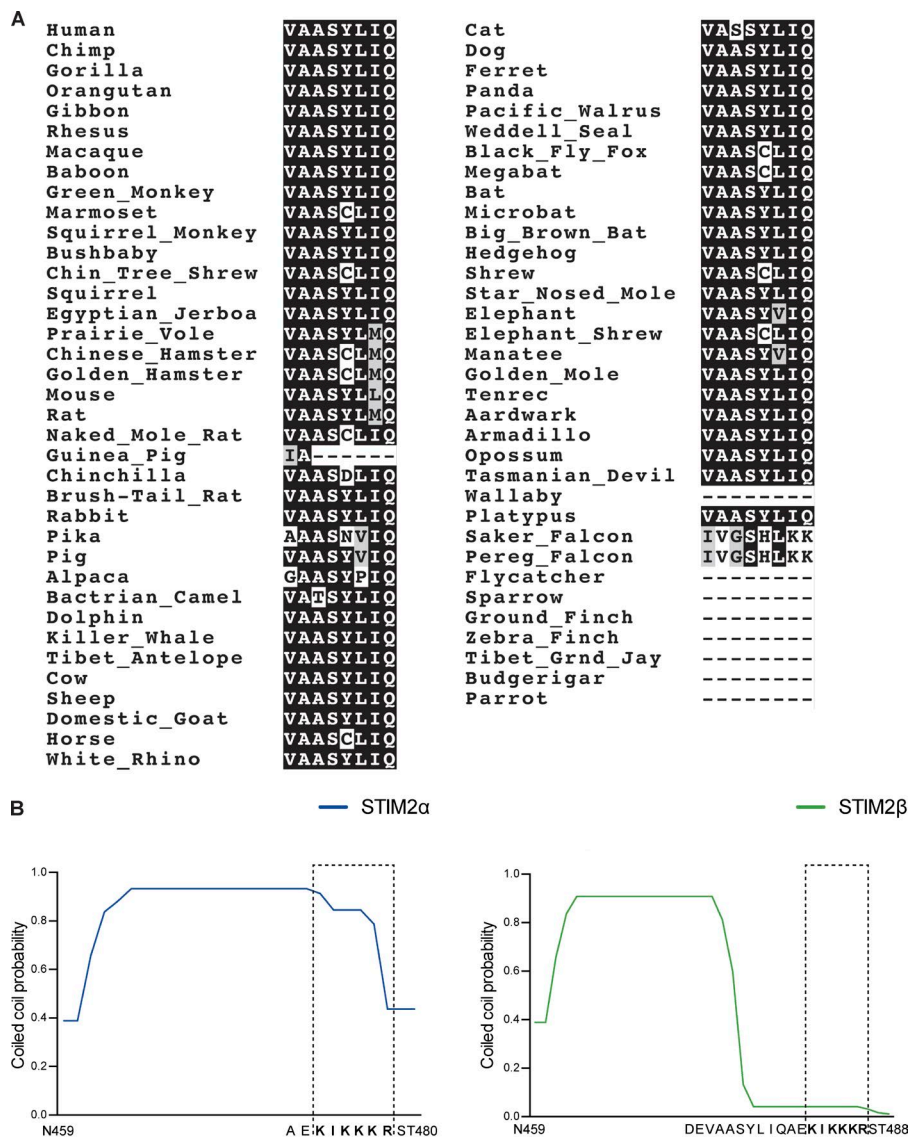


Rana et al., <http://www.jcb.org/cgi/content/full/jcb.201412060/DC1>

**Figure S1. The STIM2 $\beta$  insert sequence and its predicted effect on STIM2 structure.** (A) Alignment of STIM2 $\beta$  insert sequences across available vertebrate genomes from the MultiZ tool of the University of California, Santa Cruz genome browser ([genome.ucsc.edu](http://genome.ucsc.edu)). Sequence identity and similarity are shown in black and gray, respectively. The  $\beta$  insert sequence is present and conserved in mammalian species. STIM2 exon 9 is not annotated in the genomes of nonmammalian vertebrates, but it is unclear whether this indicates the absence of this exon in these species. (B) Coiled-coil forming propensity of STIM2 $\alpha$ - and STIM2 $\beta$ -CAD predicted by COILS. The critical basic residues (KIKKKR, highlighted in bold) involved in Orai binding show large differences between STIM2 $\alpha$  and STIM2 $\beta$ . A window size of 14 was applied; window sizes of 21 and 28 gave qualitatively similar results. Residue numbers are based on the reference sequence in the Materials and methods.

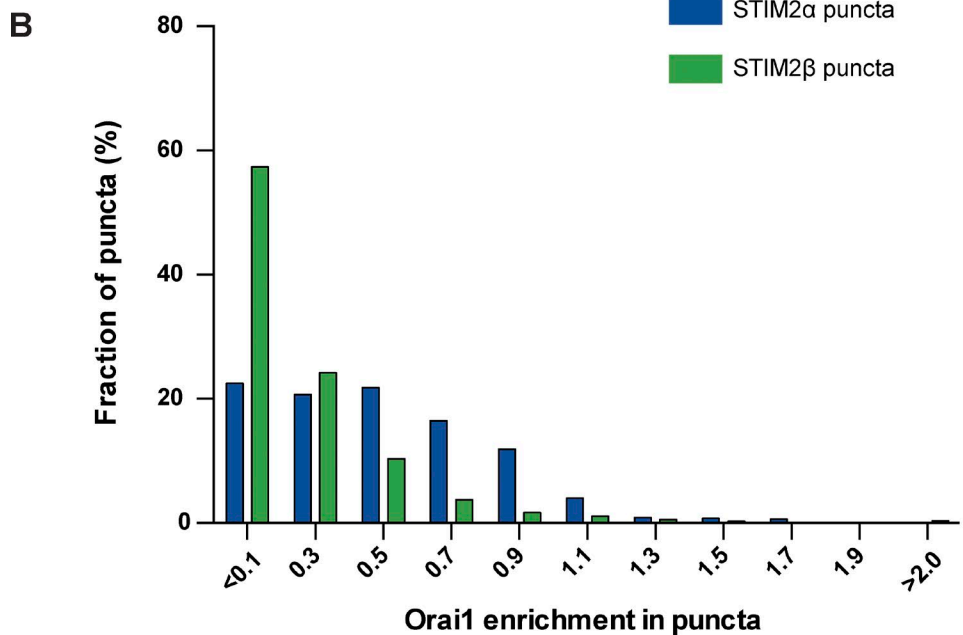
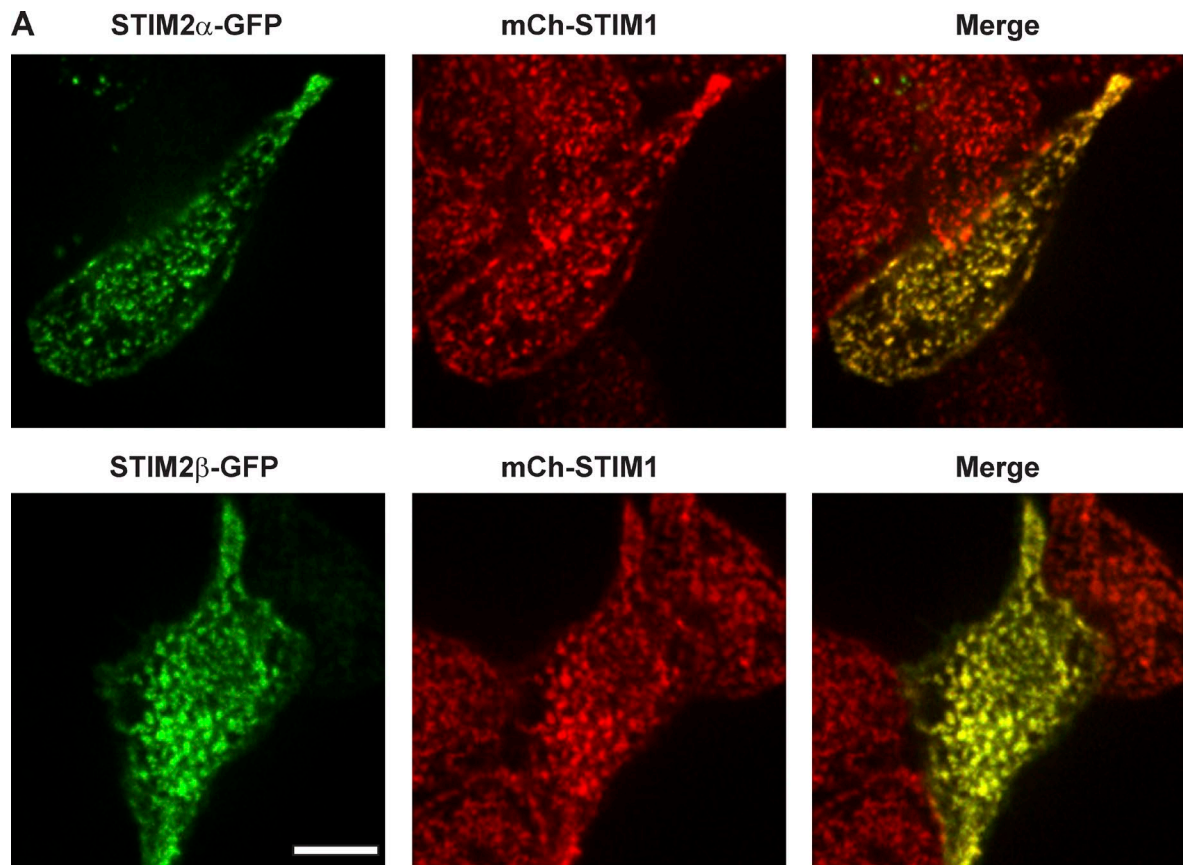


Figure S2. **STIM2 $\beta$  accumulates at ER-PM junctions but only weakly recruits Orai1.** (A) STIM2 $\beta$  puncta coincide with STIM1 puncta. HEK293 cells expressing mCherry (mCh)-STIM1 with either STIM2 $\alpha$ - or STIM2 $\beta$ -GFP were fixed after store depletion and imaged by confocal microscopy. Bar, 10  $\mu$ m. (B) Distribution of mCherry-Orai1 intensities in puncta formed by STIM2 $\beta$ - and STIM2 $\alpha$ -GFP in store-depleted HEK293 cells ( $n > 750$  puncta from  $>15$  cells for each). STIM puncta were identified (see Materials and methods) and relative Orai1 enrichment in each punctum was calculated as (Orai1 intensity in punctum – mean Orai1 intensity in cell)/(mean Orai1 intensity in cell). Approximately 60% of all STIM2 $\beta$  puncta show  $\leq 10\%$  Orai1 enrichment, whereas the remaining puncta show much less enrichment than seen in STIM2 $\alpha$  puncta. Cells expressing similar levels of Orai1, STIM2 $\alpha$ , or STIM2 $\beta$  were selected for analysis.

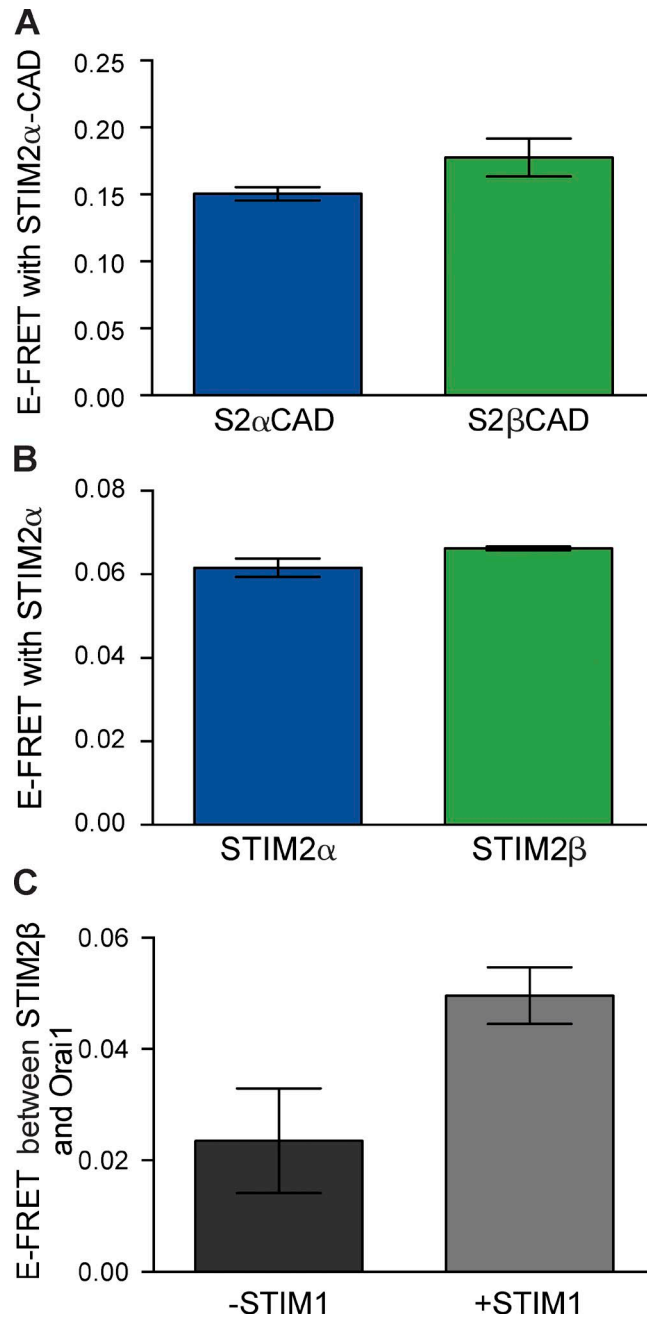


Figure S3. **STIM2 $\beta$  can heterodimerize with STIM2 $\alpha$ .** (A and B) FRET measurements between CFP-STIM2 $\alpha$ -CAD and YFP-tagged STIM2 $\alpha$ - or STIM2 $\beta$ -CAD (A), and STIM2 $\alpha$ -YFP and STIM2 $\alpha$ - or STIM2 $\beta$ -CFP (B). The interaction between full-length STIM2 $\beta$  and STIM2 $\alpha$ , as well as their CAD domains, is comparable to the homodimerization of STIM2 $\alpha$  with itself. (C) Coexpression of STIM1 increases the FRET between STIM2 $\beta$ -YFP and CFP-Orai1 ( $n \geq 8$  cells per bar,  $P = 0.034$ , two-tailed  $t$  test). Error bars show means  $\pm$  SEM.

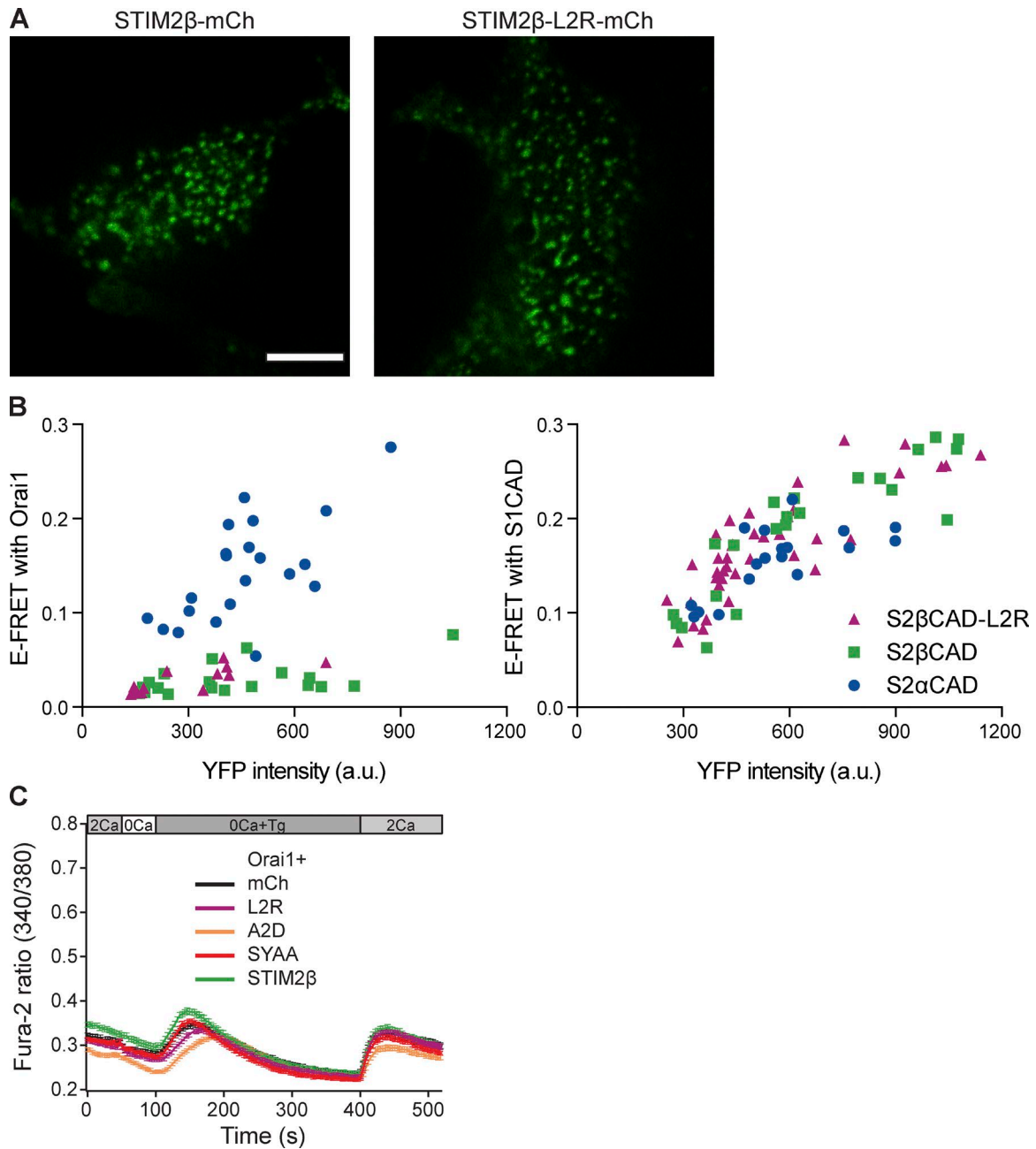


Figure S4. **Analysis of Orai1 binding, STIM1-CAD binding, and puncta formation by the STIM2 $\beta$ -L2R mutant.** (A) The STIM2 $\beta$ -L2R mutant forms puncta after store depletion. In HEK293 cells, mCherry (mCh)-tagged STIM2 $\beta$ -L2R mutant (right) localizes to the ER and forms puncta after store depletion similarly to STIM2 $\beta$  (left), suggesting that it is not grossly misfolded. Bar, 10  $\mu$ m. (B) Single-cell analysis of FRET between YFP-tagged CAD domains from STIM2 $\alpha$ , STIM2 $\beta$ , or the STIM2 $\beta$ -L2R mutant and CFP-tagged Orai1 (left,  $n > 11$  cells each) or STIM1-CAD (right,  $n > 17$  cells each) in single cells. a.u., arbitrary unit. (C) Coexpression of STIM2 $\beta$  mutants with Orai1 does not restore SOCE in Neuro2A cells ( $n > 50$  cells for each curve). Error bars show means  $\pm$  SEM.

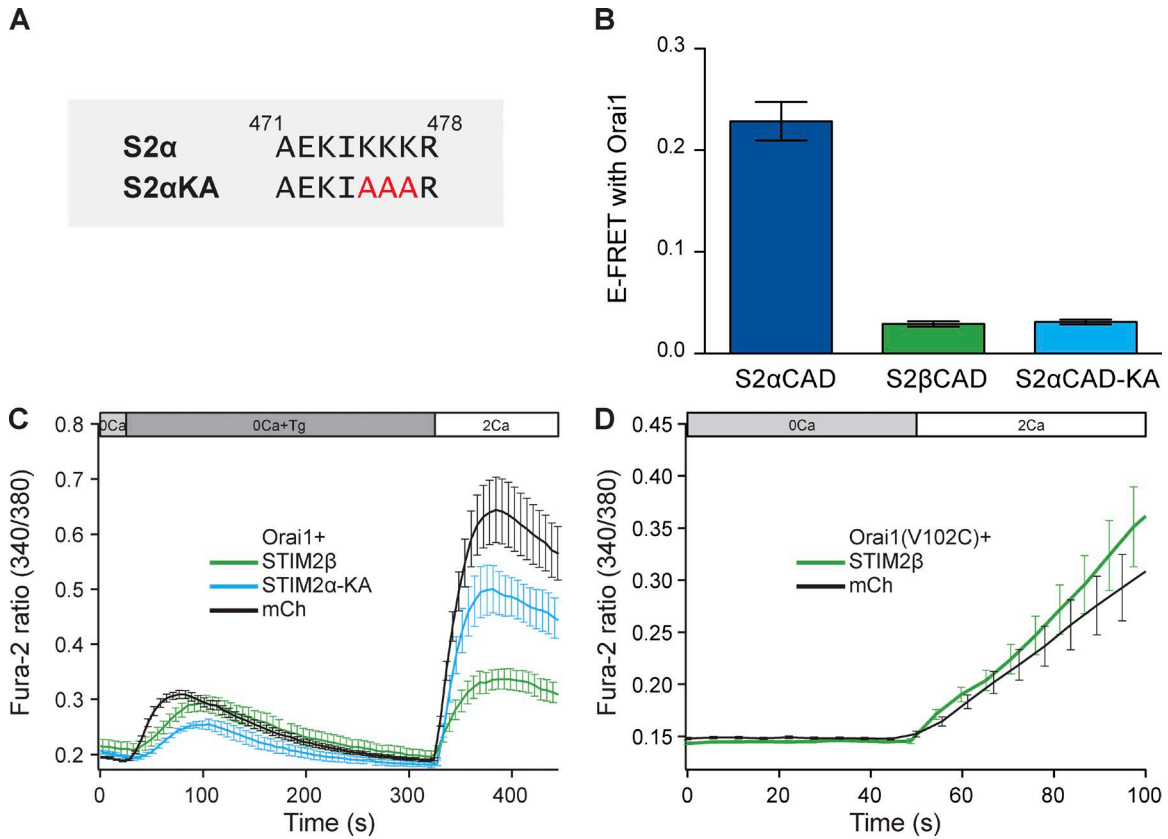


Figure S5. **The STIM2 $\alpha$ -KA mutant shows negligible binding and activation of Orai1.** (A) Sequence of the KA mutant. Residue numbers are based on the reference sequence in the Materials and Methods. (B) FRET between CFP-Orai1 and YFP-CAD domains from STIM2 $\alpha$ , STIM2 $\beta$ , and the STIM2 $\alpha$ -KA mutant. STIM2 $\alpha$ -KA mutant shows no detectable FRET above background. (C) Tg-induced SOCE in cells coexpressing Orai1 and STIM2 $\alpha$ -KA was measured using fura-2. STIM2 $\alpha$ -KA is unable to activate SOCE as assessed from the peak  $[Ca^{2+}]_i$  after  $Ca^{2+}$  readdition. (D) STIM2 $\beta$  does not inhibit Orai1(V102C) when expressed independently ( $n > 30$  cells for each curve). To prevent activation of endogenous Orai1 or any tethering of STIM2 $\beta$  to Orai1(V102C) by endogenous STIM1, the experiment was performed in store-replete cells. High expression levels of STIM2 $\beta$  were used to ensure that significant amounts of STIM2 $\beta$  are present at ER-PM junctions even in the store-replete state. Error bars show means  $\pm$  SEM.