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Supplemental information

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SUPPLEMENTAL INFORMATION

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Orai channel C-terminal peptides are key modulators of STIM-Orai coupling and calcium signal generation

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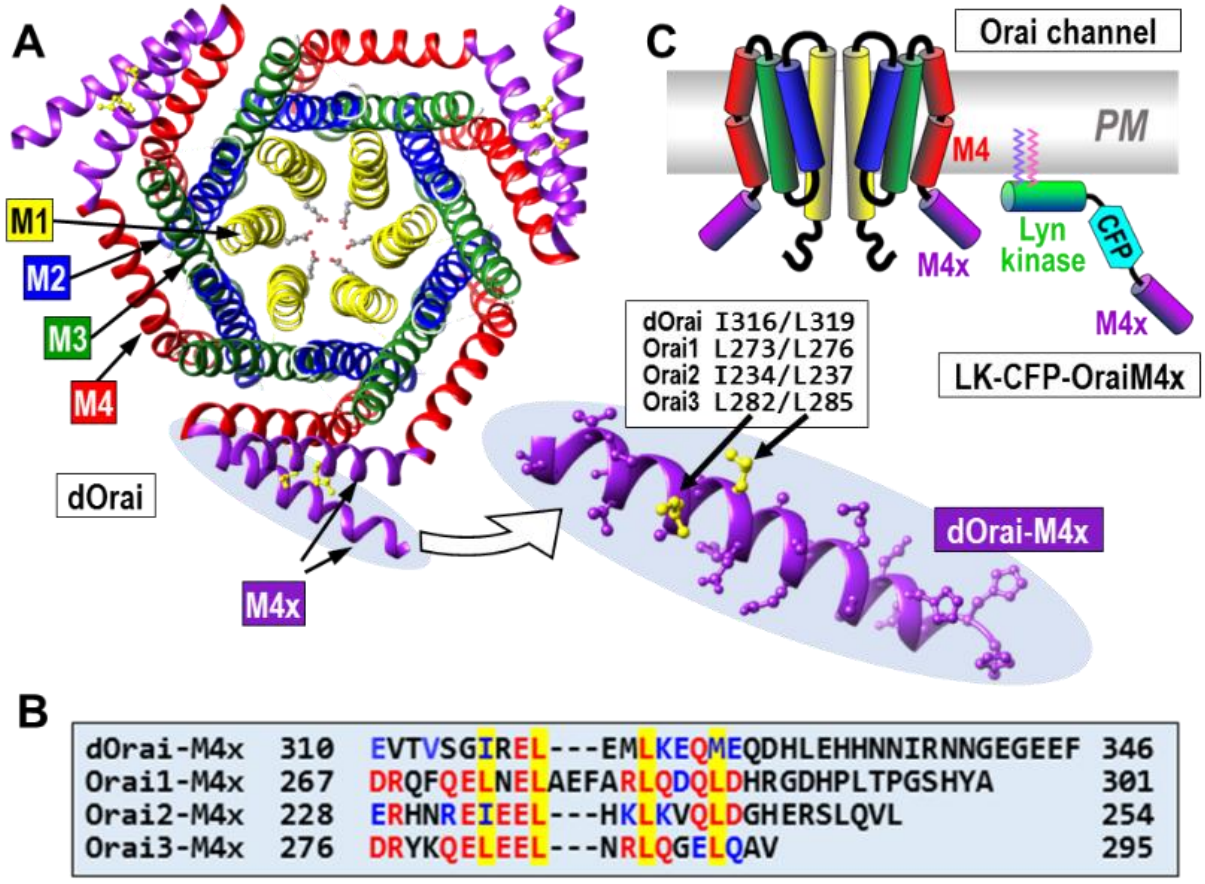

Figure S1, related to Figure 1. Development of M4x peptide constructs

(A) Crystallographic structure of the cytoplasmic face of the *Drosophila* Orai channel (dOrai), revealing six-fold symmetry for the pore-forming M1, as well as the M2, M3, and M4 helices (Hou et al, 2012). Pairs of outer M4 extension helices (M4x) from adjacent dOrai subunits were seen to undergo an asymmetric, antiparallel, hydrophobic pairing mediated by interactions between the I316 and L319 residues, giving a 3-fold outer symmetry. The dOrai1 M4x region is magnified, and the corresponding hydrophobic residues are shown for Orai1, Orai2, and Orai3.
(B) The M4x sequences of different Orai channels retain a pattern of recurring hydrophobic residues (L, I, or M) with intervening polar/charged residues, forming an amphipathic helix thought previously to form a binding pocket for STIM proteins. Note Orai1 has an additional 3 residues as shown
(C) Engineered M4x peptide constructs comprising a PM-tethering Lyn kinase receptor sequence (LK), fluorophore (CFP), and the M4x region from either of the three mammalian Orai subtypes (LK-CFP-Orai-M4x). Constructs included the M4x peptide from either Orai1, Orai2, Orai3, or a negative control without the Orai peptide (not shown).

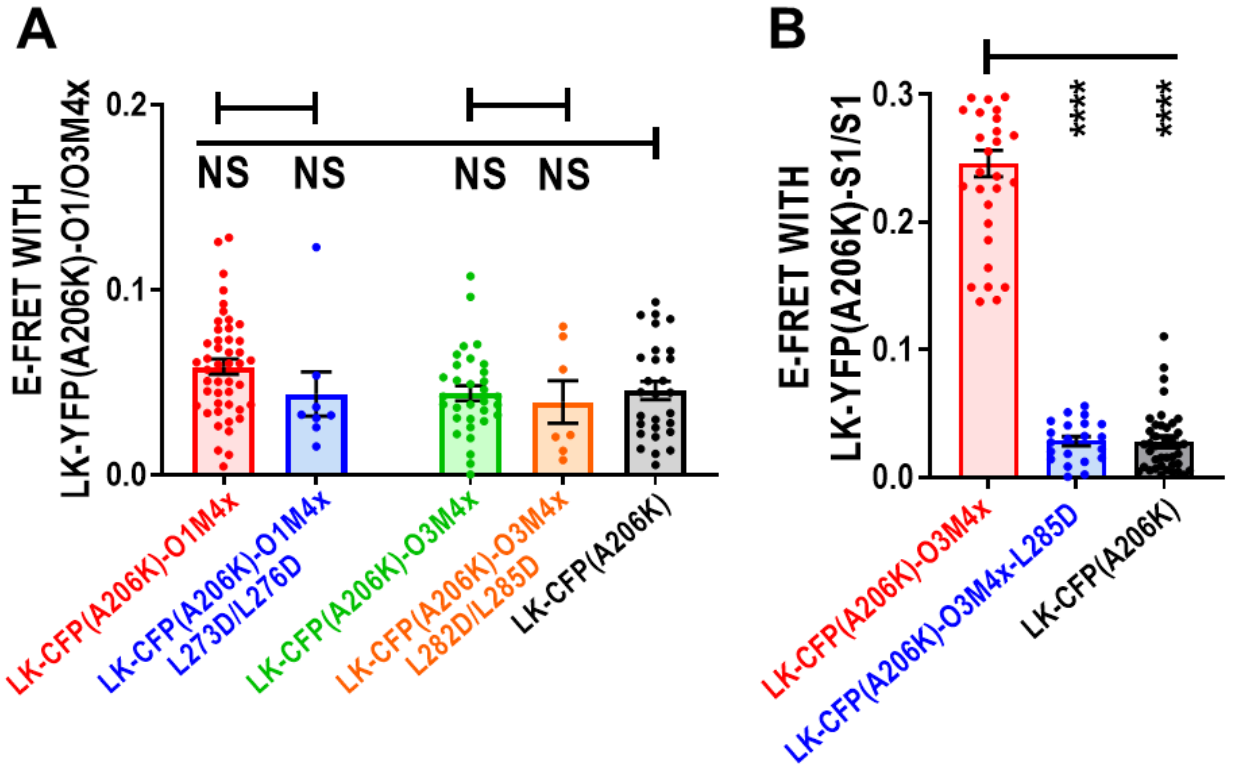

Figure S2, related to Figure 2. M4x peptide-peptide and peptide-SOAR interactions are not affected using fluorophore mutants that prevent dimerization (A206K)

(A) E-FRET analysis of self-interaction between LK-YFP-Orai-M4x and LK-CFP-Orai-M4x peptides bearing the A206K mutation within the YFP and CFP fluorophores, which prevents weak fluorophore inter-dimerization (Zacharia et al, 2005). LK-YFP(A206K)-Orai1-M4x WT transiently co-expressed with either LK-CFP(A206K)-Orai1-M4x WT (red) or Orai1-M4x bearing the L273D/L276D double mutant (blue). LK-YFP(A206K)-Orai3-M4x WT transiently co-expressed with either LK-CFP(A206K)-Orai3-M4x WT (green), Orai3-M4x bearing the double L282D/L285D mutation (orange), or LK-CFP(A206K) control, without Orai peptide (black).
(B) E-FRET measurements between YFP-SOAR dimer containing the YFP-A206K mutation and either LK-CFP(A206K)-Orai3-M4x WT (red), L282D/L285D double mutant (blue), or LK-CFP(A206K) control (black). All constructs were transiently co-expressed in HEK293 cells. One-way ANOVA analysis was performed on all E-FRET results (****$P < 0.0001$). Welch's unpaired t-tests were performed between both LK-CFP(A206K)-Orai1-M4x and LK-CFP(A206K)-Orai3-M4x counterparts as noted in (A). Results are means ± SEM, representative of at least two independent experiments. E-FRET analyses were undertaken on cells expressing a narrow range of LK-CFP-Orai-M4x fluorescence to assure accuracy of E-FRET values.

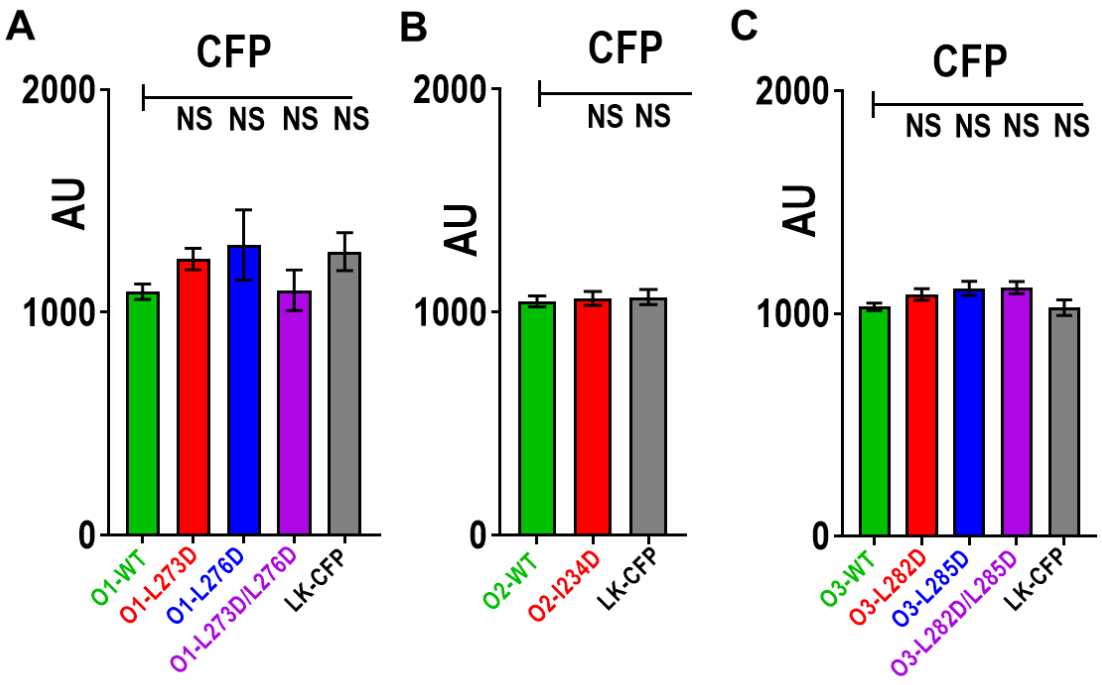

Figure S3, related to Figure 1. Expression levels of Orai M4x peptides in FRET analyses.
CFP intensity (AU) for the E-FRET analysis represented in Figure 1, between the LK-CFP-Orai-M4x peptide constructs and the YFP-SOAR dimer transiently expressed in HEK-Orai1/2/3^{TKO} cells. In all cases, CFP fluorescence was kept in a tight, narrow range.
(A) CFP intensity of LK-CFP-Orai1-M4x peptides represented in Figure 1D, with either wildtype Orai1-M4x (O1-WT), Orai1-M4x-L273D (O1-L273D), Orai1-M4x-L276D (O1-L276D), Orai1-M4x-L273D/L276D (O1-L273D/L276D), or the control (LK-CFP) with no M4x peptide.
(B) CFP intensity of LK-CFP-Orai2-M4x peptides represented in Figure 1E, with either wildtype Orai2-M4x (O2-WT), Orai2-M4x-I234D (O2-I234D), or the control (LK-CFP) with no M4x peptide.
(C) CFP intensity of LK-CFP-Orai3-M4x peptides represented in Figure 1F, with either wildtype Orai3-M4x (O3-WT), Orai3-M4x-L282D (O1-L282D), Orai3-M4x-L285D (O1-L285D), Orai3-M4x-L282D/L285D (O1-L282D/L285D), or the control (LK-CFP) with no M4x peptide.
One-way ANOVA analysis was performed on E-FRET results (****$P < 0.0001$). Results shown are means ± SEM of at least three independent experiments.

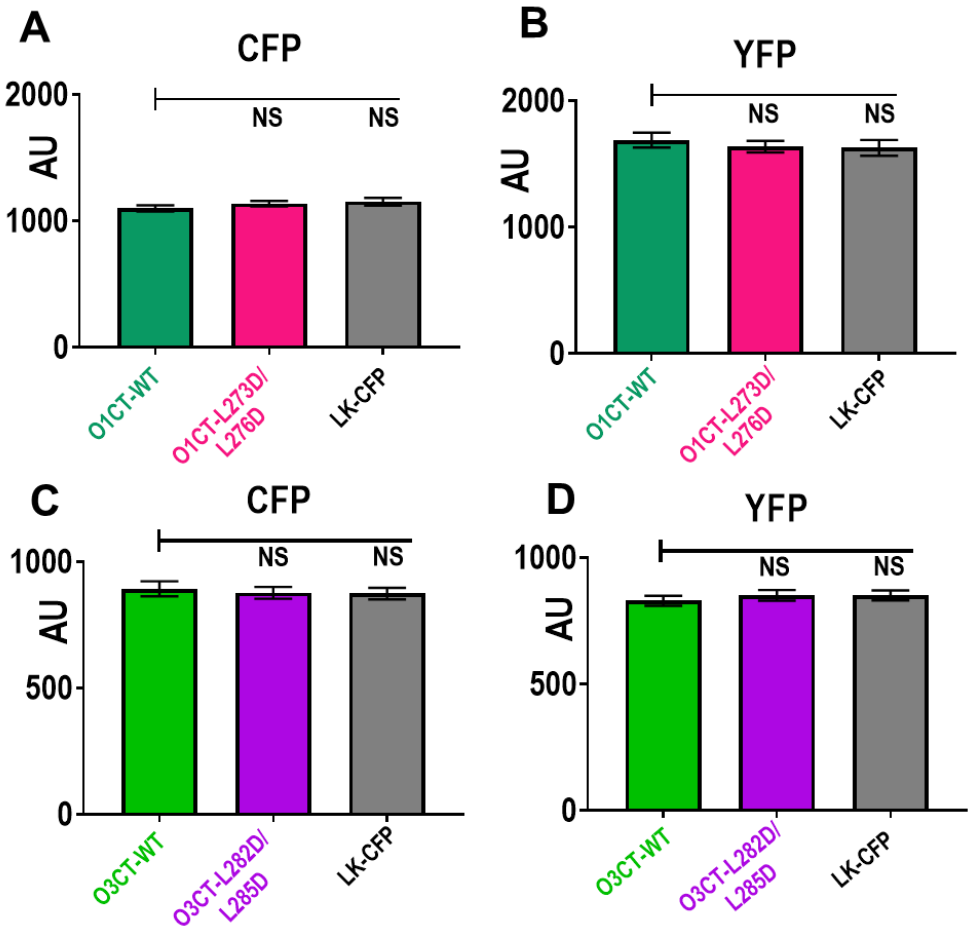

Figure S4, related to Fig. 2. CFP and YFP Intensity used for M4x peptide-peptide E-FRET analysis.

CFP intensity and YFP intensity (AU) for the E-FRET analysis shown in Figure 2D and E, between the LK-CFP-Orai-M4x peptides and the LK-YFP-Orai-M4x peptides transiently expressed in HEK-Orai1/2/3^{TKO} cells. CFP and YFP for regions of interest were kept within a narrow range for FRET analysis.

(A) CFP intensity of LK-CFP-Orai1-M4x peptides represented in Figure 2D, with either wildtype Orai1-M4x (O1-WT), Orai1-M4x-L273D/L276D (O1-L273D/L276D), or the control (LK-CFP) with no M4x peptide.

(B) YFP intensity of LK-YFP-Orai1-M4x peptide transiently co-expressed with constructs noted in (A).

(C) CFP intensity of LK-CFP-Orai3-M4x peptides represented in Figure 1F, with either wildtype Orai3-M4x (O3-WT), Orai3-M4x-L282D/L285D (O1-L282D/L285D), or the control (LK-CFP) with no M4x peptide.

(D) YFP intensity of LK-YFP-Orai3-M4x peptide transiently co-expressed with constructs noted in (C).

One-way ANOVA analysis was performed on E-FRET results. Results shown are means ± SEM of at least three independent experiments.

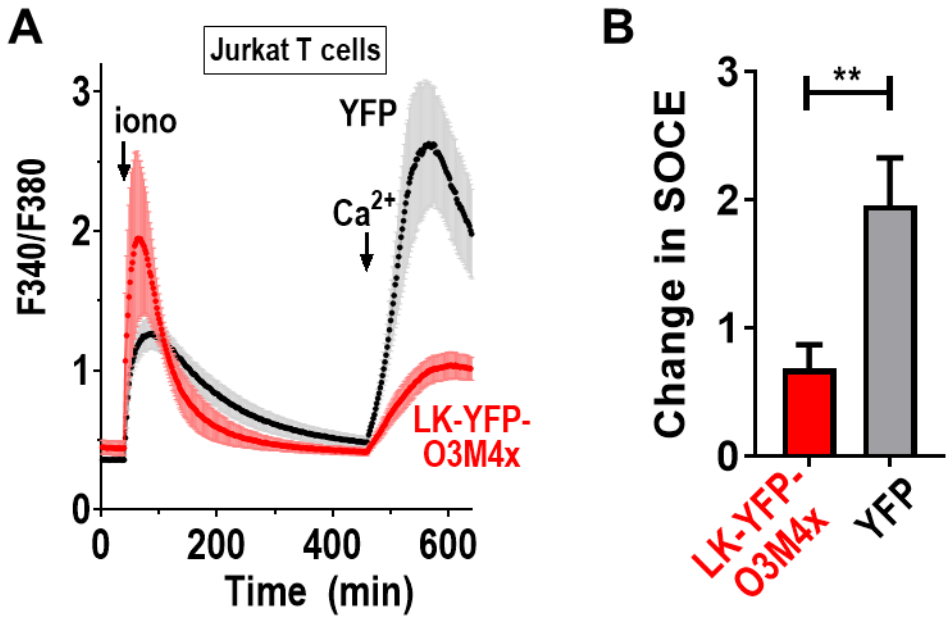

Figure S5, related to Figure 5. The Orai3 M4x peptide inhibit endogenous Jurkat store-operated Ca^{2+} entry

(A) Representative traces for Cytosolic Ca^{2+} signals that were measured by fura-2 ratiometric Ca^{2+} imaging in Jurkat WT cells transiently expressing either LK-YFP-Orai3-M4x WT (red) or YFP control (green). Cells in nominally Ca^{2+}-free medium, were treated with 2.5 μM ionomycin followed by add-back of 1 mM Ca^{2+}.

(B) Summary statistics for change from baseline (before Ca^{2+} addback) to peak (post Ca^{2+} addback) for three independent store-operated Ca^{2+} entry experiments.

Representative results are typical of at least three independent experiments. Welch's unpaired t-tests were performed between both LK-YFP-Orai3-M4x and YFP as noted in (B) (**$P < 0.01$). Results are means ± SEM, representative of at least three independent experiments.

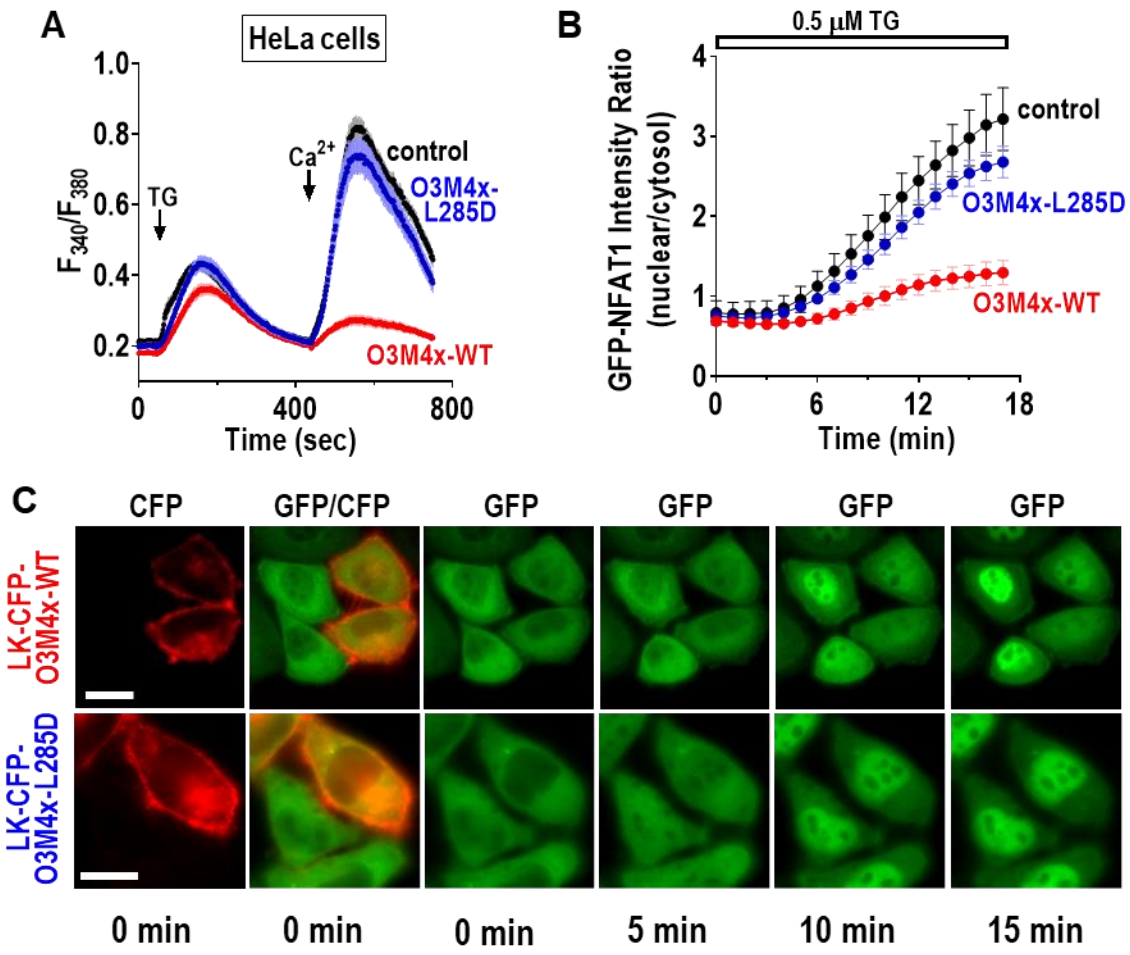

Figure S6, related to Figure 5. The Orai3 M4x peptide modulates Ca^{2+} signaling in HeLa cells

(A) Ca^{2+} responses in HeLa cells transiently expressing LK-CFP-Orai3-M4x WT (red), Orai3-M4x L285D mutant (blue), or non-transfected control (black). Cells in nominally Ca^{2+}-free medium, were treated with 0.5 µM thapsigargin (TG) followed by add-back of 1 mM Ca^{2+}.
(B and C) HeLa cells stably expressing GFP-NFAT1 were transiently transfected with either LK-CFP-O3M4x (red), or LK-CFP-O3M4x-L285D mutant (blue). Representative images were taken before (time point 0) and after 0.5 µM thapsigargin treatment. Images chosen have non-transfected neighbor cells adjacent to Orai3-M4x expressed counterpart (red fluorescence) and GFP-NFAT1 (green fluorescence) (C). GFP-NFAT1 translocation was measured as an intensity ratio of nuclear and cytosolic levels (B).
One-way ANOVA analysis were undertaken on results in (C). Ca^{2+} entry results shown are means ± SEM (****$P < 0.0001$), and representative results are typical of at least three independent experiments. Scale bars represent 5 µm.

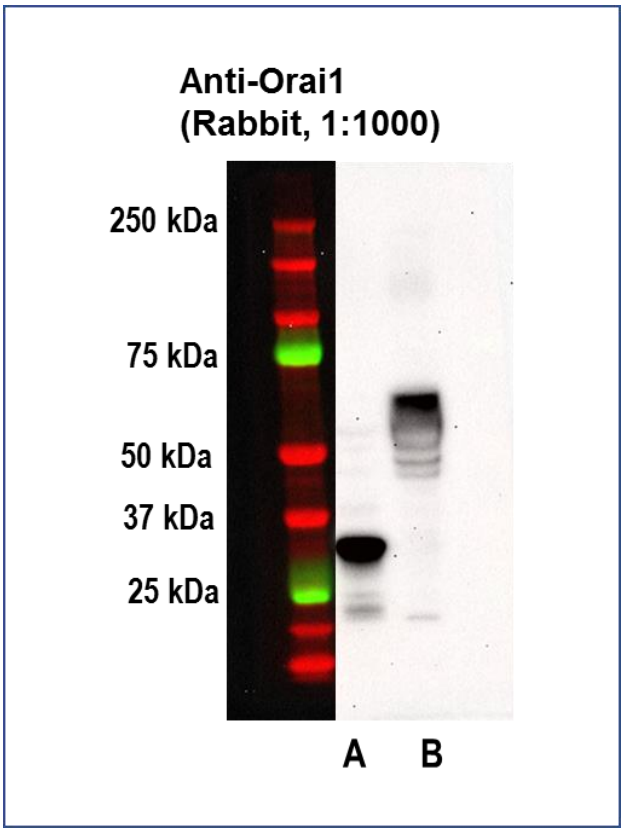

Figure S7, related to Figure 1. Western blot to show Orai1-M4x peptide protein expression.

Transient expression of either the LK-CFP-Orai1-M4x peptide (Lane A) or CFP-Orai1 WT (Lane B) in HEK-Orai1/2/3TKO cells. Anti-Orai1 antibody was used (1:1000 dilution) and was able to demonstrate expression of our M4x peptide within the cells. Note: This widely used Orai1 Ab is against the C-terminal 288-301 segment of the M4x peptide (Sigma Chemicals, product # O8264). The multiple bands have been typically seen with this Ab and reflect the α and β forms of Orai1, with and without glycosylation. The antibody recognizes the M4x peptide construct as essentially a single band of approximately 33 kDa, corresponding to CFP (27 kDa) plus the LK/linker/M4x region (~6 kDa).

Oligonucleotides (Purchased from IDT)		
O1-M4x L273D Forward: CGACAGTTCCAGGAGGACAACGAGCTGGCG	This paper	N/A
O1-M4x L273D Reverse: CGCCAGCTCGTTGTCCTCCTGGAACTGTCG	This paper	N/A
O1-M4x L276D Forward: CCAGGAGCTCAACGAGGACGCGGAGTTTGCCCGC	This paper	N/A
O1-M4x L276D Reverse: GCGGGCAAACTCCGCGTCCTCGTTGAGCTCCTGG	This paper	N/A
O1-M4x L273D/L276D Forward: CCGACAGTTCCAGGAGGACAACGAGGACGCGGAGTTTGCCCGC	This paper	N/A
O1-M4x L273D/L276D Reverse: GCGGGCAAACTCCGCGTCCTCGTTGTCCTCCTGGAACTGTCGG	This paper	N/A
O2-M4x I234D Forward: GCGCCACCGCGAGGACGAGGAGCTCCACAAGC	This paper	N/A
O2-M4x-I234D Reverse: GCTTGTGGAGCTCCTCGTCCTCGCGGTTGTGGCGC	This paper	N/A
O2-M4x L237D Forward: CCGCGAGATCGAGGATCACAAGCTCAAGGTCCAGC	This paper	N/A
O2-M4x L237D Reverse: GCTGGACCTTGAGCTTGTGATCCTCCTCGATCTCGCGG	This paper	N/A
O2-M4x I234D/L237D Forward: GCGCCACAACCGCGAGGACGAGGAGGATCACAAGC	This paper	N/A
O2-M4x I234D/L237D Forward: GCTTGTGATCCTCCTCGTCCTCGCGGTTGTGGCGC	This paper	N/A
O3-M4x L282D Forward: GACCGCTACAAGCAGGAAGACCAGGAACTGAATCGCCTGC	This paper	N/A
O3-M4x L282D Reverse: GCAGGCGATTCAGTTCCTCGTCTTCCTGCTTGTAGCGGTC	This paper	N/A
O3-M4x L285D Forward: GCAGGAACTAGAGGAAGACAATCGCCTGCAGGGGG	This paper	N/A
O3-M4x L285D Reverse:	This paper	N/A

CCCCCTGCAGGCGATTGTCTT CCTCTAGTTCCTGC		
O3-M4x L282D/L285D Forward: GCAGGAAGACGAGGAAGACAA TCGCCTGCAGGGGG	This paper	N/A
O3-M4x L282D/ L285D Reverse: CCCCCTGCAGGCGATTGTCTT CCTCGTCTTCCTGC	This paper	N/A
O3-M4x L285M Forward: GCAGGAACTAGAGGAAATGAA TCGCCTGCAGGGGG	This paper	N/A
O3-M4x L285M Reverse: CCCCCTGCAGGCGATTCATTTC CTCTAGTTCCTGC	This paper	N/A
O3-M4x L285F Forward: GCAGGAACTAGAGGAATTCAAT CGCCTGCAGGGGG	This paper	N/A
O3-M4x L285F Reverse: CCCCCTGCAGGCGATTGAATT CCTCTAGTTCCTGC	This paper	N/A
O3-M4x L285Y Forward: GCAGGAACTAGAGGAATACAAT CGCCTGCAGGGGG	This paper	N/A
O3-M4x L285Y Reverse: CCCCCTGCAGGCGATTGTATTC CTCTAGTTCCTGC	This paper	N/A
O3-M4x L288D Forward: GAGGAACTGAATCGCGACCAG GGGGAGCTGCAGGC	This paper	N/A
O3-M4x L288D Reverse: GCCTGCAGCTCCCCCTGGTCG CGATTCAGTTCCTC	This paper	N/A
O3-M4x L292D Forward: CGCCTGCAGGGGGAGGACCA GGCTGTGTGAGCG	This paper	N/A
O3-M4x L292D Reverse: CGCTCACACAGCCTGGTCCTC CCCCTGCAGGCG	This paper	N/A

Table S1, related to STAR Methods. Forward and reverse primers used to develop LK-CFP-O1/2/3 mutants.

Forward and reverse primers used to develop LK-CFP-Orai1-3 M4x peptide mutations that were utilized for this current study. All primers were designed using SnapGene viewer and NIH primer-blast (http://ncbi.nlm.nih.gov/tools/primer-blast), and purchased from IDT.