**Ms# 2019-15386 by Son et al**

**Supplemental Figure Legends**

**Fig. S1.** (**A**) $Ca^{2+}$ release and $Ca^{2+}$ influx measured using $Ca^{2+}$-addback assay in control cells (Control) and cells treated with siSTIM2 (siS2). **(B)** Bar graphs show the increase in 340/380 ratio at peak $Ca^{2+}$ influx time point ($F-F_0$). **(C)** Relative increase in nuclear NFAT1 fluorescence in cells following stimulation with either 25 μM CPA or 1 μM Tg, without and with siSTIM2 treatment (CPA+siS2 or Tg+siS2 respectively). **(D)** Bar graph showing fold changes in nuclear NFAT1 fluorescence at the 30 min time point in all cells. The addition of CPA or Tg is indicated by an arrow on the graphs. **(E)** Relative increase in nuclear NFAT1 fluorescence in control and Synta66-treated cells, as well as unstimulated cells. The addition of CPA is indicated by an arrow on the graphs. **(F)** Bar graph showing fold changes in nuclear NFAT1 fluorescence at the 30 min time point in all sets of cells. **(G)** Western blot showing knockdown of STIM2 protein by siSTIM2 (upper panel); lower panels show levels of STIM1 and Orai1 respectively in the samples. (**H**) $Ca^{2+}$ release and $Ca^{2+}$ influx in cells expressing Orai1+STIM1 (O1+S1), without (control) and with siSTIM2 (siS2) treatment. **(I)** Bar graphs show the increase in 340/380 ratio in stimulated cells, 10 min time point ($F-F_0$). **(J)** Scatter plot obtained from TIRFM images representing fluorescence of STIM1 (S1) and Orai1 (O1) in puncta detected after CPA stimulation of cells (O1+S1) control or with siSTIM2 (siS2) treatment. **(K)** Relative decreases in SOCE, NFAT1 translocation, and S1 and O1 fluorescence in puncta induced by siSTIM2 in control and O1+S1 expressing cells. All values in the bar graphs are presented as mean ± SEM. Statistical tests were done using either Students t-tests for two groups or ANOVA with Sidak multiple comparisons test for ≥ two groups. Statistical significance was presented as $*P < 0.05$ and $***P < 0.001$. For both SOCE and NFAT



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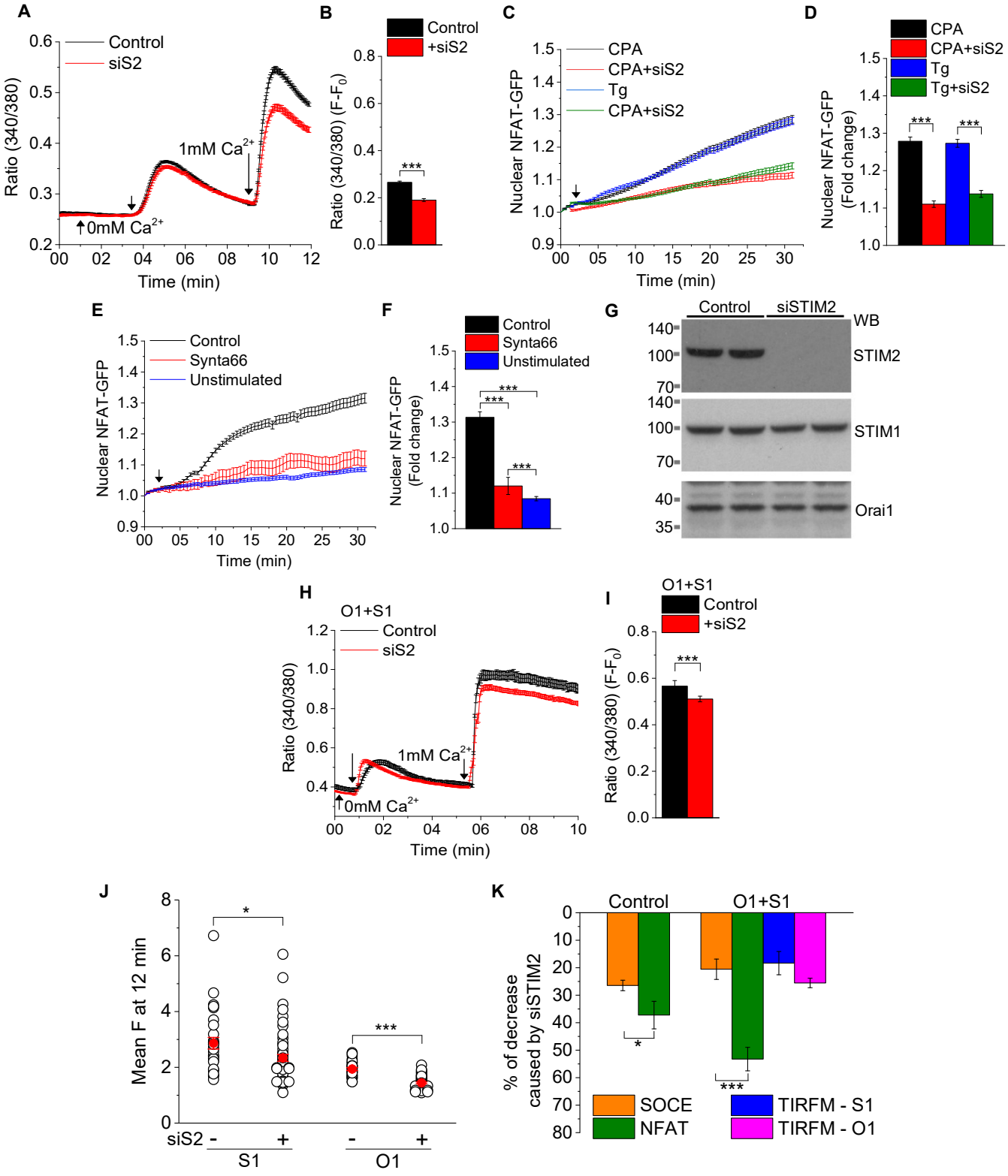
experiments, the graphs show averaged data from n = 100 to 300 cells per group. For TIRFM data, the scatter plot and bar graph show averaged data of n = 30 to 60 cells from 4 experiments.

**Fig. S2. (A, B)** Effects of $PIP_2$ depletion on YFP-Tubby, as shown by TIRFM images (before and after rapamycin addition) and whole cell fluorescence over time. Arrow on the graph indicates the addition of rapamycin. **(C, D)** Effects of $PIP_2$ depletion on CPA-induced $Ca^{2+}$ release from the ER. Arrow on the graph indicates the addition of CPA in $Ca^{2+}$-free medium. Bar graphs show the increase in 340/380 ratio ($F-F_0$) at peak increase. **(E-G)** Effects of $PIP_2$ depletion on the clustering of Orai1 (O1), STIM1 (S1) and STIM2 (S2). Bar graph shows the decrease (%) in puncta fluorescence for Orai1, STIM1 and STIM2 in cells where $PIP_2$ was depleted, as compared to control. **(H, I)** Long-term SOCE responses in control cells and cells expressing Orai1+STIM1 (O1+S1) and Orai1+STIM1ΔK (O1+SΔK). Arrow on the graph indicates the addition of 25 μM CPA. Bar graphs show the increase in 340/380 ratio at the 10 min time point ($F-F_0$) in both sets of cells. All values in the bar graphs are presented as mean ± SEM. Statistical tests were done using either Students t-tests for two groups or ANOVA with Sidak multiple comparisons test for ≥ two groups. Statistical significance was presented as ***$P < 0.001$ and n.s. (not significant, $P > 0.05$). For SOCE experiments, the graphs show averaged data from n = 100 to 300 cells per group. The TIRFM images represent data from 3 experiments, while the bar graph shows averaged data of n = 20 cells.

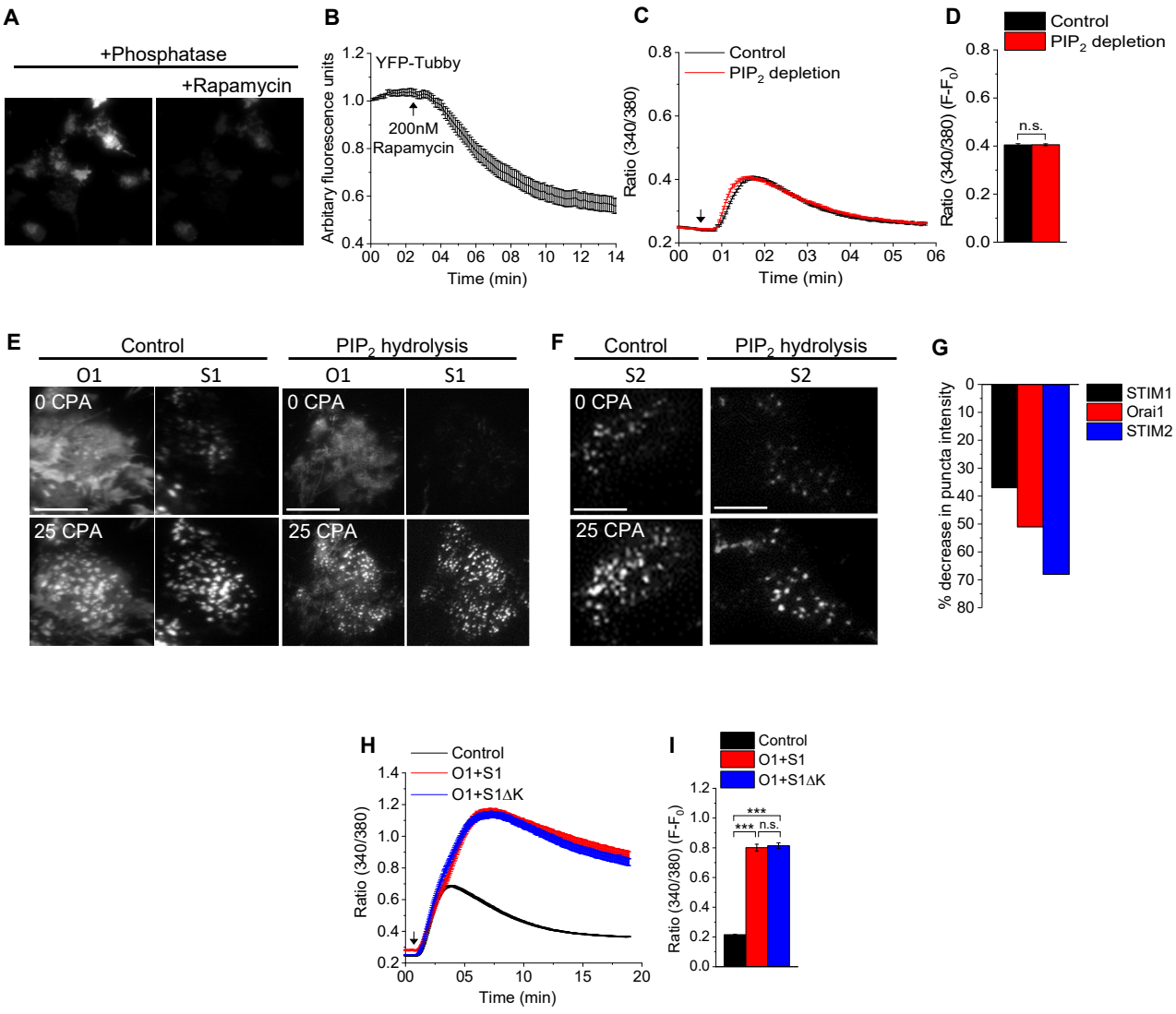
**Fig. S3.** Images acquired by TIRFM of cells expressing (**A**) YFP-STIM1 (S1); **(B**) YFP-STIM1 (S1), Orai1-CFP (O1), and mCherry-ER3 (ER); (**C**) YFP- STIM1ΔK; and **(D)** Orai1-CFP (O1), YFP- STIM1ΔK (S1ΔK) and mCherry-ER3 (ER). All TIRFM images show a representative cell

before and 10 min after 25 μM CPA stimulation (0 and 25 CPA respectively). For overlay images, images pairs are as indicated and pseudo colored green (g) or red (r). The scale bar shown is 10 μm. **(E)** Bar graph shows fluorescence increase in cells expressing S1 or S1ΔK with O1, 10 min after stimulation ($F-F_0$). TIRFM images of cell expressing **(F)** CFP-STIM2 (S2), YFP- STIM1ΔK (S1ΔK) and mCherry-ER3 (ER) and **(G)** Orai1-CFP (O1), YFP- STIM1ΔK (S1ΔK) and mCherry-STIM2 (S2), before and 10 min after 25 μM CPA stimulation (0 and 25 CPA respectively). For overlay images, images pairs are as indicated and pseudo colored green (g) or red (r). The scale bar shown is 10 μm. (**H**) Line scans through the image (position shown by orange line) show clustering of the two indicated proteins in unstimulated (0 CPA; upper panel) and stimulated (25 CPA; bottom panel) cells shown in G. **(I)** CPA-induced $Ca^{2+}$ responses in control and cells expressing either Orai1+STIM1 (O1+S1) or Orai1+STIM1+STIM2 (O1+S1+S2). **(J)** Bar graphs show the increase in 340/380 ratio 10 min time point in CPA-stimulated cells ($F-F_0$) for all conditions in I. **(K)** Relative increase (fold changes) in NFAT in the nucleus of cells expressing O1+S1 or O1+S1+S2 in response to 25 μM CPA stimulation. **(L)** Bar graphs show the nuclear fluorescence values at the 30 min time point. **(M)** Blot showing the effect of Orai1 knockdown using siOrai1 on the interaction between Myc-tagged STIM2 and endogenous AKAP79. Co-IP was done with anti-myc (1:1000 dilution) and immunoblotting was done with anti-AKAP79 at 1:1000 dilution. All values in the bar graphs are presented as mean ± SEM. Statistical significance was assessed using Students t-tests for two groups and presented as n.s. (not significant, $P > 0.05$). For both SOCE and NFAT experiments, the graphs show averaged data from n = 100 to 300 cells per group. For TIRFM images, images represent data from 4 experiments and the bar graph shows averaged data of n = 30 to 60 cells.

# Supplemental Figure 1



# Supplemental Figure 2



# Supplemental Figure 3