Microtubule-associated histone deacetylase 6 supports the calcium store sensor STIM1 in mediating malignant cell behaviors

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Abstract

Stromal-interaction molecule 1 (STIM1) is an endoplasmic reticulum Ca$^{2+}$ storage sensor that promotes cell growth, migration and angiogenesis in breast and cervical cancers. Here we report that the microtubule-associated histone deacetylase 6 (HDAC6) differentially regulates activation of STIM1-mediated store-operated Ca$^{2+}$ entry (SOCE) between cervical cancer cells and normal cervical epithelial cells. Confocal microscopy of living cells indicated that microtubule integrity was necessary for STIM1 trafficking to the plasma membrane and interaction with Orai1, an essential pore subunit of SOCE. Cancer cells overexpressed both STIM1 and Orai1 compared with normal cervical epithelial cells. HDAC6 upregulation in cancer cells was accompanied by hypoacetylated α-tubulin. Tubastatin-A, a specific HDAC6 inhibitor, inhibited STIM1 translocation to plasma membrane and blocked SOCE activation in cancer cells but not normal epithelial cells. Genetic or pharmacological inhibition of HDAC6 blocked STIM1 membrane trafficking and downstream Ca$^{2+}$ influx, as evidenced by total internal reflection fluorescent images and intracellular Ca$^{2+}$ determination. In contrast, HDAC6 inhibition did not affect interactions between STIM1 and the microtubule plus-end-binding protein EB1. Analysis of surgical specimens confirmed that most cervical cancer tissues overexpressed STIM1 and Orai1, accompanied by hypo-acetylated α-tubulin. Together, our results identify HDAC6 as a candidate target to disrupt STIM1-mediated SOCE as a general strategy to block malignant cell behavior.
**Key words:** Histone deacetylase 6, Microtubule, Store-operated Ca$^{2+}$ entry, Stromal-interaction molecule 1, $\alpha$-tubulin acetylation
Introduction

Modulation of cytosolic Ca\textsuperscript{2+} levels provides versatile and dynamic signaling that mediates fundamental cellular functions, such as proliferation, migration, gene regulation and apoptosis (1). Increase in cytosolic Ca\textsuperscript{2+} concentrations occurs as a result of Ca\textsuperscript{2+} entry from the extracellular space and Ca\textsuperscript{2+} release from intracellular stores, mainly from the endoplasmic reticulum (ER). Store-operated Ca\textsuperscript{2+} entry (SOCE) is a major Ca\textsuperscript{2+} entry pathway in nonexcitable cells (2, 3). The SOCE activation includes several steps: (a) Stimulation of G proteins or protein tyrosine kinases activates phospholipase C, which hydrolyzes phosphatidylinositol bisphosphate to release the second messenger inositol-1, 4, 5-trisphosphate (IP3). (b) Binding of IP3 to IP3 receptor in the ER membrane causes rapid and transient Ca\textsuperscript{2+} release from ER lumen. (c) The decrease of ER luminal Ca\textsuperscript{2+} activates store-operated Ca\textsuperscript{2+} (SOC) channels in the plasma membrane, leading to a sustained influx of extracellular Ca\textsuperscript{2+} across the plasma membrane (2, 4). Two genes, STIM1 (stromal-interaction molecule 1) and Orai1, are responsible for SOCE activation (2). STIM1 functions as an ER Ca\textsuperscript{2+} sensor that detects store depletion. Once ER Ca\textsuperscript{2+} depleted, STIM1 aggregates into multiple punctae that translocate to the close proximity of plasma membranes. Orai1, an essential pore-forming component of SOC channel, translocates to the same STIM1-containing structures during ER Ca\textsuperscript{2+} depletion and opens to mediate Ca\textsuperscript{2+} entry.

STIM1 is required for the development and function of regulatory T cells (5, 6) and
STIM1-deficiency causes several autoimmune diseases and myopathy in human subjects and mouse models (7). The emerging importance of STIM1 in tumor biology has been highlighted in breast and cervical cancer (8-10). Inhibiting STIM1-mediating Ca\(^{2+}\) influx impaired focal adhesion turnover of breast cancer cells, which could be rescued by the small GTPases Ras and Rac (8). The reduction of Orai1 or STIM1 by RNA interference in highly metastatic human breast cancer cells or treatment with a pharmacological SOCE inhibitor decreased tumor metastasis in animal models. Our previous study demonstrated that epidermal growth factor (EGF) induced the aggregation and trafficking of STIM1 towards the proximity of the plasma membrane, where STIM1 specifically interacted with Orai1 to mediate SOCE (9). The SOCE was necessary for the activation of Ca\(^{2+}\)-dependent protease calpain and tyrosine kinase Pyk2 in migratory cervical cancer cells (9). The activation of calpain and Pyk2 regulated multiple signaling events crucial for focal adhesion turnover and cervical cancer cell migration. More importantly, our findings from analyzing surgical specimens suggested that the pathological significance of tumor STIM1 overexpression could be proposed to benefit the locomotion of cancer cells (9). Another study based on the microarray data analyses of breast cancer patients showed that transcriptionally-defined basal-like tumors, which are featured with a poor prognosis and a lack of effective therapies, are characterized by high STIM1 and low STIM2 mRNA expressions (10). To inhibit STIM1-dependent Ca\(^{2+}\) signaling by specifically targeting STIM1 activation and translocation in cancer cells is thus a potential
target for cancer therapy.

The precise localization and trafficking of intracellular proteins mostly depend on the integrity of cytoskeleton (11). The morphology of ER is maintained through a tight interaction with microtubules (12). The microtubule property and function are critically regulated by the post-translational modifications occurring on tubulin subunits, such as acetylation, detyrosination, polyglutamylation and polyglycylation (13). Reversible acetylation of Lys40 on α-tubulin is important for regulating microtubule stability and function and seems to have a role in cell motility. Histone deacetylase 6 (HDAC6), a unique cytoplasmic member of histone deacetylase family, has been identified as a microtubule-associated protein and could function as the prominent α-tubulin deacetylase (14-16). Here we test the hypothesis that the modulation of microtubule dynamics by HDAC6-mediated α-tubulin deacetylation plays an important role in controlling STIM1-dependent Ca$^{2+}$ signaling. The results showed that microtubule-associated histone deacetylase 6 is a potential target to interfere with Ca$^{2+}$ store sensor STIM1-mediated cancer malignant behaviors.
Materials and Methods

Surgical specimens. We enrolled 6 cases with pair frozen tissues of cervical carcinoma and adjacent non-cancer epithelia for immunoblotting analyses and another 21 cases of paraffin blocks containing tissues of cervical carcinoma and adjacent non-cancer epithelia for immunofluorescent (IF) staining. All these cases are patients with early stage (International Federation of Gynecology and Obstetrics staging Ib) cervical cancer who underwent radical hysterectomy and pelvic lymphadenectomy at National Cheng Kung University Hospital (NCKUH), Taiwan. Normal cervical epithelial cells were derived from the portio surface and transformation zone of the cervix uteri obtained from 35- to 45-year-old women with no history of cervical dysplasia and who underwent hysterectomy for benign uterine diseases at NCKUH. For explant culture of cervical carcinoma cells, tissue fragments of about 10 mm$^3$ were obtained from the colposcopy-guided cervical biopsy to minimize the contamination of normal cervical epithelium at the Department of Obstetrics and Gynecology, NCKUH. The collection of surgical specimens was approved by the institutional review board of National Cheng Kung University Hospital.

Cell culture, transfection and RNA interference. Cultures of normal cervical epithelial cells, primary cancer cells, cervical cancer cell lines (SiHa, HT-3, CaSki and HeLa) and stable pools of cervical cancer cells overexpressing EGFP-STIM1 were prepared as previously described (17, 18). Cell lines were authenticated by the short tandem repeats analysis using the Promega
StemElite™ ID System (GeneLabs Life Science Corp., Taiwan). Primary cancer cells from two subjects were tested positive for type 16 and 58 human papillomavirus (HPV) genome respectively, using the EASYCHIP® HPV genotyping array (King Car Yuan Shan Institute, Taiwan). The EASYCHIP® HPV blot simultaneously detects 39 types of HPV on the basis of reverse blot hybridization. The sequences of RNAi targeting human HDAC6 and EB1 are listed in Supplementary Materials and Methods.

**Antibodies, chemicals and immunoblotting.** The detailed information of chemicals and antibodies are listed in Supplementary Materials and Methods. Immunoblots were detected with affinity-purified antibodies against various molecules and horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch). Bands in the immunoblots were quantified using Vision WorksLS software (UVP).

**Immunofluorescence and confocal images.** IF stainings were done using affinity-purified antibodies against various molecules and AlexaFluor-conjugated secondary antibodies (Invitrogen). The fluorophores were excited by laser at 405, 488, or 543 nm and detected by a scanning confocal microscope (FV-1000, Olympus). The intensity and distribution of IF staining of STIM1, Orai1 and acetyl-α-tubulin were graded as high or low in each surgical specimen. High grade indicates that IF staining intensity and distribution are more than 30% of tumor area, whereas low grade indicates that the staining intensity and distribution are less than 30% of tumor area. A pixel-by-pixel analysis by the colocalization algorithm of FV-1000
software was used to assess the colocalization of different molecules in confocal images. Cell margin was visualized by differential interference contrast (DIC) images and by immunostaining with the endogenous plasma membrane marker Na-K-2Cl cotransporter 1 (NKCC1) (19). The “juxta-plasma membrane region” was defined as 2 μm distances to the plasma membrane and STIM1 intensity within this region was defined as STIM1 membrane trafficking accordingly.

**Single cell [Ca^{2+}]_{i} measurement.** Intracellular Ca^{2+} concentration ([Ca^{2+}]_{i}) was measured at 37°C with the Fura-2 fluorescence ratio method on a single-cell fluorimeter as previously described (9, 20). The Fura-2 was excited alternatively between 340 nm (I_{340}) and 380 nm (I_{380}) using the Polychrome IV monochromator (Till Photonics) and images were detected by the Olympus IX71 inverted microscope equipped with a xenon illumination system and an IMAGO CCD camera (Till Photonics). The fluorescence intensity of excitation at 510 nm was monitored to calculate [Ca^{2+}]_{i} by TILLvisION 4.0 program (Till Photonics).

**Statistics.** All values were reported as mean±SEM (standard error of the mean). Student’s pair t test or unpaired t test was used for statistical analyses. Differences between values were considered significant when P < 0.05.
Results

**Microtubule is necessary for STIM1 membrane trafficking.** Serial Z-section of confocal images from living cells suggested that STIM1 scattered at the middle or bottom plane prior to EGF stimulation (Supplementary Fig. S1A). Upon EGF stimulation, the general increase in STIM1 fluorescent signals was observed in the serial Z-sections. The formation and redistribution of STIM1 punctae towards plasma membrane was more apparent at the bottom planes. Disruption of actin filaments by cytochalasin D showed no effect on STIM1 puncta formation and trafficking (Supplementary Fig. S1B). In contrast, STIM1 trafficking was significantly inhibited following the collapse of microtubule network induced by colcemid (Supplementary Fig. S1C). Consequently, the association between STIM1 and Orai1 at the proximity of plasma membrane was significantly attenuated (Supplementary Fig. S1D-F), suggesting that the microtubule integrity is necessary for STIM1 trafficking and the subsequent interaction with the SOC channel Orai1.

**HDAC6 inhibition induces the differential α-tubulin acetylation between normal and cancer cells.** We studied whether the modulation of microtubule dynamics by HDAC6 plays an important role in STIM1-dependent cancer malignant behaviors. HDAC6 was localized exclusively in the cytoplasm of cervical cancer cells, where it was mainly associated with microtubules instead of actin cytoskeleton (Supplementary Fig. S2A). To differentiate
the post-translational modifications of microtubules between normal and cancer cells, we analyzed the expression patterns of STIM1, Orai1, HDAC6 and acetyl-α-tubulin in cervical epithelial cells with different malignant potential (Fig. 1A). The expression levels of STIM1, Orai1, HDAC6 and acetyl-α-tubulin were similar among the primary cultures from different normal subjects. Cervical cancer HT3 and SiHa cell lines clearly overexpressed STIM1 and Orai1, compared to normal subjects. Interestingly, HDAC6 was upregulated in cervical cancer cells, which was accompanied by hypo-acetyl-α-tubulin. The coincident overexpression of HDAC6 and STIM1 in cervical cancer cells implies a possible role of α-tubulin acetylation in regulating STIM1 function. More importantly, tubastatin-A, a selective HDAC6 inhibitor (21), showed a differential effect on α-tubulin acetylation between cervical cancer cells and normal cervical epithelial cells (Fig. 1B-1D). Incubation with a low concentration of tubastatin-A (0.5 μM) for a short time period (< 5 hours) induced a significant increase in acetyl-α-tubulin of cervical cancer SiHa cells, whereas that of normal cervical epithelial cells was not affected (Fig. 1B and 1D). Moreover, incubation with a higher concentration of tubastatin-A (5 μM) for 5-10 hours remarkably caused the acetylation of α-tubulin in cervical cancer SiHa cells, but only modestly induced that of normal cervical epithelial cells.

**Active SOCE with hypoacetylated α-tubulin in cervical cancer.** To study the clinical relevance, the expression pattern of STIM1, Orai1, HDAC6 and acetyl-α-tubulin were
examined in the surgical specimens of cervical cancer (Fig. 2A-2E). Compared to that of non-cancerous tissues, the expression level of STIM1 in tumor tissues was increased in all cases analyzed by immunoblotting (Fig. 2A), which is consistent with our previous study (9). STIM1 overexpression in tumor tissues was accompanied by HDAC6 upregulation in most cases (67%) (Fig. 2A). We also enrolled another 21 cases with paraffin blocks for immunofluorescent staining (Fig. 2B-2E). Cervical cancer tissues clearly expressed STIM1 and Orai1, whereas these proteins were rarely detected in the adjacent non-cancerous cervical epithelia. On the other hand, acetyl-α-tubulin was more obvious in non-cancerous cervical epithelia, when compared to cervical cancer tissues (Fig. 2C). We graded the tumor tissues by the distribution and intensity of immunofluorescent staining (Fig. 2D and 2E). The majority of these enrolled cases showed low grade of acetyl-α-tubulin and high grade of STIM1 and Orai1. We have tested the selectivity of Orai1 antibody by using an Orai1 blocking peptide (Supplementary Fig. S2B-S2C). Evidence from images and immunoblotting suggests that the Orai1 antibody is more selective with immunofluorescent staining than immunoblotting.

A key question remained unanswered is “if there is a difference in ER Ca^{2+} release and SOCE between normal cervical cells and cervical cancer cells.” To answer this question and to avoid bias from the clonal effect of cell lines, we did the primary tissue cultures from normal subjects and cervical cancer patients. Primary normal and cancer cells were cultured in the same condition for three days before experiments. As shown in Figure 2F-2G,
TG-induced Ca\textsuperscript{2+} release from ER store was not significantly different between normal cervical cells and primary cervical cancer cells. Both normal cervical cells and primary cervical cancer cells displayed SOCE that was sensitive to SOCE inhibitor SKF96365. However, the SOCE of primary cervical cancer cells was more active, which is consistent with the molecular evidences showing overexpressed STIM1 and Orai1 in cervical cancer tissues.

**HDAC6 is involved in SOCE activation of cervical cancer cells.** We further studied whether HDAC6 is involved in the regulation of SOCE. As shown in Fig. 3A, tubastatin-A dose-dependently induced \(\alpha\)-tubulin acetylation in cervical cancer SiHa and CaSki cell lines as well as primary cancer cells. TG- or EGF-induced SOCE activation in cervical cancer cells was also inhibited by tubastatin-A in a dose-dependent manner (Fig. 3B-3E). Importantly, normal cervical epithelial cells displayed less active SOCE, which was insensitive to tubastatin-A (Fig. 3F and 3G). We also studied the role of other HDAC members on SOCE activation. Neither entinostat (MS275, an inhibitor for HDAC1, 2 and 3) nor sodium butyrate (NaB, a broad spectrum inhibitor for HDACs excluding HDAC6 and 10) affected \(\alpha\)-tubulin acetylation and SOCE activation in cervical cancer cells (Supplementary Fig. S3A-S3C). These results suggest that HDAC6, rather than other HDACs, is critically involved in the regulation of SOCE activation of cervical cancer cells.
**HDAC6 regulates STIM1 membrane trafficking.** The mechanism by which HDAC6 regulates SOCE was studied. TG or EGF stimulated the aggregation and trafficking of STIM1 proteins towards the proximity of plasma membrane in cervical cancer SiHa, CaSki and HeLa cells (Fig. 4, Supplementary Fig. S4, Supplementary Fig. S5A and S5B), without altering the acetylation level of α-tubulin (Supplementary Fig. S2D). HDAC6 inhibitor tubastatin-A caused apparent STIM1 aggregations in cytosol, which was not correlated with STIM1 movement towards juxta-membrane region induced by TG or EGF (Fig. 4, Supplementary Fig. S4, Supplementary Fig. S5A-S5B). In contrast, neither MS275 nor NaB affected the puncta formation and membrane trafficking of STIM1 (Supplementary Fig. S3D). These results indicate that STIM1 movement towards plasma membrane is critically regulated by HDAC6.

To dissect the role of microtubule dynamics in STIM1 trafficking in a more quantitative way, we monitored EGFP-STIM1 movements by the total internal reflection fluorescence (TIRF) microscopy (Supplementary Movies 1 and 2), which visualizes the fluorescence restricted to within ~100 nm from the plasma membrane of living cells (22). STIM1 was clearly located in the TIRF plane. EGF stimulated STIM1 trafficking towards the cell periphery, which was abolished by tubastatin-A (Supplementary Fig. S4A). The [Ca^{2+}]_i measurement by TIRF also demonstrated that EGF-induced Ca^{2+} spikes was inhibited by tubastatin-A in cervical cancer SiHa cells (Supplementary Fig. S4B).

The important role of HDAC6 on SOCE activation and STIM1 trafficking was further
demonstrated by the knockdown approaches (Fig. 5 and Supplementary Fig. S5C-S5E). HDAC6 knockdown by different duplexes of siRNA in cervical cancer SiHa and CaSki cells was accompanied by increasing α-tubulin acetylation (Fig. 5A and Supplementary Fig. S5C). Moreover, the SOCE activation was significantly inhibited by HDAC6 silencing (Fig. 5B, 5C, Supplementary Fig. S5D and S5E). In the presence of HDAC6-specific siRNAs, EGF or TG caused obvious STIM1 puncta without trafficking towards juxta-plasma membrane (Fig. 5D and 5E). Importantly, the inhibition of STIM1 membrane trafficking by HDAC6 knockdown or inhibitor decreased the proliferation and migration of cervical cancer cells (Supplementary Figure S5F and S5G), suggesting that STIM1 membrane trafficking is a potential target to manipulate STIM1-mediated cancer malignant behaviors.

**Microtubule plus-end tracking proteins are necessary for STIM1 trafficking.** The interaction between STIM1 and the microtubule plus-end-binding protein EB1 at the growing plus end of microtubules plays an important role in the remodeling of ER morphology (23-25). We studied the role of microtubule plus-end tracking complexes on STIM1 membrane trafficking and the subsequent SOCE activation in cervical cancer SiHa, CaSki and HeLa cells. SOCE activation was significantly inhibited by the EB1-specific siRNA (Fig. 6A-6C). Consistently, EB1 silencing blocked STIM1 trafficking towards plasma membrane and resulted in the accumulation of STIM1 aggregation in cytosol (Fig. 6D and 6E). However, the
association between STIM1 and EB1 was not affected by tubastatin-A (Fig. 7). These results suggest that the association between STIM1 and microtubule plus-end tracking complex is necessary for STIM1 trafficking and the subsequent SOCE activation, but HDAC6 is not involved in the binding of STIM1 with EB1.
Discussion

This study highlights an important role of microtubule-associated HDAC6 that is required for SOCE activation by optimizing the localization of ER Ca\(^{2+}\) sensor STIM1 towards plasma membrane. This conclusion is supported by the following evidences. (a) The interaction between STIM1 and the SOC channel Orai1 at the proximity of plasma membrane was significantly attenuated by the disruption of microtubule network. (b) The siRNA-mediated knockdown of HDAC6 expression or the pharmacological blockade of HDAC6 activity significantly decreased STIM1 membrane trafficking and the subsequent SOCE. We also found that Ca\(^{2+}\) depletion from ER store enhanced the interaction between STIM1 and the microtubule plus-end-binding protein EB1, which was required for STIM1 trafficking towards plasma membrane. α-tubulin acetylation is a post-translational modification of stabilized microtubules (13, 26). This is the first study showing the important function of HDAC6-mediated α-tubulin deacetylation in regulating STIM1-dependent SOCE, as evidenced by confocal images, TIRF images and single cell [Ca\(^{2+}\)]\(_i\) measurement.

STIM1-dependent Ca\(^{2+}\) signaling plays an important role in controlling cancer cell growth, migration, and angiogenesis (9). STIM1 involves the activation of Ca\(^{2+}\)-regulated calpain and Pyk2 which regulates the focal-adhesion dynamics of migratory cervical cancer cells. Because of an increase of p21 protein levels and a decrease of Cdc25C protein levels, STIM1-silencing in cervical cancer cells significantly inhibits cell proliferation by arresting
the cell cycle at the S and G2/M phases. STIM1 also regulates the release of vascular endothelial growth factor from cervical cancer cells. Interference with STIM1 expression or blockade of SOCE activity inhibits tumor angiogenesis and growth in animal models, confirming the crucial role of STIM1-mediated Ca\(^{2+}\) influx in aggravating tumor development \textit{in vivo}. To block STIM1-dependent Ca\(^{2+}\) signaling is thus an alternative for cancer adjuvant therapy. To target STIM1-mediated cancer malignant behaviors could be achieved by RNAi-mediated reduction of STIM1 and Orai1, the pharmacological inhibition of SOCE activity, or the interference with STIM1 trafficking towards Orai1-containing juxta-plasma membrane area. Although RNAi holds a hope as a novel nucleic acid-based therapeutic against a wide variety of diseases, to date most RNA therapeutics are still in early clinical trials and only the modest and circumscribed successes have been demonstrated in animals \cite{27}. Furthermore, the physiological and pathophysiological functions of STIM1-mediated SOCE have been implicated in several tissues \cite{2, 28}, including immune system and cardiovascular system. The systemic administration of SOC channel blockers to cancer patients may induce unexpected adverse effects. Therefore, to specifically target STIM1 membrane trafficking of cancer cells is a feasible strategy to inhibit STIM1-mediated cancer malignant behaviors.

Our clinical study has demonstrated that STIM1 overexpression was noted in cervical cancer tissues and the tumor expression level of STIM1 was associated with the clinical
outcome of early-stage cervical cancer (9). Here we studied the cell type-specific regulation of STIM1-dependent $\mathrm{Ca}^{2+}$ influx by using normal cervical epithelial cells and cervical cancer cells as the pair. Consistent with the previous findings in surgical specimens, STIM1 and Orai1 were differentially expressed between normal cervical epithelial cells and cervical cancer cell lines. Consequently, cervical cancer cells exhibited upregulated SOCE activity, compared with normal cervical epithelial cells. Moreover, the close association between SOCE activity, $\alpha$-tubulin acetylation and HDAC6 abundances suggest the possibility that HDAC6-mediated $\alpha$-tubulin deacetylation contributes to the cancer cell-specific regulation of STIM1 activation. Interestingly, normal cervical epithelial cells and cervical cancer cells showed a different sensitivity to HDAC6 inhibitor. The differential inhibitory effects of tubastatin-A on SOCE activation can be explained by the fact that tubastatin-A induced the different $\alpha$-tubulin acetylation between normal and cancer cells. Most importantly, cervical cancer tissues overexpressed STIM1 as well as HDAC6 that provides a niche to inhibit the interaction between STIM1 and Orai1 through microtubule-associated HDAC6 pathways.

In conclusion, the microtubule-associated HDAC6 is necessary for STIM1 trafficking towards juxta-membrane regions. Tubastatin-A blocks STIM1 trafficking and inhibits SOCE activation in cervical cancer cells but not in normal cervical epithelial cells. This makes the HDAC6-mediated STIM1 recruitment towards the plasma membrane as an attractive target to interfere with STIM1-mediated cancer malignant behaviors.
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Author contributions

Y.T.C., Y.F.C., W.T.C., K.Y.L., J.Y.C., H.C.C., and M.R.S. designed research; Y.T.C., Y.F.C., W.T.C., K.Y.L., and Y.L.L performed research; Y.T.C., Y.F.C., W.T.C. and M.R.S. analyzed data; and Y.T.C., Y.F.C., and M.R.S. wrote the paper.
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Figure legends

Figure 1. Tubastatin-A differentially induces α-tubulin acetylation in normal cervical cells and cervical cancer cells. **A,** Expression pattern of STIM1, Orai1, HDAC6 and acetyl-α-tubulin in normal cervical epithelial cells and cervical cancer cells. Expression of STIM1, Orai1 and HDAC6 were normalized against β-actin. Acetyl-α-tubulin was normalized against α-tubulin. **B-D,** Tubastatin-A shows a differential effect on α-tubulin acetylation between cervical cancer SiHa cells and normal cervical epithelial cells. *Tyr-α-tubulin,* tyrosinated α-tubulin. **B,** Cells were incubated with a low dose of tubastatin-A (0.5 μM) for a short time period (2-5 hr). **C,** Cells were incubated with a higher dose of tubastatin-A (5 μM) for a long time period (5-10 hr). **D,** Densitometric quantification of acetyl-α-tubulin levels, mean±SEM (n = 3).

Figure 2. Active SOCE with hypo-acetyl-α-tubulin in cervical cancer. **A,** Expression pattern of STIM1 and HDAC6 in early-stage cervical cancer. Cervical cancer with pair frozen tissues of carcinoma and adjacent non-cancer epithelia were analyzed by immunoblotting. **N,** non-cancer epithelia. **T,** tumor tissues. Expression of STIM1 and HDAC6 were normalized against β-actin. **B & C,** Immunofluorescent stainings of STIM1, Orai1 and acetyl-α-tubulin in surgical specimens of cervical cancer. Representative images of 21 different cases. *Scale bar,* 50 μm. **D & E,** Tumor grade of STIM1, Orai1 and acetyl-α-tubulin in immunofluorescent
stainings. Case numbers are indicated in parentheses beside each point. **F & G**, Representative 

[Ca\(^{2+}\)]\(_i\) measurement in normal cervical cells (from Subject 5) and primary cervical cancer cells (from Subject 2). Mean traces of [Ca\(^{2+}\)]\(_i\) measurement from at least 30 different cells in each subject. The SOCE amplitude indicates the rise of [Ca\(^{2+}\)]\(_i\), in replenishment of [Ca\(^{2+}\)]\(_o\) from 0 to 2 mM. Each value represents mean±SEM of at least 30 cells. Similar results were observed in primary tissue cultures from 6 cases of normal cervix and 2 cases of cervical carcinoma. *Arrow*, adding 2 μM thapsigargin (TG); SKF96365: SOCE inhibitor; *P<0.01.

**Figure 3. HDAC6 is important for SOCE activation in cervical cancer cells.**

* A, Tubastatin-A induces α-tubulin acetylation in different types of cervical cancer cells. Cells were incubated with different concentrations of tubastatin-A for 10 hours. Acetyl-α-tubulin was normalized against α-tubulin levels. **B & C**, Tubastatin-A inhibited TG-associated SOCE in cervical cancer SiHa cells with a concentration-dependent manner. Mean traces for [Ca\(^{2+}\)]\(_i\) measurement from at least 80 different cells. *Arrow*, adding 2 μM thapsigargin (TG). Each point in the dose-response curve (C) represents mean±SEM from at least 3 different experiments. **D & E**, Tubastatin-A dose-dependently inhibits EGF-associated SOCE in cervical cancer SiHa and CaSki cell lines and primary cervical cancer cells from tissue cultures. **D**, Mean traces for [Ca\(^{2+}\)]\(_i\) measurement from at least 60 different cells. *Arrow*, adding 100 ng/mL EGF. **E**, Quantitative analyses of changes in Ca\(^{2+}\) levels (Δ[Ca\(^{2+}\)]\(_i\)). Each
value represents mean±SEM of at least 60 cells. *P<0.01. \textbf{F & G}, Tubastatin-A showed no effect on SOCE activation in normal cervical cells. \textbf{F}, Mean traces for [Ca$^{2+}$]$_i$ measurement from at least 30 different cells. \textbf{G}, Quantitative analyses of Δ[Ca$^{2+}$]$_i$. Each value represents mean±SEM of at least 30 cells. *P<0.01.

**Figure 4. HDAC6 inhibitor blocks STIM1 membrane trafficking.** \textbf{A}, Cervical cancer SiHa cells overexpressing EGFP-STIM1 were pre-incubated with 0.1% DMSO or 5 μM tubastatin-A for 10 hours prior to TG (2 μM, 10 minutes) or EGF (100 ng/mL, 20 minutes) stimulation. Nuclei: Hoechst 33258 (blue). Na-K-2Cl cotransporter 1 (NKCC1): an endogenous plasma membrane marker. DIC: cell morphology in differential interference contrast images. \textit{Right panel}, the enlargement of areas indicated by rectangles in whole cell images. \textit{Dashed line}, the area for quantitative analyses of STIM1 fluorescent intensity (F.I.) shown in \textbf{B}. Representative confocal images from at least 3 different experiments. \textit{Scale bar}, 10 μm. \textbf{B}, Quantitative analysis of STIM1 fluorescent intensity along the dashed line in \textbf{A}. \textit{Grey rectangle}, 2 μm distances to the plasma membrane were defined as the “juxta-plasma membrane region”. \textbf{C}, Quantitative analyses of STIM1 fluorescent intensity at juxta-plasma membrane region. Each value represents mean±SEM from at least 30 different cells. *P<0.01.

**Figure 5. HDAC6 regulates STIM1 trafficking and SOCE activation.** \textbf{A}, HDAC6
knockdown by two independent siRNA duplexes in cervical cancer SiHa cells. B & C, HDAC6 silencing inhibits EGF or TG-induced SOCE activation. Mean traces of \([\text{Ca}^{2+}]_i\) measurement from at least 60 different cells. Arrow, adding EGF (100 ng/mL) or TG (2 \(\mu\)M). Right panel, Quantitative analyses of \(\Delta[\text{Ca}^{2+}]_i\). Each value represents mean±SEM of at least 60 cells. *P<0.01. D & E, HDAC6 silencing inhibits EGF or TG-induced membrane trafficking of STIM1. Lower panel, the enlargement of areas indicated by rectangles in whole cell images. Arrow head, STIM1 puncta at juxta-plasma membranes. Arrow, the aggregation of STIM1 in cytosol. Dashed line, the area for quantitative analyses of STIM1 fluorescent intensity (F.I.) shown in E. The representative confocal images from at least 5 different experiments. Scale bar, 10 \(\mu\)m. E, Quantitative analysis of STIM1 fluorescent intensity along the dashed line in A. Grey rectangle, 2 \(\mu\)m distances to the plasma membrane were defined as the “juxta-plasma membrane region”.

Figure 6. Microtubule end-binding protein EB1 is necessary for SOCE activation and STIM1 membrane trafficking. A, Representative immunoblots showing siRNA-mediated EB1 knockdown in cervical cancer SiHa and HeLa cells. B & C, EB1 silencing inhibits TG-induced SOCE activation. Mean traces of \([\text{Ca}^{2+}]_i\) measurement from at least 60 different cells. C, Quantitative analyses of \(\Delta[\text{Ca}^{2+}]_i\). Each value represents mean±SEM from at least 60 cells from 3 different experiments. D & E, EB1 silencing inhibits EGF or TG-induced STIM1
membrane trafficking in cervical cancer SiHa cells. Nuclei were stained with Hoechst 33258 (blue). **Lower panel,** the enlargements of areas indicated by rectangles in whole cell images. **Arrow head,** STIM1 puncta at juxta-plasma membranes. **Arrow,** the aggregation of STIM1 in cytosol. **Dashed line,** cell periphery. **Scale bar,** 10 μm. **E,** Quantitative analyses of STIM1 fluorescent intensity at juxta-plasma membranes. Each value represents mean±SEM from at least 50 different cells. *P<0.01.

**Figure 7.** HDAC6 inhibitor tubastatin-A does not alter the interaction between STIM1 and microtubule end-binding protein EB1. Cervical cancer SiHa (A-C) and CaSki (D-F) cells were pre-incubated with 0.1% DMSO or 5 μM tubastatin-A for 10 hours prior to TG (2 μM, 10 minutes) or EGF (100 ng/mL, 20 minutes) stimulation. Nuclei were stained with Hoechst 33258 (blue). **Lower panels in (A) & (D),** the enlargements of areas indicated by rectangles in whole cell images. Representative confocal images from at least 3 different experiments. **Arrow head,** STIM1 puncta at juxta-plasma membranes. **Arrow,** the aggregation of STIM1 in cytosol. **Scale bar,** 10 μm. **Dashed lines in (A) & (D),** the area for quantitative analyses of STIM1 fluorescent intensity (F.I.) shown in (B) & (E). **Grey rectangle in (B) & (E),** 2 μm distances to the plasma membrane were defined as the “juxta-plasma membrane region”. **(C) & (F),** Colocalization ratio between STIM1 and EB1 with pixel-by-pixel analyses. Each value represents mean±SEM from at least 30 different cells. *P<0.01.
### Fig. 1

#### A

<table>
<thead>
<tr>
<th></th>
<th>Normal cervical cells</th>
<th>Cervical cancer cells</th>
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<tbody>
<tr>
<td><strong>STIM1</strong></td>
<td>1.0 1.0 1.1 1.1</td>
<td>1.4 2.7 fold</td>
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<tr>
<td><strong>Orai1</strong></td>
<td>1.0 1.0 1.0 1.1</td>
<td>1.9 2.4 fold</td>
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<tr>
<td><strong>HDAC6</strong></td>
<td>1.0 1.2 1.0 1.1</td>
<td>1.8 2.3 fold</td>
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<tr>
<td><strong>Acetyl-(\alpha)-tubulin</strong></td>
<td>1.0 1.0 0.9 1.0</td>
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<td><strong>(\alpha)-tubulin</strong></td>
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<td></td>
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<tr>
<td><strong>(\beta)-actin</strong></td>
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#### B

- **Cervical cancer cells**
  - 0.5 \(\mu\)M Tubastatin-A
  - Acetyl-\(\alpha\)-tubulin
  - Tyr-\(\alpha\)-tubulin
  - \(\alpha\)-tubulin
  - \(\beta\)-actin

- **Normal cervical cells**
  - 0 2 5 0 2 5 hr
  - Acetyl-\(\alpha\)-tubulin
  - Tyr-\(\alpha\)-tubulin
  - \(\alpha\)-tubulin
  - \(\beta\)-actin

#### C

- **Cervical cancer cells**
  - 5 \(\mu\)M Tubastatin-A
  - Acetyl-\(\alpha\)-tubulin
  - Tyr-\(\alpha\)-tubulin
  - \(\alpha\)-tubulin
  - \(\beta\)-actin

- **Normal cervical cells**
  - 0 5 10 0 5 10 hr
  - Acetyl-\(\alpha\)-tubulin
  - Tyr-\(\alpha\)-tubulin
  - \(\alpha\)-tubulin
  - \(\beta\)-actin

#### D

- **Cervical cancer SiHa cells**
- **Normal cervical cells**

- **0.5 \(\mu\)M Tubastatin-A**
- **5 \(\mu\)M Tubastatin-A**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Acetyl-(\alpha)-tubulin (relative of control)</th>
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**Fig. 2**

**A**

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<td>1.2</td>
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</table>

**B**

- **STIM1/Orai1/Nucleus**
  - Epithelia
  - Non-cancer tissue
  - Cancer tissue

- **Acetyl-α-tubulin/Orai1**
  - Epithelia
  - Non-cancer tissue
  - Cancer tissue

**D**

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<th>Tumor grade in Orai1</th>
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<table>
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<th>n=2</th>
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**E**

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<th>n=15</th>
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<tbody>
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<td></td>
</tr>
<tr>
<td>High</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

**F**

- Normal cervical cells#5
  - $[\text{Ca}^{2+}]_i$: 0 mM, 2 mM
  - Control
  - SKF96365 (50 μM)

- Primary cancer cells#2
  - $[\text{Ca}^{2+}]_i$: 0 mM, 2 mM
  - Control
  - SKF96365 (50 μM)

**G**

- Changes in $[\text{Ca}^{2+}]_i$ levels ($\Delta[\text{Ca}^{2+}]$)
  - Normal cervical cells#5
  - Primary cancer cells#2
  - Control
  - SKF96365 (50 μM)
Microtubule-associated histone deacetylase 6 supports the calcium store sensor STIM1 in mediating malignant cell behaviors

Ying-Ting Chen, Yih-Fung Chen, Wen-Tai Chiu, et al.

Cancer Res  Published OnlineFirst May 22, 2013.