Supplementary Information

Functional Communication between IP_3R and STIM2 at sub-threshold stimuli is a critical checkpoint for initiation of SOCE

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Supplemental Materials and Methods

Cell culture, plasmids, RNAi transfection and reagents. HEK293 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin, incubation at 37^{o}C under 10% CO_2. All the siRNAs used in this study are from Dharmacon (Lafayette, CO, USA) and Life Technologies (Carlsbad, CA, USA). Lipofectamine RNAiMAX (Life Technologies) was used for siRNA transfections. Mock transfections of control cells were conducted using negative control siRNA (Life Technologies). Cells were typically used 48 h post-transfection before imaging. The mCherry-ER3, mCerulean-MAPPER, IP_3R1-mCh, pore-dead (G2506R) and phosphorylation deficient (S1589A and S1755A; Phos-Mutant) mutant of IP_3R1, TK-Orai1-CFP, TK-Orai1-mCh, TK-mCherry-STIM1, ER-LAR-GECO1, TK-YFP-STIM2, TK-YFP-STIM1, TK-YFP-S2N-S1C, TK-YFP-S1N-S2C plasmids were transfected using electroporation following the protocol recommended by Lonza (Walkersville, MD, USA). Mouse monoclonal antibody of STIM1 and STIM2 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal antibody to Orai1 was produced against C-terminal and purified by ProSci (Poway, CA, USA) and used described previously (1, 2). All other reagents used were of molecular biology grade obtained from Sigma-Aldrich (St Louis, MO, USA) unless mentioned otherwise.

CRISPR/Cas9 approach for mVenus Knock-in at C-terminus of STIM2 gene. The standard method for CRISPR/Cas9 based Knock-in approach was followed (3). Both 5' and 3' homology repair template were generated by PCR, synthesized mVenus fragments were assembled using HiFi Infusion Kit (New England Biolabs, Ipswich, MA) into linearized pUC19 vector (Takara Bio USA, Inc) and confirmed by sequencing. Multiple gRNA was designed by GPP sgRNA Designer of the Broad Institute and Benchling software (Benchling, San Francisco, USA). gRNA sequence was cloned in pX330 vector obtained from Addgene (Watertown, MA). Electroporation of HDR template and gRNA in and HEK293 cell line and fluorescence microscopy was performed after 2-3 days. Fluorescent cell sorting was done to enrich the fluorescent population. Single-cell clones were obtained by sorting a single cell in each well of a 96 well plate. At least five different clones were further confirmed by PCR, sequencing, and endogenous protein tagged with mVenus expression was confirmed by western blot analysis with GFP antibody. A homozygous single cell population was used in this study.

Plasmids and Cell lines. The mCherry-ER3 plasmid was obtained from Addgene (Watertown, MA). To generate the TK promoter-driven constructs, the following plasmids were used as templates: Orai1-LL-CFP (Tamas Balla, NICHD, NIH, Bethesda, MD, USA), YFP-STIM2 (Tobias Meyer, Stanford University) and mCherry-STIM1 (Richard Lewis, Stanford University, Stanford, CA, USA). Briefly, the CMV promoter in these plasmids were substituted with the TK promoter. To generate mCerulean-MAPPER, the fluorescent tag in GFP-MAPPER (Jen Liou, UT Southwestern, Dallas, TX) was substituted with mCerulean (Addgene). These constructs were generated and then sequenced to confirm proper substitutions of the promoter or fluorescent tag by Mutagenex Corporation (Suwanee, GA). mCh-IP3R1, as well as IP3R-TKO (Null), IP3R-

TKO/hIP3R1, IP3R1-TKO/R1G2506R, IP3R-TKO/IP3R1-Phos-Mutants are characterized previously (4-6).

$[Ca^{2+}]_i$ measurements. Fura-*2* fluorescence was measured in cells cultured for 24 h in collagen-coated glass-bottomed MatTek tissue culture dishes (MatTek Corp. Ashland, MA, USA) and transfected as required. All experiments were done 24–48 h post-transfection. Wells were loaded with 1μM Fura-2AM (Life Technologies) for 30 min at 37 °C. Fluorescence was measured with the Olympus IX50 microscope (Olympus, Center Valley, PA), Polychrome V (Till Photonics LLC, Pleasanton, CA), and EM-CCD camera (Hamamatsu, Tokyo, Japan). Image acquisition and data processing were done using MetaFlour (Molecular Devices, Downingtown, PA). Additions of carbachol (1, 10 or 100μM) and 1mM $CaCl_2$ are indicated by arrows on the graphs.

Confocal Microscopy. The FV3000 confocal laser scanning microscope (Olympus) was used to acquire live cell images of the STIM2-KI cell line (expressing mV-STIM2). All cells were imaged at 37°C using a Plan-Apochromat 63×1.4 NA oil-immersion objective lens. The imaging software, FluoView, was used to excite mVenus using the 514nm laser and collect the subsequent emitted light. Images were further analyzed using Fiji/ImageJ2.

ZEISS AiryScan Imaging. Airyscan imaging was performed with LSM880, a confocal laser scanning microscope ZEISS LSM 880 (Carl Zeiss AG, Oberkochen, Germany) equipped with an Airyscan detection unit. Airyscan technology is an improved high-resolution imaging, which can acquire images with up to 8x improvement in Signal to Noise ratio and 1.7x higher resolution than a conventional confocal. To maximize the resolution enhancement, 63X/1.46 Oil Corr M27 or Plan-Apochromat 63X/1.40 Oil Corr M27 objectives (Zeiss) with Immersol 518 F immersion media (n_e = 1.518 (23 °C); Carl Zeiss) was used for the imaging.

TIRF Microscopy (TIRFM) Imaging. TIRF microscopy experiments were using HEK293 cells cultured for 24h on glass-bottomed dishes coated with collagen (MatTek) and transfected/electroporated as required. All experiments were done 24-48h post-transfection. TIRFM was performed using an Olympus IX81 motorized inverted microscope (Olympus) with a TIRF-optimized Olympus Plan APO 60× (1.45 NA) oil immersion objective and Lambda 10-3 filter wheel (Sutter Instruments, Novato, CA, USA) containing 480-band pass (BP 40 m), 540-band pass (BP 30 m), and 575lp emission filters (Chroma Technology, Bellows Falls, VT). Images were collected using a Hamamatsu ORCA-Flash4.0 camera (Olympus) and the MetaMorph imaging software (Molecular Devices), which were then analyzed as described below.

Image Analysis. The Zen Black 2.1 software (Carl Zeiss) was used to acquire and process images on the Airyscan microscope imaging system. Data acquired by each of the 32 Airy detector channels were processed separately by performing filtering, deconvolution and pixel reassignment in order to obtain images with enhanced SNR and improved spatial resolution. For TIRFM imaging, both MetaMorph and Fiji/ImageJ2 were used in the analysis. For STIM1 and STIM2, the image stacks were first processed in MetaMorph using a region-of-interest (ROI)-based background subtraction method. For Orai1, the image stack was first processed using a Gaussian Blur filter (sigma = 5) and then used to generate an average intensity projection of the entire stack, which was then subtracted from each image in the stack. All background-corrected image stacks were analyzed in Fiji/ImageJ2 using macros developed in-house to obtain two measurement parameters: the number of clusters detected and mean fluorescence intensity. The image stack was run through the FeatureJ/Laplacian filter (7) and auto-thresholded using the Otsu method to generate a binary image stack where each cluster was displayed as white particles on a black background. Using the Set Measurements and Analyze Particles commands, ROIs were drawn

around each white particle and saved as a ROI set specific for each binary image. The ROI sets were then applied to the background-corrected image to measure mean fluorescence intensity. The STIM2 images were used to generate these ROI sets, which were then applied to other images of STIM1 and/or Orai1 if these proteins were also co-expressed in the same cell. The number of ROIs generated for each image was calculated as number of clusters detected. The quantitative measurements for each image were saved as a comma separated value-formatted files, which were then imported into Origin (OriginLab Corporation, Northampton, MA, USA) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA) for further analyses. Whole Cell ROI was also performed, wherever indicated in result section, to quantify the whole cell fluorescence intensity from basal to stimulated cells.

Two approaches were used to identify clusters that are stable for at least 1 min. Two frames of 1 min interval were pseudo-colored green and magenta to create overlay images. Mobile clusters will be either green or magenta, while immobile clusters will be white. Numeric time values (in min, labeled as m) stated on the images are counted up from the start of imaging. For example, '0/1m' represent images from the start of the experiment (0 min) and a min later (1 min). Line scans were performed in Fiji/ImageJ2 by drawing a line across the image and importing the underlying plot data into Graphpad Prism for graphing. The Image Calculator tool in Fiji was used to apply a logical AND on consecutive binary images (at 1 min intervals) to identify objects having the same position in both images. The Set Measurements and Analyze Particles commands were then used as described above to calculate the number of stable clusters detected. Overlay images were analyzed using Coloc2 plugin in Fiji/ImageJ2 to get Pearson's Correlation Coefficient (PCC) and Mander's Overlap Coefficient (MOC) values.

Co-immunoprecipitation (Co-IP) and Western blotting. Wild-type (WT) and STIM2-KI HEK293 cells were treated as indicated in the legend, washed with 1× phosphate-buffered saline and lysed in Pierce IP lysis buffer supplemented with protease inhibitors (all materials were from Thermo Fisher Scientific, Waltham, MA). Cell lysates were centrifuged (10,000 g, 10 min at 4 °C) and quantified by BCA protein assay kit (Thermo Scientific). Co-IP experiments were done using anti-Myc magnetic beads, or Protein A Sepharose CL-4B (GE Healthcare Life Sciences, Marlborough, MA) as described earlier unless indicated otherwise. For Myc-tagged proteins, Pierce anti-Myc magnetic beads (Thermo Scientific) were used following the manufacturer's instruction. After wash the immunoprecipitants were eluted in elution buffer or in gel-loading buffer by heating at 95 °C for 5-10 min and resolved in 4–12% NuPAGE gels (Life Technologies), followed by Western blotting. Proteins of interest were immunoblotted using anti-IP_3R1 and anti-Myc, anti-STIM2 (Cell Signaling Technologies, Danvers, MA), anti-β-actin (Abcam, Cambridge, MA) antibodies.

Statistics. Data analysis was performed using Origin (OriginLab Corporation) and GraphPad (GraphPad Software). Relative values have been expressed in comparison to the basal condition (average value of time frame before stimulation in timelapse analysis) or to the WT counterpart (in static analysis). Smoothened curves were generated using GraphPad to help visualize trends where appropriate and were not used for any statistical analysis. Experimental values are expressed as mean ± SEM. Statistical comparisons between two groups were made using the Student's t-test, whereas comparisons of multiple groups were made using one-way ANOVA followed by Sidak multiple comparisons test. Statistical significance was shown as significant at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, or non-significant at $P > 0.05$ (n.s.).

Supplementary Figure Legends

Fig. S1. Generation and validation of mVenus-STIM2 knock-in cell line (STIM2-KI) using the CRISPR/Cas9 strategy. (*A*) Schematic of plasmid construct showing gRNA and cas9 expression cassette. (*B*) Schematic of donor plasmid as repair template for knock-in of mVenus immediately after the signal peptide of STIM2. Site-specific cleavage of *Stim2* by the Cas9-sgRNA complex in wild-type (WT) HEK293 cells is repaired through homology-directed repair with homology arms that flank an in frame mVenus open reading frame. (*C, D*) Flow cytometry-based sorting of mVenus-STIM2 v(mV-STIM2)-positive cells (STIM2-KI cells) and subsequently single cell cloning using a 96-well plate to generate a monoclonal population. (*E*) mVenus-STIM2 expression; in STIM2-KI without (S2-Cont) and with siSTIM2 treatment (siS2). The images are representative of data obtained from 3 experiments. (*F*) Western blots showing expression of STIM2, mV-STIM2 (endogenous STIM2), STIM1, and Orai1 in wild-type (WT) and STIM2-KI (KI) cells using anti-STIM1, anti-STIM2 (same blot used after detection of STIM1), anti-GFP (also detects mVenus-STIM2) and anti-Orai1 antibodies. Red arrows indicate location of the respective proteins on the blots. (*G-J*) Fura-2 fluorescence measurements using the Ca^{2+} addback assay where cells were stimulated with cyclopiazonic acid (CPA; 25µM) in Ca^{2+}-free medium and then with 1 mM $CaCl_2$ (Ca^{2+}) in (*G, H*) WT HEK293 cells and (*I, J*) STIM2-KI (S2-KI) cells. All additions are as indicated by the black arrows. Black traces are control cells, while red and blue traces indicate cells treated with siSTIM1 and siSTIM2 respectively. (*H, J*) Bar graphs showing calcium entry components ($F-F_0$) in the cells shown in *G* and *H*. Statistical significance was assessed using ANOVA for multiple groups and presented as not significant (n.s.; $P > 0.05$) and significant (***$P < 0.001$). Graphs show averaged data (mean ± SEM) obtained from n >300 cells from at least 3 experiments.

Fig. S2. Mobility analysis of mVenus-STIM2 in STIM2-KI cells. (*A*) mVenus-STIM2 (mV-STIM2, labeled as S2) clusters in STIM2-KI cells without stimulation (basal condition, upper panels) and stimulated with 1μM CCh (lower panels). Images in each panel for (i, ii) were acquired at the start of experiment (0 m) and at the 1, 3 and 4 min time points (1, 3, and 4m). Images in (iii) were overlays of 0 and 1 min (0/1m), as well as 3 and 4 min (3/4m), time points. Images in (iv) are enlargements of the region marked by square in (iii). Overlapping clusters at the two time points appear white and indicate immobile mV- STIM2 clusters in basal condition and after CCh stimulation. (*B*) Images shown are in a similar order as in (*A*) except that cells were stimulated with 100μM CCh. All TIRFM images are representative of data obtained from 3 experiments. CCh was added at the 2 min time point for all experiments. Scale bar, 5μm.

Fig. S3. Analysis of TK-Orai1-CFP expression in HEK293 cells. (*A*) HEK293 cells showing TK-Orai1-CFP (TK-O1) without (upper panel) and with (siS2) siSTIM2 treatment, before (basal) and after 1μM CCh stimulation. (*B*) STIM2-KI cells expressing TK-Orai1-CFP (TK-O1) before (Basal; top) and after stimulation (1μM CCh; bottom). From left to right: mVenus-STIM2 (S2; Green); TK-O1 (Red); overlay of both proteins; enlarged area of overlay; and corresponding line scans. (*C-F*) of mV-STIM2 and TK-Orai1-CFP before (basal; 0/1m overlay) and after (3/4m overlay) 1μM CCh stimulation. Immobile clusters found at the same position in both frames appear as white. (*G*) HEK293 cells expressing TK-O1 during unstimulated (basal) conditions and after 1μM CCh stimulation, which is representative protein localization in majority of the cells (no preclusters). (*H*) TK-O1 clusters in wild-type HEK293 cells following stimulation with 1μM CCh. Images at the 5 and 6 min time points were pseudo-colored green (5m) and magenta (6m) were overlaid, with immobile clusters appearing white (5/6m). All TIRFM images are representative of

data obtained from 3 experiments. CCh was added at the 2 min time point for all experiments. Scale bar, 5μm. "a.u": arbitrary units.

Fig. S4. Analysis of TK-mCh-STIM1 expression in HEK293 cells. (*A*) STIM2-KI cell expressing TK-mCh-STIM1 (TK-S1), before (top) and after 1μM CCh stimulation (bottom). For each panel, the images are in the order of S2 (Green), TK-S1 (Red), overlay of both proteins, enlarged image and corresponding line-scans. (*B*) TK-mCh-STIM1 in HEK293 cells (upper panel) and siSTIM2-treated HEK293 cells (middle panel) before (basal) and after stimulation with 1μM CCh stimulation. Lower panel show representative cell treated with siSTIM2 in basal condition (lower left panel) and after 100 μM CCh (lower right panel). (*C*) Images show response to 1μM CCh in siSTIM1(siS1)-treated STIM2-KI cells. (*D*) Overlays of unstimulated STIM2-KI (S2, green) cells expressing TK-mCh-STIM1 (TK-S1, magenta) with the box showing the region used for scan analysis. (*E-H*) Overlay images of the same cell in (*B*) following addition of 1μM CCh: 2 min (4/5m overlay) and 4 min after stimulation (6/7m overlay) for (j, l) mV-STIM2 and (k, m) TK-STIM1-CFP. Immobile clusters found at the same position in both frames are white. All TIRFM images are representative of data obtained from 3 experiments. CCh was added at the 2 min time point for all experiments. *(I-J*) TIRFM images showing mCh-E-Syt2 (*I*) or mCh-E-Syt3 (*J*) expressed in control STIM2-KI cells (upper images) or STIM2-KI cells treated with siE-Syts2/3 (lower images). Scale bar, 5μm. "a.u": arbitrary units.

Fig. S5. FSK-stimulated local $[Ca^{2+}]_{ER}$ depletion in IP_3R-TKO cells and lack of Co-IP of STIM2 with IP_3R1. (*A*) Immunoprecipitation (IP) of IP_3R1 from HEK293 cells expressing Myc-STIM1 and Myc-STIM2. Immunoblotting (IB) was done using the anti-IP_3R1 and anti-Myc antibody, respectively, for IP fractions (top and second blots) and input lysates (third and bottom blots). IB antibodies are shown next to each blot. (*B*) Reverse IP using the same cell lysate as in

(*A*), but using anti-Myc antibody to pull down STIM1 and STIM2. The blots are representative of data obtained from 2 experiments. (*C*) HEK293 and (D) IP_3R-TKO cells expressing genetically encoded Ca^{2+} indicators, ER-LAR-GECO1 in basal (unstimulated condition), FSK (cells stimulated with Forskolin, 5µM). Enlargements of the region marked by square are shown for visible comparison. All images are TIRF micrographs and representative of data obtained from >3 experiments. FSK was added at the 2 min time point for all experiments. Scale bar, 5µm. (*E*) Bar graphs showing ER-LAR-GECO1 fluorescence (F/F_0) before and after stimulation with 5µM FSK in wild-type (WT) HEK293, IP_3R-TKO, and STIM2-KI (S2KI) cells. The graph shows averaged data (mean ± SEM) obtained from n = 16 Cells (WT), 13 Cells (IP_3R-TKO), and 6 Cells (S2KI). (*F*) Basal Ca^{2+} entry measured in STIM2-KI, STIM2-KI+MAPPER, and STIM2-KI cells+MAPPER+siSTIM2 cells.

Supplemental Video Legends

Supplemental Video 1: mVenus-STIM2 images in STIM2-KI cells were acquired every 10 s using TIRFM, video runs 5 frames/s. Frames 1-12 represent basal condition. 1µM CCh was added at frame 12.

Supplemental Video 2: mVenus-STIM2 images in STIM2-KI cells were acquired every 15 s using TIRFM, video runs 4 frames/s. Frames 1-8 represent basal condition with 1µM CCh addition at frame 9.

Supplemental Video 3: mVenus-STIM2images were acquired at every 10 s in siE-Syts2/3 treated STIM2-KI cells using TIRFM. Video runs 6 frames/s. Frames 1-12 represent basal condition, frames 12-36 with 1µM CCh, and frames 37-61 with 100µM CCh.

Supplemental Video 4: mVenus-STIM2 images were acquired every 10 second in siIP_3Rs (all three subtypes were knocked down) treated STIM2-KI cells using TIRFM. Video runs 5 frames/s. Frames 1-12 represent basal condition and frames 12-36 with 100µM CCh.

Supplemental Video 5: mVenus-STIM2images were acquired every 10 s in STIM2-KI cells using TIRFM. Video runs 5 frames/s. Frames 1-6 represent basal condition and frames 7-31 with 5µM Forskolin.

Supplemental Video 6: TK-YFP-STIM2 expressed in HEK293 cells was imaged using TIRFM. Images were acquired at every 10 s. Video runs 5 frames/s. Frames 1-12 represent basal condition and frames 7-31 with 5µM Forskolin.

Supplemental Video 7: TK-YFP-STIM2 expressed in IP_3R-TKO cells was imaged using TIRFM. Images were acquired at every 10 s. Video runs 5 frames/s. Frames 1-12 represent basal condition and frames 7-31 with 5µM Forskolin.

Supplemental Video 8: TK-YFP-STIM2 expressed in IP_3R-TKO+IP_3R1 cells was imaged using TIRFM. Images were acquired at every 10 s. Video runs 5 frames/s. Frames 1-12 represent basal condition and frames 7-31 with 5µM Forskolin.

Supplemental Video 9: TK-S2 and TK-S1N-S2C chimera (side by side) in unstimulated cells. Imaging was performed using TIRFM and each frame was acquired at every 10 s upto 2 min

Supplemental Video 10: TK-S1 and TK-S2N-S1C chimera (side by side) in unstimulated cells. Imaging was performed using TIRFM and each frame was acquired at every 10 s upto 2 min.

Supplemental References

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7. E. Meijering (FeatureJ: An ImageJ Plugin Suite for Image Feature Extraction. (FeatureJ: An ImageJ Plugin Suite for Image Feature Extraction https://imagescience.org/meijering/software/featurej/ (2018)

Fig. S1

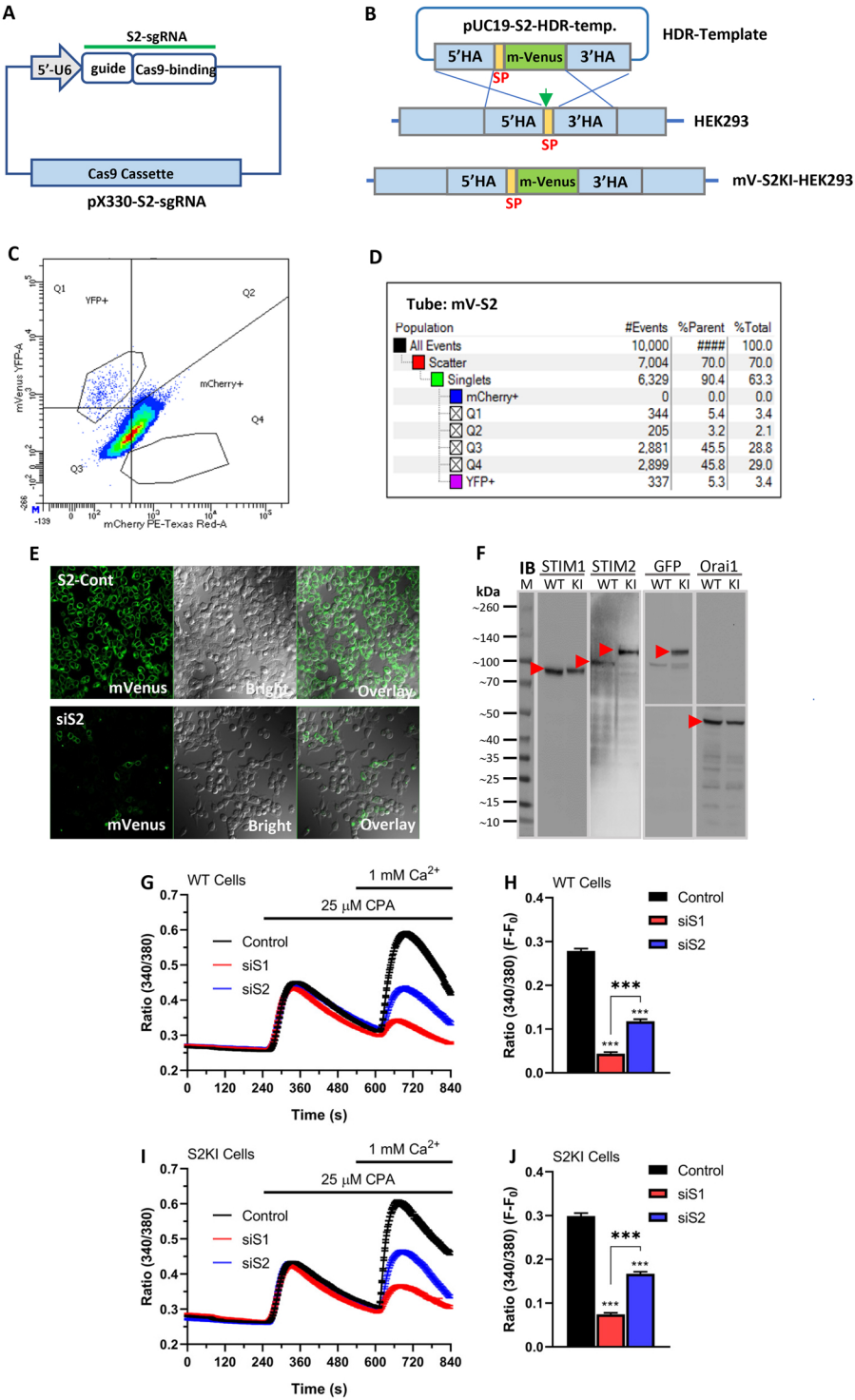
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Fig. S2

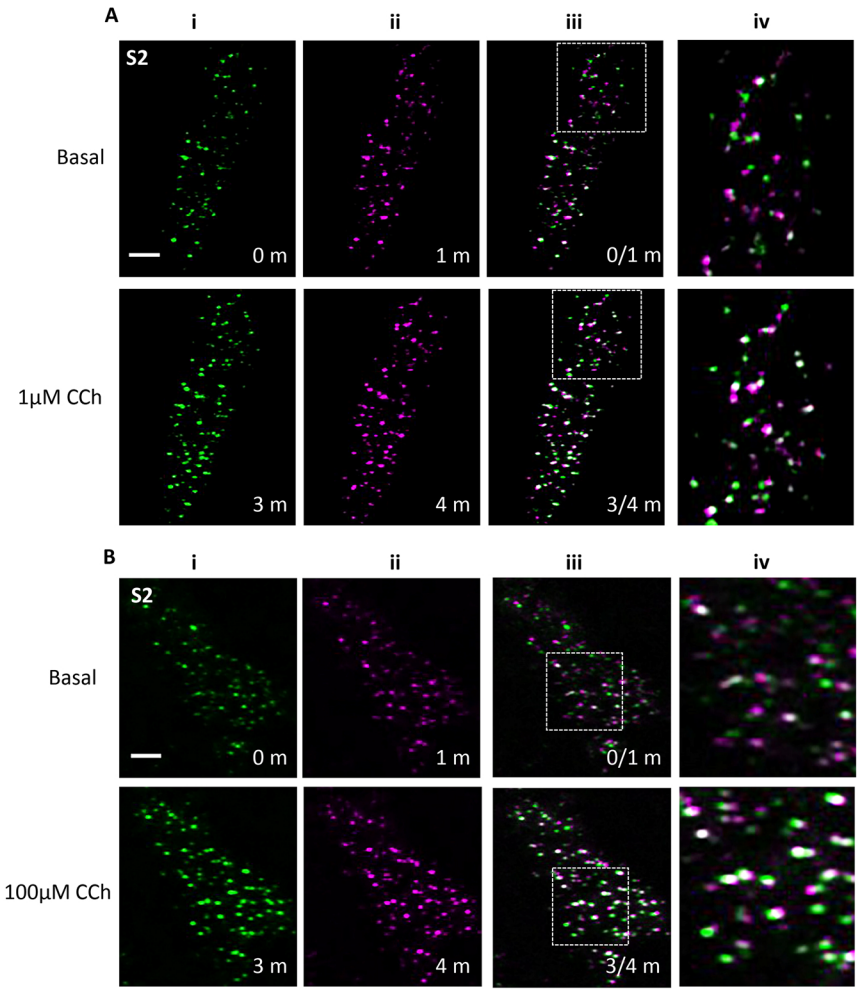
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Fig. S3

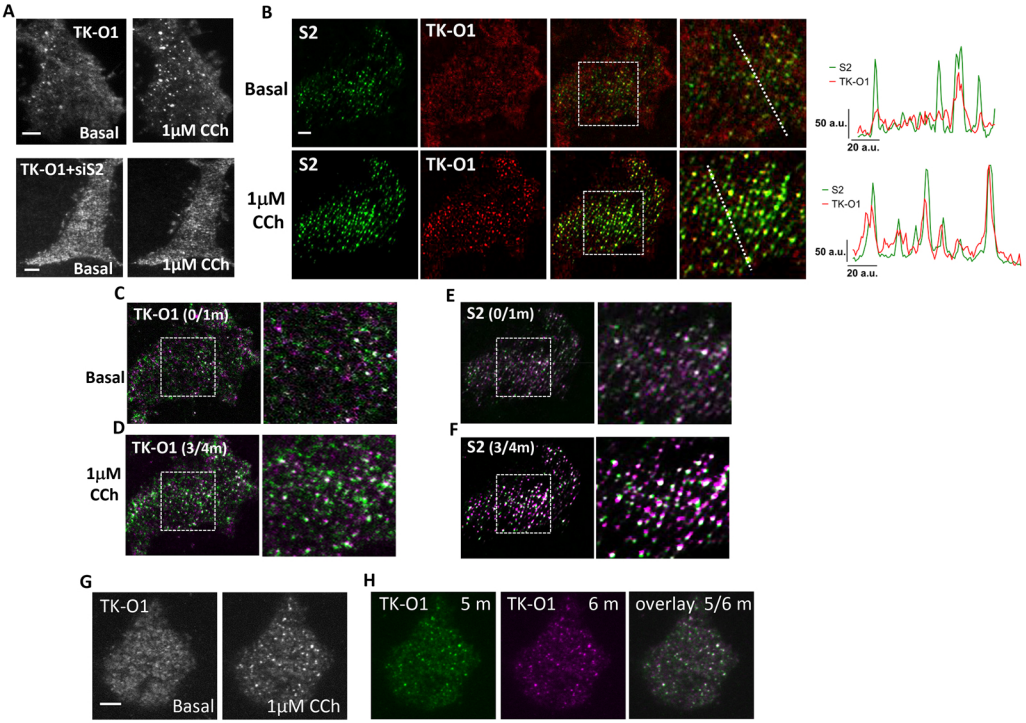
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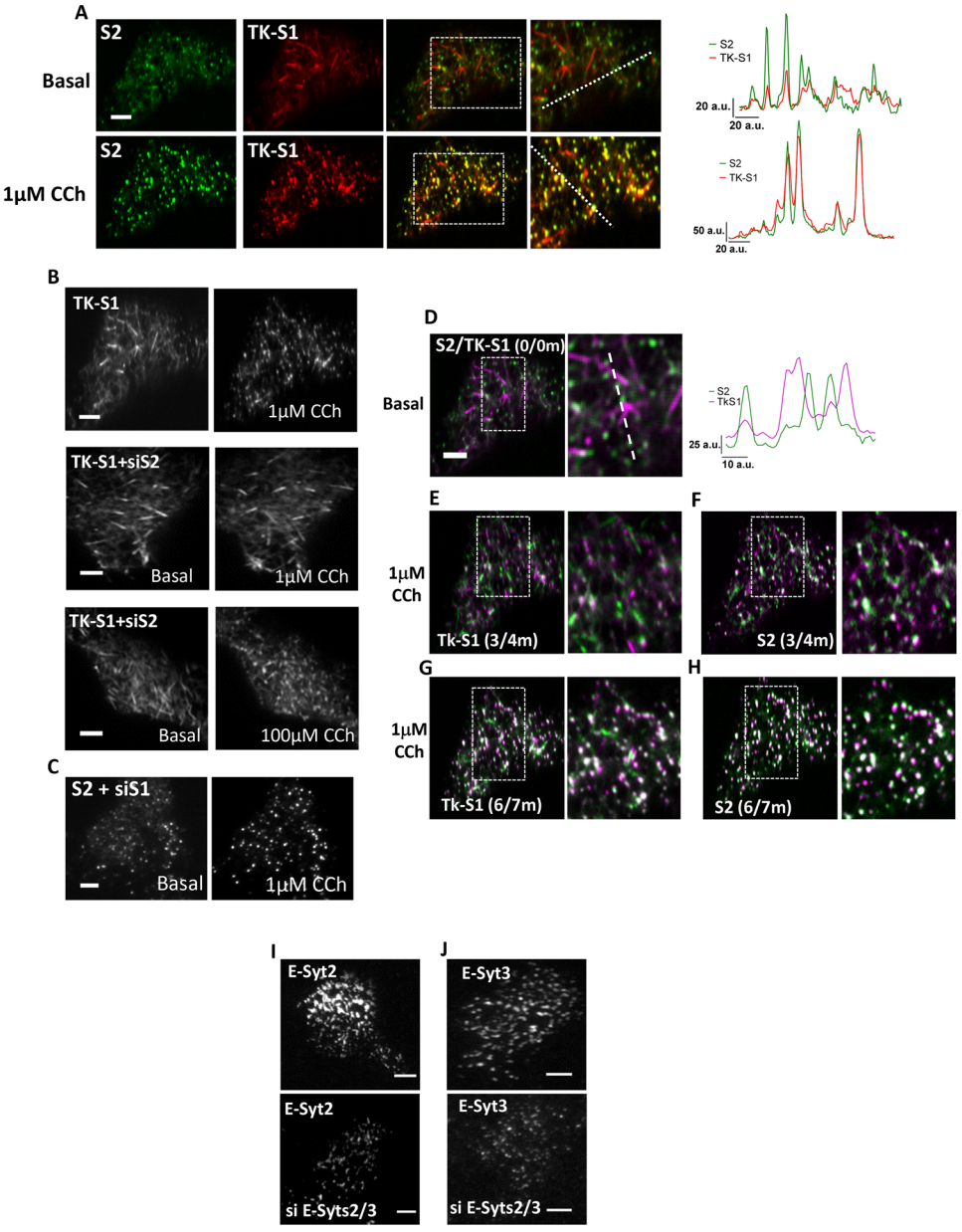
Fig. S4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Basal
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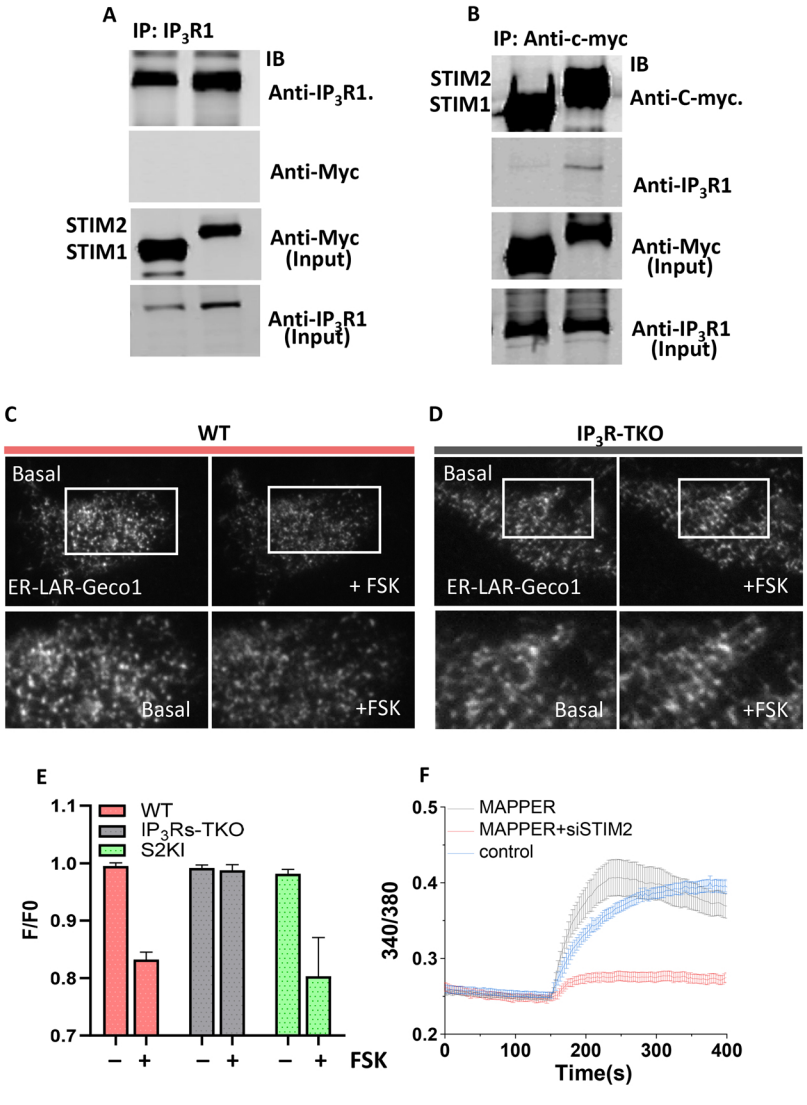
Fig.S5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IP3R-TKO
Basal
ER-LAR-Geco1
+FSK
Basal
+FSK
E
WT
IP3Rs-TKO
S2KI
F/F0
1.1
1.0
0.9
0.8
0.7
– + – + – +
FSK
F
MAPPER
MAPPER+siSTIM2
control
340/380
0.5
0.4
0.3
0.2
0
100
200
300
400
Time(s)