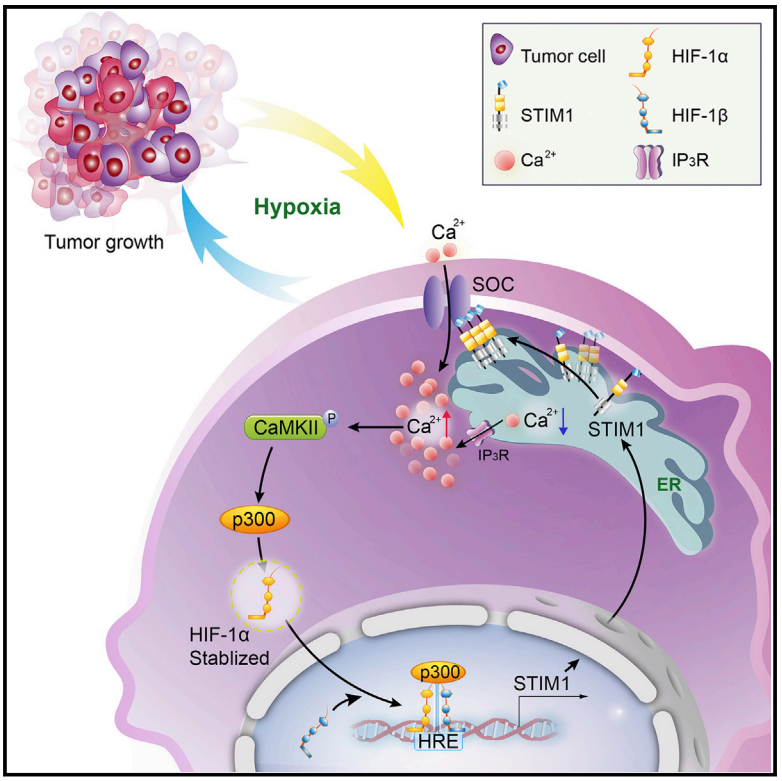
Report

Cell Reports

STIM1 Mediates Hypoxia-Driven Hepatocarcinogenesis via Interaction with HIF-1

Graphical Abstract



Authors

Yongsheng Li, Bo Guo, Qichao Xie, ..., Ying Zhu, Hongxiang Chen, Bo Zhu

Correspondence

yli@tmmu.edu.cn (Y.L.), b.davis.zhu@gmail.com (B.Z.)

In Brief

SOCE (STIM1 mediated) and HIF-1 are important regulators of tumorigenesis; however, the relationship between them, as well as the Ca^{2+} mobilization route in cancer cells during hypoxia, is unknown. Li et al. report a regulatory circuit involving HIF-1 and STIM1 that is required for SOCE and growth of hypoxic HCCs.

Highlights

- STIM1 elevation correlates with HIF-1α during hypoxic tumor growth
- HIF-1 transcripts STIM1 and promotes SOCE in hypoxic HCCs
- STIM1-mediated SOCE feedforward contributes to HIF-1 accumulation in hypoxic HCCs
- HIF1-STIM1 circuit orchestrates hypoxic hepatocarcinogenesis

Li et al., 2015, Cell Reports *12*, 388–395
July 21, 2015
http://dx.doi.org/10.1016/j.celrep.2015.06.033

CellPress

OPEN ACCESS
CellPress

Cell Reports
Report

STIM1 Mediates Hypoxia-Driven Hepatocarcinogenesis via Interaction with HIF-1

Yongsheng Li,[1,2,5,*] Bo Guo,[1,5] Qichao Xie,[1] Duyun Ye,[3] Dongxin Zhang,[3] Ying Zhu,[1] Hongxiang Chen,[4] and Bo Zhu[1,*]
[1]Institute of Cancer, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, China
[2]Department of Anesthesia, Perioperative and Pain Medicine, Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA
[3]Department of Pathophysiology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China
[4]Department of Dermatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China
[5]Co-first author
*Correspondence: yli@tmmu.edu.cn (Y.L.), b.davis.zhu@gmail.com (B.Z.)
http://dx.doi.org/10.1016/j.celrep.2015.06.033

SUMMARY

Hypoxia and intracellular Ca^{2+} transients are fundamental traits of cancer, whereas the route and regulation of Ca^{2+} mobilization in hypoxic tumorigenesis are unknown. Here, we show that stromal-interaction molecule 1 (STIM1), an ER Ca^{2+} sensor, correlates with elevated hypoxia-inducible factor-1 alpha (HIF-1α) in hypoxic hepatocarcinoma cells (HCCs) and is upregulated during hepatocarcinoma growth. HIF-1 directly controls STIM1 transcription and contributes to store-operated Ca^{2+} entry (SOCE). STIM1-mediated SOCE is also required for HIF-1 accumulation in hypoxic HCCs via activation of Ca^{2+}/calmodulin-dependent protein kinase II and p300. Administration of YC-1, a HIF-1 inhibitor, or knockdown of *HIF1A* significantly diminishes hypoxia-enhanced STIM1 and suppresses tumorigenesis. Moreover, ectopic expression of STIM1 or HIF-1α partially reverses impaired growth of tumors treated with YC-1. These results suggest a mutual dependency and regulation of STIM1 and HIF-1 in controlling Ca^{2+} mobilization and hypoxic tumor growth and highlight a potential target for early hypoxia-related intervention.

INTRODUCTION

Hypoxia is a common feature within the tumor mass and contributes to tumor growth (Harris, 2002). The transcription factor hypoxia-inducible factor-1 (HIF-1), by binding the hypoxia-responsive elements (HREs), plays a central role in hypoxic tumorigenesis (Semenza, 2003). Understanding the oxygen-sensing processes and the mechanisms through which HIFs regulates the cellular response to hypoxia is of great importance for therapeutically targeting hypoxic tumor growth.

Ca^{2+}-mediated signaling pathways are implicated in tumor development (Monteith et al., 2007). Stromal interaction molecules (STIMs), the single-pass type I membrane proteins, are ER Ca^{2+} sensors that control the intracellular Ca^{2+} concentration and mediate the activation of store-operated Ca^{2+} entry (SOCE), the major route for Ca^{2+} influx in nonexcitable cells (Vig et al., 2006; Zhang et al., 2005). Recent studies reported the importance of STIM1-mediated SOCE in tumor invasion, metastasis, and angiogenesis (Tsai et al., 2014; Yang et al., 2009). Moreover, STIM1 upregulation is potentially valuable for diagnosis and prognosis of cancer (Mayr et al., 2002; van de Vijver et al., 2002). STIM2 has also been found to contribute to the invasion and metastasis of cancer (Sobradillo et al., 2014; Stanisz et al., 2014). Nonetheless, the significance of STIMs in cancer development under hypoxic conditions remains unclear. In the present study, we addressed the regulation of STIM1 by HIF-1 and determined Ca^{2+} mobilization in hypoxic hepatocarcinogenesis. We also present the functional significance that STIM1 mediates HIF-1 upregulation and hypoxia-driven tumorigenesis. Our findings shed light on the role of STIM1 in hypoxic tumor growth and highlight a potential target for early hypoxia-related intervention.

RESULTS

STIM1 Upregulation Correlates with HIF-1α during Hypoxic Tumor Growth

We first evaluated whether STIM1 expression correlates with hepatocarcinoma and HIF-1 by analyzing ten sample sets from clinical liver cancer patients (as listed in Table S1). To differentiate between the neoplastic and normal regions, tissue sections were stained with H&E and validated by a pathologist. The scraped tissues from neoplastic and adjacent area were collected for HIF-1α and STIM1 staining. HIF-1α and STIM1 expression was significantly higher in the tumor tissues than in the adjacent tissues (Figures 1A–1C; $p < 0.001$). Moreover, the expression of STIM1 and HIF-1α was positively correlated (Figure 1D; $r = 0.8881$, $p < 0.01$).

Prompted by the above-mentioned results, we hypothesized that STIM1 plays a role in hypoxic hepatocarcinoma growth. To test this, we established an orthotopic hepatocarcinoma

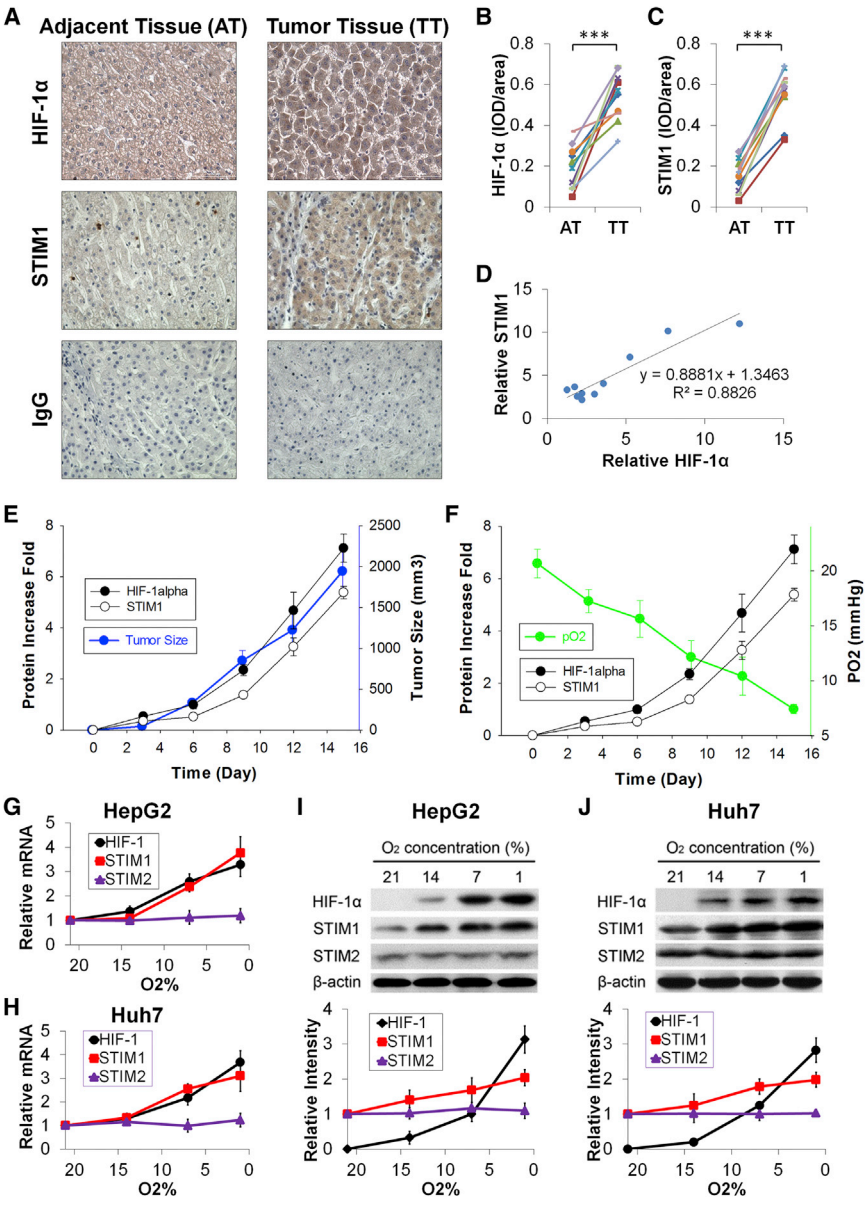


Figure 1. Elevated STIM1 Correlates with HIF-1α and Hypoxic Tumor Growth

(A–D) Immunohistochemistry of HIF-1α and STIM1 in the tumor and adjacent tissues of hepatocarcinoma patients. (A) The presented images (400×) were from the same patient. Statistical analysis of normalized expression of HIF-1α (B) and STIM1 (C) (integrated optical density [IOD] against immunoglobulin G [IgG]) in the tumor and adjacent tissues of ten hepatocarcinoma patients. ***$p < 0.001$, adjacent versus tumor. (D) Correlation between relative increase fold (against the adjacent tissues) of HIF-1α and STIM1 in the above tumor subjects.

(E and F) HepG2 cells (2×10^6 cells/100 μl) were injected into the left hepatic lobe of CD-1 nude mice. Tumor size, pO_2, and relative expression of HIF-1α and STIM1 (against β-actin and day 0) in the tumor tissues were determined as tumor growth. Results are expressed as mean ± SEM (n = 6).

(G–J) After being cultured in hypoxia (with different O_2 concentrations) for 24 hr, mRNA (G and H) and protein (I and J) expression of HIF-1α and STIM1 in HepG2 and Huh7 cells was determined with qPCR and western blot, respectively. The results were analyzed and normalized against expression under normoxia and are expressed as mean ± SEM (n = 4 independent experiments).

See also Figure S1.

xenograft nude mice model using HepG2 hepatocarcinoma cell (HCC) transplantation. We found that expression levels of both HIF-1α and STIM1 correlated with tumor size (Figure 1E). The tissue partial pressure of oxygen (pO_2) decreased with tumor growth, whereas the expression of HIF-1α and STIM1 increased (Figures 1F and S1A), suggesting an invert correlation between STIM1 expression and pO_2.

We next compared the expression of STIM1 and STIM2 in human HepG2 and Huh7 cells after exposure to different concentrations of oxygen for 24 hr in vitro. Consistent with the in vivo results, hypoxia exposure enhanced HIF-1α and STIM1, rather than STIM2, expression in HCCs in an O_2-concentration-dependent manner (Figures 1G–1J). These results indicate that STIM1 is upregulated in hypoxic tumor growth and correlates with HIF-1 accumulation.

HIF-1 Regulates STIM1 Expression and SOCE under Hypoxia

The above findings led us to speculate that STIM1 is regulated by HIF-1 under hypoxic conditions. Employing Vista software (Frazer et al., 2004), we found three HIF-1 potential binding sites in the promoter of *STIM1* (Figure 2A; A1: 3862321–3862340, A2: 3877087–3877107, and A3: 3877324–3877344 on the negative strand of chromosome 11). However, we could not find any potential HIF-1 binding sites in *STIM2* promoter. Using a chromatin immunoprecipitation (ChIP) assay, we found that HIF-1 bound to the *STIM1* promoter at the above-predicted three sites in hypoxic HepG2 cells (Figure 2B), whereas HIF-1 could not be co-immunoprecipitated with *STIM1* gene within normoxic HCCs. Among the three potential binding sites, only A2 contained an HRE sequence (5′-CCGTG-3′; see the green arrow in Figure 2A). To identify which site contributed to the transactivation of STIM1, we transfected the promoter luciferase reporters with respective site deletion into HepG2 cells and found that only A2 deletion resulted in a reporter significantly less responsive to hypoxia (Figure 2C). These results demonstrate that the A2 site, which includes putative HREs, is necessary for the transcriptional response to hypoxia.

To investigate the relationship between HIF-1 and STIM1, four small hairpin RNAs (shRNAs) against each of them were applied.

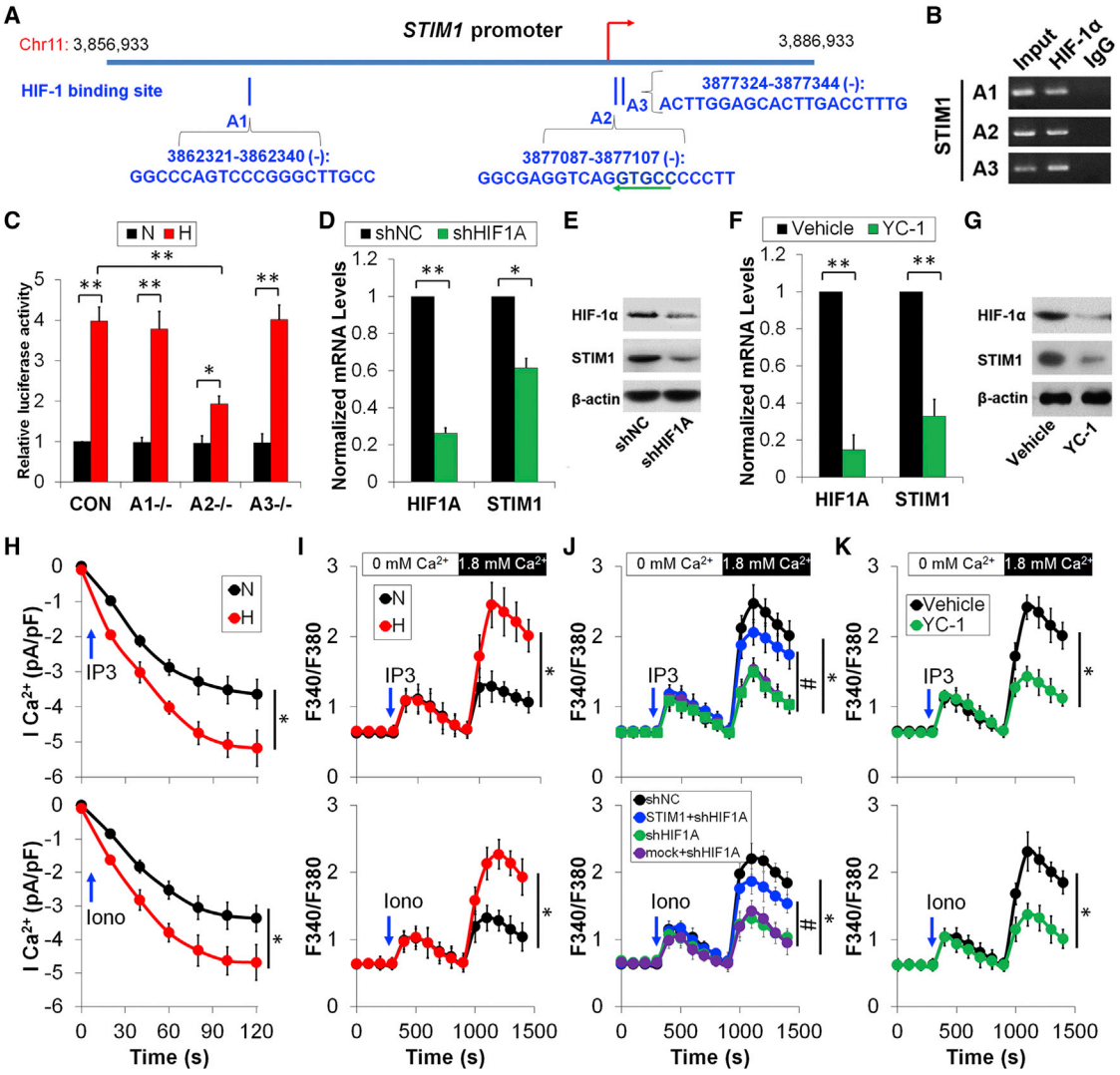


Figure 2. HIF-1 Transcripts STIM1 and Mediates Hypoxia-Enhanced SOCE in HCCs

(A) Vista predicted binding sites of HIF-1 in the promoters of *STIM1*. Red arrow, transcription start site; −, negative strand.

(B) ChIP assay with HIF-1α or control IgG antibody. The immunoprecipitated DNA was purified from HepG2 cells after exposure to hypoxia (1% O_2) for 24 hr, and the regions from sites A1, A2, and A3 of the human *STIM1* promoter as shown in (A) were amplified by PCR.

(C) After transfecting HepG2 cells with luciferase reporters of the STIM1 promoter and the promoter with A1, A2, or A3 deletion, cells were cultured under normoxia (N) or hypoxia (H; 1% O_2) for 24 hr. Luciferase activity of the reporters was determined and normalized with the control group under normoxia.

(D and E) HepG2 cells were transfected with shNC (negative control) or shHIF1A for 48 hr, then cultured in hypoxia (1% O_2) for 24 hr. The mRNA (D) and protein (E) expression of HIF-1α, STIM1, and β-actin was assayed with qPCR and western blot, respectively. Results of qPCR are expressed as mean ± SEM (n = 4 independent experiments). *p < 0.05 and **p < 0.01, shNC versus shHIF1A.

(F and G) HepG2 cells were pretreated with PBS (vehicle) or YC-1 (20 μM) for 1 hr and then exposed to hypoxia (1% O_2) for 24 hr. mRNA (F) and protein (G) expression of HIF-1α, STIM1, and β-actin was assessed with qPCR and western blot, respectively. Results of qPCR are expressed as mean ± SEM; n = 4 independent experiments. **p < 0.01, shNC versus shHIF1A.

(H) Time course of Ca^{2+} current activation after treatment with IP3 (20 μM) or Iono (1 μM) in normoxia (N) or after 1% O_2 (24 hr) treatment (H). The amplitudes are expressed as mean ± SEM of n = 11 independent cells in each group. *p < 0.05, N versus H.

(I) Ca^{2+} mobilization in HepG2 cells upon IP3 (20 μM) or Iono (1 μM) challenge under normoxia or after exposure to hypoxia for 24 hr (H). The intracellular Ca^{2+} concentration is expressed as mean ± SEM of n = 5 independent cells each group. *p < 0.05, N versus H.

(J and K) HepG2 cells were transfected with shNC, shHIF1A, mock, or STIM1 (J) or pretreated with PBS (vehicle) or YC-1 (20 μM) for 1 hr (K) and exposed to hypoxia (1% O_2) for 24 hr. Ca^{2+} mobilization in HepG2 cells upon IP3 (20 μM) or Iono (1 μM) challenge was assessed. All kinetic curves are the mean ± SEM of values observed in 30 cells from five experiments. *p < 0.05, shNC versus shHIF1A; #p < 0.05, shHIF1A versus STIM1 plus shHIF1A.

See also Figure S2.

Among them, STIM1-shRNA2 and HIF1A-shRNA1 showed the most potent inhibition on the respective target genes in both HepG2 and Huh7 cells (Figures S1B and S1C); hence, we renamed them shSTIM1 and shHIF1A and used them for the functional experiments. To address whether HIF-1 is required for STIM1 upregulation under hypoxia, we knocked down HIF-1α in HepG2 cells with shHIF1A and found that hypoxia-enhanced STIM1 was attenuated at both the mRNA and protein levels when compared with scrambled shRNA (shNC)-transfected cells (Figures 2D and 2E). Furthermore, the administration of YC-1, a pharmacological inhibitor of HIF-1 (Yeo et al., 2003), significantly reduced STIM1 under hypoxia (Figures 2F and 2G). These results indicate that HIF-1 contributes to STIM1 production in hypoxic HCCs.

Since STIM1 functions as an ER Ca^{2+} sensor for SOCE (Zhang et al., 2005), its upregulation under hypoxia and its positive correlation with HIF-1 led us to hypothesize that SOCE may mediate the Ca^{2+} transient in hypoxic HCCs. To identify the route of Ca^{2+} mobilization in hypoxic cancer cells, a whole-cell patch-clamp technique was performed with HepG2 cells (Figures 2H, S2A, and S2B). The Ca^{2+} current in resting hypoxic cells was not significantly altered (~−0.2 to 0.3pA/pF) compared with that in normoxic HCCs. The amplitude of the inward and fast-inactivated current in response to 20 μM of inositol 1,4,5-trisphosphate (IP3) or 1 μM ionomycin (Iono) was lower than that in the hypoxia group (Figure 2H and S2A). Pretreatment with the SOCE inhibitors 2-aminoethoxydiphenyl borate (2-APB) and $GdCl_3$ significantly blunted the currents elicited by IP3 in both normoxic and hypoxic HCCs (Figure S2A), which were consistent with the *I–V* recordings (Figure S2B).

It is well known that hypoxia can trigger reactive oxygen species generation, which contributes to the activation of phospholipase C (PLC) (Yadav et al., 2013), a hydrolase of phosphatidylinositol 4,5-bisphosphate (PIP2) (Wong et al., 2005). PIP2 is the precursor of IP3, which is a classical activator of SOCE (Parekh and Putney, 2005; Putney, 2009). Of interest, we found that the PLC inhibitor U73122 significantly inhibited acute-hypoxia-enhanced PIP2 hydrolysis and SOCE in both HepG2 and Huh7 cells (Figures S2C and S2D). These results indicate that IP3 may be the activator of hypoxia-enhanced SOCE.

To further assess the route of Ca^{2+} mobilization in hypoxic HCCs, a Ca^{2+}-image technique was applied (Figure 2I). In the absence of extracellular Ca^{2+}, both IP3 and Iono induced gradual increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which decayed to pre-stimulation levels in 8–10 min. The increase in $[Ca^{2+}]_i$ did not significantly differ among normoxia- and hypoxia-treated HepG2 cells, suggesting equal depletion of Ca^{2+} stores. Further replenishment of Ca^{2+} (1.8 mM) to the extra-cellular solution induced a sustained rise in $[Ca^{2+}]_i$, where SOCE increased upon hypoxia pre-exposure in a time-dependent manner (Figure 2I). These results suggest that hypoxia exposure enhanced Ca^{2+} mobilization via SOCE, but not through promoting Ca^{2+} release from the store.

We next determined the effect of HIF-1 on SOCE in HCCs after hypoxic treatment for 24 hr. While there was no significant change in stored Ca^{2+} release, the extracellular Ca^{2+} influx was much lower in the shHIF1A and shSTIM1 groups when compared to shNC (Figures 2J, S2E, and S2F). Ectopic expression of STIM1 rescued SOCE in shHIF1A-transfected HCCs (Figure 2J). YC-1 treatment also significantly decreased IP3- and Iono-initiated SOCE in HepG2 cells after 24-hr hypoxia exposure (Figure 2K). Together, these results suggest that HIF-1 is required for hypoxia-enhanced STIM1 and SOCE in HCCs.

STIM1 Feed-Forward Regulates HIF-1 in Hypoxic HCCs

Prompted by above results, we next hypothesized that STIM1 may feed-forward regulate HIF-1 via SOCE. Interestingly, both Iono and IP3 enhanced the expression of hypoxia-induced HIF-1α and its downstream target, vascular endothelial growth factor A (VEGF-A). Knockdown of STIM1 in HCCs significantly suppressed hypoxia-induced HIF-1α and VEGF-A, which could not be restored by Iono (Figure 3A). These results suggest that STIM1-mediated SOCE promotes HIF-1 expression in hypoxic HCCs.

Elevated $[Ca^{2+}]_i$ has been identified to activate Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) that phosphorylates p300, thereby increasing HIF-1 transcriptional activity and stability under hypoxia (Yuan et al., 2005). To further address the signaling pathway by which STIM1 regulates HIF-1, Iono and inhibitors against SOCE, CaMKII, and p300 were administered to HepG2 cells under hypoxia for 24 hr. We found that Iono enhanced the expression of hypoxia-induced HIF-1α and VEGF-A, which were significantly suppressed by pre-treatment with the SOCE inhibitor 2-APB, the CaMKII inhibitor KN93 (Yuan et al., 2005), and the p300 inhibitor C646 (Bowers et al., 2010) (Figures 3B and S3A), suggesting that STIM1-mediated SOCE upregulates HIF-1α during hypoxia via CaMKII and p300 activation.

The accumulation of HIF-1 in hypoxic HCCs may be attributed to elevated transcription or impaired degradation. Using shNC- and shSTIM1-transfected Huh7 cells, we found that STIM1 is required for an increase in *HIF1A* mRNA under hypoxia (Figure 3C). Moreover, the enhanced mRNA expression of *HIF1A* was blunted by 2-APB, KN93, and C646 (Figure 3D). On the other hand, 2-APB, KN93, and C646 also accelerated HIF-1α degradation upon the administration of the translation inhibitor cycloheximide (CHX) (Figures 3E and S3B). These results suggest that STIM1-mediated SOCE feed-forward regulates HIF-1 in hypoxic HCCs by upregulating its transcription and preventing degradation.

To further establish the relevance of STIM1 in tumorigenesis and HIF-1 accumulation, we employed orthotopic xenograft hepatocarcinoma nude mice. Compared with the shNC group, knockdown of STIM1 significantly suppressed malignant invasion and expression of HIF-1α and VEGF-A in tumor tissues (Figures 3F and S3C). These results demonstrate that STIM1 is critical for tumor progression and HIF-1 elevation in hypoxic HCCs.

STIM1 Mediates Hypoxia-Driven Tumor Growth

In order to investigate whether STIM1 is a HIF-1-downstream, functionally important target promoting tumor growth, we compared tumor size and expression of STIM1 and HIF-1α with a respective shRNA-transfected HepG2 cell xenograft mice model in vivo. Consistently, HepG2 cells transfected with shSTIM1 or shHIF1A grew much slower than those transfected

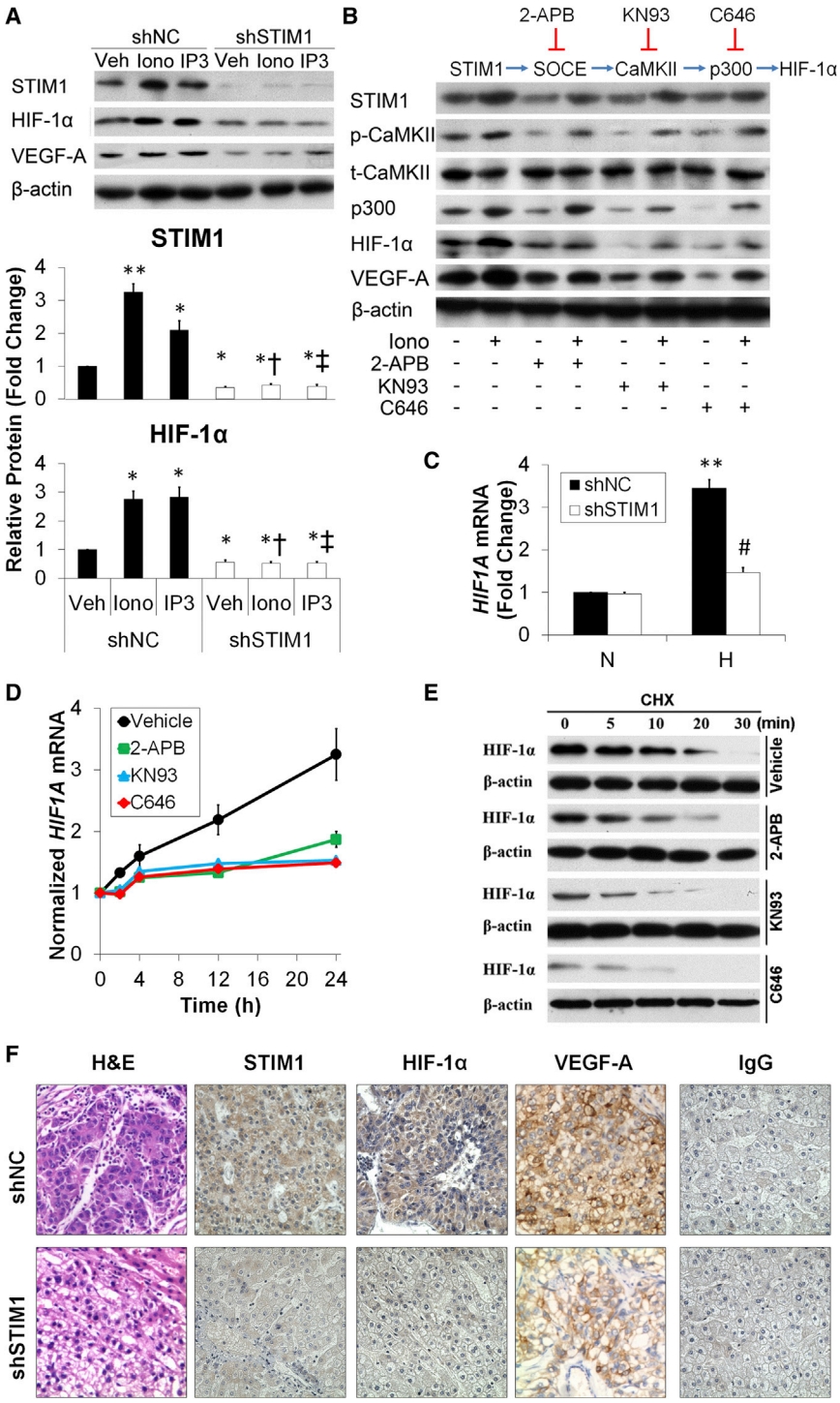


Figure 3. STIM1 Upregulates HIF-1 in Hypoxic HCCs

(A) After shNC- and shSTIM1-transfected HepG2 cells were exposed to hypoxia (1% O_2) with Iono (1 μM) or IP3 (20 μM) for 24 hr, STIM1, HIF-1α, VEGF-A, and β-actin protein levels were assessed with western blot. Quantitative results of STIM1 and HIF-1α are expressed as mean ± SEM of three independent experiments. *$p < 0.05$ and **$p < 0.01$ compared with shNC vehicle; †$p < 0.05$ compared with shNC + Iono; ‡$p < 0.05$ compared with shNC + IP3.

(B) HepG2 cells were treated with Iono (2 μM), 2-APB (50 μM), KN93 (10 μM), and C646 (30 μM) and exposed to hypoxia (H; 1% O_2) for 24 hr. Protein levels of STIM1, p-CaMKII, t-CaMKII, p300, HIF-1α, VEGF-A, and β-actin were assessed with western blot.

(C) The shNC or shSTIM1 transfected Huh7 cells were incubated under normoxia (N) or hypoxia (H, 1% O2) for 24 hr, *HIF1A* mRNA levels were assessed with qPCR and expressed as mean ± SEM of four independent experiments. **$p < 0.05$, shNC plus N versus shNC plus H; #$p < 0.05$, shNC plus H versus shSTIM1 plus H.

(D) Huh7 cells were exposed to hypoxia (H; 1% O_2) with vehicle (PBS), 2-APB (50 μM), KN93 (10 μM), and C646 (30 μM) for 24 hr. *HIF1A* mRNA was assessed with qPCR and expressed as mean ± SEM of four independent experiments.

(E) Huh7 cells were exposed under hypoxia with vehicle (PBS), 2-APB (50 μM), KN93 (10 μM), and C646 (30 μM) for 24 hr and subsequently treated with 20 μg/ml cycloheximide (CHX) under hypoxia for 0–30 min. Cell lysates were used for the assessment of HIF-1α expression.

(F) shNC- or shSTIM1-transfected HepG2 cells were transplanted into the left hepatic lobe. H&E staining and immunohistochemistry (400×) of STIM1, HIF-1α, and VEGF-A and control IgG in the tumor tissues at day 15 post-HCC transplantation were assessed.

See also Figure S3.

with shNC in the recipient nude mice (Figure 4A). Knockdown of *HIF1A* significantly reduced STIM1 and VEGF-A expression in tumor cells, while STIM1 deficiency lead to reduced HIF-1α and VEGF-A (Figures S4A and S4B). The proliferation in shSTIM1-transfected hypoxic HepG2 cells was also remarkably reduced when compared with shNC group in vitro (Figure S4C).

On the other hand, overexpression of STIM1 or HIF-1α promoted, while the HIF-1 inhibitor YC-1 effectively inhibited, tumor growth and STIM1 expression in tumor cells in the xenografts. The ectopic expression of STIM1 or HIF-1α substantially restored tumor growth and expression of STIM1, HIF-1α, and VEGF-A in HCCs treated with YC-1 (Figures 4B–4E, S4D, and S4E). Of note, overexpression of HIF-1α or STIM1 significantly accelerated *p*O2 reduction within the tumor tissues (Figure 4F). Since both HIF-1 and Ca^{2+} are key regulators of oxygen consumption (Degasperi et al., 2006; Semenza, 2003), our results indicate that the accumulation of HIF-1 and STIM1 aggravate hypoxic conditions in the tumor loci. Together, these findings support the notion that STIM1 mediates hypoxia-driven tumorigenesis.

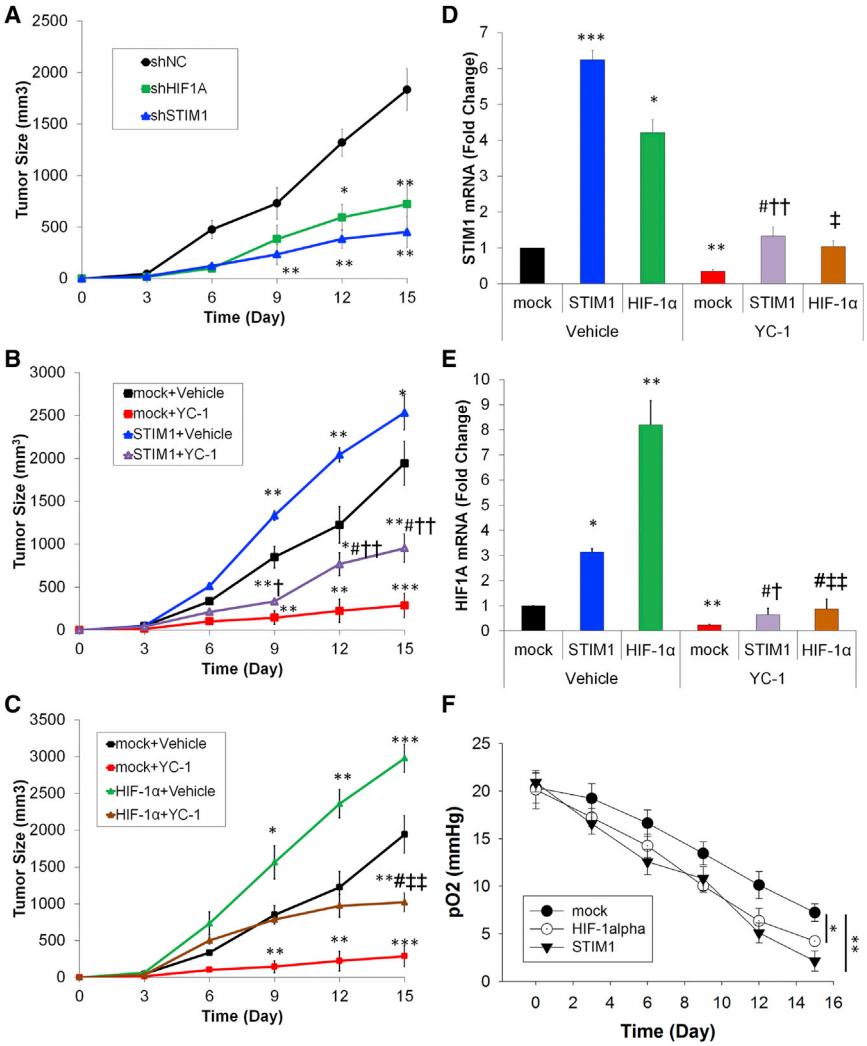


Figure 4. STIM1 Orchestrates HIF-1-Driven Tumorigenesis

(A) shNC-, shHIF1A-, and shSTIM1-transfected HepG2 cells (2×10^6 cells/100 μl) were injected into the left hepatic lobe of CD-1 nude mice, and the tumor size was examined for tumor growth. Results were expressed as mean ± SEM; n = 10. *p < 0.05 and **p < 0.01, shNC versus shSTIM1.

(B–E) After the mock-, *STIM1*-, or *HIF1A*-transfected HepG2 cells were transplanted into the left hepatic lobe, CD-1 nude mice received daily intraperitoneal injections of vehicle (PBS) or YC-1 (30 mg/g) for 2 weeks. Tumor size was assessed along with tumor growth (B and C). mRNA expression of *STIM1* (D) and *HIF1A* (E) in the fluorescence-activated cell-sorted GFP$^+$-HCCs from tumor tissues at day 15 post-HCC transplantation. Results were normalized against β-actin and are expressed as mean ± SEM of n = 10 mice per group. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to mock plus vehicle; $^{\#}$p < 0.05 compared to mock plus YC-1; †p < 0.05 and ††p < 0.01 compared to STIM1 plus vehicle; ‡p < 0.05 and ‡‡p < 0.01 compared to HIF-1 plus vehicle.

(F) After the mock-, *STIM1*-, or *HIF1A*-transfected HepG2 cells were transplanted into the left hepatic lobe, pO_2 in the tumor tissues was determined as a function of tumor growth. Results are expressed as mean ± SEM; n = 10. *p < 0.05 and **p < 0.01 compared to mock.

See also Figure S4.

DISCUSSION

STIM1 is a well-known ER Ca^{2+} sensor that mediates SOCE activation and promotes tumor invasion and migration (Tsai et al., 2014; Yang et al., 2009; Zhang et al., 2005). Signaling cascades initiated or regulated by HIF-1 are essential for the processes of tumorigenesis (Semenza, 2003). However, there is no general consensus about the relationship between HIF-1 and STIM1. Also, the significance of STIM1, as well as the route of Ca^{2+} mobilization in cancer cells during hypoxia, remain unknown. In our present study, we identified STIM1 overexpression in both human and mouse xenograft hepatocarcinoma tissues that was accompanied by tumor growth and steadily reduced pO_2. Hypoxia-induced HIF-1 promoted STIM1 expression and SOCE in HCCs by directly binding to the STIM1 promoter. Knockdown of STIM1 significantly blunted, while overexpression of STIM1 promoted, tumor growth. Moreover, administration of YC-1, an agent that inhibited HIF-1, significantly diminished STIM1 and suppressed xenograft tumorigenesis. Our results indicate that STIM1 plays a crucial role in hypoxic tumor growth.

Hypoxia is an integral part of tumor growth and has been suggested to regulate Ca^{2+} in tumor cells (Toescu, 2004). So far, an emerging paradigm for the action of hypoxia on Ca^{2+} signaling was described. For example, hypoxic pulmonary vasoconstriction and STIM1 expression were greater in distal than in proximal pulmonary arteries (Weigand et al., 2005). 2-APB, an inhibitor of STIM1 puncta formation and SOCE, was reported to block the induction of HIF-1α in a concentration-dependent manner (Yuan et al., 2008). Constitutive activity of HIF-1α resulted in accelerated uptake of cytoplasmic Ca^{2+} into intracellular stores and diminished Ca^{2+} response due to increased SERCA pump expression (Neumann et al., 2005). The gene downstream of HIF-1, such as VEGF, induced Ca^{2+} signaling and IP3 formation (Faehling et al., 2002). In the present study, we found that the hypoxia-induced Ca^{2+} transient was store operated, which is fast inactivated (at a pA range) and could be inhibited by 2-APB or $GdCl_3$ or by knockdown of STIM1 in HepG2 cells. The Ca^{2+} currents elicited by IP3 or Iono were enhanced after hypoxia exposure. Importantly, we identified a HIF-1 binding site on the promoter of *STIM1*, which contained an HRE. Moreover, HIF-1α reduction by knockdown or YC-1 administration impaired the rise of SOCE and STIM1 expression in hypoxic HCCs in vitro and in vivo. These results elucidate the mechanism by which hypoxia enhances SOCE, i.e., via HIF-1 upregulating STIM1.

The impact of Ca^{2+} on the regulation of HIF-1 is also a research hotspot in recent years. For instance, 2-APB inhibited the hyperphosphorylation of p300 and blocked the induction of HIF-1α in a dose-dependent manner. Hypoxia-evoked HIF-1α accumulation was blocked by the Ca^{2+} chelator 1,2-bis-(o-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) (Yuan et al., 2005). These findings suggest that SOCE contributes to HIF-1 expression, although the underlying mechanisms are not revealed. In the present study, we found that knockdown of STIM1 decreased the expression of HIF-1 and its proangiogenic downstream target, VEGF-A, in both hypoxic HCCs in vitro and the tumor tissues in vivo. In addition, we found that hypoxia- and Iono-enhanced p300 phosphorylation and HIF-1 accumulation (both mRNA and protein) could be inhibited by inhibitors of SOCE, CaMKII, and p300. Since p300 contributes to the stability and translocation of HIF-1α, and because HIF-1 transcripts several targets, including NF-κB, which also upregulate HIF-1α (Jiang et al., 2015), our findings suggest that STIM1-mediated SOCE promotes HIF-1α transcription and prevents HIF-1α degradation. Of interest, YC-1 significantly reduced STIM1 expression in HCCs and suppressed tumorigenesis, which could be partially reversed by STIM1 or HIF-1α overexpression. Ectopic expression of STIM1 or HIF-1α also accelerated the reduction of pO_2 in the tumor tissues in situ. These results suggest that STIM1 orchestrates hypoxic tumorigenesis via feed-forward regulation of HIF-1α.

In conclusion, this study proposes a regulatory circuit involving STIM1 and HIF-1 under hypoxic conditions that is required for tumor growth and hypoxia-enhanced Ca^{2+} mobilization in HCCs. As the tumor rapidly grows, the oxygen concentration is significantly lower than in healthy tissues, resulting in the transactivation of HIF-1 and the transcription of STIM1. The hypoxia-elevated STIM1 promotes Ca^{2+} mobilization via mediating SOCE, which contributes to the stabilization of HIF-1α by activating CaMKII and p300. This regulatory circuit aggravates the hypoxic microenvironment and accelerates tumor growth. Together, our findings contribute to a better understanding of the orchestration of hypoxia and intracellular Ca^{2+} on tumorigenesis and highlight therapeutic options for early cancer intervention.

EXPERIMENTAL PROCEDURES

Patients

Paraffin-embedded primary hepatocarcinoma tissues and corresponding adjacent nontumorous liver samples were obtained from patients at Xinqiao Hospital (Chongqing, China). Clinicopathological characteristics of hepatocarcinoma patients were summarized in Table S1. The use of clinical specimens and commercially obtained samples in this study was approved by the Xinqiao Hospital ethics committee of the Third Military Medical University.

STIM1 Promoter Luciferase Assay

Plasmid vectors of the STIM1 promoter and the promoter with A1, A2, or A3 deletion were synthesized by Promega (Madison) and transfected into HepG2 cells as described above. Cells were incubated at 21% O_2 (N) or 1% O_2 for 24 hr. A luciferase assay was then performed as (Semenza and Wang, 1992). Relative luciferase activity was expressed as normalized with the control group under normoxia.

Orthotopic HepG2 Nude Mice Model

Male CD-1 nude mice (6 weeks old, Charles River Laboratories International) were housed under specific-pathogen-free conditions. Wild-type HepG2 cells or cells that stably transfected with lentiviral vectors expressing mock, STIM1, or shRNAs against blank control, STIM1, or HIF-1A (2×10^6 cells/100 μl each) were slowly injected into the left hepatic lobe of CD-1 nude mice. The tumor volume was calculated according to the formula: tumor volume = (largest diameter × perpendicular height^2)/2. After measuring tumor volume, the tumors were removed from the liver for respective experiments. In some experiments, GFP^+ cells were fluorescence-activated cell sorted from the tumor tissues for specific protein determination. The experimental protocol was approved by the experimental animal care commission of Huazhong University of Science and Technology and the Third Military Medical University.

Determination of pO_2 in the Liver

Bi-modality magnetic resonance imaging/electron paramagnetic resonance imaging (MRI/EPRI) was performed in a 300-MHz single-point-imaging scanner to measure pO_2 as described previously (Hyodo et al., 2012). Briefly, Oxo63 was injected into the mice via tail vein catheter. The pO_2 mapping relied on the linear relationship between pO_2 and line width of the triarylmethyl (Oxo63). To quantify the line width in a voxel, images with increasing readout delay were obtained. From the images with successively increasing readout delay, the line width of Oxo63 was mapped over the tissue region. Finally, using the line-width map, pO_2 was calculated from a calibration curve calculated in vitro.

Statistical Analysis

Statistical analysis was performed using the statistical program SPSS 19.0 for windows (SPSS). All data are presented as mean ± SEM and were analyzed by one-way ANOVA. p values < 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.06.033.

AUTHOR CONTRIBUTIONS

Y.L. and B.Z. designed research. Y.L., B.G., Q.X., D.Z., Y.Z., and H.C. performed experiments and analyzed the data. D.Y. analyzed the data. Y.L., B.G., and B.Z. wrote the manuscript.

ACKNOWLEDGMENTS

We thank Prof. James W. Putney, Jr. (NIH), Prof. Monika Vig (Washington University), and Dr. Yisong Wan (University of North Carolina) for helpful discussions and valuable comments during manuscript preparation. We also appreciate Ms. Fei Yang (Third Military Medical University) for her technical support with immunohistochemistry. This work was supported by the National Natural Science Foundation of China (grants 81472435, 81171495, and 81222031) and the National Key Basic Research Program of China (973 program, grant 2012CB526603).

Received: November 27, 2014
Revised: April 30, 2015
Accepted: June 8, 2015
Published: July 9, 2015

REFERENCES

Bowers, E.M., Yan, G., Mukherjee, C., Orry, A., Wang, L., Holbert, M.A., Crump, N.T., Hazzalin, C.A., Liszczak, G., Yuan, H., et al. (2010). Virtual ligand screening of the p300/CBP histone acetyltransferase: identification of a selective small molecule inhibitor. Chem. Biol. *17*, 471–482.

Degasperi, G.R., Velho, J.A., Zecchin, K.G., Souza, C.T., Velloso, L.A., Boreckỳ, J., Castilho, R.F., and Vercesi, A.E. (2006). Role of mitochondria in the immune response to cancer: a central role for Ca2+. J. Bioenerg. Biomembr. *38*, 1–10.

Faehling, M., Kroll, J., Föhr, K.J., Fellbrich, G., Mayr, U., Trischler, G., and Waltenberger, J. (2002). Essential role of calcium in vascular endothelial growth factor A-induced signaling: mechanism of the antiangiogenic effect of carboxyamidotriazole. FASEB J. *16*, 1805–1807.

Frazer, K.A., Pachter, L., Poliakov, A., Rubin, E.M., and Dubchak, I. (2004). VISTA: computational tools for comparative genomics. Nucleic Acids Res. *32*, W273–W279.

Harris, A.L. (2002). Hypoxia—a key regulatory factor in tumour growth. Nat. Rev. Cancer *2*, 38–47.

Hyodo, F., Davis, R.M., Hyodo, E., Matsumoto, S., Krishna, M.C., and Mitchell, J.B. (2012). The relationship between tissue oxygenation and redox status using magnetic resonance imaging. Int. J. Oncol. *41*, 2103–2108.

Jiang, Y., Zhu, Y., Wang, X., Gong, J., Hu, C., Guo, B., Zhu, B., and Li, Y. (2015). Temporal regulation of HIF-1 and NF-κB in hypoxic hepatocarcinoma cells. Oncotarget *6*, 9409–9419.

Mayr, N.A., Taoka, T., Yuh, W.T., Denning, L.M., Zhen, W.K., Paulino, A.C., Gaston, R.C., Sorosky, J.I., Meeks, S.L., Walker, J.L., et al. (2002). Method and timing of tumor volume measurement for outcome prediction in cervical cancer using magnetic resonance imaging. Int. J. Radiat. Oncol. Biol. Phys. *52*, 14–22.

Monteith, G.R., McAndrew, D., Faddy, H.M., and Roberts-Thomson, S.J. (2007). Calcium and cancer: targeting Ca2+ transport. Nat. Rev. Cancer *7*, 519–530.

Neumann, A.K., Yang, J., Biju, M.P., Joseph, S.K., Johnson, R.S., Haase, V.H., Freedman, B.D., and Turka, L.A. (2005). Hypoxia inducible factor 1 alpha regulates T cell receptor signal transduction. Proc. Natl. Acad. Sci. USA *102*, 17071–17076.

Parekh, A.B., and Putney, J.W., Jr. (2005). Store-operated calcium channels. Physiol. Rev. *85*, 757–810.

Putney, J.W. (2009). Capacitative calcium entry: from concept to molecules. Immunol. Rev. *231*, 10–22.

Semenza, G.L. (2003). Targeting HIF-1 for cancer therapy. Nat. Rev. Cancer *3*, 721–732.

Semenza, G.L., and Wang, G.L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol. Cell. Biol. *12*, 5447–5454.

Sobradillo, D., Hernández-Morales, M., Ubierna, D., Moyer, M.P., Núñez, L., and Villalobos, C. (2014). A reciprocal shift in transient receptor potential channel 1 (TRPC1) and stromal interaction molecule 2 (STIM2) contributes to Ca2+ remodeling and cancer hallmarks in colorectal carcinoma cells. J. Biol. Chem. *289*, 28765–28782.

Stanisz, H., Saul, S., Müller, C.S., Kappl, R., Niemeyer, B.A., Vogt, T., Hoth, M., Roesch, A., and Bogeski, I. (2014). Inverse regulation of melanoma growth and migration by Orai1/STIM2-dependent calcium entry. Pigment Cell Melanoma Res *27*, 442–453.

Toescu, E.C. (2004). Hypoxia sensing and pathways of cytosolic Ca2+ increases. Cell Calcium *36*, 187–199.

Tsai, F.C., Seki, A., Yang, H.W., Hayer, A., Carrasco, S., Malmersjö, S., and Meyer, T. (2014). A polarized Ca2+, diacylglycerol and STIM1 signalling system regulates directed cell migration. Nat. Cell Biol. *16*, 133–144.

van de Vijver, M.J., He, Y.D., van't Veer, L.J., Dai, H., Hart, A.A., Voskuil, D.W., Schreiber, G.J., Peterse, J.L., Roberts, C., Marton, M.J., et al. (2002). A gene-expression signature as a predictor of survival in breast cancer. N. Engl. J. Med. *347*, 1999–2009.

Vig, M., Peinelt, C., Beck, A., Koomoa, D.L., Rabah, D., Koblan-Huberson, M., Kraft, S., Turner, H., Fleig, A., Penner, R., and Kinet, J.P. (2006). CRACM1 is a plasma membrane protein essential for store-operated Ca2+ entry. Science *312*, 1220–1223.

Weigand, L., Foxson, J., Wang, J., Shimoda, L.A., and Sylvester, J.T. (2005). Inhibition of hypoxic pulmonary vasoconstriction by antagonists of store-operated Ca2+ and nonselective cation channels. Am. J. Physiol. Lung Cell. Mol. Physiol. *289*, L5–L13.

Wong, R., Hadjiyanni, I., Wei, H.C., Polevoy, G., McBride, R., Sem, K.P., and Brill, J.A. (2005). PIP2 hydrolysis and calcium release are required for cytokinesis in Drosophila spermatocytes. Curr. Biol. *15*, 1401–1406.

Yadav, V.R., Song, T., Joseph, L., Mei, L., Zheng, Y.M., and Wang, Y.X. (2013). Important role of PLC-γ1 in hypoxic increase in intracellular calcium in pulmonary arterial smooth muscle cells. Am. J. Physiol. Lung Cell. Mol. Physiol. *304*, L143–L151.

Yang, S., Zhang, J.J., and Huang, X.Y. (2009). Orai1 and STIM1 are critical for breast tumor cell migration and metastasis. Cancer Cell *15*, 124–134.

Yeo, E.J., Chun, Y.S., Cho, Y.S., Kim, J., Lee, J.C., Kim, M.S., and Park, J.W. (2003). YC-1: a potential anticancer drug targeting hypoxia-inducible factor 1. J. Natl. Cancer Inst. *95*, 516–525.

Yuan, G., Nanduri, J., Bhasker, C.R., Semenza, G.L., and Prabhakar, N.R. (2005). Ca2+/calmodulin kinase-dependent activation of hypoxia inducible factor 1 transcriptional activity in cells subjected to intermittent hypoxia. J. Biol. Chem. *280*, 4321–4328.

Yuan, G., Nanduri, J., Khan, S., Semenza, G.L., and Prabhakar, N.R. (2008). Induction of HIF-1alpha expression by intermittent hypoxia: involvement of NADPH oxidase, Ca2+ signaling, prolyl hydroxylases, and mTOR. J. Cell. Physiol. *217*, 674–685.

Zhang, S.L., Yu, Y., Roos, J., Kozak, J.A., Deerinck, T.J., Ellisman, M.H., Stauderman, K.A., and Cahalan, M.D. (2005). STIM1 is a Ca2+ sensor that activates CRAC channels and migrates from the Ca2+ store to the plasma membrane. Nature *437*, 902–905.