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Effects of rapamycin-induced oligomerization of parvalbumin, Stim1 and Orai1 in puncta formation

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ABSTRACT

Elevations of cytosolic Ca^{2+} from the endoplasmic reticulum (ER) regulate a diverse range of cellular processes. When these luminal stores become depleted, the transmembrane ER protein Stim1 oligomerizes and translocates within the ER membrane to puncta junctions to couple with Orai1 channels, activating store-operated calcium entry (SOCE). Stim1 oligomerization and puncta formation have generally been associated with its luminal domains, however, studies have implicated that the cytoplasmic domains may contribute to this oligomerization. Studies have also suggested that intermediate or regulating elements may be required to fine-tune puncta formation and activation of SOCE. Here we made fusion proteins of Stim1 and Orai1 with FRB and FKBP12 domains that associate in the presence of rapamycin. Rapamycin-induced coupling of Stim1 to Stim1, Orai1 to Orai1 and Stim1 to Orai1 was found to be insufficient for puncta formation. Rapamycin was then used to recruit the cytosolic Ca^{2+} buffer protein parvalbumin (Pav) to Stim1 in order to buffer the local cytosolic Ca^{2+} near the ER membrane. Interestingly, Pav buffering near the ER caused puncta formation that was indistinguishable from those caused by thapsigargin. Our results suggest that Stim1 oligomerization and puncta formation may be additionally regulated either by local Ca^{2+} levels near the ER membrane or by as yet unidentified Ca^{2+}-dependent proteins interacting with the cytoplasmic domains of Stim1.

1. Introduction

Intracellular Ca^{2+} signals regulate many physiological processes including cell development, proliferation, apoptosis, gene transcription, as well as cardiac excitation-contraction coupling [1]. Elevations of cytosolic Ca^{2+} originate from intracellular stores such as the endoplasmic reticulum (ER). However, internal stores are finite so prolonged Ca^{2+} signaling relies on an influx of external Ca^{2+} across the plasma membrane (PM). In many cell types intracellular store depletion of Ca^{2+} causes an influx of extracellular Ca^{2+} through store-operated calcium entry (SOCE), which is mediated by Stim1 (stromal interaction molecule 1) on the ER membrane and Orai1 (also known as CRACM1) on the PM [2–5]. Stim1 is a transmembrane ER protein that senses the depletion of luminal Ca^{2+} and self-oligomerizes. Following oligomerization, Stim1 oligomers translocate within the ER membrane network to ER-PM junctions called puncta [6]. Orai1 channels translocate along the PM to these same puncta junctions [7,8], where coupling to Stim1 opens Orai1 channels to Ca^{2+} entry.

The coordinated molecular rearrangement of Stim1 and Orai1 as well as the regulation of this process continues to be extensively studied. Previously, Luik et al. [9] used rapamycin-induced protein heterodimerization to assess the role of Stim1 oligomerization in SOCE activation. Rapamycin-induced heterodimerization is well established in mammalian cell culture and whole organisms such as mice, with quick response times on the order of minutes [10]. This lipid-permeable drug is used to noncovalently bind and oligomerize the 12 kDa FK506- and rapamycin-binding protein (FKBP12) with the FKBP-rapamycin binding (FRB) domain of the mTOR protein. Rapamycin has been used to conditionally activate FRB and FKBP12 fusion proteins of cell surface proteins such as fibroblast growth factor receptors [11], cytosolic proteins such as Akt [12], and to recruit signaling molecules such as phosphoinositide 5-phosphatase to the PM to stimulate signaling [13]. In particular, Luik et al. [9] replaced the luminal Ca^{2+}-sensing domain of Stim1 with either FKBP12 or FRB domains. In the presence of rapamycin, these fusion proteins oligomerized and accumulated at puncta ER-PM junctions without the need for Ca^{2+} depletion from the ER. This work and others have confirmed that oligomerization of Stim1 can be uncoupled from store depletion

Abbreviations: Stim1, stromal interaction molecule 1; SOCE, store-operated calcium entry; Pav, parvalbumin B; FKBP12, 12 kDa FK506- and rapamycin-binding protein; FRB, FKBP-rapamycin binding domain.

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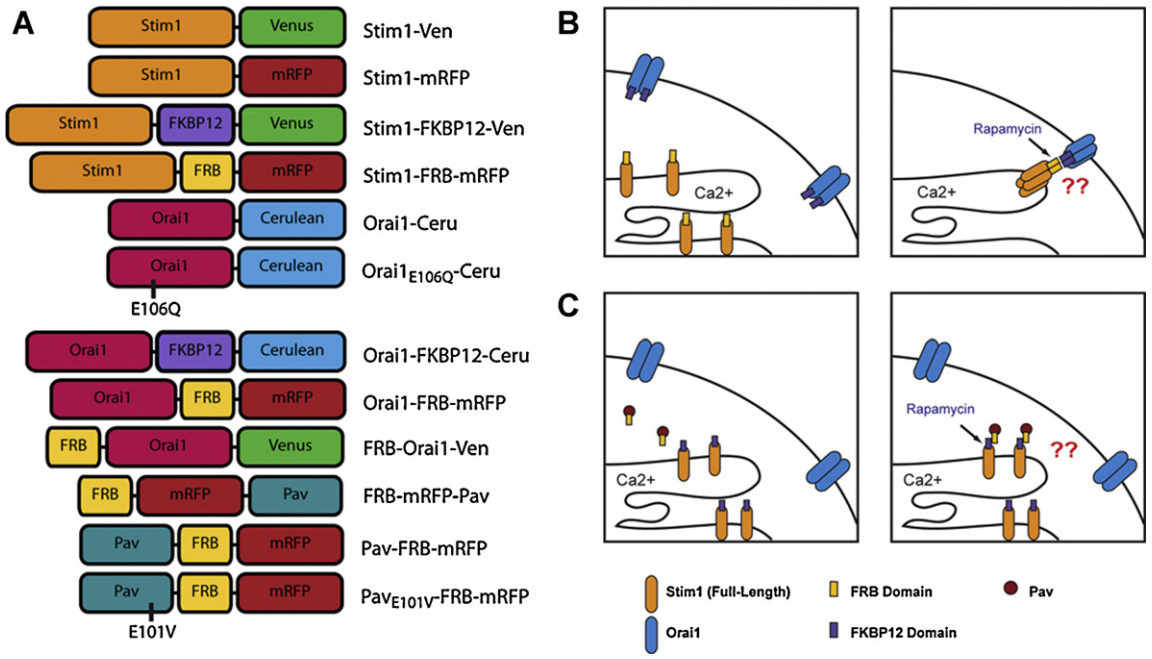

Fig. 1. Rapamycin-induced coupling of Stim1 and Orai1, and recruitment of Pav to Stim1. (A) Pav, Stim1 and Orai1 were fused to FRB and FKBP12 domains and tagged with Venus, Cerulean and mRFP using methods previously described [40,41]. (B) Rapamycin will be used to induce the interaction between Stim1-FRB and Orai1-FKBP12 to determine whether direct coupling of Stim with Orai1 (without store depletion) is sufficient to cause puncta formation. (C) Rapamycin will also be used to recruit the cytoplasmic protein Pav-FRB to Stim1-FKBP12 to study the effects of recruiting Pav, a Ca^{2+} buffer protein, to Stim1 and the ER membrane.

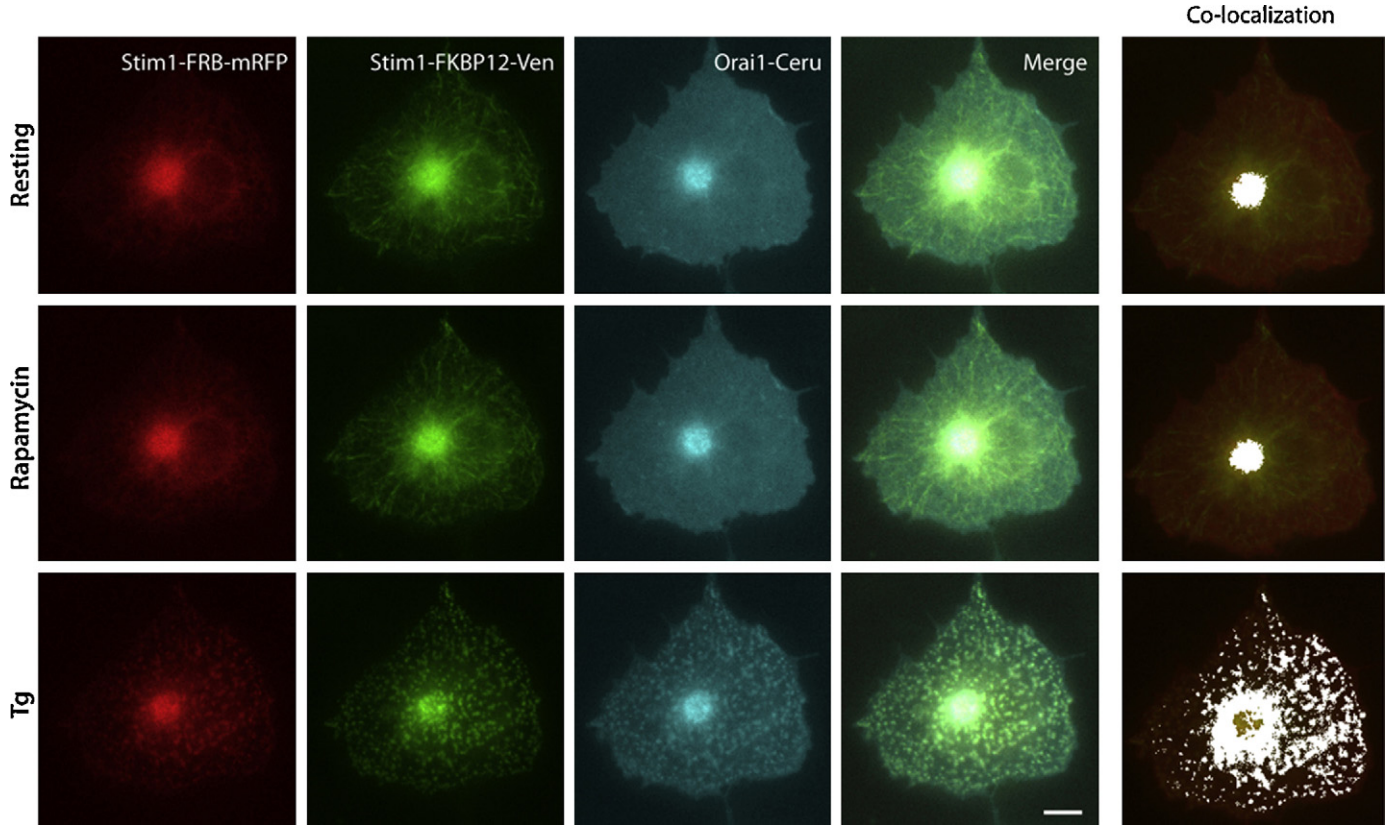

Fig. 2. Rapamycin-induced association of Stim1 fusion proteins did not induce puncta formation. Stim1-FRB-mRFP and Stim1-FKBP12-Ven were distributed on the ER and Orai1-Ceru was localized at the PM in resting Cos-7 cells. Addition of 1 μM rapamycin did not induce changes in this distribution. In contrast, a subsequent addition of 1 μM Tg induced puncta formation of both Stim1 and Orai1 fusion proteins ($n = 9/9$ cells observed). Scale bar is 30 μm. Images are false color, with white overlays indicating sites of co-localization between Stim1-FKBP12-Ven and Orai1-Ceru. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

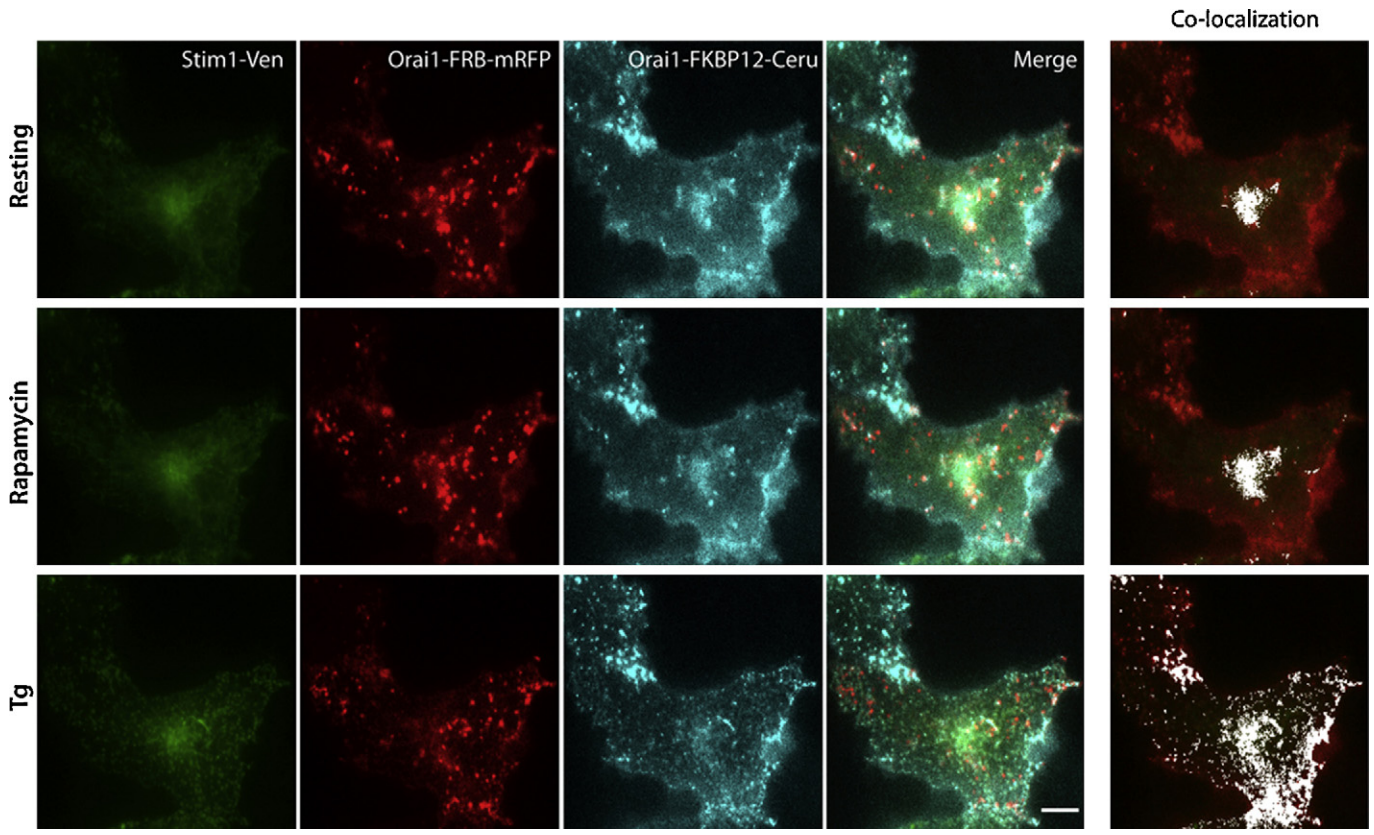

Fig. 3. Rapamycin-induced association of Orai1 fusion proteins did not induce puncta formation. Orai1-FRB-mRFP and Orai1-FKBP12-Ceru were localized at the PM in resting Cos-7 cells. Addition of 1 μM rapamycin did not induce puncta formation. In contrast, a subsequent addition of 1 μM Tg induced puncta formation of both co-expressed Stim1-Ven and Orai1 fusion proteins ($n = 9/9$ cells observed). Scale bar is 30 μm. Images are false color, with white overlays indicating sites of co-localization between Stim1-Ven and Orai1-FKBP12-Ceru. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and this oligomerization of Stim1 subsequently leads to puncta formation [6,9,14].

Although Stim1 oligomerization has generally been associated with its luminal EF-hand and sterile alpha motif (EF-SAM) domain [15,16], the cytosolic coiled-coil domains have also been shown to contribute to Stim1 oligomerization [17,18]. Association between the coiled-coil domains of Stim1 and other molecules may provide ER-retention signals and protein stability [19,20]. It is important to note that puncta formation indicates Stim1 oligomerization and accumulation, but this does not necessarily indicate that Orai1 channels (and hence SOCE) are fully activated [21,22]. Gwozdz et al. [22] have suggested that additional intermediate or regulating elements may be required to fine-tune puncta formation, molecular coupling between Stim1 and Orai1, and signal transduction from the ER to the PM. Furthermore, Malli et al. [23] has shown that cytosolic Ca^{2+} tunes the formation and disassembly of Stim1 clusters.

While both Stim1 and Orai1 are essential for SOCE, it remains unclear whether these proteins are additionally regulated by other proteins or local cytoplasmic Ca^{2+} fluxes. Here we studied the effects of rapamycin-induced coupling of Stim1 and Orai1. By fusing FRB and FKBP12 domains to Stim1 and Orai1, rapamycin could be used to bring these fusion proteins together in order to study the effects of drug-induced interactions (Fig. 1A). We also looked at the effects of recruiting parvalbumin B (Pav), a cytosolic Ca^{2+} buffer, to Stim1 and Orai1 through FRB and FKBP12 heterodimerization (Fig. 1B and C). While Pav is not known to have any natural binding partners, it is known to buffer Ca^{2+} for modulating the magnitude, duration and diffusion of Ca^{2+} signals [24–26]. Studies have used Pav in different subcellular regions to buffer localized Ca^{2+}. For example, Rodrigues et al. [27] localized Pav in the nucleus and cytoplasm to differentially buffer nucleoplasmic versus cytoplasmic Ca^{2+}, respectively. By recruiting Pav to Stim1 and Orai1, cytosolic Ca^{2+} levels near the ER and PM can be locally buffered (Fig. 1B and C). In particular, we found that rapamycin-induced Pav buffering of Ca^{2+} near the ER on the cytoplasmic side led to puncta formation, but not Ca^{2+} influx.

2. Materials and methods

Please see Supplementary Materials.

3. Results

3.1. Thapsigargin, but not rapamycin, caused puncta formation of Stim1 and Orai1

Addition of 1 μM rapamycin did not induce puncta formation of Stim1-Ven both in the absence and presence of co-expressed Orai1-Ceru. In Cos-7 cells, Stim1 tagged with Venus (Stim1-Ven), a variant of yellow fluorescent protein, showed ER localization while Orai1 tagged with Cerulean (Orai1-Ceru), a variant of cyan fluorescent protein, showed PM localization (Supp. Fig. 1). The effects of rapamycin on the localization and distribution of Stim1-Ven and Orai1-Ceru were first verified in Cos-7 cells. As expected, this localization did not change nor did puncta form upon addition of 1 μM rapamycin. In contrast, subsequent addition of 1 μM thapsigargin (Tg), which depletes ER Ca^{2+} levels by blocking the activity of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), caused puncta formation of Stim1-Ven throughout the cells. Similarly, 1 μM Tg but not 1 μM rapamycin induced co-localized puncta of Stim1-Ven

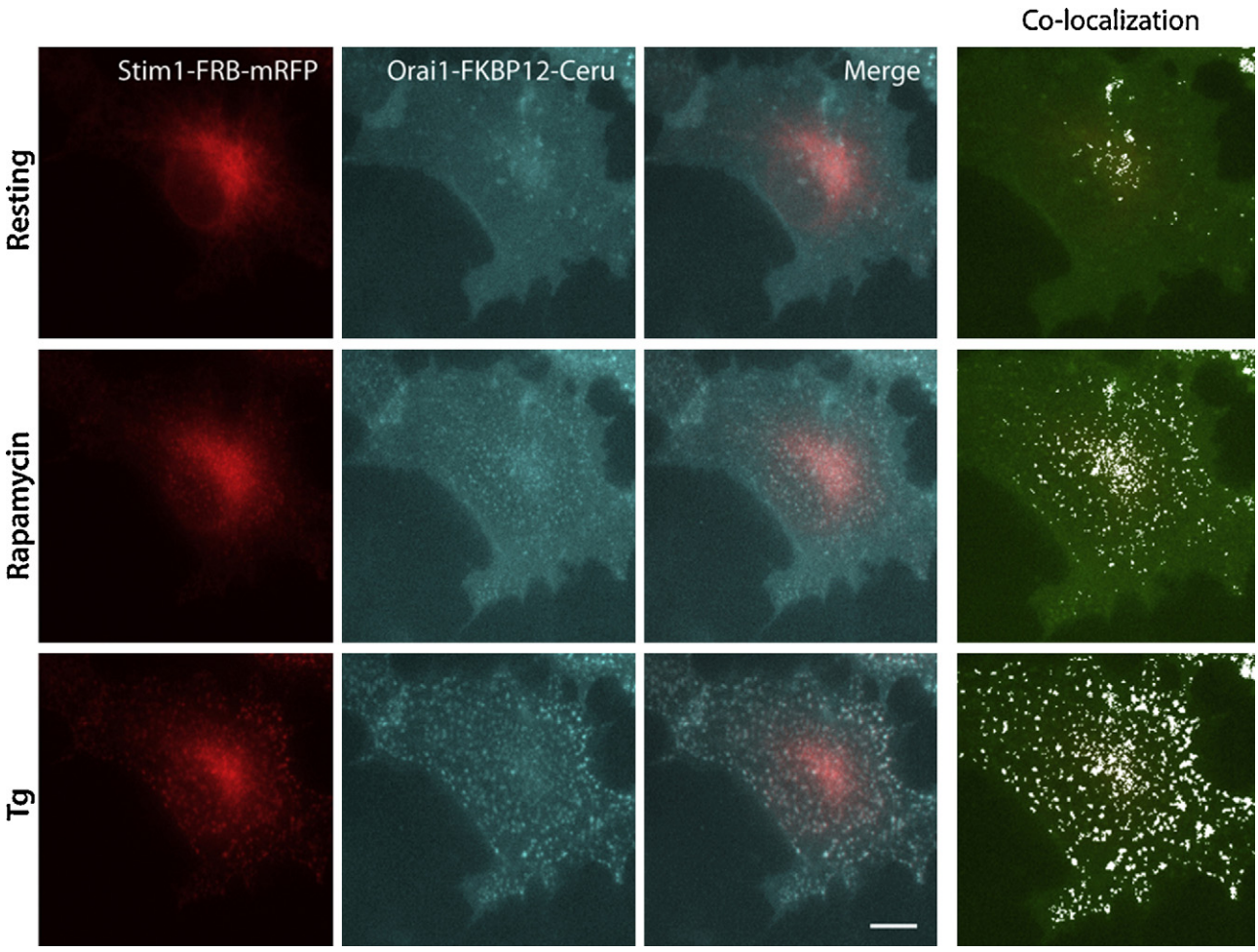

Fig. 4. Rapamycin-induced coupling of Stim1-FRB-mRFP and Orai1-FKBP12-Ceru formed small co-localized clusters but not fully-formed puncta. In resting cells, Stim1-FRB-mRFP was distributed on the ER and Orai1-FKBP12-Ceru was localized at the PM. Addition of 1 μM rapamycin lead to the formation of small co-localized clusters that remained unchanged for 30 min. A subsequent addition of 1 μM Tg, however, induced further puncta formation within 10 min ($n = 9/9$ cells observed). Scale bar is 30 μm. Images are false color, white overlays indicate sites of co-localization between Stim1-FRB-mRFP and Orai1-FKBP12-Ceru. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and Orai1-Ceru ($n = 9/9$ cells). Consistent with visual observations, rapamycin caused a minimal increase (1.9%) in co-localization between Stim1-Ven and Orai1-Ceru. In contrast, Tg caused an increase of 11.9%.

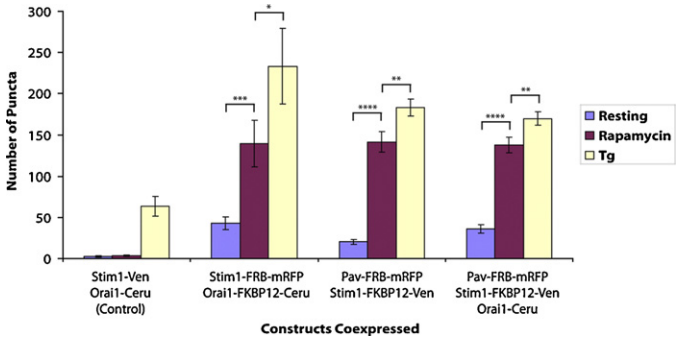

Fig. 5. Rapamycin-induced recruitment of Pav-FRB-mRFP to Stim1-FKBP12-Ven resulted in a similar number of puncta compared to Tg-induced puncta formation. The number of puncta formed was counted for resting cells, after addition of 1 μM rapamycin, and after addition of 1 μM Tg. Rapamycin-induced coupling of Stim1-FRB-mRFP and Orai1-FKBP12-Ceru led to an increase in puncta formation that was smaller than that attainable with Tg stimulation. Rapamycin-induced recruitment of Pav-FRB-mRFP to Stim1-FKBP12-Ven led to formation of comparable numbers of puncta compared to Tg-induced puncta formation in the same cells (Error bars, s.e.m.; $^{*}p = 0.053$, $^{**}p = 0.01$, $^{***}p < 0.005$, $^{****}p < 0.0001$, $n = 9$, using Student's t-test).

3.2. Rapamycin-induced association of Stim1 and Orai1 was insufficient for puncta formation

Since the rapamycin-induced heterodimerization of Stim1 proteins consisting of FRB or FKBP12 domains in its luminal Ca^{2+} sensing region was able to oligomerize and translocate Stim1 proteins to puncta, we were interested in studying the effects of fusing the FRB or FKBP12 domains at the cytoplasmic C-terminal end of Stim1. In resting Cos-7 cells, Stim1-FRB-mRFP and Stim1-FKBP12-Ven were co-localized along the ER. In both the presence and absence of co-expressed Orai1-Ceru at the PM, Stim1-FRB-mRFP and Stim1-FKBP12-Ven did not form puncta upon addition of 1 μM rapamycin. However, addition of 1 μM Tg led to puncta formation within 10 min ($n = 9/9$ cells) (Fig. 2).

Similarly, Orai1-FRB-mRFP and Orai1-FKBP12-Ceru did not form puncta upon addition of 1 μM rapamycin. In contrast, when co-expressed with Stim1-Ven, these constructs redistributed and co-localized into puncta upon addition of 1 μM Tg ($n = 9/9$ cells) (Fig. 3). Both Orai1-FRB-mRFP and Orai1-FKBP12-Ceru could be found in vesicles but these vesicles were not puncta because they did not co-localize with Stim1-Ven and moved between frames. Likewise, rapamycin-induced oligomerization of FRB-Orai1-Venus and Orai1-FKBP12-Ceru did not cause puncta formation (Supp. Fig. 2). Taken together, these results confirm that rapamycin-induced association was insufficient for puncta formation. Nonetheless,

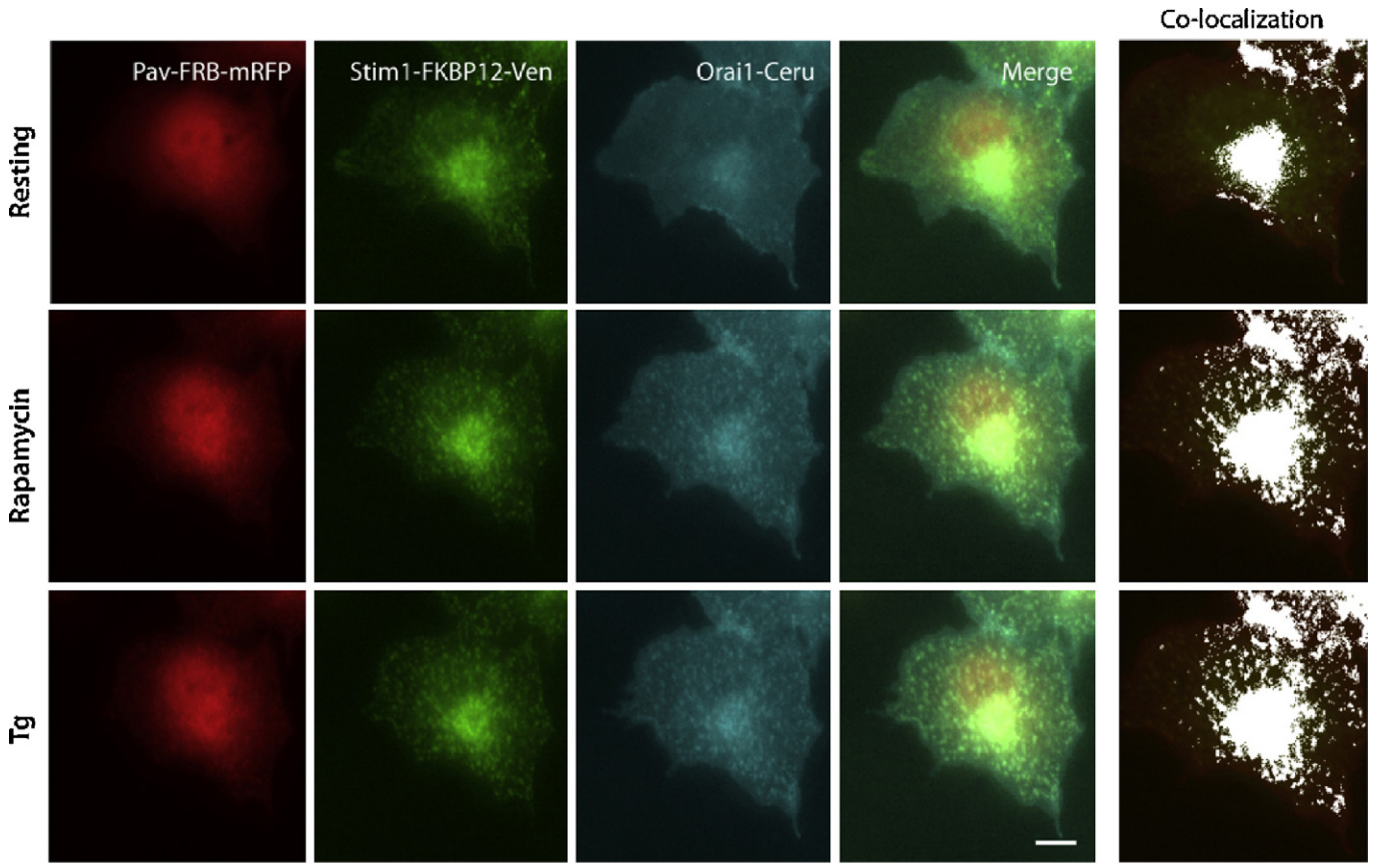

Fig. 6. Rapamycin-induced recruitment of Pav to Stim1 led to puncta formation of Orai1-Ceru. When co-expressed, Orai1-Ceru was localized at the PM in resting cells. With the addition of 1 μM rapamycin to induce Pav-FRB-mRFP recruitment to Stim1-FKBP12-Ven and puncta formation, Orai1-Ceru was also redistributed to these puncta junctions. These puncta remained unchanged after an additional 30 min and with a subsequent addition of 1 μM Tg (n = 9/9 cells observed). Scale bars are 30 μm. Images are false color, with white overlays indicating sites of co-localization between Stim1-FKBP12-Ven and Orai1-Ceru. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fusion of FRB and FKBP12 domains at the C-termini of Stim1 and Orai1 did not affect the ability of Stim1 and Orai1 fusion proteins to oligomerize and form puncta under Tg stimulation.

We next determined that the rapamycin-induced coupling of Stim1-FRB-mRFP and Orai1-FKBP12-Ceru did not lead to full puncta formation. Upon addition of 1 μM rapamycin, Stim1-FRB-mRFP and Orai1-FKBP12-Ceru formed small co-localized clusters. These clusters did not change size or shape even after an additional 30 min of imaging. Subsequent addition of 1 μM Tg, however, induced an obvious increase in puncta formation within 10 min (n = 9/9 cells) (Fig. 4). Consistent with visual observations, rapamycin caused an increase in co-localization between Stim1-FRB-mRFP and Orai1-FKBP12-Ceru of only 5.8%, while Tg caused a greater increase of 11.8%. By counting the number of puncta observed in resting cells, cells stimulated with 1 μM rapamycin and cells stimulated with 1 μM Tg, the number of rapamycin-induced puncta formed was compared with the maximum number attainable using Tg stimulation (Fig. 5). In cells co-expressing Stim1-FRB-mRFP and Orai1-FKBP12-Ceru, there was an increase in puncta formation after addition of rapamycin. However, fewer puncta were formed relative to the larger increase in puncta formation after addition of Tg.

3.3. *Rapamycin-induced recruitment of parvalbumin to Stim1 caused puncta formation but did not trigger an influx of Ca^{2+}*

To study the effects of locally buffering Ca^{2+} near the ER, Pav-FRB-mRFP was recruited to Stim1-FKBP12-Ven through rapamycin-induced binding. This caused puncta formation of Stim1-FKBP12-Ven which was additionally verified by co-localization of Orai1-Ceru to these puncta junctions. In resting Cos-7 cells expressing Pav-FRB-mRFP and Stim1-FKBP12-Ven, Pav-FRB-mRFP was distributed throughout the cytoplasm while Stim1-FKBP12-Ven was localized to the ER. Upon addition of 1 μM rapamycin, Pav-FRB-mRFP was recruited to Stim1-FKBP12-Ven and caused puncta formation within 10 min (n = 9/9 cells) (Supp. Fig. 3). When co-expressed, Orai1-Ceru was also redistributed to these puncta junctions after addition of 1 μM rapamycin (n = 9/9 cells) (Fig. 6). These puncta were visually indistinguishable from those formed by the subsequent addition of 1 μM Tg. Changes in co-localization coefficients showed an increase in co-localization between Stim1-FKBP12-Ceru and Orai1-Ceru of 6.1% upon rapamycin-induced recruitment of Pav-FRB-mRFP, similar to the increase of 7.1% due to subsequent Tg addition. Using time-lapse imaging, we compared the puncta formation of 1 μM rapamycin-induced Pav-mRFP recruitment to Stim1-FKBP12Ven to that of control cells expressing Stim1-Ven and Orai1-Ceru under 1 μM Tg. Visually, the formation of puncta in either case is indistinguishable (Supplemental Movies 1 and 2). For confirmation, by calculating PC values for each frame of the time-lapse video, the change in extent of co-localization can be plotted (Supp. Fig. 4). Fitting the data to exponential rise, time constants (τ) were determined: τ (control) = 4 min and τ (Pav recruitment) = 5 min. Furthermore, rapamycin-induced recruitment of Pav-FRB-mRFP to Stim1-FKBP12-Ven showed an increase in puncta formation that was comparable to the maximum number of puncta that can be formed using Tg, both in the absence and presence of

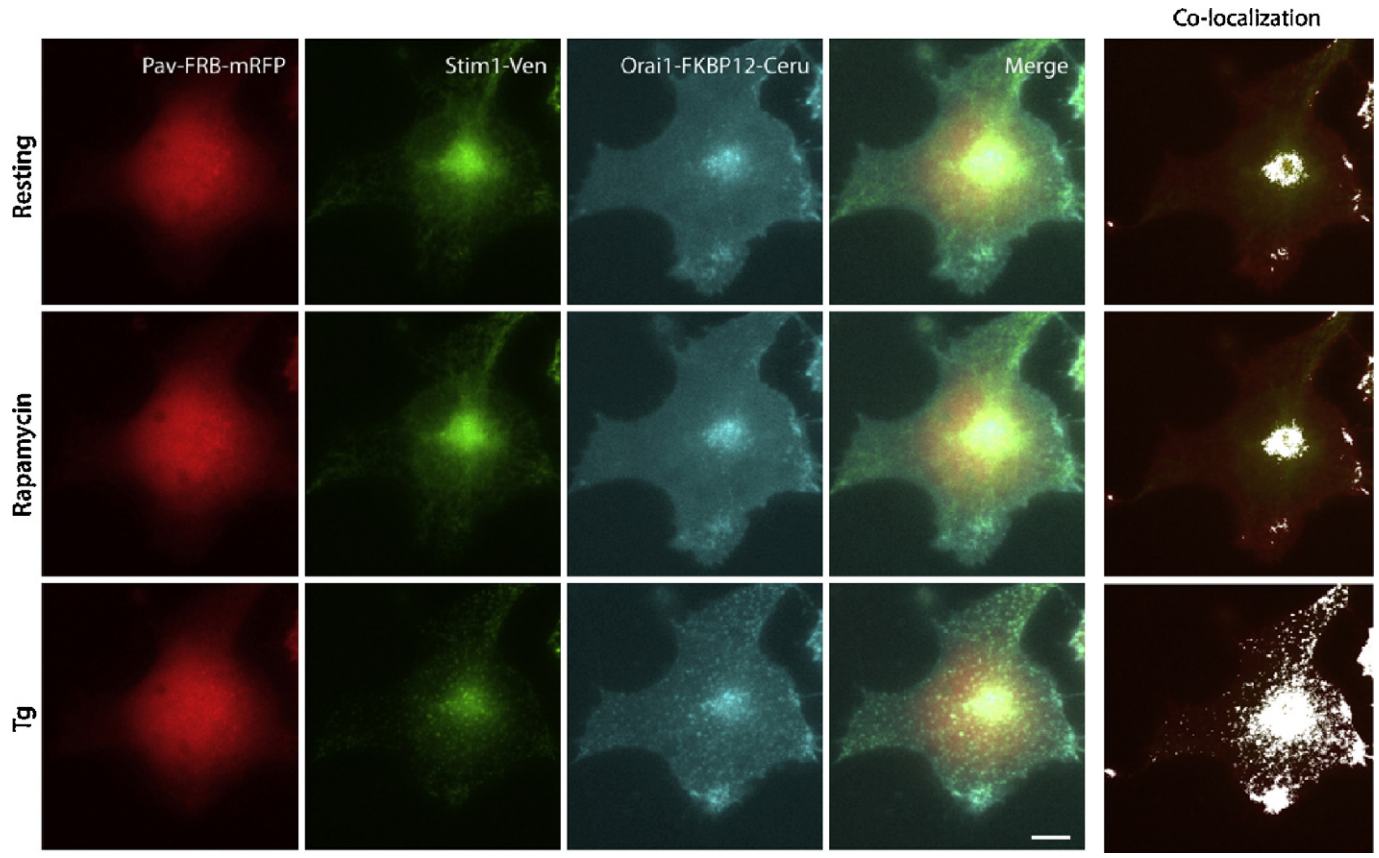

Fig. 7. Rapamycin-induced recruitment of Pav to Orai1 did not lead to puncta formation. Pav-FRB-mRFP was distributed throughout the cytoplasm, Stim1-Ven on the ER and Orai1-FKBP12-Ceru on the PM in resting Cos-7 cells. Addition of 1 μM rapamycin co-localized Pav-FRB-mRFP with Orai1-FKBP12-Ceru but did not lead to puncta formation. In contrast, a subsequent addition of 1 μM Tg induced puncta formation ($n = 9/9$ cells observed). Scale bar is 30 μm. Images are false color, with white overlays indicating sites of co-localization between Stim1-Ven and Orai1-FKBP12-Ceru. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Orai1-Ceru (Fig. 5). Similarly, rapamycin-induced recruitment of FRB-mRFP-Pav to Stim1-FKBP12-Ven showed an increase in puncta formation (Supp. Fig. 2).

As controls, we verified that rapamycin-induced recruitment of FRB-mRFP to Stim1-FKBP12-Ven did not form puncta (Supp. Fig. 5). Similarly, when Pav-FRB-mRFP was co-expressed with Stim1-Ven, Pav-FRB-mRFP remained cytoplasmic and did not cause puncta formation of Stim1-Ven upon addition of 1 μM rapamycin (Supp. Fig. 6). This suggests that the local recruitment of Pav to Stim1 (and not the presence of cytosolic Pav itself) was responsible for puncta formation.

In order to determine that puncta formation was due to the local Ca^{2+} buffering effects of Pav, we constructed the mutant variant Pav_{E101V} containing an inactivating substitution at position E101V essential for Ca^{2+} binding at the EF Ca^{2+}-binding site. This resulted in Pav_{E101V} having a reduced binding affinity for Ca^{2+} [28]. In resting Cos-7 cells expressing Pav_{E101V}-FRB-mRFP and Stim1-FRB-mRFP, Pav_{E101V}-FRB-mRFP was distributed throughout the cytoplasm similar to Pav-FRB-mRFP. Upon addition of 1 μM rapamycin, Pav_{E101V}-FRB-mRFP was recruited to Stim1-FKBP12-Ven within 5 min. However, puncta did not form after 30 min of additional imaging and the co-expressed Orai1-Ceru did not redistribute and remained at the PM. In contrast, the subsequent addition of 1 μM Tg induced puncta formation of Stim1-FKBP12-Ven within 10 min with Orai1-Ceru redistributing to these puncta junctions ($n = 9/9$ cells) (Supp. Fig. 7). This suggests that the Ca^{2+} buffering function of Pav was responsible for puncta formation. In addition, recruiting Pav-FRB-mRFP to Orai1-FKBP12-Ceru with 1 μM rapamycin also did not cause any puncta formation even in the presence of co-expressed Stim1-Ven ($n = 9/9$ cells) (Fig. 7). As expected, recruiting FRB-mRFP-Pav to Orai1-FKBP12-Ceru did not cause puncta formation (Supp. Fig. 2). Lastly, to determine if Ca^{2+} entry from Orai1 played a role in puncta formation, the mutant variant $Orai_{E106Q}$ was created that contains a substitution at position E106Q to abolish Ca^{2+} entry [29]. In Cos-7 cells expressing Pav-FRB-mRFP, Stim1-FKBP12-Ven and $Orai_{E106Q}$-Ceru, the addition of 1 μM rapamycin similarly induced puncta formation (Supp. Fig. 2). This suggests that local Ca^{2+} concentrations near the ER on the cytosolic side play a role in puncta formation.

3.4. Rapamycin-induced recruitment of Pav to Stim1 did not trigger an influx of Ca^{2+}

We next used TNXL [30], a Ca^{2+} biosensor based on fluorescence resonance energy transfer, to determine whether puncta formation due to the recruitment of Pav-FRB-mRFP to Stim1-FKBP12-Ven would cause a Ca^{2+} influx through Orai1-Ceru. Interestingly, however, a Ca^{2+} influx was not observed upon rapamycin-induced puncta formation ($n = 3/3$ cells) (Fig. 8A). Likewise, rapamycin-induced recruitment of Orai1-FRB-mRFP to Stim1-FKBP12-Ven did not result in Ca^{2+} entry ($n = 3/3$ cells) (Fig. 8B). To verify that Stim1-FKBP12-Ven and Orai1-Ceru fusion proteins were still able to mediate Ca^{2+} influx through SOCE, control Cos-7 cells expressing TNXL, Pav-FRB-mRFP, Stim1-FKBP12-Ven and Orai1-Ceru were pre-incubated with 1 μM rapamycin for 30 min in Ca^{2+}-free PBS. Cells were then stimulated with 1 μM Tg to deplete

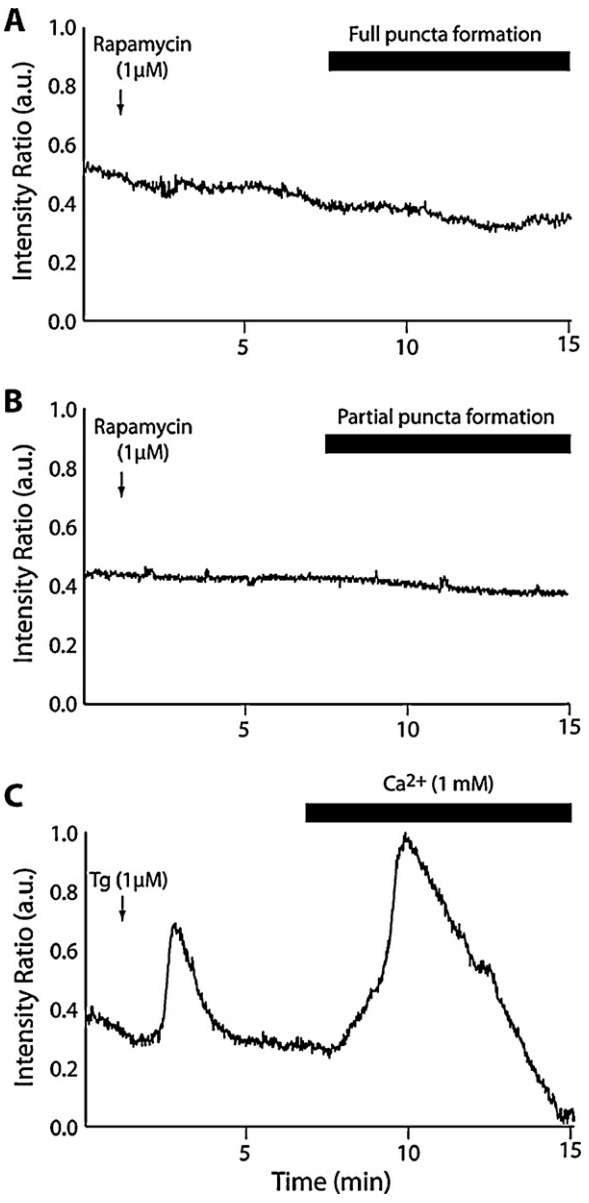

Fig. 8. Rapamycin-induced recruitment of Pav-FRB-mRFP or Orai1-FRB-mRFP to Stim1-FKBP12-Ven did not trigger an influx of Ca^{2+}. (A) In Cos-7 cells expressing TNXL, Pav-FRB-mRFP, Stim1-FKBP12-Ven and Orai1-Ceru, 1 μM rapamycin-induced puncta formation did not lead to a subsequent influx of Ca^{2+} ($n = 3/3$ cells). The bar indicates when partial puncta formation was completed (i.e. no changes in appearance of puncta). (B) In Cos-7 cells expressing TNXL, Orai1-FRB-mRFP and Stim1-FKBP12-Ven, 1 μM rapamycin-induced partial puncta formation did not lead to a subsequent influx of Ca^{2+} ($n = 3/3$ cells). Both experiments were performed in PBS with 1 mM calcium chloride. The bar indicates when full puncta formation was completed. (C) Cos-7 cells expressing TNXL, Pav-FRB-mRFP, Stim1-FKBP12-Ven and Orai1-Ceru were pre-incubated with 1 μM rapamycin to induce binding of Pav-FRB-mRFP to Stim1-FKBP12-Ven. The experiments were initially performed in PBS with no Ca^{2+}. Cells were then stimulated with 1 μM Tg to deplete intracellular Ca^{2+} stores. An influx of Ca^{2+} was observed upon addition of 1 mM Ca^{2+} to the extracellular medium ($n = 2/2$ cells observed). The bar indicates when Ca^{2+} was present.

intracellular Ca^{2+} stores. Upon addition of 1 mM Ca^{2+}, a corresponding Ca^{2+} influx was observed ($n = 2/2$ cells) (Fig. 8C). These results confirm that fusion proteins Stim1-FKBP12-Ven and Orai1-Ceru retained the ability to mediate Ca^{2+} influx. These results also suggest that the rapamycin-induced recruitment of Pav-FRB-mRFP to Stim1-FKBP12-Ven did not sterically interfere with the interaction between Stim1 and Orai1.

4. Discussion

In the present study, we have demonstrated that local Ca^{2+} buffering near the ER by Pav on the cytoplasmic side can induce puncta formation of Stim1 and Orai1, a previously unknown means of puncta formation. Pav is a Ca^{2+} buffer protein that has a 150–250 nM dissociation constant with Ca^{2+} in the presence of physiological concentrations of Mg^{2+} (0.5–1 mM) [31]. Since resting concentration of cytosolic Ca^{2+} is between 20 and 100 nM [31], Pav is mostly in the Ca^{2+}-free form and ready to bind Ca^{2+} when it increases. This, in effect, slows diffusion of Ca^{2+} ions. At the ER, Ca^{2+} is dynamically exported and imported in a homeostasis that can be disrupted when Tg inhibits Ca^{2+} import mechanisms leading to a rapid increase of cytosolic Ca^{2+} (Fig. 8C). The rapamycin-induced recruitment of Pav to the ER dynamically altered the flow of Ca^{2+} in and out of the ER, which led to puncta formation. Pav overexpression alone did not cause puncta formation because the homeostasis of ER Ca^{2+} transport likely occurred prior to imaging. Thus, the dynamic change in the local Ca^{2+} buffering was important for puncta formation. Our results suggest that Stim1 oligomerization and puncta formation may be additionally regulated by local Ca^{2+} levels near the ER membrane or by other Ca^{2+}-dependent proteins that interact with the cytoplasmic domains of Stim1. This is in line with studies that have shown a modulatory role of cytosolic Ca^{2+} for clustering of Stim1 [23] and reports suggesting the binding of C-terminal domains of Stim1 to auxiliary proteins [19,22,32]. Some binding partners of Stim1 have recently been determined including the microtubule-associated protein EB1 [33] and extracellular-signal-regulated kinases (ERK), which phosphorylate target sequences on Stim1 [34]. There may be as yet unidentified Ca^{2+}-dependent binding partners contributing to the regulation of puncta formation and SOCE activation.

Although the rapamycin-induced recruitment of Pav to Stim1 was sufficient to induce puncta formation of both Stim1 and Orai1 that resembled those formed with Tg, an influx of Ca^{2+} was not observed. Full activation of Orai1 and SOCE may require contribution from the luminal end of Stim1, which depends on store depletion and has been shown to control the extent of oligomerization [9,17]. Structurally, the luminal Ca^{2+}-sensing region consists of an EF-SAM domain [15]. The folded and unfolded state of this EF-SAM is important for regulating the oligomerization state of Stim1 [16]. With Ca^{2+} bound, the EF-SAM domain is bound tightly in a stable configuration. When ER stores are depleted, Ca^{2+} dissociates from the EF-SAM domain causing it to unfold and expose hydrophobic residues, which leads to rapid oligomerization of destabilized EF-SAM domains on different Stim1 proteins. Further aggregation and stability of Stim1 proteins is then mediated through the cytoplasmic C-terminal coiled-coil domains, which also contain a polybasic motif that targets Stim1 oligomers to puncta junctions [8,14]. Recent studies have demonstrated an autoinhibitory mechanism within the C-terminus of Stim1 [35–37]. This intramolecular occlusion prevents the interaction between Orai1 and the Stim1 Orai1-activating region (SOAR) domain. Korzeniowski et al. [36] suggest that it is likely conformational changes of Stim1 due to luminal Ca^{2+} changes regulate the unmasking of SOAR upon store depletion. Future studies may reveal the extent of contribution of the luminal and cytoplasmic domains to SOCE activation [8,14].

The rapamycin-induced coupling of mutant Stim1 proteins containing FRB or FKBP12 domains in place of the luminal Ca^{2+} sensing domains has been shown to cause oligomerization and puncta formation [9]. In contrast, we have shown that rapamycin-induced Stim1 association on the C-terminal end did not cause puncta formation, while the direct rapamycin-induced coupling of Stim1-FRB-mRFP and Orai1-FKBP12-Ceru led to the formation of small co-localized clusters that could subsequently fully form puncta with the addition of Tg. Studies have shown that Stim1

self-associates in cells with replete Ca^{2+} stores [9,18]. The cytosolic domains play roles in forming both resting oligomers and higher-order oligomers when stores are depleted [32]. However, resting oligomers do not spontaneously form puncta or activate Orai1. The observed small co-localized clusters of Stim1-FRB-mRFP and Orai1-FKBP12-Ceru in the presence of rapamycin may be small resting oligomers of Stim1 positioned close enough to the PM for rapamycin-induced coupling of the FRB and FKBP12 domains.

In summary, this study demonstrates the previously unknown effect of locally buffering cytoplasmic Ca^{2+} near the ER to induce puncta formation. Future studies to identify other Stim1-associated proteins will help to examine regulatory mechanisms that fine-tune the oligomerization and puncta formation of Stim1. Further information concerning regulatory mechanisms will also provide insights into why Stim1 puncta formation is not always correlated to the subsequent activation of Orai1 and SOCE. These regulatory mechanisms may also provide targets for therapeutic drugs for Ca^{2+}/SOCE-related pathologies [38,39].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ceca.2012.01.007.

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