STIM1- and Orai1-mediated Ca\textsuperscript{2+} oscillation orchestrates invadopodium formation and melanoma invasion

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Ca\textsuperscript{2+} signaling has been increasingly implicated in cancer invasion and metastasis, and yet, the underlying mechanisms remained largely unknown. In this paper, we report that STIM1- and Orai1-mediated Ca\textsuperscript{2+} oscillations promote melanoma invasion by orchestrating invadopodium assembly and extracellular matrix (ECM) degradation. Ca\textsuperscript{2+} oscillation signals facilitate invadopodial precursor assembly by activating Src. Disruption of Ca\textsuperscript{2+} oscillations inhibited invadopodium assembly. Furthermore, STIM1 and Orai1 regulate the proteolysis activity of individual invadopodia. Mechanistically, Orai1 blockade inhibited the recycling of MT1–matrix metalloproteinase (MMP) to the plasma membrane and entrapped MT1-MMP in the endocytic compartment to inhibit ECM degradation. STIM1 knockdown significantly inhibited melanoma lung metastasis in a xenograft mouse model, implicating the importance of this pathway in metastatic dissemination. Our findings provide a novel mechanism for Ca\textsuperscript{2+}-mediated cancer cell invasion and shed new light on the spatiotemporal organization of store-operated Ca\textsuperscript{2+} signals during melanoma invasion and metastasis.

Abbreviations used in this paper: CI, confidence interval; IDX, invadopodium degradation index; IP, immunoprecipitation; MESNA, 2-mercaptoethane sulfonate; MMP, matrix metalloproteinase; NMDA, N-methyl-D-aspartate receptor; SOCE, store-operated calcium entry.

Introduction

Focalized proteolysis by invasive cells is essential for the remodeling of ECM in multiple physiological processes, including bone resorption, immune surveillance, and organ development (Gimona et al., 2008). This feature is exploited by malignant cells to promote invasion and metastasis during cancer progression (Sabeh et al., 2009; Murphy and Courtneidge, 2011). Invadopodia are actin-rich membrane protrusions mediating focal ECM degradation in malignant cancer cells (Linder, 2007; Wolf et al., 2007; Murphy and Courtneidge, 2011). The assembly of invadopodia is initiated in response to the focal generation of phosphatidylinositol-3,4-biphosphate and the activation of the nonreceptor tyrosine kinase Src, which recruits adaptor protein TKS5 and cortactin to initiate assembly of the actin core of invadopodium (Seals et al., 2005; Artym et al., 2006; Oikawa et al., 2008; Oser et al., 2009; Yamaguchi and Oikawa, 2010). Upon maturation, invadopodia recruit and secrete proteinases such as membrane type 1 (MT1)–matrix metalloproteinase (MMP), MMP2, and MMP9 to degrade ECM and facilitate invasion (Artym et al., 2006; Clark et al., 2007; Clark and Weaver, 2008; Oser et al., 2009). Signaling molecules downstream of the ubiquitous secondary messenger Ca\textsuperscript{2+} have been previously implicated in invadopodium regulation (Baldassarre et al., 2003; Alexander et al., 2008; Cortesio et al., 2008). However, the role of Ca\textsuperscript{2+} signaling in invadopodium modulation is not known.

Store-operated calcium entry (SOCE) is a Ca\textsuperscript{2+}-entry mechanism regulated by extracellular stimuli (Putney, 1986). SOCE is induced in response to the activation of plasma membrane...
receptors and subsequent Ca²⁺ release from the endoplasmic reticulum (Hogan et al., 2010). Upon Ca²⁺ release, the endoplasmic reticulum Ca²⁺ sensor STIM1 oligomerizes and translocates to the junction between plasma membrane and endoplasmic reticulum to activate the plasma membrane pore-forming unit Orai1, which induces SOCE (Liou et al., 2005; Roos et al., 2005; Feske et al., 2006; Vig et al., 2006).

We previously reported that store-operated calcium channel proteins STIM1 and Orai1 were critical for breast cancer cell migration, invasion, and metastasis (Yang et al., 2009), and there was accumulating evidence suggesting that hyperactive SOCE promotes cancer progression (Berry et al., 2011; Chen et al., 2011, 2013a,b; Hou et al., 2011; Hu et al., 2011; Huang et al., 2011; Chang et al., 2012; Fedida-Metula et al., 2012; Wang et al., 2012, 2015; Chantôme et al., 2013). More recently, N-methyl-d-aspartate receptor (NMDA)– and TRPM7-mediated Ca²⁺ signals were shown to promote cancer invasion and metastasis (Middelbeek et al., 2012; Li and Hanahan, 2013; Davis et al., 2014). However, it remains unclear how Ca²⁺ signals are organized, spatially or temporally, to mobilize cancer invasion machinery and to promote metastasis.

Here, we examine the hypothesis that dysregulated Ca²⁺ signals in cancer cells promote invasion through focalized proteolysis and ECM remodeling. We unexpectedly discovered that STIM1- and Orai1-mediated SOCE in melanoma cells was organized in the form of persistent Ca²⁺ oscillations and regulated both the assembly and activity of invadopodium. Our findings bring insight into spatiotemporal organization of Ca²⁺ signals during cancer invasion and metastasis and shed new light on the role of dysregulated Ca²⁺ signals in cancer malignancy.

**Results**

**Ca²⁺ is required for invadopodium formation and ECM degradation**

When plated on gelatin-coated coverslips, WM793 human melanoma cells assembled invadopodia within 4 h (Fig. S1, A and B). To investigate the role of Ca²⁺ in invadopodium regulation, WM793 cells were treated with the membrane-permeable Ca²⁺ chelator BAPTA-AM. Buffering of cytosolic Ca²⁺ remarkably reduced the number of invadopodia in WM793 cells and focalized gelatin degradation, suggesting that Ca²⁺ signals were a critical regulator of invadopodium formation and function (Fig. S1 C and Fig. 1, A and B). Similar inhibition of invadopodium formation and activity was observed when the extracellular Ca²⁺ was buffered with 0.5 mM EGTA (Fig. S1 C and Fig. 1, A and B). Treatment with nifedipine (L-type voltage-gated Ca²⁺ channel blocker), APV (NMDA blocker), and CNQX (AMPA receptor blocker) had no noticeable effects on invadopodium number or gelatin degradation (Fig. S1 C and Fig. 1, A and B). In contrast, the SOCE inhibitor 2-APB significantly decreased invadopodium number in WM793 cells and almost abolished focalized proteolysis by WM793 cells (Fig. S1 C and Fig. 1, A and B). The importance of SOCE in the modulation of invadopodium formation and ECM degradation was further confirmed by using a different SOCE blocker (SKP96365) in CHL-1 and WM245 melanoma cell lines (Fig. S1, D–G). Collectively, these data implied that Ca²⁺ influx mediated by SOCE channels is a critical regulator of invadopodium formation and ECM degradation.

**SOCE mediates Ca²⁺ oscillation to regulate the assembly of invadopodial precursor**

Invadopodia are assembled as actin-rich invadopodial precursors, which recruit MT1-MMP upon maturation to degrade ECM (Artym et al., 2006). When stimulated with 10% FBS, serum-starved WM793 cells started assembling invadopodial precursor...
Figure 1. **STIM1 and Orai1 are crucial for invadopodium formation and activity.** (A and B) Scattered dot plot showing the effects of Ca^2+^ chelators and Ca^2+^ blockers on invadopodium number per cell (A) and gelatin degradation area per cell (B). Horizontal bars represent means ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001 as calculated by two-tailed Mann-Whitney test. NS indicates not statistically significant. (C) Representative fluorescence micrographs showing effects of STIM1 and Orai1 shRNA on invadopodium formation and focalized proteolysis in WM793 cells. (D) Scattered dot plot showing that depletion of STIM1 and Orai1 with shRNAs decreased the mean number of invadopodia per cell when compared with WM793 cells expressing control shRNA. (E) Orthogonal views of confocal z stacks showing Orai1 shRNA inhibited gelatin degradation and penetration into Alexa Fluor 488–gelatin coating by invadopodia (arrowheads) in WM793 cells, with WM793 cells expressing control shRNA as a control. Bars: (horizontal) 10 µm; (vertical) 2 µm. (F and G) Scattered dot plot showing that inhibition of SOCE with STIM1 shRNA and Orai1 shRNA inhibited the focalized proteolysis activity of individual invadopodium when compared with control shRNA, as determined by the quantification of IDX. (H) Representative fluorescence micrographs showing that ectopic STIM1 overexpression promoted invadopodium formation and focalized proteolysis of Alexa Fluor 488–gelatin. (I) Scattered dot plot showing that ectopic STIM1 increased numbers of invadopodia per cell, which was abrogated by 100 µM 2-APB; n = 51, 47, 52, and 52 for control, STIM1, control + 2-APB, and STIM1 + 2-APB, respectively. (J) Effects of STIM1 overexpression on the degradation activity of individual invadopodium in WM793 cells. Insets in C and H are magnified views of the boxed areas in the main images. RU, relative unit. Bars (main images) 10 µm; (insets) 2 µm. Two-tailed p-values were determined by Mann-Whitney test or by unpaired Student’s t test after log transformation. Horizontal bars represent means ± SEM. The numbers of cells used for quantitation are indicated in the parenthesis of respective figure labeling, and representative results from at least three similar independent experiments are presented. Ctrl sh, control shRNA.
Figure 2. SOCE-mediated Ca²⁺ oscillations regulate invadopodial precursor assembly. (A) WM793 cells were starved overnight, and invadopodial (Invado.) precursor assembly were initiated by stimulation with 10% FBS. Data are presented as means ± SEM (n = 33). (B, C, and E) Representative traces from single cell Ca²⁺ imaging experiments. WM793 cells were stimulated with 10% FBS in the presence of 2 mM Ca²⁺ (B, n = 307), 0 mM Ca²⁺ (C, n = 178), or 2 mM Ca²⁺ and 20 µM SKF96365 (E, n = 364). (D) Representative traces showing restoring the extracellular Ca²⁺ from 0 to 2 mM also restored Ca²⁺ oscillations (n = 150). (F) Scattered dot plot showing quantification of Ca²⁺ oscillation frequencies in control (2 mM Ca²⁺; B), 0 mM Ca²⁺ (C, n = 178), or 2 mM Ca²⁺ and 20 µM SKF96365 (E, n = 364). (G) Representative images showing侵cis precursor assembly at 0, 15, 30, 45, and 60 min after stimulation with 10% FBS. (H) Bar graphs showing quantification of invadopodial precursor assembly at 30 and 60 min after stimulation. (I, J, and L) Representative traces showing Ca²⁺ oscillations in cells treated with control shRNA, STIM1 shRNA, or Orai1 shRNA. (K) Scattered dot plot showing quantification of Ca²⁺ oscillation frequencies in control shRNA (n = 434), STIM1 shRNA (n = 364), and Orai1 shRNA (n = 305).
10–15-min after stimulation (Fig. 2 A). Intriguingly, serum treatment also stimulated immediate but transient Ca\(^{2+}\) release and sustained Ca\(^{2+}\) signals in the form of Ca\(^{2+}\) oscillation (Fig. 2 B and Video 1). Buffering extracellular Ca\(^{2+}\) with EGTA didn’t affect the transient Ca\(^{2+}\) release but almost eliminated the Ca\(^{2+}\) oscillation signals (Fig. 2 C and Video 2). Importantly, Ca\(^{2+}\) oscillation was restored after adding back Ca\(^{2+}\) to the extracellular medium, strongly suggesting that Ca\(^{2+}\) influx was crucial for Ca\(^{2+}\) oscillation (Fig. 2 D and Video 3). To determine whether SOCE was responsible for Ca\(^{2+}\) oscillation, we also examined the effects of SKF96365 on serum-stimulated Ca\(^{2+}\) oscillations in WM793 cells (Fig. 2 E and Video 4). EGTA buffering and SOCE blockade with SKF96365 significantly reduced the Ca\(^{2+}\) oscillation frequencies from 18.6 ± 1.4/h to 3.3 ± 0.1/h and 7.2 ± 0.4/h, respectively, suggesting that SOCE was a major mediator of serum-stimulated Ca\(^{2+}\) oscillation (Fig. 2 F). We further determine the role of Ca\(^{2+}\) oscillation in the assembly of invadopodial precursor through live cell imaging and fluorescent staining on fixed cells (Fig. 2, G and H; and Videos 5–7). Inhibition of Ca\(^{2+}\) oscillations with EGTA or SKF96365 remarkably decreased the numbers of invadopodia precursor assembly (Fig. 2, G and H). To further critically evaluate the role of SOCE in Ca\(^{2+}\) oscillation and invadopodia precursor assembly, we used shRNAs to knockdown STIM1 and Orai1 in WM793 cells. As expected, STIM1 and Orai1 shRNA significantly decreased the frequency of calcium oscillation when compared with control shRNA-expressing cells (P < 0.001; Fig. 2, I–K). Inhibition of Ca\(^{2+}\) oscillation with STIM1 or Orai1 shRNA also decreased the number of serum-stimulated invadopodial precursor (Fig. 2 L). Collectively, our data suggested that SOCE-mediated Ca\(^{2+}\) oscillations are critical for assembly of invadopodial precursors.

To determine whether SOCE regulate invadopodium lifetime, WM793 cells stably expressing Lifeact-mAPPLE were stimulated with 10% FBS after overnight starvation, and the assembly and disassembly of invadopodia were recorded by time-lapse live cell imaging. The effects of SOCE manipulation on invadopodium lifetime were analyzed by Kaplan–Meier survival analysis (Fig. S2). Neither SOCE activation (through STIM1 overexpression) nor inhibition (through 2-APB treatment or STIM1 and Orai1 knockdown) had a significant effect on invadopodium lifetime in WM793 cells. SOCE blocks calcium-dependent plasma membrane localization of MT1-MMP

We used WM793 cells expressing cortactin-GFP to investigate the effects of SOCE blockade on invadopodium assembly and ECM degradation kinetics. WM793 cells started assembling invadopodia ~60–90 min after being plated onto gelatin-coated glass coverslips. The cortactin-GFP signals in invadopodial precursor increased steadily after initiation, reaching a plateau at around 20 min (Fig. 4, A and B). An accelerated ECM degradation phase was detected when the cortactin-GFP signal reached the plateau, suggesting the recruitment of proteinase and maturation around this time. Intriguingly, although SOCE blockade with 2-APB dramatically reduced the number of invadopodium precursors, 2-APB treatment had no obvious effect on the kinetics of the invadopodium assembly (Fig. 4, A and C). Importantly, no degradation of gelatin was detected in the 2-APB–treated cells even 60 min after the cortactin-GFP signal reached plateau, suggesting that SOCE blockade also impaired invadopodium maturation (Fig. 4, A and C).

To understand how SOCE regulates the proteolysis activity of invadopodia, we investigated the effects of SOCE blockade on levels of secreted soluble MMPs and membrane-bound...
SOCE blockade with 2-APB or STIM1 and Orai1 double knockdown had no noticeable effect on total protein levels of MT1-MMP (Fig. 5B). However, surprisingly, SOCE blockade reduced the plasma membrane fraction of MT1-MMP (biotinylated MT1-MMP) by 50–70% (Fig. 5, B and C), suggesting that SOCE regulates invadopodium activity mainly through the subcellular localization of MT1-MMP. Indeed, when stably expressed in WM793 cells, MT1-MMP–EGFP localized to the plasma membrane (Fig. 5). There were very little MMP9 present in the conditioned medium, suggesting that WM793 cells secrete mostly MMP2 instead of MMP9. SOCE blockade with STIM1 and Orai1 knockdown had only very modest effect on the levels of secreted MMP2, indicating SOCE regulates invadopodium activity through mechanisms other than soluble MMP (Fig. 5 A).

Next, we investigated the effect of SOCE blockade on MT1-MMP total protein levels and subcellular localization. SOCE blockade with 2-APB or STIM1 and Orai1 double knockdown had no noticeable effect on total protein levels of MT1-MMP (Fig. 5B). However, surprisingly, SOCE blockade reduced the plasma membrane fraction of MT1-MMP (biotinylated MT1-MMP) by 50–70% (Fig. 5, B and C), suggesting that SOCE regulates invadopodium activity mainly through the subcellular localization of MT1-MMP. Indeed, when stably expressed in WM793 cells, MT1-MMP–EGFP localized to the plasma membrane (Fig. 5).

Figure 3. STIM1 and Orai1 regulate invadopodia through Src. [A] Effects of ectopic STIM1 and Orai1 on the levels of pY416 Src, pT308 Akt, and pY397 FAK in WM793 cells. Adherent WM793 cells expressing control vector, STIM1, or STIM1 plus Orai1 were lysed on plates, and 50 µg total cell lysate was loaded onto each lane. Western blotting was performed with respective antibodies as indicated, with GAPDH as a loading control. (B) Effects of ectopic STIM1 and Orai1 on pY416 Src levels in MCF-7 and NMuMG cells; 50 µg total cell lysate was loaded onto each lane. (C) Induction of Ca2+ influx with A23187 and thapsigargin (TG) increased Src activity. Adherent WM793 cells were treated with 5 µM A23187 and 2 µM thapsigargin for 30 min before being lysed. (D) Inhibition of SOCE with STIM1 shRNA, 100 µM 2-APB, or 0.5 mM EGTA inhibited Src activity. (E) Scattered dot plot showing that STIM1-mediated invadopodia formation was abrogated by treatment with 2.5 nM dasatinib; n = 51, 50, 51, and 53 for control, STIM1, control + dasatinib, and STIM1 + dasatinib, respectively. (F) Scattered dot plot showing ectopic expression of v-Src in WM793 cells rescued invadopodium formation in STIM1 knockdown cells; n = 49, 46, 49, and 48 for control shRNA, STIM1 shRNA, control shRNA + v-Src, and STIM1 shRNA + v-Src, respectively. (G) Ectopic expression of v-Src rescued the inhibition of WM793 cell invasion by STIM1 knockdown. The relative levels of p-Src, p-Akt, and p-FAK in A–D were determined through densitometry using ImageJ software. Horizontal bars in E–G represent means ± SEM. Two-tailed p-values are determined by Mann–Whitney test. The numbers of cells used for quantitation were indicated in the parenthesis of respective figure labeling, and representative results from at least three similar independent experiments were presented. sh, shRNA.
was removed with 2-mercaptoethane sulfonate (MESNA; Fig. 5 H, lanes 1–3). MESNA treatment before endocytosis completely removed biotinylation on MT1-MMP, confirming the efficacy of this approach (Fig. 5 H, lane 1). After 30-min endocytosis at 37°C, MESNA was able to only partially remove biotin, suggesting endocytosis of plasma membrane MT1-MMP (Fig. 5 H, lanes 2 and 3). The biotinylated MT1-MMP in the endocytic compartment were then allowed to be recycled back to the plasma membrane in the presence of SOCE blocker or vehicle control (Fig. 5 H, lanes 4–7). After 60-min (or 30 min) recycling, there were $\geq 20\%$ (or 30%) of biotinylated MT1-MMP retained in the intracellular compartments of the control cells (Fig. 5 H, lanes 4 and 6). In contrast, when SOCE was blocked with 2-APB, $>40\%$ (or 50%) of biotinylated MT1-MMP remained intracellular after a 60-min (or 30 min) recycling, indicating inhibition of MT1-MMP recycling by SOCE blockade. Collectively, our data suggest that SOCE blockade entraps MT1-MMP in the endocytic compartment through interfering with its recycling to the plasma membrane, which inhibits the ECM degradation activity of invadopodia.

**SOCE is critical for melanoma metastasis**

Gaining invasiveness is one of the first and most critical steps of metastasis (Fidler, 2003; Nürnberg et al., 2011). Prognosis for melanoma patients significantly worsens with deeper levels of dermal invasion and when melanoma progresses from a radial growth phase to vertical growth phase (Clark, 1991). Our data suggested that dysregulated SOCE may promote melanoma metastasis and progression. To examine this hypothesis, we first determined the expression of STIM1 and Orai1 in a panel of melanoma cell lines. As shown in Fig. 4, SOCE blockade inhibits invadopodium maturation. (A) Live cell imaging showing invadopodium precursor assembly and gelatin degradation by mature invadopodia in control (top) and 100 µM 2-APB–treated (bottom) cells. Arrowheads indicate nascent invadopodia. Bars: (main images) 10 µm; (magnified views) 5 µm. (B and C) Quantification of nascent invadopodium assembly (cortactin-GFP fluorescence intensity) and ECM degradation by nascent invadopodia in control (B) or 2-APB–treated cells (C). Lines in B and C are curve fittings of cortactin-GFP intensity and gelatin degradation index data. The data shown are representative of three experimental repeats. RU, relative unit.
There was 10-fold higher frequency of medium to high levels of STIM1 expression in malignant melanoma (46%, 25 out of 54 cases) compared with benign nevi (5%, 1 out of 21 cases; Fig. 6 D), suggesting dysregulation of SOCE in melanomas. To define the role of SOCE in melanoma metastasis, we used a luciferase reporter to label 1205Lu cells expressing control shRNA or STIM1 shRNA.

STIM1 knockdown did not affect the proliferation of 1205Lu cells in vitro (Fig. 6 E). Luciferase-labeled cells were injected via tail vein into nude mice, and lung metastasis of 1205Lu melanoma cells was monitored using bioluminescence imaging. As shown in Fig. 6 (F and G), we detected significant lung metastasis of 1205Lu control cells by bioluminescence imaging 36 d after mouse xenografting, which was consistent with the highly metastatic nature of this WM793 subline. The lung melanoma cell lines. As shown in Fig. 6 A, STIM1 and Orai1 were overexpressed in most melanoma cells when compared with normal epidermal human melanocytes. The expression levels of STIM1 in melanoma cells derived from metastatic melanoma were higher than cells from primary melanoma (Fig. 6 A). We further compared the STIM1 and Orai1 expression levels between WM793 cells and its highly metastatic 1205Lu subline, which was selected based on its ability to consistently metastasize to the lung in a spontaneous metastasis mouse model (Juhasz et al., 1993). Orai1 levels were similar between the two cell lines, but STIM1 protein expression was further increased by three- to fourfold in 1205Lu cells, implicating a role for STIM1 in melanoma metastasis (Fig. 6 B).

To evaluate the clinical significance of SOCE in melanoma progression, we examined a melanoma tissue microarray for levels of STIM1 expression (Fig. 6 C). There was 10-fold higher frequency of medium to high levels of STIM1 expression in malignant melanoma (46%, 25 out of 54 cases) compared with benign nevi (5%, 1 out of 21 cases; Fig. 6 D), suggesting dysregulation of SOCE in melanomas. To define the role of SOCE in melanoma metastasis, we used a luciferase reporter to label 1205Lu cells expressing control shRNA or STIM1 shRNA. STIM1 knockdown did not affect the proliferation of 1205Lu cells in vitro (Fig. 6 E). Luciferase-labeled cells were injected via tail vein into nude mice, and lung metastasis of 1205Lu melanoma cells was monitored using bioluminescence imaging. As shown in Fig. 6 (F and G), we detected significant lung metastasis of 1205Lu control cells by bioluminescence imaging 36 d after mouse xenografting, which was consistent with the highly metastatic nature of this WM793 subline. The lung
metastasis of 1205Lu cells were reduced by >80% after STIM1 knockdown, as determined by measuring normalized photon flux from luciferase-labeled melanoma cells on day 8 and day 36 (Fig. 6, F and G). The inhibition of melanoma lung metastasis by STIM1 shRNA was further confirmed by hematoxylin and eosin staining of the lungs harvested from nude mice (Fig. 6 H). Collectively, our data suggested that STIM1 overexpression in melanoma was critical for melanoma metastasis.

**Constitutive Ca\(^{2+}\) influx is not able to promote invadopodium formation and melanoma invasion**

Our data demonstrated that SOCE signals in melanoma cells were organized in oscillatory patterns to regulate invadopodia. It was not clear, however, whether oscillatory organization per se was needed or whether oscillatory frequency was merely indicative of the requirement of net increase in cytosolic Ca\(^{2+}\) during a certain time period. Because constitutive Ca\(^{2+}\) influx, induced by thapsigargin and A23187, was sufficient to activate Src activity (Fig. 3 C), we set out to determine the effects of constitutive Ca\(^{2+}\) influx on invadopodium formation and melanoma invasion (Fig. 7, C and D). These data indicated that the oscillatory organization is required for SOCE to coordinate invadopodium assembly and ECM remodeling.

**Discussion**

In this study, we present evidence showing that STIM1- and Orai1-mediated SOCE promotes melanoma invasion and ECM degradation by increasing invadopodium formation and activity. Using a novel image quantification method, we were able to demonstrate that SOCE regulates the proteolysis activity of individual metastatic cells by promoting invadopodium formation.

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**Figure 6. SOCE is critical for melanoma metastasis.** (A) Expression levels of STIM1 and Orai1 in human melanocytes and melanoma cell lines derived from primary melanoma or distant metastases. The relative levels of STIM1 and Orai1 were determined through densitometry using ImageJ software. (B) STIM1 expression was up-regulated in the highly metastatic 1205Lu subline of WM793 cells. (C) Immunohistochemistry staining showing STIM1 overexpression in malignant melanoma when compared with benign nevus. Staining intensity and percentage of positively stained lesion (in magenta) and melanoma T stages (T1–T4, in black) are indicated. (D) Proportions of benign nevi (n = 21) and malignant melanoma (n = 53) with medium to high expression of STIM1; P < 0.001 as determined by two-tailed Fisher’s exact test. (E) STIM1 knockdown had no effect on the proliferation of 1205Lu cells in vitro. Data are shown as means ± SD from triplicates. The data shown are from a single experiment out of three repeats. (F–H) Inhibition of 1205Lu cell lung metastasis by STIM1 knockdown. (F) Representative bioluminescence images showing the lung metastasis of 1205Lu cells stably expressing control shRNA or STIM1 shRNA. (G) Quantification of 1205Lu cell lung metastasis using bioluminescence imaging. Normalized photon flux showed the fold increase in bioluminescence signals (on day 8 and day 36, respectively) over day 0. Box and whiskers are 25–75th and 10–90th percentiles, respectively. *, P < 0.05; ***, P < 0.001. (H) Representative hematoxylin and eosin staining showing the presence of 1205Lu metastases in the lung of nude mice. M, melanoma metastases in the lung; H, heart; sh, shRNA.
Moreover, because constitutive increase in cytosolic Ca\(^{2+}\) is deleterious and may lead to cell death (Orrenius et al., 2003), Ca\(^{2+}\) oscillations provide the Ca\(^{2+}\) signals necessary for invadopodia formation and activity without causing cytotoxicity. It is also conceivable that invadopodia formation and function require coordinated cycles of high and low calcium signals provided by oscillation, as recently demonstrated in the case of mast cell exocytosis (Wollman and Meyer, 2012). Although constitutive Ca\(^{2+}\) increase was effective in the activation of Src, it inhibited invadopodia formation and melanoma invasion. This is consistent with the notion that temporal oscillation is critical for Ca\(^{2+}\) signals to coordinate invasion and ECM degradation. The SOCE activated by repetitive discharge of store Ca\(^{2+}\) during oscillation also creates subplasmalemmal Ca\(^{2+}\) microdomains to regulate enzyme activity and gene transcription (Chang et al., 2008; Di Capite et al., 2009). Future effort to determine spatial and temporal organization of subplasmalemmal Ca\(^{2+}\) microdomains during melanoma invasion and ECM remodeling is warranted.

Invasion is a critical step during melanoma progression. The prognosis of melanoma patients significantly worsens with deeper levels of invasion, after melanoma breaches the basement membrane separating epidermis from dermis and progresses from radial growth phase to vertical growth phase (Clark, 1991). Our data suggested that SOCE was hyperactivated during melanoma progression, with STIM1 expression increased in malignant melanomas when compared with benign nevi. The overexpression of STIM1 and hyperactivation of SOCE in melanoma likely promoted melanoma invasion and metastasis. Indeed, when STIM1 was knocked down in 1205Lu cells, the lung metastasis of this highly metastatic melanoma cell was dramatically reduced.

![Figure 7](image_url)

**Figure 7. Inhibition of invadopodium formation and melanoma cell invasion by constitutive increase in cytosolic Ca\(^{2+}\).** (A and B) Representative traces showing the effects of 2 μM thapsigargin (A) and 5 μM A23187 (B) on cytosolic Ca\(^{2+}\) (n > 100, from three independent experiments). Arrows indicate addition of 10% FBS and thapsigargin or A23187. (C and D) Effects of thapsigargin and A23187 induced constitutive Ca\(^{2+}\) increases on invadopodium formation (C) and Matrigel invasion (D) in WM793 cells. The numbers of cells used for quantitation in C are indicated in the parenthesis. Data presented are means ± SE. Representative results from at least three similar independent experiments are presented. *, P < 0.05; **, P < 0.01. F/F0 is defined as ratio between fluorescence at a given time [F] and fluorescence at time 0 [F0]. TG, thapsigargin.
data, together with recently reported findings in breast and cervical cancer (Yang et al., 2009; Chen et al., 2011; Chantôme et al., 2013), suggested that hyperactive SOCE pathways in various cancer might be targeted to inhibit metastasis and progression.

Materials and methods

Antibodies

We used the following antibodies in this study: STIM1 antibody for immuno-histochemistry [MA1-19451; Thermo Fisher Scientific], STIM1 antibody for Western blotting (mouse monoclonal; 610954; BD), Orai1 (rabbit polyclonal; O28264; Sigma-Aldrich), GAPDH (mouse monoclonal; G8795; Sigma-Aldrich), Src (mouse monoclonal; clone GD11 05-184; EMD Millipore), p-Src (Tyr416) (rabbit polyclonal; 2101; Cell Signaling Technology), Akt (mouse monoclonal; 2966; Cell Signaling Technology), p-AKT (Ser473) (rabbit monoclonal; 1931H2; Cell Signaling Technology), FAK (rabbit polyclonal; 3285; Cell Signaling Technology), p-FAK (Y397) (rabbit polyclonal; 2328; Cell Signaling Technology), Tubulin (mouse monoclonal; T6199; Sigma-Aldrich), cortactin (mouse monoclonal; clone 4F11 05-180; EMD Millipore), and p230 trans-Golgi (mouse monoclonal; 611219; BD).

Cell culture

The cell culture media used were RPMI 1640 (for all melanoma cells) and DMEM (for MCF-7 and NMuMG). All cell culture media were supplemented with 10% FBS and penicillin/streptomycin.

RNA interference

RNA interference of STIM1 and Orai1 was performed using pSUPER.Retro. puro vector [Oligoengene] encaging shRNA. The target sequences were as follows: 5′-AGAGAGGACCTAGACTCTCAGC-3′ (STIM1 sh1), 5′-TCGGCTCT-GACATCTATCCT-3′ (Orai1 sh1), and 5′-CCAGCAGTGAAGTGTGACA-3′ (Orai1 sh2). To efficiently knockdown Orai1, two shRNAs targeting two different regions of the same gene were used simultaneously. In some experiments, two previously described STIM1 shRNA (sh2 and sh3), targeting 5′-GGCTCT-GGATACAGTGCTC-3′ and 5′-GGATGCTGTCATTTTTTGA-3′ (Yang et al., 2009) and a new Orai1 sh3 (targeting 5′-GGCTCTGGATACAGTGCTC-3′) were used to confirm STIM1 and Orai1 knockdown effects.

cDNA constructs

The retrovirus vectors encoding STIM1 and Orai1 were constructed by subcloning human STIM1 and Orai1 fragments into plNCX2 vector (cytomegalovirus promoter; Takara Bio Inc.) between Bgl II and Stu I, as described previously [Yang et al., 2009]. Lifeact-APPLE was generated by first subcloning mAPPLE into plNCX2 between HindIII and StuI and then subcloning Lifeact between Xho I and Hind III. MT1-MMP–EGFP in the pEGFP-N1 vector was a gift from M. Montoya (Spanish Nacional Cancer Research Center, Madrid, Spain; Bravo-Cordero et al., 2007). pEGFP–MT1-MMP was digested with Hind III and Not I, and the MT1-MMP–EGFP fragment was inserted between Hind III and Not I sites in pPCX vector (cytomegalovirus promoter; Takara Bio Inc.). mRFP–Rab5 in pmRFP-C3 backbone was obtained from Addgene (14437).

Fluorescent gelatin labeling

Fluorescent gelatin was synthesized by incubating bovine skin gelatin with amine reactive fluorescent dyes. Alexa Fluor 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester (Invitrogen) was dissolved in DMSO at 10 mg/ml. Dye solution (50 µl) with amine reactive fluorescent dyes. Alexa Fluor 488 carboxylic acid, Alexa Fluor 594– or Alexa Fluor 488–labeled phalloidin (1:100 dilution from a 20U/ml stock solution) for 30 min. Extensive washes with PBS were performed after each step. The coverslips were mounted onto slides in mounting medium (150 mM Tris, pH 8.0, and 90% glycerol). Fluorescent micrographs were obtained with an upright fluorescence microscope (Axio Observer.Z1; Carl Zeiss) equipped with 63x oil immersion objective.

Gelatin degradation was quantified using ImageJ software [National Institutes of Health] by setting signal threshold for gelatin fluorescence in each individual cell. A region of interest was selected along the outline of each individual cell with phallolidin staining, and the degraded area with gelatin fluorescence signal below the set threshold was measured by ImageJ. We quantified 20–30 cells from five random 63x fields. Data are shown as scattered dots, with each dot representing an area (in square micrometers) degraded in a single melanoma cell.

To evaluate the degradation activity of individual invadopodium, we quantified the IDX. A region of interest was selected around the actin core of an invadopodium to include all the degraded area, and the mean gelatin fluorescence intensity (F) and total area (A) of the region (in pixels) were quantified using ImageJ. Mean fluorescence intensity in a nodegradation reference area [F] in the vicinity was also measured.IDX of each invadopodium was calculated according to the following equation: IDX = (F - F0) / A. IDX is proportional to the total amount of gelatin degraded by individual invadopodium and is comparable between different coverslips assuming similar gelatin coating thickness. Data are presented as scatter dots, with each dot representing the ID of a single invadopodium.

Immunofluorescence staining

Melanoma cells (9 × 104) in RPMI 1640 growth medium were plated onto gelatin-coated glass coverslips for 12 h and fixed with 4% paraformaldehyde. The cells were then permeabilized in antibody diluting buffer (2% BSA and 0.1% Triton X-100 in PBS) and incubated with mouse anti-cortactin (clone 4F11 05-180; EMD Millipore) at 1:1,000 dilution, for 1 h. The cells were incubated with Alexa Fluor 488–conjugated anti-mouse IgG (1:300) or anti-rabbit IgG (1:300) and Alexa Fluor 594–labeled phalloidin (2% BSA and 0.1% Triton X-100 in PBS) and incubated with mouse anti-cortactin (clone 4F11 05-180; EMD Millipore) at 1:1,000 dilution, for 1 h. The cells were incubated with Alexa Fluor 488–conjugated anti-mouse IgG (1:300) or anti-rabbit IgG (1:300) and Alexa Fluor 594–labeled phalloidin (1:100) for 30 min. An extensive wash was performed between each step. The coverslips were then mounted onto slides in mounting medium (Invitrogen). Consecutive fluorescent microscope (Axio Observer.Z1) or laser scanning confocal microscope (TCS SP5 Acousto-Optical Beam Splitter; Leica).

To quantify the number of invadopodia per cell, melanoma cells stained with cortactin antibody (clone 4F11 05-180) and phalloidin were visualized under an upright fluorescence microscope (Axio Observer.Z1) with a 63x oil immersion objective. Invadopodia were defined as round dots positive for both cortactin staining and actin staining on the ventral side of cells (Yamaguchi et al., 2005; Artyom et al., 2006). Typically, invadopodia numbers from around 50 cells from 10 random 63x fields were counted for each experimental group. Because essentially all of the ventral actin dots were positively for cortactin, in some experiments, only the actin channel was used for quantification. Data are presented as scattered dots, with each dot representing the number of invadopodia in a single cell.

Live cell time-lapse recording

WM793 cells stably expressing cortactin-EGFP were plated on Texas red gelatin-coated glass-bottomed 35-mm tissue culture dishes (MatTek Corporation) 4 h before imaging. The culture medium was changed at 4 h and the solution supplemented with 100 mM Hepes buffer, pH 7.4. OxyFluo...
Invadopodial precursor quantitation
To quantitate invadopodial precursor formation, WM793 cells were plated onto Alexa Fluor 488–gelatin-coated coverslips in growth medium (10% FBS) and allowed to attach for 1 h. The medium was then changed to starvation medium (1% FBS), and the cells were starved overnight. To induce assembly of invadopodial precursor, FBS were added directly to the well to the final concentration of 10%. Cells were fixed with 4% PFA 30 or 60 min after induction and stained with phalloidin. Invadopodial precursors were defined as invadopodium–like actin dots at the ventral side of the cell without gelatin degradation activity.

For live imaging of invadopodial precursors, WM793 cells expressing Lifeact–mAPPLE were plated on gelatin-coated glass-bottom 35-mm tissue culture dishes for 4 h. Cells were then washed by RPMI 1640 containing 1% FBS and incubated overnight. Invadopodia was induced by adding 10% FBS, and cells were subjected to live cell imaging in a live cell incubator (XL S1 full enclosure incubator; Carl Zeiss) immediately. The invadopodium formation process was recorded for 60 min at 30-s intervals. The time-lapse recordings were examined frame by frame to identify newly assembled invadopodial precursors.

Membrane protein biotinylation assay
WM793 cells grown to 90% confluence were washed twice with ice-cold PBS, and freshly prepared 1 mM EZ-link sulfo-NHS-S-biotin (Thermo Fisher Scientific) in ice-cold PBS was added. After incubation on ice for 30 min, cells were washed twice with ice-cold PBS. The unreacted biotin was quenched for 10 min by incubation with ice-cold PBS containing 50 mM glycine. Cells were then washed three times with ice-cold PBS and lysed in 0.5 ml immunoprecipitation (IP) buffer (PBS, pH 7.4, 5 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, 50 mM NaF, 1 mM NaVO₃, and 1% Triton X-100). Cell lysate was briefly sonicated (5 s) and centrifuged at 14,000 rpm on a 4°C bench top centrifuge. The supernatant was incubated with NeutrAvidin beads (Thermo Fisher Scientific) at 4°C for 3 h. The beads were extensively washed with IP buffer, eluted with SDS-PAGE loading buffer, and subjected to Western blotting.

To examine MT1-MMP endocytosis, quenched cells were incubated at 37°C in cell cultural incubator for 30 min in the presence of 100 µM 2-APB or vehicle control to allow the internalization of biotinylated MT1-MMP. The internalization was stopped by washing the cells with ice-cold PBS for three times. Biotin present on the cell surface was cleaved off by a second MESNA treatment. Finally, cells were washed with PBS and then lysed for affinity purification.

For confocal assay, MESNA-treated cells were incubated in a cell culture incubator at 37°C for 30 or 60 min to allow recycling of the endocytosed biotinylated MT1-MMP. Recycling was stopped by washing the cells with ice-cold PBS for three times. Biotin reexposed to the cell surface was cleaved off by a second MESNA treatment. Finally, cells were washed with ice-cold PBS for three times and lysed in IP buffer for affinity purification.

Calcium assay
The effects of STIM1 shRNA, Orai1 shRNA, and ectopic STIM1 overexpression on store-operated Ca²⁺ influx were measured using Fluo4-AM as the Ca²⁺ indicator using a fluorescence microplate reader as described previously (Yang and Huang, 2005; Yang et al., 2009). In brief, Fluo4-AM–loaded cells in a 96-well plate were stimulated with 2 µM thapsigargin (in the presence of 0 mM extracellular Ca²⁺), and the change in fluorescence was measured using a fluorescence microplate reader for 300 s. Extracellular Ca²⁺ was then added to a final concentration of 2 mM, and the change in Fluo4 fluorescence was monitored for another 300 s.

Calcium imaging
Calcium imaging was performed with a confocal microscope (LSM710; Carl Zeiss) with a 1.4 NA oil immersion objective and sampling speed of 1-0.7 s/frame (512 x 512 pixels). For chemical Ca²⁺ indicator loading, WM793 cells were incubated with 5 µmol/liter Fluo4-AM (Invitrogen) for 10 min at room temperature. For measurement of Fluo4, excitation was at 488 nm, and fluorescence emission was collected at 490–540 nm.

Cell invasion assay
Cell invasion assay were performed using Matrigel-coated cell invasion insert (6.5-mm diameter and 8-µm pore size; BD) as previously described (Yang et al., 2009; Sun et al., 2011). In brief, melanoma cells (10⁵) suspended in starvation medium were added to the upper chamber of the invasion insert, and the insert was placed in a 24-well dish containing medium with or without 10% serum. Invasion assays were performed for 10 h, and cells were fixed with 3.7% formaldehyde. Cells were stained with crystal violet staining solution, and cells on the upper side of the insert were removed with a cotton swab. Three randomly selected fields (10× objectives) on the lower side of the insert were photographed, and the cells on the lower surface of the insert were counted.

Immunohistochemistry staining
Melanoma tissue microarray was purchased from Biomax. Tissue microarray slides were baked in a 60°C oven for 60 min, deparaffinized, and hydrated to deionized H₂O. Epitope was retrieved by incubating with 0.21% citrate buffer, pH 6, in a 99°C water bath for 30 min. After epitope retrieval, slides were washed in running water for 2–3 min, incubated with 3% H₂O₂ for 5 min, and washed with deionized H₂O and PBS. After slides were incubated with normal serum (1:20, diluted in 2% BSA-PBS) for 10 min at room temperature in a humidity chamber, slides were then incubated overnight with 150 µl monoclonal anti-STIM1 antibody (1:300; Thermo Fisher Scientific) at 4°C in a humidity chamber. Slides were then washed with PBS (PBS with Tween 20; three times, 5 min each) and incubated with biotinylated anti–mouse secondary antibody (1:500) at room temperature for 60 min. The slides were then incubated with avidin biotin complex (alkaline phosphatase conjugated) for 45 min. After three washes with PBS (5 min each), slides were incubated with Vulcan Fast red for 10–15 min, washed in deionized H₂O, and then counterstained using Harris modified hematoxylin (Thermo Fisher Scientific). Slides were then dehydrated and mounted. The tissue microarray was examined by a board-certified dermatopathologist (R. Mathew) for STIM1 staining intensity (on a 0–3 scale) and percentage of STIM1-positive cells (0–100%). A final STIM1 staining score for each sample was calculated by multiplying intensity score by percentage score. Melanoma samples with STIM1 staining score <1 were stratified to the “STIM1 negative or low” group, whereas samples with a score equal to or >1 were stratified to the “STIM1 medium or high” group.

1205Lu melanoma metastasis experiment
All animal work was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Moffitt Cancer Center. Nu/Nu immunodeficient mice were used for experimental lung metastasis experiments. 1205Lu human melanoma cells expressing the luciferase reporter were trypsinized and washed with PBS. Subsequently, 10⁵ cells in 0.2 ml PBS were injected into the lateral tail vein. Luciferase-based noninvasive bioluminescent imaging and analysis were performed as described previously with an imaging system (IVIS 200; Xenogen) by injecting 100 µl chloroform (2.5 µg/ml) via intraperitoneal injection to each mouse (Yang et al., 2009, 2012). The mice were imaged on day 0 and then on a weekly basis thereafter. At the end of the metastasis experiment, lungs were harvested from euthanized mice, fixed in paraformaldehyde, and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin.

Data analyses
Data are expressed as means ± SD or means ± SEM as indicated. Two-tailed p-values were determined by Student’s t-test, Mann–Whitney test, or Fisher’s exact test as indicated, with P < 0.05 defined as statistically significant.

Microscopy
For wide-field fluorescence microscopy, samples were examined with an automated upright microscope (Axiovert Z1; Carl Zeiss) through a 63×/1.4 NA Plan Apochromat oil immersion objective. Images were captured using the AxioCam MRM3. AxioVision version 4.7 software suite (Carl Zeiss), Realtime live cell microscopy was performed with an automated inverted microscope (Observer Z1; Carl Zeiss) outfitted with full enclosure incubator (37°C, 5% CO₂; XL S1) in RPMI 1640 medium. Images were captured using the charge-coupled camera (AxioCam MRM3) through a 40×, 1.4 NA Plan Apochromat oil objective. AxioVision version 4.7 software suite (Carl Zeiss) was used to manage the acquired images. Definite focus was used to ensure the stability of the focal plane over time.

For confocal microscopy, samples were viewed with an inverted microscope (DMi6000; Leica), confocal scanner (TCS SPS; Leica), and a 63×, 1.4 NA Plan Apochromat oil immersion objective (Leica). Argon 488 and HeNe 594 laser lines were applied to excite the samples, and a tuneable acousto-optical beam splitter was used to minimize cross talk between fluorochromes. Gain, offset, pinhole, and lookup table settings were identical.
for all samples. Images were captured with photomultiplier detectors and prepared with the LAS AF software version 1.6.0 build 1016 [leica].

Online supplemental material
Fig. S1 presents effects of Ca2+ influx blockade on invadopodium formation and ECM degradation. Fig. S2 shows the lack of effect of STIM1 overexpression, 2-APB treatment, and STIM1 and Orai1 knockdown on invadopodium lifetime. Fig. S3 demonstrates the role of Src in SOCE-mediated Ca2+ release and ECM degradation. Fig. S2 shows the lack of effect of STIM1 overexpression, 2-APB treatment, and STIM1 and Orai1 knockdown on invadopodium lifetime. Fig. S3 demonstrates the role of Src in SOCE-mediated Ca2+ release and ECM degradation. Fig. S2 shows the lack of effect of STIM1 overexpression, 2-APB treatment, and STIM1 and Orai1 knockdown on invadopodium lifetime.

References
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