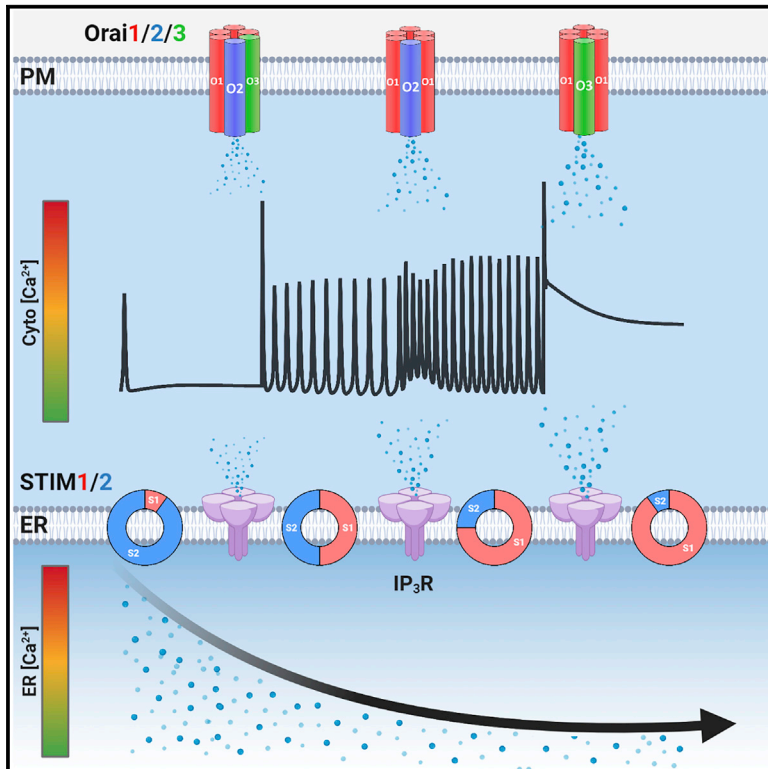


Omnitemporal choreographies of all five STIM/Orai and IP₃Rs underlie the complexity of mammalian Ca²⁺ signaling

Graphical Abstract



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In brief

Ca²⁺ signals are crucial for cell function. Emrich et al. show that the five store-operated STIM/Orai proteins are non-redundant. Physiological store-operated channel activities are omnitemporal choreographies of all five STIM1/2 and Orai1/2/3 proteins and their functional interactions with IP₃Rs, ensuring the fidelity and diversity of Ca²⁺ signaling and NFAT activation.

Highlights

- All five STIM/Orai proteins are always required together during Ca²⁺ signaling
- Unactivated STIM1/2 inhibit IP₃R and activated STIM1/2 cooperatively activate Orai1/2/3
- STIM1 contribution increases and that of STIM2 decreases as agonist intensifies
- Graded IP₃R disinhibition and Orai activation tailor agonist strength to NFAT activity



Article

Omnitemporal choreographies of all five STIM/Orai and IP₃Rs underlie the complexity of mammalian Ca²⁺ signaling

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SUMMARY

Stromal-interaction molecules (STIM1/2) sense endoplasmic reticulum (ER) Ca²⁺ depletion and activate Orai channels. However, the choreography of interactions between *native* STIM/Orai proteins under physiological agonist stimulation is unknown. We show that the five STIM1/2 and Orai1/2/3 proteins are non-redundant and function together to ensure the graded diversity of mammalian Ca²⁺ signaling. Physiological Ca²⁺ signaling requires functional interactions between STIM1/2, Orai1/2/3, and IP₃Rs, ensuring that receptor-mediated Ca²⁺ release is tailored to Ca²⁺ entry and nuclear factor of activated T cells (NFAT) activation. The N-terminal Ca²⁺-binding ER-luminal domains of unactivated STIM1/2 inhibit IP₃R-evoked Ca²⁺ release. A gradual increase in agonist intensity and STIM1/2 activation relieves IP₃R inhibition. Concomitantly, activated STIM1/2 C termini differentially interact with Orai1/2/3 as agonist intensity increases. Thus, coordinated and omnitemporal functions of all five STIM/Orai and IP₃Rs translate the strength of agonist stimulation to precise levels of Ca²⁺ signaling and NFAT induction, ensuring the fidelity of complex mammalian Ca²⁺ signaling.

INTRODUCTION

Ca²⁺ is a universal second messenger that regulates a myriad of physiological responses, including immune function, exocrine secretion, and muscle contraction (Ahuja et al., 2020; Berridge, 2016; Trebak and Kinet, 2019). The endoplasmic reticulum (ER) luminal-free Ca²⁺ is 10,000-fold higher than the cytosol (Bagur and Hajnóczky, 2017). ER-free Ca²⁺ is tightly controlled through the opposing functions of the inositol 1,4,5-trisphosphate receptor (IP₃R) channels, which release Ca²⁺ into the cytosol and the sarcoplasmic/ER Ca²⁺-ATPases (SERCA), which pump Ca²⁺ from the cytosol into the ER (Bagur and Hajnóczky, 2017; Berridge, 2016; Yule, 2001). Agonist stimulation of plasma membrane (PM) receptors coupled to phospholipase C (PLC) produces diffusible IP₃, leading to activation of IP₃Rs and depletion of ER Ca²⁺ stores (Putney and Tomita, 2012). Store depletion triggers the activation of ER resident stromal-interaction molecule (STIM) proteins to couple with and gate PM Orai channels and drive Ca²⁺ entry through the ubiquitous store-operated Ca²⁺ entry (SOCE) pathway

(Hogan and Rao, 2015; Prakriya and Lewis, 2015; Trebak and Putney, 2017), mediated by the Ca²⁺ release-activated Ca²⁺ (CRAC) current (Hoth and Penner, 1992). SOCE plays a critical dual role in the acute refilling of ER Ca²⁺ to support Ca²⁺ release and in generating local Ca²⁺ signals that activate Ca²⁺-dependent nuclear factor of activated T cells (NFAT) transcription factors (Gwack et al., 2007; Trebak and Putney, 2017). Dysregulation of SOCE contributes to the pathogenesis of multiple immune, muscle, exocrine, and metabolic disorders (Feske, 2019).

Stimulation of cells with either SERCA blockers (e.g., thapsigargin) or high-agonist concentrations induces massive depletion of ER Ca²⁺ and robust cytosolic Ca²⁺ plateaus. Under these conditions, STIM1 and Orai1 have emerged as the major participants in SOCE. However, maximal activation of SOCE represents only the extreme end of diverse and graded SOCE activities in response to the full range of agonist concentrations. Stimulation of mammalian cells with relatively low physiological concentrations of agonists results in the generation of cytosolic Ca²⁺ oscillations (Dupont et al., 2011). In lymphocytes, Ca²⁺ oscillations are driven by



oscillating SOCE activity independently of IP₃R (Dolmetsch and Lewis, 1994; Lewis and Cahalan, 1989). In most cells, however, Ca²⁺ oscillations are initiated by IP₃R-mediated Ca²⁺ release and maintained by ER Ca²⁺ refilling through SOCE (Bird and Putney, 2005). We recently demonstrated that Orai1 is dispensable for sustaining regenerative Ca²⁺ oscillations in HEK293 cells (Yoast et al., 2020b). Surprisingly, endogenous Orai2 and Orai3, which contribute an almost undetectable fraction of SOCE, support oscillations, suggesting that miniscule amounts of SOCE are enough to support sustained Ca²⁺ oscillations (Yoast et al., 2020b).

Although structurally similar, STIM1 and STIM2 are activated by distinct levels of ER Ca²⁺ depletion (Brandman et al., 2007; Liou et al., 2005). By comparison to STIM1, STIM2 is a weak activator of Orai channels and is triggered by more modest levels of ER Ca²⁺ depletion (Brandman et al., 2007; Wang et al., 2014; Zheng et al., 2018a). The contributions and precise choreographies of native STIM1 and STIM2 translocation to ER-PM junctions and interactions with Orai1/2/3 over the range of physiological agonist concentrations are unknown. A number of studies have used knockdown to investigate STIM1/2 in regulating Ca²⁺ oscillations (Ong et al., 2015; Thiel et al., 2013; Wedel et al., 2007) but with conflicting results. Earlier reports suggested that STIM1 is solely required for Ca²⁺ oscillations (Bird et al., 2009; Wedel et al., 2007), while more recent studies suggested that STIM2 is the key regulator of Ca²⁺ oscillations (Ong et al., 2015; Thiel et al., 2013). Further complicating this picture, evidence has emerged that STIM2 actively recruits STIM1 to ER-PM junctions under conditions of modest ER Ca²⁺ depletion (Ong et al., 2015; Son et al., 2020; Subedi et al., 2018), highlighting the complex function of STIM1/2 in Ca²⁺ signaling.

Here, we show that, in response to various agonist concentrations, both STIM isoforms are always required together to ensure the fidelity of Ca²⁺ signaling. The distinct C termini of STIM1 and STIM2 coordinate the wide range of Ca²⁺ oscillation frequencies in response to low- and mid-range agonist concentrations, while the C terminus of STIM1 exclusively drives sustained Ca²⁺ plateaus in response to high-agonist concentrations. STIM1/STIM2 cooperation is required for optimal nuclear translocation of NFAT1 and NFAT4. The Ca²⁺ binding N-terminal EF hand of unactivated (resting) STIMs is required for STIM1/2-mediated negative regulation of IP₃R, likely setting a threshold for IP₃ activation of IP₃R. As the ratio of activated/unactivated STIMs increases with increasing agonist concentrations, IP₃R inhibition by STIMs is relieved concomitantly with increased CRAC activity, effectively matching Ca²⁺ release to Ca²⁺ entry. This ensures that graded Ca²⁺ signals and NFAT activities are tailored to their matching physiological agonist intensities. Our data challenge the current oversimplified model of mammalian CRAC channel activation involving either STIM2/Orai1 or STIM1/Orai1 interactions under basal and agonist stimulation, respectively. Physiological CRAC channel activities are *Omni tempore* choreographies of all five STIM/Orai proteins and their functional interaction with IP₃R.

RESULTS

Both native STIM1 and STIM2 support Ca²⁺ oscillations

We generated several clones of HEK293 cells lacking STIM1 (STIM1-KO), STIM2 (STIM2-KO), or both (STIM1/2-KO) using

CRISPR/Cas9 (Figure 1A). Western blots showed no compensatory change in either STIM protein (Figure 1A) or the three isoforms of IP₃R (Figures S1D–S1G) in these knockout cells; HEK293 cells in which all three isoforms of IP₃R were knocked out were used as controls (Alzayady et al., 2016). There was no difference in Ca²⁺ release between parental wild-type (WT)-HEK293 and STIM-KO cells in response to store depletion by 2 μM thapsigargin (Figure 1B; see also Figure 4F for ER measurements). As expected, however, SOCE was slightly reduced in STIM2-KO cells, mostly reduced in STIM1-KO cells, and completely abrogated in STIM1/2-KO cells (Figures 1B and 1C). Orai1/2/3 triple-knockout cells (Orai-TKO), which show complete abrogation of SOCE (Yoast et al., 2020b), were included as controls. Previously, we determined that 10 μM of the muscarinic agonist carbachol (CCh) mostly induces Ca²⁺ oscillations (Yoast et al., 2020b). For each condition, five representative cells are shown (Figures 1D–1H), and data from several independent experiments with two independent CRISPR/Cas9-KO clones per condition are statistically analyzed (Figures 1I–1L), including Ca²⁺ oscillations frequency, the percentage of oscillating cells, cells with Ca²⁺ plateaus, and non-responding cells (either 0 or 1 spike) as defined in Figures S1A–S1C.

Stimulation of WT-HEK293 cells with 10 μM CCh in the presence of 2 mM Ca²⁺ produced an average of 6 oscillations/14 min (Figure 1I). At this concentration, WT-HEK293 cells demonstrated an average of 79% oscillating cells, 16% plateau cells, and 5% non-responders (Figures 1J–1L). While STIM1-KO cells demonstrated a similar oscillation frequency compared to WT-HEK293 (Figure 1I), 95% of those cells responded with Ca²⁺ oscillations and 0% with plateaus (Figures 1J and 1K). STIM2-KO cells produced an increased average of ~6.9 oscillations/14 min, as well as an increased percentage of plateau cells (24%) compared to WT-HEK293 (Figures 1J–1L). These results suggest that, while both STIM1 and STIM2 can independently support Ca²⁺ oscillations, Ca²⁺ plateaus strictly require STIM1 and that STIM2 negatively regulates plateau responses.

Unexpectedly, STIM1/2-KO cells showed rapid oscillations in the first 5 min of CCh stimulation, followed by a progressive decrease in peak amplitude and subsequent rundown (Figure 1G). STIM1/2-KO cells showed a significantly increased percentage of non-responding cells (36%) and no cells eliciting a plateau response (Figures 1J–1L). The rapid oscillatory phenotype of STIM1/2-KO cells was unrelated to Ca²⁺ entry as inhibition of SOCE with 5 μM Gd³⁺ (Trebak et al., 2002) reduced the frequency of Ca²⁺ oscillations in WT-HEK293 but not in STIM1/2-KO cells (Figures S1L–S1P). Furthermore, Orai-TKO cells have a significantly reduced frequency of oscillations with only ~3 oscillations/14 min (Figures 1H and 1I; see also Yoast et al., 2020b). These findings suggest that STIM proteins exert control over ER Ca²⁺ release through IP₃R. This behavior of STIM1/2-KO cells will be addressed further below.

Interestingly, we also observed a qualitative change in the behavior of Ca²⁺ oscillations between WT-HEK293 cells and STIM-KO cells as summarized in Figures S1H–S1K. A significant portion of WT-HEK293 and STIM2-KO cells typically responded as in Figures S1H and S1J, respectively, with 44.6% of WT-HEK293 cells stimulated with 10 μM CCh showing a rapid oscillatory response at first, which gradually transitioned into sustained

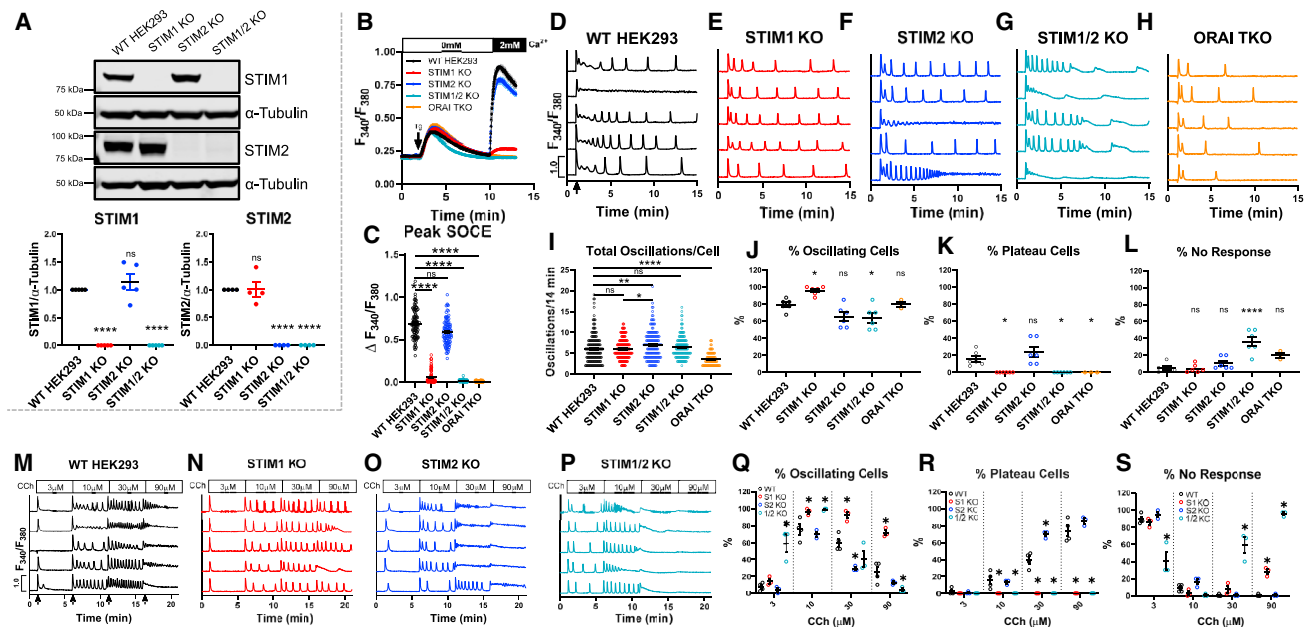


Figure 1. Native STIM1/STIM2 mediate unique Ca^{2+} oscillation profiles and agonist sensitivities

(A) Top: western blot of STIM1 and STIM2 in WT-HEK293 and STIM-KO cells. Bottom: quantification (mean \pm SEM) of STIM1/2 band intensities normalized to α -tubulin. Each point is a biological replicate (STIM1, $n = 5$; STIM2, $n = 4$).
 (B) Fura2 Ca^{2+} measurements upon store depletion with 2 μ M thapsigargin in 0 mM Ca^{2+} and re-addition of 2 mM Ca^{2+} .
 (C) Quantification (mean \pm SEM) of SOCE from (B). From left to right $n = 100, 101, 100, 99,$ and 120 cells.
 (D–H) Representative Ca^{2+} oscillation traces from 5 cells/condition upon stimulation with 10 μ M CCh at 1 min (arrow in D) in 2 mM Ca^{2+} .
 (I) Quantification (mean \pm SEM) of total oscillations in 14 min. From left to right $n = 189, 230, 156, 152,$ and 96 cells.
 (J–L) Mean \pm SEM of percentage of (J) oscillating, (K) plateau, and (L) non-responding cells for (D)–(H). From left to right $n = 6, 6, 6,$ and 3 independent experiments.
 (M–P) Representative Ca^{2+} traces in response to increasing CCh concentrations in 2 mM Ca^{2+} for 5 min each (arrows in M).
 (Q–S) Mean \pm SEM of percentage of (Q) oscillating, (R) plateau, and (S) non-responding cells for (M)–(P). From left to right $n = 4, 3, 3,$ and 3 independent experiments. For all figures, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

oscillations that return to baseline (Figure S1H). 21.3% of STIM2 KO cells showed a reversal of this phenotype with oscillations that become rapid and tetanize into moderate plateaus (Figure S1J); only 1.6% of WT-HEK293 cells responded as in Figure S1J, and only 0.8% of STIM2-KO cells responded as in Figure S1H. STIM1-KO and STIM1/2-KO cells showed consistent behavior throughout recordings (Figures S1I and S1K). Thus, while endogenous STIM1 and STIM2 can independently support Ca^{2+} oscillations, they likely function together under physiological conditions. Quantification of oscillation data from two independent CRISPR/Cas9 knockout clones per condition is presented in Figures S2A–S2H.

STIM1/STIM2 cooperate across the range of agonist concentrations

Although STIM1 and STIM2 are highly homologous, the EF-hand domain of STIM2 has lower affinity for Ca^{2+} (Zheng et al., 2018a). Current models suggest that basal unstimulated SOCE activity is controlled by STIM2, while STIM1 is increasingly recruited with increasing agonist concentrations (Ong et al., 2015; Thiel et al., 2013). We measured Ca^{2+} signals to increasing CCh concentrations ranging from 3 to 90 μ M. At 3 μ M CCh, WT-HEK293, STIM1-KO, and STIM2-KO cells were almost entirely refractory, while nearly 60% of

STIM1/2-KO cells demonstrated Ca^{2+} oscillations (Figures 1M–1S). At 10 μ M CCh, WT-HEK293 and STIM2-KO cells showed 75% and 70% of oscillating cells, respectively, with 100% of STIM1-KO and STIM1/2-KO cells oscillating. Interestingly, STIM2-KO cells demonstrated increased sensitivity by exhibiting plateaus at 30 μ M CCh (70%) compared to WT-HEK293 cells (40%), suggesting that STIM2 negatively regulates STIM1 and SOCE, in agreement with an earlier study (Soboloff et al., 2006). At 90 μ M CCh, WT-HEK293 and STIM2-KO cells reached comparable percentages of cells responding with plateaus (~80%). Importantly, STIM1-KO cells failed to support plateaus at all CCh concentrations (Figures 1Q–1S), further documenting that plateaus are exclusively mediated by STIM1. These data suggest that STIM1 and STIM2 work collaboratively across the range of stimulus intensities to tailor Ca^{2+} responses to their respective agonist concentrations.

STIM C termini determine the diversity of receptor-activated Ca^{2+} signaling events

We utilized STIM1/2-KO cells to determine whether STIM1 and STIM2 re-expression can rescue the phenotype of STIM1/2-KO cells. To achieve near endogenous protein levels, we expressed YFP-tagged STIM constructs driven by the relatively

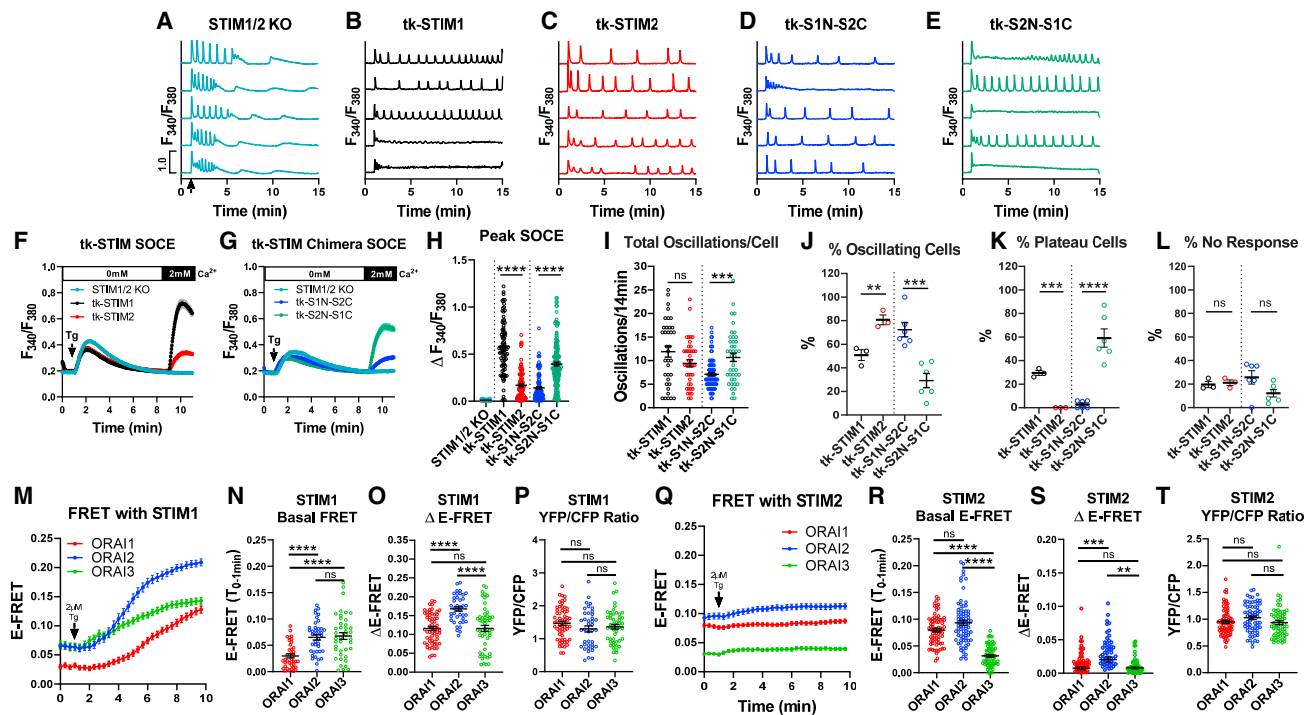


Figure 2. STIM1/STIM2 have distinct interactions with Orai1/2/3

(A–E) Ca^{2+} traces of (A) non-transfected STIM1/2-KO or STIM1/2-KO cells individually rescued with (B) tk-YFP-STIM1, (C) tk-YFP-STIM2, (D) tk-YFP-S1N-S2C, and (E) tk-YFP-S2N-S1C from 5 representative cells/condition upon stimulation with $10 \mu\text{M}$ CCh at 1 min (arrow in A) in 2 mM Ca^{2+} . (F) SOCE in response to $2 \mu\text{M}$ thapsigargin in Ca^{2+} -free followed by re-addition of 2 mM Ca^{2+} in STIM1/2-KO or STIM1/2-KO cells rescued with tk-YFP-STIM1 or tk-YFP-STIM2. (G) SOCE in STIM1/2-KO cells rescued with tk-YFP-S1N-S2C or tk-YFP-S2N-S1C. (H) Maximal SOCE (mean \pm SEM) for (F) and (G). From left to right $n = 90, 88, 96, 116,$ and 154 cells. (I) Total oscillations (mean \pm SEM) in 14 min for (A)–(E). From left to right $n = 36, 41, 87,$ and 43 cells. (J–L) Percentage (mean \pm SEM) of (J) oscillating, (K) plateau, and (L) non-responding cells for (B)–(E); from left to right $n = 3, 3, 6,$ and 6 independent experiments. (M) E-FRET plotted as mean \pm SEM between STIM1-YFP and each CFP-Orai expressed in Orai-TKO cells at baseline and after $2 \mu\text{M}$ thapsigargin in 2 mM Ca^{2+} . (N–P) Scatterblots (mean \pm SEM) quantifying basal E-FRET, (O) change in E-FRET after thapsigargin, and (P) YFP/CFP fluorescence ratios from data in (M). From left to right $n = 60$ (Orai1), 40 (Orai2), and 51 (Orai3) cells. (Q) E-FRET presented as mean \pm SEM between STIM2-YFP and each CFP-Orai expressed in Orai-TKO cells before and after thapsigargin. (R–T) Scatterblots (mean \pm SEM) quantifying basal E-FRET (R), change in E-FRET after thapsigargin (S), and YFP/CFP fluorescence ratios (T) from data in (Q). From left to right $n = 71$ (Orai1), 67 (Orai2), and 64 (Orai3) cells. For all figures, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

weak thymidine kinase (tk) promoter (Korzeniowski et al., 2010) (Figure S3A shows re-expression of STIM1/2 to near endogenous levels). Both tk-STIM1 and tk-STIM2 rescued SOCE activated by store depletion with $2 \mu\text{M}$ thapsigargin. As expected, tk-STIM1 supported significantly larger SOCE compared to tk-STIM2 (Figures 2F and 2H). Furthermore, $10 \mu\text{M}$ CCh induced sustained oscillations and plateaus in STIM1/2-KO cells expressing tk-STIM1 with a similar percentage to those of STIM2-KO cells (Figures 2A–2C and 2J–2L). Similarly, tk-STIM2 expression in STIM1/2-KO cells supported oscillations with a percentage similar to that of STIM1-KO cells, with 0% of cells displaying plateaus (Figures 2A–2C and 2J–2L). Frequencies of oscillations were generally higher in these rescue experiments with tk-STIM1 and tk-STIM2 supporting ~ 12.6 and 9 oscillations/14 min, respectively (Figure 2I), suggesting that, while average tk-STIM1 and tk-STIM2 protein levels within cell populations is similar to endogenous STIM levels (Figure S3A), tk-STIM1 and tk-STIM2 protein levels in individual cells are likely higher compared to native levels.

Next, we utilized two STIM chimeric constructs that we recently developed (Emrich et al., 2019). The first chimera consists of the N-terminal and transmembrane domains of STIM1 combined with the C-terminus of STIM2 (tk-S1N-S2C), while the second is the N-terminal and transmembrane domains of STIM2 combined with the C-terminus of STIM1 (tk-S2N-S1C). Both STIM chimeras rescued SOCE triggered by thapsigargin when expressed in STIM1/2-KO cells with tk-S2N-S1C demonstrating significantly higher Ca^{2+} entry compared to tk-S1N-S2C (Figures 2G and 2H). Whole-cell patch-clamp recordings in STIM1/2-KO cells co-expressing Orai1 with either STIM chimera (driven by the robust CMV promoter) demonstrated that S2N-S1C supported a larger CRAC current density compared to S1N-S2C (Figures S3C and S3D). In terms of frequency of Ca^{2+} oscillations, percentage of oscillating cells, plateau cells, and non-responding cells, the behavior of these chimeras was solely determined by their C termini, with tk-S2N-S1C and tk-S1N-S2C supporting similar responses to those of tk-STIM1 and tk-STIM2, respectively (Figures 2I–2L).

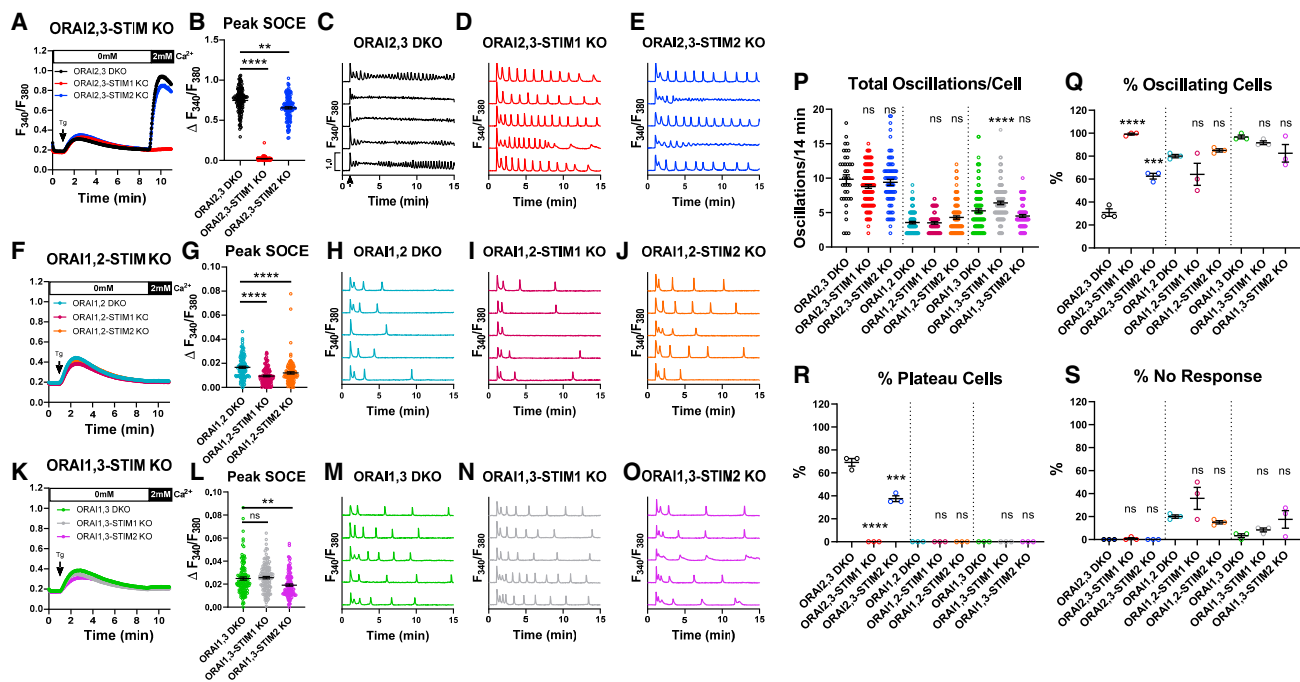


Figure 3. Coordinated STIM/Orai interactions enhance the diversity of Ca²⁺ signaling events

(A) SOCE measurement upon store depletion with 2 μM thapsigargin in 0 mM Ca²⁺ followed by re-addition of 2 mM Ca²⁺ in Orai2,3-STIM KO cells. (B) Quantification (mean \pm SEM) of maximal SOCE for (A). From left to right n = 120, 120, and 120 cells. (C–E) Representative Ca²⁺ traces from 5 cells/condition upon stimulation with 10 μM CCh at 1 min (arrow in C) in 2 mM Ca²⁺. (F and G) SOCE in Orai1,2-STIM KO cells (F). (G) SOCE quantification (mean \pm SEM) from (F). From left to right n = 120, 120, and 120 cells. (H–J) Representative Ca²⁺ traces of Orai1,2-STIM KO cells as in (C)–(E). (K and L) SOCE in Orai1,3-STIM KO cells (K). (L) SOCE quantification (mean \pm SEM) from (K). From left to right n = 120, 121, and 120 cells. (M–O) Representative Ca²⁺ oscillation in Orai1,3-STIM KO cells as in (C)–(E). (P) Quantification (mean \pm SEM) of oscillations/14 min for (C)–(E), (H)–(J), and (M)–(O). From left to right n = 37, 119, 75, 96, 76, 102, 116, 110, and 99 cells. (Q–S) Quantification (mean \pm SEM) of percentage of (Q) oscillating, (R) plateau, and (S) non-responding cells for (C)–(E), (H)–(J), and (M)–(O). n = 3 independent experiments. For all figures, **p < 0.01, ***p < 0.001, ****p < 0.0001.

These experiments further demonstrate that STIM1 and STIM2 can independently support Ca²⁺ oscillations and that the C-terminus of STIM1 is the primary driver of plateau responses.

STIM/Orai isoform interactions shape the diversity of Ca²⁺ signaling events

We recently proposed that native CRAC channels are heteromers of all three Orai isoforms. Without Orai2/3, physiological Ca²⁺ oscillations in response to low/mid-range agonist concentrations are mostly replaced with Orai1-driven Ca²⁺ plateaus (Yoast et al., 2020b). Here, we performed side-by-side Förster resonance energy transfer (FRET) experiments with either STIM1-YFP or STIM2-YFP co-expressed with each CFP-Orai isoform in Orai-TKO cells (Figures 2M–2T). STIM1 exhibited significant interaction under basal conditions with Orai2 and Orai3 in comparison to Orai1 (Figures 2M and 2N), and the interaction between STIM1 and Orai isoforms was enhanced after store depletion (Figures 2M–2P). In contrast, STIM2 demonstrated significant basal interactions with Orai1 and Orai2 compared to Orai3 (Figures 2Q and 2R), and the interaction of STIM2 with each Orai isoform only marginally increased after store depletion (Figures 2Q–2T). One potential interpretation of these data is that STIM2 interaction with Orai1 at rest mediates SOCE

at low-agonist concentrations, while interactions of STIM1 with Orai3 dampens SOCE at high-agonist concentrations. The interaction of both STIM1 and STIM2 with Orai2 likely fine-tunes SOCE across the full spectrum of physiological agonist concentrations.

We used CRISPR/Cas9 to generate cells expressing combinations of one native Orai and one native STIM. We generated six different combinations consisting of the double Orai knockout (Orai2,3-KO, Orai1,2-KO, and Orai1,3-KO), in which we knocked out either STIM1 or STIM2 (Figure S3B). STIM2 knockout in Orai2,3-KO cells caused a slight reduction in SOCE, while STIM1 knockout caused a near complete loss of SOCE (Figures 3A and 3B). In agreement with our previous data (Yoast et al., 2020b), relieving the negative regulatory effects of Orai2 and Orai3 on native Orai1 results in enhanced percentage (~70%) of Orai2,3-KO cells responding with Ca²⁺ plateaus upon stimulation with 10 μM CCh (Figures 3C and 3Q–3S; cf. to 16% of WT-HEK293 cells in Figure 1K). Importantly, in the absence of STIM1, 100% of Orai2,3-STIM1-KO cells responded with Ca²⁺ oscillations (Figures 3D and 3Q–3S). Interestingly, Orai2,3-STIM2-KO cells presented an intermediary phenotype with ~40% of cells responding with plateaus and ~60% of cells responding with Ca²⁺ oscillations (Figures 3E and 3Q–3S). Within oscillating cells,

deletion of either STIM1 or STIM2 did not affect the frequency of Ca^{2+} oscillations per cell (Figure 3P).

Orai1,2-KO cells have an almost complete abrogation of SOCE activated by thapsigargin (Figure 3F). Yet, knockout of either STIM1 or STIM2 in these Orai1,2-KO cells demonstrated an additional very small reduction in SOCE, which only became evident after analyzing many cells (Figures 3F and 3G). Consistent with Ca^{2+} plateaus requiring Orai1 (Yoast et al., 2020b), Orai1,2-KO cells lacking either STIM isoform or neither responded strictly with Ca^{2+} oscillations upon stimulation with 10 μM CCh (Figures 3H–3J and 3Q–3S). Orai1,2-STIM1-KO cells showed an increased percentage of non-responding cells compared to the parental Orai1,2-KO cells, although this increase was not significant (Figure 3S). While Orai1,2-KO and Orai1,2-STIM1-KO cells displayed a similar oscillation frequency (~ 3.5 oscillations/14 min), oscillations in Orai1,2-STIM2-KO cells were slightly enhanced (~ 4.2 oscillations/14 min), although this did not reach significance (Figure 3P).

Although Orai1,3-KO cells showed an almost complete abrogation of SOCE activated by thapsigargin, the miniscule remaining SOCE was significantly larger than in Orai1,2-KO cells (Figures 3K and 3L), consistent with Orai2 displaying higher channel activity than Orai3 (Yoast et al., 2020b). STIM2 knockout in Orai1,3-KO cells caused an appreciable decrease of this miniscule SOCE, while STIM1 knockout did not (Figure 3L). Orai1,3-KO cells lacking either STIM isoform or neither responded strictly with Ca^{2+} oscillations upon 10 μM CCh stimulation (Figures 3M–3O and 3Q–3S). Orai1,3-STIM2-KO cells showed an increased in percentage of non-responding cells compared to the parental Orai1,3-KO cells, although this was not significant (Figure 3S). The oscillatory frequency of Orai1,3-KO cells was significantly higher than Orai1,2-KO cells (Figure 3P). STIM1 knockout in Orai1,3-KO cells significantly enhanced the oscillatory frequency (from ~ 5.25 to ~ 6.4 oscillations/14 min), while STIM2 knockout caused a small decrease (to ~ 4.5 oscillations/14 min) that was not significant (Figure 3P).

Unactivated STIM inhibit IP_3R -mediated Ca^{2+} release

The rapid oscillatory response of STIM1/2-KO cells (Figures 1G and 1P) was not observed in Orai-TKO cells (Figure 1H), arguing that this is independent of the loss of SOCE. The frequency of Ca^{2+} oscillations in STIM1/2-KO cells was unchanged in the presence of 5 μM Gd^{3+} (Figures S2A–S2E), which blocks all Orai channel isoforms (Yoast et al., 2020b; Zhang et al., 2020), ruling out contributions from STIM-independent Ca^{2+} influx through Orai channels. Blockade of Ca^{2+} entry and Ca^{2+} extrusion by 1 mM Gd^{3+} , termed lanthanide insulation (Wedel et al., 2007), did not alter the Ca^{2+} oscillatory frequencies of WT-HEK293, STIM1-KO, and STIM2-KO cells in response to 10 μM CCh (Figures 4A–4C and 4E; cf. Figure 4E to Figure 1I). Importantly, although the Ca^{2+} oscillations of STIM1/2-KO cells were sustained due to the lanthanide insulation, their frequency remained rapid (Figures 4D and 4E), suggesting that the rapid Ca^{2+} oscillations of STIM1/2-KO cells is independent of both Ca^{2+} entry and Ca^{2+} extrusion and is likely the result of enhanced ER Ca^{2+} release. Of note, while 10 μM CCh stimulation of STIM1/2-KO cells failed to produce Ca^{2+} plateaus (Figure 1K), when the

lanthanide insulation protocol was used $\sim 40\%$ of STIM1/2-KO cells responded with plateaus (Figures S4A–S4C), suggesting that the combination of enhanced ER Ca^{2+} release and abrogation of Ca^{2+} extrusion increase cytosolic Ca^{2+} enough to produce Ca^{2+} plateaus in STIM1/2-KO cells.

We performed direct ER Ca^{2+} measurements using the genetically encoded R-CEPIA1er. WT-HEK293 cells and their STIM-KO counterparts were stimulated with a supramaximal concentration of CCh ($>100 \mu\text{M}$) in 0 mM external Ca^{2+} to deplete ER Ca^{2+} , followed by restoration of 2 mM Ca^{2+} without CCh to allow ER refilling (Figure 4F). Interestingly, both STIM1-KO and STIM2-KO cells displayed a slight increase in the rate of ER Ca^{2+} depletion compared to WT-HEK293 that was not significant. However, we observed a significant increase in the rate of ER Ca^{2+} depletion in STIM1/2-KO cells (Figure 4G). The rate of ER Ca^{2+} refilling was significantly reduced in STIM1-KO cells while only marginally reduced in STIM2-KO cells (Figure 4H). As expected, acute ER Ca^{2+} refilling was essentially abrogated in STIM1/2-KO cells (Figures 4F and 4H). While these data suggest that acute ER refilling at the timescale of minutes is STIM dependent in agreement with previous work (Zheng et al., 2018b), STIM1/2-KO cells eventually refill their ER Ca^{2+} as documented by the state of their stores at the beginning of recordings. This STIM-independent refilling likely involves alternative, and perhaps slower, Ca^{2+} entry routes.

We utilized two variants of STIM1 and STIM2 that are incapable of activating Orai channels and mediating SOCE. The first is STIM1 with a point mutation (STIM1-F394H) (Wang et al., 2014) within the C-terminal STIM-Orai activating region (SOAR) (Park et al., 2009; Yuan et al., 2009). The second is a naturally occurring STIM2 splice variant called STIM2.1, which does not activate Orai channels (Miederer et al., 2015; Rana et al., 2015; Zhou et al., 2018). STIM1-F394H tagged with CFP and STIM2.1 tagged with YFP were expressed individually and together in STIM1/2-KO cells, and ER Ca^{2+} depletion and replenishment were analyzed. Interestingly, expression of either STIM1-F394H or STIM2.1 reduced the rate of ER Ca^{2+} depletion compared to STIM1/2-KO cells, and this effect was further enhanced with co-expression of both STIM variants (Figures 4I and 4J). As expected, expression of these STIM variants, which are unable to engage and activate Orai proteins, individually or together, failed to support acute ER refilling (Figure 4I). In response to 10 μM CCh, STIM1/2-KO cells rapidly oscillate and then cease this activity by ~ 5 min. Strikingly, rescue with either STIM1-F394H or STIM2.1 prolonged the time of rundown, thereby increasing total oscillation number (Figures 4K–4N). This effect was further enhanced when STIM1-F394H and STIM2.1 were co-expressed in STIM1/2-KO cells (Figures 4K and 4O). Obviously, because these cells lack both functional Orai-activating STIM1/2, Ca^{2+} oscillations eventually cease. Rescue with either STIM variant, or both, did not substantially alter the percentage of non-responders, plateau, or oscillating cells (Figures S4J–S4L). Under lanthanide insulation, co-expression of STIM1-F394H and STIM2.1 in STIM1/2-KO cells caused a reduction in the frequency of oscillations and a decrease in the percentage of plateau cells (Figures S4D–S4I), strongly arguing that STIM1/2 N termini negatively regulate Ca^{2+} release from the ER.

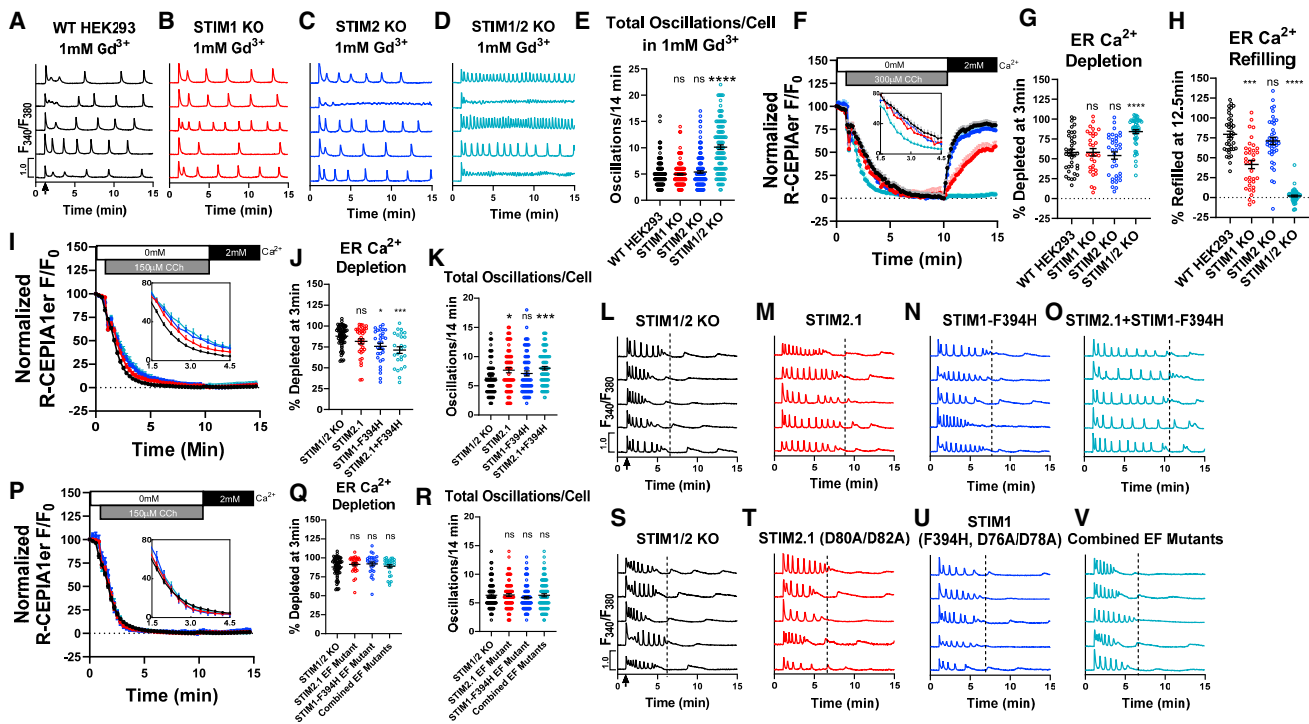


Figure 4. N-terminal EF-hands of STIM1/2 regulate receptor-mediated Ca^{2+} release

(A–D) Representative Ca^{2+} traces from 5 cells/condition with 10 μM CCh stimulation at 1 min (arrow in A) with 1 mM Gd^{3+} and 2 mM Ca^{2+} in bath. (E) Total oscillations/14 min (mean \pm SEM) from (A)–(D). Left to right, $n = 173, 191, 166,$ and 123 cells. (F) ER Ca^{2+} measurements in cells using R-CEPIA1er. Cells were stimulated with 300 μM CCh in 0 mM Ca^{2+} followed by 2 mM Ca^{2+} at 10 min (without CCh). Inset: zoomed in from 1.5–4.5 min. (G and H) ER Ca^{2+} depletion rate (G; mean \pm SEM) in (F) at 3 min after CCh in 0 mM Ca^{2+} . (H) ER Ca^{2+} refilling rate (mean \pm SEM) in (F) at 12.5 min in 2 mM Ca^{2+} . Left to right $n = 37, 35, 41,$ and 53 cells. (I and J) ER Ca^{2+} measurements in STIM1/2-KO or STIM1/2-KO cells rescued with YFP-STIM2.1, STIM1-F394H-CFP, or STIM2.1 + STIM1-F394H (I). Inset: zoomed in from 1.5–4.5 min. (J) ER Ca^{2+} depletion rate (mean \pm SEM) in (I) at 3 min after CCh stimulation in 0 mM Ca^{2+} . Left to right $n = 67, 36, 30,$ and 25 cells. (K) Total oscillations/14 min (mean \pm SEM) from STIM1/2-KO or STIM1/2-KO cells rescued with YFP-STIM2.1, STIM1-F394H-CFP, or combined STIM2.1 + STIM1-F394H. Cells were stimulated with 10 μM CCh in 2 mM Ca^{2+} . Left to right $n = 81, 64, 67,$ and 88 cells. (L–O) Representative Ca^{2+} traces from 5 cells/condition from (K). (P) ER Ca^{2+} measurements in STIM1/2-KO or STIM1/2-KO cells rescued with YFP-STIM2.1-(D80A/D82A), STIM1-(F394H/D76A/D78A)-CFP, or STIM2.1+STIM1-F394H EF-hand mutants. Inset: zoomed in from 1.5–4.5 min. (Q) ER Ca^{2+} depletion rate (mean \pm SEM) in (P) at 3 min after CCh stimulation in 0 mM Ca^{2+} . Left to right $n = 67, 31, 26,$ and 29 cells. (R) Total oscillations/14 min (mean \pm SEM) from (Q) after stimulation with 10 μM CCh in 2 mM Ca^{2+} . Left to right $n = 108, 97, 101,$ and 93 cells. (S–V) Ca^{2+} traces from 5 cells/condition from (R). For all figures, $*p < 0.05, ***p < 0.001, ****p < 0.0001$.

We mutated key residues within the EF-hand of STIM1-F394H (D76A/D78A) and STIM2.1 (D80A/D82A) to abolish Ca^{2+} binding and mimic STIM activation (Brandman et al., 2007; Liou et al., 2005). The expression of these EF-hand mutants in STIM1/2-KO cells either individually or together failed to alter the rate of ER Ca^{2+} depletion in response to supramaximal concentration of CCh (Figures 4P and 4Q) and failed to alter the oscillatory profile in response to 10 μM CCh (Figures 4R–4V), suggesting that unactivated STIMs inhibit Ca^{2+} release. Rescue with either STIM1-F394H (D76A/D78A) and STIM2.1 (D80A/D82A) EF-hand mutants or both did not substantially alter the percentage of non-responders, plateau, or oscillating cells (Figures S4M–S4Q). STIM EF-hand domains are not simply regulating Ca^{2+} release through buffering of ER Ca^{2+} . Indeed, ER Ca^{2+} measurements after passive store depletion with the reversible SERCA

pump blocker cyclopiazonic acid (CPA, 25 μM) showed that the rate of ER Ca^{2+} depletion by CPA, but not refilling, was identical among WT-HEK293, STIM1-KO, STIM2-KO, and STIM1/2-KO cells (Figures S5A–S5C).

We then measured the elementary Ca^{2+} signals generated by IP_3Rs (called Ca^{2+} puffs) in WT-HEK293 cells and STIM1/2-KO cells using total internal reflection fluorescence (TIRF) microscopy. Cells were loaded with caged IP_3 (ci- IP_3/PM), and Ca^{2+} puffs were recorded after photolysis of ci- IP_3 for 60 s at a rate of 166 frames/s. Ca^{2+} puffs were detected following photolysis of ci- IP_3 in both WT-HEK293 and STIM1/2-KO cells (Figures 5A and 5B). The number of puffs and number of puff sites were significantly higher in STIM1/2-KO cells compared to WT-HEK293 cells (Figures 5C and 5D; Videos S1 and S2). The increased number of Ca^{2+} puffs and number of puff sites are

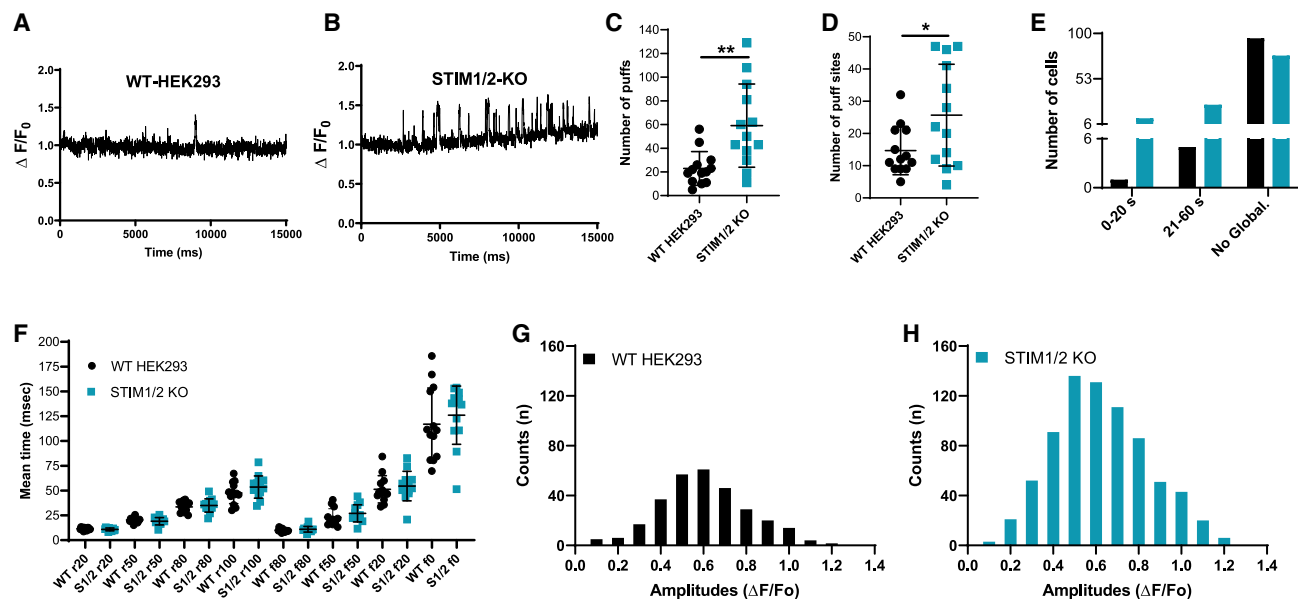


Figure 5. STIM1/STIM2 regulate IP₃R-mediated Ca²⁺ puffs in response to IP₃ uncaging

(A and B) Representative traces of Cal-520 fluorescence ratios ($\Delta F/F_0$) from the center of a single puff site ($1.3 \times 1.3 \mu\text{m}$) evoked by photolysis of ci-IP₃ at 3 s in WT-HEK293 ($n = 12$ experiments, 101 cells) and STIM1/2-KO ($n = 10$ experiments, 115 cells), respectively.

(C and D) 13 cells from each condition were randomly selected to quantify the number of puffs (C) and puff sites (D). (C) $n = 768$ puffs in 13 STIM1/2-KO cells when compared to $n = 298$ puffs in 13 HEK293 cells. (D) $n = 334$ puff sites in 13 STIM1/2-KO cells when compared to $n = 191$ puff sites in 13 HEK293 cells.

(E) From a total of 101 HEK293 cells and 115 STIM1/2-KO cells, a bar graph shows the proportions of WT-HEK293 and STIM1/2-KO cells in which the Ca²⁺ signals either did not globalize or globalized within 0–20 or 21–60 s.

(F) Mean rise and decay times of fluorescence of Ca²⁺ puffs when it increases or decreases to 20%, 50%, 80%, and 100% from 13 cells each of HEK293 and STIM1/2-KO.

(G and H) Amplitudes distribution of the Ca²⁺ puffs in WT-HEK293 (G) and STIM1/2-KO cells (H). For all figures, * $p < 0.05$, ** $p < 0.01$.

consistent with the rapid oscillatory behavior and the increased sensitivity of STIM1/2-KO cells to low CCh concentrations (Figures 1G and 1P). Further, Ca²⁺ signals globalized in $\sim 33\%$ of STIM1/2-KO cells compared to $\sim 6\%$ of WT-HEK293 cells within 60 s (Figure 5E), consistent with increased sensitivity of IP₃R in STIM1/2-KO cells. The mean rise (r) and fall (f) time of the Ca²⁺ puffs was similar between WT-HEK293 and STIM1/2-KO cells (Figure 5F), indicating that the fundamental biophysical properties of IP₃R clusters were unaltered. The amplitude distribution of puffs did not differ between WT-HEK293 and STIM1/2-KO cells with most puffs ranging from 0.3 to 0.9 peak amplitude (Figures 5G and 5H). Similar results were obtained when Ca²⁺ puffs were evoked by 1 μM CCh (Figures S5D–S5I). The number of puffs and number of puff sites were significantly greater in STIM1/2-KO as compared to WT-HEK293 cells. While Ca²⁺ signals globalized rarely within 90 s in WT cells ($\sim 9\%$), $\sim 45\%$ of STIM1/2-KO cells globalized within this period. The amplitude distribution and mean rise and fall time of Ca²⁺ puffs were similar between WT-HEK293 and STIM1/2-KO cells (Figures S5G–S5I).

Mathematical modeling supports STIM1/2, Orai1/2/3, and IP₃Rs shape receptor-evoked Ca²⁺ signaling

We constructed a simplified model of how SOCE is controlled by the interaction of STIMs (defined as STIM_{*i*} where $i = 1, 2$) with Orai dimers (defined as Orai_{*j,k*} where $j, k = 1, 2, 3$). This Orai model was refined from our recent study (Yoast et al., 2020b) by the

addition of STIM1 and STIM2 interactions with Orai_{*j,k*}. The model makes five assumptions that are described in the STAR Methods. The model qualitatively reproduces the range of Ca²⁺ oscillations and plateaus in response to increasing concentrations of agonist (represented by “ p ”; Figures 6A–6D; solid graphs). Specifically, the model predicts the following experimental data: (1) both STIM1 and STIM2 support oscillations and are both involved across the full range of agonist concentrations. However, in the absence of STIM1, even high-agonist concentrations do not lead to sustained plateaus (Figures 6A–6C; solid graphs). (2) High sensitivity to agonist and faster oscillatory frequency of STIM1/2-KO cells (Figure 6D; solid graphs), in support of the negative regulation of IP₃Rs by unactivated STIM proteins. (3) The Gd³⁺ insulation protocol (i.e., closed cell; Figures S6A–S6D): STIM1/2-KO cells readily respond by Ca²⁺ plateaus when Ca²⁺ extrusion is abrogated in the closed cell condition (Figure S6D). (4) The ER Ca²⁺ measurements (Figures 6E and 6F; depicting normalized and absolute ER Ca²⁺ levels, respectively). (5) Data obtained with Orai double-knockout/STIM single-knockout cells with either thapsigargin stimulation, first in 0 mM Ca²⁺ and then in Ca²⁺-containing external solution or with application of increasing concentrations of agonist in Ca²⁺-containing external solution (Figures S7A–S7C). Native STIM1 and Orai1 are essential for mediating Ca²⁺ plateaus in response to high-agonist concentrations with STIM2, Orai2, and Orai3 regulating SOCE across the full range of agonist concentrations but have negligible contributions to plateaus on their own

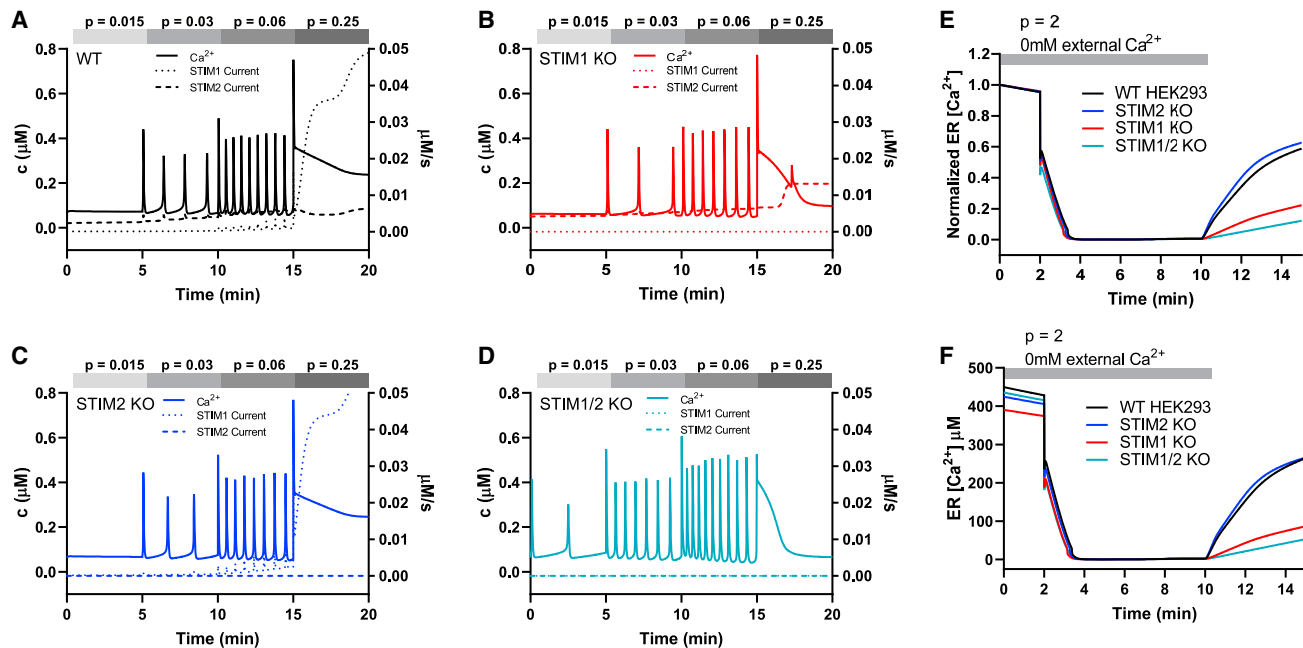


Figure 6. Mathematical modeling supports coordinated STIM1/STIM2 functions over the diverse range of Ca^{2+} signaling events

(A–D) Simulation of responses to different agonist concentrations ($p = 0.015, p = 0.03, p = 0.06, p = 0.25$) applied sequentially in that order, each for 5 min, to WT- and STIM-KO cells in 2 mM Ca^{2+} . Solid lines are Ca^{2+} concentrations plotted against the left axis, while the dotted and dashed lines are STIM-mediated currents, which are plotted against the right axis.

(E and F) Simulation of normalized (E) and absolute (F) ER [Ca^{2+}] in response to high-agonist concentration ($p = 2$), in the absence of external Ca^{2+} , followed (at 10 min) by restoration of external Ca^{2+} and removal of agonist. In (F), the ER starts at different concentrations due to changes in the leak current (called α_0 in the model).

(Figures S7A–S7C). Consistent with these results, model simulations show that, in WT-HEK293 cells, at all agonist concentrations, STIM1 interaction with the Orai1,1 homodimer mediates the great majority of SOCE activity (Figure S7D). Finally, the model reproduces the dynamic change of oscillation properties in response to agonist application in WT-HEK293 cells, as the oscillations initially start faster but gradually change to slower oscillations on a lower baseline (see, e.g., Figure S1H), a known feature of Ca^{2+} oscillation models (Sneyd et al., 2004, 2017).

The experimental data show that, at the beginning of recordings, ER Ca^{2+} stores of STIM1/2-KO cells are full (Figures 4F, 4I, and 4P; Figures 5E and 5F; see also Zheng et al., 2018b), suggesting the presence of an unknown alternative pathway that refills ER Ca^{2+} in hours or days. As such, the model predicts that, under basal conditions (i.e., without agonist), only 28% of the Ca^{2+} influx into the cell is through STIM/Orai channels; 7% (of this 28%) of STIM/Orai-mediated current is mediated by STIM1, with the remaining 93% mediated by STIM2 (Figure 6; dotted graphs). For low-agonist concentrations, this proportion is maintained. At higher agonist concentrations, 92% of the Ca^{2+} influx into the cell is mediated by STIM/Orai channels, and, of that, 93% is mediated by STIM1 and 7% by STIM2.

Coordinated STIM1/STIM2 functions tailor receptor activation to NFAT induction

SOCE is a critical regulator of NFAT isoform nuclear translocation (Feske et al., 2006; Gwack et al., 2007; Rao, 2009; Smedler

and Uhlén, 2014; Tomida et al., 2003; Zhang et al., 2019). The nuclear translocation of NFAT1 is relatively slow and requires robust SOCE activity, while NFAT4 nuclear translocation is more rapid and requires relatively low SOCE activity (Srikanth et al., 2017; Srinivasan and Frauwrith, 2007; Yissachar et al., 2013; Yoast et al., 2020b; Zhang et al., 2019). We expressed GFP-tagged NFAT1 and NFAT4 reporter constructs in WT-HEK293 and STIM-KO cells. These four cell types were then stimulated with either low ($10\ \mu\text{M}$) or high ($300\ \mu\text{M}$) concentrations of CCh and NFAT nuclear translocation was monitored. In WT-HEK293 cells, stimulation with $10\ \mu\text{M}$ CCh caused a slow and gradual accumulation of NFAT1 in the nucleus (Figures 7A and 7E). NFAT1 nuclear translocation was significantly reduced in STIM1-KO and STIM2-KO cells and essentially abolished in STIM1/2-KO cells (Figures 7A, 7B, and 7E). When WT-HEK293 cells were stimulated with $300\ \mu\text{M}$ CCh, NFAT1 nuclear translocation was more robust (Figure 7C). While NFAT1 nuclear translocation was completely inhibited in STIM1/2-KO cells, it was only slightly inhibited in STIM2-KO cells and substantially reduced in STIM1-KO cells (Figures 7C, 7D, and 7F). The nuclear translocation of NFAT4 in WT-HEK293 cells was significantly faster by comparison to that of NFAT1 at both agonist concentrations (Figures 7G–7L). NFAT4 nuclear translocation in response to either 10 or $300\ \mu\text{M}$ of CCh was significantly reduced in STIM1-KO and STIM2-KO cells and essentially abrogated in STIM1/2-KO cells (Figures 7G–7L). These results demonstrate that both STIM1 and STIM2 are required for optimal NFAT

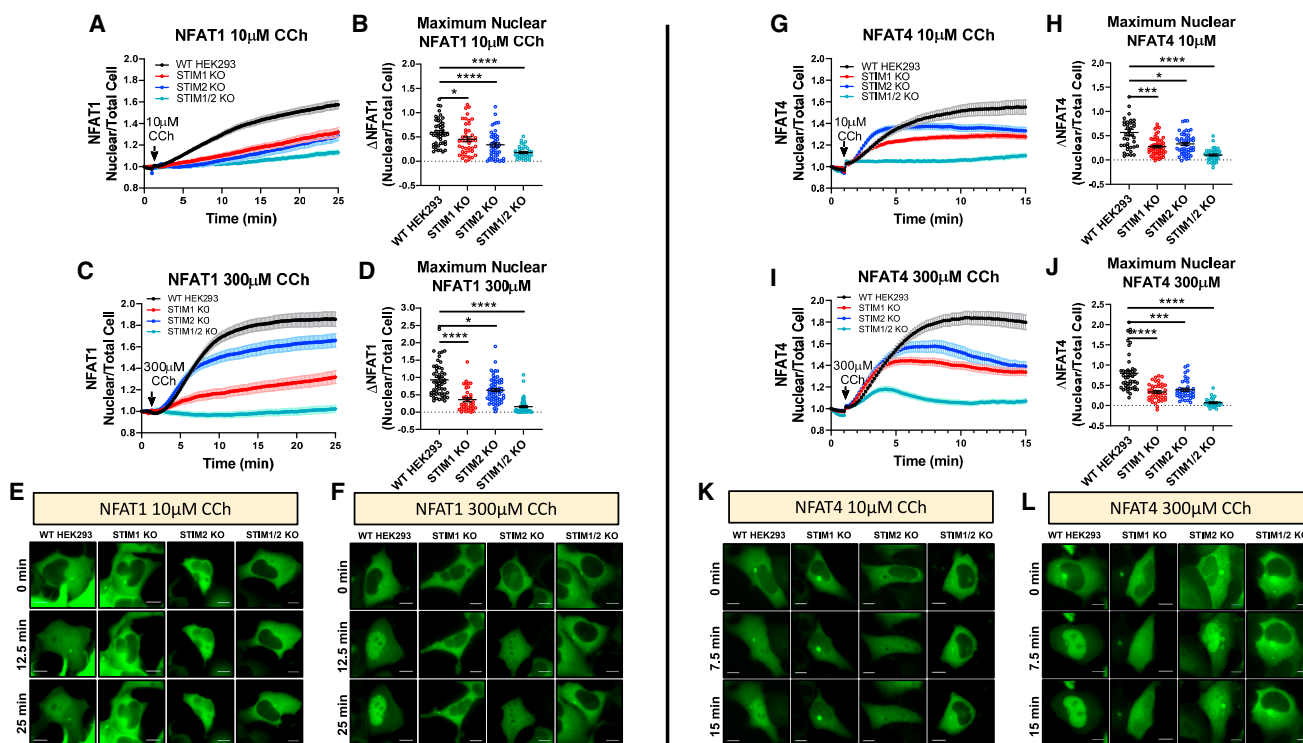


Figure 7. Both STIM1 and STIM2 are required for optimal NFAT1/4 activation

(A) Time-lapse quantification of NFAT1-GFP nuclear translocation in response to 10 μM CCh in 2 mM Ca^{2+} as determined by the ratio of nuclear/total GFP fluorescence.

(B) Mean \pm SEM of nuclear NFAT1 fluorescence for (A) at 25 min. From left to right $n = 46, 43, 44,$ and 36 cells.

(C) Mean \pm SEM of NFAT1-GFP nuclear translocation in response to 300 μM CCh.

(D) Mean \pm SEM of nuclear NFAT1 fluorescence from (C) at 25 min. From left to right $n = 55, 36, 62,$ and 59 cells.

(E and F) Representative time-lapse images of NFAT1-GFP-expressing cells after 10 μM CCh (E) or 300 μM CCh (F).

(G) Same as in (A) but utilizing NFAT4-GFP-expressing cells.

(H) Mean \pm SEM of nuclear NFAT4 fluorescence for (G) at 15 min. From left to right $n = 39, 53, 49,$ and 40 cells.

(I) Mean \pm SEM of NFAT4-GFP nuclear translocation in response to 300 μM CCh.

(J) Mean \pm SEM of nuclear NFAT4 fluorescence from (I) at 15 min. From left to right $n = 46, 40, 36,$ and 29 cells.

(K and L) Representative time-lapse images of NFAT4-GFP-expressing cells after 10 μM CCh (K) or 300 μM CCh (L). Scale bar, 10 μm . For all figures, * $p < 0.05,$ ** $p < 0.01,$ *** $p < 0.001,$ **** $p < 0.0001.$

nuclear translocation under both low- and high-agonist stimulus intensities.

DISCUSSION

SOCE requires STIM1 and Orai1, and the molecular mechanisms underlying their coupling have been intensively investigated (Fahrner et al., 2017; Lewis, 2020; Yeung et al., 2020; Zhou et al., 2017, 2019). STIM2 is a weaker SOCE activator and its recruitment is triggered under basal conditions, when ER Ca^{2+} depletion is modest (Brandman et al., 2007). Recently, we showed that all three Orai isoforms oligomerize to form native CRAC channels, mediating the diversity of Ca^{2+} signaling events in response to the full range of stimulus intensities (Yoast et al., 2020a, 2020b). Here, we addressed the choreography of interactions between native STIM1/2 and Orai1/2/3. Multiple caveats have hindered the understanding of the contributions of each native STIM/Orai to Ca^{2+} signaling. First,

ectopic expression has been performed on a wild-type background, such that the interference of endogenous SOCE is unknown. Ectopic expression typically produces higher protein levels than native levels (Zhang et al., 2019), altering stoichiometry and channel activity. In most of these overexpression studies, only STIM1 and Orai1 were considered. Second, measurements of native SOCE activity have been mostly performed upon maximal store depletion. Under physiological conditions, stimulation of cells with relatively low or moderate agonist concentrations induces concomitant IP_3R -mediated Ca^{2+} oscillations and ER Ca^{2+} replenishment through SOCE, resulting in only minor and transient fluctuations of ER Ca^{2+} . Third, prior reports investigating native STIM1 and STIM2 in Ca^{2+} oscillations have relied on small interfering RNA (siRNA) knockdown (Bird et al., 2009; Ong et al., 2015; Thiel et al., 2013; Wedel et al., 2007), producing conflicting results with variations in knockdown efficiencies between studies as likely confounding factors.

To overcome these limitations, we studied multiple clones of HEK293 cells devoid of STIM1 or STIM2 proteins individually and in combination. Both STIM1-KO and STIM2-KO cells maintain oscillation frequencies like WT-HEK293 cells. However, loss of STIM1 completely abolished Ca^{2+} plateaus. STIM2-KO cells exhibit plateaus at lower concentrations of agonist in comparison to WT-HEK293, suggesting that STIM2 negatively regulates SOCE activity and is required for enhancing the repertoire of Ca^{2+} signaling events. Ca^{2+} plateaus are driven exclusively by the C terminus of STIM1. Alterations in Ca^{2+} oscillation profiles are predicted to have important downstream functional consequences, particularly in the activation of the Ca^{2+} -dependent transcription factors, NFAT (Gwack et al., 2007; Vaeth and Feske, 2018; Zhang et al., 2019). By investigating the activation of NFAT1 and NFAT4, which have distinct nuclear translocation requirements (Srikanth et al., 2017; Yissachar et al., 2013), we demonstrate that loss of either STIM1 or STIM2 impairs NFAT1 and NFAT4 translocation in response to both low- and high-agonist concentrations, suggesting that the presence of both native STIM1 and STIM2 is always required for optimal activation of both NFAT1 and NFAT4 at all agonist concentrations. The combined functions of STIM1 and STIM2 are required to tailor the Ca^{2+} signal to the strength of agonist stimulation and NFAT transcriptional activity. STIM1 plays the major role for NFAT1 nuclear translocation in response to high concentrations of agonist. This is consistent with the known properties of NFAT1, which requires robust Ca^{2+} concentrations for activation and with STIM1 function described herein as the exclusive driver of Ca^{2+} plateaus. We recently revealed that both endogenous STIM1 and STIM2 have major contributions to SOCE in the colorectal cancer cell line HCT116 (Emrich et al., 2019). Recent studies proposed that STIM2 enhances Ca^{2+} signals by recruiting STIM1 to Orai1 channels under conditions of modest store depletion (Ong et al., 2015; Son et al., 2020; Subedi et al., 2018), in agreement with our findings. Whether STIM1/2 function as homodimers or heterodimers requires further investigations. Native CRAC channels are likely heterohexamers of all three OraIs. STIM1 displays stronger basal interactions with Orai2 and Orai3 compared to Orai1 (Yoast et al., 2020b; Zhang et al., 2013). Interestingly, STIM2 shows stronger basal interaction with Orai1 and Orai2 compared to Orai3. Combinations of single STIM-KO/double Orai-KO and modeling showed that Ca^{2+} plateaus strictly require the STIM1/Orai1 duo, which also mediate the diversity of Ca^{2+} oscillation frequencies in combination with the other five STIM/Orai duos.

Although STIM1/2-KO cells completely lack SOCE, their oscillation profile does not phenocopy that of Orai-TKO cells. STIM1/2-KO cells are more sensitive to IP_3 -mediated Ca^{2+} release, and we show that STIM1/2 negatively regulate IP_3 R channel activity. We failed to detect physical interactions between STIM and IP_3 R proteins with co-immunoprecipitations assays (data not shown). While the mechanisms of this IP_3 R regulation by STIMs require further experimentation, our mutagenesis, ER Ca^{2+} measurements, and modeling data suggest it is the unactivated STIMs that inhibit IP_3 R channels, likely setting the threshold of activation by IP_3 . As STIM proteins unfold and become increasingly activated (a state mimicked by EF-hand mutants), the inhibition of IP_3 Rs is lifted with concomitant and equivalent CRAC channel

activation, thus precisely coordinating the levels of Ca^{2+} release and Ca^{2+} entry. Taken together, our work clarifies the complex and dynamic choreography of native interactions between IP_3 Rs, two STIM, and three Orai proteins and how this set of proteins provides the mammalian cell with an effective toolkit to enhance the diversity of Ca^{2+} signaling events and subsequent transcriptional control in response to the full spectrum of agonist intensities.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2021.108760>.

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AUTHOR CONTRIBUTIONS

S.M.E., R.E.Y., D.I.Y., and M.T. designed research; S.M.E., R.E.Y., P.X., V.A., and L.E.W. performed research; J.S. conceived and performed mathematical simulations; D.I.Y., N.H., and D.L.G. contributed new reagents/analytic tools; S.M.E., R.E.Y., V.A., D.I.Y., and M.T. analyzed data; and S.M.E. and M.T. wrote the paper with input from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|----------------------------------|-----------------------------------|
| Antibodies | | |
| Mouse monoclonal anti-GAPDH | MilliporeSigma | Cat# MAB374; RRID: AB_2107445 |
| Rabbit monoclonal anti-STIM1 | Cell Signaling Technologies | Cat# 5668; RRID: AB_10828699 |
| Rabbit polyclonal anti-STIM2 | Cell Signaling Technologies | Cat# 4917; RRID: AB_2198021 |
| α -IP3R1 | Yule Lab | N/A |
| α -IP3R2 | Yule Lab | N/A |
| Anti-IP3R-3 | Becton, Dickinson (BD) | Cat# 610313; RRID: AB_397705 |
| α -Tubulin (DM1A) Mouse mAb | Cell Signaling Technologies | Cat# 3873S; RRID: AB_1904178 |
| 680 CW goat anti-Mouse | LI-COR | Cat# 926-68070; RRID: AB_10956588 |
| 800 CW donkey anti-Rabbit | LI-COR | Cat# 926-32213; RRID: AB_621848 |
| Chemicals, peptides, and recombinant proteins | | |
| Thapsigargin | Calbiochem | Cat#586005 |
| Carbachol | MilliporeSigma | Cat#212385 |
| Fura-2 AM | Invitrogen | Cat#F1201 |
| BAPTA-tetracesium Salt | Santa Cruz Biotechnology | Cat#480436-84-8 |
| Intercept® (TBS) Blocking Buffer | LI-COR | Cat#927-60001 |
| Halt Protease and Phosphatase Inhibitor | Thermo Scientific | Cat#PI78443 |
| RIPA Buffer | Sigma-Aldrich | Cat#R0278-50ML |
| Gadolinium(III) Chloride | ACROS Organics | Cat#AC383560050 |
| Cyclopiazonic Acid | Alomone Labs | Cat#C-750 |
| Lipofectamine 2000 Transfection Reagent | Thermo Scientific | Cat#11668027 |
| Cal-520, potassium salt | AAT Bioquest | Cat#21140 |
| ci-IP3/PM | Tocris | Cat#6210 |
| EGTA, AM | Invitrogen | Cat#E1219 |
| Critical commercial assays | | |
| Guide-it Mutation Detection Kit | Clontech Laboratories | Cat#631443 |
| Cell line Nucleofector Kit | Lonza | Cat#VCA-1003 |
| In-Fusion® HD EcoDry Cloning System | Clontech Laboratories | Cat#639688 |
| Pierce Rapid Gold BCA Protein Assay Kit | Thermo Scientific | Cat#A53225 |
| Deposited data | | |
| http://data.mendeley.com/login?redirectPath=/datasets/9dtvhkzdz/draft?a=c43ec1d3-e142-4fd0-b29f-86660a9ecbe4 | Trebak Lab, this paper | N/A |
| Experimental models: cell lines | | |
| HEK293 cells | ATCC | Cat#CRL-1573 |
| STIM1 KO cells | Trebak Lab (Emrich et al., 2019) | N/A |
| STIM2 KO cells | Trebak Lab (Emrich et al., 2019) | N/A |
| STIM1/2 KO cells | Trebak Lab (Emrich et al., 2019) | N/A |
| Orai2,3 DKO cells | Trebak Lab (Yeast et al., 2020b) | N/A |
| Orai1,3 DKO cells | Trebak Lab (Yeast et al., 2020b) | N/A |
| Orai1,2 DKO cells | Trebak Lab (Yeast et al., 2020b) | N/A |
| Orai TKO cells | Trebak Lab (Yeast et al., 2020b) | N/A |
| Orai2,3-STIM1 KO cells | Trebak Lab, this paper | N/A |

(Continued on next page)

| <i>Continued</i> | | |
|---|---|------------|
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| Orai2,3-STIM2 KO cells | Trebak Lab, this paper | N/A |
| Orai1,3-STIM1 KO cells | Trebak Lab, this paper | N/A |
| Orai1,3-STIM2 KO cells | Trebak Lab, this paper | N/A |
| Orai1,2-STIM1 KO cells | Trebak Lab, this paper | N/A |
| Orai1,2-STIM2 KO cells | Trebak Lab, this paper | N/A |
| IP3R TKO cells | Yule Lab(Alzayady et al., 2016) | N/A |
| Oligonucleotides | | |
| Forward primer for CMV-YFP-STIM2.1 (D80A/D82A) 5'-ACAAATGGCTGATGCCAAAGATGGTGAATTG-3' | Trebak Lab, this paper | N/A |
| Reverse Primer for CMV-YFP-STIM2.1 (D80A/D82A) 5'-TGGCATCAGCCATTTGTTTATGTATTGTTTGAAG-3' | Trebak Lab, this paper | N/A |
| Forward primer for CMV-STIM1-F394H (D76A/D78A)-YFP 5'- ACTGATGGCCGATGCTGCCAATGGTGTGTGG-3' | Trebak Lab, this paper | N/A |
| Reverse primer for CMV-STIM1-F394H (D76A/D78A)-YFP 5'- CAGCATCGGCCATCAGTTTGTGGATGTTACGG-3' | Trebak Lab, this paper | N/A |
| ORAI1 g1 CRISPR/Cas9 Guide RNA 5'- GTTGCTCACCGCCTCGATGT-3' | Trebak Lab, this paper | N/A |
| ORAI2 g1 CRISPR/Cas9 Guide RNA 5'- ACGACAGGGCCTGTACCGAG-3' | Trebak Lab, this paper | N/A |
| ORAI2 g3 CRISPR/Cas9 Guide RNA 5'- CTCATGCGGGGACTCGCTGA-3' | Trebak Lab, this paper | N/A |
| ORAI3 g2 CRISPR/Cas9 Guide RNA 5'- GTTCTGTCACCGCGGCTACC-3' | Trebak Lab, this paper | N/A |
| ORAI3 g4 CRISPR/Cas9 Guide RNA 5'- CCAGAGACTGCACCGCTACG-3' | Trebak Lab, this paper | N/A |
| STIM1 g3 CRISPR/Cas9 Guide RNA 5'- TGATGAGCTTATCCTCACCA-3' | Trebak Lab, this paper | N/A |
| STIM2 g1 CRISPR/Cas9 Guide RNA 5'- AGATGGTGAATTGAAGTAG-3' | Trebak Lab, this paper | N/A |
| Recombinant DNA | | |
| HA-NFAT1(4-460)-GFP | Addgene | Cat#11107 |
| HA-NFAT4(3-407)-GFP | Addgene | Cat#21664 |
| LentiCRISPR v2 | Addgene | Cat#52961 |
| pU6-(Bbs1)-mCherry | Addgene | Cat#64324 |
| pSpCas9(BB)-2A-GFP (PX458) | Addgene | Cat#48138 |
| pCMV R-CEPIA1er | Addgene | Cat#58216 |
| CMV-CFP-ORAI1 | Gill Lab | NA |
| CMV-CFP-ORAI2 | Gill Lab | NA |
| CMV-CFP-ORAI3 | Gill Lab | NA |
| CMV-STIM1-YFP | Gill Lab | NA |
| CMV-STIM2-YFP | Gill Lab | NA |
| CMV-YFP-S1N-S2C | Trebak Lab(Emrich et al., 2019) | NA |
| CMV-YFP-S2N-S1C | Trebak Lab(Emrich et al., 2019) | NA |
| TK-YFP-S1N-S2C | Trebak Lab, this paper | NA |
| TK-YFP-S2N-S1C | Trebak Lab, this paper | NA |
| TK-STIM1-YFP | Trebak Lab, this paper | NA |
| TK-STIM2-YFP | Trebak Lab, this paper | NA |

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Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------------------|------------------------|------------|
| CMV-YFP-STIM2.1 | Gill Lab | NA |
| CMV-STIM1-F394H-YFP | Gill Lab | NA |
| CMV-YFP-STIM2.1 (D80A/D82A) | Trebak Lab, this paper | NA |
| CMV-STIM1-F394H (D76A/D78A)-YFP | Trebak Lab, this paper | NA |

Software and algorithms

| | | |
|-------------------|--|---|
| Graphpad Prism 8 | GraphPad | https://www.graphpad.com/scientific-software/prism/ |
| Clampfit 10.3 | Molecular Devices, LLC. | https://www.moleculardevices.com/ |
| ImageJ | (Schneider et al., 2012) | https://imagej.nih.gov/ij/ |
| LAS X | Leica | https://www.leica-microsystems.com/ |
| SlideBook 6.0 | Intelligent Imaging Innovations, Inc. | https://www.intelligent-imaging.com/slidebook |
| MAXCHELATOR | Chris Patton: cpatton@stanford.edu | https://somapp.ucdmc.ucdavis.edu/pharmacology/bbers/maxchelator/ |
| Image Studio Lite | LI-COR | https://www.licor.com/bio/image-studio-lite/download |

RESOURCE AVAILABILITY

Lead contact

Requests for resources and reagents should be directed and fulfilled by the Lead Contact, Dr. Mohamed Trebak (mtrebak@psu.edu).

Materials availability

All unique/stable reagents generated in the study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

Raw data, unprocessed micrographs, and statistics of all data included in the manuscript are available here:

<https://data.mendeley.com/datasets/9dtvhhkzdz/draft?a=c43ec1d3-e142-4fd0-b29f-86660a9ecbe4>

This study did not generate any unique software or code sets.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture and transfection

All HEK293 cell lines were cultured in high glucose (4.5g/L) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1X Antibiotic-Antimycotic (Thermo Scientific), and cultured at 37°C with 5% CO₂. For CMV-driven STIM and Orai constructs, HEK293 cells were transfected using an Amaxa Nucleofector II (Amaxa Biosystems) with the Nucleofector Kit V (VVCA-1003) and program Q-001. For TK-driven and NFAT reporter constructs, cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Following each transfection cells were plated on 25mm glass coverslips and incubated for 24 hours before use in experiments.

Generation of STIM and Orai CRISPR/Cas9 knockout cell lines

HEK293 Orai and STIM knockout cell lines were generated as previously described (Emrich et al., 2019; Yoast et al., 2020b; Zhang et al., 2019). Briefly, sequence specific guide RNAs (gRNA) targeting *STIM1*, *STIM2*, *Orai1*, *Orai2*, or *Orai3* were cloned into one of two fluorescent vectors (pSpCas9(BB)-2A-GFP, Addgene #48138; pU6-(BbsI)_CBh-Cas9-T2A-mCherry, Addgene #64324). For genes with commercially available validated antibodies (*STIM1*, *STIM2*, and *Orai1*) a single gRNA was utilized to reduce off target effects. For genes without validated antibodies (*Orai2* and *Orai3*), two gRNA sequences were used to excise the entire gene. Parental HEK293 cell lines were transfected with each fluorescent CRISPR vector and individual single or double GFP/mCherry positive cells were FACS sorted (BD FACS Aria SORP) into 96-well cell culture plates 24 hours after transfection. Individual knockout clones were validated using western blot analysis, Sanger sequencing, and functional Ca²⁺ imaging experiments. Each gRNA sequence used to generate single and double Orai and STIM knockout cell lines was as follows:

- *Orai1* g1: 5'-GTTGCTCACCGCCTCGATGT-3'
- *Orai2* g1: 5'-ACGACAGGGCCTGTACCGAG-3'

- *Orai2* g3: 5'-CTCATGCGGGGACTCGCTGA-3'
- *Orai3* g2: 5'-GTTCGTGCACCGCGGCTACC-3'
- *Orai3* g4: 5'-CCAGAGACTGCACCGCTACG-3'
- *STIM1* g3: 5'-TGATGAGCTTATCCTCACCA-3'
- *STIM2* g1: 5'-AGATGGTGAATTGAAGTAG-3'

METHOD DETAILS

DNA constructs

All novel tk-driven constructs (tk-STIM1, tk-STIM2, tk-S1N-S2C, tk-S2N-S1C) and STIM1/STIM2 EF-hand mutants were generated with primers compatible with the In-Fusion cloning system (Takara Bio Inc.). Primers used to create the D80A/D82A substitutions in YFP-STIM2.1 were 5'-ACAAATGGCTGATGCCAAAGATGGTGAATTG-3' and 5'-TGGCATCAGCCATTTGTTTATGTATTGTTTGAAG-3'. Primers used to create the D76A/D78A substitutions in STIM1-F394H-YFP were 5'-ACTGATGGCCGATGCTGCCAATGGTGATG TGG-3' and 5'-CAGCATCGGCCATCAGTTTGTGGATGTTACGG-3'. CMV-CFP-Orai1, CMV-CFP-Orai2, CMV-CFP-Orai3, STIM1-YFP, STIM2-YFP, YFP-STIM2.1, and STIM1-F394H-YFP were from Dr. Don Gill. HA-NFAT1(4-460)-GFP (Addgene #11107) and HA-NFAT4(3-407)-GFP (Addgene #21664) were from Dr. Anjana Rao. pCMV R-CEPIA1er was from Dr. Masamitsu Iino (Addgene #58216).

Fluorescence imaging

HEK293 cells (1.5×10^5) were plated onto 25mm glass coverslips and allowed to attach overnight. For cytosolic Ca^{2+} measurements, coverslips were mounted in Attofluor cell chambers (Thermo Scientific) and cells were incubated in complete DMEM containing $2 \mu\text{M}$ Fura-2AM (Molecular Probes) for 30 minutes at 37°C . Following Fura-2 loading, cells were washed 3 times with a HEPES-buffered saline solution (HBSS) containing the following components: 120mM NaCl, 5.4mM KCl, 0.8mM $MgCl_2$, 2mM $CaCl_2$, 20mM HEPES, 10mM D-glucose, and adjusted to pH 7.4 with NaOH. Chambers were mounted on a Leica DMI8 fluorescence microscope and Fura-2 fluorescence was measured every 2 s by excitation at 340nm and 380nm using a fast shutter wheel and the emission at 510nm was collected through a 20X fluorescence objective. Fluorescence data was collected from individual cells on a pixel by pixel basis and processed using Leica Application Suite X. All cytosolic Ca^{2+} concentrations are presented as the ratio of F_{340}/F_{380} and plotted as means \pm SEM from at least three independent experiments.

For ER Ca^{2+} measurements, HEK293 cell lines were transfected by Nucleofection with $1 \mu\text{g}$ of R-CEPIA1er and imaged 24 hours later on a Leica TCS SP8 confocal microscope with a 40X oil objective. RFP fluorescence was excited with the 552nm laser at 1% output at room temperature. Images were collected every 10-15 s and traces were plotted as means \pm SEM after normalization to the first time point. Prior to the start of each recording, cells were switched from HBSS containing 2mM $CaCl_2$ to a HBSS solution nominally free of $CaCl_2$.

For NFAT nuclear translocation, HEK293 cell lines were transfected with $1 \mu\text{g}$ of NFAT1-GFP or NFAT4-GFP reporter constructs using Lipofectamine 2000. Cells were imaged 24 hours post transfection using a Leica DMI8 fluorescence microscope equipped with a Hamamatsu Flash 4 camera with a 40X oil objective. GFP fluorescence from the whole cell and nucleus was collected every 10 s using a 488nm fast filter wheel with emission captured through a corresponding GFP filter cube. NFAT1 and NFAT4 nuclear translocation was calculated using the equation $NFAT \text{ translocation} = \left(\frac{Nuclear F_{510}}{total cell F_{510}} \right)$ and plotted as a function of time in response to carbachol stimulation.

Förster resonance energy transfer (FRET) measurements

Measurement of FRET between STIM1 and STIM2 with each Orai isoform was performed as previously described (Yoast et al., 2020b). Briefly, Orai-TKO cells were transfected by Nucleofection with $2 \mu\text{g}$ STIM1-YFP in combination with $3 \mu\text{g}$ CFP-Orai1, $4 \mu\text{g}$ CFP-Orai2 or $5 \mu\text{g}$ CFP-Orai3 and $2 \mu\text{g}$ STIM2-YFP together with $3 \mu\text{g}$ CFP-Orai1, $4 \mu\text{g}$ CFP-Orai2, or $5 \mu\text{g}$ CFP-Orai3. Imaging was performed on a Leica DMI 6000B inverted automated fluorescence microscope equipped with a 40X oil objective with simultaneous collection of CFP, YFP, and FRET images every 20 s. Exposure times for STIM1 and STIM2 FRET were 1000ms (CFP), 250ms (YFP), and 1000ms (FRET). Three channel FRET was calculated with SlideBook 6.0 software (Intelligent Imaging Innovations) using the formula $F_C = I_{DA} - \frac{F_D}{D_D} \times I_{DD} - \frac{F_A}{D_A} \times I_{AA}$ where, I_{DD} , I_{AA} , and I_{DA} were the respective fluorescent intensities of CFP, YFP, and FRET channels after taking individual CFP and YFP background correction images. Calculation of E-FRET was performed as previously described using the formula $E_{app} = F_C \frac{F_C}{F_C + G \times I_{DD}}$ (Zal and Gascoigne, 2004).

Detection and analysis of Ca^{2+} puffs using TIRF microscopy

Parental WT-HEK293 cells or STIM1/2-KO cells were grown on 15-mm glass coverslips coated with poly-D-lysine (100 $\mu\text{g}/\text{ml}$) in a 35-mm dish for 2 days. Prior to imaging, the cells were washed three times with imaging buffer (137 mM NaCl, 5.5 mM glucose, 0.56 mM $MgCl_2$, 4.7 mM KCl, 1.26 mM $CaCl_2$, 10 mM HEPES, 1 mM Na_2HPO_4 at pH 7.4). The cells were subsequently incubated with Cal520-AM (5 μM ; AAT Bioquest #21130) and 6-O-[(4,5-Dimethoxy-2-nitrophenyl)methyl]-2,3-O-(1-methylethylidene)-D-*myo*-Inositol 1,4,5-tris[bis[(1-oxopropoxy)methyl]phosphate] (ci-IP₃/PM; 1 μM , Tocris #6210) in imaging buffer with 0.01% BSA in the dark at room temperature. After 1 hour incubation, the cells were washed three times with imaging buffer and incubated in imaging buffer containing EGTA-AM (5 μM , Invitrogen #E1219). After 45 minutes incubation, the media was replaced with fresh imaging buffer and incubated for additional 30 minutes at room temperature to allow for de-esterification of loaded reagents (Lock et al., 2015).

Following loading, the coverslip was mounted in a chamber and imaged using an inverted Olympus IX83 total internal reflection fluorescence microscope (TIRFM) equipped with an oil-immersion PLAPO OTIRFM 60x objective lens (1.45 numerical aperture). The cells were illuminated using a 488 nm laser to excite Cal-520 and the emitted fluorescence was collected through a band-pass filter by a Hamamatsu ORCA-Fusion CMOS camera. The angle of the excitation beam was adjusted to achieve TIRF with a penetration depth of ~ 140 nm. Images were captured from a field of view by directly streaming into RAM. To photorelease IP₃, UV light from a 405 nm laser was introduced to uniformly illuminate the field of view. Both the intensity of the UV flash (~ 200 μ W) and the duration (1000 msec) for uncaging IP₃ were optimized to prevent spontaneous puff activity in the absence of loaded ci-IP₃. TIRF images were captured using 4 X 4 pixel binning (433.333 nm/pixel) from equal field of views for WT-HEK293 and STIM1/2-KO cells at a rate of ~ 166 frames per second. After visualizing images with the cellSens [Ver.2.3] life science imaging software (Olympus), images were exported as vsi files. Images, 10 s before and 60 s after flash photolysis of ci-IP₃, were captured.

The vsi files were converted to tif files using Image J (Fiji) and further processed using FLIKA, a Python programming based tool for image processing (Ellefsen et al., 2014). From each recording, 500 frames (~ 3 s) before photolysis of ci-IP₃ were averaged to obtain a ratio image stack (F/F₀) and standard deviation for each pixel for recording up to 20 s following photolysis. The image stack was Gaussian-filtered, and pixels that exceeded a critical value (1.0 for our analysis) were located. The 'Detect-puffs' plug-in was utilized to detect the number of clusters (puff sites), number of events (number of puffs), amplitudes and durations of localized Ca²⁺ signals from individual cells. All the puffs identified automatically by the algorithm were manually confirmed before analysis (Lock et al., 2018; Mataragka and Taylor, 2018). The results from FLIKA were saved as excel and graphs were plotted using GraphPad Prism 8.

A similar protocol for loading was followed to test the effect of CCh on Ca²⁺ puffs. In this case, the cells were not loaded with ci-IP₃. Cells were stimulated by perfusing 1 μ M CCh to generate IP₃-induced Ca²⁺ release.

Western blot analysis

HEK293 cell lines were harvested and lysed for 15 minutes in ice cold RIPA buffer (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0; Sigma) containing 1X Halt protease/phosphatase inhibitors (Thermo Scientific). Following lysis, samples were clarified by centrifugation at 15,000xg for 10 minutes at 4°C. Supernatants were collected and protein concentration was determined using the Pierce Rapid Gold BCA Protein Assay Kit (Thermo Scientific). Equal concentrations of protein extract were loaded into 4%–12% NuPAGE BisTris gels (Life Technologies) and transferred to PVDF membranes utilizing the Transblot Turbo Transfer System (Bio-Rad). Membranes were blocked for 1 hour at room temperature in Odyssey Blocking Buffer in TBS (LI-COR) and incubated overnight at 4°C with primary antibody. The following antibodies and dilutions were used: STIM1 (1:2000; 4916, Cell Signaling Technology), STIM2 (1:1000; 4917, Cell Signaling Technology), α -tubulin (1:5000; 3873, Cell Signaling Technology), GAPDH (1:5000; MAB374, Sigma), IP3R-1 (1:1000, Yule Lab), IP3R-2 (1:1000, Yule Lab), and IP3R-3 (1:1000; 610313, BD Biosciences). Membranes were washed with TBST and incubated for 1 hour at room temperature with the following secondary antibodies: IRDye 680RD goat anti-mouse (1:10,000 LI-COR) or IRDye 800RD donkey anti-rabbit (1:10,000 LI-COR). Membranes were imaged on an Odyssey CLx Imaging System (LI-COR) and analysis performed in Image Studio Lite version 5.2 (LI-COR) and ImageJ.

Patch clamp electrophysiology

For measurement of I_{crac}, STIM1/2-KO cells were transfected by Nucleofection with 3.5 μ g of each YFP-tagged STIM chimera (YFP-S1N-S2C or YFP-S2N-S1C) along with 1.5 μ g of CFP-Orai1. Cells were seeded onto 30mm glass coverslips and patch clamp recordings were performed 24 hours after transfection. Patch-clamp recordings were performed using an Axopatch 200B along with a Digidata 1440A (Molecular Devices). Pipettes were pulled from borosilicate glass capillaries using a P-1000 Flaming/Brown micropipette puller (Sutter Instrument Company) and a DMF1000 (World Precision Instruments) was used for polishing. Glass electrodes with a resistance of 2 to 4 M Ω were utilized. Recordings were only performed when a > 16 G Ω seal was formed and when whole cell series resistance was < 8 M Ω . Patch Master (HEKA) was used for data acquisition and analysis. For recording of CRAC currents, cells were maintained at a +30mV holding potential and reverse voltage ramps from +100 to -140 mV for 250ms at 2 s intervals were performed. After establishing the whole-cell configuration, intracellular stores were depleted using 20mM BAPTA. The following pipette and bath solutions were utilized:

Pipette solution: 145 mM cesium methanesulfonate, 20 mM BAPTA, 10 mM HEPES, 8 mM MgCl₂, and 25 μ M IP₃ (pH to 7.2 with CsOH).

Bath solution: 115 mM Na-methanesulfonate, 10 mM CsCl, 1.2 mM MgSO₄, 10 mM HEPES, 20 mM CaCl₂, and 10 mM glucose (pH 7.4 with NaOH).

Mathematical modeling

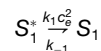
The majority of the model parameters remain unchanged from (Yoast et al., 2020b). The only additional parameters are the equilibrium constants of the STIMi-Oraij,k interactions and the unitary current through a single STIMi-Oraij,k complex.

The following model assumptions are important for obtaining the correct qualitative behavior. There is flexibility in the precise numbers used, but the qualitative trends are critical.

1. There is a background STIM-independent influx of Ca^{2+} into the cell, which we call a leak. Knockout of STIM changes the size of this leak, which in turn sets the resting ER Ca^{2+} concentration. However, changes in the leak are not always sufficient to maintain 100% of the basal ER Ca^{2+} concentration when STIM1 or STIM2 are knocked out. STIM2-KO, STIM1-KO and STIM1,2-KO cells have resting ER Ca^{2+} of, respectively, 94%, 86% and 97% of basal. We propose no mechanism for how the leak changes. However, the counterintuitive assumption that STIM1,2-DKO cells have a higher resting ER Ca^{2+} than the STIM1-KO cells is consistent with the findings of Zheng et al. (Zheng et al., 2018b).
2. Unactivated STIM1 and STIM2 inhibit IP_3R . This is modeled by a phenomenological function (see Modeling Methods below) that increases IP_3R activity as the amount of unactivated STIM1 and/or STIM2 decreases, as a result of activation or knockout of STIM. This is a positive feedback loop (agonist stimulation decreases ER Ca^{2+} , which activates STIM, which decreases the amount of unactivated STIM, which removes inhibition from the IP_3R , which lowers ER Ca^{2+} further), but plays no significant role in the generation of single oscillations. Its main function is to increase oscillation frequency in a STIM1,2 DKO cell that has been closed by 1mM Gd^{3+} (see Figure 4D).
3. Binding ratios to the Orai homodimers (for example, the ratio of STIM1/Orai1,1 to STIM1/Orai2,2 binding, and so on) are determined from the FRET data in Figures 2M and 2Q; see details below in Modeling methods.
4. There is three times more STIM1 than STIM2.
5. In most cases, the unitary current through a STIM2-Oraij,k complex is about 1/5 of the current through the analogous STIM1-Oraij,k complex. The two exceptions are the unitary currents through STIM2-Orai2,2 and STIM2-Orai3,3 which are ten times the current through STIM1-Orai2,2 and STIM1-Orai3,3 respectively.

Modeling methods

The models of Sneyd et al. (2017) and Yoast et al. (2020b) are combined with a simple STIM model. Let S_1 denote the concentration of STIM1 and let S_1^* denote the concentration of activated STIM1, with analogous notation for STIM2. Assuming that S_1 is activated in the reaction



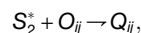
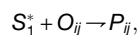
it follows that, at steady state,

$$\frac{S_1^*}{S_1^T} = \frac{K_{e1}^2}{K_{e1}^2 + c_e^2} = \phi_1(c_e),$$

where S_1^T is the maximal concentration of STIM1, and where $K_{e1}^2 = \frac{k_{-1}}{k_1}$. Similarly,

$$\frac{S_2^*}{S_2^T} = \frac{K_{e2}^2}{K_{e2}^2 + c_e^2} = \phi_2(c_e).$$

The STIM bind to the various Orai dimers as follows,



and thus, at steady state,

$$P_{ij} = p_{ij} \phi_1(c_e) O_{ij},$$

$$Q_{ij} = q_{ij} \phi_2(c_e) O_{ij},$$

for some constants p_{ij} and q_{ij} , which determine how well each STIM binds to the Orai dimers. Each p_{ij} and q_{ij} is a combination of the equilibrium binding constant of the relevant Orai and STIM and the ratio of S_1^T/S_2^T .

The ratios of the equilibrium binding constants are estimated from the data in Figures 3M and 3Q. This gives $\frac{p_{11}}{p_{22}} = 1/2$, $\frac{p_{11}}{p_{33}} = 2/3$, $\frac{q_{11}}{q_{22}} = 4/5$, $\frac{q_{11}}{q_{33}} = 2$. In other words, STIM1 binds Orai2 twice as well as it binds Orai1, and binds Orai3 with an intermediate efficacy. Similarly STIM2 binds Orai1 twice as well as it binds Orai3, and binds Orai2 better still. For want of a better choice, each binding constant p_{ij} with $i \neq j$ was set to $(p_{ii} + p_{jj})/2$. The relative sizes of p_{ij} and q_{ij} are then adjusted by choice of S_1^T/S_2^T . We chose $\frac{S_1^T}{S_2^T} = 3$, in order to get qualitative agreement with the data presented here.

The inward Ca current, J_{in} , is given by

$$J_{in} = \alpha_0 + \sum (\alpha_{ij} P_{ij} + \beta_{ij} Q_{ij}) = \alpha_0 + \phi_1 \sum \alpha_{ij} P_{ij} + \phi_2 \sum \beta_{ij} Q_{ij}.$$

for some constants α_{ij} and β_{ij} which control the amount of current through each open STIM-Orai_{i,j} complex. The α_{ij} were taken from (Yoast et al., 2020b), and the β_{ij} were chosen to obtain qualitative agreement with the data presented here. Reasonable agreement was obtained by setting $\beta_{ij} = \alpha_{ij}/5$, with the exceptions $\beta_{22} = 10\alpha_{22}$ and $\beta_{33} = 10\alpha_{33}$. Hence, the current through STIM2-Orai_{i,j} is generally only 20% of the current through the analogous STIM1-Orai_{i,j}, with the exception of Orai_{2,2} and Orai_{3,3}. The parameter α_0 is the STIM-independent leak.

We also have a conservation equation for Orai1,

$$2(O_{11} + P_{11} + Q_{11}) + O_{12} + P_{12} + Q_{12} + Q_{13} + P_{13} + Q_{13} + O_1 = I_1,$$

with analogous conservation equations for Orai2 and Orai3. Given known values of c_e , p_{ij} , q_{ij} , α_{ij} , β_{ij} the conservation equations can be solved to determine O_{ij} , and thus determine J_{in} . This needs to be done for every time step of the differential equations for the calcium concentration, which are taken from (Sneyd et al., 2017).

To model the inhibition of the IP₃R by unactivated STIM, we multiply the usual IP₃R current (Sneyd et al., 2017) by the factor

$$F = 1 + 2 \frac{K^n}{K^n + (1 - S_1)^n} \frac{K^n}{K^n + (1 - S_2)^n}$$

where $K = 0.005$ and $n = 6$, and where S_i denotes the normalized concentration of activated STIM_i. Under basal conditions, S_1 and S_2 are much less than 1, and F is thus close to 1. As S_1 and S_2 increase, F also increases, to a maximum value of 3. Thus, in the absence of any unactivated STIM, the current through the IP₃R is three times greater than the current would be in the absence of feedback from STIM.

QUANTIFICATION AND STATISTICAL ANALYSIS

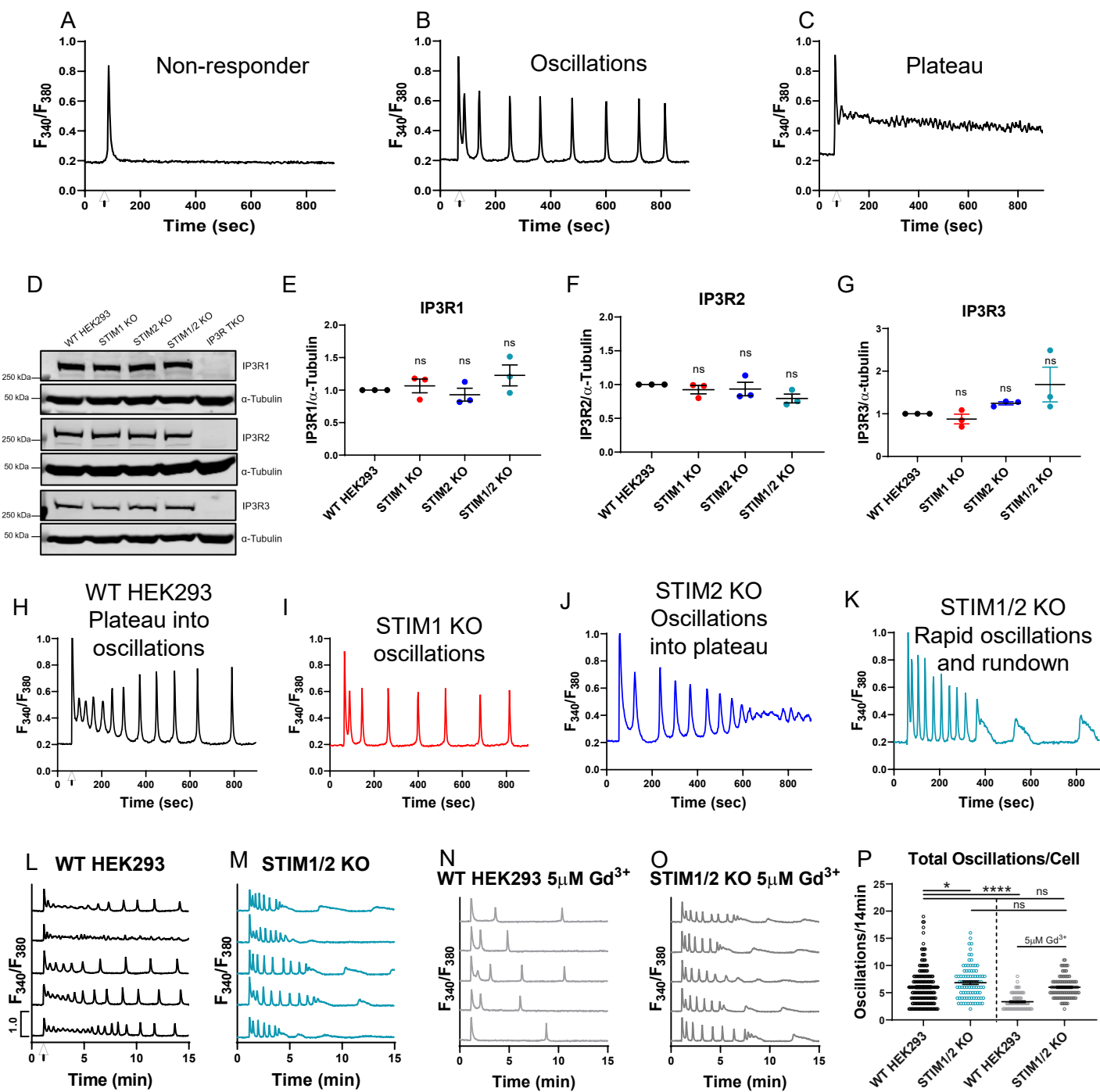
All statistical tests were conducted using GraphPad Prism 8 and data presented as mean \pm SEM. N numbers are included in each figure legends. An unpaired, nonparametric Mann-Whitney t test was used when comparing two sample groups. For comparisons of greater than two groups, the Kruskal-Wallis test with Dunn's multiple comparison was used. Specifically, for Figure 1. Data analyzed with the Kruskal-Wallis one-way ANOVA with multiple comparisons to WT-HEK293 cells. For Figure 2. (H) and (I) are analyzed with the Mann-Whitney U test. (J-L) are analyzed with an unpaired Student's t test. (N-P) and (R-T) are analyzed with the Kruskal-Wallis one-way ANOVA with multiple comparisons. For Figure 3. (B, G, L) and (P) are analyzed with the Kruskal-Wallis one-way ANOVA with multiple comparisons to the respective parental cell line. (Q-S) are analyzed with one-way ANOVA with multiple comparisons to the respective parental cell line. For Figure 4. Data analyzed with the Kruskal-Wallis one-way ANOVA with multiple comparisons to WT-HEK293 (E, G, H) or non-transfected STIM1/2-KO controls (J, K, Q, R). For Figure 5. Data analyzed with Student's t test (unpaired, two-tailed). For Figure 7. Data analyzed with the Kruskal-Wallis one-way ANOVA with multiple comparisons to WT-HEK293. For representation of statistical significance, *, **, ***, and **** indicate p values of < 0.05, < 0.01, < 0.001, and < 0.0001 respectively. ns; not significant. Exact p values for all comparisons performed in the study along with all raw data are posted online on Mendeley Data.

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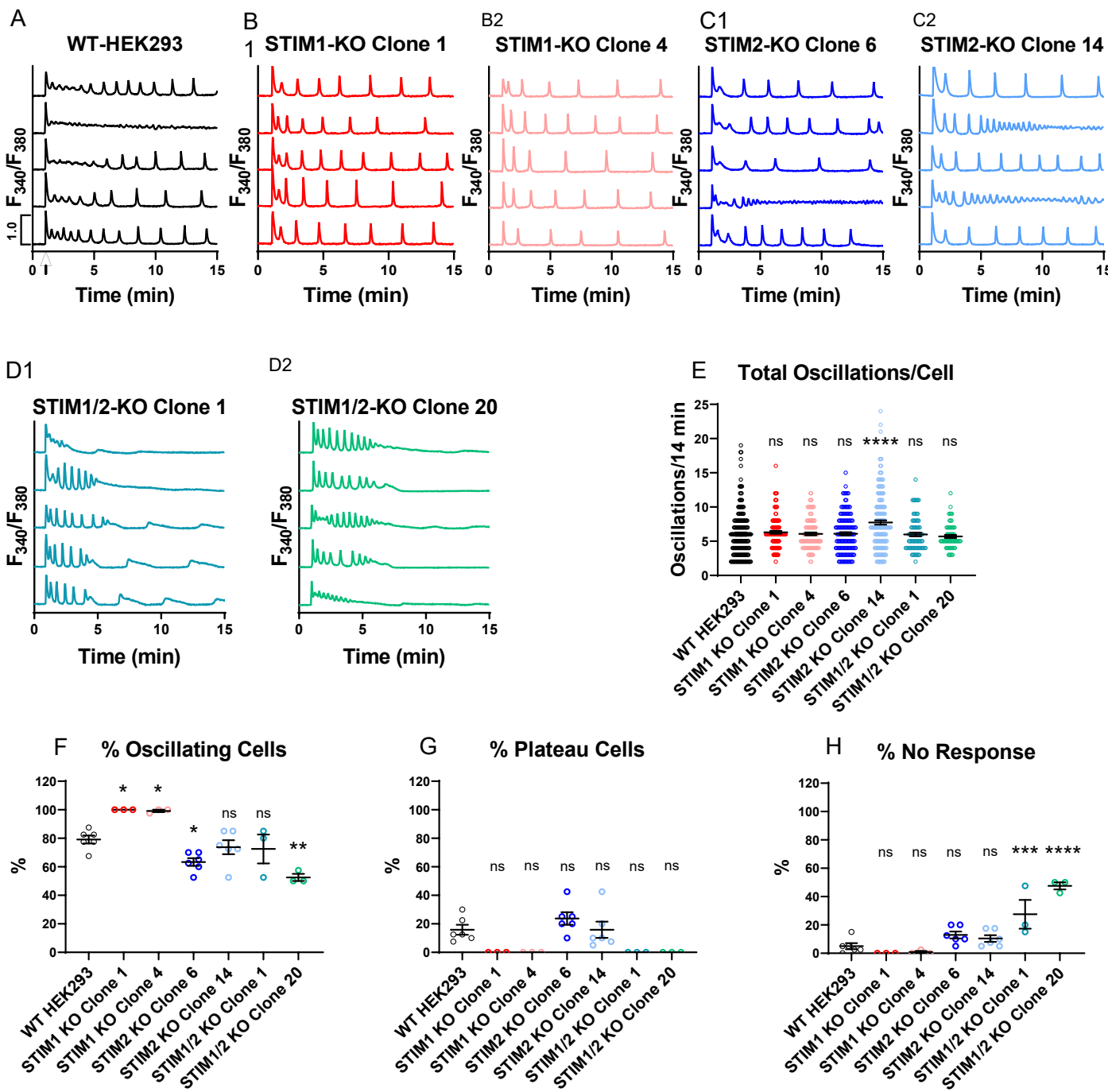
Supplemental information

**Omnitemporal choreographies of all five
STIM/Orai and IP₃Rs underlie the
complexity of mammalian Ca²⁺ signaling**

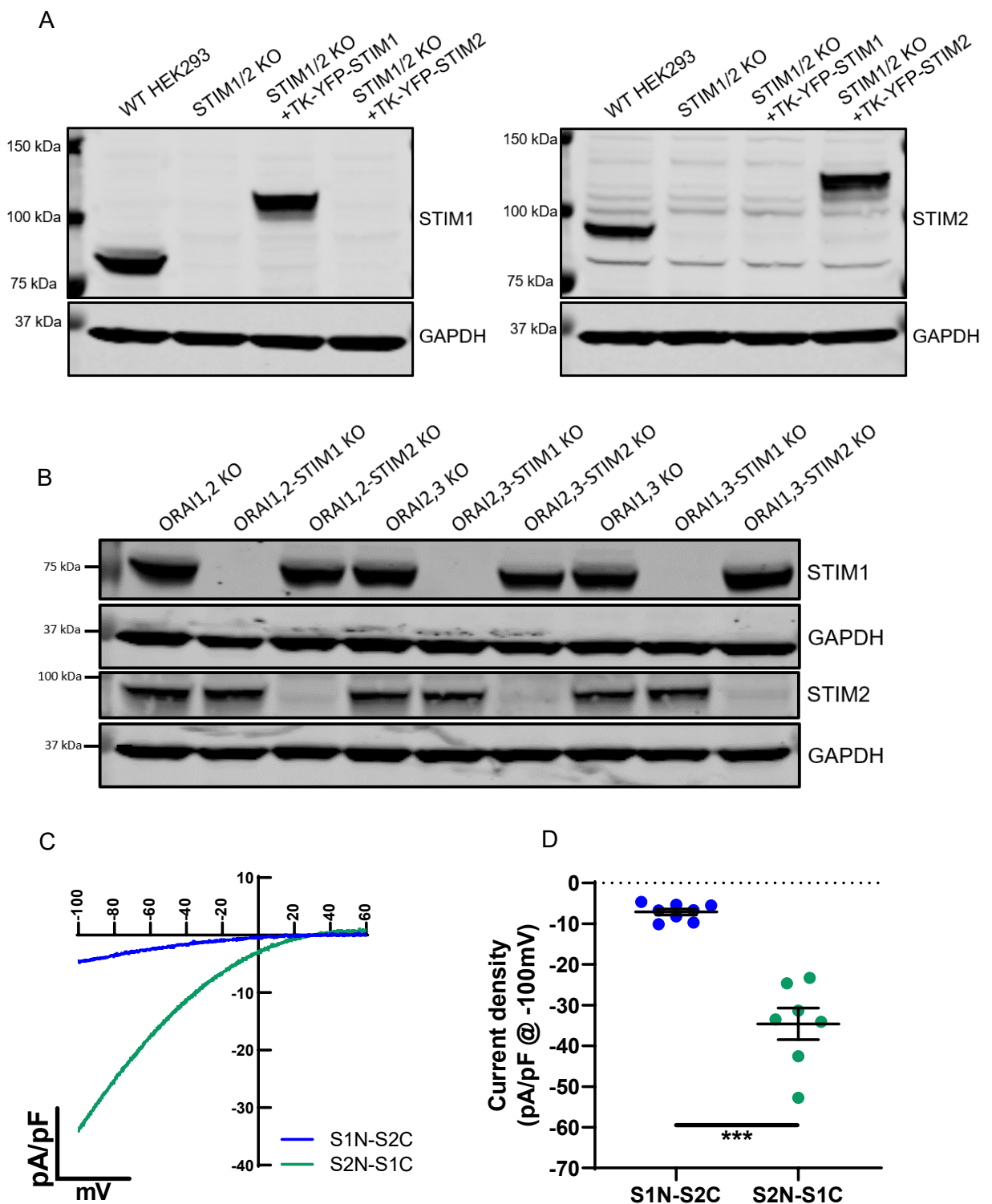
Scott M. Emrich, Ryan E. Yoast, Ping Xin, Vikas Arige, Larry E. Wagner, Nadine Hempel, Donald L. Gill, James Sneyd, David I. Yule, and Mohamed Trebak



Supplementary Figure 1. IP₃R isoform expression, and effect of Gd³⁺ on Ca²⁺ oscillation profiles in WT HEK293 and STIM-KO cells (Related to Figure 1). Traces representing the three oscillatory profiles quantified throughout the manuscript. (A) Non-responding cells demonstrate no response or a single initial spike that returns to baseline following agonist stimulation. (B) Cells showing regenerative oscillations that return to baseline prior to each subsequent spike for the duration of the recording. (C) Plateau cells with sustained elevation of cytosolic Ca²⁺ for the duration of the recording. The arrow in each trace represents the time of addition of 10 μ M carbachol (CCh). (D) Western blot analysis of IP₃R1/2/3 isoforms in WT HEK293 and STIM knockout cell lines. (E) Quantification of IP₃R1 intensity normalized to α -tubulin loading control. Each point represents an individual biological replicate ($n = 3$ each for each condition). (F) Quantification of IP₃R2 intensity ($n = 4$ each for each condition). (G) Quantification of IP₃R3 intensity ($n = 3$ each for each condition). (H) Characteristic Ca²⁺ oscillation profiles in WT HEK293, (I) STIM1-KO, (J) STIM2-KO, and (K) STIM1/2-KO cell lines in response to 10 μ M CCh stimulation (represented by arrow in H). (L) Ca²⁺ traces from 5 representative cells/condition upon stimulation with 10 μ M carbachol (CCh) at 1 minute (indicated by arrow in L) in the presence of 2mM external Ca²⁺. WT HEK293 cells were stimulated in the absence or (N) presence of 5 μ M Gd³⁺. (M) Representative Ca²⁺ traces of STIM1/2-KO cells in the absence or (O) presence of 5 μ M Gd³⁺. (P) Quantification of total oscillations in 14 minutes from (L-O). From left to right $n = 189, 90, 78,$ and 113 individual cells. Scatter plot in (P) is presented as mean \pm SEM and analyzed with the Kruskal-Wallis one-way ANOVA with multiple comparisons to WT HEK293 cells (* $p < 0.05$; **** $p < 0.0001$; ns, not significant). Comparison between STIM1/2-KO cells in the absence or presence of 5 μ M Gd³⁺ was performed with the Mann-Whitney U test (ns, not significant).

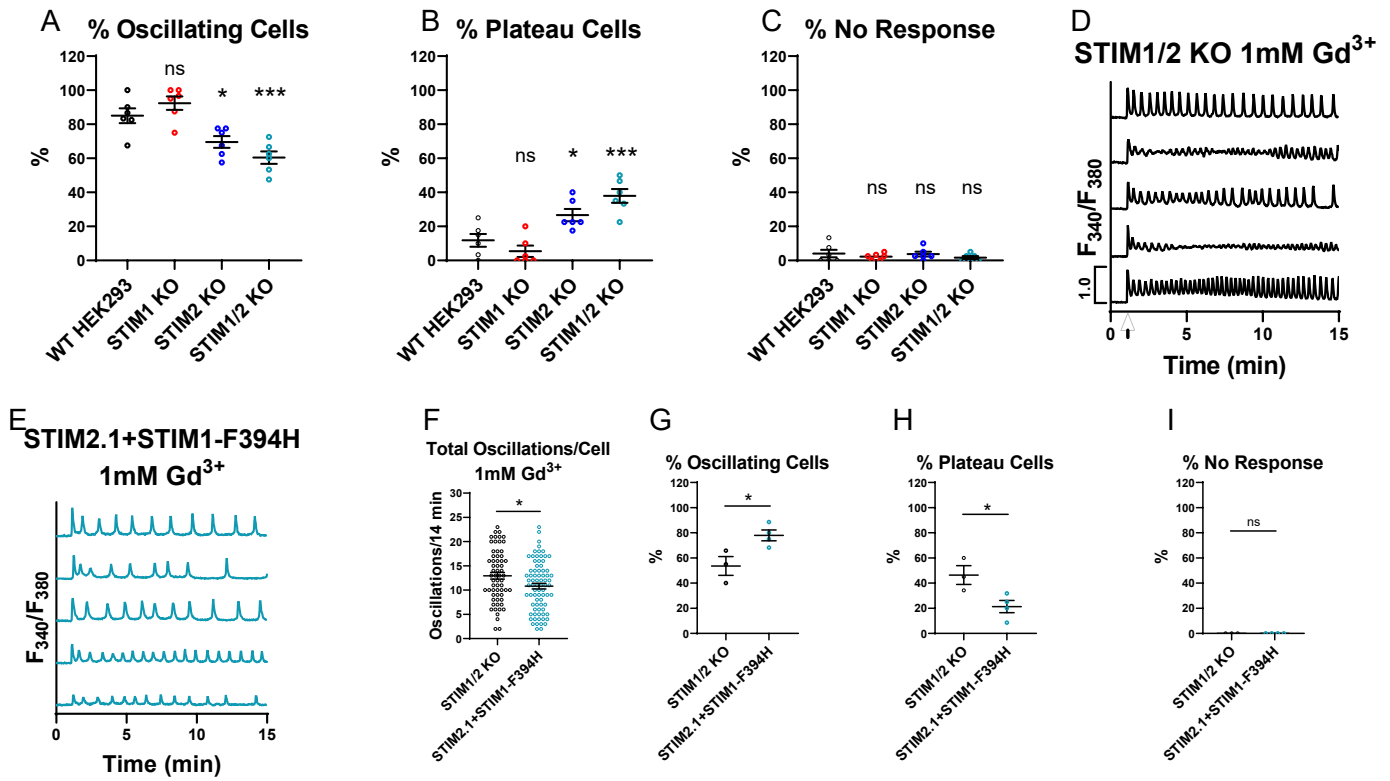


Supplementary Figure 2. Ca²⁺ oscillations from two independent clones for each of HEK293 STIM1-KO, STIM2-KO, and STIM1/2-KO cells (Related to Figure 1). (A) WT HEK293 Ca²⁺ traces from 5 representative cells/condition upon stimulation with 10 μ M carbachol (CCh) at 1 minute (indicated by arrow in A) in the presence of 2mM external Ca²⁺. Same conditions as in (A) but for (B) STIM1-KO clones# 1 and 4, (C) STIM2-KO clones# 6 and 14, and (D) STIM1/2-KO clones# 1 and 20. (E) Quantification of total oscillations in 14 minutes. From left to right $n = 189, 120, 119, 150, 175, 86,$ and 62 individual cells. Oscillation frequency data in (Fig. 1I) is pooled from the two clones presented in (B-D) for each condition. (F) % oscillating cells, (G) % plateau cells, and (H) % non-responding cells for each cell line shown in (A-D). For (F-H) from left to right $n = 6, 3, 3, 6, 6, 3$ and 3 independent experiments. Scatter plots of oscillation frequency data (E) are presented as mean \pm SEM and analyzed with the Kruskal-Wallis one-way ANOVA with multiple comparisons to WT HEK293 cells (**** $p < 0.0001$; ns, not significant). Scatter plots in (F-H) are presented as mean \pm SEM and analyzed with an ordinary one-way ANOVA with multiple comparisons to WT HEK293 cells (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant).

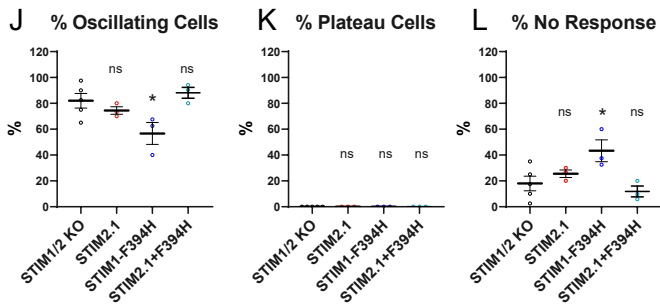


Supplementary Figure 3. STIM1/2 Western blots in double Orai-STIM knockout cell lines, and CRAC currents mediated by STIM chimeric constructs (Related to Figures 2 and 3). (A) *Left*: Representative western blots of WT HEK293, STIM1/2-KO cells, and STIM1/2-KO cells rescued with tk-YFP-STIM1 or tk-YFP-STIM2 and probed with primary antibodies against STIM1 and GAPDH. *Right*: Same samples shown in *Left*, but probed with a primary antibody against STIM2. (B) Representative western blots of double Orai-KO/single STIM-KO HEK293 cells probed with primary antibodies against STIM1, STIM2, and GAPDH. (C) Representative I/V curves of whole-cell patch clamp currents in STIM1/2-KO cells expressing CFP-Orai1 with either YFP-S1N-S2C or YFP-S2N-S1C chimeric STIM constructs. In these recordings, store depletion was achieved through dialysis of 20mM BAPTA through the patch pipette. (D) Quantification of peak current density at -100mV from (C). For each condition $n = 8$ (S1N-S2C + Orai1) and $n = 7$ (S2N-S1C + Orai1) individual cells. Scatter plot in (D) is presented as mean \pm SEM and analyzed with the Mann-Whitney U test (*** $p < 0.001$).

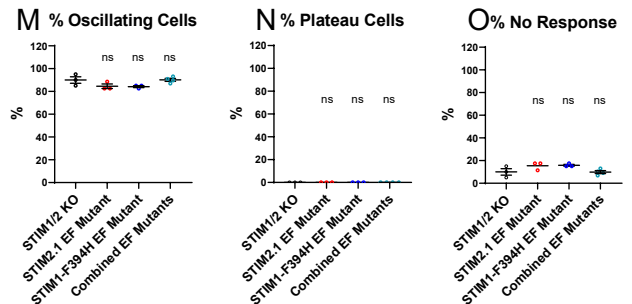
STIM KO cells: Oscillations in 1mM Gd³⁺



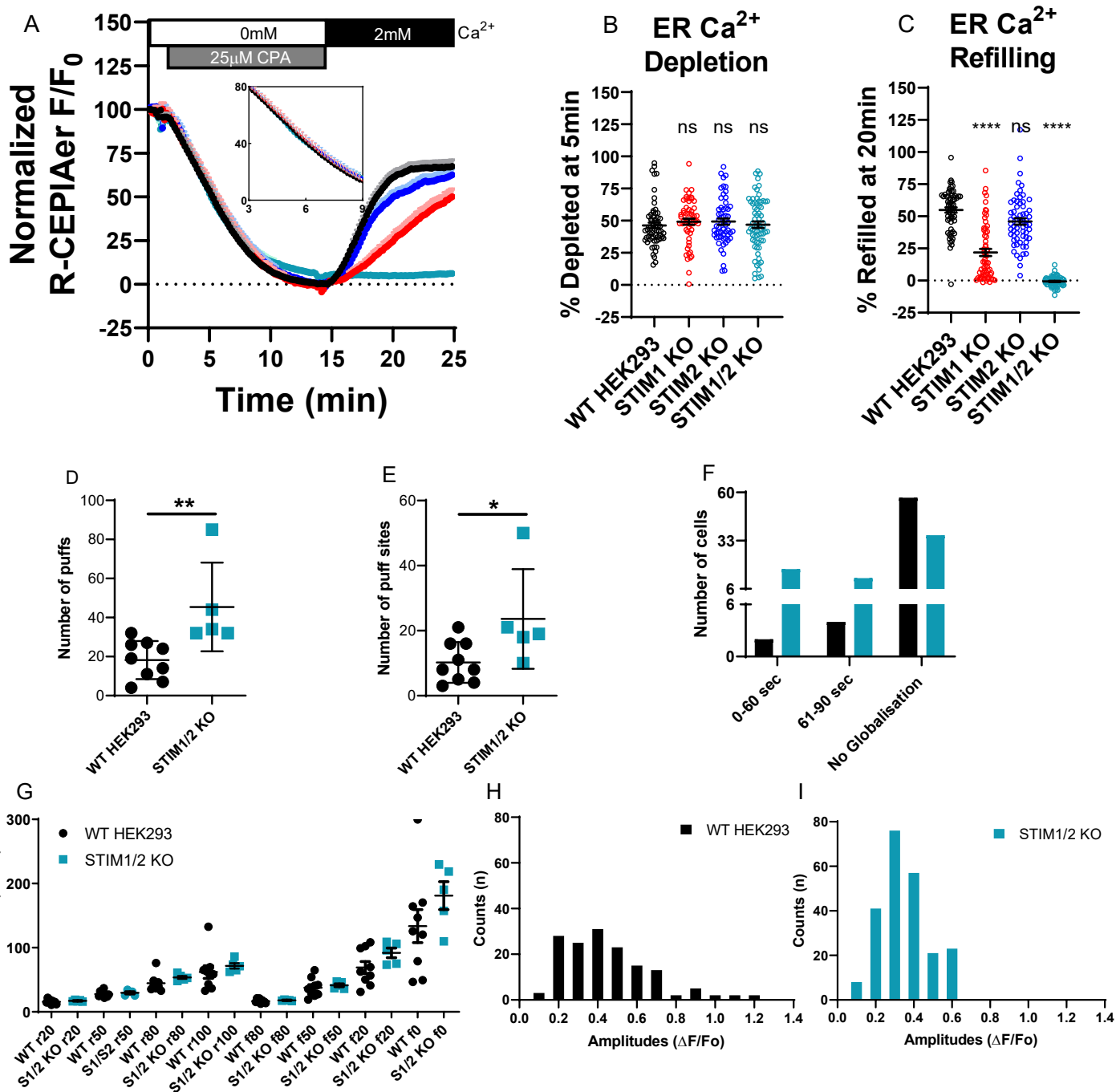
Oscillations in STIM KO cells rescued by STIM2.1/STIM1-F394H



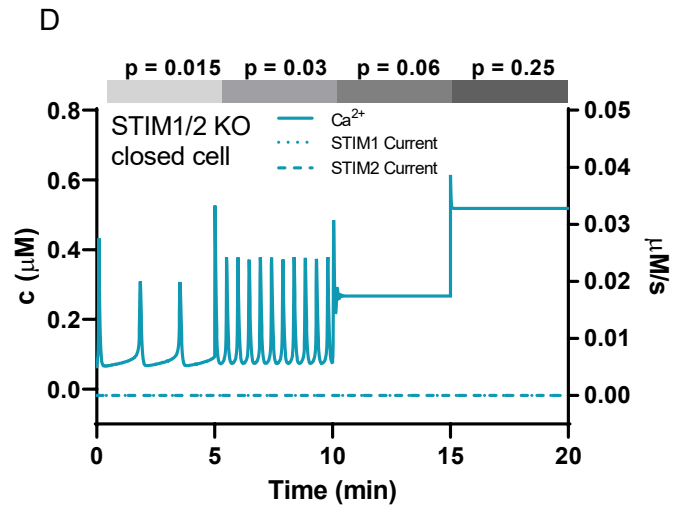
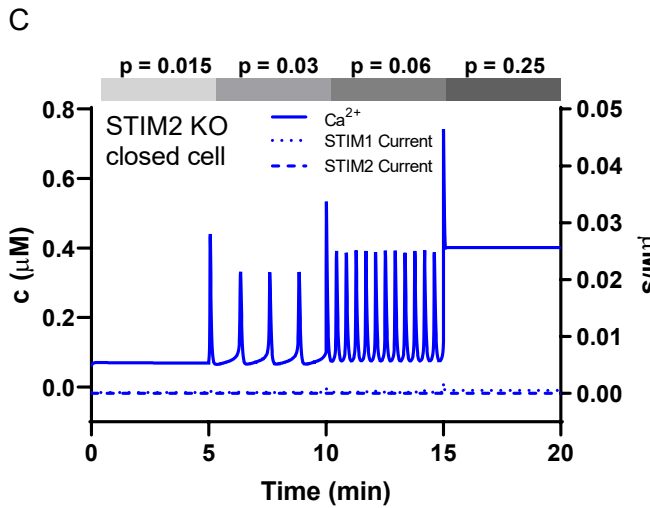
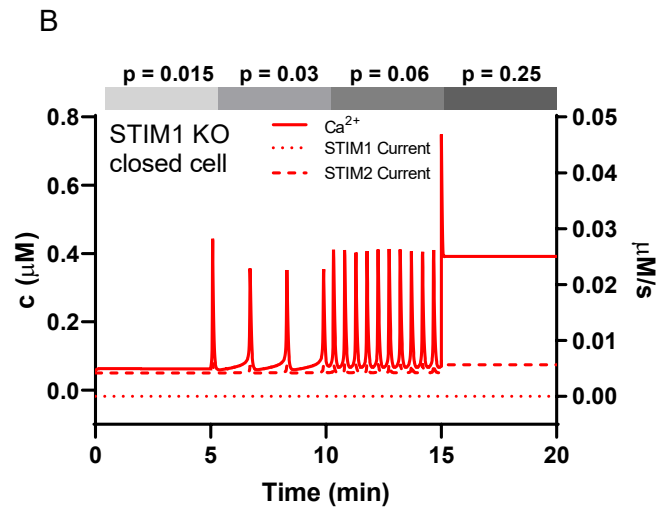
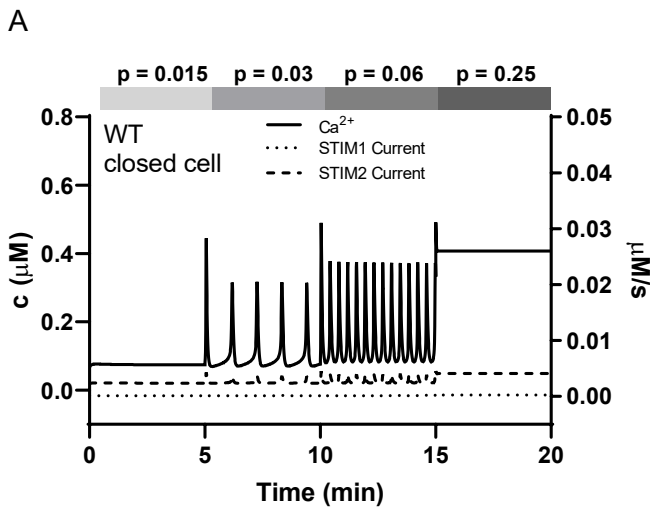
Oscillations in STIM KO cells rescued by EF-Hand Mutants of STIM2.1/STIM1-F394H



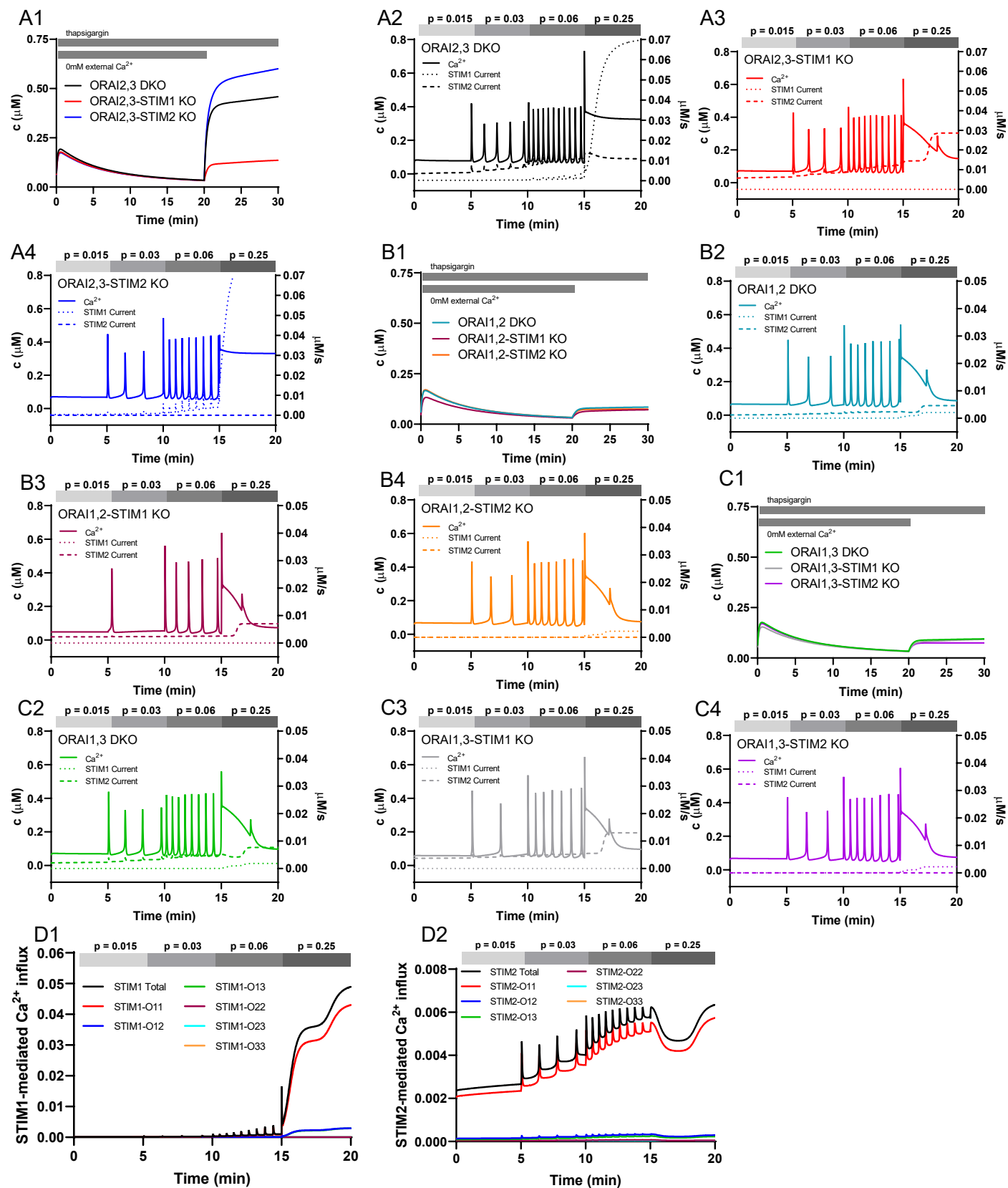
Supplementary Figure 4. Quantifications of Ca²⁺ signaling events in STIM-KO cells with the lanthanide insulation protocol or rescue with the non-agonist STIM mutants (Related to Figure 4). Quantification of (A) % oscillating cells, (B) % plateau cells, and (C) % non-responding cells for each cell type shown in (Fig. 4A-D). From left to right $n = 6, 6, 6,$ and 6 independent experiments. (D) Representative Ca²⁺ oscillation traces of STIM1/2-KO cells and (E) STIM1/2-KO cells rescued with combined YFP-STIM2.1 + STIM1-F394H-CFP and stimulated with $10 \mu\text{M}$ carbachol (CCh) at 1 minute (indicated by arrow in D) in the presence of 1mM Gd³⁺ and 2mM external Ca²⁺. (F) Quantification of total oscillations in 14 minutes from (D-E). From left to right $n = 62$ (STIM1/2-KO) and $n = 77$ (STIM2.1+STIM1-F394H) individual cells. (G) % oscillating cells, (H) % plateau cells, and (I) % non-responding cells for each cell type shown in (D-E). For (G-I) from left to right $n = 3$ independent experiments for both conditions. Quantification of (J) % oscillating cells, (K) % plateau cells, and (L) % non-responding cells for each cell line shown in (Fig. 4K-O). From left to right $n = 5, 3, 3,$ and 3 independent experiments. Quantification of (M) % oscillating cells, (N) % plateau cells, and (N) % non-responding cells for each cell line shown in (Fig. 4R-V). From left to right $n = 3, 3, 3,$ and 4 independent experiments. Scatter plots in (A-C, J-L, M-O) are presented as mean \pm SEM and analyzed with an ordinary one-way ANOVA with multiple comparisons to WT HEK293 (A-C) or STIM1/2-KO (J-L, M-O) where (* $p < 0.05$; *** $p < 0.001$; ns, not significant). Scatter plots in (F-I) are presented as mean \pm SEM and analyzed with an unpaired Student's t-test (F) or Mann-Whitney U test (* $p < 0.05$; ns, not significant).



Supplementary Figure 5. STIM1/STIM2 regulate IP₃R-mediated Ca^{2+} puffs in response to carbachol stimulation (Related to Figure 5). (A) Measurement of ER Ca^{2+} in HEK293 STIM-KO cells using R-CEPIA1er. Cells were stimulated with 25 μ M CPA in a 0mM Ca^{2+} bath solution followed by re-addition of 2mM Ca^{2+} at 15 min (without CPA). Inset shows zoomed in trace from 3-9 min (B) Quantification of ER Ca^{2+} depletion rate in (A) at 5 min after CPA stimulation in 0mM external Ca^{2+} . (C) Quantification of ER Ca^{2+} refilling rate in (A) at 20 min after restoration of 2mM external Ca^{2+} . From left to right $n = 63, 56, 57,$ and 70 individual cells. Scatter plots of ER Ca^{2+} depletion and refilling quantifications are presented as mean \pm SEM and analyzed with the Kruskal-Wallis one-way ANOVA with multiple comparisons to WT HEK293 (**** $p < 0.0001$; ns, not significant). (D) Number of Ca^{2+} puffs were elicited by addition of 1 μ M CCh to WT-HEK293 ($n = 9$ independent experiments with 63 cells) and STIM1/2-KO cells ($n = 5$ independent experiments with 65 cells). The number of puffs were significantly higher in STIM1/2-KO (227 in 65 cells) when compared to HEK293 cells (164 in 63 cells). Similarly, (E) The number of puff sites were significantly higher in STIM1/2-KO (118 in 65 cells) when compared to HEK293 cells (92 in 63 cells). (F) Bar graph showing the number of WT-HEK293 and STIM1/2-KO cells in which the calcium signals globalize within 0-60 seconds or 61-90 seconds following CCh perfusion. Also, shown are the number of cells in which the calcium signals do not globalize. (G) Mean rise and decay times for the fluorescence to increase or decrease to 20%, 50%, 80%, 100% of Ca^{2+} puffs evoked by CCh from HEK293 ($n = 63$) and STIM1/2-KO ($n = 65$) cells are plotted. (H, I) Histograms representing the amplitudes distribution of the Ca^{2+} puffs following stimulation with CCh of WT-HEK293 (H) and STIM1/2-KO cells (I). Data presented as mean \pm SEM. Statistical significance was determined by student's t-test (unpaired, two-tailed). * $p < 0.05$, ** $p < 0.01$ with respect to HEK293. ns, not significant.



Supplementary Figure 6. Mathematical model simulations of Ca²⁺ responses to increasing agonist concentrations under the Gd³⁺ insulation protocol in WT and STIM-KO cells (Related to Figure 6). Responses to four different agonist concentrations ($p = 0.015$, $p = 0.03$, $p = 0.06$, $p = 0.25$) applied sequentially in that order, each for 5 min. In each panel the solid line is the Ca²⁺ concentration, and is plotted against the left axis, while the dotted and dashed lines are the STIM-mediated currents, and are plotted against the right axis. Panels A-D; Ca²⁺ responses in a closed cell system, mimicking the 1mM Gd³⁺ insulation protocol (i.e., with no membrane influx of extrusion of Ca²⁺) with all three Orai isoforms present. The STIM1/2-KO cell is the most effective oscillator, which results from the model assumption that the IP₃R is inhibited by unactivated STIM. Under this Gd³⁺ insulation protocol (closed cell), the absence of STIM1 (B and D) does not hinder the cell from responding with a raised plateau of Ca²⁺ at high agonist concentrations.



Supplementary Figure 7. Mathematical model simulations of Ca^{2+} responses to thapsigargin and increasing agonist concentrations in double Orai-KO/single STIM-KO cells (Related to Figure 6). Responses to thapsigargin (panels A1, B1 and C1) and responses to increasing steps of agonist concentration ($p = 0.015$, $p = 0.03$, $p = 0.06$, $p = 0.25$) applied sequentially in that order, each for 5 min (other panels). The left panels (A1, A2, A3, A4) are for an Orai2,3-DKO cell, the middle panels (B1, B2, B3, B4) are for an Orai1,2-DKO cell, and the right panels (C1, C2, C3, C4) are for an Orai1,3-DKO cell. For each column of panels, the color coding described in the top panel refers also to the lower panels. These responses reproduce the qualitative features seen in the experimental data and cells are unable to maintain Ca^{2+} plateaus in the absence of STIM1. Simulations of STIM1-mediated (D1) and STIM2-mediated (D2) Ca^{2+} currents in response to increasing concentrations of agonist (denoted by p) in various Orai $_{i,k}$ conditions.