CHAPTER FOUR

On the Stoichiometry of Resting and Activated CRAC Channels

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Contents

Abstract

STIM1 and Orai1 are two essential components of the calcium release-activated calcium (CRAC) channel. Recently, distinct stoichiometries of STIM1 to Orai1 in assembling CRAC channels are proposed based on different techniques, such as single-molecule bleaching, biochemistry, crystallography, and concatenated constructs for electrophysiological experiments. Here, we review in detail these experiments as well as the strength and weakness of methods used. We propose that the tetrameric Orai1 is the pore for the resting and activated CRAC channel, where from two to eight STIM1 proteins open the channel in a graded manner.

Ca^{2+} is a versatile signal that mediates many important cellular processes, including synaptic transmission, muscle contraction, and gene expression (Berridge, Bootman, & Roderick, 2003). Among the modules of the Ca^{2+} signaling system, store-operated calcium (SOC) entry is one of the most intriguing calcium entry pathways (Putney, 2009). Activation of surface membrane receptors causes sustained extracellular Ca^{2+} entry into the cell interior, and this phenomenon was named SOC because the channel permeability to Ca^{2+} increases as the agonist-sensitive Ca^{2+} store is depleted (Putney, 1986). Using the whole-cell patch clamp technique, Hoth and

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Penner (1992) identified an intracellular Ca^{2+} store depletion-activated calcium current in mast cells. They named it the calcium (store) release-activated calcium (CRAC) channel. It should be mentioned that the CRAC channel is only one type of SOC, which may include other channels such as TRP channels (Smyth et al., 2006).

Similar to voltage-gated calcium channels (VGCCs) in secretory cells, the SOC/CRAC channel has important physiological functions in a number of nonsecretory cells. For example, Ca^{2+} entry via SOC regulates cytokine secretion in mast cells and T cells, modulates contraction in muscle cells, regulates fertilization and cell division in oocytes, and activates transcription factors in T cells (Hogan, Lewis, & Rao, 2010). Despite the importance and the electrophysiological fingerprints of this ion channel, the molecular mechanism of its function remains elusive. The opening of the membrane SOC/CRAC channel is regulated by the status of the endoplasmic reticulum (ER) Ca^{2+} store in a feedback manner (Lewis, 2007). By designing RNA interference (RNAi) vectors targeting limited numbers of presumed Ca^{2+}-binding proteins in HeLa and *Drosophila* S2 cells, two groups searched for the ER Ca^{2+} sensor of the CRAC channel; they independently identified STIM1 (Stim), a trans-membrane protein residing on the ER membrane, to be crucial for the activation of SOC entry (Liou et al., 2005; Roos et al., 2005). In 2006, three groups independently identified Orai1/CRACM1 as the CRAC ion channel pore via whole-genome RNAi screening (Feske et al., 2006; Vig, Peinelt, et al., 2006; Zhang et al., 2006). Coexpressing STIM1 with Orai1 reconstitutes the "monster" CRAC current in mammalian cells that are devoid of endogenous CRAC current (Peinelt et al., 2006; Prakriya et al., 2006; Vig, Beck, et al., 2006; Yeromin et al., 2006), and reconstitution of Orai1 with STIM1 fragments in the liposome membrane generates significant Ca^{2+} influx (Zhou et al., 2010). These findings indicate that STIM1 and Orai1 are necessary and sufficient for the assembly and activation of the CRAC channel complex.

As Ca^{2+} stores are depleted, ER STIM1 aggregates, possibly via its luminal SAM domain and its cytoplasmic coil-coiled domain (Baba et al., 2006; Stathopulos, Li, Plevin, Ames, & Ikura, 2006), and it migrates toward the ER–plasma membrane junction to interact with Orai1 on the opposing plasma membrane (Wu, Buchanan, Luik, & Lewis, 2006; Xu et al., 2006). During store refilling, both ER luminal Ca^{2+} and cytoplasmic Ca^{2+} modulate the dissociation of STIM1 and Orai1 (Malli, Naghdi, Romanin, & Graier, 2008), after which the proteins revert to their original status (Liou, Fivaz, Inoue, & Meyer, 2007). An L-type VGCC on the plasma

membrane physically interacts with ryanodine receptor on the opposing sarcoplasmic reticulum membrane, which leads to coordinated calcium entry and release in cardiomyocytes (Franzini-Armstrong, 2004). This physical arrangement resembles the assembly of CRAC channels in stimulated cells. However, compared to the static structure formed by the VGCC/ryanodine complex, the STIM1/Orai1 channel complex formation is a dynamic process that is spatiotemporally regulated. Therefore, to understand the stoichiometry of the CRAC channel and how it is regulated by store depletion is fundamental to the understanding of CRAC channel activation.

1. THE STOICHIOMETRY OF Orai1

Functional ion channels often form from subunits assembled into multimers. When Orai1 was initially identified, association of Orai1 proteins with each other *in vivo* was reported (Gwack et al., 2007; Li et al., 2007; Vig, Beck, et al., 2006). The first study that investigated this homo-oligomerization used a technique previously used to determine the composition of mammalian potassium channels (Liman, Tytgat, & Hess, 1992). Mignen, Thompson, and Shuttleworth (2008) coexpressed STIM1 and preassembled tandem multimeric Orai1 constructs in HEK293 cells, which generated macroscopic CRAC-like currents. The current was inhibited when a dominant-negative $Orai1_{E106Q}$ mutant was coexpressed with up to three tandem Orai1 and STIM1. When cells were transfected with an Orai1 tetramer plus STIM1, the CRAC current was insensitive to coexpression of a monomeric $Orai1_{E106Q}$ mutant. Based on these data, the authors proposed that Orai1 must assemble into a tetramer to form the channel pore, so the monomeric $Orai1_{E106Q}$ mutant was ineffective in reducing the current (Mignen et al., 2008). However, in their study, coexpression of tandem Orai1 and STIM1 only reconstituted a relatively small CRAC current (<10 pA/pF) compared to the usually huge CRAC current reported in other studies (Li et al., 2007; Peinelt et al., 2006). In addition, the amplitude of CRAC currents was correlated with the ratios and amounts of STIM1 and Orai1 vectors expressed, which were not held constant in their experiments. About the same time, we and others explored the functions of STIM1 and Orai1 using a different approach (Ji et al., 2008; Penna et al., 2008). By counting the number of bleaching steps of single GFP targeted to Orai1 multimers activated by the C-terminal cytosolic domain of STIM1, both of our groups showed that the activated CRAC channels contain Orai1 tetramers. However, regarding Orai1 in quiescent cells, different hypotheses have been proposed.

By counting the bleaching steps of EGFP tagged to different tandem Orai1 vectors coexpressed with STIM1 in fixed HEK293 cells, we found that the distribution of number of steps fits nicely with a tetrameric configuration of resting Orai1 (Ji et al., 2008). In contrast, Penna et al. observed that most of the overexpressed *Drosophila* GFP-Orai in live oocytes bleached in two steps. Complimentary to a predominance of Orai1 dimers found in resting S2 cells using biochemical assays, they concluded that resting Orai is a dimer (Penna et al., 2008). This -meric difference could be due to the different species of origin of Orai used (human vs. *Drosophila*) or to fixed-cell bleaching versus live-cell bleaching. Later, different sets of experiments were conducted to resolve such discrepancies. Madl et al. (2010) approached the problem using single-molecule tracking in combination with brightness analysis. They prebleached a rectangular region of surface membrane and tracked the surrounding Orai1 punctae as they diffused back into the prebleached region in resting cells. The histogram of Orai1 puncta brightness was best fit by a 4-Gaussian model, again supporting a tetramer configuration of resting Orai1 (Madl et al., 2010). Demuro et al. (2011) performed single-molecule bleaching in fixed HEK293 cells. By showing that paraformaldehyde (PFA) application led to a small cytoplasmic Ca^{2+} increase from ER stores, they concluded that the fixation led to a shift of bleaching pattern of Orai1 from dimer to tetramer due to store depletion. However, we also performed single-molecule bleaching experiments in HEK293 cells expressing Orai1 alone. No open CRAC channel was observed in these cells, but most of the Orai1 punctae still bleached in three or four steps (Fig. 4.1B and D). Furthermore, by coexpressing Orai1-mKO with 1, 2, or 3 tandem Orai1-EGFP multimers in HEK293 cells, we observed robust fluorescence resonance energy transfer (FRET) that was not changed by PFA fixation under TIRF illumination, arguing against an Orai1 aggregation induced by PFA fixation (Ji et al., 2008). A final addition to this controversy is the recently resolved crystal structure of Orai1 multimers, which revealed a striking hexamer structure (Hou, Pedi, Diver, & Long, 2012).

Because dimer, tetramer, and hexamer structures of Orai1 are all proposed based on different methods, we will try to list their strengths and weaknesses below in an objective manner. Single-molecule bleaching of EGFP in live or fixed cells was used to support a dimeric or a tetrameric configuration of Orai1. Although EGFP has been used for single-molecule tracking experiments for many years (Sako, Minoghchi, & Yanagida, 2000), a large portion of the bleaching events of Orai1-EGFP punctae

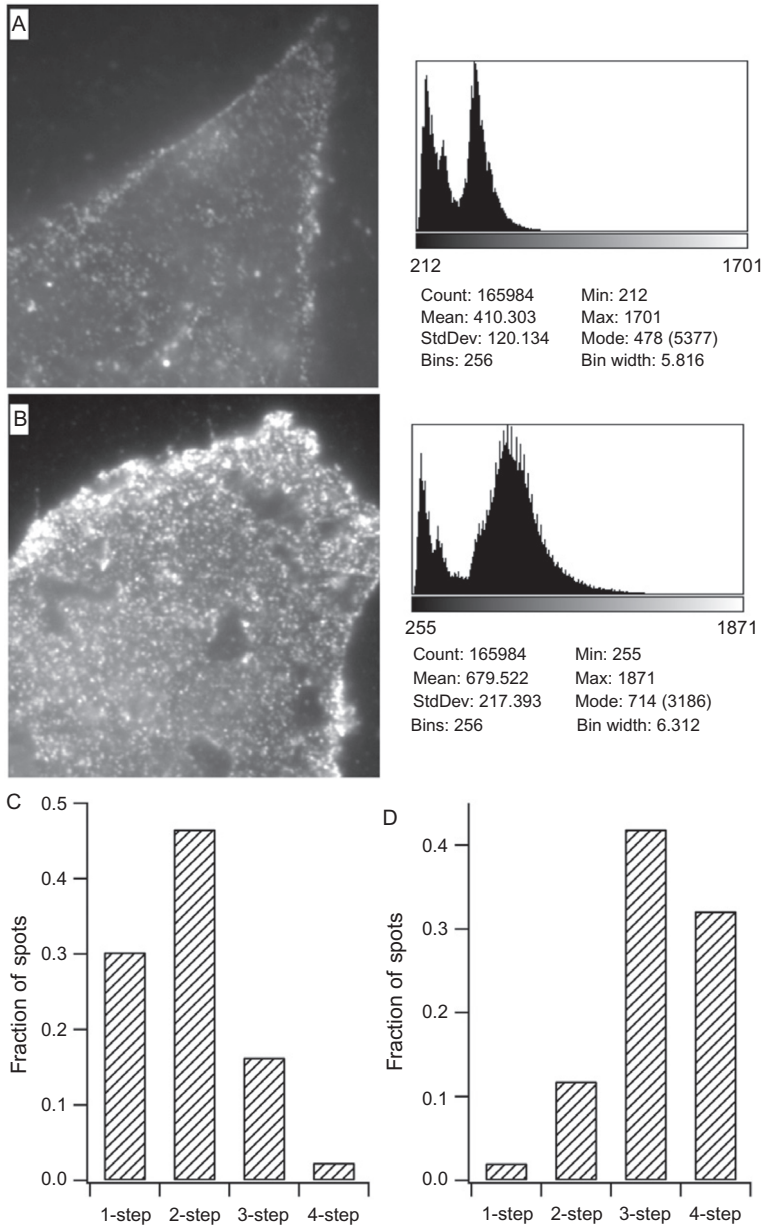

Figure 4.1 Distribution of EGFP bleaching steps seen with TIRF in fixed HEK293 cells transiently expressing Orai1-EGFP alone for ~6 h. Cells used in (A) and (B) were from the same chamber, excited and captured with the same configuration. Each image is a projection of 1000 time-lapse images of the individual cell together to give better visual contrast. It took longer time to find the right focus to image the cell A. Therefore,

(Continued)

(~80%) in mammalian cells cannot be reliably analyzed (Ji et al., 2008). However, considerably less ambiguity (50% of traces) was found in experiments with Orai1 tandems, which usually exhibited only one or two bleaching steps. Individual EGFP molecules may transit between the excited and dark states rapidly (Dickson, Cubitt, Tsien, & Moerner, 1997), which contributes to the strong on-and-off blinking of fluorescence over baseline and the ambiguities in dissecting multiple bleaching steps. In live cells, rapid movement of the majority of Orai1 punctae on the cell surface membrane is observed (Madl et al., 2010), and a vesicular population of Orai1 is also found in PC-12 cells (Dickson, Duman, Moody, Chen, & Hille, 2012). Therefore, the rapid lateral and axial movement of Orai1 under TIRF illumination adds another noise source to the fluorescence intensity, preventing bleaching steps from being reliably detected in live-cell imaging. Another requirement of single-molecule imaging is the ability to delicately adjust protein expression to see individual EGFP molecules. Under such conditions, it is important to find the right focus for the transfected cell without delay. As shown in Fig. 4.1A and C, delays in finding the right cell or right focus lead to excess light exposure that apparently changes the histogram of bleaching steps (as compared to Fig. 4.1B and D). Due to these limitations of single-molecule bleaching, we believe that it is important to compare histograms of bleaching steps from different tandem vectors to confirm the bleaching results of monomeric Orai1. In addition, both STIM1 and Orai1 form large aggregates at ER–plasma membrane junctions in cells that overexpress STIM1 and Orai1, which may be different from single-molecule bleaching experiments where little Orai1 is available. In this regard, the ideal experiment to study stoichiometry in live cells with excessive exogenous Orai1 is to detect the interaction between functional tandem Orai1-EGFP multimers and Orai1-mKO with FRET imaging. We and others have shown that Orai1-mKO failed to interact with 4-tandem Orai1-EGFP (Ji et al., 2008; Madl et al., 2010), similar to the electrophysiological experiments in cells coexpressing $Orai1_{E106Q}$ and tandem Orai1 (Mignen et al., 2008). However, this type of experiment requires the design of concatenated constructs that are physically connected by linkers. Large

Figure 4.1—Cont'd as compared to the cell B, the fluorophore in the cell A was prebleached before imaging, as could be seen from the intensity histogram shown in the right. (C and D) The frequency distribution of one-step and multistep bleaching events shown in the cell A and the cell B, respectively.

vectors tend to exhibit decreased expression and reduced delivery to the cell surface membrane (Ji et al., 2008), and different linker lengths or sequences may lead to insertion of endogenous subunits or exclusion of linked subunits from channel pores (McCormack, Lin, Iverson, Tanouye, & Sigworth, 1992). Nevertheless, by carefully choosing the linker length and sequence, this type of strategy has been successfully used to characterize a number of channels (Baur, Minier, & Sigel, 2006; Groot-Kormelink, Broadbent, Beato, & Sivilotti, 2006; Minier & Sigel, 2004).

Despite all these differences in methods and results, it seemed generally agreed that open CRAC channels are tetramers until Hou et al. showed a hexamer crystal structure of activated Orai. It remains possible that the insertion of endogenous Orai1 subunits into concatenated vectors masked the correct configuration of Orai1 in the FRET and electrophysiological experiments (Ji et al., 2008; Mignen et al., 2008). The failure of our group and Cahalan's group to observe a significant number of 5- and 6-step bleachings can also be explained as a result of discarding 80% of irresolvable bleachings of punctae (Ji et al., 2008; Penna et al., 2008). On the other hand, the crystal structure experiment could be misleading as well. Although Hou et al. verified that their protein complex was capable of conducting Ca^{2+}, they failed to do electrophysiological experiments to confirm that this Ca^{2+} entry exhibited CRAC-like characteristics. In fact, it is shown recently that the overexpression of a concatenated hexameric Orai1 construct reconstitutes an non-selective cation current, in contrast to the highly calcium selective current reconstituted in cells expressed tetrameric Orai1 vector (Thompson & Shuttleworth, 2013). Moreover, the Orai1 construct they used lacked an N-terminal cytoplasmic domain, which is not essential for channel activation but important for the maximal opening of Orai1 by STIM1 (Li et al., 2007). Therefore, without the 8–10 nm protrusion of the truncated cytoplasmic domain of Orai1 (Maruyama et al., 2009; Varnai, Toth, Toth, Hunyady, & Balla, 2007), it is unclear whether the crystal structure represented the assembly of CRAC channel *in vivo*. Moreover, membrane proteins have to be detergent-solubilized to be crystallized for X-ray diffraction, which may lead to nonphysiological assembly of protein complexes. For example, the *S. aureus* mechanosensitive channel of large conductance proteins generates variable stoichiometries *in vitro*, in contrast to the pentamer structure *in vivo* (Dorwart, Wray, Brautigam, Jiang, & Blount, 2010). Therefore, whether hexameric Orai1 functions as a CRAC channel pore in live cells remains to be determined.

2. THE STOICHIOMETRY OF STIM1

Resting STIM1 on the ER membrane is a monomer or dimer. As ER Ca^{2+} drops, STIM1 proteins interact with each other to form large aggregates (Liou et al., 2007), possibly via their luminal SAM domain and cytoplasmic coiled-coil domain (Baba et al., 2006; Stathopulos et al., 2006). The migration of STIM1 oligomers to the ER–plasma membrane junction opens the Orai1 channel (Luik, Wu, Buchanan, & Lewis, 2006; Wu et al., 2006; Xu et al., 2006). Because the interaction of STIM1 and Orai1 is dynamically regulated, it is difficult to assess how many STIM1 are required to open one Orai1 channel. Expressing the C-terminal fragment of STIM1 (STIM1-C) led to constitutive activation of the endogenous CRAC channel and increased SOC entry (Ji et al., 2008; Penna et al., 2008). By transfecting HEK293 cells with STIM1-C-EGFP, we found that EGFP punctae bleached in either one or two steps. If we increased the amount of STIM1-C-EGFP transfected, more punctae bleached in one but not two steps; if we coexpressed exogenous Orai1 with STIM1-C-EGFP, more punctae bleached in two steps. Therefore, we propose that two bleaching steps represent the population of STIM1-C that interacts with Orai1 and opens the channel (Ji et al., 2008). Obviously, this is an indirect demonstration of two STIM1-C proteins opening one Orai1 channel. Moreover, STIM1-C does not form high-order oligomers, and coexpressing STIM1-C with Orai1 only generates a marginal CRAC current. Therefore, it is unclear whether full-length STIM1 activates Orai1 in the same manner.

In 2009, different groups pinpointed the minimal C-terminal domain of STIM1 that maximally activates Orai1 channels without depletion of ER Ca^{2+} stores (Muik et al., 2009; Park et al., 2009; Yuan et al., 2009). Therefore, we explored the stoichiometry of STIM1 by directly linking different numbers of functional STIM1 domains to Orai1 constructs (Li et al., 2011). An initial attempt using the minimal functional domain of STIM1 was not successful because the linked vector tended to form aggregates and failed to be delivered to the surface membrane. Finally, we selected the dimeric STIM1 fragment (residues 336–485), termed the S domain, which activates the Orai1 channel efficiently (Yuan et al., 2009). By linking either one or two S domains of STIM1 to the cytoplasmic C-terminal of Orai1, we showed that the vector can be correctly expressed and targeted to the surface membrane; HEK293 cells that overexpressed the Orai1-S vector exhibited a constitutively active CRAC current with an amplitude of ~50 pA, which

was further enhanced upon coexpressing Orai1-S with cytoplasmic SS vector. On the other hand, cells transfected with Orai1-SS vector exhibited a large constitutive CRAC-like current that could not be further increased by coexpressing cytosolic SS vector. The LQ347/348AA mutation in STIM1 abolishes the ability of STIM1 to activate Orai1 (Yuan et al., 2009). Introducing the LQ347/348AA mutation into either S domain within Orai1-SS-eGFP effectively reduced the current by half. These results indicate that both S domains are required for the maximal activation of a single Orai1 subunit so that eight S domains are required for the activation of the whole channel pore. Because different numbers of STIM1 may lead to different levels of Orai1 activation, we tried to establish the number of S domains that interacted with Orai1 to the open probability of CRAC channel. We used a L273D mutation in the cytoplasmic C-terminal domain of Orai1 that cannot interact with the linked S domain of STIM1 (Muik et al., 2008). This allowed us to generate a series of constructs containing one ($Orai1_{L273D}$-3 × Orai1-SS), two (2 × $Orai1_{L273D}$-2 × Orai1-SS), three (3 × $Orai1_{L273D}$-Orai1-SS), and four mutated subunits (4 × $Orai1_{L273D}$-SS). Except for the 4 × $Orai1_{L273D}$-SS, HEK293 cells expressing these vectors exhibited comparable small CRAC-like currents. In cells transfected with $Orai1_{L273D}$-3 × Orai1-SS or 2 × $Orai1_{L273D}$-2 × Orai1-SS, coexpressing the cytoplasmic SS domain further increased the constitutive CRAC current; this was not observed in cells cotransfected with 3 × $Orai1_{L273D}$-Orai1-SS and cytoplasmic SS vector (Li et al., 2011). Therefore, these data suggest that two SS domains are capable of opening the Orai1 channel pore, whereas more SS domains open the channel in a graded fashion. Next, we constructed a series of four-tandem heterozygous mutant/WT Orai1 constructs that contained different numbers of $Orai1_{L273D}$ subunits, and coexpressed them with full-length STIM1. When the ER Ca^{2+} store was depleted in these cells, the reconstituted CRAC current increased in a graded manner as the number of functional Orai1 subunits in the channel pore increased. Based on these experiments, we proposed a model to describe the sequential activation of Orai1 by STIM1 (Li et al., 2011). As shown in Fig. 4.2, one STIM1 dimer can interact with one Orai1 subunit in the pore and open the channel, whereas the presence of more STIM1 dimer leads to activation of more Orai1 subunits and a superlinear increase in channel current. It is possible that our conclusion is affected by the linker between the concatenated subunits, which may perturb the channel activation kinetics or their cooperativity. However, the current reconstituted by the concatenated vectors shared all known characteristic of the endogenous CRAC current, and a longer linker between the Orai1 and the SS domain

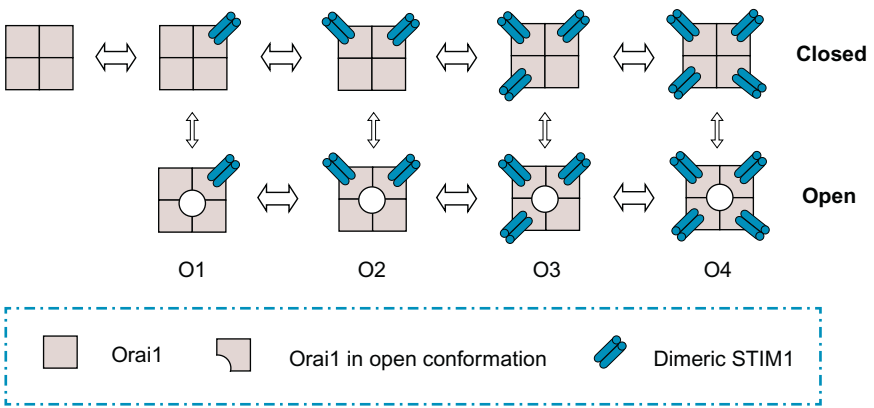

Figure 4.2 Sequential activation model of the CRAC channel. Dimeric STIM1 molecules activate one Orai1 subunit. Different numbers of Orai1 subunits being activated result in multiple opening states (O1–O4). The opening activity maximizes when all four Orai1 subunits are activated by eight STIM1 molecules.

(44 amino acids) generated currents with similar amplitudes to the original Orai1-SS, suggesting the current linker length does not restrict the interaction between the S domain and Orai1. Moreover, the maximal CRAC current occurred in cells with a STIM1:Orai1 ratio at the ER–plasma membrane junction of ~2:1 (Hoover & Lewis, 2011), again confirming our conclusion that eight STIM1 maximally open the Orai1 channel.

3. SUMMARY

Despite the several different methods and experiments used, the stoichiometry of Orai1 and STIM1 remains controversial. Although a structural study revealed a hexameric structure of Orai1, we favor the idea that tetrameric Orai1 forms open the CRAC channel pore because this has been supported by different methods, including single-molecule imaging, FRET imaging, and electrophysiology experiments. We also favor a tetrameric Orai1 in the resting state. If dimeric Orai1 indeed forms a tetramer upon interaction with STIM1 during calcium store depletion as proposed (Demuro et al., 2011; Penna et al., 2008), whether and how Orai1 tetramers disassemble into dimers after ER store replenishment will be an interesting topic to explore in the future. From dimer to octamer, larger STIM1 oligomers open the Orai1 channel pore in a graded manner, which allows for the fine-tuning of CRAC-related calcium signaling processes. For example, various physiological stimuli may bring different ratios of STIM1 and Orai1 to the ER–plasma membrane junctions, leading to local Ca^{2+} entry and specific oscillation patterns required for differential responses and gene expression in immune cells (Dolmetsch, Lewis, Goodnow, & Healy, 1997; Dolmetsch, Xu, & Lewis, 1998). Finally, the mechanism for the formation and disassembly of the different Orai1/STIM1 complexes awaits future investigation.

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