Remodeling of ER–plasma membrane contact sites but not STIM1 phosphorylation inhibits Ca\(^{2+}\) influx in mitosis

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Edited by Pietro De Camilli, Howard Hughes Medical Institute and Yale University, New Haven, CT, and approved April 10, 2019 (received for review December 17, 2018)

Store-operated Ca\(^{2+}\) entry (SOCE), mediated by the endoplasmic reticulum (ER) Ca\(^{2+}\) sensor stromal interaction molecule 1 (STIM1) and the plasma membrane (PM) channel Orai1, is inhibited during mitosis. STIM1 phosphorylation has been suggested to mediate this inhibition, but it is unclear whether additional pathways are involved. Here, we demonstrate using various approaches, including a nonphosphorylatable STIM1 knock-in mouse, that STIM1 phosphorylation is not required for SOCE inhibition in mitosis. Rather, multiple pathways converge to inhibit Ca\(^{2+}\) influx in mitosis. STIM1 interacts with the cochaperone BAG3 and localizes to autophagosomes in mitosis, and STIM1 protein levels are reduced. The density of ER–PM contact sites (CSs) is also dramatically reduced in mitosis, thus physically preventing STIM1 and Orai1 from interacting to activate SOCE. Our findings provide insights into ER–PM CS remodeling during mitosis and a mechanistic explanation of the inhibition of Ca\(^{2+}\) influx that is required for cell cycle progression.

Significance

The mechanisms blocking Ca\(^{2+}\) influx in mitosis are complex and involve a decrease in stable endoplasmic reticulum (ER)–plasma membrane (PM) contact sites and degradation of the ER Ca\(^{2+}\) sensor stromal interaction molecule 1 (STIM1) but not its phosphorylation. This challenges the current view that STIM1 phosphorylation is essential for mitotic store-operated Ca\(^{2+}\) entry inhibition and sheds light on the dynamics of ER–PM contact sites and of Ca\(^{2+}\) influx in mitosis.

Author contributions: F.Y., J.G., C.W.T., and K.M. designed research; F.Y., S.Z.H., L.S., E.A.-A., R.K., and A.M.B. performed research; S.C. and C.W.T. contributed new reagents/analytic tools; F.Y., S.Z.H., L.S., E.A.-A., R.K., A.M.B., and K.M. analyzed data; and F.Y. and K.M. wrote the paper.

The authors declare no conflict of interest.

This article is PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1821399116/-/DCSupplemental.

Published online May 7, 2019.
The C-terminal end of STIM1 contains an Ser/Thr-rich region with 10 residues that match the minimal consensus motif for the primary mitotic kinase cyclin-dependent kinase 1, Cdk1 (S/T-P). STIM1 phosphorylation has been argued to underlie SOCE inhibition in mitosis, since mutating the Ser/Thr residues to Ala (STIM1-10A) was reported to prevent the inhibition of SOCE (31). In contrast, STIM1 phosphorylation does not play a role in SOCE inhibition in oocyte meiosis based on both Ala and phosphomimetic substitutions (29). These findings suggest that STIM1 may be regulated differentially in mitosis vs. meiosis, which is surprising given the conserved kinase cascades driving M phase.

Herein, we demonstrate that phosphorylation of STIM1 is not required for SOCE suppression in mitosis. Rather, multiple processes converge to prevent SOCE during mitosis, including STIM1 degradation through the autophagy pathway and its inability to cluster in response to store depletion. Another contributing factor to inhibition of Ca\(^{2+}\) influx is a dramatic reduction in ER–PM CSs in mitosis, thus physically preventing direct interaction between STIM1 and Orai1. These findings underlie a previously unrecognized complexity and highlight the evolutionary necessity to enlist redundant mechanisms to ensure complete inhibition of Ca\(^{2+}\) influx in mitosis.

**Results**

**SOCE Is Inhibited in Mitosis in Primary and Cultured Cells.** SOCE has been shown to be inhibited in various cultured cell lines in mitosis (20, 24, 25). We confirm SOCE inhibition in mitosis in both HEK293 and Jurkat cells using the standard protocol of depleting Ca\(^{2+}\) stores with the sarcoplasmic/ER Ca\(^{2+}\)-ATPase (SERCA) inhibitor thapsigargin (Fig. 1A and B). To test whether SOCE inhibition in mitosis extends to primary cells, we isolated CD4\(^{+}\) T cells from mouse spleen and lymph nodes and expanded them under neutralizing condition (Th\(_{nec}\)), leading to their reentry into the cell cycle (SI Appendix, Fig. S1A). Mitotic CD4\(^{+}\) T cells were enriched by treating with nocodazole and sorting for the G2/M-phase population (SI Appendix, Fig. S1B). SOCE was inhibited in mitotic CD4\(^{+}\) T cells to a similar extent to what is observed in the HEK293 and Jurkat cell lines (Fig. 1B; SI Appendix, Fig. SIC shows enrichment of Jurkats in mitosis). To rule out potential alterations in SERCA expression or function in mitosis, we depleted stores with ionomycin and observe a similar inhibition of SOCE (SI Appendix, Fig. S1 D and E).

**Constitutive Activation of SOCE in Mitosis Results in Mitotic Catastrophe.** SOCE is universally inhibited in M phase, and reversing this inhibition in oocyte meiosis disrupts cell cycle progression and the egg-to-embryo transition (26). However, the effects of an active SOCE in mitosis are not known. We constitutively activated SOCE at low levels by expressing mCherry-Orai1 with the activating region from STIM1 (Ca\(^{2+}\) release activated channel activation domain) (YFP-CAD) (32, 33) or YFP as a control and assessed cell viability using a membrane-impermeant dye that penetrates dead cells with damaged PM but not live cells with intact PM (Materials and Methods). YFP expression alone did not affect cell viability in either interphase or mitosis (Fig. 1C). In contrast, although SOCE activation was tolerated in interphase cells, with over 65% of the cell population surviving, it caused significant cell death of over 80% of...
the population in mitosis (Fig. 1C). This shows that a constitutively active SOCE in mitosis results in mitotic catastrophe (Fig. 1C), and argues that suppression of SOCE is an adaptive response that insulates mitotic cells from Ca\(^{2+}\) influx to allow progression through mitosis.

**Orai1 Plasma Membrane Residence Is Not Altered in Mitosis.** Orai1 is internalized during oocyte meiosis, thus contributing to SOCE inhibition (28, 29). To assess whether Orai1 is trafficked in a similar fashion during mitosis, we measured the steady-state levels of Orai1 at the PM in mitosis compared with interphase in a cell line stably expressing Orai1 with an HA tag in the second extracellular loop and a YFP tag on the cytosolic N terminus (Fig. 1D, cartoon) (34). To detect PM Orai1, we stained nonpermeabilized cells with anti-HA antibodies and used DAPI to identify cells in mitosis (Fig. 1D). Confocal sections show Orai1 localizing to the PM in both interphase and mitosis as reported by anti-HA (Fig. 1D). To obtain a more quantitative measure of Orai1 levels at the PM, we used flow cytometry on nonpermeabilized cells stained with anti-HA antibodies to label PM Orai1, while total Orai1 was reported by YFP. In addition, to isolate cells in mitosis, we stained with an antiphosphorylated histone H3 at Ser28 (pH3Ser28) antibody. Histone H3 is phosphorylated in a mitosis-specific fashion and as such, distinguishes mitotic cells within the G2/M population (35). The steady-state levels of PM Orai1 estimated as the ratio of HA to YFP were indistinguishable in mitotic (YFP\(^+\)pH3Ser28\(^+\)) and interphase (YFP\(^+\)pH3Ser28\(^-\)) cells (Fig. 1E). Quantitative Western blots confirm that there was no change in Orai1 expression in mitosis (Fig. 1F). These results indicate that SOCE inhibition in mitosis does not involve modulation of Orai1 expression or PM residence.

**Nonphosphorylatable STIM1 Does Not Prevent Mitotic Suppression of SOCE.** We next examined the role of STIM1 phosphorylation in SOCE inhibition in mitosis. Truncation of the C-terminal end of STIM1, which contains the Cdk1 consensus sites typically phosphorylated in mitosis, or mutating to Ala the 10 Ser/Thr consensus sites has been documented to prevent SOCE inhibition in

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**Fig. 2.** STIM1 phosphorylation is not required for SOCE suppression in mitosis. (A) Representative SOCE traces from asynchronous (Asyn) and mitotic (Mito) cells transfected with mCh-STIM1 (WT) or mCh-STIM1-10A mutant (10A; Left). Summary data of SOCE levels (Right; \(n = 4\); means \(\pm\) SEM, paired \(t\) test, \(P < 0.0001\) for WT and \(P = 0.0006\) for 10A). Tg, thapsigargin. (B) Western blot analyses of STIM1, Orai1, and phosphohistone H3 (Ser10) in HEK293, Jurkat, and CD4\(^+\) T cells. Representative of three similar experiments. (C) Schematic of the genomic region of the control STIM1-10A-KI mouse line that expresses WT STIM1. The targeting construct contained a floxed region consisting of a WT cDNA containing exons 10–12 followed by an FRT-flanked PGK promoter-driven Neo cassette upstream of the target exon coding for the WT to A substitutions. Cre-mediated deletion replaces WT STIM1 with the 10A mutant. (D) Detection of endogenous STIM1 (Top) and Orai1 (Bottom) in splenocytes from control KI (Ctrl) and 10A-KI mice. STIM1 was detected using either the N-terminal Ab, which detects total STIM1, or the C-terminal mAb, which does not recognize the 10A mutant. (E) Western blots from asynchronous and mitotic HEK293 and CD4\(^+\) T cells from the 10A-KI mice. β-actin was used as loading control. (F and G) Representative traces and summary data of SOCE in CD4\(^+\) T cells from Ctrl (F) and 10A-KI mice (G; \(n = 12\) from three different mice; mean \(\pm\) SEM, paired \(t\) test, \(P < 0.0001\)). **P ≤ 0.001.
mitosis. We, therefore, measured SOCE in cell populations expressing either WT STIM1 or the 10A mutant. In both cases, SOCE was inhibited in mitosis to similar levels (Fig. 2A). We confirmed that both WT STIM1 and the C-terminal truncation (STIM1-482) are functional, as they both increased SOCE levels when coexpressed with Orai1 (SI Appendix, Fig. S2A and C). This argues, in contrast to previous studies (25, 31), that overexpression of the nonphosphorylatable STIM1 does not prevent SOCE inhibition in mitosis.

We confirmed that the inhibition of SOCE in mitosis in cells expressing STIM1 or the 10A mutant was not due to perturbation of the STIM1–Orai1 ratio, since SOCE was also suppressed in mitosis when Orai1 was coexpressed with STIM1, the 10A mutant, or the STIM1-482 truncation (SI Appendix, Fig. S2B and C). Interestingly as well, neither the 10A nor the 10E mutants were detected by the anti-STIM1 C-terminal mAb directed against the phosphorylation-rich region of STIM1 (SI Appendix, Fig. S3B; SI Appendix, Fig. S3 C–E shows additional validation of the anti-STIM1 antibodies).

**STIM1 Is Not Phosphorylated in Primary CD4+ T Cells or Macrophages in Mitosis.** STIM1 from mitotic HEK293 and Jurkat cells exhibits a slower mobility on SDS/PAGE due to its phosphorylation (Fig. 2B). Mitosis and interphase were distinguished by the phosphorylation status of histone H3 (Fig. 2B). However, no shift in STIM1 electrophoretic mobility was detected in mitotic primary CD4+ T cells (Fig. 2B) or bone marrow–derived macrophages (SI Appendix, Fig. S4A), suggesting that the phosphorylation status of mitotic STIM1 may be different in primary cells vs. cell lines. Alternatively, mouse STIM1 may not be as susceptible to the phosphorylation-induced mobility shift on SDS/PAGE, since the mobility of endogenous mouse oocyte STIM1 does not change in meiosis, while the mobility of exogenously expressed human STIM1 does (30).

To differentiate between these possibilities, we assessed STIM1 phosphorylation status and mapped phosphorylated residues on endogenous STIM1 from mitotic CD4+ T cells and Jurkat cells by MS-based shotgun proteomics. Smyth et al. (25) identified S668 as the critical mitosis-specific STIM1 phosphorylation site, whereas Yu et al. (29) identified S519, S575, S618, S620/621, and S668 in meiosis. Of these sites, both S618 and S668 were phosphorylated in mitotic Jurkat cells (SI Appendix, Fig. S3F), whereas only S575 was detected in mitotic CD4+ T cells (SI Appendix, Fig. S3F). Both S519 and S575 have been shown to be phosphorylated in interphase cells (25, 36). Therefore, no mitosis-specific phosphorylation of STIM1 could be detected in CD4+ T cells. This in conjunction with the lack of the mobility shift associated with STIM1 phosphorylation in mitosis indicates that STIM1 is not phosphorylated in mitosis in primary CD4+ T cells.

**STIM1 Phosphorylation Is Not Required for SOCE Suppression in Mitosis.** To further assess the role of STIM1 phosphorylation in mitosis, we generated an STIM1-EGFP-KI HeLa cell line that stably expresses H2B-RFP, which paints chromosomes and allows identification of mitotic cells (Fig. 3A). Using this line, we confirmed the loss of ∼50% of the endogenous STIM1 protein in mitosis by quantifying EGFP fluorescence (Fig. 3C). We further confirmed degradation of endogenous STIM1 in mitosis by stable isotope labeling of amino acids in cell culture (SILAC) labeling and quantitative MS (SI Appendix, Fig. S5C), which also showed close to a 50% decrease in STIM1 expression in mitosis. Collectively, these different approaches establish that STIM1 is degraded in mitosis.

**Mitotic STIM1 Targets the Autophagy Pathway and Associates with the BAG3 Chaperone.** How is STIM1 targeted for degradation during mitosis? We first explored the role of the ubiquitin–proteasome system as a well-established pivotal player in cell cycle progression. We immunoprecipitated STIM1 from asynchronous and mitotic HEK293 and Jurkat cells and from mitotic cells treated with the proteasome inhibitor MG132 (SI Appendix, Fig. S5B). This confirms that the STIM1-10A replaced the endogenous STIM1 alleles in the STIM1-10A-KI mice. Furthermore, the expression of Orai1 was not altered in splenocytes from the STIM1-10A-KI line (Fig. 2D). As expected, STIM1 in mitotic CD4+ T cells from the 10A-KI mice does not show the slower electrophoretic mobility observed in mitotic HEK293 cells (Fig. 2E).

To determine the effect of a nonphosphorylatable STIM1 on SOCE, we isolated CD4+ T cells from control and STIM1-10A-KI strains and measured SOCE in interphase and mitosis. SOCE was similar in interphase CD4+ T cells from WT and STIM1-10A-KI strains (SI Appendix, Fig. S4 D and E). Nonetheless, SOCE was suppressed to similar extents in mitotic CD4+ T cells isolated from the STIM1-10A strain (inhibited by 74.33 ± 0.76%) or from the congenic control KI mouse line expressing WT STIM1 (66.65 ± 0.96%) (Fig. 2 F and G). We conclude that, even when examined at the whole-animal level, inhibition of SOCE in mitosis does not require phosphorylation of STIM1.

**STIM1 Is Down-Regulated in Mitosis.** Since SOCE inhibition in mitosis is not likely due to modulation of Orai1 or to STIM1 phosphorylation, additional mechanisms must be at play. Since proteolysis and ER remodeling have important roles in cell division, we considered whether they modulate SOCE in mitosis. We therefore, measured STIM1 expression using quantitative Western blots in HEK293 (SI Appendix, Fig. S5A), Jurkat, and primary CD4+ T cells (SI Appendix, Fig. S5B). In all three cell types, endogenous STIM1 expression decreased by ∼50% in mitosis, with no change in Orai1 protein levels (SI Appendix, Fig. S5B).

To confirm degradation of endogenous STIM1 in mitosis, we tagged one of the endogenous STIM1 alleles with EGFP using CRISPR-Cas9–mediated gene editing in HeLa cells. We refer to this cell line as STIM1-EGFP-KI. SOCE activates normally in this cell line and is suppressed in mitosis (Fig. 3A). Both the WT and tagged STIM1 proteins exhibit the typical mobility shift in mitosis due to phosphorylation (Fig. 3B). Furthermore, expression of both proteins was decreased by ∼50% in mitosis (Fig. 3B), supporting the conclusion that STIM1 is degraded in mitosis.

In the experiments described so far, mitotic populations were isolated after nocodazole treatment. We were concerned that prolonged arrest due to nocodazole could indirectly affect STIM1 expression. To allow identification of mitotic cells without perturbing the cell cycle, we generated an STIM1-EGFP-KI HeLa cell line that stably expresses H2B-RFP, which paints chromosomes and allows identification of mitotic cells (Fig. 3C). Using this line, we confirmed the loss of ∼50% of the endogenous STIM1 protein in mitosis by quantifying EGFP fluorescence (Fig. 3C). We further confirmed degradation of endogenous STIM1 in mitosis by stable isotope labeling of amino acids in cell culture (SILAC) labeling and quantitative MS (SI Appendix, Fig. S5C), which also showed close to a 50% decrease in STIM1 expression in mitosis. Collectively, these different approaches establish that STIM1 is degraded in a mitosis-specific fashion.

We further considered whether other proteins that contribute to ER–PM Cxs (37–41) might also be degraded in mitosis. However, expressions of extended synaptotagmin 1, oysterolysin, or the protein–related protein-8, and GRAM Domain Containing 2A were similar in asynchronous and mitotic cells (SI Appendix, Fig. S6 D–I). In contrast, STIM2 expression increased in mitosis (SI Appendix, Fig. S6 A–C). These data argue that STIM1 is selectively degraded in mitosis.

**Mitotic STIM1 Targets the Autophagy Pathway and Associates with the BAG3 Chaperone.** How is STIM1 targeted for degradation during mitosis? We first explored the role of the ubiquitin–proteasome system as a well-established pivotal player in cell cycle progression. We immunoprecipitated STIM1 from asynchronous and mitotic HEK293 and Jurkat cells and from mitotic cells treated with the proteasome inhibitor MG132 (SI Appendix, Fig. S5B).
As expected, MG132 treatment in mitosis increased the levels of ubiquitinated proteins (SI Appendix, Fig. S7A). However, there is no discernable increase in STIM1 polyubiquitination in mitosis independent of MG132 treatment (SI Appendix, Fig. S7A), arguing that proteasomal degradation is not involved in STIM1 degradation in mitosis. Consistent with a previous report (42), we did detect monoubiquitinated STIM1 in interphase as well as in mitosis (SI Appendix, Fig. S7A).

Selective autophagy also plays important roles in mitosis (43). We, therefore, tested whether STIM1 is targeted to autophagosomes in mitosis. We imaged the distribution of endogenous STIM1 and the autophagosome marker LC3 in asynchronous and mitotic HEK293 cells stably expressing LC3-GFP (Fig. 3D). Pearson colocalization analysis show a high colocalization index in mitosis but not in interphase cells (Fig. 3D), implying that STIM1 targets to autophagosomes in mitosis. Inhibition of autophagy using bafilomycin A1 (44) prevented STIM1 degradation in mitosis (SI Appendix, Fig. S7B).

To further assess the mechanisms targeting STIM1 to autophagosomes in mitosis, we focused on the cochaperone BAG3, which plays a critical role in mitosis progression by targeting proteins for selective autophagy (45, 46). We show that STIM1 interacts preferentially with BAG3 in mitotic cells. DSP cross-linked Asyn and Mito HEK293 lysates were immunoprecipitated with either STIM1 C-terminal Ab (HEK; Top) or N-terminal Ab (HEK (Middle) and Jurkat (Bottom)). Western blots analysis with BAG3 and STIM1 antibodies shows preferential interaction in mitosis. Images are representative of two independent experiments. IP, immunoprecipitation. *P < 0.05; **P ≤ 0.001.
care to visualize STIM1 clusters only in cells expressing endogenous or near-endogenous levels of STIM1. In response to store depletion with either thapsigargin (Fig. 4A) or ionomycin (SI Appendix, Fig. S7B), STIM1 clusters and forms stable puncta in interphase but not in mitosis in STIM1-EGFP-H2B-RFP HeLa cells. To corroborate this finding, we generated a stable cell line expressing human STIM1-YFP using STIM1-knockout mouse embryonic fibroblast (MEF) cells (48) such that the only STIM1 expressed is the YFP-tagged version. In this case as well, store depletion leads to STIM1 puncta formation in interphase but not mitotic cells (Fig. 4B). These results show that STIM1 does not form stable puncta in response to store depletion in mitosis. Consistent with these findings, overexpressed STIM1 was shown not to form puncta in response to store depletion in oocyte meiosis and in mitosis (25, 29).

**ER–PM Junctions Are Down-Regulated During Mitosis.** Mitosis is associated with remodeling of the cytoskeleton and organelles, including the ER (49). Furthermore, mitotic cells have increased internal hydrostatic pressure and surface tension that drive cell rounding, which is important for spindle orientation and chromosome segregation (50). We expressed GFP-membrane-attached peripheral ER (MAPPER), a well-characterized probe for ER–PM junctions that does not discernably modify them (51), in H2B-RFP stable HEK293 cells to identify mitotic cells (Fig. 4C). As expected, MAPPER localizes in a punctate pattern—indicative of ER–PM CS—preferentially at the PM focal plane compared with the cell equator in interphase cells (Fig. 4C). In contrast, GFP-MAPPER is diffusely distributed in mitotic cells (Fig. 4C). A 3D reconstruction of the cell volume with punctate GFP-MAPPER pseudocolored as gray dots and diffuse MAPPER in green in Fig. 4D, highlights the loss of ER–PM CS in mitosis (Fig. 4D). Quantification of the density of ER–PM CS marked by MAPPER shows a 16-fold decrease in mitosis: 0.178 ± 0.010 puncta per 1 μm² in interphase vs. 0.011 ± 0.002 puncta per 1 μm² in mitosis (Fig. 4E). Combined total internal reflection fluorescence (TIRF) and epifluorescence imaging of GFP-MAPPER distribution in the same cells confirms the loss of ER–PM CS in mitosis (SI Appendix, Fig. S7D).

A version of MAPPER with a shorter cytosolic linker (SI Appendix has details) reveals a similar down-regulation of ER–PM CS in mitosis (SI Appendix, Fig. S8). This shows that the length of...
the linker, which would be expected to bring the ER and PM closer together (51), has no bearing on the loss of ER–PM CSs in mitosis. Collectively, these results argue that ER remodeling in mitosis is associated with loss of ER–PM CSs.

We also assessed ER–PM CS remodeling at the ultrastructural level in Jurkat cells by EM on sections near the cell equator. We identified interphase cells by their diffuse DNA and intact nuclear envelope and mitotic cells by their condensed chromosones and absence of a nuclear envelope (Fig. 5 A and B). We defined ER–PM CS as areas where the distance between the ER and PM was ≤30 nm. The density of ER–PM CSs was significantly reduced in mitosis (0.098 ± 0.012 in interphase to 0.02 ± 0.0027 in mitosis) (Fig. 5C). Even when present, ER–PM CSs were slightly but significantly wider in mitosis (19.37 ± 0.57 nm, n = 117) compared with interphase (17.4 ± 0.36 nm, n = 346) (Fig. 5D). Furthermore, ER–PM CSs were, on average, longer in interphase (122.8 ± 5.7 nm) than during mitosis (99.17 ± 7 nm) (Fig. 5E) due primarily to the loss of long ER–PM CS (>300 nm) in mitosis (Fig. 5E).

These analyses show that, in addition to the significant reduction in the number of ER–PM CS in mitosis, those that remain are shorter and wider.

Interestingly, ER–PM CSs in interphase were of two distinct classes based on the orientation of the ER toward the PM, with the ER tubule either running parallel to the PM or approaching the PM at an angle (Fig. 5A). In mitosis, the ER invariably was oriented parallel to the PM (Fig. 5B).

We also measured distances between the PM and the closest ER tubule, which for interphase cells, represents the ER–PM CS (Fig. 5F). In mitosis, most sections lacked ER–PM CS, with ER tubules instead appearing fragmented, consistent with remodeling of the ER. Most of these near-PM ER tubules run parallel to the PM but at some distance from it (103.5 ± 2.65 nm, n = 775) (Fig. 5B, arrowheads), with only a small portion coming close enough to the PM to create an ER–PM CS in mitosis (Fig. 5B, arrows). Therefore, most ER in mitotic cells is too far from the PM to allow for direct STIM1–Orai1 interactions and SOCE.

Discussion

Cells have evolved mechanisms to remodel Ca²⁺ signals during cell division to ensure genomic and cell cycle integrity. A conserved aspect of this regulation is the inhibition of SOCE to allow cell division to proceed. Indeed, constitutive low-level activation of SOCE, although well tolerated in interphase, causes mitotic catastrophe (Fig. 1C). Herein, we elucidate multiple mechanisms that combine to effectively inhibit SOCE in mitosis and resolve a long-standing controversy in the field by showing that this inhibition does not involve STIM1 phosphorylation.

Based in part on overexpression of nonphosphorylatable STIM1 mutants, STIM1 phosphorylation was argued to be sufficient to inhibit SOCE in mitosis (25, 31). We were unable to replicate these findings (Fig. 2A and SI Appendix, Fig. S2). One potential technical discrepancy is that, in the previous studies, mitotic cells were identified based on cell rounding, which may include cells at the G2/M transition not yet committed to mitosis, thus skewing the results toward functional SOCE. Furthermore, the phosphorylation state of STIM1 does not correlate with

![Fig. 5](https://example.com/fig5.png)

**Fig. 5.** Ultrastructural analysis of ER–PM CS in interphase (Inter) and mitotic (Mito) cells. (A and B) Representative EM images from Inter (A) and Mito (B) Jurkat cells. A representative whole cell in shown at low magnification for Inter and Mito, with several zoomed in magnifications of ER–PM CS in Inter (arrows in A). For Mito (B), in addition to ER–PM CS (arrows), the regions of the closest ER to the PM are shown (arrowheads). (Scale bar: whole-cell images in A and B, 1 μm; zoomed-in regions in A and B, 100 nm.) (C–F) Quantification of ER–PM CS (MCS) density (C), junctional gap distance (D), and ER–PM CS length (E) in Inter and Mito. (F) Distance from the closest ER tubules to the PM in high-magnification EM images. For Inter, that distance is by default that of ER–PM CS. **P ≤ 0.01; ***P ≤ 0.001.
SOCE inhibition, since SOCE is inhibited in primary CD4+ T cells and macrophages in the absence of STIM1 phosphorylation (Fig. 2B and SI Appendix, Figs. S3F and S4A). KI mice, where STIM1 was replaced by a nonphosphorylatable STIM1-10A (Fig. 2), are viable and reproduce normally. SOCE is inhibited during mitosis in primary CD4+ T cells isolated from STIM1-10A mice (Fig. 2 D and E). Collectively, these results show conclusively that STIM1 phosphorylation does not mediate SOCE inhibition in mitosis.

SOCE is activated through direct physical interaction of STIM1 and Orai1, with the stoichiometry of the proteins playing a critical role (52). Maximal SOCE current is observed with an STIM1:Orai1 stoichiometry of 2:1: that is, 12 STIM1 molecules per functional Orai1 hexameric channel. Hence, loss of STIM1 protein in mitosis (~50%) (Fig. 3 A–C and SI Appendix, Fig. S5) is likely to inhibit SOCE even if STIM1 and Orai1 were able to interact across the ER–PM CS.

Interestingly, STIM1 is not degraded through the proteasomal pathway in mitosis, as we were unable to detect polyubiquitination of STIM1, even in the presence of a proteasome inhibitor (SI Appendix, Figs. S1 and S2). We finally show that the density of ER–PM CSs is reduced in mitosis using MAPPER (Fig. 4) and ultrastructural studies to directly visualize ER–PM CS (Fig. 5). SOCE depends on the integrity of ER–PM CS to allow contact between STIM1 and Orai1. Therefore, the down-regulation of ER–PM junctions in mitosis is likely to be a major contributor to SOCE inhibition by physically preventing STIM1 from interacting with Orai1.

The average distance between the PM and the closest ER in mitosis is ~100 nm (Fig. S5), consistent with a previous EM study on mitotic HeLa cells that showed peripheral ER being excluded from approaching the PM by cortical F-actin at a distance of 150–200 nm (55). In addition, cortical actin contributes to the spatial organization of ER–PM junctions in interphase (56). The actin cortex reorganizes during mitosis to regulate cell surface tension (50, 57). Thus, we speculate that remodeling of the actin cytoskeleton in mitosis contributes to preventing ER–PM cross-talk.

Several studies document ER restructuring in M phase (55, 58–65), suggesting a transition from a tubular structure in interphase cells to a patchy cisternal organization. This report shows a decrease in ER–PM CS in mitosis, which would have implications not only on SOCE and Ca2+ signaling but also, on lipid metabolism and membrane structure.

Collectively, our findings provide a comprehensive view of the mechanisms underlying SOCE inhibition in mitosis. They show that cells have enlisted autophagy, protein–protein interaction, and remodeling of ER–PM CS to block Ca2+ influx in mitosis. These overlapping mechanisms highlight the critical need to inhibit SOCE to allow for tight regulation of Ca2+ dynamics and ensure homeostasis during cell division.

Materials and Methods

Mouse Strains. STIM1-10A-KI mice and control KI mice were generated by Ozgene Pty Ltd. Details are provided in SI Appendix. All animals were bred and/or maintained in the Well Cornell Medicine Qatar pathogen-free animal facility. All experimental procedures and colony maintenance were approved by the Well Cornell Medicine Institutional Animal Care and Use Committee (protocol 2014-0043).

Gene Editing of STIM1 in HeLa Cells. CRISPR-Cas9 was used to introduce mono-meric EGFP (A206K) through a short linker (RILQSTVPRARDDPVPAT) in frame at the C-terminus of one of the STIM1 alleles in HeLa cells (STIM1-EGFP-KI) as described in SI Appendix, SI Methods.

Primary Cell Culture and Cell Lines. For ex vivo cultured CD4+ T cells, total CD4+ T cells from mouse spleen and lymph nodes were purified using CD4 (L347) Microbeads (Milteny Biotech Inc.). Naïve CD4+ T cells were purified by cell sorting for the CD4+CD26+CD25− population. CD4+ T cells were stimulated with plate-bound anti-CD3 (145-2C11) and anti-CD28 (37.51) antibodies and cultured in neutralizing condition (ThemoFisher) in the presence of 50 U/mL human interleukin-2 (PeproTech) as previously described (66). YFP-HA-Orai1 stable CHO cells were described previously (34). STIM1 knockdown MEF cells were a gift from Masatsugu Oh-hora, Tokyo Medical and Dental University, Tokyo (48). To generate hSTIM1-YFP stable STIM1 knockdown MEF cells, STIM1-KO-MEF cells were infected with retrovirus expressing hSTIM1-YFP (1975A; Addgene) and then, selected using puromycin.

To generate HEK293 and STIM1-EGFP-KI HeLa cell lines stably expressing the mitotic marker H2B-RFP, cells were infected with lentivirus expressing H2B-RFP (26001; Addgene) (67). H2B-RFP-expressing cells were enriched for the RFP+ population on a cell sorter (BD FACSAria II).

To generate GFP-LC3 stable HEK293 cells, cells were infected with retrovirus expressing GFP-LC3 (22405; Addgene) (68) and then, enriched for the GFP+ population (BD FACSAria II).

For mitotic synchronization, cells were treated with 100 ng/mL nocodazole (Sigma) for 16–18 h. For the experiments assessing STIM1 ubiquitination, HEK293 and Jurkat cells were treated with nocodazole for 16 h followed by incubation with 25 μM MG-132 (Selleckchem) for an additional 4 h.

Plasmids. YFP-Bag3 plasmid (EX-U1468-M15) was purchased from GeneCo-poeia. YFP-CAD (33) and mcherry-STIM1 (69) plasmids were a gift from Rich Lewis, Stanford University, Stanford, CA, and the YFP-STIM1-482 plasmid (25) was a gift from James Putney, NIH, Bethesda, MD.

To construct human STIM1-10A (S486A, S492A, S575A, S600A, S608A, S618A, S621A, T626A, S628A, S668A) and STIM1-10E mutant, pSGEM-mcherry-STIM1-7A and pSGEM-mcherry-STIM1-7E plasmids (29) were used as templates for site-directed mutagenesis of S575, S600, and S608 for either A or E substitution using the Quickchange mutagenesis kit (Agilent Technologies). The primer pairs used are listed in SI Appendix.

Cell Sorting and FACS Analysis. To isolate the G2/M population from nocodazole-treated, ex vivo-cultured CD4+ T cells (mitotic CD4+ T cells), cells were stained with either NuclearRed for Ca2+ imaging or Hoechst33342 for Western blots and MS. Cells were sorted on an FACSaria II (BD Biosciences) and collected into a medium containing nocodazole. Jurkat cells were stained with Hoechst33342 and analyzed for cell cycle profile on a BD Fortessa. When the G2/M population was above 85% of total cells, cells were used for additional analysis. Mitotic HEK293 cells were obtained by nocodazole treatment followed by mitotic shake off.

To isolate asynchronous and mitotic HEK293 cells expressing mcherry-STIM1-EGFP-KI, for Ca2+ imaging or mcherry-STIM1-EGFP-KI, asynchronous or mitotic shake-off cells transiently expressing mcherry-STIM1-WT or -10A were subjected to cell sorting for the mcherry+ population. Cells were collected into the medium containing nocodazole.

For cell viability analysis, cells were stained with Fixable Viability Dyes eFluor 506 (ThermoFisher), fixed, permeabilized, and stained with Alexa Fluor 647 Rat anti-histone H3 (pS28) antibody (clone HTA28; 558217; BD), a well-known marker for mitosis (70). eFluor 506 permeates only dead cells with damaged PM and is not viable with an intact PM. It further increases its fluorescence dramatically on binding cellular proteins, thus allowing differentiation between the live and dead cell populations. The dye is fixable, allowing retention of the fluorescence before additional processing.

For localization analysis, cells were harvested and stained with Fixable Viability Dyes eFluor 506 (ThermoFisher) and mouse anti-HA antibody (Chemicon, A5406) followed by staining with secondary Goat anti-Mouse Alexa Fluor 405 (ThermoFisher Scientific) before fixation. Flow cytometry data were analyzed by the Flowjo v10 software (Tree Star).

Confocal Microscopy. Cells were cultured on 35-mm glass-bottom dishes (MatTek) at 37 °C with 5% CO2 and imaged in Ringer solution containing 155 mM NaCl, 4.5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM Na-phosphate, and 5 mM Na-Hepes, pH 7.4. For mitotic Ca2+ free Ringer, CaCl2 was replaced with MgCl2. Imaging was performed on a Zeiss LSM 880 confocal microscope using a Plan Apo 63×/1.4 oil DIC II objective with pinhole 150 nm or fully open as indicated.
Antibodies and reagents used for staining were STIM1 N-terminal rabbit polyclonal antibody (11565–1-AP; lot no. 00016319; Proteintech), α-tubulin (MAB4512; Invitrogen), β-actin (M0251; Sigma-Aldrich), Alexa Fluor 633 goat anti-mouse IgG antibodies, and phalloidin Alexa Fluor 633 (Invitrogen).

Images were analyzed using ZEN Black software, and figures were compiled using Adobe Photoshop or Illustrator; 3D reconstruction of confocal stacks and ER–PM junction analysis were performed using the Imaris package (Bitplane).

TIRF and Epifluorescence Imaging. Live cell TIRF and epifluorescence imaging were performed on a Zeiss 21 AxioObserver inverted fluorescence microscope with a TIRF slider using an alpha Plan-Apochromat 63×/1.46 Oil Corr M27 TIRF objective at 37 °C. Images were processed using Zeiss Zeno Blue software.

Ca²⁺ Imaging. Thapsigargin, ionomycin, and Fura2-AM were purchased from Invitrogen. Equal numbers of cells were loaded with Fura2-AM for 30 min at 37 °C, washed with Ca²⁺–free Ringer solution (120 mM NaCl, 5 mM KCl, 3 mM MgCl₂, 20 mM Hepes, pH 7.4), and transferred into the glass-bottom 96-well plate (3603; Corning Costar). The plate was centrifuged at 1,109 × g for 3 min at room temperature. Ca²⁺ imaging was performed on a FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices) by recording fluorescence (≥500 nm) after alternate excitation at 340 and 380 nm (71). SOCE was calculated by subtracting fluorescence values (F₃₄₀/F₃₈₀) before Ca²⁺ addition from the maximum fluorescence from the restoration of extracellular Ca²⁺. Graphs were analyzed in Prism 6 software (GraphPad).

Samples were analyzed by liquid chromatography–tandem MS using an analytical platform consisting of an EASY nLC-1200 interfaced to a Q-Exactive HF orbitrap mass spectrometer (Thermo Fisher). MS raw data were searched using the MaxQuant suite of algorithms (72, 73) (version 1.5.2.80) against the Homo sapiens UniprotKB database (canonical and isoforms) as well as 20151012; 48460) (74) using default parameters with carbamidomethylated cysteine as fixed and acetyl protein N termini, oxidated methionine, and phosphorylated serine/threonine/tyrosine as fixed and variable post-translational modifications.

Statistics. Data are presented as mean ± SEM. Groups were compared using the Prism 6 software (GraphPad) using either paired or unpaired two-tailed Student t test or the Wilcoxon nonparametric test as indicated. Statistical significance is indicated by P values.

ACKNOWLEDGMENTS. We thank colleagues who shared valuable reagents: Stefan Feske (New York University) for the STIM1flox/flox mouse line; Masatsugu Oh-hora (Tokyo Medical and Dental University) for the STIM1 KO MEF cell line; Richard S. Lewis (Stanford) for the nChanery-STIM; YFP-HA-ORAI1 and YFP-CAV plasmids; Jian Liu (University of Texas Southwestern) for GFP-MAPPER; and Jim Putney (NIH) for YFP-STIM1–482 plasmid. We thank the Microscopy Core; Noha Goswani and Hisham Ben Hamidane at the Proteomic Core; the vivalirium core staff at Well Cell Cornell Medicine (Qatar) (H. and L.); and Lee Cohen-Gould for the imaging core at Well Cornell Medicine for their technical support and help. We also thank Ms. Aleksandra Lberska for help with the cell-sorting studies. This work and the WCM-Q Cores were supported by the Biomedical Research Program at the WCM-Q, a program funded by Qatar Foundation. S.C. and C.W.T. were supported by Wellcome Senior Investigator Award 102444 (to C.W.T.). The statements made herein are solely the responsibility of the authors.

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