The Mitochondrial Ca\textsuperscript{2+} uniporter is a central regulator of interorganellar Ca\textsuperscript{2+} transfer and NFAT activation

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Mitochondrial Ca\textsuperscript{2+} uptake tailors the strength of stimulation of plasma membrane phospholipase C–coupled receptors to that of cellular bioenergetics. However, how Ca\textsuperscript{2+} uptake by the mitochondrial Ca\textsuperscript{2+} uniporter (MCU) shapes receptor-evoked interorganellar Ca\textsuperscript{2+} signaling is unknown. Here, we used CRISPR/Cas9 gene knockout, subcellular Ca\textsuperscript{2+} imaging, and mathematical modeling to show that MCU is a universal regulator of intracellular Ca\textsuperscript{2+} signaling across mammalian cell types. MCU activity sustains cytosolic Ca\textsuperscript{2+} signaling by preventing Ca\textsuperscript{2+}-dependent inactivation of store-operated Ca\textsuperscript{2+} release–activated Ca\textsuperscript{2+} channels and by inhibiting Ca\textsuperscript{2+} extrusion. Paradoxically, MCU knockout (MCU-KO) enhanced cytosolic Ca\textsuperscript{2+} responses to store depletion. Physiological agonist stimulation in MCU-KO cells led to enhanced frequency of cytosolic Ca\textsuperscript{2+} oscillations, endoplasmic reticulum Ca\textsuperscript{2+} refilling, nuclear translocation of nuclear factor for activated T cells transcription factors, and cell proliferation, without altering inositol-1,4,5-trisphosphate receptor activity. Our data show that MCU has dual counterbalancing functions at the cytosol–mitochondria interface, whereby the cell-specific MCU-dependent cytosolic Ca\textsuperscript{2+} clearance and buffering capacity of mitochondria reciprocally regulate interorganellar Ca\textsuperscript{2+} transfer and nuclear factor for activated T cells nuclear translocation during receptor-evoked signaling. These findings highlight the critical dual function of the MCU not only in the acute Ca\textsuperscript{2+} buffering by mitochondria but also in shaping endoplasmic reticulum and cytosolic Ca\textsuperscript{2+} signals that regulate cellular transcription and function.

In addition to their well-established role in cellular energy production and metabolism, mitochondria play a critical role in cellular signaling pathways that regulate gene transcription, cell survival, and function (1, 2). In particular, mitochondria are active participants in cellular Ca\textsuperscript{2+} signaling (3–8). Mitochondrial Ca\textsuperscript{2+} uptake, which is driven by the steep voltage gradient across the inner mitochondrial membrane, occurs through a protein complex containing the pore-forming mitochondrial Ca\textsuperscript{2+} uniporter (MCU) protein (9, 10). MCU forms a Ca\textsuperscript{2+}-selective tetrameric channel in the inner mitochondrial membrane that is regulated by the gate-keeping function of Ca\textsuperscript{2+}-binding MICU1/2 protein dimers (11–16). MICU1/2 dimers keep the MCU channel closed under resting levels of free cytosolic Ca\textsuperscript{2+}. Increased Ca\textsuperscript{2+} concentration in the vicinity of MCU and Ca\textsuperscript{2+} binding to the EF-hand domains of MICU1/2 disinhibits MCU channels and enhances mitochondrial Ca\textsuperscript{2+} uptake (13, 14). Ca\textsuperscript{2+} extrusion from the mitochondrial matrix to the cytosol occurs through independent transporters, which include the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCLX) (17) and possibly the Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger Letm1 (18). The gatekeeping of MCU channel activity by MICU1/2 is relieved only when cytosolic Ca\textsuperscript{2+} concentration is high (above ~1.3 μM and above 500 nM when only MICU1 is present (13), but see also (14) where calculated Kd for Ca\textsuperscript{2+} binding to MICU1/2 dimers is ~650 nM). As such, mitochondrial Ca\textsuperscript{2+} uptake is thought to take place at specialized microdomains where cytosolic Ca\textsuperscript{2+} concentrations are high. Such microdomains include the mitochondria-associated membranes (MAMs) where the endoplasmic reticulum (ER) membrane is within 10 to 30 nm from the outer mitochondrial membrane and Ca\textsuperscript{2+} is transferred to mitochondria through closely apposed inositol-1,4,5-trisphosphate receptor (IP\textsubscript{3}R) channels within ER membranes (19).

Activation of plasma membrane (PM) receptors that couple to isoforms of phospholipase C by hormones, neurotransmitters, and growth factors causes the breakdown of membrane-associated phosphatidylinositol-4,5-bisphosphate into two second messengers: the membrane-bound diacylglycerol and the diffusible IP\textsubscript{3} (20). Activation of IP\textsubscript{3}R channels located in the ER causes ER Ca\textsuperscript{2+} store depletion. Upon Ca\textsuperscript{2+} store depletion, ER-resident stromal interaction molecules 1 and 2
Ca\textsubscript{2+} entry through Orai/CRAC channels leads to the activation of STIM puncta independently of mitochondria. This reciprocal regulation by MCU ne-tunes cytosolic Ca\textsuperscript{2+} extrusion, and mitochondrial Ca\textsuperscript{2+} uptake in inhibiting CRAC channel slow CDI and sustaining cytosolic Ca\textsuperscript{2+} signaling. Our data show that MCU has dual counterbalancing functions at the cytosol–mitochondria interface. MCU activity can enhance the CRAC-mediated increase of cytosolic Ca\textsuperscript{2+} to increase NFAT activity and can also inhibit NFAT activity through the “Ca\textsuperscript{2+} sink” effect of mitochondria. This reciprocal regulation by MCU fine-tunes interorganellar Ca\textsuperscript{2+} transfer and NFAT activation during receptor-evoked signaling.

**Results**

**MCU knockout enhances cytosolic Ca\textsuperscript{2+} despite promoting Ca\textsuperscript{2+} extrusion and CRAC channel slow CDI**

We performed CRISPR/Cas9 gene knockout of MCU in six cell lines from different tissues and species (Fig. 1). A seventh cell line (HeLa) and its corresponding MCU CRISPR/Cas9 knockout clone were kindly provided by Dr Suresh Joseph (Thomas Jefferson University) (Fig. S1). The cell lines are human HeLa, embryonic kidney (HEK293), colorectal cancer HCT116 and DLD1, and Jurkat T cells, rat basophilic leukemia (RBL-1) mast cells, and mouse A20 B cells. These MCU knockout cell lines provided a clean background to analyze the role of mitochondrial Ca\textsuperscript{2+} uptake in shaping intracellular Ca\textsuperscript{2+} signaling. To alleviate potential off-target effects of CRISPR/Cas9 KO, we studied two independent MCU-KO clones for each cell line (except for HeLa cells, of which we obtained only one clone). These clones were identified; validated for MCU-KO with genomic sequencing, Western blot, and mitochondrial membrane potential fluorescence measurements in a permeabilized cell system (Fig. S2). The addition of a bolus 10 \mu M Ca\textsuperscript{2+} to the bath of permeabilized parental cells resulted in rapid mitochondrial uptake, which did not occur in MCU-KO cells.
Figure 1. MCU-KO enhances cytosolic Ca\(^{2+}\) upon thapsigargin stimulation. Western blot documenting MCU protein knockout in two independent CRISPR/Cas9 MCU-KO clones of HEK293 cells and quantification of MCU protein band densitometry relative to GAPDH from three independent experiments (A). Ca\(^{2+}\) measurements in HEK293 parental cells and MCU-KO clones in response to stimulation with 2 μM thapsigargin (Tg) in Ca\(^{2+}\)-free buffer followed by restoration of 2 mM extracellular Ca\(^{2+}\) to determine the magnitude of SOCE (B). Quantification of SOCE (Δ SOCE) from at least three independent experiments are also shown (B) (n = 120; each point represents one cell). Identical experiments to (A and B) were performed in human Jurkat T cells (C and D) (n = 167–178), mouse A20 B cells (E and F) (n = 114–119), rat RBL-1 mast cells (G and H) (n = 82–115), human DLD1 colorectal carcinoma cells (I and J) (n = 104–112), and human HCT116 colorectal carcinoma cells (K and L) (n = 203–229). All Western blot data (A, C, E, G, I, and K) are representative of three independent runs (n = 3). Statistical significance was determined using an ordinary one-way ANOVA where ****p < 0.0001. SOCE data are represented as the mean ± SEM and were statistically analyzed using a Kruskal-Wallis one-way ANOVA with multiple comparisons with their corresponding parental cell line where ****p < 0.0001; ns, not significant p > 0.05. MCU, mitochondrial Ca\(^{2+}\) uniporter; SOCE, store-operated Ca\(^{2+}\) entry.

Demonstrating that acute mitochondrial Ca\(^{2+}\) uptake was abrogated in MCU-KO cells (Fig. S2).

The predominant receptor-activated Ca\(^{2+}\) entry pathway in all seven nonexcitable cell lines considered herein is the SOCE pathway, which biophysically manifests as the CRAC current encoded by STIM1/2 and Orai1/2/3 proteins. To maximally activate SOCE, we irreversibly blocked the sarcoplasmic/ER Ca\(^{2+}\) ATPase (SERCA) using thapsigargin (Tg; 2 μM), which causes passive depletion of ER Ca\(^{2+}\) stores (recorded in Ca\(^{2+}\)-free bath solutions) and activation of SOCE, which manifests upon addition of 2 mM Ca\(^{2+}\) to the extracellular bath (Fig. 1, B, D, F, H, J, and L). Surprising, the use of this protocol revealed that MCU-KO led to a significant increase in apparent SOCE (37). CRAC currents were also inhibited by these drugs, when recorded with either the perforated patch-clamp technique or the whole-cell technique with pipette solutions containing a relatively low Ca\(^{2+}\) buffer (1.2 mM EGTA) but including metabolites that promote energized mitochondria (36). These findings showed that a healthy mitochondrial membrane potential supports mitochondrial Ca\(^{2+}\) uptake, which in turn limits slow Ca\(^{2+}\)-dependent inhibition (CDI) of CRAC channels to sustain SOCE.

Therefore, we performed SOCE measurements in response to thapsigargin in parental HEK293, Jurkat, and RBL-1 cells and their MCU-KO counterparts and tested the effect of 5 μM of the protonophore trifluoromethoxy carbonylnicarbonyl cyanide phenyldiazonitrophenylhydrazide (FCCP) on SOCE. As seen in Figure 2, A–C, when added after SOCE was initiated, FCCP significantly inhibited SOCE in both parental cells and their MCU-KO counterparts. The inhibition of SOCE in parental Jurkat (Fig. 2B) and RBL-1 (Fig. 2C) cells was slower than in their MCU-KO counterparts and was preceded by a transient increase in Ca\(^{2+}\), presumably reflecting bigger mitochondrial Ca\(^{2+}\) stores in the parental cells. In HEK293 cells, the rate of SOCE inhibition by FCCP was similar between wildtype and MCU-KO cells (Fig. 2A), suggesting a bigger contribution of mitochondria as a reservoir for Ca\(^{2+}\) in Jurkat and RBL-1 cells compared with HEK293 cells. Addition of 1 μM ionomycin to HEK293 and Jurkat cells (red arrow; Fig. 2, A and B) to release
any remaining Ca\textsuperscript{2+} from thapsigargin-independent stores (presumably mitochondria) showed a small mitochondrial Ca\textsuperscript{2+} release in wildtype Jurkat cells but not in wildtype HEK293 cells. We subsequently added 10 μM ionomycin (blue arrow) in the presence of 2 mM Ca\textsuperscript{2+} to confirm that maximal Fura2 signal is equal between MCU-KO and parental cells. Overall, these data argue that inhibition of SOCE by FCCP cannot be explained simply by lack of mitochondrial Ca\textsuperscript{2+} buffering through MCU. One recurring observation in our recordings is the enhanced rate of decline of SOCE steady-state plateaus in Jurkat and RBL-1 MCU-KO cells compared with parental cells (Fig. 2, B and C), suggesting that mitochondrial Ca\textsuperscript{2+} buffering by MCU inhibits PM Ca\textsuperscript{2+} ATPase–mediated Ca\textsuperscript{2+} extrusion, as shown previously in Jurkat T cells (39). Indeed, inhibition of SOCE with 5 μM Gd\textsuperscript{3+} revealed that the rate of Ca\textsuperscript{2+} extrusion is significantly faster in MCU-KO cells compared with Jurkat (Fig. 3, A and B) and RBL-1 parental cells (Fig. 3, C and D).

We then sought to determine whether increased SOCE upon MCU-KO is due to increased net CRAC currents across the PM by using the whole-cell patch clamp technique. We chose to record from two widely studied cell lines that produce the largest native CRAC currents, namely, Jurkat and RBL-1 cells. We recorded both Ca\textsuperscript{2+} CRAC currents in 20 mM Ca\textsuperscript{2+} bath solutions and performed rapid switches to divalent-free bath solutions to record Na\textsuperscript{+} CRAC currents, which are larger in size. We measured whole-cell CRAC currents from Jurkat and RBL-1 cells under two different conditions. The first condition uses a pipette solution with a low buffering capacity (1 mM EGTA) that includes 30 μM IP\textsubscript{3} to empty the stores and contains a cocktail of mitochondria-energizing metabolites (see Methods) that can reveal potential mitochondrial effects on CRAC channel slow CDI. The second condition uses a pipette solution with a very strong buffer (50 mM BAPTA) that would negate any potential effect of mitochondrial Ca\textsuperscript{2+} buffering on CRAC currents. Native Ca\textsuperscript{2+} and Na\textsuperscript{+} CRAC currents under both weak and strong buffer conditions in MCU-KO clones of both Jurkat and RBL-1 cells were statistically of similar magnitude compared with currents from their corresponding parental cells (Fig. 4). To accurately determine if MCU deletion alters store-independent CRAC channel slow CDI, we used an established patch clamp protocol where Jurkat cells were preincubated with thapsigargin in the absence of extracellular Ca\textsuperscript{2+} and ICRAC was recorded on introduction of 20 mM Ca\textsuperscript{2+} to the bath solution (34). I\textsubscript{CRAC} was recorded in both Jurkat MCU-KO and parental cells with a pipette solution containing a low buffer (1.2 mM EGTA, 0.66 mM Ca\textsuperscript{2+} (34); see Experimental procedures) either with or without supplementation with the mitochondria-energizing cocktail (Fig. 5). Of interest, even in the absence of the mitochondria-energizing cocktail, MCU-KO cells showed enhanced slow CDI compared with control cells (Fig. 5, A–C). Furthermore, when the pipette solution included the mitochondria-energizing cocktail, I\textsubscript{CRAC} slow CDI of parental Jurkat cells was inhibited on average by ~90%, whereas I\textsubscript{CRAC} slow CDI in MCU-KO cells remained unchanged (Fig. 5, D–F). CDI recordings with and without mitochondria-energizing cocktail
in each individual wildtype Jurkat and MCU-KO cell are shown in Figures S5 and S6, respectively.

Taken together, these data indicate that, although abrogation of MCU-mediated mitochondrial Ca\(^{2+}\) uptake promotes Ca\(^{2+}\) extrusion and CRAC channel CDI, its net effect is an increase, rather than a decrease, in cytosolic Ca\(^{2+}\) levels. Our findings suggest that preventing mitochondria from buffering its allocated share of cytosolic Ca\(^{2+}\) could lead to accelerated ER Ca\(^{2+}\) refilling and enhanced frequency of agonist-evoked cytosolic Ca\(^{2+}\) oscillations, NFAT activation, and cell proliferation. These concepts are explored further below.

**MCU knockout enhances the frequency of cytosolic Ca\(^{2+}\) oscillations without affecting IP\(_{3}\)R activity**

We determined the effect of MCU-KO on Ca\(^{2+}\) signaling triggered by physiological agonist stimulation. To induce cytosolic Ca\(^{2+}\) oscillations, Jurkat cells were stimulated with a relatively low concentration (0.125 μg/ml) of anti-CD3 antibody, which is commonly used to activate the T cell receptor and drive clonal expansion and interleukin 2 (IL-2) production. Under these conditions, essentially all Jurkat cells that responded displayed regenerative Ca\(^{2+}\) oscillations (Fig. 6, A and B) with MCU-KO Jurkat cells displaying enhanced frequency of Ca\(^{2+}\) oscillations compared with the parental cells (Fig. 6, A–C). Furthermore, Ca\(^{2+}\) oscillations in MCU-KO Jurkat cells were of longer duration compared with those of the parental cells (Fig. 6, D and E).

Stimulation of HEK293 with a low concentration of carbachol (CCh) (10 μM CCh), in the presence of 2 mM extracellular Ca\(^{2+}\), evoked regenerative Ca\(^{2+}\) oscillations in the majority of cells (Fig. 6, F and I–K). Note that HEK293 cells were the only cell line studied where SOCE in response to thapsigargin was not significantly enhanced by MCU-KO. Yet, MCU-KO HEK293 cells showed a higher proportion of cells responding with repetitive Ca\(^{2+}\) oscillations compared with their parental counterparts (Fig. 6, I–K). Furthermore, when
oscillating cells are considered MCU-KO HEK293 cells showed a higher frequency of cytosolic Ca\textsuperscript{2+} oscillations than their parental counterparts (Fig. 6H), suggesting that MCU-KO leads to higher cytosolic Ca\textsuperscript{2+}. It is not obvious why the cytosolic Ca\textsuperscript{2+} signal in response to thapsigargin is similar between MCU-KO HEK293 cells and their parental counterparts (despite clear differences in Ca\textsuperscript{2+} oscillations triggered by agonist). We reasoned that differences in mitochondrial and/

Figure 4. Effect of MCU-KO on CRAC currents. A, CRAC current development taken at −100 mV in (from left to right) parental RBL-1 mast cells, MCU-KO clone #1, and MCU-KO clone #2 of the same cells. Recordings were initiated after break-in with a pipette solution containing 1 mM EGTA and a mitochondria-energizing cocktail (see Experimental procedures). B and C, I-V relationship taken from traces in (A) were indicated by color-coded $ signs (B), and quantification of peak CRAC current density (C) (n = 10–12), similar data to (B and C) but for Na\textsuperscript{+} CRAC currents recorded in divalent-free (DVF) solutions and I-V curves taken were indicated by the # signs. F–J, similar recordings and data analysis to (A–E) for parental Jurkat T cells and their MCU-KO clone #1 and clone #4. K–T, (n = 6–12), similar recordings and data analysis in parental and MCU-KO RBL-1 and Jurkat cells to (A–J) but using a pipette solution containing 50 mM BAPTA. All CRAC current data were analyzed using a Kruskal–Wallis one-way ANOVA with multiple comparisons to the corresponding parental cell line where ns, not significant p > 0.05. CRAC, Ca\textsuperscript{2+}-release-activated Ca\textsuperscript{2+}; MCU, mitochondrial Ca\textsuperscript{2+} uniporter.

Figure 5. MCU-KO promotes slow CDI of CRAC currents. Slow CDI of CRAC currents recorded in parental Jurkat cells (A and D) and their MCU-KO counterparts (B and E) with a pipette solution containing 1.2 mM EGTA and 0.66 mM Ca\textsuperscript{2+} in either the presence (D and E) or absence (A and B) of the mitochondria-energizing cocktail. Percentage of CDI is reported for all conditions in (C, n = 8; F, n = 13–14). All CRAC current data are representative of at least three independent experiments and were analyzed using a Mann–Whitney test where **p < 0.01 and ****p < 0.0001. CDI, Ca\textsuperscript{2+}-dependent inactivation; CRAC, Ca\textsuperscript{2+}-release-activated Ca\textsuperscript{2+}; MCU, mitochondrial Ca\textsuperscript{2+} uniporter.
or cytosolic volumes between these different cells might play a role. We performed high-resolution three-dimensional multi-spectral confocal microscopy to generate volumetric reconstructions of the cytosol and mitochondria of Jurkat, A20, RBL-1, and HEK293 cell lines (Fig. 7, A–E). Our volumetric measurements showed that HEK293 cells have a higher surface area and cytosolic and cell volume compared with all other cell lines (Fig. 7, A–G). However, we found no obvious correlation between the mitochondrial volume or the mitochondria/cell ratio and the differences in SOCE in response to thapsigargin between parental and MCU-KO cells (Fig. 7, D and E). Of interest, side-by-side comparisons of mitochondrial Ca$^{2+}$

Figure 6. MCU-KO enhances the frequency of Ca$^{2+}$ oscillations and ER refilling. A–E, Ca$^{2+}$ oscillations were elicited in wildtype Jurkat cells (A) and their MCU-KO counterparts (B) by stimulation with 0.125 μg/ml of anti-CD3 antibody in Ca$^{2+}$-containing (2 mM) buffer. The number of Ca$^{2+}$ oscillations/14 min (C; n = 114–122), overlaid representative spikes (D), and quantification of oscillation duration in seconds (E; n = 40–81) in Jurkat cells and their MCU-KO variant are represented. Both oscillation frequency and duration data were analyzed using a Mann–Whitney test where *p < 0.05 and **p < 0.01. F–K, Ca$^{2+}$ oscillations were elicited in wildtype HEK293 cells (F) and their MCU-KO counterparts (G) by stimulation with 10 μM carbachol (CCh) in Ca$^{2+}$-containing (2 mM) buffer. H, (n = 58–186) quantification of the number of oscillations/14 min in wildtype HEK293 cells and their MCU-KO clones #6 and #7. Quantification of the percentage of HEK293 cells and MCU-KO cells that respond with either repetitive Ca$^{2+}$ oscillations (I; n = 5–8) or sustained Ca$^{2+}$ plateaus (J; n = 5–8) as well as cells that show no response (K; n = 5–8). All HEK293 oscillation data were analyzed for statistical significance using the Kruskal–Wallis test where **p < 0.01; and ns, not significant p > 0.05. L–N, direct measurements of ER Ca$^{2+}$ levels using CEPIAer in HEK293 cells and their MCU-KO counterparts. Cells were stimulated with 300 μM CCh in Ca$^{2+}$-free buffer to elicit ER Ca$^{2+}$ depletion followed by CCh washout and replenishment of 2 mM Ca$^{2+}$ to measure ER refilling (L). Data in (L) are normalized in (M) and rates of ER depletion (N) and refilling (O). Rates of ER depletion are represented as ±SEM of n = 43 to 45 individual cells; resulting data from these traces were analyzed using an unpaired t test where *p < 0.05, ****p < 0.0001, and ns, not significant p > 0.05. All data are representative of at least three independent experiments. ER, endoplasmic reticulum; MCU, mitochondrial Ca$^{2+}$ uniporter.

Figure 7. Cell and mitochondrial volume and Ca$^{2+}$ extrusion differ between different cell lines. A, representative three-dimensional rendering of mitochondrial volume (red) relative to total cell volume in wildtype Jurkat, A20, RBL-1, and HEK293 cells. Cytosolic (B), cell (C), and mitochondrial (D) volumes, mitochondrial/cell volume ratio (E), surface area (F), and surface area/volume ratios (G) were calculated and statistically analyzed using a one-way ANOVA where **p < 0.01, ****p < 0.0001, and ns, not significant p > 0.05. For all volume measurements n = 44 to 209. Mitochondrial Ca$^{2+}$ extrusion was quantified for HEK293 comparing Jurkat (H), A20 (I), and RBL-1 (J) cells using a permeabilized cell system. Bolus 10 μM Ca$^{2+}$ was added to allow Ca$^{2+}$ uptake, followed by addition of the MCU inhibitor RU360 (1 μM) to determine the rate of mitochondrial Ca$^{2+}$ extrusion. The NCLX inhibitor, CGP37157 (10 μM), was added at the end of the recordings. (K; n = 3), Mitochondrial Ca$^{2+}$ extrusion was calculated and statistically analyzed using a one-way ANOVA where ****p < 0.0001. All data are representative of at least three independent experiments.
extrusion between wildtype HEK293, Jurkat, A20, and RBL-1 cells using the permeabilized cell preparation showed that HEK293 cells have significantly enhanced mitochondrial Ca\(^{2+}\) extrusion compared with all other cell types (Fig. 7, H–K). The enhanced mitochondrial Ca\(^{2+}\) extrusion in HEK293 cells suggests a moderate role of mitochondria in buffering cytosolic Ca\(^{2+}\) in these cells (see also Fig. 2A where FCCP effect is similar between parental and MCU-KO HEK293 cells). This might contribute to the blunting of small differences in the Ca\(^{2+}\) signal between MCU-KO and parental HEK293 cells under the thapsigargin protocol. Additional studies are needed to resolve this issue.

We also performed measurements of ER Ca\(^{2+}\) stores in MCU-KO and wildtype HEK293 cells by transiently expressing the genetically encoded Ca\(^{2+}\) sensor R-CEPIAer. Treatment of cells with supramaximal concentration of carbachol (300 \(\mu\)M CCh) in Ca\(^{2+}\)-free solution caused a significantly bigger drop of CEPIAer fluorescence in MCU-KO cells compared with parental cells (Fig. 6L), suggesting a higher ER Ca\(^{2+}\) content in MCU-KO cells compared with parental cells. These CEPIAer fluorescence measurements are represented as normalized data in Figure 6M. We did not resolve a difference in the rate of ER Ca\(^{2+}\) depletion between wildtype and MCU-KO HEK293 cells (Fig. 6N). However, the rate of ER Ca\(^{2+}\) refilling after CCh wash-off and addition of 2 mM Ca\(^{2+}\) to the bath was significantly faster in MCU-KO HEK293 cells (Fig. 6O), suggesting that MCU function limits cytosolic Ca\(^{2+}\) and ER Ca\(^{2+}\) content.

To determine whether enhanced Ca\(^{2+}\) oscillatory frequency of MCU-KO cells is due to increased IP\(_3\)R activity, we used total internal reflection fluorescence (TIRF) microscopy to measure the elementary Ca\(^{2+}\) signals called Ca\(^{2+}\) puffs, which are mediated by IP\(_3\)Rs. We loaded parental HEK293 and MCU-KO cells with a Ca\(^{2+}\) dye and caged IP\(_3\) (ci-IP\(_3\)/PM; see Experimental procedures), and Ca\(^{2+}\) puffs were recorded at a rate of 166 frames/s upon photolysis of ci-IP\(_3\). We detected Ca\(^{2+}\) puffs in both parental HEK293 and MCU-KO cells following photolysis of ci-IP\(_3\) (Fig. 8). However, the number of puffs and the number of puff sites recorded in either Ca\(^{2+}\)-free or Ca\(^{2+}\)-containing bath solutions were not significantly different between MCU-KO cells and parental HEK293 cells (Fig. 8, A, B, F, and G). The amplitude distribution of puffs did not differ between HEK293 and MCU-KO cells with most puffs ranging from 0.2 to 0.7 peak amplitude (Fig. 8, C and H). The mean rise (r) and fall (f) time of the Ca\(^{2+}\) puffs were also similar between HEK293 and MCU-KO cells (Fig. 8, D and I), indicating that the fundamental biophysical properties of IP\(_3\)R clusters were not different between these two groups of cells. Furthermore, there was no difference between both groups of cells in the proportion of cells that showed a globalized Ca\(^{2+}\) signal within 60 s (Fig. 8, E and J). These data suggest that MCU knockout does not alter IP\(_3\)R channel activity.

**MCU knockout enhances NFAT1/4 activation**

Given IP\(_3\)R activity is unaltered by MCU-KO, the simplest explanation for enhanced Ca\(^{2+}\) oscillations in MCU-KO cells is that MCU-KO causes extramitochondrial Ca\(^{2+}\) buildup as mitochondria are unable to buffer their allocated share of cytosolic Ca\(^{2+}\). Hence, cytosol-ER-mitochondrial Ca\(^{2+}\) transfer would be backed up in MCU-KO cells, leading to faster ER

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**Figure 8. MCU-KO has no effect on inositol-1,4,5-trisphosphate receptor-mediated Ca\(^{2+}\) puffs.** A–E, Ca\(^{2+}\) puffs were measured in Ca\(^{2+}\)-containing buffer (1.3 mM Ca\(^{2+}\)) using Cal-520 fluorescence ratios (ΔF/F\(_0\)) from the center of single puff sites (1.3 × 1.3 \(\mu\)m) evoked by photolysis of ci-IP\(_3\) in wildtype HEK293 (n = 20 independent experiments; 153 cells) and their MCU-KO counterparts (n = 19 independent experiments; 132 cells). A and B, thirteen cells from each condition were randomly selected to quantify the number of puffs (A) and puff site (B). C, amplitudes distribution of the Ca\(^{2+}\) puffs in wildtype HEK293 and MCU-KO cells. D, mean rise and decay times of fluorescence of Ca\(^{2+}\) puffs when it increases (r) or decreases (f) to 20%, 50%, 80%, and 100% from 13 cells each of HEK293 and MCU-KO. E, bar graph showing the proportions of wildtype HEK293 and MCU-KO cells in which the calcium signals globalized within 60 s. F–J, similar experiments and data representation to (A–E) in wildtype HEK293 (n = 11 independent experiments; 93 cells) and MCU-KO (n = 12 independent experiments; 109 cells) where Ca\(^{2+}\) puffs were measured in Ca\(^{2+}\)-free buffer supplemented with 1 mM EGTA. All data in (A, B, E, F, G, and J) were subjected to an unpaired t test where ns, not significant p > 0.05. MCU, mitochondrial Ca\(^{2+}\) uniporter.
refilling and/or increased net cytosolic Ca$^{2+}$. We tested whether the net increase in global cytosolic Ca$^{2+}$ we observed in MCU-KO cells is associated with enhanced NFAT activity, which is controlled by Ca$^{2+}$ microdomains near CRAC channels. NFAT activity was significantly enhanced in MCU-KO cells of both HEK293 (Fig. 9, A–C) and Jurkat cells (Fig. 9, D–F) compared with their respective controls. We determined the effect of MCU-KO on NFAT4 nuclear translocation in HEK293 cells using a reporter NFAT4-GFP construct coupled to fluorescence microscopy (Fig. 9, A–C). In Jurkat cells, we determined the effect of MCU-KO on native NFAT1 nuclear translocation using an NFAT1-specific antibody and ImageStream (Fig. 9, D–F). Stimulation with 10 μM CCh caused a significantly bigger nuclear translocation of NFAT4 in MCU-KO HEK293 cells when compared with parental controls (Fig. 9, A–C). Using a multiphoton imaging flow cytometer (ImageStream) we tracked native NFAT1 nuclear translocation in response to ER store depletion with 2 μM thapsigargin for 30 min in populations of tens of thousands of MCU-KO Jurkat cells and their parental counterparts. MCU-KO Jurkat cells showed a significantly more robust nuclear translocation of endogenous NFAT1 compared with the parental Jurkat cells (Fig. 9, D–F). These data suggest that the global cytosolic Ca$^{2+}$ increase triggered by deletion of MCU leads to sufficient Ca$^{2+}$ buildup at the vicinity of CRAC channels to enhance NFAT nuclear translocation.

**Tissue-specific MCU knockdown in mice enhances lymphocyte SOCE and proliferation**

To determine if the increase in cytosolic Ca$^{2+}$ upon MCU-KO also occurs in primary cells and is not restricted to cell lines, we generated tissue-specific knockdown (KD) mice for Cd4$^+$ T cells and B cells (see Fig. S7 for mice genotyping). We used a 4-hydroxytamoxifen (4-OHT)-inducible CD4 Cre line to generate mice with Cd4$^+$ T cell–specific MCU-KD (Fig. 10, A–G). The B cell–specific MCU-KD mice were generated using the noninducible MB1 Cre line (Fig. 10, H–M). The LoxP-Cre system led to reduction in MCU protein expression by ~60% to 80% (Fig. 10, B–D, H, and I).

Primary Cd4$^+$ T cells were isolated with negative selection from spleens of MCU$^{flx/flx}$ mice (MCU$^{flx/flx}$ Cd4$^{Cre(-)}$) and MCU$^{flx/flx}$ Cd4$^{Cre(+)}$ mice (MCU$^{flx/flx}$ Cd4$^{Cre(+)}$), activated in vitro using anti-CD3/anti-CD28 conjugated beads and mouse interleukin-2 (IL-2) and treated with (Z)-4-Hydroxytamoxifen (4-OHT) to induce Cre activity for 96 h before experimenta- tion. Control cells for both MCU$^{flx/flx}$ Cd4$^{Cre(-)}$ and MCU$^{flx/flx}$ Cd4$^{Cre(+)}$ conditions originated from the same mice but were not subjected to 4-OHT treatment. We verified that the

![Image](image-url)

**Figure 9. MCU-KO enhances NFAT nuclear translocation.** A–C, HEK293 cells and their MCU-KO counterparts were transfected with an NFAT4-GFP construct, stimulated with 10 μM CCh, and nuclear translocation of NFAT4-GFP was monitored over time using a fluorescence microscope (A; see Experimental procedures, the scale bar represents 10 μm). The ratio of nuclear/total fluorescence of NFAT4-GFP (B) and difference between maximal and basal ratios (ΔNFAT4; C) are represented for both groups of cells and were statistically analyzed using a Mann–Whitney test where *p < 0.01. (D–F), ImageStream analysis of native NFAT1 nuclear translocation using flow cytometry in wildtype and MCU-KO Jurkat cells stimulated with 2 μM thapsigargin (Tg) for 30 min. Native NFAT1 was labeled with a fluorescently tagged specific antibody, and NFAT1 nuclear translocation was determined by colocalization of NFAT1 with DAPI nuclear staining (D). Histograms showing the distribution (E) and intensity (F) of nuclear NFAT1 fluorescence from n = 3 independent experiments with 10,000 cells/run in wildtype Jurkat and MCU-KO cells. The results of these experiments were analyzed using an unpaired t test where ****p < 0.0001. MCU, mitochondrial Ca$^{2+}$ uniporter; NFAT, nuclear factor for activated T cells.
administration of 4-OHT does not alter MCU protein levels in MCUfl/fl CD4cre(-) mice (Fig. 10C). Stimulation of cells with 2 μM thapsigargin showed that only MCU-KD CD4+ T cells, namely, CD4+ T cells isolated from MCUfl/fl CD4cre(-) mice and treated with 4-OHT, displayed significantly higher SOCE (Fig. 10, E–G). Because the B cell–specific MB1Cre line is noninducible, primary B cells were isolated with negative selection from spleens of MCUfl/fl B1cre(-) mice either with MB1Cre (MCUfl/fl B1cre(+)) or without (MCUfl/fl B1cre(-)) and immediately used for Ca2+ imaging to measure SOCE. Stimulation of B cells with 2 μM thapsigargin showed that B cells isolated from MCUfl/fl B1cre(-) showed significantly higher SOCE compared with B cells isolated from MCUfl/fl B1cre(-) mice (Fig. 10, F and K).

The induction of MCU-KO with 4-OHT for 5 days in CD4+ T cells from MCUfl/fl CD4cre(+), and treated with 4-OHT, displayed significantly enhanced SOCE compared with B cells isolated from MCUfl/fl B1cre(-) and MCUfl/fl B1cre(-) mice. B cell proliferation induced by weak B cell receptor (BCR) stimulation can be enhanced by Ca2+-independent signals such as costimulation with anti-CD40 or stimulation of Toll-like receptors by lipopolysaccharides (LPSs) (40, 41). Therefore, B cells were stimulated via their BCR with anti-IgM (Fig. 10L), costimulated with anti-IgM + anti-CD40 (Fig. 10M) or stimulated with LPS alone (Fig. S8C), or stimulated with anti-CD40 alone as a control (Fig. S8D) and B cell proliferation was monitored using the CFSE dye (Fig. S8A). There were no differences in cell viability between different groups of cells and stimulatory conditions (Fig. S8B). Primary B cells from MCUfl/fl B1cre(-) mice (MCU-KD B cells) showed significantly enhanced proliferation in response to BCR stimulation with anti-IgM compared with control MCUfl/fl B1cre(-) B cells as documented by dilution of the CFSE dye with increased proportion of B cells that underwent a third cycle of division (Fig. 10L). As expected, this difference in B cell proliferation was not observed when cells were costimulated with anti-IgM + anti-CD40 (Fig. 10M) or when stimulated with LPS (Fig. S8C). Stimulation with anti-CD40 alone had marginal to no effect on B cell proliferation (Fig. S8D).
Mathematical modeling supports MCU-mediated regulation of cytosolic Ca\(^{2+}\)

First, we tested how mitochondrial Ca\(^{2+}\) transport alone (i.e., without assuming the existence of microdomains that affect CRAC channel activity through CDI) can change the properties of cytosolic Ca\(^{2+}\) rise activated by passive store depletion with thapsigargin as well as Ca\(^{2+}\) oscillations activated by agonist stimulation. We took an existing model of Ca\(^{2+}\) oscillations (25, 42, 43), slightly simplified the description of the Ca\(^{2+}\) influx pathways, and added to it six different models, of increasing complexity, of mitochondrial Ca\(^{2+}\) transport.

- Model 1 assumes that mitochondria act like a simple Ca\(^{2+}\) buffer, with highly simplified models of Ca\(^{2+}\) uptake by the MCU and Ca\(^{2+}\) extrusion via NCLX.
- Model 2 assumes that the MCU and NCLX fluxes are modeled in a more complex manner, following (44) (itself based on the earlier work of (45, 46)) but omitting the mitochondrial membrane potential and metabolism.
- Model 3 is the same as model 2 but includes the mitochondrial membrane potential and metabolism.
- Model 4 is the model of (44) with no changes to the equations or parameters. It differs from model 3 principally in the choice of IP\(_3\)R model and includes models of the mitochondrial membrane potential and metabolism, although it does not include MAMs.
- Model 5 extends model 3 to include MAMs, following (47).
- Model 6 is the model of (47) with no changes to parameters or equations. It differs from model 5 principally in the choice of IP\(_3\)R model.

Our six models thus combine in various ways different models of the IP\(_3\)R, the mitochondrial potential, mitochondrial metabolism, and MAMs. Model 1 is considerably simpler than the others, whereas models 5 and 6 are the most complex.

When we simulate the effects of MCU-KO, NCLX-KO, and MCU-KO + NCLX-KO on thapsigargin-activated cytosolic Ca\(^{2+}\) rise, we found that MCU-KO and MCU-KO + NCLX-KO cause similar increases in cytosolic [Ca\(^{2+}\)], whereas NCLX-KO leads to a slight decrease (Fig. 11, A–E), in agreement with our experimental data with MCU and previous work with NCLX (48). This enhancement of cytosolic [Ca\(^{2+}\)] upon MCU or NCLX knockout is due simply to the absence of the MCU pathway that removes Ca\(^{2+}\) from the cytosol. Ca\(^{2+}\) influx to the cytosol remains almost unchanged in the model, but Ca\(^{2+}\) removal is downgraded, so an enhancement of cytosolic [Ca\(^{2+}\)] necessarily occurs.

These simulations were performed for models 2, 3, and 5 with three variations on model 5 representing different percentages of total IP\(_3\)Rs located within MAMs (10%, 50%, or 70%; Fig. 11, C–E). These thapsigargin simulations could not be performed for models 1, 4, and 6 as discussed in more detail in Experimental procedures.

Figure 11. Mathematical modeling shows that MCU-KO increases cytosolic Ca\(^{2+}\) but has complex and nonlinear effects on Ca\(^{2+}\) oscillations. A–E, various models of the cytosolic Ca\(^{2+}\) signal in response to maximal store depletion with Tg in the absence then presence of 2 mM external Ca\(^{2+}\). In model 5, which considers MAM regions of close contact between the endoplasmic reticulum and mitochondria, computations are shown when 10%, 50%, or 70% of total cellular inositol-1,4,5-trisphosphate (IP\(_3\)) receptors are located within these MAMs. F–L, period of Ca\(^{2+}\) oscillations (1/Frequency) as a function of relative concentrations of IP\(_3\) ([IP\(_3\)]\(_0\) is the lowest value of [IP\(_3\)] for which oscillations exist in the model with MCU) for the six different models described in the Experimental procedures. For model 5, computations are shown when either 10% or 70% of total cellular inositol-1,4,5-trisphosphate receptors are located within the MAMs. MAM, mitochondria-associated membrane; MCU, mitochondrial Ca\(^{2+}\) uniporter.
When considering agonist-evoked \( \text{Ca}^{2+} \) oscillations, the qualitative results from each model differ significantly. For each model, the oscillation period is plotted against the relative \([\text{IP}_3]\), which is a model proxy for agonist stimulation (Fig. 11, F–L). In each case, increasing agonist concentration increases the oscillation frequency (i.e., the period decreases). Knockout of the MCU results in oscillations that, in some models, are slightly faster but in others are slightly slower. In some cases (models 2 and 4), the effect on oscillations changes as agonist stimulation increases. Furthermore, in all cases, for a small range of high agonist concentrations, MCU-KO can turn a plateau response into an oscillatory response (this is the region where the red “MCU-KO” curves overlap the black wildtype “WT” curves). It follows that mitochondrial Ca\(^{2+}\) transport does not have a simple, monotonic effect on the properties of Ca\(^{2+}\) oscillations. Instead, the effects of mitochondrial Ca\(^{2+}\) transport are critically dependent on the exact details of each mitochondrial Ca\(^{2+}\) flux, as well as other cellular parameters, such as the choice of \(\text{IP}_3\)-R model, the relative mitochondrial volume, or the proportion of the ER membrane that is closely associated with the mitochondrial membrane. In particular, it is not correct to say that “buffering” by the mitochondria will necessarily increase oscillation period; there is no clear relationship between mitochondrial Ca\(^{2+}\) transport and oscillation frequency.

Next, we constructed a model that considers MCU activity within a functional ICRAC microdomain (IM model) whereby MCU activity prevents slow CDI of ICRAC. This model also considers that 10% of \(\text{IP}_3\)-Rs are within MAMs but does not include the equations for mitochondrial metabolism and membrane potential. The inclusion of the mitochondrial metabolism equations makes no qualitative difference to the results. Thus, the IM model is just model 2 with the inclusion of \(I_{\text{CRAC}}\) CDI and MAMs with all other parameters being unchanged from model 2. We modeled CDI of \(I_{\text{CRAC}}\) after (34), and initial tests of this IM model gave results that were qualitatively consistent with (34) (Fig. S9). We then simulated the effects of MCU-KO, NCLX-NO, and MCU-KO + NCLX-KO on thapsigargin-activated cytosolic Ca\(^{2+}\) rise and agonist-evoked Ca\(^{2+}\) oscillations with three variations of this model that consider 10%, 36%, or 70% of the incoming Ca\(^{2+}\) through \(I_{\text{CRAC}}\) enters mitochondria (Fig. 12). The model showed that NCLX-KO leads to a slight decrease in SOCE activated by thapsigargin, whereas MCU-KO and MCU-KO + NCLX-KO cause a similar increase in SOCE that recedes to a lower plateau equivalent to that of wildtype cells. Ca\(^{2+}\) oscillations in wildtype and MCU-KO cells in response to increasing concentrations of \(\text{IP}_3\) were not altered by 10%, 36% or 70% variations in the IM model (Fig. 12).

In summary, our mathematical modeling predicts that all the experimental results presented here are consistent with the hypothesis that the MCU affects SOCE activated by thapsigargin and Ca\(^{2+}\) oscillations evoked by \(\text{IP}_3\)-producing agonists only via its effect on mitochondrial Ca\(^{2+}\) transport, and that no microdomains connected to CRAC channels or \(\text{IP}_3\)-R (MAMs) are required to explain the experimental data.

![Figure 12](image-url)
Discussion

Through Ca\(^{2+}\) uptake and extrusion, mitochondria can shape receptor-activated intracellular Ca\(^{2+}\) signaling. Mitochondria rapidly take up Ca\(^{2+}\) during receptor-evoked Ca\(^{2+}\) signaling and then extrude it much more slowly (49). Mitochondrial Ca\(^{2+}\) regulates metabolic activity, through modulation of the activity of three dehydrogenases of the TCA cycle (50, 51). Mitochondria can take up cytosolic Ca\(^{2+}\) within the MAM regions of close apposition between IP3R in the ER and mitochondria (52). In addition, respiring mitochondria can buffer Ca\(^{2+}\) from a distant site functionally connected to CRAC channels, thus reducing the extent of slow CDI of these channels to sustain SOCE and cytosolic Ca\(^{2+}\) signaling (38, 53).

In this study, we performed genetic knockout of the MCU in several nonexcitable cell lines and in mice to determine the function of MCU in regulating receptor-evoked Ca\(^{2+}\) signaling. We reveal that MCU-KO enhances cytosolic Ca\(^{2+}\) signaling and downstream activation of NFAT transcription factors. Our data show that store depletion-mediated activation of SOCE and agonist-evoked cytosolic Ca\(^{2+}\) oscillations are enhanced when MCU-mediated mitochondrial Ca\(^{2+}\) uptake is abrogated. However, this enhancement of cytosolic Ca\(^{2+}\) signals is not due to increased activities of either IP3R or CRAC channels. We measured IP3R Ca\(^{2+}\) puffs in intact MCU-KO and wildtype control cells. The number of IP3R-mediated Ca\(^{2+}\) puffs and number of puff sites were similar between MCU-KO and wildtype cells. We recorded whole-cell CRAC currents from two cell lines under low physiological cytosolic Ca\(^{2+}\) buffering conditions with energized mitochondria and under strong cytosolic Ca\(^{2+}\) buffering and show that CRAC currents are of similar density in MCU-KO and wildtype parental controls. However, evaluation of CRAC channel slow CDI in the presence of thapsigargin showed that MCU activity prevents store-dependent slow CDI of IC\(_{\text{CRAC}}\) in agreement with previous studies (36, 37). Our data argue that MCU does not alter the activity of native IP3R. Although MCU function can affect CRAC channel activity by preventing its slow CDI and can inhibit cytosolic Ca\(^{2+}\) extrusion, the net result of MCU-KO is an enhancement of cytosolic Ca\(^{2+}\) after store depletion or agonist stimulation compared to control cells. Our findings have a simple and straightforward interpretation: abrogation of MCU-mediated Ca\(^{2+}\) uptake in response to receptor stimulation prevents the transfer of incoming Ca\(^{2+}\) through CRAC channels from the cytosol to mitochondria, causing cytosolic Ca\(^{2+}\) accumulation, increased ER Ca\(^{2+}\) refilling and enhanced NFAT nuclear translocation.

Using TIRF imaging, Korzeniowski et al. (54) showed that on store depletion, mitochondria were remotely distant, or ad minimum excluded, from the sites of SOCE, which were visualized by STIM1 puncta and that mitochondrial Ca\(^{2+}\) uptake was independent of the distance between STIM1 and mitochondria. Naghdi et al. (55) used as MAKAP-RFP-CAAX construct to immobilize mitochondria by linking them to the plasma membrane of endothelial cells and showed that this strategy has no effect on SOCE. Nevertheless, slow CDI of CRAC channels is mediated by a site that is hundreds of nm away, consistent with the reported localization of mitochondria within ~200nm from Quintana and Hoth (56). Previous studies predating the discovery of MCU showed that abrogation of mitochondrial Ca\(^{2+}\) buffering using drugs such as the potent uncoupler of oxidative phosphorylation, FCCP, or the inhibitor of Complex III of the electron transport chain, Antimycin A1, inhibited SOCE (37). This decrease in SOCE resulted, at least partially, from evoking CRAC channel slow CDI (36). Our own findings revealed that mitochondrial depolarization with FCCP indeed inhibits SOCE, in agreement with these studies. However, FCCP equally inhibited SOCE in MCU-KO and wildtype cells, suggesting that, although FCCP promotes IC\(_{\text{CRAC}}\) slow CDI, the drug likely inhibits CRAC channels through additional MCU-independent mechanisms. Because these drugs are known to generate hydrogen peroxide (57, 58), their inhibitory effect on SOCE might be mediated by oxidation of Orai1 (48, 59).

Extensive studies by Hoth and coworkers showed that mitochondria are critical for T cell activation. Upon stimulation, mitochondria move closer to, within 200 nm of, the immune synapse to sequester Ca\(^{2+}\). The presence of mitochondria near the immune synapse coincides with enhanced Ca\(^{2+}\) concentration near the PM and inhibited cytosolic Ca\(^{2+}\) export through the PM Ca\(^{2+}\) ATPase, thus enhancing the efficiency of NFAT activity and T cell activation (39, 56, 60, 61). Therefore, both MCU function and its deletion can enhance NFAT activity. The former through inhibition of cytosolic Ca\(^{2+}\) export and CRAC channel slows CDI at the PM, whereas the latter through enhanced global cytosolic Ca\(^{2+}\). Samanta et al. (38) showed blunted SOCE and cytosolic Ca\(^{2+}\) oscillations in RBL-1 cells treated with siRNA against MCU and suggested that cytosolic Ca\(^{2+}\) uptake by MCU prevents CDI of both IP3R and CRAC channels. This discrepancy cannot be explained by differences between the MCU knockdown performed by Samanta et al. and MCU knockout in our study. We obtained similar results in primary splenic CD4+ T cells and B cells isolated from mice subjected to LoxP/Cre-mediated knockdown, which confers 60% to 80% MCU knockdown as documented by Westerns on isolated CD4+ T and B cells. Samanta et al. (53) also reported that NCLX knockdown and Na+ depletion have no effect on CRAC channels, results that are at odds with previous findings (37, 48). Therefore, the reasons for the discrepancy between the studies by Samanta et al. and ours are not clear. However, these investigators reported results from a single cell line, used one single siRNA, did not document protein or mRNA knockdown of MCU or NCLX, and did not rule out off-target effects on STIM–Orai expression in their system (38, 53).

Although NFAT nuclear translocation is driven by Ca\(^{2+}\)/CaM-dependent activation of calcineurin at the vicinity of CRAC channels, NFAT activity also depends on other signaling regulators, including several kinases that differentially regulate the nuclear export of various NFAT isoforms (62). We cannot exclude the possibility that other mechanisms of NFAT regulation are altered in MCU-KO cells and further studies are required to further clarify this issue. We show that B cells isolated from B cell–specific MCU
knockdown mice have enhanced proliferation compared with B cells isolated from littermate controls. However, previous studies in HeLa cells and vascular smooth muscle cells showed that MCU knockdown or knockout halts cell cycle progression and reduces proliferation (63, 64). The discrepancy between these previous studies and ours can likely be explained by the differential requirement for MCU by cancer cells and proliferative smooth muscle cells versus B cells. The contribution of mitochondrial Ca$_{2+}$ to bioenergetics in cancer cells and proliferative smooth muscle cells is likely more prominent than in hematopoietic cells, which rely heavily on glycolysis. The effect of MCU-KO on enhanced NFAT activity in cancer cells and proliferative smooth muscle cells is likely largely offset by the disruption of mitochondrial metabolism as a result of MCU loss.

Our mathematical modeling support previous computational work showing that the effects of mitochondrial Ca$_{2+}$ transport on cytosolic Ca$_{2+}$ oscillations are complex and nonintuitive (65). Although mitochondria take up and release cytosolic Ca$_{2+}$ in a manner superficially similar to that of a traditional Ca$_{2+}$ buffer, the complexities and nonlinearities inherent to these transport processes mean that the downstream effects on oscillation frequency cannot be easily predicted. In some cases, oscillation frequency is increased by mitochondrial transport; in other cases, oscillation frequency is decreased. Thus, the common understanding that inhibition of mitochondrial Ca$_{2+}$ buffering decreases oscillation frequency is not supported by our modeling. Our models showed that MCU-KO enhances, whereas NCLX-KO decreases, cytosolic Ca$_{2+}$ rise in response to store depletion by thapsigargin. Our modeling predicts that MCU-KO mediates its effects only through changes in mitochondrial Ca$_{2+}$ transport and that no Ca$_{2+}$ microdomains that can alter CDI of Ca$_{2+}$ channels (CRAC or IP$_{3}$R) are required to explain our experimental data. Ishii et al. (66) showed that, during Ca$_{2+}$ oscillations in HeLa cells Ca$_{2+}$ shuttles between the ER and mitochondria and that Ca$_{2+}$ uptake by mitochondria occurs at the expense of refilling of the ER, in agreement with our observations. Our model of NCLX-KO is also consistent with previous experimental findings from our group and others. Hoth et al. (37) showed that SOCE was reduced when NCLX was inhibited in cells subjected to Na$^+$ depletion and Naghdi et al. (55) showed reduction in SOCE when cells were treated with CGP37157, a pharmacological inhibitor of the NCLX. We previously reported that molecular knockdown of NCLX or Na$^+$ depletion inhibits SOCE and CRAC channel activity in HEK293 cells and primary vascular smooth muscle cells (48).

In summary, in addition to preventing CRAC channel inactivation and inhibiting cytosolic Ca$_{2+}$ extrusion to enhance NFAT activity, mitochondrial Ca$_{2+}$ uptake by MCU and Ca$_{2+}$ storage by mitochondria is concomitantly critical for negative regulation of cytosolic Ca$_{2+}$ and NFAT induction. These two concomitant and reciprocal functions of MCU at the cytosol–mitochondria interface fine-tune interorganellar Ca$_{2+}$ transfer and NFAT activation during receptor-evoked signaling.

**Experimental procedures**

**Cell culture**

Parental HEK293, Jurkat E6-1, RBL-1, DLD1, HCT116, and A20 cell lines were purchased directly from ATCC (Catalog # CRL-1573, TIB-152, CRL-1378, CCL-221, CCL-247, and TIB-208). HEK293, RBL-1, and HeLa cells were cultured in high-glucose (4.5 g/l) Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and 1× Antibiotic–Antimycotic solution. Jurkat, A20, and DLD1 were maintained in RPMI 1640 with L-glutamine supplemented with 10% heat-inactivated fetal bovine serum and 1× Antibiotic–Antimycotic solution. HCT116 were cultured in McCoy’s 5A medium supplemented with 10% heat-inactivated fetal bovine serum and 1× Antibiotic–Antimycotic solution. All cell lines were stored in a heated (37 °C) humidified incubator under standard cell culture conditions (5% CO$_{2}$; 95% air). The absence of mycoplasma contamination was verified for all cell lines using a sensitive PCR-based detection kit (ABM: G238).

**Fluorescence imaging**

Adherent and semiadherent cell lines were seeded onto round 25-mm glass coverslips 24 h prior to imaging at a concentration of 1.5 × 10$^6$ and 2.5 × 10$^6$ cells, respectively. Alternatively, A20, Jurkat E6-1, and primary CD4$^+$ T cells were seeded onto round Poly-L-lysine (0.01%; mol wt 150,000–300,000)-treated 25-mm glass coverslips 30 min prior to dye loading. Once attached, HEK293, HeLa, HCT116, and DLD1 cells were incubated in Dulbecco’s modified Eagle’s medium containing the ratiometric Ca$_{2+}$ indicator Fura-2 AM (2 μM) for 30 min. RBL-1 cells were loaded with 4 μM Fura-2AM following the same procedure. Jurkat and A20 cells were incubated with 2 μM Fura-2 AM in Hepes-buffered salt solution (HBSS; 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl$_2$, 20 mM Hepes, 10 mM glucose adjusted to pH 7.4 with NaOH) supplemented with 2 μM Ca$_{2+}$ for 30 min at room temperature. Before imaging, all coverslips were transferred from a standard six-well plate into an Attofluor cell chamber and washed 3× with Ca$_{2+}$-containing HBSS to remove excess Fura-2 AM. Using a fast shutter wheel (Sutter Instruments), cytosolic Ca$_{2+}$ was measured by alternatively exciting Fura-2 with 340- and 380-nm wavelengths and recording the resulting 510-nm dye emission on a pixel-by-pixel basis through a 20× fluorescence objective paired with a Hamamatsu Flash 4 camera and processed using Leica Application Suite X software.

Measurement of NFAT4 nuclear translocation in HEK293 cells was achieved by transient overexpression of NFAT4-GFP (Addgene #21664) as previously described (32, 42, 43). Briefly, 1 × 10$^6$ cells were incubated with a solution containing 1 μg of NFAT4-GFP and 3 μl Lipofectamine 2000 for 3 h. One day later, coverslips containing transfected cells were transferred to imaging chambers and washed with Ca$_{2+}$-containing HBSS as described above. Using a Leica imaging system and a 40× oil immersion objective, NFAT4-GFP was excited at 488 nm, and emission spectra were selectively captured through a GFP filter.
cube. Real-time analysis of NFAT4-GFP nuclear translocation in response to 10 μM Cch stimulation was calculated using the equation:

\[
\text{NFAT4 Translocation} = \left( \frac{\text{Nuclear}_{F10}}{\text{Whole Cell}_{F10}} \right)
\]

Quantification of ER Ca\textsuperscript{2+} store depletion and refilling was done by transfecting parental and MCU-KO HEK293 cells with red R-CEPI1er (Addgene: #58216) using Lipofectamine done by transfecting parental and MCU-KO HEK293 cells formally illuminate the

from a 405-nm laser at 50% power was introduced to uniformly illuminate the field of view. Both the intensity of the UV flash (~2.4 mW) and the duration (1000 ms) for uncaging IP\textsubscript{3} were optimized to prevent spontaneous puff activity in the absence of loaded ci-IP\textsubscript{3}. TIRF images were captured using 4 × 4 pixel binning (433.333 nm/pixel) from equal field of views for WT-HEK293 and MCU-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-IP\textsubscript{3}, were captured.

The vsi files were converted to tif files using Fiji and further processed using FLIKA, a Python programming-based tool for image processing (68). From each recording, 500 frames (~3 s) before photolysis of ci-IP\textsubscript{3} were averaged to obtain a ratio image stack (F/Fo) and standard deviation for each pixel for recording up to 20 s following photolysis. The image stack was Gaussian filtered, and pixel that exceeded a critical value (1.0 for our analysis) was 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 Ca\textsuperscript{2+} signals from individual cells. All the puffs identified automatically by the algorithm were manually confirmed before analysis (69, 70). The results from FLIKA were saved as excel, and graphs were plotted using GraphPad Prism8.

**Patch clamp electrophysiology**

RBL-1 cells were seeded onto 30-mm round glass coverslips 12 h before recording; Jurkat cells were seeded onto Poly-L-lysine-coated coverslips 1 h before recording. Native I\textsubscript{CRAC} recordings were carried out using an Axopatch 200B and Digidata 1440A (71–73). Pipettes were pulled from borosilicate glass capillaries (World Precision Instruments) with a 100 MΩ tip, and were filled with the following solutions: Pipette solution 1 (high buffer): 135 mM Na-methanesulfonate, 10 mM CsCl, 1.2 mM MgSO\textsubscript{4}, 10 mM Hepes, 20 mM CaCl\textsubscript{2}, and 10 mM glucose (pH 7.4, adjusted with NaOH).

Divalent-free bath solution:

155 mM Na-methanesulfonate, 10 mM EDTA, 1 mM HEDTA, and 10 mM Hepes (pH 7.4, adjusted with NaOH).

Pipette solution 1 (low buffer):

135 mM Cs-methanesulfonate, 1 mM EGTA, 8 mM MgCl\textsubscript{2}, and 10 mM Hepes, 0.03 mM IP\textsubscript{3}, 2 mM pyruvic acid, 2 mM malic acid, 1 mM NaH\textsubscript{2}PO\textsubscript{4}, 2 mM Mg-ATP (pH 7.2 adjusted with CsOH).
Pipette solution 2 (high buffer):
85 mM Cs-methanesulfonate, 50 mM Cs-1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (Cs-BAPTA), 8 mM MgCl₂, and 10 mM Hepes (pH adjusted to 7.2 with CsOH).

Recordings of CRAC channel store-independent slow CDI were performed according to (36). Cells were incubated in the bath solution without Ca²⁺ and in the presence of 2 μM thapsigargin for 3 to 5 min before beginning of recordings. The bath and pipette solutions were as follows:

Bath solutions:
- 0 mM Ca²⁺ bath solution (in mM): 155 NaCl, 4.5 KCl, 3 MgCl₂, 10 D-glucose, and 5 Hepes (pH = 7.4 with NaOH).
- 20 mM Ca²⁺ bath solution (in mM): 125 NaCl, 4.5 KCl, 20 CaCl₂, 3 MgCl₂, 10 D-glucose, and 5 Hepes (pH = 7.4 with NaOH).

Pipette solutions:
- Low buffer pipette solution (in mM): 140 Cs-methanesulfonate, 2 MgCl₂, 0.66 CaCl₂, 1.2 EGTA, 10 Hepes (pH = 7.2 with CsOH).
- Low buffer + mitochondrial cocktail pipette solution (in mM): 140 Cs-methanesulfonate, 2 MgCl₂, 0.66 CaCl₂, 1.2 EGTA, 10 Hepes, 2.5 mM malic acid, 2.5 mM pyruvate acid, 1 mM NaH₂PO₄, 5 mM Mg-ATP, 0.5 mM Na-GTP (pH = 7.2 with CsOH).

Cytosolic and mitochondrial volume determination
To determine the cytosolic volume of all cell lines pEGFP-N1 (Addgene# 6085-1) was transiently overexpressed. One day before cytosolic volume measurement all cell lines were electroporated using the Amaxa Nucleofector II. Cells were first resuspended in the transfection reagent supplemented with pEGFP-N1 (1 μg). Cell line–specific protocols Q-001, X-001, T-030, and L-013 were used to electroporate HEK293, Jurkat, RBL-1, and A2O cells, respectively. One day later, live cells were stained with MitoTracker Deep Red (200 nM) for 15 min at room temperature in the dark before imaging on a Leica DMI8 confocal microscope. Stained and washed cells were observed through a 40x oil immersion objective. Both GFP and MitoTracker signals were then collected from randomly selected fields of view by compiling Z-Stacks of the lower and upper bounds of all cells. MitoTracker and GFP channels were then used to reconstruct 3D surfaces using IMARIS Cell Biology software (Oxford Instruments). Once both cytosolic (GFP) and mitochondrial (MitoTracker) surfaces were reconstructed, volume measurements were recorded individually.

ImageStream native NFAT1 nuclear translocation assay
Jurkat cells were first counted and transferred to 1.5-ml Eppendorf tubes at a concentration of 1.2 × 10⁶ cells per replicate. Cells were then centrifuged and media replaced with HBSS supplemented with 2 mM Ca²⁺ to eliminate the possibility of serum-mediated activation. Experimental cells were then stimulated with α-CD3 (0.125 μg/ml) or treated with Tg (2 μM) to induce cytosolic Ca²⁺ oscillations or evoke SOCE. Following stimulation, cells were immediately fixed and permeabilized using a transcription factor staining kit according to the manufacturer protocol (Tonbo Biosciences Cat: TNB-0607-KIT). After fixation and permeabilization, cells were spun down, the supernatant was removed, and stained with a primary conjugated NFAT1–FITC antibody (Cell Signaling #14324S at 1:100) for 1 h at room temperature protected from light. Unbound NFAT1 antibody was then washed away and cells were resuspended in 40 μL of PBS. Nuclear staining was performed immediately before image acquisition by adding 10 μL of the nuclear stain Dapi (5×) to give a final concentration of (0.25 μg/ml).

Images were acquired using a 10-color Amnis ImageStream X Mk II imaging cytometer. Channels 1 and 9 (430–480 nm) were used to obtain bright-field images, channel 2 was used for FITC (Ex:488 Em:480–560 nm), and Dapi through channel 7 (Ex:405 Em:430–505 nm). Prior to final image acquisition cells were gated to exclude debris and doublets and Ex laser power was adjusted to prevent pixel saturation. From each stained and unstained sample 3000 to 5000 in-focus single-cell events were collected. After running all samples single color compensation samples were run for FITC then Dapi. All data were analyzed using IDEAS software version 6.2 (Amnis Corp). During the analysis in-focus cells were first identified, then doublets were excluded, and finally cells positive for both DAPI and NFAT1 were selected for further analysis. The extent of nuclear NFAT1 was then determined using the nuclear translocation module. Raw nuclear similarity scores were then exported to Prism GraphPad resulting in the final figures.

Generation of transgenic MCUfl/fl CD4Cre(ERT2) and MCUfl/fl MB1Cre mice
We generated inducible MCU-KO CD4⁺ T cells by breeding MCUfl/fl mice (generous gift from Dr John W. Elrod, Temple University) (74) with CD4CreER12 (B6(129X1)-Tg(Cd4-cre/ERT2)11Gnri/J) purchased from Jackson laboratory (75). The resulting CD4CreER12-positive MCUfl/fl mice from this cross were then bred with MCUfl/fl mice to generate all control and experimental cohorts. B cell–specific knockout of MCU was achieved by breeding MB1-Cre (B6.C(Cg)-Cd79atm1(Cre)Rth/J) mice from Jackson laboratory with MCUfl/fl mice. From that cross MCUfl/fl/MB1Cre/fl mice were then bred with MCUfl/fl mice to generate the experimental and control groups (76). PCR-based genotyping was performed on all mice to identify experimental and control mice. Western blots were used to validate MCU-KO at the protein level. Both male and female mice were utilized in equal numbers to reduce the possibility of sex-dependent variation biasing the results. All mice were maintained in the specific pathogen-free barrier animal facility at the Pennsylvania State University College of Medicine animal facility. Prior to any breeding or experimentation all protocols used were approved by the Pennsylvania State Institutional Animal Care and Research Advisory Committee (IACUC) to ensure all animals were handled and cared for according to ethical guidelines.
Primary T and B cell isolation and culture

About 6 to 8-week-old MCU^{fl/fl} CD4^{Cre+} or MCU^{fl/fl} MB1^{Cre+} mice and control counterparts were humanely sacrificed immediately prior to immune cell isolation. Superficial cervical and inguinal lymph nodes were first collected followed by the spleen. All lymphoid tissues were then disrupted by passing the tissue through a 70-μm mesh screen. The resulting single-cell suspension was then washed 10 ml of cold RPMI and resuspended in 1 ml of Robosep buffer (STEMCELL technologies) prior to the negative selection of B or T cells. Using the corresponding negative selection kit primary mouse CD4^{+} T cells and B cells were isolated using the magnetic beads and antibody cocktail as recommended by the manufacturer.

Isolated CD4^{+} T cells were then cultured ex vivo to increase the efficiency of CreERT2 activity. Naïve primary CD4^{+} T cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1× Antibiotic-Antimycotic, 1× Glutamax, mIL2 (30 U/ml), and Mouse T-Activator anti-CID3/CD28 Dynabeads (Thermo Fisher) at a concentration of one bead per cell. Experimental mice (MCU^{fl/fl} CD4^{Cre+}) and control (MCU^{fl/fl} CD4^{Cre-}) mice were both treated with 2 μM 4-OHT for 4 days. As a control, the second group of (MCU^{fl/fl} CD4^{Cre-}) mice was only administered vehicle (dimethyl sulfoxide). Cells were subsequently washed and beads removed.

Genetic knockout of MCU in multiple cell lines using CRISPR/Cas9

To generate MCU-KO HEK293 and HCT116 cells, human MCU gRNA3 (hMCU gRNA1) was subcloned into the lentivector v2 backbone (Addgene, Plasmid #52961) and transfected into wildtype HEK293 cells via nucleofection as described. Forty-eight hours after transfection, CRISPR/Cas9-positive cells were selected by treating the culture medium with puromycin (2 μg/ml) (Gemini Bio Products). After 6 days of selection, cells were trypsinized and seeded at a concentration of 0.5 cells per well into a 96-well plate. Resulting colonies were then screened using the Guide-it Mutation Detection Kit (Clontech Laboratories, 631443). Knockout was further confirmed via Western blot, functional analysis, and genomic sequencing.

An alternative method was used for all other cell lines to avoid any potential effects of constitutive Cas9 expression. The same two human gRNAs (hMCU gRNA1and 2) were used to generate all Jurkat and DLD1 MCU-KO clones. Two independent mouse-specific gRNAs (mMCU gRNA 1 and 2) were used to generate MCU-KO A20 cells, whereas a single gRNA (rMCU gRNA1) was used to generate MCU-KO RBL-1 cells. All gRNA sequences can be found in Table S1.

gRNAs for all cell lines were cloned into one of two fluorescent vectors to allow for the identification of transiently transfected cells using fluorescence-activated cell sorting (pSpCas9_BB-2A-GFP and pU6-(BbsI)_CBh-Cas9_T2A-mCherry: Addgene). One day after transfection with the corresponding gRNA single cells with high gRNA expression were sorted into the wells of a 96-well plate using a fluorescence-activated cell sorting Aria SORP high-performance cell sorter. After sorting, cells were maintained in complete medium until colonies began to form ~2 to 3 weeks depending on the cell line. Visible colonies were collected and analyzed by Western blot to identify knockout clones. Positive clones were functionally analyzed, and those clones that showed no mitochondrial Ca^{2+} uptake were used for all experiments.

Furthermore, all clones were sequenced to validate gRNA efficiency. In HEK293 MCU-KO cells we observed two different genomic deletions (11 and 1 nt; exon 2) that resulted in a genomic frameshift and the formation of early stop codons. In Jurkat MCU-KO cells we also observed two different mutations. The first was a 7-nt insertion into exon 3, and the second caused multiple indels that resulted in a genetic frameshift and formation of an early stop codon in exon 3. In RBL-1 we observed two different deletions from exon 3 (16 and 400 nt) that both resulted in genomic frameshifts. Two different deletion events were also observed in A20 cells; the first was a single-nucleotide deletion and the second a 7-nt deletion from exon 1. We also observed two different deletions in HCT116, the first being a 16-nt deletion and the second a 19-nt deletion; both occurred in exon2 and resulted in frameshifts. In DLD1 cells, two unique 2-nt deletions were observed within exon 4 that both resulted in genomic frameshifts and the introduction of an early stop codon.

Western blot analysis

Cells were collected from culture flasks or isolated from mice, washed with ice-cold phosphate-buffered saline, and pelleted via centrifugation prior to lysis in ice-cold RIPA buffer (150 mM NaCl, 1.0% IGEPEAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0; Sigma) supplemented with protease and phosphatase inhibitor (Halt; Thermo Scientific) for 15 min on ice. Crude protein lysates were clarified by centrifugation (15,000g/12 min at 4 °C) and the supernatants collected. Total protein concentration was determined using the Pierce BCA assay as described prior to loading a NuPAGE precast 4% to 12% Bis-Tris gel. After subjecting the samples to 120 V for 1.5 h proteins were transferred to a polyvinylidene difluoride membrane using a Bio-Rad Criterion blotter and 1× NuPAGE Transfer Buffer (Invitrogen). After 1 h of transferring at 95 V the membrane was removed and blocked with LI-COR Tris-buffered saline (TBS) buffer for 1 h at room temperature. Primary antibodies were added to blocked membranes overnight at 4 °C in a shaker. On the next day, PVDF membranes were then washed 3× with TBST and probed with the corresponding species-specific LI-COR secondary antibody for 1 h at room temperature. Then the membrane was washed 3× with TBST and immediately imaged using a LI-COR Odyssey imaging system and fluorescence quantified using Image Studio lite software (LI-COR).

Measurement of mtΨ and mtCa^{2+} uptake in permeabilized cells

HEK293 (6 × 10^6), Jurkat (10 × 10^6), A20 (10 × 10^6), and RBL-1 (7 × 10^6) cells were washed with PBS immediately
before resuspension in an intracellular medium consisting of 10 mM NaCl, 120 mM KCl, 1 mM KH2PO4, 20 mM Hepes-Tris, pH 7.2, and 2 μM of the SERCA pump inhibitor thapsigargin. Just before beginning the measurement 2 mM sucrose was added to the intracellular medium. Cells were permeabilized with 40 μg/ml digitonin, and simultaneous measurements of mitochondrial membrane potential (mTP) and extramitochondrial Ca2+ were achieved by loading permeabilized cells with the ratiometric mTP dye JC-1 (800 nM) and the fluorescent Ca2+ indicator Fura2-FF (0.5 μM). Both dyes were excited, and emissions were recorded using a dual-wavelength excitation and emission fluorimeter (DeltaRam, PTI).

qRT-PCR analysis

Total mRNA was isolated from HEK293 (2 × 10⁶) and Jurkat cells (4 × 10⁶) parental and MCU-KO cell lines using the RNeasy Mini Kit (Qiagen). Isolated RNA was then analyzed using a NanoDrop spectrophotometer (Thermo Scientific), and 1 μg of DNase I-treated (Thermo Fisher) RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Total cDNA (1 μl) was diluted 1:5 and then combined with target-specific primers, SYBR Green qPCR Master Mix (Applied Biosystems), and mQH2O resulting in a 10-μl total reaction. All targets were subjected to the same PCR protocol that began with an initial activation step for 2 min at 95 °C followed by a 95 °C for 2 min melt step. The initial melt steps were then followed by 40 cycles of 95 °C for 15 s, a 15-s annealing step at 54.3 °C, and target amplification at 72 °C for 30 s. Once these steps were complete, a standard melt curve was generated to ensure primer specificity. Analysis of target and control samples was carried out using the comparative Ct method. All samples were normalized to the average of the two reference genes, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and NONO (Non-POU Domain Containing Octamer Binding). All samples were run in triplicate to ensure reproducibility.

Mathematical modeling

Models of the IP3R, SERCA, and the PM ATPase were taken directly from (42, 43), with unchanged parameters. The model of Ca2+ influx, however, was simplified from that of (42, 43) by omitting the majority of the complexity generated by two different forms of STIM and three different forms of ORAI. Instead, we modelled Ca2+ influx by

\[ J_{in} = A \left( \frac{1 - (1 - 1/v_1)c_e}{K_{e1} + c_e} \right) \left( 1 - \frac{c_e^2}{K_{e2} + c_e} \right) + a_0, \]

where A = 0.2 μM/s, v1 = 3, K_{e1} = 50 μM, K_{e2} = 200 μM, and a_0 = 0.005 μM/s. The variable c_e denotes ER [Ca2+].

To incorporate mitochondrial transport, the base model was supplemented by the addition of three terms each representing a mitochondrial Ca2+ flux: a flux through the MCU (J_{MCU}), a flux through the NCLX (J_{NCLX}), and (following (44)) a background leak term (J_x). The four different models of mitochondrial Ca2+ transport are as follows.

Model 1. The mitochondria are modeled as simple buffers. Thus

\[ J_{MCU} = k_{on}(c_m - c), \]

\[ J_{NCLX} = k_{off}c_m, \]

\[ J_x = 0, \]

where k_{on} = 3 μM⁻¹s⁻¹, k_{off} = 5/s, b = 5 μM were chosen so as to give a physiological resting value of c_m and to ensure that the buffering was close to linear. The variable c_m denotes the mitochondrial [Ca2+].

Model 2. The mitochondrial fluxes are modeled following (44) but with the omission of the mitochondrial membrane potential and the mitochondrial metabolism. Thus

\[ J_{MCU} = V_{MCU} \phi_1, \]

\[ \phi_1 = \left( \frac{c}{K_1} \right) \left( 1 + \left( \frac{c}{K_1} \right)^3 \right), \]

\[ \phi_2 = \left( 1 + \left( \frac{c}{K_1} \right)^4 \right) + \frac{L}{1 + (c/K_2)^2}; \]

\[ J_{NCLX} = V_{NCLX}(c_m / c), \]

\[ J_x = k_x(c_m - c), \]

where V_{MCU} = 15 μM/s, K_1 = 6 μM, K_2 = 0.38 μM, V_{NCLX} = 0.005 μM/s, L = 50, and k_x = 0.01/s. The variable c denotes the cytosolic [Ca2+].

Model 3. The mitochondrial fluxes are modeled following (44) (and use the same parameter values), including the mitochondrial membrane potential and the equations describing mitochondrial metabolism. As with the previous models, the model of the IP3R is taken from (25, 42, 43), and thus the results from model 3 are not identical to those of (44).

Model 4 is identical to the model of (44).

Model 5. The mitochondrial fluxes are identical to those of model 3, but now MAMs are included, in the manner of (47) (i.e., using a compartmental model approach and not including any spatially distributed compartments). However, the IP3R model is the same as in model 3, and thus models 5 and 6 give
significantly different outcomes. To maintain a physiological resting $c_{\text{m}}$, $V_{	ext{MUC}}$ was increased to 150 μM/s.

Model 6 is identical to the model of (47).

Model for calcium-dependent inactivation of $I_{\text{CRAC}}$

A specified fraction (10%, 36%, or 90%) of $I_{\text{in}}$ was assumed to enter the cell directly into an IM formed between the PM and the mitochondrial membrane, containing only $I_{\text{CRAC}}$ and MCU. Inclusion of NCLX and/or PM Ca$^{2+}$ pumps into the IM makes no qualitative difference to the results. To describe calcium-dependent and time-dependent inactivation of $I_{\text{CRAC}}$ we used one of the simplest possible models. A new time-dependent variable, $h_{\text{icrac}}$, was introduced, obeying the differential equation

$$\tau_{\text{icrac}} \frac{dh_{\text{icrac}}}{dt} = h_{\text{icrac,0}} - h_{\text{icrac}},$$

where

$$h_{\text{icrac,0}} = \frac{K_{\text{icrac}}^2}{K_{\text{icrac}} + x^2}.$$

Here, $x$ is either the cytosolic or IM [Ca$^{2+}$] depending on whether we are modeling the influx into the IM or into the cytosol. Finally, $I_{\text{in}}$ was replaced by $h_{\text{icrac,0}}$, so that an increase in [Ca$^{2+}$] at the IM causes inhibition of the CRAC channel, with time constant $\tau_{\text{icrac}}$. Calcium moves from the IM to the cytosol at a rate proportional to the concentration difference of the two compartments, with rate constant 0.001/min. Other parameter values ($K_{\text{icrac}} = 0.05$ mM, $\tau_{\text{icrac}} = 5$ min) were chosen to give qualitative agreement with Figure 2A of (34) (as shown in Fig. S9).

Knockout simulations

The KO simulations of Figure 11 could not be performed on models 1, 3, and 6 for technical reasons.

Model 1 models mitochondria as a simple Ca$^{2+}$ buffer with the usual buffering kinetics and with a fixed total amount of buffer (i.e., with no proper MCU or NCLX modeling). Since model 1 contains no specific models of either the MCU or the NCLX, simulations of MCU or NCLX KO would be arbitrary and unreliable at best. Model 3 is a closed-cell model (i.e., with no Ca$^{2+}$ transport across the PM), which again is incompatible with KO experiments that change the total Ca$^{2+}$ in the cell, making KO simulations impossible. Finally, model 6 is unable to simulate MCU/NCLX KO owing to resultant instabilities in the model. We do not yet understand the source of these instabilities, which remain a topic of current investigation.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 9. Statistically significant differences between groups were identified within the figures where *, **, *** and **** indicate $p$-values of <0.05, <0.01, <0.001, and <0.0001, respectively. Statistical significance or lack thereof are reported within the figure legends, and the type of statistical tests performed and resulting $p$-values from that test are recorded in supporting information.

Data availability

Statistical analyses and raw data of all experiments herein, including images of unprocessed Westerns, are included in a separate file. All original cell lines reported herein are available from the lead investigator upon request and completion of an MTA.

Supporting information—This article contains supporting information.

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Abbreviations—The abbreviations used are: 4-OHT, 4-hydroxytamoxifen; BCR, B cell receptor; CDI, Ca$^{2+}$-dependent inactivation; CRAC, Ca$^{2+}$ release–activated Ca$^{2+}$; CCh, Carbachol; ER, endoplasmic reticulum; FCCP, trifluoromethoxy carbonylcyanide phenylhydrazone; IM, $I_{\text{CRAC}}$ microdomain; IP$_{3}$, inositol-1,4,5-trisphosphate; IP$_{3}$R, IP$_{3}$ receptor; LPS, lipopolysaccharide; MAM, mitochondria-associated membrane; MCU, mitochondrial Ca$^{2+}$ uniporter; NCLX, mitochondrial Na$^{+}$/Ca$^{2+}$ exchanger; NFAT, nuclear factor for activated T cells; PM, plasma membrane; RBL, rat basophilic leukemia; SERCA, sarcoplasmic/ER Ca$^{2+}$ ATPase; SOCE,
store-operated Ca²⁺ entry; STIM, stromal interaction molecule; Tg, thapsigargin; TIRF, total internal reflection fluorescence.

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