Fig. 4). While TRPM7 allows Ca^{2+} sparks in invasive cancer cells, the channel regulates metastatic processes (*i.e.* migration and invasion) by Ca^{2+} -independent mechanisms in endogenous system or without receptor stimulation. TRPM7 is part of a mechanosensory complex that translates mechanical forces into intracellular signaling. TRPM7 could trigger biochemical signals involved in metastatic processes through its kinase activity and/or through Mg^{2+} entry. To support this hypothesis, it has been shown that Mg^{2+} but not Ca^{2+} stimulates TRPM7 kinase domain activity (Matsushita et al. 2005; Ryazanova et al. 2004). Nevertheless, the understanding of TRPM7-dependent mechanisms that regulate cancer migration and invasion processes remains a challenge for future researches.

3. Multi-level regulation of constitutive Ca^{2+} entry by Orai and TRP channels.

Many studies have reported Orai1 and TRP channel contributions to receptor-induced Ca^{2+} entry (R-SOCE and/or ROCE). Based on the fact that Orai and TRPs coexist in many tissues, and that their activities are triggered in parallel following receptor stimulation, speculative models have been proposed as to how TRP-ROCE might affect Orai1 R-SOCE and *vice versa*. Saul et al. have very nicely detailed these models in a recent review (Saul et al. 2014).

Regarding constitutive Ca^{2+} entry, as extensively described in this review, the same considerations can be made. Therefore, it makes sense to speculate also about models explaining how TRP and Orai channels may interplay in the specific context of basal Ca^{2+} entry (Fig. 5) controlling normal or pathological steady-state basal calcium levels.

Model 1: *Activity of B-SOCE and TRPs contribute independently to constitutive Ca^{2+} entry.*

The ubiquitous Orai1 B-SOCE co-exists in certain cell types with other types of basal influx pathways. In PCa cells for example, high STIM2 expression might enhanced Orai1 B-SOCE (see section 2.2) but TRPV2 and TRPV6 are also present and active at rest (see section 2.3.2). In the simplest view, one can imagine that constitutive Ca^{2+} entry in these cells consist of the mere addition of all these different pathways.

It is however very unlikely that these Ca^{2+} entry pathways are acting in parallel without any interference between them considering the large body of evidence showing that it is often not the case in the context of stimulated Ca^{2+} entry.

Alternative models: *Reciprocal regulation of B-SOCE and TRP-dependent constitutive calcium entries*

All the channels participating in B-SOCE or TRP-dependent constitutive Ca^{2+} entries are often found localized in similar PM structures/sub-compartments or even linked to the same multiprotein complexes (Deliot and Constantin 2015). Thus, opening of one type of channel probably influence the activity of the neighbouring others. This functional cross-talk can be direct through protein-protein interaction induced allosteric modulations, or indirect, either by triggering Ca^{2+} -dependant feedback/trafficking mechanisms or by inducing local changes in

the driving force for Ca^{2+} entry. One can therefore speculate on two alternative models accounting for opposite types of reciprocal regulation.

Model 2: *TRPs basal activity decrease B-SOCE.*

While B-SOCE seems to play a housekeeping role in the maintenance of $[\text{Ca}^{2+}]_c$ at normal values in healthy resting cells, in cancer cells, additional constitutive Ca^{2+} entry pathways are often presents due for example to the *de novo* expression of TRPV2, TRPV6 and/or TRPM7 and tend to stably increase $[\text{Ca}^{2+}]_c$ (see sections 2.3.2&2.3.3). As a consequence, more free Ca^{2+} ions are available to maintain $[\text{Ca}^{2+}]_{ER}$ in resting conditions, reducing the probability of STIM2-regulated B-SOCE's contribution (see section 2.2). Additionally, by allowing a significant entry of monovalent cations, the presence of constitutively active channel subunits with low divalent/monovalent cation permeability such as TRPV2 and TRPCs, depolarizes the membrane potential to less negative values and thereby might reduce the driving force for Ca^{2+} entry through other channels at close proximity such as Orai1 or TRPV6. Note that as previously described, Ca^{2+} -activated K^+ channels could balance this phenomenon (see section 2.1.1). Heteromerization of TRP channels can also affect their physiological and biological properties, and for example, TRPC1 was shown to act as a negative regulator of TRPV6 by formation of heterocomplexes (Schindl et al. 2012).

Model 3: *B-SOCE amplifies TRPs activity.*

Conversely, B-SOCE might be required for TRPV-dependent constitutive Ca^{2+} entry. Supporting this hypothesis, it has been very recently shown that TRPV6 translocates to the PM via an Orai1/TRPC1-mediated Ca^{2+} /Annexin I/S100A11 pathway in PCa cells (Raphael et al. 2014). This feature could be also shared by TRPV2, as Ca^{2+} has been described as a factor fueling TRPV2 trafficking towards the PM (Boels et al. 2001) (see section 2.3.2).

Note that all these models of coordination are not mutually exclusive. In a single cell, basal calcium level is control by multimodal constitutive Ca^{2+} entries with actors of which, likely coordinate their action in a complex and tangled interplay.

4. Conclusion and future directions.

In conclusion, recent and accumulating evidence indicate that in addition to Ca^{2+} channels that are activated by a specific cell stimulus (SOCs, VOCs, SMOCs, ARCs) some Ca^{2+} channels could be active at rest, without applying any external stimulation, inducing constitutive Ca^{2+} entry. Nevertheless, additional research is required to determine the mechanisms underlying the constitutive activation of these Ca^{2+} channels and their regulation. Since these channels are activated mostly in pathological conditions, there is a need to identify the molecular roles of these channels in pathologies such as cancer. Future work will have to focus on the characterization of their role in tumor development (primary tumor and/or metastatic development) and on their clinical relevance. Many questions remain unanswered: What are the difference in signalplex responsible for Ca^{2+} constitutive entries between normal and tumor cells in term of molecular composition and regulation? Are these channels responsible for basal Ca^{2+} entry linked to particular membrane structures (e.g. lipid raft) associated with either malignant transformation or tumor microenvironment? Answering these questions will help to develop potent and specific inhibitors of these channels in order to design specific therapeutic approaches targeting tumoral cells.

Conflict of interest statement

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Measurements of intracellular Ca^{2+} concentration following removal (0 mM Ca) and addition (2 mM Ca) of external Ca^{2+} concentration in the SK3 expressing cells MDA-MB435s cells. The suppression of Ca^{2+} from external solution reversely decrease intracellular Ca^{2+} concentration.

Figure 2. Orai1 channel mediates constitutive Ca^{2+} entries in breast cancer and non-cancer cells. Left, in MDA-MB435s cells cancer cell line Orai1 channel associate with SK3 channel. SK3 channel hyperpolarize plasma membrane and increase the driving force for Ca^{2+} leading to constitutive Ca^{2+} entry through Orai1 channel. Right, Orai1 physically associate with SPCA2 and promotes a constitutive Ca^{2+} entry in breast cancer and non-cancer epithelial cells.

Figure 3. STIM2 mediates constitutive Ca^{2+} entry to regulate basal Ca^{2+} level in cancer and non-cancer cells.

STIM2 regulates basal Ca^{2+} influx even in the absence of external receptor stimulation and with only small decreases in ER Ca^{2+} concentration. **(A)** Meyer's group initially identified STIM2 as a positive regulator of basal store operated Ca^{2+} influx (B-SOCE) in a siRNA screening using a Dicer-generated siRNA library of the human signaling proteome. STIM2 α (*STIM 2.2*) senses very small ER Ca^{2+} depletion through its luminal EF-hand and trigger plasma membrane Ca^{2+} influx through Orai1. In basal conditions, STIM2 translocate to ER-PM junctions with only small decreases in ER Ca^{2+} concentration. At basal ER Ca^{2+} levels, most of the STIM2 molecules are constitutively localized at ER/PM junctions and coupled to Orai1 in a store-independent manner. **(B)** Graham *et al.* (2011) proposed that a full-length

pre-protein STIM2 (preSTIM2) with an intact signal peptide, escape ER targeting and interacts with Orai1 in a store-independent manner to regulate basal Ca^{2+} influx and $[\text{Ca}^{2+}]_c$. (C) Recently identified STIM2 β (*STIM2.1*), a spliced isoform of STIM2 α , negatively regulates the resting $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_{ER}$ through a sequence-specific allosteric interaction with Orai1. STIM2 β is also constitutively activated and formed puncta in resting cells even without store depletion.

Figure 4. TRPM7 is involved in cancer cell proliferation, migration and invasion through different mechanisms.

In cancer cells, TRPM7 knockdown experiments show that the channel regulates basal proliferation, migration and invasion without receptor stimulation and through different mechanisms. (A) TRPM7 regulates cell proliferation by promoting constitutive Ca^{2+} influx in some cancer types including retinoblastoma, head and neck, ER+ breast cancer and neuroblastoma. (B) TRPM7 regulates pancreatic ductal adenocarcinoma cell migration by Mg^{2+} entry. (C) TRPM7 regulates ER- breast cancer cell migration and invasion, and neuroblastoma cell invasion through its kinase domain. Constitutive Mg^{2+} entry through TRPM7 could enhance cancer cell invasion since kinase domain activation is stimulated by intracellular Mg^{2+} .

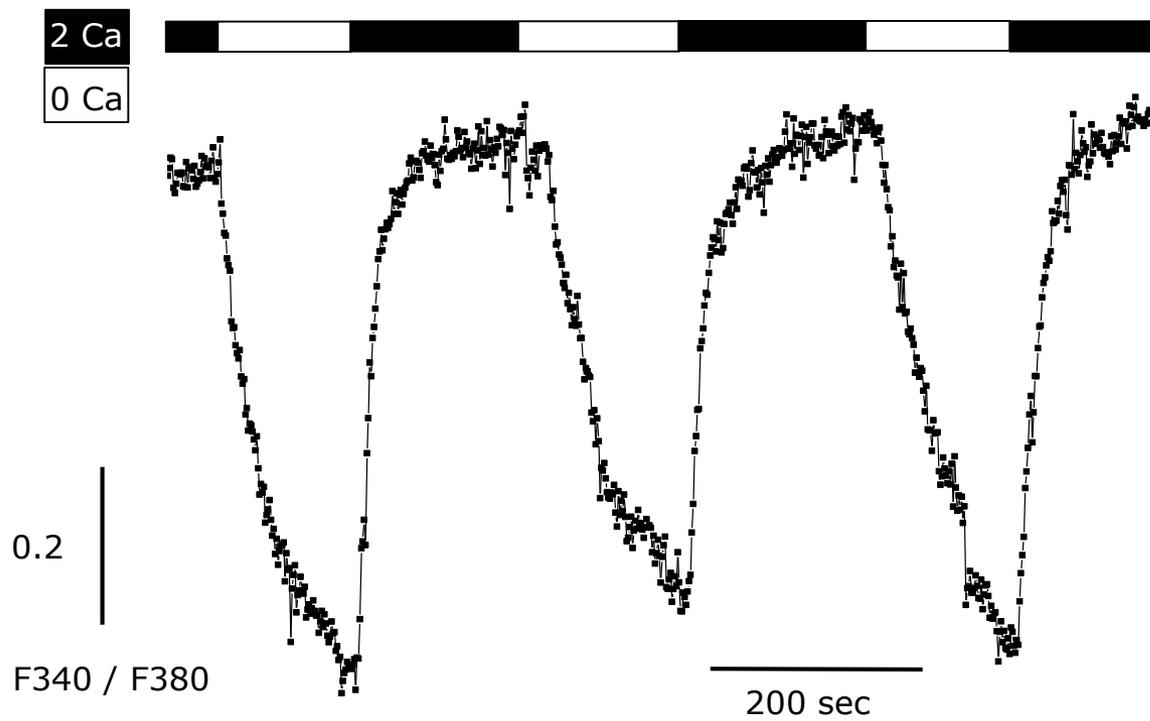
Figure 5. Orai and TRP multi-levels regulation of constitutive Ca^{2+} entry.

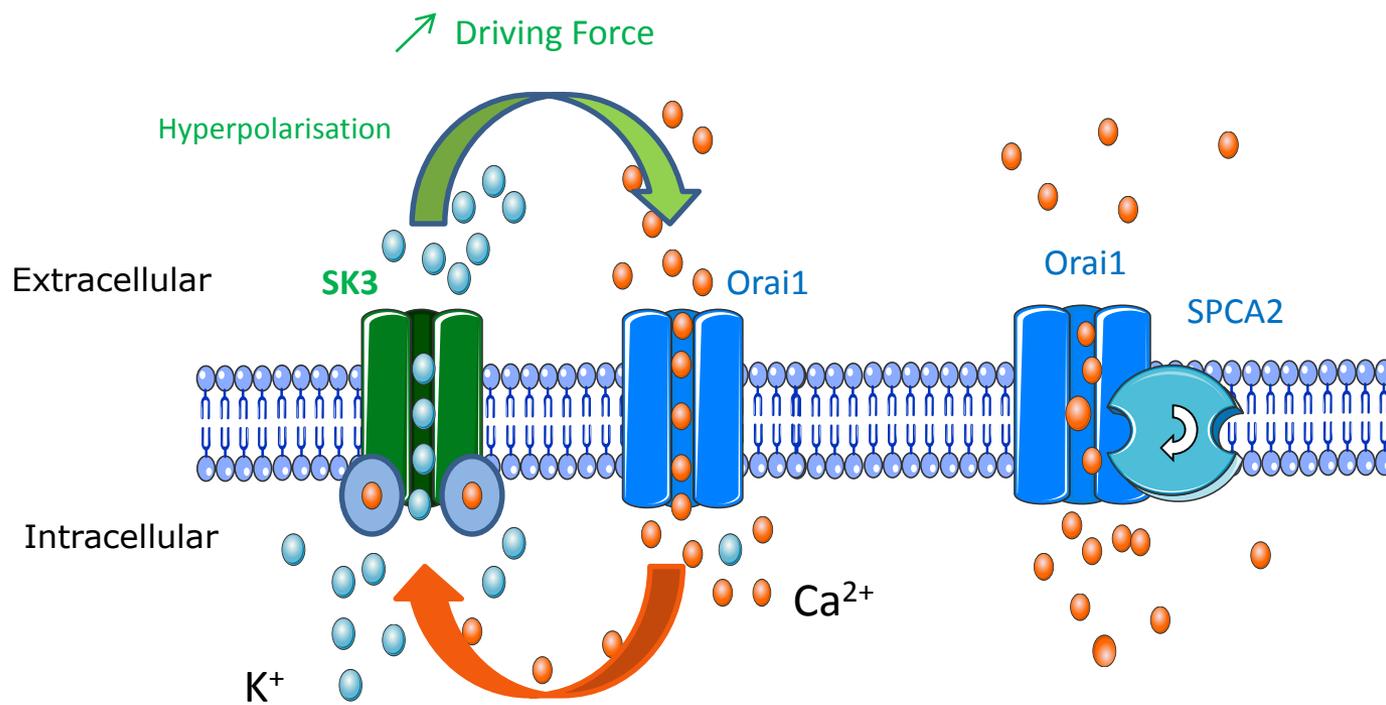
Different speculative models can be proposed for the regulation of constitutive Ca^{2+} entry by Orai and TRP channels. **In model 1**, B-SOCE (STIM2/Orai1) and constitutively active TRPs subunits contribute independently to constitutive Ca^{2+} entry, thus total constitutive Ca^{2+} entry can be viewed as the sum of the different pathways acting in parallel. Alternatively, **models 2 & 3** are based on a coordinated regulation of constitutive Ca^{2+} entry, taking into account possible functional cross-talks between B-SOCE and TRP-dependent pathways. **Model 2**

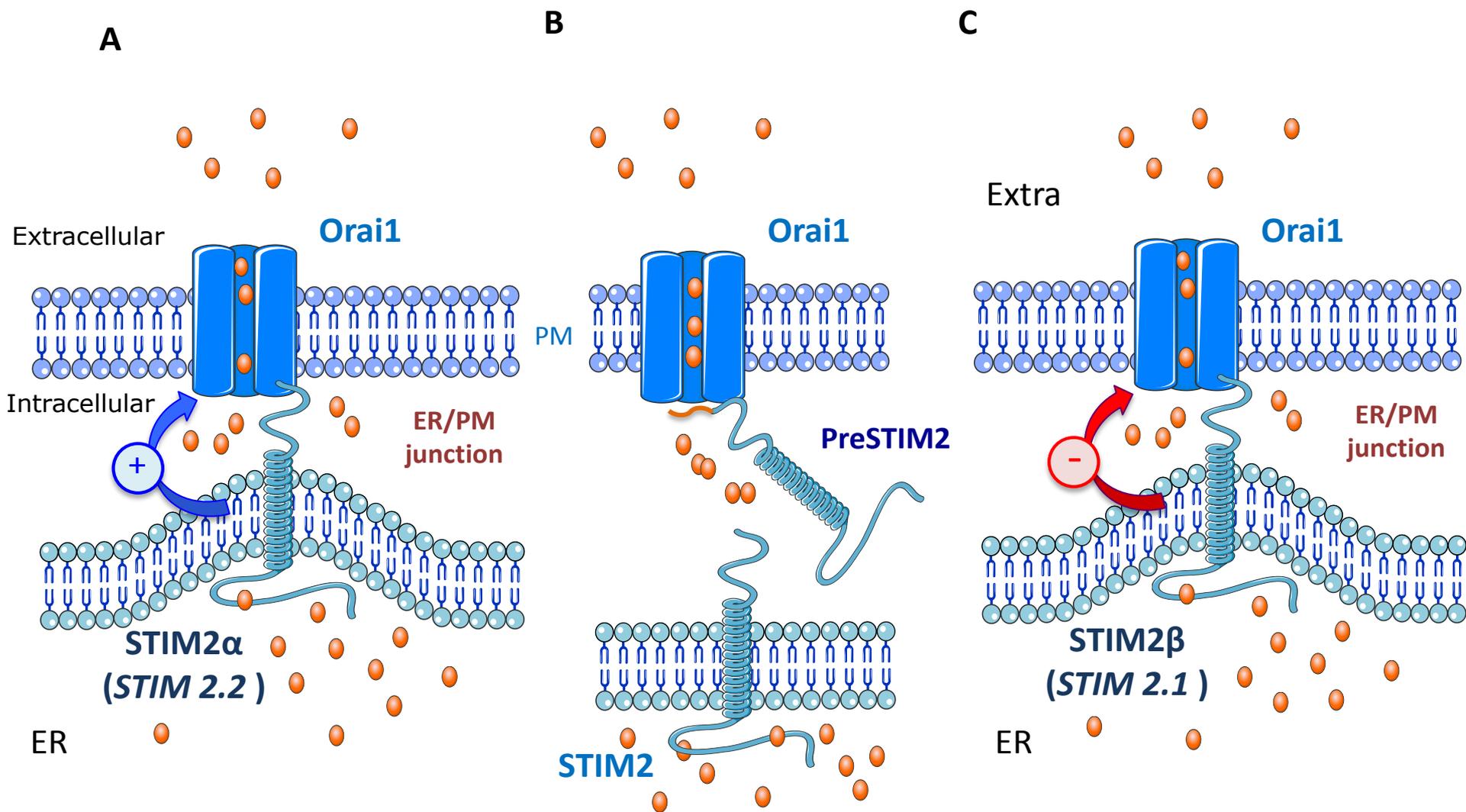
illustrates two possible negative feedback of TRPs constitutive activity on B-SOCE: 1) The stable increase in $[Ca^{2+}]_c$ observed cancer cells often results from the appearance of TRPVs-dependent constitutive Ca^{2+} entries. This elevated $[Ca^{2+}]_c$ favor the maintenance of a high ER Ca^{2+} content and decreases the probability of STIM2 activation to mediate B-SOCE; 2) Most TRPs are non-specific and, besides Ca^{2+} , let enter monovalent cations into the cell reducing the driving force for Ca^{2+} entry. This might decrease Ca^{2+} fluxes through other pathways such as Orai1 B-SOCE. **Model 3** shows how B-SOCE could amplify TRPs-dependent constitutive Ca^{2+} entries by facilitating the PM targeting of constitutively active TRPs subunits (TRPV2/TRPV6).

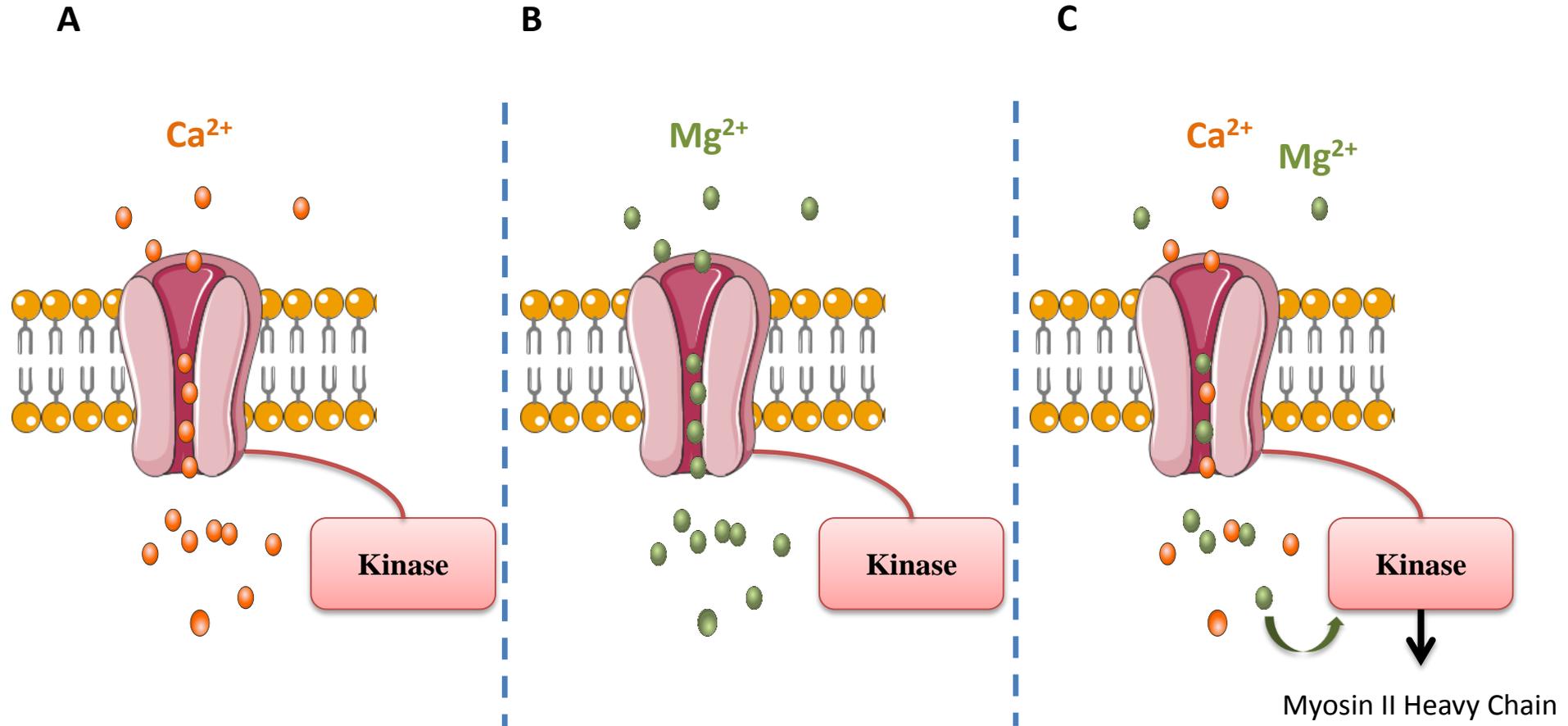
Note that in all models, *i*) the basal Ca^{2+} level results from an equilibrium between constitutive Ca^{2+} entry into the cytosol and active extrusion/ER storage by transporters/pumps; *ii*) the driving force for Ca^{2+} entry can be modulated by other ion conductance, especially K^+ channels.

For more details please refer to section 3 of the text. Freely adapted from Saul *et al.*, (Saul et al. 2014).



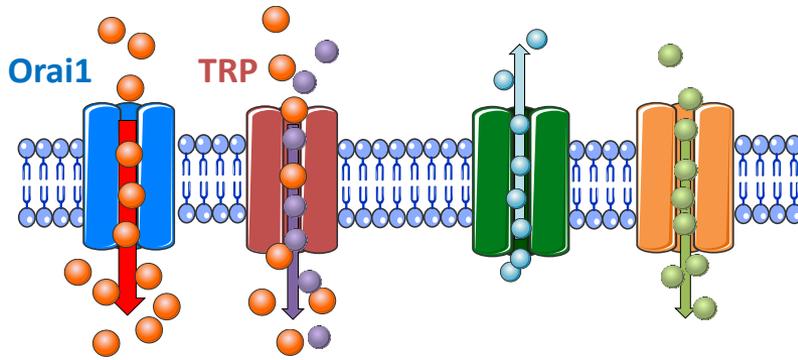






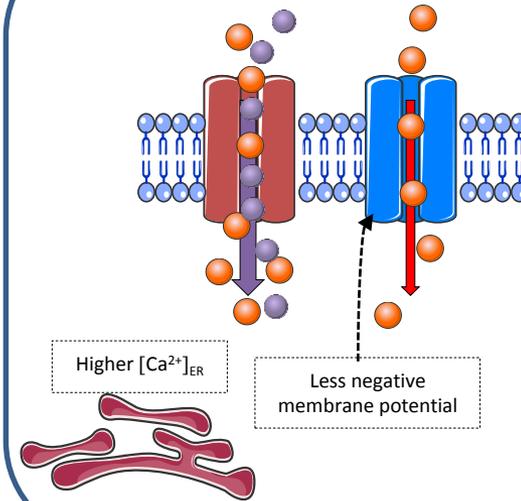
Model 1:

TRP-dependent Ca^{2+} influx and B-SOCE contribute independently to constitutive Ca^{2+} Entry

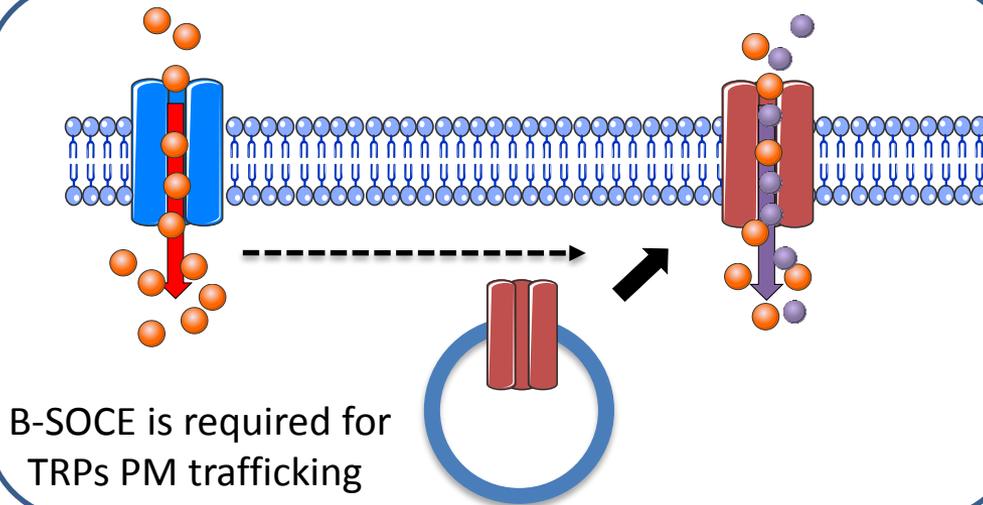


Model 2:

TRP's decrease B-SOCE



Model 3:



● Ca^{2+} ● Na^+ ● K^+ ● Cl^-