Supporting Information

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SI Text

Materials and Methods

Molecular Biology and Electrophysiology. pSGEM-mCherry-STIM1 was obtained by subcloning the mCherry-STIM1 fragment from pCMV6-XL5-mCherry-STIM1into the NotI site of pSGEM, and pSGEM-GFP-Orai1 was constructed by subcloning NheI/Klenow-XhoI GFP-Orai1 fragment from pEX-GFP-Myc-Orai1 into EcoRV-XhoI in pSGEM. For pSGEM-C-Terminus-STIM1-mCherry plasmid, C-terminus STIM1 (235–685) fragment was amplified using the following primers: forward 5′- CTCGAGCTCAAGCTTATGCGTTAC TCCAAGGAGCACATGAAG; reverse 5′- GACTGCAGAATTCGCTTCTTAAGAGGCTTCTTAAAG, and subcloned into the HindIII/EcoRI of pCherry-N1 plasmid, which was generated by substituting the GFP fragment between the BamHI/NotI sites in pEGFP-N1 plasmid (Addgene) with mCherry obtained by PCR. The CT-STIM1-mCherry fragment was generated by PCR using primers: forward 5′-CAATTGGCGGCCGCATGCGTTACTCCAAGGAGCACATGAAG, reverse 5′- CTGATGCTCGAGTTACTTGTACAGCTCGTCCATGCCG, and into NotI/XhoI site of pSGEM. All point mutants were generated using the Quickchange mutagenesis kit from Stratagene. All mutants and constructs were verified by DNA sequencing and by analytical endonuclease restriction enzyme digestion. For in vitro transcription, after linearization with NheI, capped RNAs of GFP-Orai1, C-terminal-STIM1-Cherry, Cherry-STIM1 wild-type and mutants were transcribed *in vitro* using T7 RNA polymerase with T7 mMESSAGE mMACHINE kit (Ambion). Oocytes were injected with 1ng RNA of GFP-Orai1 and 5ng RNA of C-terminal-STIM1-Cherry, Cherry-STIM1 wild-type or mutants for electrophysiological recording; injected with 2ng RNA of GFP-Orai1 and 10ng RNA of C-terminal-STIM1-Cherry, Cherry-STIM1 wild-type and mutants for confocal imaging. Cells were kept at 18°C incubator for 1–3 days after injection. For the pSGEM-mCherry-xCaveolin-1 plasmid, a BamHI- XhoI fragment containing the *Xenopus* Caveolin-1 cDNA (from ATCC) was subcloned into the corresponding site in pSGEM to generate pSGEM-xCaveolin-1. Then mCherry was PCR amplified using primers: forward 5′- ACGCGGCCGCATGGTGAGCAAGGGCGA, reverse 5′- ACGGATCCGCGGCTTGTACAGCTCGTCCATGCC, digested and inserted into NotI-BamHI sites of pSGEM-xCaveolin-1.

Imaging. In some experiments, the cell membrane was stained with wheat germ agglutinin (WGA)-Alexa633 (Molecular Probes) in OR2 solution (in mM: 82.5 NaCl, 2.5 KCl, 1 $CaCl_2$, 1 Na_2HPO_4, 5 HEPES, pH7.5) for 10 min at RT. Cells were normally scanned in OR2 solution; except for experiments of ER store depletion with 5mM TPEN for 10 min at RT, cells were scanned in nominally Ca^{2+} free OR2 solution. For FRAP analyses pre-bleach and recovery images were scanned (pixel dwell: 0.84 μs) at 561 nm at 1% laser power. A 25 x 25 pixels square was bleached at 60% laser power at reduced scanning speed (pixel dwell: 5.31 μs). FRAP recovery curves were derived by comparing with reference unbleached area, subtracting the background, and fitting with a monoexponential decay function.

Immunoprecipitation and Western Blotting. For STIM1 immunoprecipitation, uninjected oocytes or cells injected with mCherry-STIM1 WT or mutants were collected, and lysed in: (in mM) 30 HEPES (pH 7.5), 100 NaCl, 100 NaF, 2 sodium vanadate, 50 β-glycerophosphate, 10 Sodium pyrophosphate, 5 EDTA, 5 EGTA, 1 DTT and protease inhibitor cocktail III). Crude lysates were centrifuged three times at 1000g at 4°C for 10 min to remove yolk. NP-40 was then added to a final concentration of 4% followed by incubation at 4°C for 2 hr with constant rotating and centrifugation at 20,000 $\times$ g for 20 min.

Primers for Mutagenesis. STIM1-D76A

Forward: 5′-CCACAAACTGATGGCCGATGATGCCAATG-3′
Reverse: 5′-CATTGGCATCATCGGCCATCAGTTTGTGG-3′

STIM1–4A (S618,S621,T626,S628A)

Forward: 5′-CCCGTTCTCACGCCCCCAGCGCCCCAGACCCAGACGCACCAGCTCCAGTTGGGG-3′
Reverse: 5′-CCCCAACTGGAGCTGGTGCGTCTGGGTCTGGGCGCTGGGGGCGTGAGAACGGG-3′

STIM1–4E (S618,S621,T626,S628E)

Forward: 5′-CCCGTTCTCACGAGCCCAGCGAGCCAGACCCAGACGAACCAGAGCCAGTTGGGG-3′
Reverse: 5′-CCCCAACTGGCTCTGGTTCGTCTGGGTCTGGCTCGCTGGGCTCGTGAGAACGGG-3′

STIM1-S486,S492A

Forward: 5′-GAGGAGATTGTGGCTCCCTTGTCCATGCAGGCCCCTAGCCTGCAG-3′
Reverse: 5′-CTGCAGGCTAGGGGCCTGCATGGACAAGGGAGCCACAATCTCCTC-3′

STIM1-S486,S492E

Forward: 5′-GAGGAGATTGTGGAGCCCTTGTCCATGCAGGAGCCTAGCCTGCAG-3′
Reverse: 5′-CTGCAGGCTAGGCTCCTGCATGGACAAGGGCTCCACAATCTCCTC-3′

STIM1-S575A

Forward: 5′-GAAACTGCCTGACGCCCCTGCCCTGGCC-3′
Reverse: 5′-GGCCAGGGCAGGGGCGTCAGGCAGTTTC-3′

STIM1-S575E

Forward: 5′-GGAGAAACTGCCTGACGAGCCTGCCCTGGCCAAGA-3′
Reverse: 5′-TCTTGGCCAGGGCAGGCTCGTCAGGCAGTTTCTCC-3′

STIM1-S668A

Forward: 5′-GAAACAGACTCCGCCCCAGGCCGGAAG-3′
Reverse: 5′-CTTCCGGCCTGGGGCGGAGTCTGTTTC-3′

STIM1-S668E

Forward: 5′-GAAACAGACTCCGAGCCAGGCCGGAAG-3′
Reverse: 5′-CTTCCGGCCTGGCTCGGAGTCTGTTTC-3′

Mass Spectrometry

In-Gel Trypsin Digestion. The sample was resuspended in SDS NuPAGE LDS sample buffer, and resolved on a 4–12% gradient Nu-PAGE gel (Invitrogen). The gel was stained with Simply BlueTM SafeStain according to the manufacturer's instructions. The gel plugs containing the proteins were then robotically (Progest,Genomic Solutions) trypsin digested using an in-gel digestion protocol (1).

Analysis of Peptides by nanoLC/MS/MS. The resulting 50-μL peptide pools were separated by reverse phase high performance liquid chromatography (RP-HPLC) using a splitless HPLC system (Nano LC-2D, Eksigent). Peptides were loaded on a 4 mm $\times$ 75 μm column packed with C12 resin (Jupiter proteo 4 μm, Phenomenex) and eluted using a gradient elution (gradient conditions were 0.1–30% B in 35 min, 30–50% B in 10 min,

50–80% B in 5 min) flowing at 200 nL/min over the analytical column (15 cm × 75 μm packed with the same resin). The column was interfaced to a 30 μm × 3 cm stainless steel emitter (Proxeon, Odense) onto which 1.8 kV was applied. The peptides were analyzed by an ion trap (LCQ Deca XP Plus, Thermo) using data-dependent acquisition. Each scan cycle consisted of one parent MS scan that used a *m/z* range of 400–1,600 followed by 4 MS/MS scans of the 4 most intense ions that had not been previously selected for fragmentation. Normalized collision activation energy was set at 35% and 3 microscans were summed following automatic-gain-control implementation which consisted of 5 × 108 ions for MS and 6 × 107 ions for MS/MS. Dynamic exclusion was set for 30 s, ensuring that peptides were only selected once for fragmentation. The total run time was 62.5 min for each analysis.

Data Searching. Data from the MS/MS analysis was searched against the nonredundant NCBI database for *Xenopus laevis* proteins using Mascot as the search engine. This process was automated by Mascot Daemon. Peptides were searched with a fixed modification of carbamidomethyl (Cys), and variable modifications of oxidation (Met), phosphorylation of serine or threonine, and phosphorylation of tyrosine. Maximum limit for missed cleavages was set at 2, peptide mass tolerance was set at 2.0 Da, and fragment ion tolerance was set at 0.6 Da. The search results were then parsed into Scaffold (Proteome Software), which verified peptide identification from Mascot and probabilistically validates these peptide identifications using PeptideProphet (2) and derives corresponding protein probabilities using ProteinProphet (3).

Phosphoprotein Identification. Candidate phosphopeptides (peptides identified by Mascot as phosphorylated) were all manually verified and annotated to ensure genuineness. The fragment ion data were vigilantly examined to ensure sufficient spectral data (both intact ion series and ion series containing the typically observed neutral loss) existed to confirm the localization of the phosphorylation site on the peptides containing multiple potential sites.

1. Edmondson RD, et al. (2002) Protein kinase C epsilon signaling complexes include metabolism- and transcription/translation-related proteins: Complimentary separation techniques with LC/MS/MS. *Mol Cell Proteomics* 1:421–433.
2. Keller A, Nesvizhskii AI, Kolker E, Aebersold R (2002) Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem* 74:5383–5392.
3. Nesvizhskii AI, Keller A, Kolker E, Aebersold R (2003) A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem* 75:4646–4658.

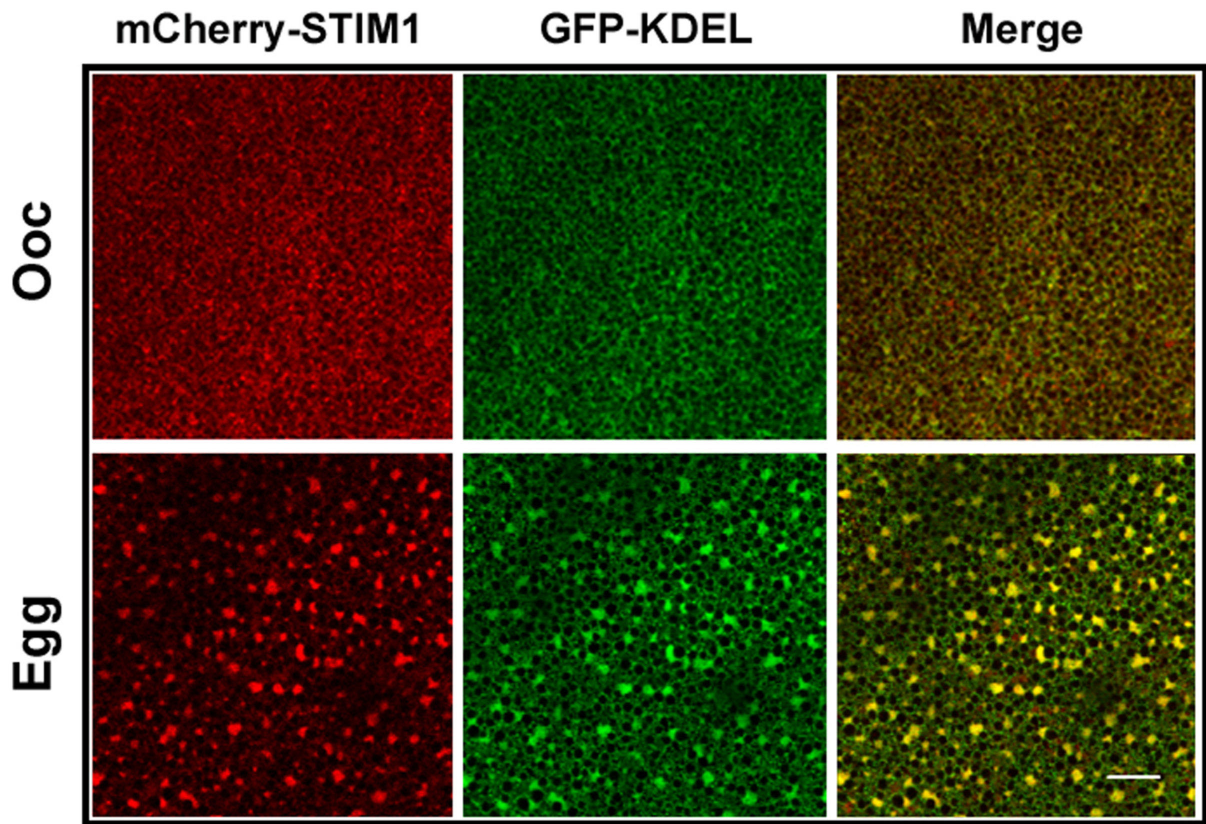

Fig. S1. Colocalization of mCherry-STIM1 (10 ng RNA injected) with GFP–KDEL (10 ng RNA injected) to mark the ER in both oocytes and eggs. Confocal images of oocytes and eggs coinjected with mCherry–STIM1 and GFP–KDEL. Note that STIM1 and GFP–KDEL colocalize to ER patches in eggs indicating ER remodeling during maturation. (Scale bar, is 10 μm.)

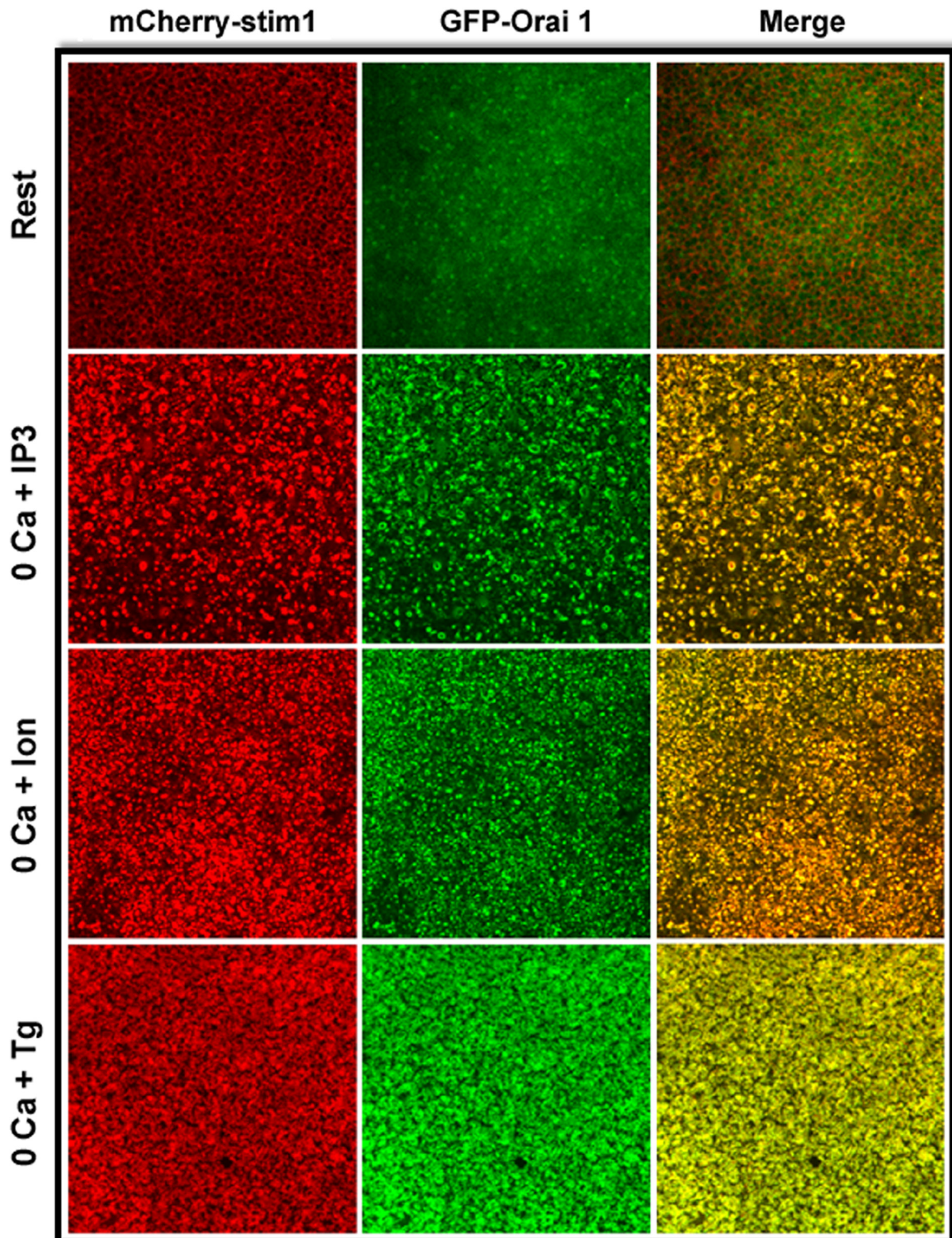

Fig. S2. Coclustering of STIM1 and Orai1 in oocytes with different ER Ca^{2+} store depletion methods. Oocytes expression both mCherry–STIM1 and GFP–Orai1 and incubated in Ca^{2+} free-OR2 solution were untreated (Rest) or stores were depleted by either IP_3 injection (2 μM) (0 Ca^{2+} +IP3), incubation with ionomycin (10 μM) for 10 min (0 Ca^{2+} +Ion), or incubation with thapsigargin (5 μM) (0 Ca^{2+} +Tg) for 3 h at room termperature. Scale bar is 10 μM.

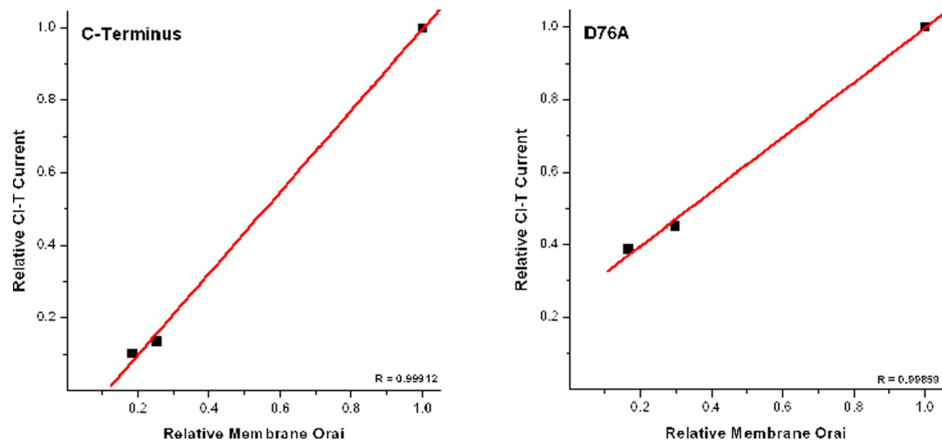

Fig. S3. Correlation between the levels of cell membrane GFP–Orai1 and Ca^{2+} influx levels as measured using $I_{Cl\text{-}T}$. Data from Fig. 3 for both GFP–Orai1 fluorescence at the cell membrane and Cl-T current levels were normalize to the values in oocytes, plotted, and fitted with linear regression.

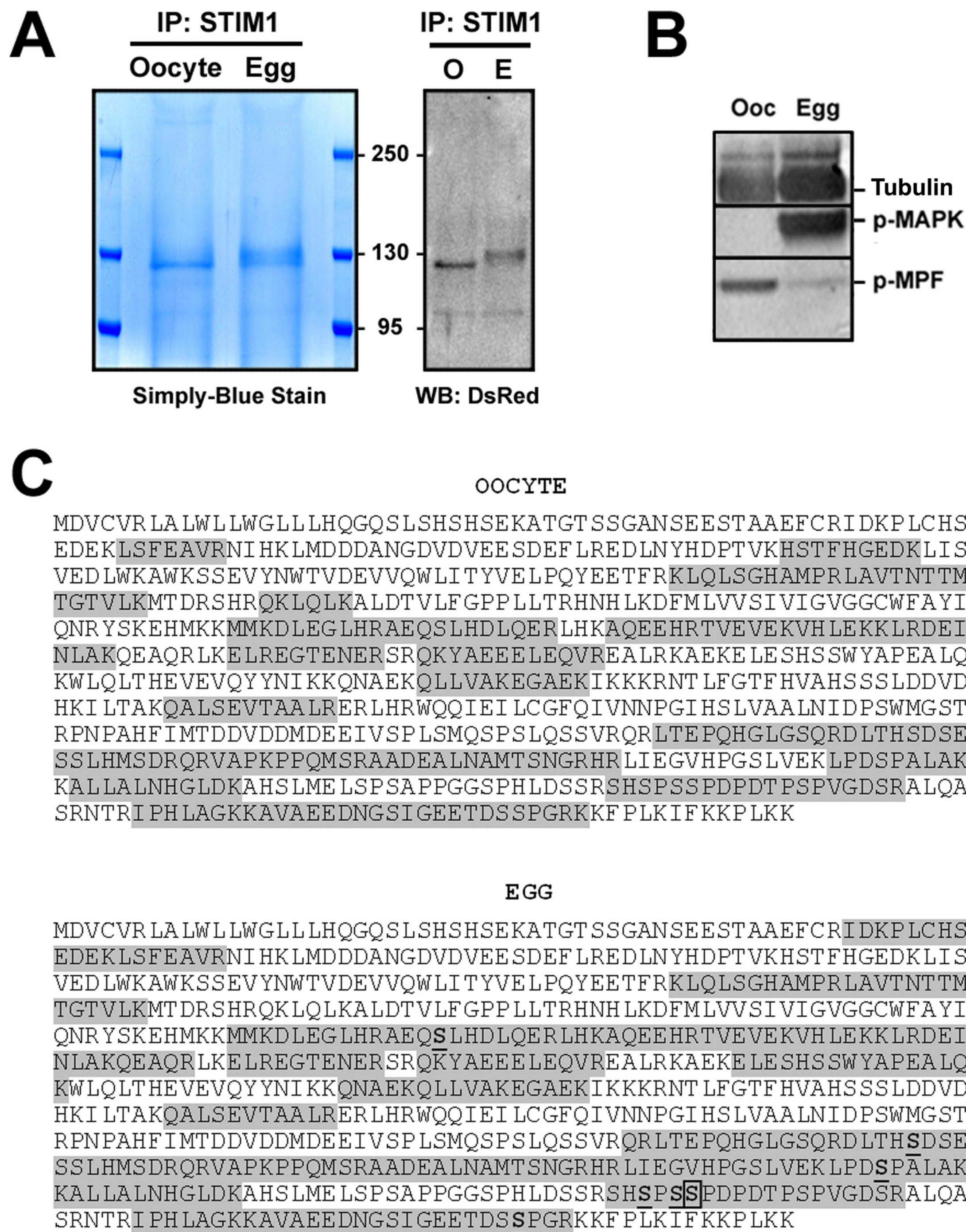

Fig. S4. Mass spectrometry analysis of stim1 phosphorylation. (*A*) Simply-blue stained gel for mass spectrometry analysis. STIM1 was immunoprecipitated (IP) from mCherry–STIM1 injected oocytes or eggs. One-fourteenth of the samples were subjected to Western blot analysis by using anti-DsRed antibody to confirm successful stim1 IP as indicated by the ≈130-kDa bands and the slower mobility in eggs. (*B*) Western blot analysis of MAPK and MPF kinase activity from samples using anti-phospho-MAPK, anti-phospho-Cdc2 antibodies. Tubulin was used as loading control. (*C*) Identification of STIM1 phosphorylation sites. STIM1 IP samples from oocytes and eggs were digested in-gel with trypsin and the resulting peptides analyzed by nanoLC/MS/MS to identify phosphorylated peptides and mapped the phosphorylated residues. The IP was performed on 2 batches of oocytes and the MS analysis performed 3 times. No phosphorylation was detected in oocytes. Residues where phosphorylation was detected in eggs are underlined. However only 1 of these residues (box) was detected as phosphorylated in more than 1 MS study, indicating that stim1 phosphorylation is hetergenous in meiosis. Overall MS coverage was 38% and 44% in oocytes and eggs respectively, whereas coverage in the cytoplasmic region of stim1, which is accessible to the kinases, was 48% and 57%, respectively, in oocytes and eggs.

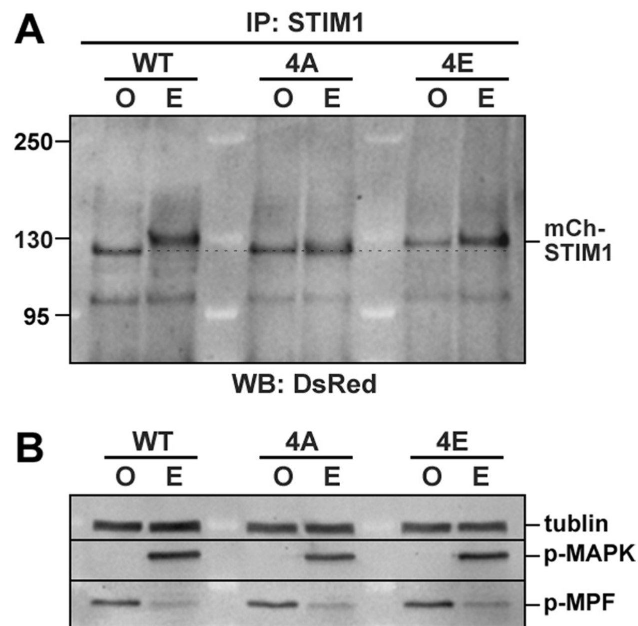

Fig. S5. Changes in electrophoretic mobility of the stim1^{4A} and stim1^{4E} mutants during meiosis. (*A*) The differential electrophoretic mobility of STIM1 in eggs versus oocytes is reversed in the stim1^{4A} and stim1^{4E} mutants. Note that the stim1^{4E} mutant runs with a slower mobility similar to that of wild-type stim1 in eggs, indicating that phosphomimetic mutations at residues S618,S621,T626,S628 is sufficient to replicate the slower mobility behavior of stim1 in eggs. (*B*) Western blot analysis of MAPK and MPF kinase activity from STIM1 WT or 4A/E mutants expressing cells using anti-phospho-MAPK, anti-phospho-Cdc2 antibodies. Tublin was used as loading control.