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Ciara M. Walsh, Michael Chvanov, Lee P. Haynes, Ole H. Petersen, Alexei V. Tepikin, et al.. Role of phosphoinositides in STIM1 dynamics and store-operated calcium entry. *Biochemical Journal*, 2009, 425 (1), pp.159-168. 10.1042/BJ20090884 . hal-00479209

**HAL Id: hal-00479209**

**<https://hal.science/hal-00479209>**

Submitted on 30 Apr 2010

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# Role of phosphoinositides in STIM1 dynamics and store-operated calcium entry

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Running title: Phosphoinositides in STIM1 translocation and  $\text{Ca}^{2+}$  entry

Key words : STIM1, ORAI1, phosphoinositides,  $\text{PIP}_2$ ,  $\text{PIP}_3$ , store-operated calcium entry,  $\text{Ca}^{2+}$  signals, ER calcium

## Abbreviations :

2-DG, 2-deoxyglucose; ER-PM junctions, endoplasmic reticulum-plasma membrane (ER-PM) junctions; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FRB, fragment of mTOR that binds FKBP12; GFP, green fluorescent protein;  $\text{IP}_3$ , inositol-1,4,5-trisphosphate; olig, oligomycin; PI3K, phosphatidylinositol 3-kinase; PI4K, phosphatidylinositol 4-kinase; rapa, rapamycin; RFP, red fluorescent protein; SOCE, store operated  $\text{Ca}^{2+}$  entry; SOC, store-operated  $\text{Ca}^{2+}$  channel; thapsi, thapsigargin.

## Summary

$\text{Ca}^{2+}$  entry through store-operated  $\text{Ca}^{2+}$  channels involves the interaction at endoplasmic reticulum-plasma membrane (ER-PM) junctions of STIM and Orai. STIM proteins are sensors of the luminal ER  $\text{Ca}^{2+}$  concentration and following depletion of ER  $\text{Ca}^{2+}$ , they oligomerise and translocate to ER-PM junctions where they form STIM puncta. Direct binding to Orai proteins activates their  $\text{Ca}^{2+}$  channel function. It has been suggested that an additional interaction of the C-terminal polybasic domain of STIM1 with plasma membrane phosphoinositides could contribute to STIM1 puncta formation prior to binding to Orai. We investigated the role of phosphoinositides in the formation of STIM1 puncta and store-operated  $\text{Ca}^{2+}$  entry (SOCE) in response to store depletion. Treatment of HeLa cells with inhibitors of the phosphatidylinositol 3-kinase (PI3K) and phosphatidylinositol 4-kinase (wortmannin and LY294002) partially inhibited formation of STIM1 puncta. Additional rapid depletion of  $\text{PtdIns}(4,5)\text{P}_2$  resulted in more substantial inhibition of the translocation of STIM1-EYFP into puncta. The inhibition was extensive at a concentration of LY294002 (50  $\mu\text{M}$ ) that should primarily inhibit PI3K consistent with a major role for  $\text{PtdIns}(4,5)\text{P}_2$  and  $\text{PtdIns}(3,4,5)\text{P}_3$  in puncta formation. Depletion of phosphoinositides also inhibited SOCE based on measurement of the rise in intracellular  $\text{Ca}^{2+}$  concentration after store depletion. Overexpression of Orai1 resulted in a recovery of translocation of STIM1 into puncta following phosphoinositide depletion and under these conditions SOCE was increased to above control levels. These observations support the idea that phosphoinositides are not essential but contribute to STIM1 accumulation at ER-PM junctions with a second translocation mechanism involving direct STIM1/Orai interactions.

## Introduction

Store operated  $\text{Ca}^{2+}$  entry (SOCE) is the major mechanism for receptor-induced  $\text{Ca}^{2+}$  influx in non-excitabile cells and is important for  $\text{Ca}^{2+}$  dependent signalling events including cell growth and differentiation, secretion, gene expression and  $\text{Ca}^{2+}$  homeostasis [1]. Physiologically, activation of phospholipase C-coupled receptors in the plasma membrane generates inositol-1,4,5-trisphosphate ( $\text{IP}_3$ ), resulting in the subsequent release of  $\text{Ca}^{2+}$  from the endoplasmic reticulum (ER) through  $\text{IP}_3$  receptors. The depletion of  $\text{Ca}^{2+}$  from the ER store subsequently triggers  $\text{Ca}^{2+}$  influx through store operated  $\text{Ca}^{2+}$  channels (SOCs) in the plasma membrane to elevate intracellular free calcium concentration ( $[\text{Ca}^{2+}]_i$ ) [1]. Importantly, the channels are activated not by the production of receptor-associated signalling molecules but by the decrease in luminal ER  $\text{Ca}^{2+}$  content [2], involving a coupling between the ER and the plasma membrane (PM).

Although SOCE and its associated current,  $I_{\text{CRAC}}$ , was discovered a number of years ago [1], only recently has the molecular mechanism responsible for this ER-PM coupling been uncovered. Recently the ER  $\text{Ca}^{2+}$  sensing protein, STIM1 [3] [4], and the plasma membrane channel, Orai1 [5] [6, 7], were identified in high throughput RNAi screens as proteins which are essential for the activation of SOCs. STIM1 is normally distributed throughout the ER [4, 8] [9] where its N-terminal EF hand binds to  $\text{Ca}^{2+}$  within the ER lumen. Depletion of  $\text{Ca}^{2+}$  from within the ER lumen triggers the release of  $\text{Ca}^{2+}$  from the EF hand of STIM1, which induces the oligomerisation of the EF-SAM domains of STIM1 within the ER [10-12] and the subsequent movement of STIM1 to ER-PM junctions where it forms aggregates known as “puncta” [4, 9]. STIM1 puncta at these sites interact with and induce the clustering of Orai1 channel subunits to form active SOC channels [13] [14]. Mutational analysis of the interacting domains of STIM1 revealed that the cytoplasmic domain of STIM1 is necessary and sufficient for the activation of SOCE [15]. Furthermore, several recent studies have identified a minimal region within the cytoplasmic domain of STIM1 which is essential for the clustering of Orai1 and the activation of SOCs [16-20]. However, the STIM1 protein also contains a short lysine-rich polybasic domain at its extreme C-terminus. Intriguingly, in some studies mutation or deletion of the STIM1 polybasic domain has been shown to reduce puncta formation, SOCE and  $I_{\text{CRAC}}$  [8, 16, 17, 21], suggesting that the polybasic domain may be involved in STIM1 targeting to the plasma membrane, possibly through an interaction with some, as yet unidentified, plasma membrane component [8, 16, 21].

There are several lines of evidence to suggest that phosphorylated inositol lipids in the plasma membrane may regulate SOCE. Firstly, depletion of phosphoinositides has been shown to block both SOCE and  $I_{\text{CRAC}}$  in several cells types [22-24]. In addition, a recent study uncovered a requirement for both  $\text{PtdIns}(4,5)\text{P}_2$  and  $\text{PtdIns}(3,4,5)\text{P}_3$  to regulate the plasma membrane localisation of many proteins which contain a cluster of polybasic amino acids within their C-termini [25]. The polybasic region of STIM1 would make it an excellent candidate as a membrane-targeting domain through binding to phosphoinositides [8], although other reports have argued against a role for

phosphoinositides in STIM1 translocation [26]. The current study aimed to investigate the role of the phosphoinositides in the translocation of STIM1 and the activation of SOCE. Our results suggest that the phosphoinositides are plasma membrane components responsible for targeting STIM1 to ER-PM junctions and reveal a role for the phosphoinositides in the activity of SOCs.

## Materials and Methods

### Plasmids

The plasmids for the rapamycin-inducible phosphatase system were gifts from Tamas Balla (NICHD, Bethesda). The PH domain of phospholipase C<sub>δ1</sub> cloned into GFP-C1 was a gift from Matilda Katan (Imperial Cancer Research Fund, London, UK). For EYFP-Rit and mCherry-Rit plasmid construction, total RNA was extracted from approximately 100 × 10<sup>6</sup> HeLa cells using Trizol reagent (Invitrogen, The Netherlands) and used as a template for reverse transcription using Improm-II reverse transcriptase (Promega, Southampton, UK) and oligo-dT<sub>(15)</sub> priming. The resulting single stranded cDNA was then used as a template in PCR reactions to amplify the full length Rit1 protein. The full length Rit1 was then used as a template to PCR amplify the polybasic Rit tail (nucleotides 770 – 847) using specific primers containing restriction endonuclease sites to permit subsequent sub-cloning into the N-terminally tagging pEYFP-C1 vector (Clontech, Palo Alto, USA) or into the N-terminally tagging mCherry-C1 vector (gift from Roger Y. Tsien, University of California at San Diego). The primers for the Rit tail were based on the human sequence with Genbank accession number: NM\_006912. For pcDNA-Orai1 plasmid construction, the previously described mCherry-Orai1 plasmid [27] was used as a template to amplify full-length Orai1 using specific primers containing restriction endonuclease sites, allowing for subsequent sub-cloning into the pcDNA3.1(-) vector (Invitrogen, USA). For pCerulean-Orai1 plasmid construction, the Cerulean colour tag was excised from the pCerulean-C1 vector (a gift from David W Piston, Vanderbilt University Medical Centre, Tennessee) by digestion using the AgeI and BsrGI restriction endonucleases and ligated into the mCherry-Orai1 vector to replace the mCherry tag. The STIM1-EYFP plasmid was described previously [27] and a STIM1-ECFP plasmid was constructed in a similar manner.

### Chemicals and Reagents

Cell culture reagents were purchased from Invitrogen (Paisley, UK), except for GeneJuice, which was purchased from Merck Biosciences (Darmstadt, Germany). Thapsigargin, oligomycin, wortmannin and rapamycin were purchased from Calbiochem (San Diego, CA, USA). Fluo4-AM was from Invitrogen/Molecular Probes. All other chemicals were purchased from Sigma (Gillingham, UK).

### Cells and Transfection

HeLa cells were maintained in DMEM supplemented with 10% FBS, 1% penicillin and streptomycin and 1% MEM nonessential amino acids. Before transfection, cells

were grown to 80-90% confluency in 35-mm Petri dishes with glass bottoms (MatTek, Ashland, MA, USA). Plasmids and transfection reagents were added directly to the petri dishes. Single transfection and co-transfections were carried out using 3  $\mu$ g plasmid(s) of interest in total per dish, supplemented with 8  $\mu$ l GeneJuice.

### Solutions

The standard extracellular solution used during all experiments contained: 140 mM NaCl, 4.7 mM KCl, 2 mM  $\text{CaCl}_2$ , 1.13 mM  $\text{MgCl}_2$ , 10 mM glucose, 10 mM HEPES (pH 7.3). For  $\text{Ca}^{2+}$  imaging experiments, cells were treated with 2  $\mu$ M thapsigargin in  $\text{Ca}^{2+}$ -free standard solution to deplete intracellular  $\text{Ca}^{2+}$  stores and subsequently perfused with standard solution containing 5 mM  $\text{Ca}^{2+}$  to permit  $\text{Ca}^{2+}$  influx. All experiments were performed at room temperature. Cells were washed three times in standard extracellular solution and mounted in a perfusion chamber on the stage of an inverted microscope prior to imaging.

### Confocal Microscopy and image analysis

Confocal images were acquired using a Leica AOBs SP2 microscope (Leica Microsystems AG, Wetzlar, Germany) equipped with a 63x water immersion objective (N.A. 1.2) or a 63x oil immersion objective (N.A. 1.4). Images of STIM1-EYFP were obtained with 496 nm excitation light and emission fluorescence selected with a 505-550 nm bandpass filter. For excitation of RFP-5-phosphatase and mCherry-Rit constructs, a 594 nm laser line was used and emission fluorescence was collected between 610-700 nm. For GFP imaging, cells were excited with the 488 nm laser line and emission was collected between 500 and 515 nm. For CFP or Cerulean tagged proteins, 405 nm excitation was used and emission fluorescence collected between 450 – 490 nm. Images were processed using the CorelDraw software package (Corel Corporation, Ottawa, Canada) or ImageJ (NIH). To determine the extent of fluorescence associated with the plasma membrane regions of interest were drawn around the outside of each cell and immediately beneath the cell periphery. Peripheral fluorescence was calculated by subtraction of cytoplasmic fluorescence from the outer region of interest. Fluorescence values were calculated on the basis of the mean fluorescence per pixel in the respective regions rather than total fluorescence to avoid problems arising from variations in the size of the selected regions of interest. For quantification of STIM1-EYFP puncta formation, the puncta were selected as spots of high fluorescence intensity ranging from approximately 0.5 – 1.0  $\mu$ m in diameter. Accuracy of puncta quantification was verified by independent blind counting.

### Measurement of intracellular free $\text{Ca}^{2+}$ concentration

HeLa cells were loaded with 5  $\mu$ M Fluo-4 AM for 30 min at room temperature to measure changes in ( $[\text{Ca}^{2+}]_i$ ) and imaged using a Leica AOBs SP2 confocal microscope. Fluo-4 was excited using a 488 nm laser and emission fluorescence was collected between 495-545 nm. To allow for comparison between different cells from different experiments, Fluo-4 fluorescence measurements for each cell were calculated as the ratio of the fluorescence at the first time point and to eliminate variability between experiments in the resting fluorescence level these values are shown as F/F0 normalised to the peak ratio for the control cells in each experiment..



## RESULTS

### **STIM1 can translocate to puncta following ATP depletion and loss of the phosphoinositides PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>**

The phosphoinositides PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> have been suggested to bind the polybasic domain of STIM1 and target it to ER-PM junctions [8]. We have shown previously that STIM1 translocation to puncta is stimulated by ATP depletion which reduced the levels of PtdIns(4,5)P<sub>2</sub> at the plasma membrane [27]. We investigated whether STIM1 puncta formation is dependent on PtdIns(3,4,5)P<sub>3</sub> by treating cells with wortmannin prior to ATP depletion. We saw translocation into the cytosol of the polybasic tail from the small-GTPase Rit linked to mCherry as a marker for plasma membrane PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> [25]. STIM1 puncta formation was, however, still observed after ATP depletion (data not shown).

### **Activation of the rapamycin-inducible phosphatase results in rapid depletion of plasma membrane PtdIns(4,5)P<sub>2</sub>**

In the previous experiment, methods were used which would gradually decrease PtdIns(4,5)P<sub>2</sub> levels over a period of time as ATP was depleted. The results could suggest that PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> are not essential for targeting STIM1 to the plasma membrane but it is possible that depletion of PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> under these conditions was slow. We, therefore, investigated the potential role of phosphoinositides in STIM1 puncta formation using a more specific and more rapid approach to deplete the levels of PtdIns(4,5)P<sub>2</sub> at the plasma membrane. To enable fast depletion of PtdIns(4,5)P<sub>2</sub> we aimed to use a previously described inducible type IV 5-phosphatase to specifically hydrolyse PtdIns(4,5)P<sub>2</sub> [25, 29]. This system is based on the rapamycin-stimulated heterodimerisation of two proteins: the fragment of mTOR that binds FKBP12 (FRB) and FKBP12 itself. In this system, FRB contains a plasma membrane localisation signal and also a CFP (PM-FRB-CFP) or RFP (PM-FRB-RFP) tag to monitor its localisation. The FKBP12 protein is fused to a type IV 5-phosphatase and an RFP tag (RFP-ptase-dom) and is distributed throughout the cytosol where it is inactive in the absence of rapamycin. In order to test the ability of this phosphatase system to alter PtdIns(4,5)P<sub>2</sub> levels in our hands, HeLa cells were co-transfected with the GFP-tagged PH domain of phospholipase C<sub>δ1</sub> (GFP-PH-PLC) to monitor PtdIns(4,5)P<sub>2</sub> at the plasma membrane, along with the PM-FRB-CFP and RFP-ptase-dom constructs (Figure 1A). Perfusion with rapamycin (1 μM) resulted in the rapid translocation of the RFP-ptase-dom from the cytosol to the plasma membrane and the dissociation of GFP-PH-PLC from the plasma membrane (Figure 1B). Quantification of the fluorescence intensity at the plasma membrane showed that dissociation of GFP-PH-PLC is complete within 2 min with no further decrease in membrane fluorescence after 2 min (Figure 1B, n = 6), suggesting that PtdIns(4,5)P<sub>2</sub> depletion is rapid and complete within this timeframe. This method therefore can be used as a robust means of specifically and rapidly depleting PtdIns(4,5)P<sub>2</sub> at the plasma membrane.

### **Neither PtdIns(4,5)P<sub>2</sub> nor PtdIns4P/PtdIns(3,4,5)P<sub>3</sub> alone are essential for targeting STIM1 to puncta**

To test whether STIM1 accumulates in puncta via an interaction with plasma membrane PtdIns(4,5)P<sub>2</sub>, cells were transfected with STIM1-EYFP along with RFP chimeras of both the PM-FRB-RFP and RFP-ptase-dom constructs. In this case, there was some plasma membrane fluorescence from the RFP-PM-FRB construct prior to the addition of rapamycin (Figure 2A, top right) but a clear translocation of the RFP-ptase-dom from the cytosol to the plasma membrane was visible following rapamycin treatment (Figure 2A bottom right). (Note that it was necessary to use RFP fusion proteins of both the plasma membrane bound FRB and the 5-ptase domain so that in future experiments we could simultaneously monitor these proteins along with the localisation of Cerulean-Orai1 (Cer-Orai1) and STIM1-EYFP proteins in the same cell.) Cells were perfused with rapamycin for 1 min to activate the phosphatase. Further perfusion with a combination of rapamycin and the SERCA pump inhibitor, thapsigargin (2  $\mu$ M), to deplete intracellular stores failed to prevent formation of STIM1 puncta (Figure 2A, bottom left, n = 8). Quantification of the number of puncta per cell indicated, however, a 40% reduction in the mean number of puncta per cell following PtdIns(4,5)P<sub>2</sub> depletion (Figure 5) indicating a partial contribution of this lipid. In cells transfected with STIM1-EYFP alone, thapsigargin-induced STIM1 translocation was observed after pre-incubation with 20  $\mu$ M wortmannin to inhibit both PtdIns 3-kinase (PI3K) and PtdIns 4-kinase (PI4K) and deplete PtdIns4P/PtdIns(3,4,5)P<sub>3</sub> (Figure 2B, right, n = 20) but the mean number of puncta was again reduced by around 40% (Figure 5) arguing that PtdIns4P and PtdIns(3,4,5)P<sub>3</sub> are not essential but also contribute to STIM1 puncta formation

### **Phosphoinositides together regulate STIM1 translocation to puncta**

Although the previous experiments suggest that PtdIns4P, PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> contribute to but are not the sole regulators of STIM1 clustering in puncta, they do not rule out the possibility that these lipids in combination can contribute to targeting STIM1 to ER-PM junctions. To test this, HeLa cells were cotransfected with STIM1-EYFP, RFP-PM-FRB and RFP-ptase-dom and pretreated with 20  $\mu$ M wortmannin for 30 mins (Figure 2C) to inhibit PI3K and PI4K. Addition of rapamycin for 1 min induced translocation of the phosphatase to the membrane. Interestingly, additionally depleting PtdIns(4,5)P<sub>2</sub> by this method appeared to essentially abolish thapsigargin-induced STIM1 translocation into puncta (Figure 2C, n = 22). Quantification of STIM1 puncta (Figure 5) indicated a 94% reduction in the mean number of puncta per cell under these conditions that was significantly larger than the inhibition due to wortmannin alone suggesting that multiple phosphoinositides have a collective role in regulating STIM1 accumulation at ER-PM junctions.

### **PtdIns(4,5)P<sub>2</sub> depletion further inhibits STIM1 translocation into puncta in cells treated with the PI3K/PI4K inhibitor LY 294002**

In order to confirm the results found with wortmannin similar experiments were carried out with the alternative lipid kinase inhibitor LY294002 using HeLa cells cotransfected with STIM1-EYFP, RFP-PM-FRB and RFP-ptase-dom. In these



experiments LY294002 was used at two different concentrations at which it has been reported to inhibit PI3K alone (50  $\mu$ M) or both PI3K and PI4K (300  $\mu$ M) [28]. Following pretreatment with the inhibitor alone translocation into puncta was still observed in response to thapsigargin treatment although the mean number of puncta per cell were reduced especially at the high concentration of LY294002 (Figure 3A, 5). When cells were additionally treated with rapamycin to deplete PtdIns(4,5)P<sub>2</sub> levels STIM1 translocation to puncta was further inhibited and indeed was barely detectable especially at the higher concentration of LY294002 (Figure 3B,C). Quantification of the number of puncta formed per cell (Figure 5) indicated a significantly larger effect after rapamycin treatment compared to LY294002 alone. There was a 75% reduction in mean number of puncta per cell at the lower concentration of LY294002 after phosphatase activation with a further reduction at the higher concentration (90% inhibition). The large effect of the low concentration of LY294002 at which PI3K would be inhibited is consistent with major contributory roles for the lipids, PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> in puncta formation.

### **Overexpression of Orai1 rescues STIM1 puncta formation following phosphoinositide depletion**

It is possible that STIM1 is targeted to phospholipids in the plasma membrane via its polybasic domain. Deletion or mutation of this domain has been reported in several studies to prevent STIM1 puncta formation and SOC activation [8, 15]. It has recently been shown, however, that overexpression of Orai1 can rescue puncta formation and SOC activation under such conditions due to interaction of a separate domain within the C-terminus of STIM1 directly with Orai1 [16]. We therefore tested the prediction that STIM1 puncta formation would be restored in cells over-expressing Orai1 in addition to STIM1 under conditions where phosphoinositides are depleted. In order to do this, HeLa cells were cotransfected with STIM1-EYFP, Cer-Orai1, RFP-PM-FRB and RFP-ptase-dom and preincubated with wortmannin (Figure 4A). Translocation of the phosphatase was then stimulated by the addition of rapamycin, followed by the perfusion of cells with a combination of rapamycin and thapsigargin to deplete internal stores. Interestingly, translocation of STIM1-EYFP now occurred despite the depletion of phosphoinositides in cells where Cer-Orai1 was also overexpressed (Figure 4(B),  $n = 21$ ) with the mean number of puncta per cell not significantly different from control untransfected cells treated with thapsigargin (Figure 5). These data agree with previous reports which support a role for the polybasic region of STIM1 in targeting STIM1 to the plasma membrane but that a second domain within Orai1 can interact with STIM1 to independently target it to the plasma membrane for SOC activation [16-18].

### **Phosphoinositides contribute to STIM1-mediated store-operated Ca<sup>2+</sup> entry**

A number of recent investigations point to a crucial role for phosphoinositides in the activity of SOCs [22, 23, 30]. To determine whether the inhibition of STIM1 puncta formation by phosphoinositide depletion affects SOCE, we monitored [Ca<sup>2+</sup>]<sub>i</sub> in HeLa cells expressing STIM1-ECFP and the RFP-PM-FRB and RFP-ptase-dom constructs by loading them with the Ca<sup>2+</sup> indicator Fluo4-AM. The cells were pretreated with wortmannin for 30 min. At the beginning of each recording, cells were perfused with

either  $\text{Ca}^{2+}$ -free solution or  $\text{Ca}^{2+}$ -free solution containing rapamycin. Thapsigargin was subsequently added to deplete intracellular stores. SOCE could be activated by reintroducing  $\text{Ca}^{2+}$  to the solution and  $[\text{Ca}^{2+}]_i$  was then compared between cells which had been treated with and without rapamycin. Thapsigargin induced efficient SOCE leading to an increase in  $[\text{Ca}^{2+}]_i$  in control cells untreated with rapamycin (Figure 6A, upper trace;  $n = 27$ ). Influx was, however, greatly reduced in cells where phosphoinositides had been depleted (Figure 6A, lower trace,  $n = 18$ ). This significant reduction was also indicated by comparison of the peak fluorescence ratio values or each trace (Figure 6A) showing a 60% reduction after phosphoinositide depletion. These data suggest that phosphoinositides at the plasma membrane contribute to SOCE under these conditions.

### **Overexpression of Orai1 restores $\text{Ca}^{2+}$ influx in cells depleted of phosphoinositides**

In Figure 4 it was shown that overexpression of Orai1 recovers STIM1 puncta formation in cells depleted of phosphoinositides. To investigate whether SOCE is also restored under these conditions, cells were cotransfected with STIM-EYFP and the RFP phosphatase system as before but in this case untagged Orai1 was overexpressed in the same cells. Cells were loaded with Fluo-4 and  $[\text{Ca}^{2+}]_i$  was recorded as described above. Surprisingly, the increase in  $[\text{Ca}^{2+}]_i$  due to  $\text{Ca}^{2+}$  influx in cells depleted of phosphoinositides was not only recovered by Orai1 overexpression but was significantly higher in rapamycin treated (Figure 6B upper trace;  $n=15$ ) than control cells (Figure 6B, lower trace;  $n=18$ ) untreated with rapamycin. These data suggest that, although phosphoinositides contribute to SOCE, they are not absolutely essential for  $\text{Ca}^{2+}$  influx and agree with previous reports that suggest that the interaction between STIM1 and Orai1 is the key for activation of SOCE. In these conditions,  $\text{Ca}^{2+}$  influx appeared to be significantly greater following depletion of phosphoinositides which may suggest an additional negative role for phosphoinositides in SOCE.

### **Phosphoinositides contribute to store-operated $\text{Ca}^{2+}$ entry mediated by endogenous STIM1.**

The experiments described above on SOCE were carried out in cells overexpressing STIM1. In order to test whether SOCE mediated by endogenous STIM1 was affected by phosphoinositides depletion, cells were transfected with only the RFP phosphatase system and pretreated with wortmannin. The response to  $\text{Ca}^{2+}$  readdition after thapsigargin treatment was monitored after treatment with rapamycin. A direct comparison was made between cells in the same microscope fields that were non-transfected (-ptase) and acted as controls and transfected (+ptase) cells. This experiment also rules out any effects of rapamycin itself as all cells were exposed to the drug. The data (Figure 7) indicated that phosphoinositide depletion also inhibited SOCE mediated by endogenous STIM1.

## DISCUSSION

Recent reports have established that the ER protein, STIM1, regulates SOCE by translocating to ER-PM junctions following intracellular store depletion and that this can be visualised as the formation of STIM1 puncta [9, 31, 32]. The site of STIM1 puncta at the plasma membrane is critical for SOCE and STIM1 clustering has been suggested to be in part determined by lipid raft domains which anchor it to the plasma membrane [33], but the constituent(s) within the plasma membrane responsible for this have not been identified. Phosphoinositides might be involved in STIM1 translocation as several studies have pointed to a role for plasma membrane phosphoinositides in the activity of SOC<sub>s</sub> [22-24].

In the present study, we investigated whether the phosphoinositides are required for STIM1 puncta formation. We used several methods to individually or simultaneously reduce PtdIns4P, PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> levels and studied the effects of phosphoinositide depletion on the formation of STIM1 puncta at ER-PM junctions triggered by Ca<sup>2+</sup> store depletion. We also investigated the consequence of phosphoinositide depletion on STIM1-mediated SOCE. Initially, depletion of phosphoinositides was carried out using a combination of both high dose wortmannin treatment at levels which have been shown to inhibit both PI3K and PI4K [28] and ATP depletion [27]. Under these conditions, STIM1 translocation still occurred even though levels of PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> were reduced as shown by dissociation of the mCherry-Rit reporter from the plasma membrane before the first signs of puncta formation (data not shown). Although this argues against a role for the phosphoinositides in the translocation of STIM1, it is possible that since ATP depletion would alter the state of phosphorylation of many proteins, this treatment may have other effects on the localisation of STIM1 such as uncovering an inhibitory mechanism which normally prevents the aggregation of STIM1 oligomers in puncta. Alternatively ATP depletion may not deplete the phosphoinositides sufficiently or rapidly enough. It was clear that a more specific and more rapid method for the depletion particularly of PtdIns(4,5)P<sub>2</sub> was required to investigate the specific roles of each of the phosphoinositides in STIM1 translocation. For this reason, we used an inducible phosphatase system, which has recently been reported to specifically and reliably deplete PtdIns(4,5)P<sub>2</sub> levels [29, 34]. The data presented here reveal a contribution of phosphoinositides to the recruitment of STIM1, since puncta formation was inhibited under conditions that would deplete PtdIns4P, PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>. Interestingly, puncta formation was partially affected by separate depletion of either PtdIns(4,5)P<sub>2</sub> or PtdIns4p/PtdIns(3,4,5)P<sub>3</sub> but depletion of all lipids was required for an essentially complete inhibition of puncta formation suggesting that STIM1 can interact with multiple phosphoinositides in the process of puncta formation.

These data complement previous reports which have shown that the STIM1 polybasic domain plays a role in STIM1 function at the plasma membrane [8, 16, 17, 21]. Several hypotheses have been put forward for the function of the polybasic domain in STIM1. Some have suggested that it interacts directly with Orai1 to regulate the activity of the channel [17], while others have proposed that it is targeted to the

plasma membrane by an interaction with some constituent(s) within the plasma membrane itself [8]. It has been suggested that the polybasic domain may direct the recruitment of STIM1 to ER-PM junctions by binding directly to the phosphoinositides PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> via electrostatic interactions as is the case for small GTPases with polybasic tails [25]. Our studies are consistent with this hypothesis.

The data presented here also show that the phosphoinositides also contribute to SOCE since depletion of phosphoinositides significantly reduced thapsigargin induced Ca<sup>2+</sup> entry. Extensive phosphoinositide depletion resulted in a 94% reduction in STIM1 puncta formation but only a 60% overall reduction in the peak Fluo4 fluorescence ratio. It should be noted, however, that the fluorescence ratio is not linearly related to [Ca<sup>2+</sup>]<sub>i</sub> leading to an underestimate of the degree of inhibition of SOCE and suggesting that phosphoinositides do play a crucial role in this conditions. The partial inhibitory effects of depletion of individual phosphoinositides on STIM1 puncta formation is consistent with previous findings on the effects of inhibition of PI3K [35] and PI4K [36, 37] on agonist-induced Ca<sup>2+</sup> influx. In addition, some studies have reported the inhibition of SOCE and *I*<sub>CRAC</sub> by the simultaneous inhibition of PI3K/PI4K [22, 24]. Broad et al [22] used high levels of wortmannin to inhibit both PI3K and PI4K and consequently blocked both Ca<sup>2+</sup> influx and *I*<sub>CRAC</sub> in rat basophilic leukemia cells. The authors noted that this effect was not due to a decrease in IP<sub>3</sub> and DAG production but could not determine the precise mechanism for the phosphoinositides in SOC activity. Others [24] used LY294002 to inhibit PI3K/PI4K activity in platelets and suggested that they are involved in mediating Ca<sup>2+</sup> entry through a mechanism which involves reorganisation of the actin cytoskeleton following store depletion. However, our data imply that it is probable that the phosphoinositides contribute to the regulation of Ca<sup>2+</sup> influx through the recruitment of STIM1 to the plasma membrane following store depletion, since STIM1 puncta formation was substantially inhibited when plasma membrane phosphoinositides are depleted by treatment with either wortmannin or LY294002 and hydrolysis of PtdIns(4,5)P<sub>2</sub> by an inducible phosphatase. The lower concentration of LY294002 that was used in the present study has been reported to have little effect on PI4K [28]. We established that at the low (50 μM) concentration of LY294002, dissociation from the Golgi complex of the PtdIns4P-specific PH domain reporter FAPPI [38] as a marker of PI4K inhibition was 2.5 fold less than at the high (300 μM) concentration (data not shown). The effectiveness of the lower concentration of LY294002 in inhibiting STIM1 puncta formation is consistent with key roles for the two lipids PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> although we cannot rule out an additional contribution of PtdIns4P.

A recent study [16] showed that a truncated STIM1 protein lacking the polybasic domain fails to form puncta when expressed alone in HEK293 or HeLa cells but does redistribute into puncta and activate *I*<sub>CRAC</sub> when coexpressed with Orail in the same cells reconciling different effects reported of deletion of this domain. This led to the proposal of two separate mechanisms for the recruitment of STIM1 to the plasma membrane. STIM1 can be anchored to the plasma membrane via a direct interaction of



a region within its C-terminal ERM domain with Orai1 [16-20]. Alternatively, STIM1 can be recruited to ER-PM junctions via an Orai1-independent mechanism involving its polybasic domain [8, 16]. It has been proposed that under physiological conditions, the polybasic domain may initially direct STIM1 oligomers to ER-PM junctions and thereby facilitate its interaction with and activation of Orai1 [16]. This dual targeting mechanism seems to be a vertebrate adaptation since the polybasic domain is present only in vertebrate STIM homologues. In this study, we observed that STIM1 can effectively form puncta in cells depleted of plasma membrane phosphoinositides when Orai1 is over-expressed in the same cells, suggesting that the binding of STIM1 to the phosphoinositides is not essential for puncta formation. We also tested whether SOCE was recovered following phosphoinositide depletion by over-expression of Orai. Note that in our experiments over-expression of both STIM1 and Orai constructs in control cells did not produce an increase in SOCE above that seen with STIM1 alone as described in other studies [39-41]. Previous observations have however been derived from study of HEK293 cells in which there is little endogenous SOCE [39-41] and presumably low levels of endogenous Orai. Our results showed that Orai over-expression resulted in a recovery of SOCE in cells depleted of phosphoinositides.

Our observations support the hypothesis that there are two mechanisms for STIM1 targeting to the plasma membrane. In addition,  $\text{Ca}^{2+}$  influx was restored to above that of controls level in these cells. These data agree with several recent reports which suggest that the interaction between the ERM domain of STIM1 and Orai1 is necessary and sufficient for the activation of SOCs and does not require the polybasic domain [16-20]. Our observations also suggest two potential functions for the phosphoinositides in SOCE. Under physiological conditions, the phosphoinositides may contribute to the activation of SOCE by enhancing recruitment of STIM1 to ER-PM junctions, thereby increasing the likelihood of an interaction between STIM1 and Orai1 for the activation of  $\text{Ca}^{2+}$  influx. Additionally, they might regulate the ability of STIM1 to activate SOCs when bound to Orai1, since  $\text{Ca}^{2+}$  influx is enhanced in the absence of the phosphoinositides in cells coexpressing Orai1. It is possible that in the absence of the phosphoinositides, the ERM domain of STIM1 is exposed, resulting in enhanced binding of the ERM domain to Orai1 and consequently increased  $\text{Ca}^{2+}$  influx. Enhanced activation of SOCS by a STIM1 protein, which lacks a functional polybasic domain has previously been observed in cells coexpressing Orai1 [17]. This regulatory mechanism may therefore be required under physiological conditions to prevent over-activation of the channels and may have evolved in vertebrates as an additional mechanism to protect cells against  $\text{Ca}^{2+}$  toxicity.

A study which appeared while this paper was in preparation, investigated the role of the phosphoinositides in STIM1 and Orai1 mediated  $\text{Ca}^{2+}$  entry and found only a small effect of phosphoinositide depletion on STIM1 puncta [42]. The experimental approach used in that study differed from that in the present paper since STIM1 translocation and puncta formation were stimulated before subsequently depleting the phosphoinositides using a combination of the  $\text{PtdIns}(4,5)\text{P}_2$ -specific inducible phosphatase system and high concentration of LY294002. It is possible that in these experiments, the preformed STIM1 puncta might render  $\text{PtdIns}(4,5)\text{P}_2$  inaccessible to



the phosphatase, thereby preventing its efficient hydrolysis. In the present report, stimulation of STIM1 puncta formation by store depletion, was only carried out after the cells were treated with a combination of either wortmannin or LY294002 and the inducible phosphatase. This would, thereby, ensure a reduction in the levels of plasma membrane phosphoinositides prior to puncta formation. This method proved to be effective in preventing STIM1 puncta formation. Korzeniowski et al [42] showed that PLC activation or PI3K/PI4K inhibition by LY294002 reduced both  $I_{CRAC}$  and SOCE in COS-7 cells, even under conditions where PtdIns(4,5)P<sub>2</sub> levels remained unchanged, suggesting that PtdIns(4)P rather than PtdIns(4,5)P<sub>2</sub> is the major phosphoinositide required for activation of SOC<sub>s</sub>. Our studies suggest that PtdIns(4,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> are the key lipids involved in targeting STIM1 to ER-PM junctions.

In summary, the present study confirm that there are two distinct modes of STIM1 translocation to the plasma membrane. One involves a direct interaction between STIM1 and Orai1, the other requiring plasma membrane phosphoinositides. We propose two functions for this interaction in the regulation of SOCE. Initially, the phosphoinositides may target STIM1 to ER-PM junctions to facilitate its subsequent interaction with Orai1 at these sites. In addition, the potential binding of the polybasic domain of STIM1 to phosphoinositides may regulate the activity of the Orai1 channel by determining the extent of STIM1-Orai1 binding. Further studies are required to confirm a direct interaction between STIM1 and the various phosphoinositides and to determine the extent to which each phosphoinositide species contributes to the activity of store operated Ca<sup>2+</sup> channels.

### Acknowledgements

This work was supported by a Wellcome Trust Prize Ph.D. studentship to C. M. W. and an MRC programme grant to A.V.T. and O.H.P. The authors are grateful to Tamas Balla (NICHD, Bethesda), Matilda Katan (Imperial Cancer Research Fund, London, UK), David W Piston (Vanderbilt University Medical Centre, Tennessee) and Roger Y. Tsien (University of California at San Diego) for the gifts of plasmids used in this work.

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## Figure Legends.

**Figure 1. Activation of the inducible phosphatase results in rapid and efficient depletion of PtdIns(4,5) $P_2$ .** HeLa cells were cotransfected with the GFP-PH-PLC, PM-FRB-CFP and RFP-ptase-dom constructs. 24h post-transfection, cells were treated with rapamycin (1  $\mu$ M). Rapamycin rapidly depleted PtdIns(4,5) $P_2$  as shown by the removal of GFP-PH-PLC from the plasma membrane to the cytosol (B). Quantification of fluorescence at the plasma membrane reveals that depletion is complete within 2mins (C). Scale bar = 10  $\mu$ m.

**Figure 2. STIM1-EYFP translocation occurs in cells following depletion of either PtdIns(4,5) $P_2$  or PtdIns4P/PtdIns(3,4,5) $P_3$  EYFP but is inhibited by the depletion of multiple phosphoinositides.** HeLa cells were transfected with either STIM1-EYFP alone or cotransfected with the STIM1-EYFP, PM-FRB-RFP and RFP-ptase-dom constructs. (A) Control cells treated only with thapsigargin are shown on the left. In cells which overexpress all three proteins, depletion of PtdIns(4,5) $P_2$  by the rapamycin-inducible phosphatase did not prevent the translocation of STIM1-EYFP stimulated by thapsigargin. (B) Control cells treated only with thapsigargin are shown on the left. Inhibition of PI3K and PI4K by wortmannin (20  $\mu$ M) pretreatment did not prevent thapsigargin-induced STIM1 translocation in cells expressing STIM1-EYFP alone. (C) Cells cotransfected with the STIM1-EYFP, PM-FRB-RFP and RFP-ptase-dom constructs and perfused with 50  $\mu$ M LY294002 for 30 min followed by addition of rapamycin. Addition of thapsigargin had little or no effect on the distribution of STIM1. Scale bars = 10  $\mu$ m.

**Figure 3. Translocation of STIM1-EYFP is inhibited by the depletion of multiple phosphoinositides in cells treated with LY204002.** HeLa cells were cotransfected with the STIM1-EYFP, PM-FRB-RFP and RFP-ptase-dom constructs. (A) Cells were perfused with no additions (controls), 50 or 300  $\mu$ M LY294002 for 30 min followed by addition of thapsigargin which resulted in some STIM1 translocation into puncta under all conditions. Note that after treatment with 300  $\mu$ M LY294002, few STIM1 puncta formed in some cells (e.g. the cell marked by an asterisk). (B) Cells were perfused with 50  $\mu$ M LY294002 for 30 min followed by addition of rapamycin. Treatment with thapsigargin had little effect on the distribution of STIM1. (C). Cells were perfused with 300  $\mu$ M LY294002 for 30 min followed by addition of rapamycin. Treatment with thapsigargin had little effect on the distribution of STIM1. Scale bar = 10  $\mu$ m.

**Figure 4. Overexpression of Orai1 rescues STIM1 puncta formation following depletion of phosphoinositides.** HeLa cells were cotransfected with STIM1-EYFP, PM-FRB-RFP and RFP-5-ptase-dom constructs and Cerulean-Orai1. The cells were treated with 20  $\mu$ M wortmannin (A) followed by addition of rapamycin to deplete PtdIns(4,5) $P_2$ . Overexpression of Orai1 rescued thapsigargin-stimulated translocation of STIM1 (B). Scale bar = 10  $\mu$ m.



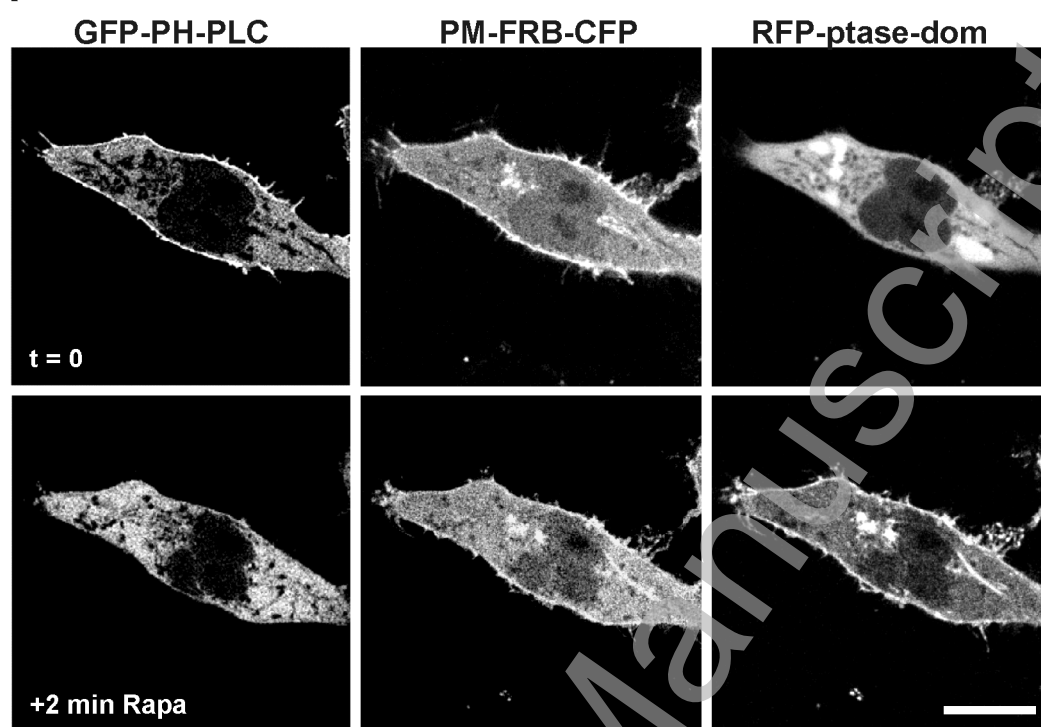
**Figure 5. Quantification of puncta formed in response to thapsigargin treatment and the effect of depletion of PtdIns(4,5) $P_2$  and inhibition of lipid kinases.** Cells from the various treatments used in the study were analysed and the number of puncta formed following thapsigargin treatment counted and expressed as mean per cell  $\pm$  SEM based on the number of cells indicated in parentheses. The statistical significance of values for all conditions compared to control thapsigargin-treated cells and indicated pairwise comparisons were determined using the two-tailed Student's t-test and statistically significant differences are indicated.

**Figure 6. Phosphoinositide depletion reduces store-operated  $Ca^{2+}$  influx but this is reversed by overexpression of Orai1.** (A) HeLa cells were transfected with STIM1-EYFP, RFP-PM-FRBP and RFP-ptase-dom. 48h post-transfection, cells were loaded with Fluo-4 (5  $\mu$ M) and incubated in wortmannin (20  $\mu$ M) for 30 min. Intracellular stores were depleted with thapsigargin (2  $\mu$ M). Readdition of  $Ca^{2+}$  to the external solution 420s later allowed the measurement of  $Ca^{2+}$  influx in cells which had been treated with or without rapamycin. Rapamycin treatment resulted in the inhibition of  $Ca^{2+}$  influx when compared to cells which had been incubated in the absence of rapamycin, as shown in the averaged traces. Quantification of the average peak values of  $Ca^{2+}$  influx from these traces revealed a significant decrease in peak  $[Ca^{2+}]_i$  values in rapamycin treated cells. (B) In a similar experiment but with HeLa cells also overexpressing Orai from pcDNA3,  $Ca^{2+}$  influx was restored in rapamycin-treated cells to levels greater than that of cells which had not been treated with rapamycin, as shown in the averaged traces and the peak  $[Ca^{2+}]_i$  values.

**Figure 7 Phosphoinositide depletion reduces store-operated  $Ca^{2+}$  influx due to endogenous STIM1.** Cells were transfected with RFP-PM-FRBP and RFP-ptase-dom. 48h post-transfection, cells were loaded with Fluo-4 (5  $\mu$ M) and incubated with wortmannin (20  $\mu$ M) for 30 min and treated with rapamycin (1  $\mu$ M) before thapsigargin treatment and readdition of  $Ca^{2+}$  to the external solution after 620s. In this experiment  $[Ca^{2+}]_i$  was monitored in transfected (+ptase, n = 22) and non-transfected cells (-ptase, n = 18) in the same microscope fields and directly compared so that all cells had been treated with rapamycin.

Figure 1

**A**



**B**

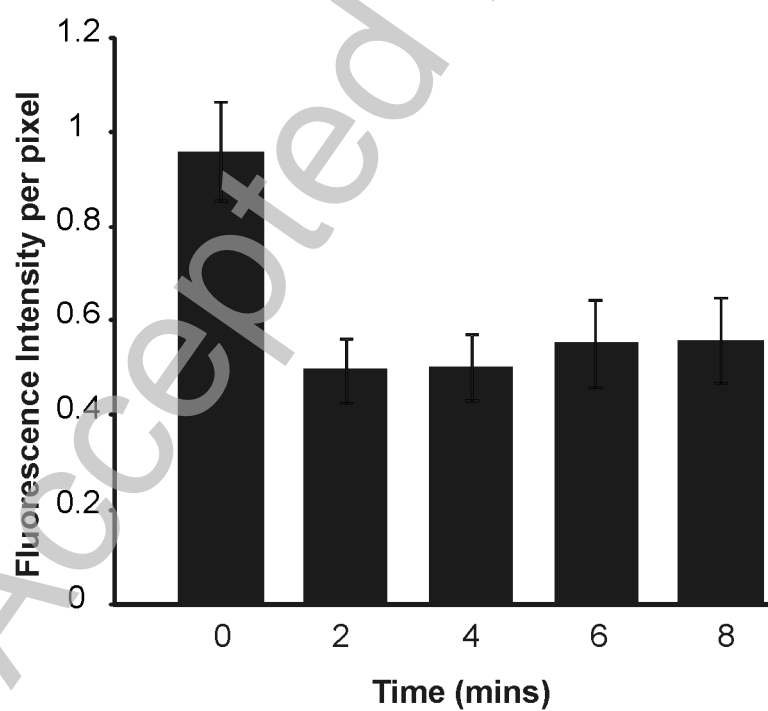


Figure 2

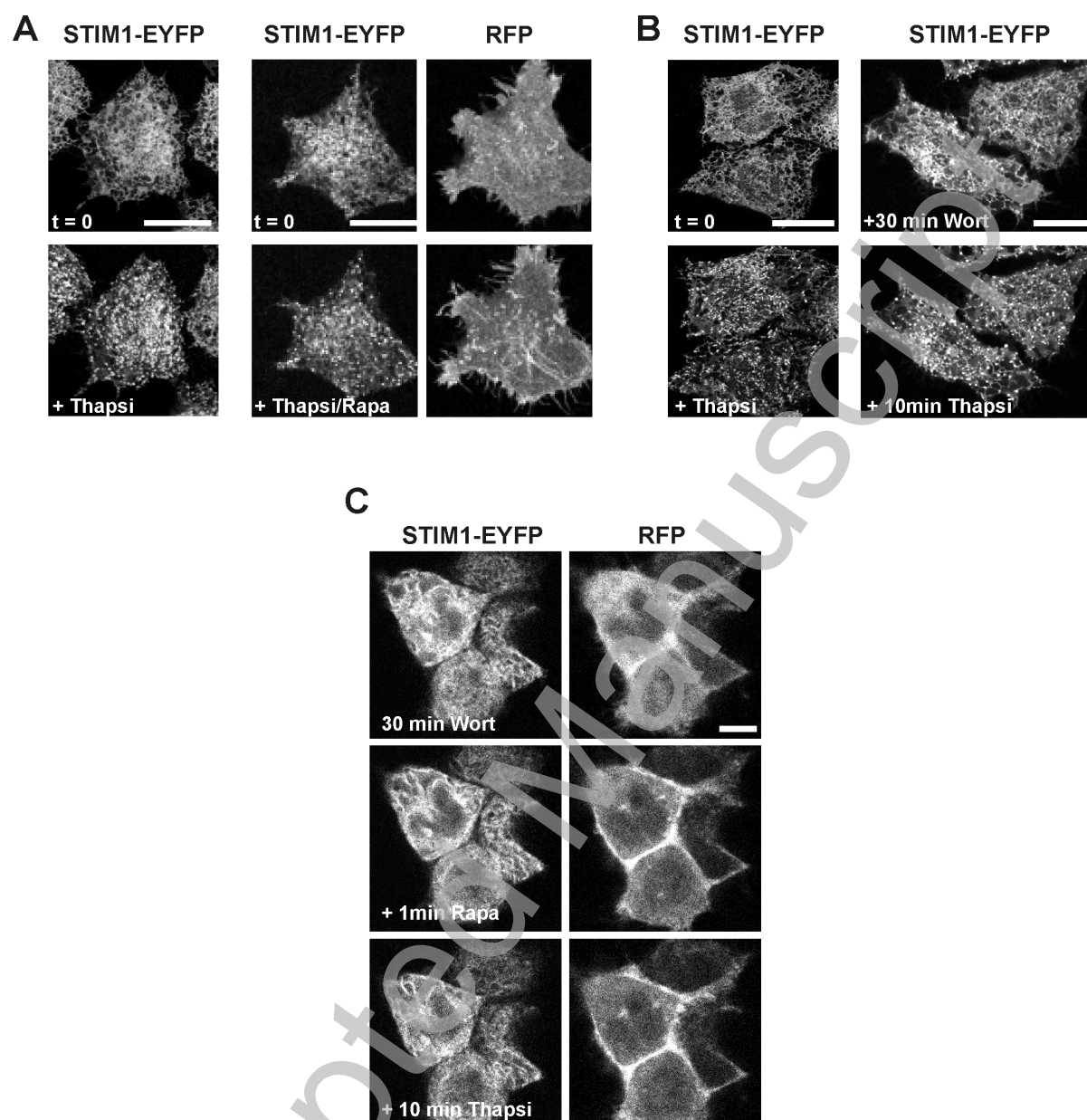
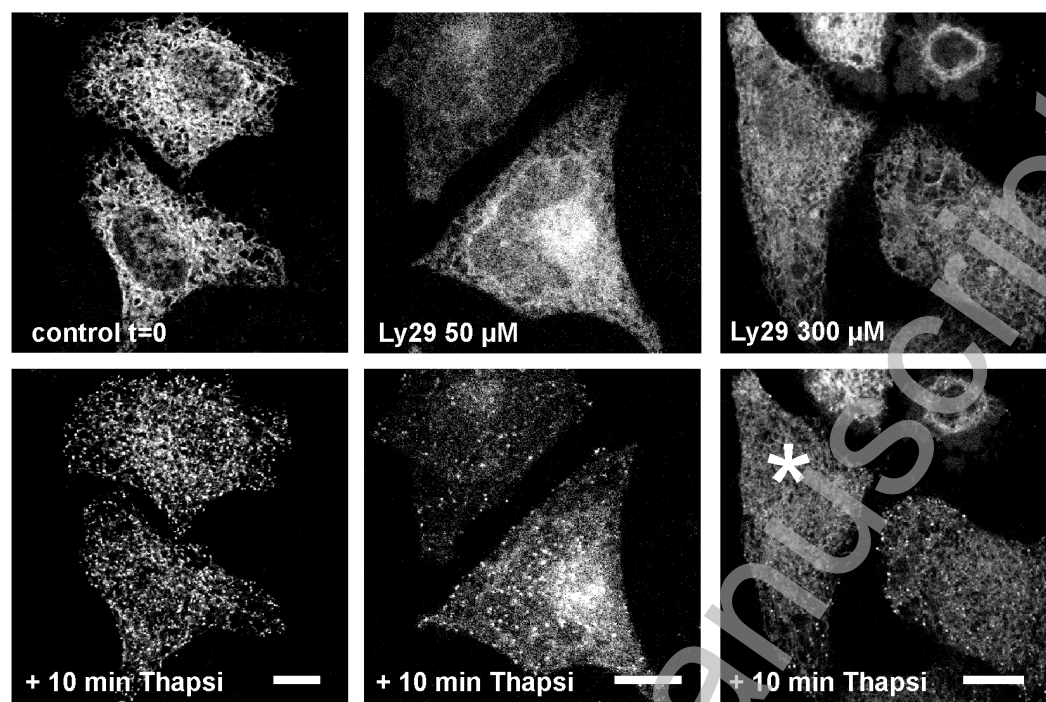


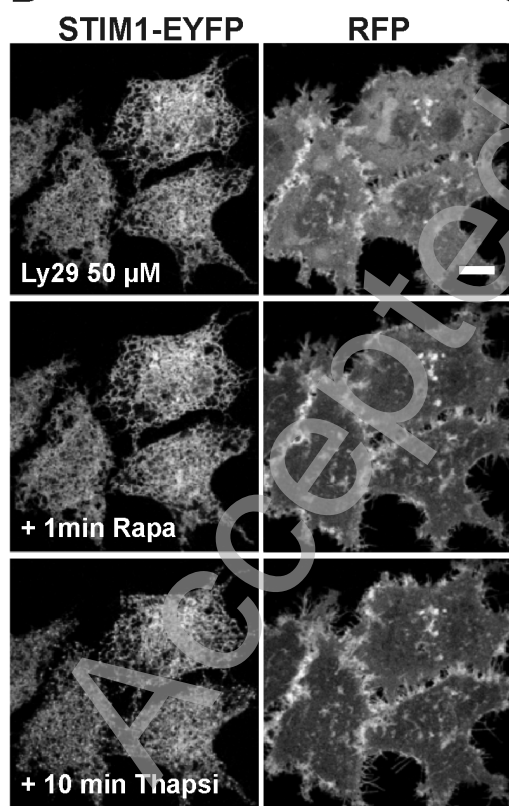


Figure 3

A



B



C

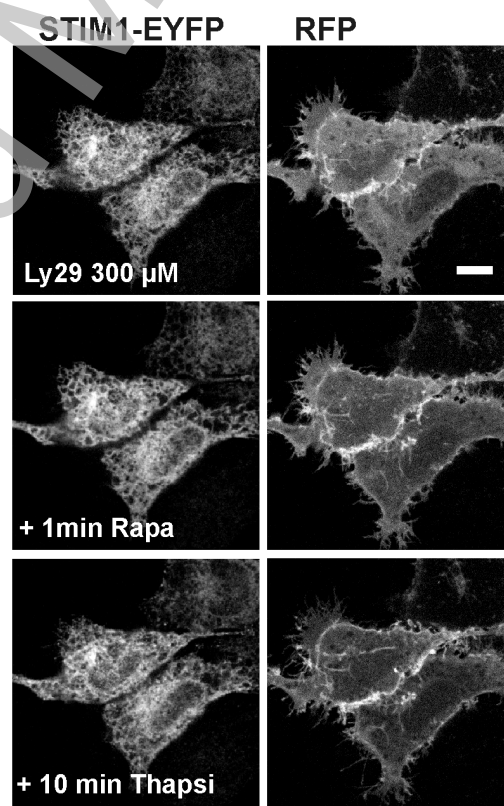


Figure 4

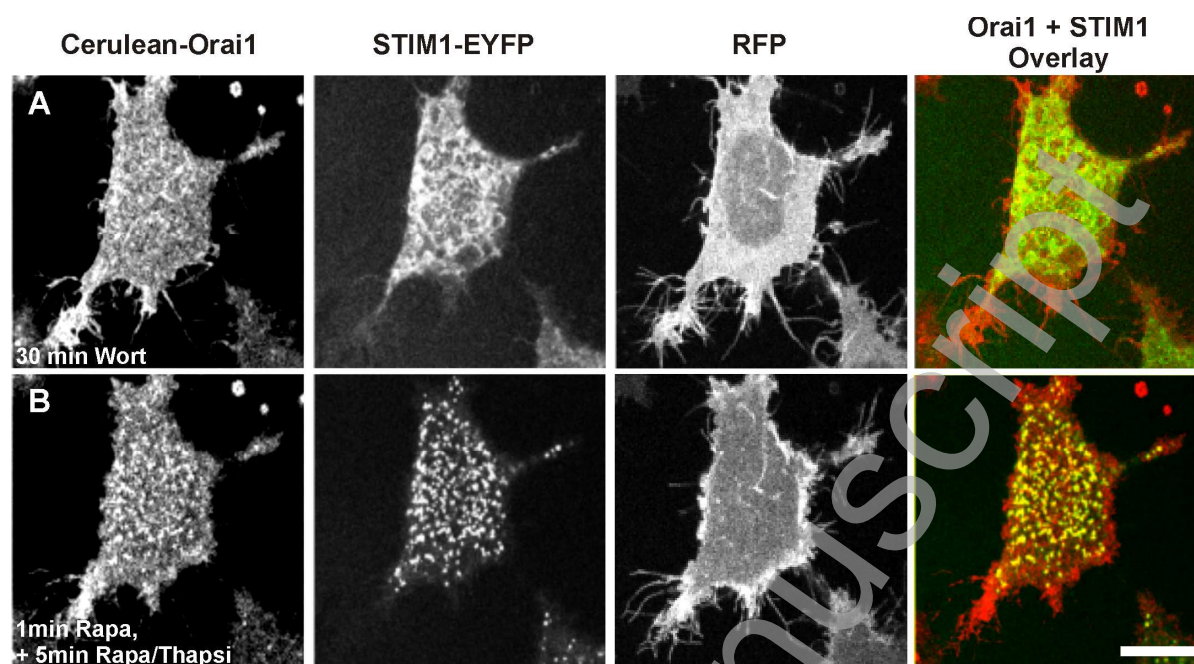




Figure 5

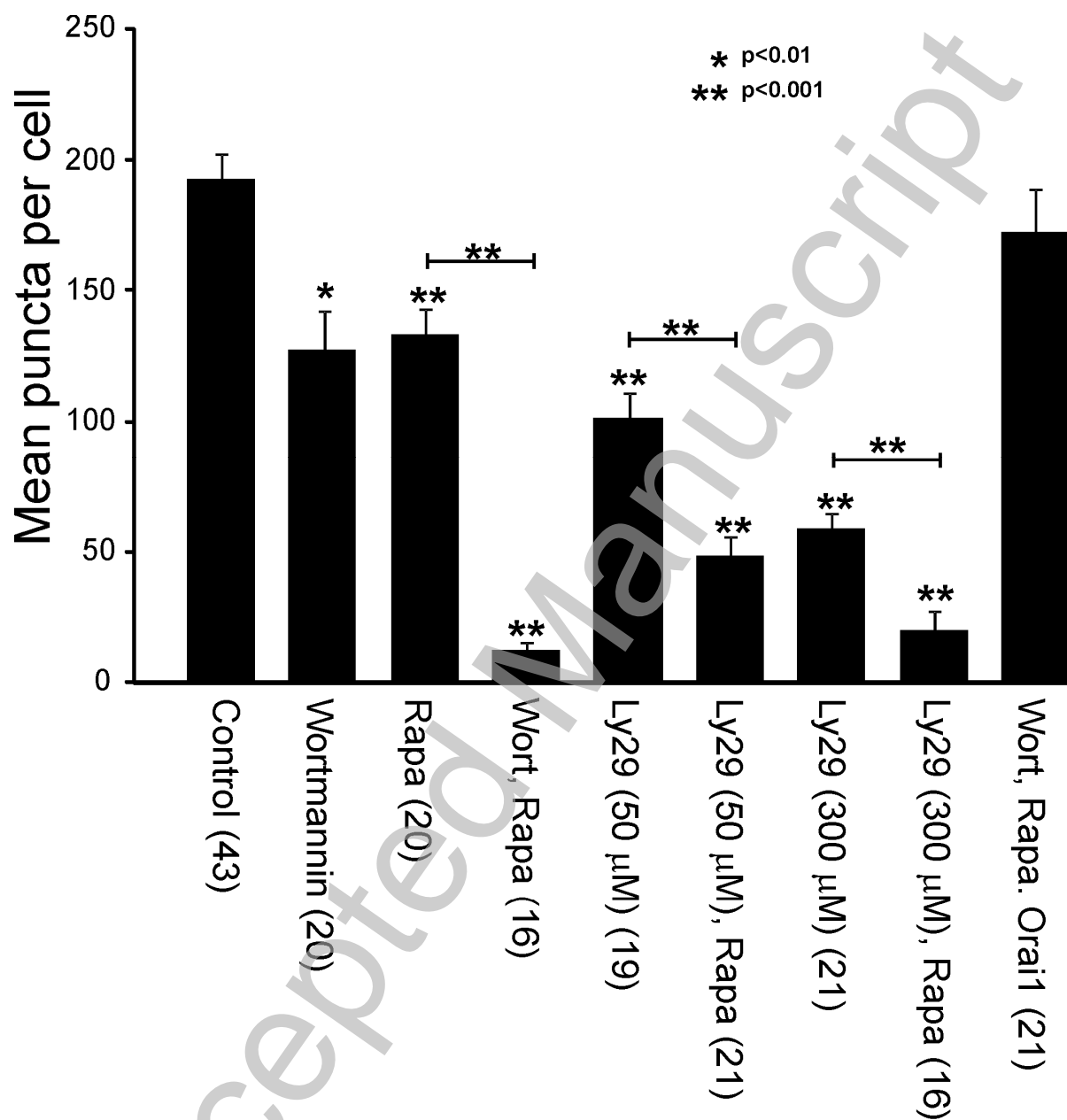


Figure 6

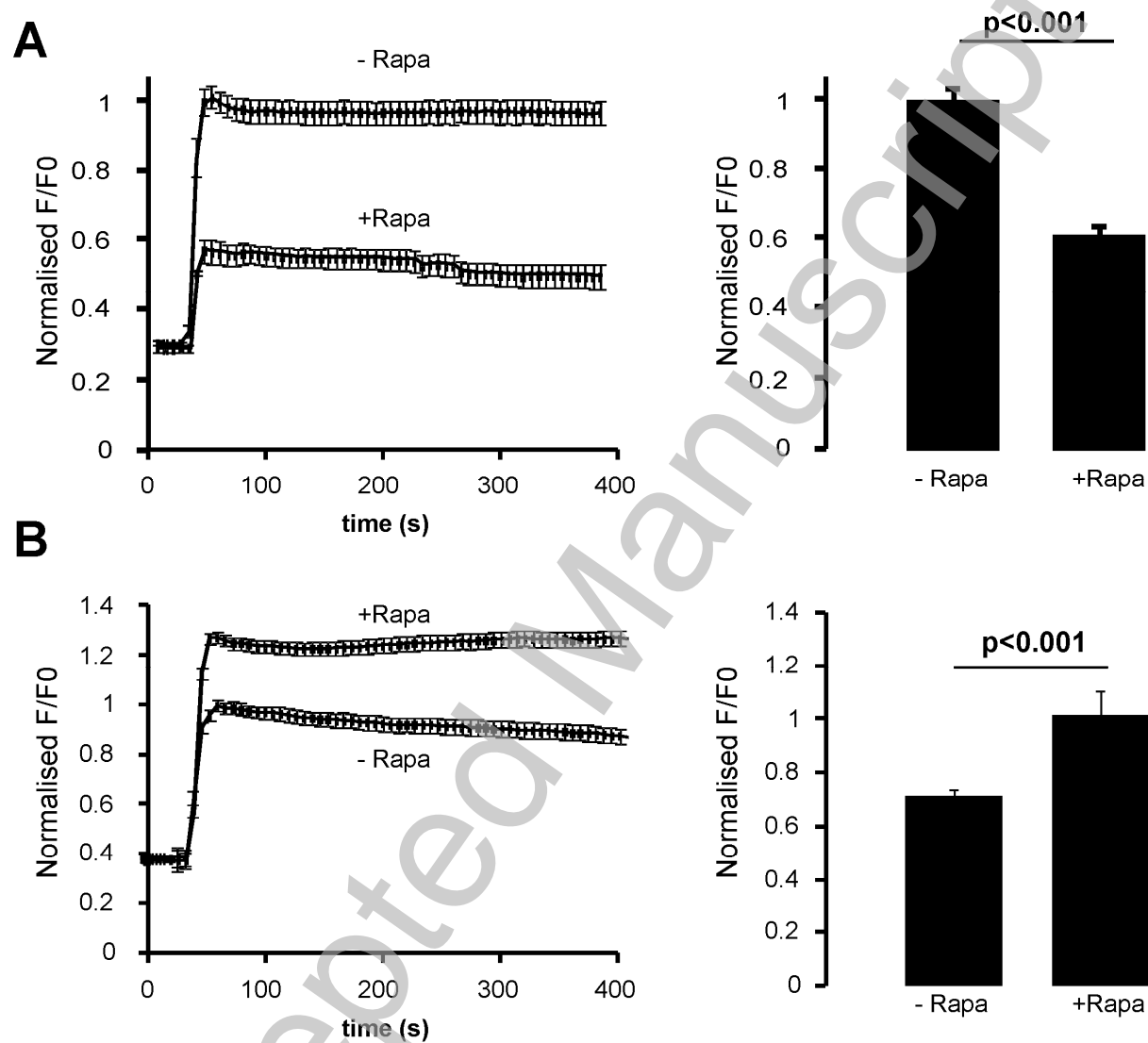


Figure 7

