Figure S1. **Characterization of Rabs and dynamin on Orai1 trafficking.** (A) Oocytes were injected with GFP-Orai1 (2 ng RNA/cell) and RFP-Rab5 [wild type (WT)] or the constitutively active Rab5 mutant, RFP-Rab5Q79L (10 ng RNA/cell), and then matured into eggs. Confocal images were taken deep into the cell (~2 µm) to visualize late endosomes in the egg. (B) Images from an oocyte and an egg expressing both GFP-Orai1 and mCherry-Rab7 taken at a deep focal plane within the cell. (C) C3 exoenzyme partially inhibits Orai1 internalization in eggs. Oocytes expressing GFP-Orai1 (2 ng RNA/cell) and x1-MEM16a (8 ng RNA/cell) were injected with C3 exoenzyme (1.7 ng/cell), allowed to rest for 1 h, and then matured into eggs. Orthogonal sections of deep plane images are shown. C3 exoenzyme induces invaginations at the cell membrane, where Orai1 and TMEM16A are colocalized. Internalized Orai1 in endosomes, as indicated by the arrows and boxes deep inside the cell, does not colocalize with TMEM16A. Positions of arrows and boxes in the orthogonal section are indicated by the matching arrow and bar. (D) Images taken at a deep focal plane within the cell for a representative oocyte and egg expressing GFP-Orai1 alone, GFP-Orai1 with dynamin (Dyn) wild type, and GFP-Orai1 with dynamin K44A. Bars: (A–C) 5 µm; (D) 2 µm.
Figure S2. **Subcellular distribution of the Cav dominant-negative mutant P168L.** (A) Sequence alignment of human (NCBI Protein database accession no. NP_001744) and Xenopus (GenBank/EMBL/DDBJ accession no. AF455042_1) Cav-1. Proline (P) 132 in human and P168 in Xenopus are shown in red. (B) Coexpression of GalNAc-GFP (10 ng RNA/cell) with mCherry-Cav (Ch-Cav; 10 ng RNA/cell) in oocytes and eggs. Images are from a z stack on each cell, with the first image representing the surface of the visible signal in the stack (Surface) and the second image taken ~2-µm deeper into the cell (Deep). In addition, orthogonal sections through the z stacks are shown. (C) Coexpression of GalNAc-GFP with mCherry-Cav-P168L (10 ng RNA/cell) in oocytes and eggs. In oocytes and eggs, GalNAc-GFP and mCherry-Cav-P168L colocalize to the ER. In some eggs, GalNAc-GFP and mCherry-Cav-P168L colocalize to the Golgi compartment (bottom). (D) Orai1 is trapped in the Golgi and ER compartments when it was coexpressed with the dominant-negative Cav mutant (P168L) in oocytes and eggs. Bars, 2 µm.
**Figure S3. Subcellular distribution of N- and C-terminal Orai1 deletions.** (A) Oocytes and eggs expressing Orai1-ΔN90-CFP (5 ng RNA/cell) and the ER marker Cherry-KDEL (ch-KDEL). Both deep focal plane images and orthogonal sections are shown. (B) Representative oocyte and egg images both at the cell surface and deep into the cytoplasm from cells expressing GFP-Orai1-ΔC267 (2 ng RNA/cell) and the ER marker Cherry-KDEL. (left) Orthogonal sections are also shown. (C) Orthogonal sections and deep focal plane images from oocytes and eggs expressing GFP-Orai1-ΔC289 with Cherry-KDEL. (D) Cav has no effect on Orai1-ΔN70-CFP internalization in eggs. Representative orthogonal sections from oocytes and eggs expressing Orai1-CFP or Orai1-ΔN70-CFP (5 ng RNA/cell) alone or with Cav (10 ng RNA/cell) as indicated. Orai1-CFP localizes to the plasma membrane in oocytes and internalizes and enriches into endosomes in eggs; coexpression of Cav does not affect Orai1 internalization in eggs (left). Orai1-ΔN70-CFP is not internalized in eggs, and coexpression of Cav does not restore its ability to internalize into endosomes in eggs (right). (E and F) Cav but not Rab5 rescues GFP-Orai1-ΔC267 internalization in eggs. Oocytes and eggs coexpressed GFP-Orai1-ΔC267 (2 ng RNA/cell) with either Cherry-Cav (10 ng RNA/cell) or RFP-Rab5 (10 ng RNA/cell). Orthogonal section and images from z stack are shown to visualize Orai1 distribution between the cell membrane and endosomal compartment. (E) In eggs, Cherry-Cav rescues GFP-Orai1-ΔC267 internalization and shifts its cellular distribution from plasma membrane to the endosomal compartment. (F) Coexpression of GFP-Orai1-ΔC267 with RFP-Rab5 does not change plasma membrane localization in eggs. Bars, 5 µm.
Figure S4. **Subcellular distribution of Orai1 C terminus point mutants.** (A and B) Oocytes and eggs expressing GFP-Orai1-L282S (A) or L273S mutants (B; 2 ng RNA/cell) with the ER marker Cherry-KDEL (ch-KDEL; 10 ng RNA/cell). Orthogonal sections are shown for all treatments with corresponding images either from a surface (cell membrane) or a deep focal plane to visualize the endosomal or ER compartments. Similar to wild type, GFP-Orai1-L282S localizes to the plasma membrane in oocytes and internalizes to the endosomal compartment in eggs. Although GFP-Orai1-L273S localizes to the ER in oocytes and eggs. The ER remodels in eggs and forms patches. Bars, 5 µm.

Figure S5. **Colocalization of GFP-Orai1 with human Cav.** (A) Images and orthogonal sections illustrating the colocalization of Orai1 with human Cav-1 (hCav) in both oocytes and eggs. (B) Mutations of two aromatic residues, F250 and F253, expected to disrupt a putative C-terminal Cav-binding site, have no effect on Orai1 trafficking in oocytes and eggs. Cells were injected with Cherry-KDEL (ch-KDEL; 10 ng RNA/cell) and GFP-Orai1-F250A,253A (2 ng RNA/cell). (C) Mutation of a consensus MAPK/MPF site (T295) in the C terminus of Orai1 to test the potential role of phosphorylation at this residue on Orai1 internalization. Mutating T295 to Ala does not affect Orai1 trafficking in oocytes or in eggs. Generating the phosphomimetic mutant T295E similarly shows no effect on Orai1 trafficking (not depicted). Bars, 5 µm.