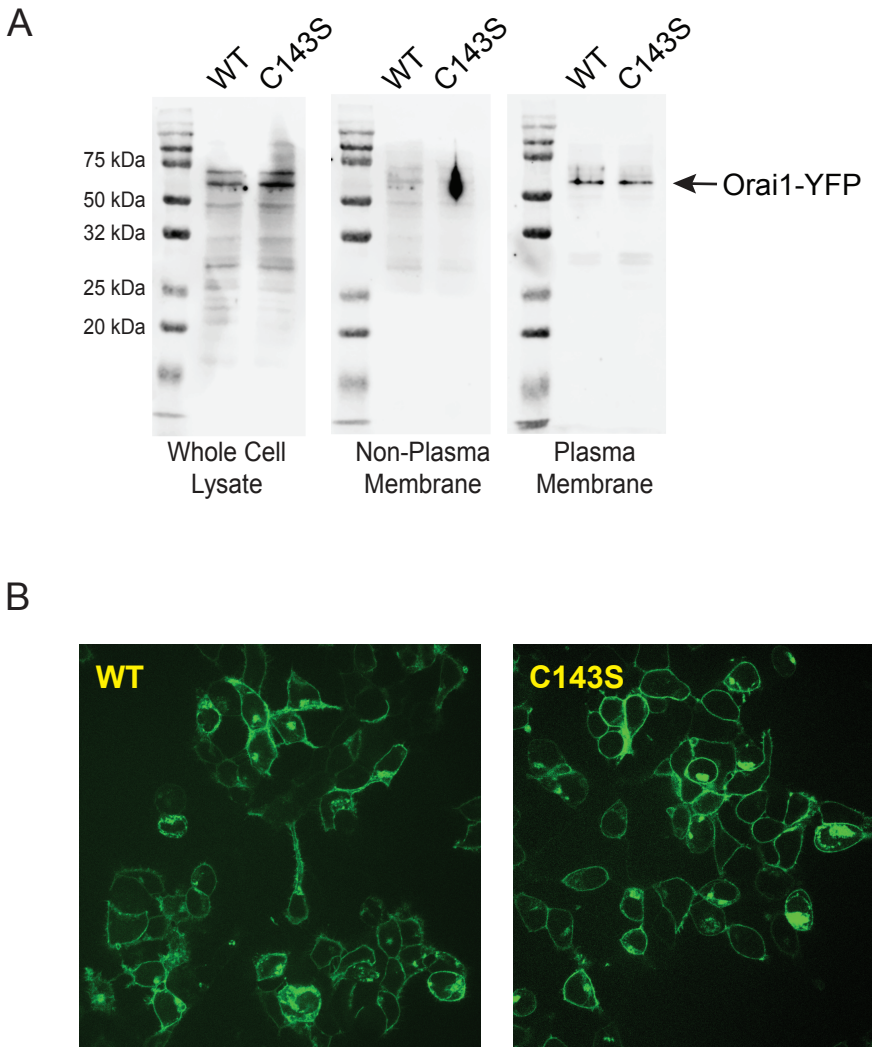


Figure S1: WT and C143S Orai1-YFP Expression. A) Cell surface biotinylation of wild-type and C143S mutant Orai1. “Non-plasma membrane” was not bound to streptavidin beads. “Plasma membrane” was eluted from the streptavidin beads. See methods for details. **B)** Confocal images of WT and C143S Orai1-YFP expressing HEK293T cells. Images were taken under identical conditions.

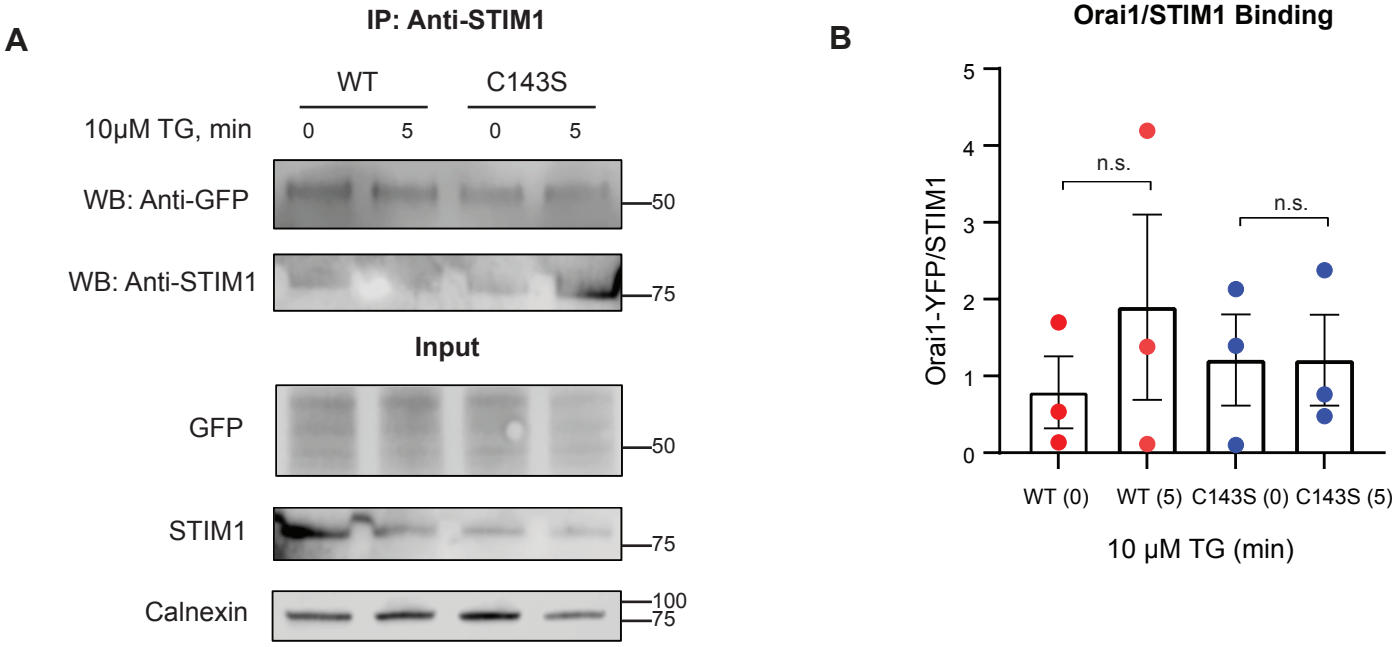


Figure S2: Co-immunoprecipitation of Orai1-YFP and STIM1. A) Representative western blot of HEK293T cells expressing either WT or C143S Orai1-YFP after co-immunoprecipitation using STIM1 antibody. Cells were treated with 10 μM thapsigargin (TG) for 0 or 5 minutes, followed by lysis, co-immunoprecipitation, and Western blot analysis. The GFP antibody cross-reacts with the YFP conjugated to Orai1. Input levels of Orai1-YFP and endogenous STIM1 are shown, along with Calnexin as a loading control. **B)** Quantification of (A), with co-immunoprecipitated Orai1-YFP normalized to co-immunoprecipitated STIM1. A two-tailed t-test showed no significant changes in Orai1/STIM1 binding after TG treatment in either WT or C143S Orai1-YFP., WT (0) vs. WT (5) p=0.4398; C143S (0) vs. C143S (5) p=0.9970. N=3.

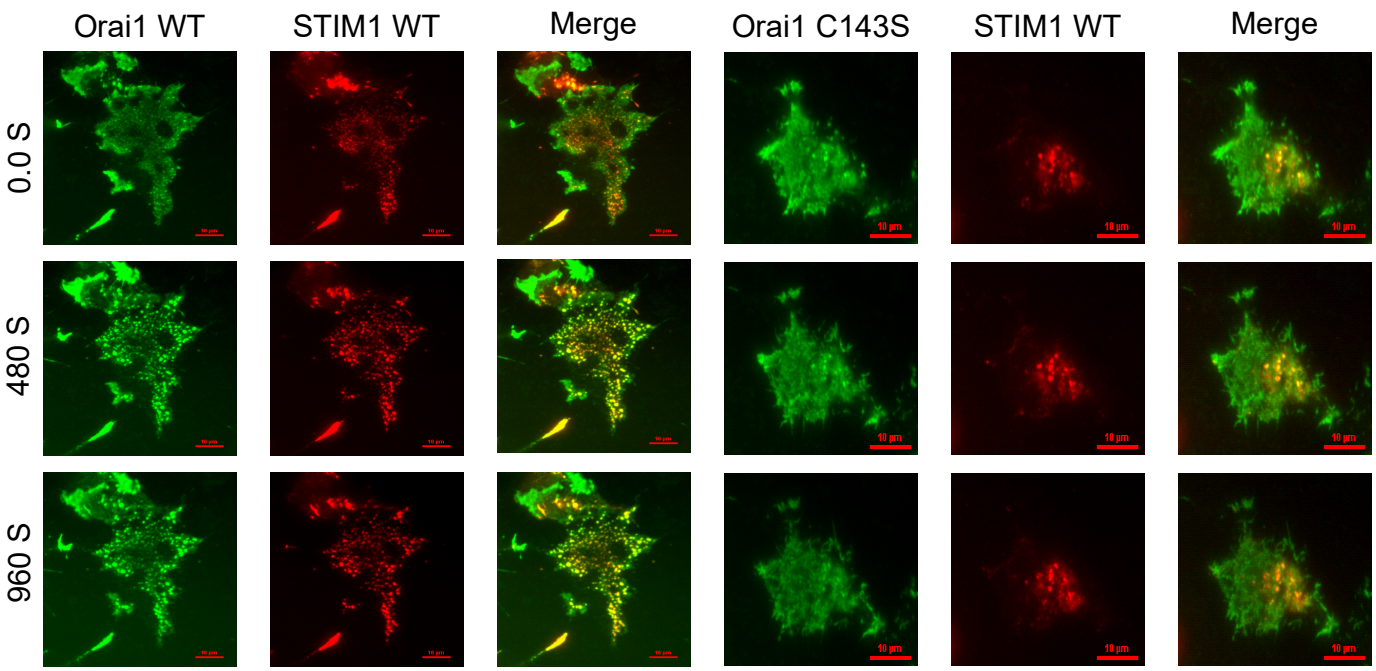


Figure S3. Individual channels from Figure 3. Shown are the individual Orai1-YFP, C143S-Orai1-YFP, and STIM1-mRFP channels obtained by TIRF microscopy as presented in Figure 3. Time (in seconds) is shown to the left of the panels. Thapsigargin (10 μM) was added at 60 seconds. A 10μm scale bar is shown.

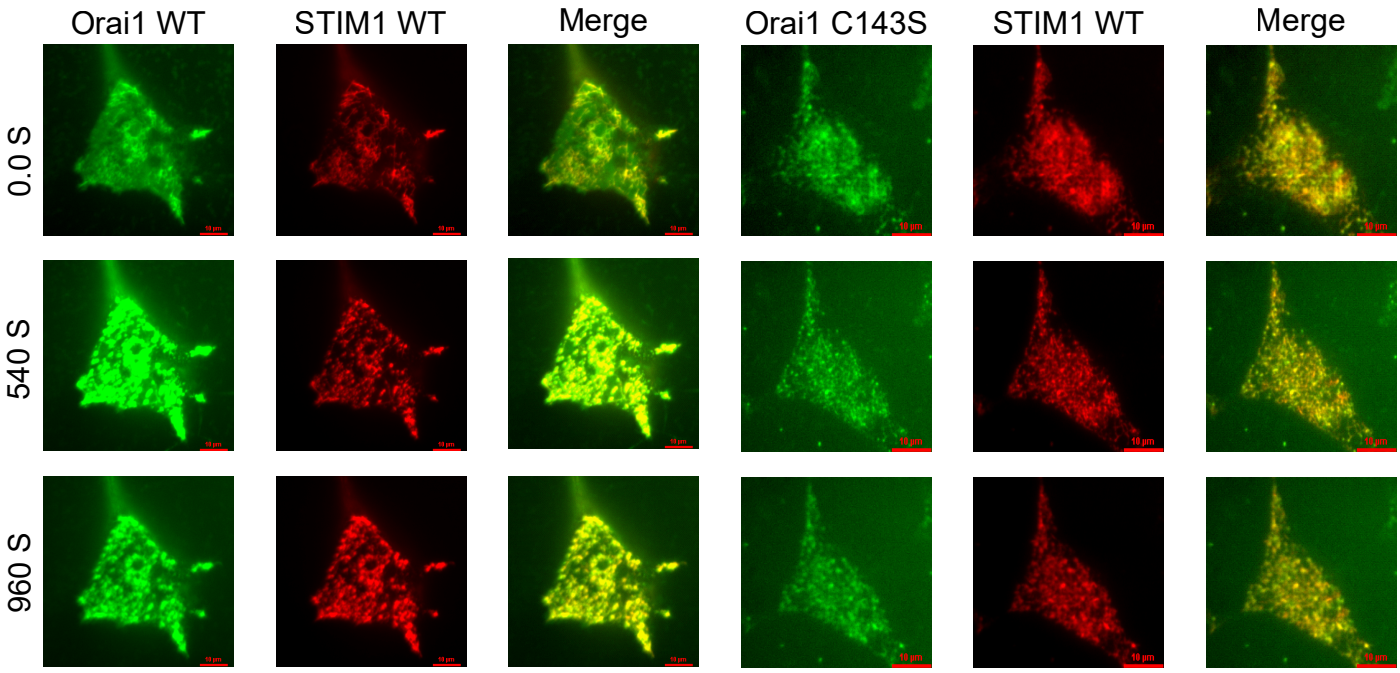
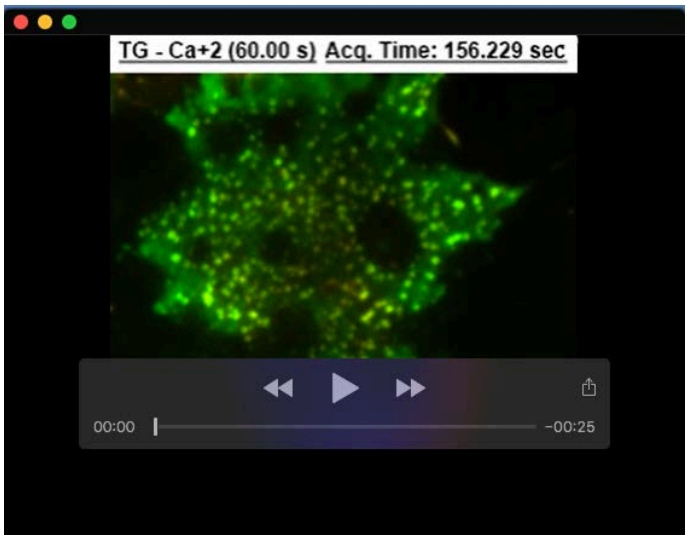
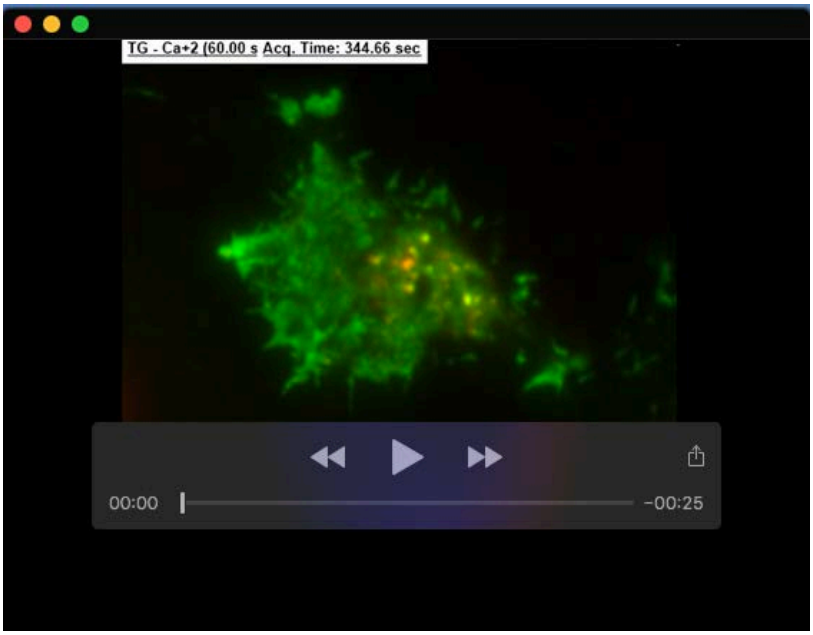


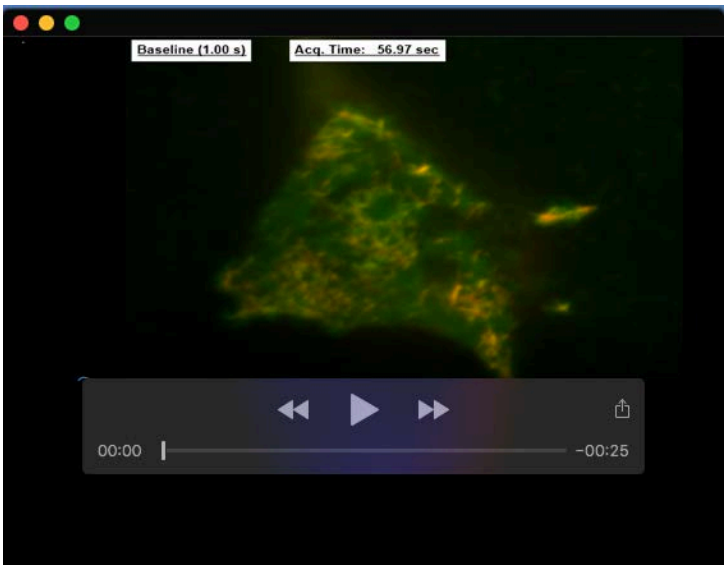
Figure S4. Individual channels from Figure 4. Shown are the individual Orai1-GCaMP6f, C143S-Orai1-GCaMP6f, and STIM1-mRFP channels obtained by TIRF microscopy as presented in Figure 3. Time (in seconds) is shown to the left of the panels. Thapsigargin (10 μM) was added at 60 seconds. A 10μm scale bar is shown.



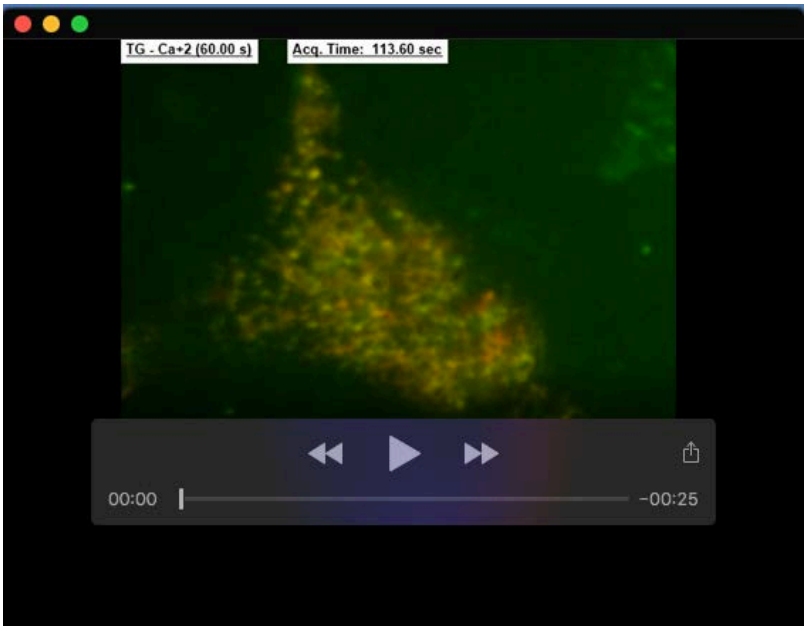
Movie 1. Movie of wild-type Orai1-YFP and STIM1-mRFP expressed in Orai1 triple knockout HEK293 cells acquired by TIRF microscopy (related to Fig. 3A). TIRF images were acquired every 10 seconds for 16 minutes. The time frame of the movie is accelerated 10-fold.



Movie 2. Movie of C143S mutant Orai1-YFP and STIM1-mRFP expressed in Orai1 triple knockout HEK293 cells acquired by TIRF microscopy (related to Fig. 3A). TIRF images were acquired every 10 seconds for 16 minutes. The time frame of the movie is accelerated 10-fold.



Movie 3. Movie of wild-type Orai1-GCaMP6s and STIM1-mRFP expressed in Orai1 triple knockout HEK293 cells acquired by TIRF microscopy (related to Fig. 4A). TIRF images were acquired every 10 seconds for 16 minutes. The time frame of the movie is accelerated 10-fold. The fluorescence of Orai1-GCaMP6s is calcium sensitive.



Movie 4. Movie of C143S mutant Orai1-GCaMP6s and STIM1-mRFP expressed in Orai1 triple knockout HEK293 cells acquired by TIRF microscopy (related to Fig. 4A). TIRF images were acquired every 10 seconds for 16 minutes. The time frame of the movie is accelerated 10-fold. The fluorescence of Orai1-GCaMP6s is calcium sensitive.

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