STIM1-Dependent Peripheral Coupling Governs the Contractility of Vascular Smooth Muscle Cells

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Short title: STIM1 forms Ca²⁺ signaling microdomains in smooth muscle cells

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Abstract

Peripheral coupling between the sarcoplasmic reticulum (SR) and plasma membrane (PM) forms signaling complexes that regulate the membrane potential and contractility of vascular smooth muscle cells (VSMCs). The mechanisms responsible for these membrane interactions are poorly understood. In many cells, STIM1 (stromal-interaction molecule 1), a single transmembrane-domain protein that resides in the endoplasmic reticulum (ER), transiently moves to ER-PM junctions in response to depletion of ER Ca^{2+} stores and initiates store-operated Ca^{2+} entry (SOCE). Fully differentiated VSMCs express STIM1 but exhibit only marginal SOCE activity. We hypothesized that STIM1 is constitutively active in contractile VSMCs and maintains peripheral coupling. In support of this concept, we found that the number and size of SR-PM interacting sites were decreased, and SR-dependent Ca^{2+} signaling processes were disrupted in freshly isolated cerebral artery SMCs from tamoxifen-inducible, SMC-specific STIM1-knockout (*Stim1-smKO*) mice. VSMCs from *Stim1-smKO* mice also exhibited a reduction in nanoscale colocalization between Ca^{2+} -release sites on the SR and Ca^{2+} -activated ion channels on the PM, accompanied by diminished channel activity. *Stim1-smKO* mice were hypotensive, and resistance arteries isolated from them displayed blunted contractility. These data suggest that STIM1 – independent of SR Ca^{2+} store depletion – is critically important for stable peripheral coupling in contractile VSMCs.

Keywords: STIM1, vascular smooth muscle, cerebral artery, peripheral coupling sites

Introduction

Subcellular Ca^{2+} -signaling microdomains formed by interactions between the sarcoplasmic reticulum (SR) and the plasma membrane (PM) are vital for many physiological processes, including regulation of the contractility of vascular smooth muscle cells (VSMCs) (1, 2). Ca^{2+} signals that occupy these compartments are typified by Ca^{2+} sparks – large-amplitude Ca^{2+} transients that reflect optically detected Ca^{2+} ions released into the cytosol from the SR through clusters of type 2 ryanodine receptors (RyR2s). Ca^{2+} sparks activate clusters of large-conductance Ca^{2+} -activated K^+ (BK) channels on the PM, generating transient, macroscopic outward K^+ currents that hyperpolarize the PM (1, 3, 4). A complementary Ca^{2+} signaling pathway that causes VSMC membrane depolarization and elevated contractility is formed by interactions between inositol 1,4,5-trisphosphate receptors (IP_3Rs) on the SR and monovalent cation-selective, Ca^{2+} -activated TRPM4 (transient receptor potential melastatin 4) channels on the PM. Ca^{2+} released from the SR through IP_3Rs activates Na^+ influx through TRPM4, causing depolarization of the PM and increased VSMC contractility (2, 5). The close association of the SR and PM creates subcellular compartments where the local Ca^{2+} ion concentration can reach the micromolar range required for activation of BK and TRPM4 channels under physiological conditions (6). In non-excitabile cells, endoplasmic reticulum (ER)-PM junctions and associated proteins have been well characterized (7, 8). In contrast, SR-PM junctional areas of VSMCs and the essential proteins that mediate these interactions remain poorly understood.

The ER-PM junctions of non-excitabile cells are highly specialized hubs for ion channel signaling cascades. These spaces are the sites of one of the most ubiquitous

86 receptor-regulated Ca^{2+} entry pathways in such cells, termed store-operated Ca^{2+} entry
87 (SOCE), which is mediated by the ER-resident Ca^{2+} -sensing protein STIM1 (stromal
88 interaction molecule 1) and Ca^{2+} -selective channels of the Orai group on the PM (9-13).
89 STIM1 is a single-pass transmembrane ER/SR protein that possesses a low-affinity
90 Ca^{2+} -sensing EF-hand facing the lumen of the ER/SR (9, 14-32). Following Ca^{2+} store
91 depletion by IP_3 -producing receptor agonists, STIM1 acquires an extended
92 conformation and migrates to ER-PM junctions, exposing a cytosolic STIM-Orai
93 activating region that physically traps and activates Orai channels on the PM (9, 10, 14,
94 20, 24, 26, 28, 31-37). The other STIM protein family member, STIM2, is structurally
95 similar to STIM1. Fully differentiated VSMCs from systemic arteries express STIM1 but
96 not STIM2 and do not exhibit detectable SOCE or its biophysical manifestation, the Ca^{2+}
97 release-activated Ca^{2+} (CRAC) current (38-40). Many species express IP_3 Rs but lack
98 STIM and Orai proteins, suggesting that receptor-evoked Ca^{2+} signaling is not always
99 complemented by the operation of STIM and Orai mechanisms (41). Evolutionary
100 evidence indicates that Orai appeared before STIM, implying that STIM might have
101 arisen to support the function of ER-PM junctions and only subsequently co-opted an
102 existing Orai for SOCE (41). Additional accumulating evidence indicates that, in addition
103 to its role in SOCE, mammalian STIM1 protein serves as an essential regulator of
104 several other ion channels and signaling pathways. STIM1 both positively and
105 negatively regulates the function of L-type voltage-gated Ca^{2+} channels (Cav1.2) (42),
106 transient receptor potential canonical (TRPC) channels (43), and arachidonate-
107 regulated Ca^{2+} (ARC) channels (44). It has also been reported to regulate the function

of Ca^{2+} pumps, such as the SR/ER Ca^{2+} ATPase (SERCA) and PM Ca^{2+} ATPase (PMCA), as well as several cAMP-producing adenylyl cyclases at the PM (45-48).

In the current study, we investigated the role of STIM1 in the formation of stable peripheral coupling sites in native, contractile SMCs from cerebral arteries. We show that STIM1 knockout disrupts the functional coupling of Ca^{2+} release sites on the SR with Ca^{2+} -dependent ion channels on the PM. We further show that this function of STIM1 is independent of Orai1 channel activity and SR Ca^{2+} store depletion and acts to sustain subcellular Ca^{2+} signaling pathways that are essential for the regulation of VSMC contractility.

Results

Stim1-smKO mice lack STIM1 protein expression in VSMCs.

Mice with *loxP* sites flanking exon 2 of the *Stim1* gene (*Stim1^{fl/fl}* mice) were crossed with myosin heavy chain 11 *Myh11^{CreERT2}* mice (49, 50), generating *Myh11^{CreERT2}; Stim1^{fl/wt}* mice, in which *Myh11* promoter-driven *Cre* expression is induced by injection of tamoxifen. Heterozygous *Myh11^{CreERT2}; Stim1^{fl/wt}* mice were then intercrossed, yielding tamoxifen-inducible *Myh11^{CreERT2}; Stim1^{fl/fl}* mice. Cre-recombinase expression was induced in male *Myh11^{CreERT2}; Stim1^{fl/fl}* mice by daily intraperitoneal injection of tamoxifen (100 μL , 10 mg/mL) for 5 days, beginning at 4–6 weeks of age to generate SMC specific *Stim1* knockout mice (*Stim1-smKO*). Controls for all experiments consisted of *Myh11^{CreERT2}; Stim1^{fl/fl}* mice injected with sunflower oil, the vehicle for tamoxifen. Mice were used for experiments one week after the final injection. The Wes capillary electrophoresis immunoassay-based protein detection system was used for qualitative and quantitative assessment of STIM1 protein in smooth muscle tissues from

Stim1-smKO and control mice. STIM1 protein was readily detected as a single band in cerebral artery, mesenteric artery, aortic, colonic, and bladder smooth muscle isolated from control mice but was virtually undetectable in smooth muscle isolated from *Stim1*-smKO mice (Figure 1A). STIM1 protein levels normalized to total protein (Figure 1-figure supplement 1A) were significantly lower in cerebral artery, aortic, colonic, and bladder smooth muscle from *Stim1*-smKO mice compared with controls (Figure 1A). In contrast, STIM1 protein expression was detected at similar levels in whole brains from both control and *Stim1*-smKO mice (Figure 1A), reflecting STIM1 expression in brain cells apart from VSMCs. Tamoxifen injection had no effect on STIM1 protein levels in *Myh11^{CreERT2}; Stim1^{wt/wt}* mice (Figure 1 – figure supplement 1B–G).

In further studies, single SMCs from cerebral arteries isolated from control and *Stim1*-smKO mice were enzymatically dispersed, immunolabeled with an anti-STIM1 primary antibody, and imaged using a GSDIM (ground state depletion followed by individual molecule return) superresolution microscopy system in epifluorescence (Figure 1B) and TIRF (total internal reflection fluorescence (Figure 1 – figure supplement 2A) modes. We previously showed that our GSDIM system has a lateral resolution of 20–40 nm (51-53). TIRF-mode GSDIM detects fluorophores at or near the plasma membrane to a depth of approximately 150 nm.

VSMCs from control mice exhibited punctate STIM1 protein clusters (Figure 1B). Frequency analyses revealed that the sizes of these clusters were exponentially distributed, with a majority of clusters (~95%) ranging in area between 400 and 7600 nm² (mean = 2135 ± 21 nm²; median = 800 nm²) (Figure 1C). STIM1 cluster density and size were significantly reduced in VSMCs isolated from *Stim1*-smKO mice (Figure 1D

and Figure 1 – figure supplement 2B and C). In addition, the number of GSDIM events detected in VSMCs isolated from *Stim1*-smKO mice was comparable to background levels observed in cells from control mice immunolabeled with secondary antibody only, providing further evidence of effective STIM1 knockdown (Figure 1- figure supplement 2D and E). Taken together, these data demonstrate selective, tamoxifen-inducible SMC-specific knockout of STIM1 expression in *Stim1*-smKO mice.

In many cells, depletion of ER/SR Ca^{2+} stores causes STIM1 to form large clusters and initiate SOCE. Here, we examined the effects of the SERCA pump inhibitor thapsigargin on STIM1 clusters in VSMCs from control mice using TIRF-mode GSDIM. This treatment had no effect on the density or size of STIM1 protein clusters at the plasma membrane (Figure 1- figure supplement 3A-C). In addition, we compared SOCE between cultured, proliferative cerebral artery VSMCs and native, contractile VSMCs. We found that proliferative VSMCs exhibit robust SOCE, whereas SOCE is virtually undetectable in contractile VSMCs (Figure 1 - figure supplement 3D and E). These data indicate that in contractile VSMCs, STIM1 cluster size and density are unaffected by the depletion of SR Ca^{2+} and that SOCE is absent from these cells.

PM and SR coupling is diminished in VSMCs from Stim1-smKO mice.

To investigate how STIM1 knockout affects PM and SR interactions, we costained native SMCs isolated from cerebral arteries of control and *Stim1*-smKO mice with Cell-Mask Deep Red and ER-Tracker green to label the PM and SR, respectively, as described in our prior publications (51, 54). Using live-cell structured illumination microscopy (SIM), we acquired Z-stack images of PM- and SR-labeled VSMCs as 0.25-

µm slices. We then reconstructed the 3D surfaces of the PM and SR from these images (Figure 2A), also generating a third surface indicating the sites of colocalization between the PM and SR (Figure 2A; Videos 1 and 2). The mean volume of the PM did not differ between *Stim1*-smKO and control mice, but the volume of the SR was smaller in cells isolated from *Stim1*-smKO mice (Figure 2B and C). The reduction in SR volume is likely due to the peripheral SR pulling away from the PM. The overall PM-SR colocalization was significantly reduced in VSMCs from *Stim1*-smKO mice compared with controls (Figure 2D). As shown in representative image galleries of individual colocalization sites (Figure 2E and F), the majority of PM-SR coupling sites in cells from both groups formed spherical surfaces, but some of the larger structures exhibited an elongated morphology. Frequency analyses showed that the volume of individual colocalization sites in cells from both groups exhibited an exponential distribution (Figure 2G). In addition, the number of coupling sites per unit volume and mean volume of individual sites were smaller in cells from *Stim1*-smKO mice compared with those from controls (Figure 2H and I). These data indicate that STIM1 maintains contact between the peripheral SR and PM, and interactions between the PM and SR are decreased by *Stim1* knockout in VSMCs.

Stim1 knockout decreases the colocalization of BK and RyR2 protein clusters.

BK channels on the PM of VSMCs are functionally coupled with RyR2s on the SR (1). Therefore, we investigated how *Stim1* knockout affects the nanoscale structure of BK-RyR2 signaling complexes using GSDIM superresolution microscopy. Freshly isolated VSMCs from *Stim1*-smKO and control mice were co-immunolabeled for RyR2

and the BK channel pore-forming subunit BK α and imaged using GSDIM in epifluorescence illumination mode. The resulting superresolution localization maps (Figure 3A, left-most panels) showed that both proteins were present as defined clusters in VSMCs. Using an object-based analysis (OBA) approach (55, 56) as described in previous publications (51, 53, 54, 57, 58), we generated new maps of RyR2 clusters that overlapped at the resolution limit of our microscope system (~20-40 nm) with the centroid of each BK cluster and BK clusters that coincided with the centroid of each RyR2 cluster (Table S1). These two maps were then merged to reveal colocalized RyR2-BK channel protein clusters in VSMCs from both groups of animals that were below the resolution of our GSDIM system (Figure 3A, middle and right-most panels). Particle analysis of these clusters showed that the density of individual BK protein clusters (number of clusters per unit area) was similar for both groups of animals (Figure 3B), whereas the density of individual RyR2 clusters was lower in VSMCs from *Stim1*-smKO mice compared with controls (Figure 3C). In both groups, the sizes of individual BK channel and RyR2 clusters followed an exponential distribution (Figure 3B and C). The mean size of individual BK clusters was smaller in VSMCs from *Stim1*-smKO mice compared with those from controls (Figure 3B); in contrast, the mean size of RyR2 clusters was slightly larger in cells from *Stim1*-smKO mice (Figure 3C). In terms of colocalization, this analysis showed a significant reduction in the density of colocalized BK-RyR2 protein clusters in VSMCs from *Stim1*-smKO mice compared with controls (Figure 3D). The mean size of colocalizing clusters from *Stim1*-smKO mice was smaller compared with those from control mice (Figure 3D), and the sizes of BK-RyR2

colocalization sites in cerebral artery SMCs from both groups exhibited an exponential distribution (Figure 3D).

Stim1 knockout decreases the colocalization of TRPM4 and IP₃R protein clusters.

TRPM4 channels on the PM are functionally coupled with IP₃Rs on the SR (2). Therefore, we also investigated how interactions between PM TRPM4 channels and SR IP₃Rs were altered by *Stim1* knockout. Freshly isolated VSMCs from control and *Stim1*-smKO mice were co-immunolabeled for TRPM4 and IP₃R and imaged using GSDIM in epifluorescence illumination mode. The resulting GSDIM localization maps showed that these proteins are present as discrete clusters in cells (Figure 4A, left-most panels).

We next used OBA to identify and map individual and colocalized TRPM4 and IP₃R protein clusters (Figure 4A, middle and right-most panels; Table S1). This analysis showed that the densities of individual TRPM4 and IP₃R clusters were similar in both groups (Figure 4B and C) and that their sizes were exponentially distributed (Figure 4B and C). The mean sizes of individual TRPM4 and IP₃R clusters were smaller in VSMCs from *Stim1*-smKO mice compared with those from controls. (Figure 4B and C). The density of colocalized TRPM4-IP₃R cluster sites did not differ between groups (Figure 4D), but the sizes of these colocalized clusters were smaller in cells from *Stim1*-smKO mice compared with those from controls (Figure 4D). Like individual clusters, colocalized clusters exhibited an exponential distribution (Figure 4D).

245 *STIM1 colocalizes with BK and TRPM4 channels*

246 To determine the location of STIM1 clusters relative to BK and TRPM4 channel
247 clusters at the plasma membrane, we imaged VSMCs from control mice that had been
248 coimmunolabeled for BK and STIM1 (Figure 5A) or TRPM4 and STIM1 (Figure 5B)
249 using TIRF-GSDIM. The frequency of colocalization of BK and STIM1 clusters and
250 TRPM4 and STIM1 clusters was determined using OBA and mapped (Figure 5A and B).
251 For comparison, new maps were generated from each original superresolution map that
252 replicated the density and cluster size distribution, but a random location was assigned
253 to each protein cluster. We then performed OBA for the simulated random distribution
254 and compared the colocalization frequency of the original maps with the colocalization
255 frequency of their randomized counterparts. The fraction of colocalized clusters in the
256 original maps was greater than its randomized counterpart for every cell (Figure 5C and
257 D). These data show that BK and STIM1 (Figure 5C) and TRPM4 and STIM1 (Figure
258 5D) colocalized more frequently than predicted if the distribution of protein clusters was
259 random, suggesting a mechanistic basis of interaction.

261 *Stim1 knockout alters the properties of Ca^{2+} sparks.*

262 To investigate how *Stim1* knockout alters fundamental Ca^{2+} signaling
263 mechanisms, we loaded freshly isolated VSMCs with the Ca^{2+} -sensitive fluorophore
264 Fluo-4-AM and imaged them using live-cell, high-speed, high-resolution spinning-disk
265 confocal microscopy. Spontaneous Ca^{2+} sparks were present in cerebral artery SMCs
266 from both control (Figure 6A; Video 3) and *Stim1*-smKO (Figure 6B; Video 4) mice. The
267 frequency of Ca^{2+} spark events did not differ between groups (Figure 6C and D).

However, the mean amplitude of Ca^{2+} spark events was significantly greater in VSMCs isolated from *Stim1*-smKO mice compared with those from controls (Figure 6E). Further analyses revealed that spatial spreads, durations, and decay times of individual Ca^{2+} spark events were significantly greater in VSMCs isolated from *Stim1*-smKO mice compared with those taken from control mice, but rise times did not differ (Figure 6F–I). To investigate the effects of *Stim1* knockout on total SR Ca^{2+} store load, we applied a bolus of caffeine (10 mM) to Fluo-4-AM-loaded VSMCs isolated from control and *Stim1*-smKO mice. The peak amplitude of caffeine-evoked global increases in cytosolic $[\text{Ca}^{2+}]$ did not differ between groups (Figure 6J), indicating that *Stim1* knockout did not alter total SR $[\text{Ca}^{2+}]$. Therefore, alterations in the properties of Ca^{2+} sparks associated with the knockout of *Stim1* are not the result of changes in SR Ca^{2+} load.

Stim1 knockout diminishes physiological BK and TRPM4 channel activity.

We next used patch-clamp electrophysiology to investigate how knockout of *Stim1* affects the activity of BK and TRPM4 channels in VSMCs. When Ca^{2+} sparks activate clusters of BK channels at the PM, they generate macroscopic K^+ currents termed spontaneous transient outward currents (STOCs) (1). Here, we recorded STOCs over a range of membrane potentials using the amphotericin B perforated patch-clamp configuration, which allows the membrane potential to be controlled without disrupting intracellular Ca^{2+} signaling pathways (51, 54). The frequencies and amplitudes of STOCs were lower in VSMCs from *Stim1*-smKO mice compared with those from controls at all membrane potentials greater than -60 mV (Figure 7A, B, and C). We measured whole-cell BK channel currents to determine if diminished STOC

activity was attributable to a decrease in the total number of BK channels available for activation at the PM. Cerebral artery SMCs isolated from *Stim1*-smKO and control mice were patch-clamped in the conventional whole-cell configuration, and whole-cell K⁺ currents were recorded during the application of voltage ramps. Using the selective BK blocker paxilline to isolate BK channel currents, we found that whole-cell BK current amplitude did not differ between VSMCs from control and *Stim1*-smKO mice (Figure 7D and E), indicating that the number of BK channels available for activation and their functionality was not altered by *Stim1* knockout. *Stim1* knockout did not alter mRNA levels of BK α - or β 1-subunits or RyR2s in cerebral arteries (Figure 7 – figure supplement 1A). In addition, the potent and selective Orai1 blocker Synta66 (59) had no effect on STOC amplitude or frequency (Figure 7 – figure supplement 1B-D). These findings indicate that diminished STOC activity following knockout of *Stim1* is not due to changes in BK and RyR2 expression and that CRAC channel activity is not required for the generation of STOCs.

TRPM4 is a Ca²⁺-activated, monovalent cation-selective channel that is impermeable to divalent cations (60). At membrane potentials in the physiological range for VSMCs (-70 to -30 mV), TRPM4 channels conduct inward Na⁺ currents that depolarize the plasma membrane in response to increases in intraluminal pressure and receptor-dependent vasoconstrictor agonists (61, 62). Under native conditions, TRPM4 channels are activated by Ca²⁺ released from the SR through IP₃Rs, generating transient inward cation currents (TICCs) (2, 63). To determine the effects of STIM1 knockout on TRPM4 activity, we recorded TICCs using the amphotericin B perforated patch-clamp configuration (63). In agreement with previous reports (5, 62), we found

that TICC activity in VSMCs from control mice was increased following application of negative pressure (-20 mmHg) through the patch pipette to stretch the plasma membrane, an effect that was attenuated by the selective TRPM4 blocker, 9-phenanthrol (Figure 7F). TICC activity and amplitude in VSMCs isolated from *Stim1*-smKO mice were significantly reduced compared with controls (Figure 7F- H). To determine if these differences were attributable to changes in TRPM4 channel function or availability, we activated TRPM4 currents in VSMCs from *Stim1*-smKO and control mice using an internal solution containing 200 μ M free Ca^{2+} and compared whole-cell TRPM4 currents in both groups by patch-clamping VSMCs in the conventional whole-cell configuration (64). The TRPM4-sensitive component of the current was isolated by applying 9-phenanthrol. We found that whole-cell TRPM4 current amplitudes did not differ between VSMCs from control and *Stim1*-smKO mice (Figure 7I and J), suggesting that the number of TRPM4 channels available for activation at the PM was not altered by *Stim1* knockout. *Stim1* knockout did not alter mRNA levels of TRPM4 subunits or any of the IP_3R subtypes (1, 2, or 3) in cerebral arteries (Figure 7- figure supplement 2A). In addition, blockade of Orai1 had no effect on TICC activity (Figure 7 – figure supplement 2B and C). These findings suggest that diminished TICC activity following knockout of *Stim1* is not due to diminished expression of TRPM4 or IP_3Rs , and that generation of TICCs is independent of Orai1 channel activity.

The contractility of resistance arteries from Stim1-smKO mice is blunted.

Knockout of *Stim1* in VSMCs decreased the activity of BK and TRPM4 channels under physiological recording conditions. These channels have opposing effects on

VSMC membrane potential, contractility, and arterial diameter, with BK channels causing dilation (1) and TRPM4 channels causing constriction (65). Thus, the overall functional impact of deficient channel activity is not immediately apparent. Therefore, to investigate the net consequences of *Stim1* knockout on arterial contractile function, we employed a series of *ex vivo* pressure myography experiments. Constrictions of intact cerebral pial arteries in response to a depolarizing concentration (60 mM) of extracellular KCl did not differ between groups (Figure 8A), suggesting that knocking out *Stim1* in cerebral artery SMCs did not grossly alter voltage-dependent Ca^{2+} influx or underlying contractile processes. Contractile responses to increases in intraluminal pressure (myogenic vasoconstriction) were evaluated by measuring steady-state luminal diameter at intraluminal pressures over a range of 5 to 140 mmHg in the presence (active response) and absence (passive response) of extracellular Ca^{2+} . Myogenic tone, calculated by normalizing active constriction to passive dilation, was significantly lower in cerebral arteries from *Stim1*-smKO mice compared with those from controls (Figure 8B and C). Contractile responses to the synthetic thromboxane A_2 receptor agonist U46619 were also significantly blunted in cerebral arteries from *Stim1*-smKO mice compared with those from vehicle-treated controls (Figure 8D and E). These data demonstrate that the ability of cerebral arteries from *Stim1*-smKO mice to contract in response to physiological stimuli is impaired. Additional investigations using 3rd-order mesenteric arteries yielded similar findings (Figure 8F–J), indicating widespread vascular dysfunction in *Stim1*-smKO mice.

Further experiments investigated the effects of the BK channel inhibitor paxilline and the TRPM4 channel inhibitor 9-phenanthrol on vasoconstriction of cerebral arteries

isolated from control and *Stim1*-smKO mice. We found that paxilline increased myogenic tone in cerebral arteries isolated from control mice, whereas this treatment had little effect on cerebral arteries from *Stim1*-smKO mice (Figure 8- figure supplement 1A and B). These findings are consistent with the patch-clamp electrophysiology data indicating low levels of BK channel activity in VSMCs from *Stim1*-smKO mice. Treatment with 9-phenanthrol abolished the myogenic tone of cerebral arteries from control mice but had little effect on cerebral arteries from *Stim1*-smKO mice (Figure 8- figure supplement 1C and D), in agreement with the patch-clamp electrophysiology studies that found low levels of TICC activity in VSMCs from *Stim1*-smKO mice.

We also found that Synta66 had no effect on KCl-induced vasoconstriction or myogenic tone of cerebral arteries from control mice, indicating that these responses are independent of Orai1 channel activity (Figure 8 – figure supplement 1E-G).

Stim1-smKO mice are hypotensive.

Age-matched *Myh11^{CreERT2}; Stim1^{fl/fl}* mice were surgically implanted with radio telemetry transmitters as previously described (66). After a recovery period (14 days), systolic and diastolic blood pressure (BP), heart rate (HR), and locomotor activity levels were recorded for 48 hours before tamoxifen injection (control). Systolic and diastolic BP, HR, and activity levels were again recorded for 48 hours, beginning 1 week after completing the tamoxifen injection protocol (*Stim1*-smKO). Normal diurnal variations were observed for all parameters (Figure 9). The mean systolic BP of *Stim1*-smKO mice was lower than that of control mice during both day and night cycles (Figure 9A), whereas diastolic BP did not differ between groups (Figure 9B). Mean arterial pressure

(MAP) (Figure 9C) was lower in *Stim1*-smKO mice compared with controls at night and tends to be lower during the day ($P = 0.056$). The pulse pressure of *Stim1*-smKO mice was lower than that of control mice during both day and night cycles (Figure 9D). HR (Figure 9E) and locomotor activity (Figure 9F) did not differ between groups. Vehicle injection did not affect BP, HR, or locomotor activity (Figure 9 – figure supplement 1). These data indicate that acute knockout of *Stim1* in VSMCs lowers BP, probably due to diminished arterial contractility and decreased total peripheral resistance.

Discussion

Junctional membrane complexes formed by close interactions of the ER/SR with the PM are critical signaling hubs that regulate homeostatic and adaptive processes in nearly every cell type. The canonical function of STIM1 is to enable SOCE via Orai channels, but mounting evidence suggests that the protein has additional, SOCE-independent functions. Here we show that STIM1 is crucial for fostering SR-PM junctions and functional coupling between SR and PM ion channels that control VSMC contractility. In support of this concept, we found that the number and sizes of SR/PM coupling sites were significantly reduced in VSMCs from *Stim1*-smKO mice. *Stim1* knockout also altered the nanoscale architecture of ion channels in Ca^{2+} -signaling complexes, transformed the properties of Ca^{2+} sparks, and diminished BK and TRPM4 channel activity under physiological recording conditions. BK and TRPM4 channel activity and vasoconstrictor responsiveness were not altered by selective inhibition of Orai1. Resistance arteries isolated from *Stim1*-smKO mice exhibited blunted responses to vasoconstrictor stimuli, and animals became hypotensive following acute knockout of *Stim1* in smooth muscle. These findings collectively demonstrate that in contractile

VSMCs, STIM1 expression is necessary for the functional coupling of Ca^{2+} release sites on the SR and Ca^{2+} -activated ion channels on the PM in a manner independent of Orai1, Ca^{2+} store depletion, and SOCE. Loss of functional coupling in VSMCs following *Stim1* knockout has profound consequences, disrupting arterial function and BP regulation.

The SR-PM signaling domains of VSMCs are less orderly compared with those in cardiac and skeletal muscle cells and remain incompletely characterized. SR-PM junctions within the transverse (T) tubules of cardiomyocytes and skeletal muscle cells have regular, repeating structures that are formed, in part, by cytoskeletal elements and proteins of the junctophilin (67-69) and triadin (70, 71) families. In VSMCs, which lack T-tubules, SR-PM interactions occur at peripheral coupling sites that form throughout the periphery with no apparent distribution pattern. Our research team has previously identified vital roles for microtubule networks (54) and junctophilin 2 (JPH2) (51) in the formation of peripheral coupling sites in VSMCs. Here, we found that knockout of *Stim1* in VSMCs with intact SR Ca^{2+} stores reduced the number and sizes of SR-PM colocalization sites. Why is STIM1 active under these conditions? A simple explanation is that resting SR $[\text{Ca}^{2+}]$ in fully differentiated, contractile VSMCs is sufficiently low to trigger constitutive activation of STIM1. This concept is supported by a report by Luik et al. (72), who showed that the half-maximal concentration ($K_{1/2}$) of ER Ca^{2+} for the activation of I_{CRAC} in Jurkat T cells is 169 μM and the $K_{1/2}$ for redistribution of STIM1 to the PM is 187 μM . These data are in close agreement with another study, which reported that the $K_{1/2}$ of ER Ca^{2+} for redistribution of STIM1 in HeLa cells was 210 μM and that for maximum redistribution was 150 μM (73). Few studies have reported SR

[Ca²⁺] measurements in native, contractile VSMCs. Using the low-affinity ratiometric Ca²⁺ indicator, mag-fura-2, one well-controlled study estimated that resting SR [Ca²⁺] in contractile SMCs was ~110 μM (4). Under these conditions, STIM1 is expected to be in a fully active configuration which is also supported by our data where thapsigargin failed to increase the number or size of STIM1 puncta in contractile VSMCs. It is also possible that regional SR [Ca²⁺] levels near active Ca²⁺-release sites (RyRs and IP₃R) are lower than global SR [Ca²⁺], which could further stimulate STIM1 activity at these sites and reinforce junctional coupling. Thus, we put forward the concept that STIM1 is in an active state in quiescent contractile smooth muscle and is necessary for forming Ca²⁺ signaling complexes that are vital for contractile function. Our data further imply that, as VSMCs transition to a proliferative phenotype during the development of disease states associated with vascular remodeling, SR Ca²⁺ levels increase, leading to STIM1 inactivation, loss of stable peripheral coupling, and acquisition of SOCE activity (74).

It would be useful to define the molecular composition of microdomains formed by the interactions of the SR and PM in VSMCs. However, we cannot image SR and PM dyes in native cells using GSDIM because the high laser levels and long exposure times required for this technique bleach the dyes. The SIM mode of our LLS instrument is ideal for imaging the dyes (due to low bleaching) but lacks the resolution of the GSDIM system needed for the nanoscale detection of protein clusters. Consequently, we cannot simultaneously image the sites of membrane interaction and protein clusters. An examination of our superresolution maps suggests that all protein clusters are uniformly distributed – there are no apparent sights of enrichment. Therefore, it seems

likely that the ion channel content of the SR:PM interacting domains does not significantly differ from regions of the PM that do not interact with the SR.

Ion channel proteins in the membranes of excitable cells form discreet clusters whose sizes are exponentially distributed, a phenomenon that has been suggested to occur through stochastic self-assembly (75). Here, we found that acute knockout of STIM1 in VSMCs reduced the mean sizes of BK, TRPM4, and IP₃R protein clusters and slightly increased the mean size of RyR2 protein clusters. According to the stochastic model proposed by Sato et al. (75), the steady-state size of membrane protein clusters is limited by the probability of removal from the PM through recycling or degradation processes, with larger clusters having a higher likelihood of removal. Thus, the smaller size of BK, TRPM4, and IP₃R clusters following STIM1 knockout is likely a consequence of an increase in the rate of channel removal from the membrane. STIM1 knockout also caused a reduction in the SR volume as it retracts from the PM, suggesting that STIM1 is necessary for maintaining contact between the peripheral SR and PM. Accordingly, we propose that by maintaining a connection between the peripheral SR and PM, STIM1 increases the dwell time of BK, TRPM4, and IP₃Rs proteins in the membrane, allowing larger clusters to form. This could be the result of direct protein-protein interactions. For example, previous studies have provided evidence of direct interactions between STIM1 and IP₃Rs (76, 77), and our data show that STIM1 interacts with BK and TRPM4 at the nanoscale, potentially influencing cluster formation. However, our data show that the majority of BK and TRPM4 protein clusters do not colocalize with STIM1. It is more likely that the intact peripheral SR partially protects membrane proteins from endocytic and/or recycling cascades, allowing larger clusters

to form before they are removed. Loss of the peripheral SR following STOM1 knockdown removes this defense, decreasing the dwell time of proteins in the PM, resulting in smaller clusters.

Knockout of *Stim1* in VSMCs significantly impacted Ca^{2+} signaling, ion channel activity, vascular contractility, and the regulation of BP. We purport that these outcomes result from nanoscale disruptions in cellular architecture. Ca^{2+} sparks occur within microdomains formed by SR/PM junctional sites. An increase in the distance between the two membranes will enlarge the area of the microdomains. This likely explains the observed increase in the spatial spread of Ca^{2+} sparks when STIM1 is knocked out. An enlargement of the microdomains also increases the distance between the source of the Ca^{2+} spark and the SERCA and PMCA pumps and Na/Ca^{2+} exchangers, which remove Ca^{2+} from the cytosol (78-80), potentially contributing to prolonged decay and increased amplitude seen in cells from *Stim1*-smKO mice. Thus, the compromised structural integrity of subcellular Ca^{2+} signaling microdomains formed by interactions of the PM and SR likely accounts for the altered properties of Ca^{2+} sparks associated with STIM1 knockout. Decreased nanoscale colocalization of BK with RyR2 and TRPM4 with IP_3Rs manifested as diminished Ca^{2+} -dependent activity of BK and TRPM4 channels (STOCs and TICC), reflecting a loss in the functional coupling of Ca^{2+} -release sites on the SR and ion channels on the PM. The smaller sizes of BK and TRPM4 protein clusters on the PM following *Stim1* knockdown may also reduce BK and TRPM4 channel currents. At the intact blood vessel level, the diminished TRPM4 and BK channel activity resulted in impaired contractility in response to physiological stimuli. This finding is interesting because our prior studies investigating the role of microtubular structures (54) and JPH2

(51) in maintaining peripheral coupling in VSMCs showed that disruption of PM-SR interactions caused cerebral arteries to become hypercontractile. In these studies, arterial hypercontractility resulted from interruption of the BK-RyR2 signaling pathway, which hyperpolarizes the VSMC membrane and balances the depolarizing and contractile influences of the TRPM4-IP₃R cascade. *Stim1* knockout, in contrast, affected both pathways, indicating that STIM1 influences peripheral coupling in a manner that differs from that of the microtubule network and JPH2 and further suggesting heterogeneity in the formation of junctional membrane complexes in VSMCs. Diminished arterial contractility following *Stim1* knockout resulted in a drop in arterial BP, probably due to decreased total peripheral resistance. This finding differs from previous reports by other groups showing that, although myogenic tone and phenylephrine-induced vasoconstriction was blunted in mesenteric arteries from a constitutive SMC-specific STIM1-knockout model, resting BP was not affected in this model (81-83). This difference is likely due to elevated levels of circulating catecholamines, which increase HR and cardiac output and thereby compensate for diminished vascular resistance (83).

In summary, our data demonstrate a vital role for STIM1 in the maintenance of critical Ca²⁺-signaling microdomains in contractile VSMCs that is independent of SR Ca²⁺ store depletion. Disruptions in cellular architecture at the nanoscale level associated with the loss of STIM1 resulted in arterial dysfunction and impaired BP regulation, highlighting the essential nature of Ca²⁺-signaling complexes formed by SR-PM interactions in cardiovascular control.

Methods

Animals

All animal studies were performed in accordance with guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Nevada, Reno. Mice were housed in cages on a 12-hour/12-hour day-night cycle with ad libitum access to food (standard chow) and water. All transgenic mouse strains were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice with *loxP* sites flanking exon 2 of the *Stim1* gene (*Stim1^{fl/fl}* mice) were crossed with myosin heavy chain 11 *Myh11^{CreERT2}* mice (49, 50), generating *Myh11^{CreERT2}; Stim1^{fl/wt}* mice. Heterozygous *Myh11^{CreERT2}; Stim1^{fl/wt}* mice were then intercrossed, yielding *Myh11^{CreERT2}; Stim1^{fl/fl}* mice.

Induction of STIM1 knockout

Male *Myh11^{CreERT2}; Stim1^{fl/fl}* mice were intraperitoneally injected at 4–6 weeks of age with 100 μ L of a 10 mg/mL tamoxifen solution once daily for 5 days to produce *Stim1*-smKO mice. Mice were used for experiments 1 week after the final injection. Littermate *Myh11^{CreERT2}; Stim1^{fl/fl}* mice injected with the vehicle for tamoxifen (sunflower oil) were used as controls for all experiments.

Wes capillary electrophoresis

Tissues isolated from mice were homogenized in ice-cold RIPA buffer (25 mM Tris pH 7.6, 150 mM NaCl, 1% Igepal CA-630, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitor cocktail (Cell Biolabs, Inc., San Diego, CA) using a mechanical homogenizer followed by sonication. The resulting homogenate was centrifuged at

14,000 rpm for 20 minutes at 4°C, and the supernatant containing proteins was collected. Protein concentration was quantified with a BCA protein assay kit (Thermo Scientific, Waltham, MA) by absorbance spectroscopy using a 96-well plate reader. Proteins were then resolved by capillary electrophoresis using the Wes system (ProteinSimple, San Jose, CA, USA) and probed with an anti-STIM1 primary antibody (S6072; Sigma-Aldrich, St. Louis, MO, USA). Total protein expression was quantified using the *Total Protein Detection Module for Wes* from Proteinsimple, which utilizes biotin labeling of all proteins that are then detected using Streptavidin-HRP chemiluminescence. Bands were analyzed using Compass for SW (ProteinSimple). STIM1 band intensities were normalized to the total protein band intensities of the respective samples.

SMC isolation

Mice were euthanized by decapitation and exsanguination under isoflurane anesthesia. Cerebral pial arteries were isolated carefully in ice-cold Mg^{2+} -containing physiological salt solution (Mg^{2+} -PSS; 5 mM KCl, 140 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose; pH 7.4, adjusted with NaOH) and then incubated in an enzyme cocktail containing 1 mg/ml papain (Worthington Biochemical Corp., Lakewood, NJ, USA), 1 mg/mL dithiothreitol (DTT; Sigma-Aldrich), and 10 mg/mL bovine serum albumin (BSA; Sigma-Aldrich) for 12 minutes at 37°C. The arteries were then washed three times with Mg^{2+} -PSS and incubated in 1 mg/mL collagenase type II (Worthington) in Mg^{2+} -PSS for 14 minutes. The arteries were washed three times with Mg^{2+} -PSS and then dissociated into single cells by triturating with a fire-polished glass Pasteur pipette.

SOCE

Contractile and proliferative VSMCs were allowed to attach to glass coverslips overnight, and coverslips were then mounted to a Teflon chamber and incubated with 4 μ M Fura2-AM in complete media at 37°C for 45 min. Loading solution included 0.1% of Pluronic F-127 to facilitate Fura2-AM loading. Cells were then washed with a Hepes-buffered salt solution (HBSS) containing 140 mM NaCl, 4.7 mM KCl, 1.13 mM MgCl₂, 10 mM HEPES, 2.0 mM CaCl₂, and 10 mM glucose (pH 7.4 adjusted by NaOH). Cells were then incubated for 10 min in HBSS at room temperature before recordings. Coverslips were then mounted to Nikon TS100 inverted microscope equipped with a 20X Fluor objective and 0.75 numerical aperture. Fura2-AM was alternately excited at 340 and 380 nm, and fluorescent emission was captured at 510 nm. Fluorescence from multiple cells (10-35) were recorded and analyzed with a digital fluorescence imaging system (InCytim2, Intracellular Imaging, Cincinnati, OH). The fluorescence ratio at 340 and to 380 was obtained for each pixel. Thapsigargin at a final concentration of 2 μ M was suspended in HBSS. Mean data are reported as the peak F_{340}/F_{380} ratio.

Visualization of PM-SR colocalization sites using SIM

Cerebral pial artery SMCs were allowed to adhere onto poly-L-lysine-coated round coverslips (5 mm diameter) during a 30-minute incubation at 37°C with the SR stain, ER-Tracker Green (Thermo Fisher Scientific; diluted 1:1000 in Mg²⁺-PSS). After incubation, ER-Tracker Green was removed, and the PM stain Cell-Mask Deep Red (Thermo Fisher Scientific; diluted 1:1000 in Mg²⁺-PSS) was added, and cells were incubated for 5 minutes at 37°C. Cell-Mask Deep Red was then removed, and cells were washed with Mg²⁺-PSS and imaged using a lattice light-sheet microscope (LLSM; Intelligent Imaging Innovations, Inc., Denver, CO) (84). Coverslips with stained cells

were mounted onto a sample holder and placed in the LLSM bath, immersed in Mg^{2+} -PSS. Imaging was performed in SR-SIM mode, set to 100-ms exposures. For each cell, 200 Z-steps were collected at a step size of 0.25 μm . Imaging was limited to no more than 30 minutes for each coverslip to prevent artifacts caused by internalization of the plasma membrane dye. Surface reconstruction and colocalization analyses of PM and SR were performed using Imaris v9.8 (Bitplane, Zurich, Switzerland) image analysis software. The Surface-Surface coloc plugin was used to visualize areas of the PM and SR that colocalized to form coupling sites. PM-SR colocalization percentage was calculated by dividing the total PM-SR colocalization site volume by PM volume and multiplying by 100. Using fluorescent beads, we determined that for the SIM modality of the LLS the resolution for 642 nm wavelength (used for PM labeling) is 250 to 335 nm and the resolution for 488 nm wavelength (used for SR labeling) is 225 to 295 nm.

GSDIM superresolution microscopy

Ground state depletion microscopy followed by individual molecule return (GSDIM) was performed as described previously (51, 53, 54, 57, 58). For epifluorescence imaging, freshly isolated cerebral pial artery SMCs were allowed to adhere onto poly-L-lysine coated glass coverslips for 30 minutes. For TIRF imaging, the coverslips were first cleaned by sonicating in 5 N NaOH for 45 minutes and then sonicated for another 45 minutes in deionized water before adding freshly isolated cerebral pial artery SMCs. The cells were then fixed for 20 minutes with 2% paraformaldehyde, quenched with 0.4 mg/mL $NaBH_4$, and permeabilized with 0.1%

611 Triton X-100. Cells were then blocked with 50% SEABLOCK blocking buffer (Thermo
612 Fisher Scientific, Waltham, MA) for 2 hours and incubated overnight at 4°C with primary
613 antibody (Anti-STIM1 – (4916) Cell Signaling Technologies, Danvers, MA; Anti-STIM1
614 (610954) BD Biosciences, Franklin Lakes, NJ; Anti-BK α 1 – (APC-021) Alomone Labs,
615 Jerusalem, Israel; Anti-RyR2 – (MA3-916) Thermo Fisher Scientific, Waltham, MA; Anti-
616 TRPM4 – (ABIN572220) antibodies-online.com, Limerick, PA; Anti-IP₃R – (ab5804)
617 Abcam, Cambridge, UK) diluted in PBS containing 20% SEABLOCK, 1% BSA, and
618 0.05% Triton X-100. Cells were washed three times with 1X PBS after each step. After
619 overnight incubation, unbound primary antibody was removed by washing four times
620 with 20% SEABLOCK, after which cells were incubated with secondary antibodies
621 (Alexa-Fluor 647– or Alexa-Fluor 568–conjugated goat anti-rabbit, goat anti-mouse,
622 donkey anti-goat or donkey anti-rabbit as appropriate) at room temperature for 2 hours
623 in the dark. After washing with 1X PBS, coverslip-plated cells were mounted onto glass
624 depression slides in a thiol-based photo-switching imaging buffer consisting of 50 mM
625 Tris/10 mM NaCl (pH 8), 10% glucose, 10 mM mercaptoethylamine, 0.48 mg/mL
626 glucose oxidase, and 58 μ g/mL catalase. Coverslips were sealed to depression slides
627 with Twinsil dental glue (Picodent, Wipperfurth, Germany) to exclude oxygen and
628 prevent rapid oxidation of the imaging buffer. Superresolution images were acquired in
629 epifluorescence or TIRF mode using a GSDIM imaging system (Leica, Wetzlar,
630 Germany) equipped with an oil-immersion 160 $\times$ HCX Plan-Apochromat (NA 1.47)
631 objective, an electron-multiplying charge-coupled device camera (EMCCD; iXon3 897;
632 Andor Technology, Belfast, UK), and 500-mW, 532- and 642-nm laser lines.
633 Localization maps were constructed from images acquired at 100 Hz for 25,000 frames

using Leica LAX software. Post-acquisition image analyses of cluster size distribution were performed using binary masks of images in NIH ImageJ software. Object-based analysis was used to establish colocalization of proteins of interest in superresolution localization maps.

Object-based colocalization analysis

Object-based analysis was used to establish the co-localization of BK channels with RyR2 and TRPM4 channels with IP₃R in superresolution localization maps. We used NIH ImageJ software with the JACoP co-localization analysis plug-in (55, 56). The JACoP plug-in was used to split the two channels representing fluorophores detected by Alexa-Fluor 568 or Alexa-Fluor 647. Contiguous objects in both channels were identified by systematically inspecting the neighboring 8 pixels (in 2D) of a reference pixel. All adjacent pixels with intensities above a user-defined threshold limit were considered part of the same structure as the reference pixel and were segmented as individual objects. The superresolution localization maps were previously thresholded by the detection algorithm incorporated into the LAX software used for image acquisition. Therefore, the threshold level in JACoP was set to 1 (nearly the minimum) for all images. After thresholding, centroids (defined as the single-pixel geometric centers of the specified objects) were determined for each object. Clusters in the other wavelength within 20 nm (resolution limit of our GSDIM system) of the centroid were considered “colocalized.” The percentage of colocalizing clusters was calculated as the number of colocalizing clusters as a percentage divided by the total number of clusters detected. This method of colocalization analysis can overcome artifacts caused due to uneven fluorescence intensities and is appropriate to use when the analyzed objects in question

are small and punctate like the protein clusters in our images (56). For comparison, new maps were generated from each original superresolution map using JACoP that replicated the density and cluster size distribution, but the location of each protein cluster was assigned to a random site. We then performed OBA for the simulated random distribution and compared the colocalization frequency of the original maps with the colocalization frequency of their randomized counterparts.

Patch-clamp electrophysiology

Freshly isolated cerebral artery SMCs were transferred to the recording chamber and allowed to adhere to glass coverslips at room temperature for 20 minutes. Recording electrodes (3–4 MΩ) were pulled on a model P-87 micropipette puller (Sutter Instruments, Novato, CA, USA) and polished using a MF-830 MicroForge (Narishige Scientific Instruments Laboratories, Long Island, NY, USA). Spontaneous transient outward currents (STOCs) and transient inward cation currents (TICCs) were recorded in Ca²⁺-containing PSS (134 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, and 10 mM glucose; pH 7.4, adjusted with NaOH). The patch pipette solution contained 110 mM K-aspartate, 1 mM MgCl₂, 30 mM KCl, 10 mM NaCl, 10 mM HEPES, and 5 μM EGTA (pH 7.2, adjusted with NaOH). Amphotericin B (200 μM), prepared on the day of the experiment, was included in the pipette solution to perforate the membrane. Currents were recorded using an Axopatch 200B amplifier equipped with an Axon CV 203BU headstage (Molecular Devices) for all experiments. Currents were filtered at 1 kHz, digitized at 40 kHz, and stored for subsequent analysis. Clampex and Clampfit (version 10.2; Molecular Devices) were used for data acquisition and analysis, respectively. For STOCs, cells were clamped at a membrane potential manually

680 spanning a range from -60 mV to 0 mV. STOCs were defined as events > 10 pA, and
681 their frequency was calculated by dividing the number of events by the time between
682 the first and last event. The potential contribution of Orai1 channels to STOCs was
683 assessed by applying Synta66 (10 μ M) to the bath solution, while STOCs were
684 recorded at a physiological membrane potential (-40 mV). Whole-cell K⁺ currents were
685 recorded using a step protocol (-100 to +100 mV in 20 mV steps for 500 ms) from a
686 holding potential of -80 mV. Whole-cell BK currents were calculated by current
687 subtraction following administration of the selective BK channel blocker paxilline (1 μ M).
688 Current-voltage (I-V) plots were generated using currents averaged over the last 50 ms
689 of each voltage step. The bathing solution contained 134 mM NaCl, 6 mM KCl, 10 mM
690 HEPES, 10 mM glucose, 2 mM CaCl₂, and 1 mM MgCl₂; pH 7.4 (NaOH). The pipette
691 solution contained 140 mM KCl, 1.9 mM MgCl₂, 75 μ M Ca²⁺, 10 mM HEPES, 0.1 mM
692 EGTA, and 2 mM Na₂ATP; pH 7.2 (KOH).

693 TICC_s, induced by membrane stretch delivered by applying negative pressure
694 (20 mmHg) through the recording electrode using a Clampex controlled pressure clamp
695 HSPC-1 device (ALA Scientific Instruments Inc., Farmingdale, NY, USA), were recorded
696 from cells clamped at a membrane potential of -70 mV. TICC activity was calculated as
697 the sum of the open channel probability (*N*Po) of multiple 1.75-pA open states (5). The
698 contribution of Orai1 channels to TICC_s activity was assessed by applying Synta66 (10
699 μ M) in the bath solution after activating TICC_s through membrane stretch. Conventional
700 whole-cell TRPM4 currents were recorded using ramp protocol consisting of a 400 ms
701 increasing ramp from -100 to +100 mV ending with 300 ms step at +100 mV from a
702 holding potential of -60 mV. A new ramp was applied every 2 s. TRPM4 whole-cell

currents were recorded in a bath solution consisting of (in mM): 156 NaCl, 1.5 CaCl₂, 10 glucose, 10 HEPES, and 10 TEA-Cl; pH 7.4 (NaOH). The patch pipette solution contained (in mM): 156 CsCl, 8 NaCl, 1 MgCl₂ 10 mM HEPES; pH 7.4 (NaOH) and 200 μM free [Ca²⁺], adjusted with an appropriate amount of CaCl₂ and EGTA as calculated using Max-Chelator software.

Quantitative droplet digital PCR

Total RNA was extracted from arteries by homogenization in TRIzol reagent (Invitrogen, Carlsbad, CA), followed by purification using a Direct-zol RNA microprep kit (Zymo Research, Irvine, CA), DNase I treatment (Thermo Fisher Scientific), and reverse transcription into cDNA using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). Quantitative droplet digital PCR (ddPCR) was performed using QX200 ddPCR EvaGreen Supermix (Bio-Rad, Hercules, CA), custom-designed primers (Supplementary Table S2), and cDNA templates. Generated droplet emulsions were amplified using a C1000 Touch Thermal Cycler (Bio-Rad), and the fluorescence intensity of individual droplets was measured using a QX200 Droplet Reader (Bio-Rad) running QuantaSoft (version 1.7.4; Bio-Rad). Analysis was performed using QuantaSoft Analysis Pro (version 1.0.596; Bio-Rad).

Imaging of Ca²⁺ Sparks

A liquid suspension (~0.2 mL) of freshly isolated VSMCs was placed in a recording chamber (RC-26GLP, Warner Instruments, Hamden, CT, USA) and allowed to adhere to glass coverslips for 20 minutes at room temperature. VSMCs were then loaded with the Ca²⁺-sensitive fluorophore, Fluo-4 AM (1 μM; Molecular Probes), in the

dark for 20 minutes at room temperature in Mg^{2+} -PSS. Cells were subsequently washed three times with Ca^{2+} -containing PSS and incubated at room temperature for 20 minutes in the dark to allow sufficient time for Fluo-4 de-esterification. Images were acquired using an iXon 897 EMCCD camera (Andor; 16 x 16 μm pixel size) coupled to a spinning-disk confocal head (CSU-X1; Yokogawa), with a 100x oil-immersion objective (Olympus; NA 1.45) at an acquisition rate of 33 frames per second (fps). Custom software (SparkAn; <https://github.com/vesselman/SparkAn>) (85) provided by Dr. Adrian D. Bonev (University of Vermont) was used to analyze the properties of Ca^{2+} sparks. The threshold for Ca^{2+} spark detection was defined as local increases in fluorescence $\geq 0.2 \Delta F/F_0$.

Pressure myography

Pressure myography experiments were conducted using current guidelines (86). Cerebral pial and 3rd order mesenteric arteries were carefully isolated in ice-cold Mg^{2+} -PSS. Each artery was then cannulated and mounted in an arteriography chamber and superfused with oxygenated (21% O_2 /6% CO_2 /73% N_2) Ca^{2+} -PSS (119 mM $NaCl_2$, 4.7 mM KCl, 21 mM $NaCO_3$, 1.18 mM KH_2PO_4 , 1.17 mM $MgSO_4$, 0.026 mM EDTA, 1.8 mM $CaCl_2$, and 4 mM glucose) at 37°C and allowed to stabilize for 15 minutes. Each artery was then pressurized to 110 mmHg using a pressure servo controller (Living Systems instruments, St. Albans City, VT, USA). Any kinks or bends were gently straightened out, the pressure was reduced to 5 mmHg, and the artery was allowed to stabilize for 15 minutes. The viability of each artery was assessed by measuring the response to high extracellular $[K^+]$ PSS (made isotonic by adjusting the $[NaCl]$, 60 mM KCl, 63.7 mM NaCl). Arteries that contracted less than 10% were excluded from further investigation.

Myogenic tone was assessed by raising the intraluminal pressure from 5 mmHg to 140 mmHg in 20-mmHg increments, with the artery maintained at each pressure increment for 5 minutes (active response). The artery was then superfused for 15 minutes at 5 mmHg intraluminal pressure with Ca^{2+} -free PSS supplemented with EGTA (2 mM) and the voltage-dependent Ca^{2+} channel blocker diltiazem (10 μM), followed by application of pressure increments from 5 mmHg to 140 mmHg (passive response). The artery lumen diameter was recorded using edge-detection software (IonOptix, Westwood, MA, USA). Myogenic reactivity at each intraluminal pressure was calculated as $[1 - (\text{Active diameter}/\text{Passive Diameter})] \times 100$. The contribution of Orai1 channels to myogenic tone was assessed by treating vessels with Synta66 (10 μM) in the superfusing bath. The effects of BK and TRPM4 channel inhibition on myogenic tone was assessed in vessels pressurized to 60 mmHg. Tone was allowed to develop before administering paxilline (1 μM) in the superfusing bath to inhibit BK channels or 9-phenanthrol (30 μM) to inhibit TRPM4 channels.

In separate arteries, the contractile response to the thromboxane A_2 receptor agonist U46619 and α_1 -adrenergic receptor agonist phenylephrine was assessed in cerebral and mesenteric arteries, respectively. Arteries were pressurized to 20 mmHg to prevent the development of myogenic tone. Cumulative concentration-response curves were produced by adding U46619 (0.01–1000 nM) or phenylephrine (0.001–100 μM) to the superfusing bath solution. Arteries were maintained at each concentration for 5 minutes or until a steady-state diameter was reached before adding the next concentration. Following the addition of the final concentration, arteries were bathed in Ca^{2+} -free PSS to obtain the passive diameter. Contraction was calculated at each

concentration as vasoconstriction (%) = [(lumen diameter at constriction – lumen diameter at baseline)/passive lumen diameter] × 100.

In vivo radiotelemetry

Stim1-smKO mice were initially anesthetized using 4–5% isoflurane carried in 100% O₂ (flushed at 1 L/min), after which anesthesia was maintained by adjusting isoflurane to 1.5–2%; preoperative analgesia was provided by subcutaneous injection of 50 µg/kg buprenorphine (ZooPharm, Windsor, CO, USA). The neck was shaved and then sterilized with iodine. Under aseptic conditions, an incision (~1 cm) was made to separate the oblique and tracheal muscles and expose the left common carotid artery. The catheter of a radio telemetry transmitter (PA-C10; Data Science International, Harvard Bioscience, Inc., Minneapolis, MN, USA) was surgically implanted in the left common carotid artery and secured using non-absorbable silk suture threads. The body of the transmitter was embedded in a subcutaneous skin pocket under the right arm. After a 14-day recovery period, baseline BP, HR, and locomotor activity were recorded in conscious mice for 48 hours using Ponemah 6.4 software (Data Science International). Parameters were measured for 20 seconds every 5 minutes. Mice were then injected with either vehicle or tamoxifen using the protocol described above; after 7 days following the final injection, baseline BP readings, HR, and locomotor activity were re-recorded in conscious mice for 48 hours.

Chemicals

Unless specified otherwise, all chemicals used were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Statistical analysis

All data are expressed as means $\pm$ standard error of the mean (SEM) unless specified otherwise. Statistical analyses were performed using paired or unpaired Student's t-test, or analysis of variance (ANOVA), as appropriate. A P-value < 0.05 was considered to indicate statistically significant differences. GraphPad Prism v9.3 (GraphPad Software, Inc., USA) was used for statistical analyses and graphical presentations.

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Competing Interests

The authors declare that they have no competing interests.

817 **Supplementary Tables**

	Control	<i>Stim1</i>-smKO
BK:RyR2	2.6±0.25%, n=20 cells	2.2±0.17, n=18 cells
RyR2:BK	1.6±0.15%, n=20 cells	1.8±0.18, n=18 cells
TRPM4:IP3R	5.8±0.39%, n=15 cells	5.2±0.46%, n=15 cells
IP3R:TRPM4	5.1±0.44%, n=15 cells	3.9±0.47%, n=15 cells

818 **Table S1** – Percentage of overlapping clusters for BK:RyR2, RyR2:BK, TRPM4:IP₃R,
819 and IP₃R:TRPM4 in control and *Stim1*-smKO cells.

820

Gene Name	Forward (5' to 3')	Reverse (5' to 3')	Accession number
<i>Kcnma1</i>	GCTTAAGCTCCTGATGATAGCC	AAGGTGGTTCCTCAGGGTTAA	NM_001253358.1
<i>Kcnmb1</i>	ACCAACAGTGCTCCTATATCCC	ACGCTGGTCTCGTTGACTTG	NM_031169.4
<i>Ryr2</i>	TGGAGGACATGCATCCAACA	TCCTATGCCTGACAAGAACTCC	NM_023868.2
<i>Trpm4</i>	TTCACGTACTCTGGCCGAAA	CGGGTAACGAGACTGTACACA	NM_175130.4
<i>Itpr1</i>	AACGTGGGCCACAACATCTA	CCAGGTTTCAGCATGGTTTGAA	NM_010585.4
<i>Itpr2</i>	CCTCAAGACAACCTGCTTCA	TGATGTGCTCCTCAAAGGAC	NM_010586.1
<i>Itpr3</i>	GCAGCGAGAAGCAGAAGAAA	GTTGTCAAACCTTGTCCTCTCC	NM_080553.3

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822 **Table S2** – Forward and reverse primer sequences used for ddPCR experiments.

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833 **Supplementary Video Legends**

834 **Video 1:**

835 PM-SR interactions in a cerebral artery SMC isolated from a control mouse. Animated
836 representation of a SIM image series reconstructed and rendered in 3D. The PM is
837 shown in red and made transparent for better visualization; the SR is shown in green,
838 and colocalized areas are shown in yellow.

839 **Video 2:**

840 PM-SR interactions in a cerebral artery SMC isolated from a *Stim1*-smKO mouse.
841 Animated representation of a SIM image series reconstructed and rendered in 3D. The
842 PM is shown in red and made transparent for better visualization; the SR is shown in
843 green, and areas of colocalization are shown in yellow.

844 **Video 3:**

845 Representative movie showing spontaneous Ca^{2+} sparks in a cerebral artery SMC
846 isolated from a control mouse.

847 **Video 4:**

848 Representative movie showing spontaneous Ca^{2+} sparks in a cerebral artery SMC
849 isolated from a *Stim1*-smKO mouse.

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Figure 1: Inducible SMC-specific *Stim1* knockout.

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(A) Representative Wes protein capillary electrophoresis experiments, presented as Western blots, showing STIM1 protein expression levels in smooth muscle tissues and brains of control and *Stim1-smKO* mice. Summary data showing densitometric analyses of STIM1 protein expression in cerebral artery smooth muscle (CA SM), mesenteric artery smooth muscle (MA SM), aortic smooth muscle, colonic smooth muscle, bladder smooth muscle, and brain, normalized to total protein (n = 3–6 mice/group; *P < 0.05, unpaired t-test). ns, not significant. **(B)** Representative epifluorescence superresolution localization maps of isolated cerebral artery SMCs from control and *Stim1-smKO* mice

1098 immunolabeled for STIM1. Insets: enlarged areas highlighted by the white squares in
1099 the main panels. Scale bars: 3 μ m (main panels) and 250 nm (inset panels). **(C)**
1100 Distribution plot of the surface areas of individual STIM1 clusters in cerebral artery
1101 SMCs isolated from control mice (n = 42726 clusters from 18 cells from 3 mice). **(D)**
1102 STIM1 cluster density in cerebral artery SMCs isolated from control and *Stim1*-smKO
1103 mice (n = 18 cells from 3 mice/group; *P < 0.05, unpaired t-test).

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Figure 1 – figure supplement 1.

(A) Representative Wes blot showing total protein levels in cerebral artery smooth muscle (CA SM), mesenteric artery smooth muscle (MA SM), aortic smooth muscle,

colonic smooth muscle, bladder smooth muscle, and whole-brain tissues isolated from control and *Stim1*-smKO mice. **(B–G)** Summary data showing STIM1 protein expression normalized to total protein levels in cerebral artery smooth muscle **(B)**, mesenteric artery smooth muscle **(C)**, aortic smooth muscle **(D)**, colonic smooth muscle **(E)**, bladder smooth muscle **(F)**, and brain **(G)** tissues isolated from vehicle-injected *Myh11^{CreERT2}: Stim1^{fl/fl}* mice and tamoxifen-injected *Myh11^{CreERT2}: Stim1^{wt/wt}* mice (n = 3–5 mice/group, unpaired t-test). ns, not significant.

Figure 1 – figure supplement 2.

(A) Representative TIRF-mode superresolution localization maps of isolated cerebral artery SMCs from control and *Stim1*-smKO mice immunolabeled for STIM1 (green). Insets: enlarged areas highlighted by the white squares in the main panels. Scale bars: 3 μm (main panels) and 250 nm (inset panels). **(B)** Cluster density (clusters per unit area) of STIM1 clusters in cerebral artery SMCs from control and *Stim1*-smKO mice. **(C)** Mean cluster size of STIM1 clusters in isolated cerebral artery SMCs from control and *Stim1*-smKO mice. (Data from 11566 clusters from 10 cells from 3 mice for control and 2968 clusters from 8 cells from 3 mice for *Stim1*-smKO, * $P < 0.05$, unpaired t-test). **(D)** Representative epifluorescence GSDIM superresolution localization maps of VSMCs from a control and *Stim1*-smKO mice immunolabeled for STIM1, and a control mouse

1144 immunolabeled with only the secondary antibody (2° Ab) used to detect STIM1 (goat
1145 anti-rabbit Alexa Fluor 647). Insets: enlarged areas highlighted by the white squares in
1146 the main panels. Scale bars: 3 μ m (main panels) and 250 nm (inset panels). **(E)** GSDIM
1147 counts detected per unit area in cerebral artery SMCs isolated from control and *Stim1*-
1148 smKO mice immunolabeled with anti-STIM1 antibody, and VSMCs isolated from control
1149 mice immunolabeled with the secondary antibody only. Data are presented as means $\pm$
1150 SEM (n = 18 cells from 3 mice in each group; *P < 0.05 vs. control, one-way ANOVA).
1151 ns, not significant.
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Figure 1 – figure supplement 3.

(A) Representative superresolution localization maps of VSMCs isolated from control mice treated with vehicle or thapsigargin (2 μM) for 10 minutes and immunolabeled for STIM1 (green). Insets: enlarged areas highlighted by the white squares in the main panels. Scale bars: 3 μm (main panels) and 250 nm (inset panels). **(B)** Density (clusters per unit area) of STIM1 clusters in vehicle and thapsigargin-treated VSMCs. **(C)** Mean cluster size of STIM1 clusters in vehicle and thapsigargin-treated VSMCs. (Data from 31779 clusters from 25 cells from 3 mice for vehicle and 18846 clusters from 19 cells from 3 mice for thapsigargin, unpaired t-test). ns, not significant. **(D)** Representative traces showing changes in intracellular [Ca^{2+}] expressed as change in ratiometric

1164 fluorescence (F_{340}/F_{380}) in proliferative and contractile VSMCs after depletion of SR
1165 Ca^{2+} by treatment with thapsigargin (2 μM) in Ca^{2+} -free solution, followed by restoration
1166 of extracellular Ca^{2+} (2 mM) to induce SOCE. **(E)** Summary data of SOCE (expressed
1167 as peak F_{340}/F_{380} ratio) in proliferative and contractile SMCs. (n = 6 cells in each group,
1168 *P < 0.05, unpaired t-test).

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Figure 2: *Stim1* knockout decreases the density and area of PM-SR coupling sites.

(A) Representative 3D surface reconstructions of cerebral artery SMCs isolated from control and *Stim1*-smKO mice labeled with PM (red) and SR (green) dyes and imaged using SIM. Representations of colocalizing PM and SR surfaces (yellow), generated from surface reconstructions. Scale bar: 5 μ m. **(B and C)** PM and SR volumes and **(D)** PM-SR colocalization (%) in cells from control and *Stim1*-smKO mice. **(E and F)** Ensemble images of all PM-SR colocalization sites in single cells from the control and *Stim1*-smKO mice shown in panel A. Scale bar: 10 μ m **(G)** Frequency distribution of the volumes of individual PM-SR colocalization sites in VSMCs isolated from control and *Stim1*-smKO mice. **(H)** Densities and **(I)** mean volumes of individual coupling sites in VSMCs from control and *Stim1*-smKO mice. Data are for 1736 colocalization sites in 19 cells from 6 mice for control and 1484 colocalization sites in 25 cells from 7 mice for *Stim1*-smKO (*P < 0.05, unpaired t-test). ns, not significant.

Figure 3: *Stim1* knockout decreases colocalization of BK and RyR2 protein clusters.

(A) Epifluorescence-mode superresolution localization maps of freshly isolated VSMCs from control and *Stim1*-smKO mice immunolabeled for BK (red) and RyR2 (green). Colocalized BK and RyR2 clusters were identified by object-based analysis (OBA) and mapped (cyan). Scale bar: 3 μ m. Panels to the right show enlarged areas of the original superresolution maps indicated by the white boxes. Scale bar: 500 nm. **(B)** Summary data showing the density (clusters per unit area), frequency distribution of sizes, and mean size of BK channel clusters. **(C)** Summary data showing the density, frequency distribution of sizes, and mean size of RyR2 clusters. **(D)** Summary data showing the density, frequency distribution of sizes, and mean size of colocalizing BK and RyR2 clusters, identified using object-based analysis. For density data, n = 20 cells from 3 mice for controls and n = 18 cells from 3 mice for *Stim1*-smKO mice. For frequency distribution and mean cluster size data: control, n = 44,340 BK channel clusters, n = 15,193 RyR2 clusters, and n = 1054 colocalizing clusters; *Stim1*-smKO: n = 30,552 BK channel clusters, n = 9702 RyR2 clusters, and n = 547 colocalizing clusters (*P < 0.05, unpaired t-test). ns, not significant.

Figure 4: *Stim1* knockout decreases colocalization of TRPM4 and IP₃R protein clusters.

(A) Epifluorescence-mode superresolution localization maps of freshly isolated VSMCs from control and *Stim1*-smKO mice immunolabeled for TRPM4 (cyan) and IP₃R (magenta). Colocalized TRPM4 and IP₃R clusters were identified by object-based analysis (OBA) and mapped (yellow). Scale bar: 3 μ m. Panels to the right show enlarged areas of the original superresolution maps indicated by white boxes. Scale bar: 500 nm. **(B)** Summary data showing the density (clusters per unit area), frequency distribution of sizes, and mean size of TRPM4 channel protein clusters. **(C)** Summary data showing the density, frequency distribution of sizes, and mean size of IP₃R clusters. **(D)** Summary data showing the density, frequency distribution of sizes, and mean size of colocalizing TRPM4 and IP₃R clusters, identified using object-based analysis. For density data, n = 15 cells from 3 mice for both control and *Stim1*-smKO mice. For frequency distribution and mean cluster size data: control, n = 64292 TRPM4 channel clusters, n = 51728 IP₃R clusters, and n = 5164 colocalizing clusters; *Stim1*-smKO mice, n = 56771 TRPM4 channel clusters, n = 45717 IP₃R, and n = 3981 colocalizing clusters (*P<0.05, unpaired t-test). ns, not significant.

and TRPM4

(A) TIRF-mode superresolution localization maps of freshly isolated VSMCs from control mice immunolabeled for BK (red) and STIM1 (green). Colocalized BK and STIM1 clusters were identified by object-based analysis (OBA) and mapped (cyan). Scale bar: 3 μ m. Panels to the right show enlarged areas of the original superresolution maps indicated by the white boxes. Arrows show examples of colocalizing clusters. Scale bar: 500 nm. **(B)** TIRF-mode superresolution localization maps of freshly isolated VSMCs from control mice immunolabeled for TRPM4 (cyan) and STIM1 (magenta). Colocalized TRPM4 and STIM1 clusters were identified by OBA and mapped (yellow). Scale bar: 2 μ m. Panels to the right show enlarged areas of the original superresolution

1263 maps indicated by the white boxes. Arrows show examples of colocalizing clusters.
1264 Scale bar: 500 nm. **(C)** Colocalization frequency of BK and STIM1 clusters in imaged
1265 cells compared to colocalization frequency of BK and STIM1 clusters in randomized
1266 maps generated from respective cells. n = 11 cells from 4 mice. (*P<0.05, paired t-test).
1267 **(D)** Colocalization frequency of TRPM4 and STIM1 clusters in imaged cells compared to
1268 colocalization frequency of TRPM4 and STIM1 clusters in randomized maps generated
1269 from respective imaged cells. (n = 11 cells from 4 mice; *P<0.05, paired t-test).
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Figure 6: *Stim1* knockout alters Ca^{2+} spark properties.

(A and B) Representative time-course images of cerebral artery SMCs isolated from a control **(A)** or *Stim1-smKO* **(B)** mouse exhibiting Ca^{2+} spark events, presented as changes in fractional fluorescence (F/F_0). The elapsed time of the event is shown in seconds (s). Scale bar: 10 μm . **(C)** Representative traces of Ca^{2+} spark events in cerebral artery SMCs isolated from a control (black trace) or *Stim1-smKO* (red trace) mice presented as changes in fractional fluorescence ($\Delta F/F_0$) vs. time. **(D–I)** Summary data showing Ca^{2+} spark frequency **(D)**, amplitude **(E)**, spatial spread **(F)**, event duration **(G)**, rise time **(H)**, and decay time **(I)** in VSMCs isolated from control and *Stim1-smKO* mice (control, n = 43 spark sites in 18 cells from 4 mice; *Stim1-smKO*, n = 41 spark sites in 19 cells from 4 mice; *P < 0.05, unpaired t-test). ns, not significant. **(J)** Summary data showing caffeine (10 mM)-evoked changes in global Ca^{2+} in cerebral

1292 artery SMCs isolated from control and *Stim1*-smKO mice. (Control, n = 8 cells from 4
1293 mice; *Stim1*-smKO, n = 8 cells from 4 mice, unpaired t-test). ns, not significant.

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Figure 7: *Stim1* knockout diminishes physiological BK and TRPM4 channel activity.

(A) Representative traces of STOCs in cerebral artery SMCs from control and *Stim1*-smKO mice, recorded by perforated patch-clamp electrophysiology over a range of membrane potentials (−60 to 0 mV). **(B and C)** Summary data showing STOC frequency **(B)** and amplitude **(C)** (control, n = 13 cells from 4 animals; *Stim1*-smKO, n = 17 cells from 5 mice; *P < 0.05, two-way ANOVA). **(D)** Representative traces of paxilline (1 μM)-sensitive BK currents in cerebral artery SMCs from control and *Stim1*-smKO mice, recorded by patch-clamping in conventional whole-cell mode during a series of command voltage steps (−100 to +100 mV). **(E)** Summary data for whole-cell BK currents (control, n = 6 cells from 3 mice; *Stim1*-smKO, n = 7 cells from 3 mice; two-way ANOVA). **(F)** Representative traces of TRPM4 currents in cerebral artery SMCs from control and *Stim1*-smKO mice voltage-clamped at -70 mV, recorded using perforated patch-clamp electrophysiology. TRPM4 currents were evoked as TICC by application of negative pressure (-20 mmHg) through the patch pipette and were blocked by bath-application of 9-phenanthrol (9-phen; 30 μM). **(G)** Summary data showing TICC activity as TRPM4 channel open probability (NP_o) and **(H)** TICC amplitude in control (n = 12 cells from 5 mice) and *Stim1*-smKO (n = 15 cells from 5 mice) mice (*P < 0.05, unpaired t-test). **(I)** Representative conventional whole-cell patch-clamp recordings of 9-phenanthrol-sensitive TRPM4 currents in cerebral artery SMCs from control and *Stim1*-smKO mice. Currents were activated by free Ca^{2+} (200 μM), included in the patch pipette solution, and were recorded using a ramp protocol from -100 to 100 mV from a holding potential of -60 mV. **(J)** Summary of whole-cell TRPM4 current density at +100

1335 mV (control, n = 5 cells from 3 mice; *Stim1*-smKO, n = 5 cells from 3 mice, unpaired t-
1336 test). ns, not significant.

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Figure 7 – figure supplement 1.

(A) mRNA expression levels (transcript copies/ng of RNA) of *Kcnma1* (BK α 1), *Kcnmb1* (BK β 1), and *Ryr2* (RyR2) in cerebral arteries from control and *Stim1-smKO* mice. Data are presented as means $\pm$ SEM ($n = 3$ mice in each group, unpaired t-test). ns, not significant. **(B)** Representative trace of STOCs recorded from a VSMC isolated from a control mouse before and after the selective Orai1 blocker Synta66 (10 μ M) was applied. **(C and D)** Summary data showing STOC amplitude **(C)** and frequency **(D)** in the absence (control) and presence of Synta66 (10 μ M) ($n = 7$ cells from 4 animals, unpaired t-test). ns, not significant.

Figure 7 – figure supplement 2.

(A) mRNA expression levels of *Trpm4* (TRPM4), *Itpr1* (IP₃R1), *Itpr2* (IP₃R2), and *Itpr3* (IP₃R3) in cerebral arteries from control and *Stim1-smKO* mice. Data are presented as means $\pm$ SEM (n = 3 mice in each group, unpaired t-test). ns, not significant.

(B) Representative trace of TICC activity evoked by application of negative pressure (-20 mmHg) through the patch pipette in a VSMCs from a control mouse voltage-clamped at -70 mV recorded by patch-clamping in the perforated-patch configuration. TRPM4 currents were blocked by bath-application of 9-phenanthrol (9-phen; 30 μ M).

(C) Summary data showing TICC activity as TRPM4 channel open probability (NP_o) in the absence (control) and presence of Synta66 (10 μ M). (n = 6 cells from 3 animals for each group. unpaired t-test). ns - not significant.

Cerebral Arteries

Mesenteric Arteries

Figure 8: Resistance arteries from *Stim1*-smKO mice are dysfunctional.

(A) Summary data showing vasoconstriction of cerebral pial arteries isolated from control and *Stim1*-smKO mice in response to 60 mM KCl (n = 12 vessels from 6 mice for both groups, unpaired t-test). ns, not significant. **(B)** Representative traces showing changes in luminal diameter over a range of intraluminal pressures (5 to 140 mmHg) in cerebral pial arteries isolated from control (black trace) and *Stim1*-smKO (red) mice. Gray traces represent passive responses (Ca²⁺-free solution) to changes in intraluminal pressure for each artery. **(C)** Summary data showing myogenic reactivity as a function of intraluminal pressure (n = 6 vessels from 3 mice for each group; *P < 0.05, two-way ANOVA). **(D)** Representative traces showing changes in luminal diameter in response to a range of concentrations (0.1 to 1000 nM) of the vasoconstrictor agonist U46619 in cerebral arteries isolated from control (black trace) and *Stim1*-smKO (red trace) mice. **(E)** Summary data showing vasoconstriction as a function of U46619 concentration (n = 6 vessels from 3 mice for each group; *P < 0.05, two-way ANOVA). **(F)** Summary data showing vasoconstriction of 3rd-order mesenteric arteries isolated from control and *Stim1*-smKO mice in response to 60 mM KCl (n = 12 vessels from 6 mice for both groups, unpaired t-test). ns, not significant. **(G)** Representative traces showing changes in luminal diameter over a range of intraluminal pressures (5 to 140 mmHg) in 3rd-order mesenteric arteries isolated from control (black trace) and *Stim1*-smKO (red) mice. Gray traces represent passive responses to changes in intraluminal pressure for each artery. **(H)** Summary data for myogenic reactivity as a function of intraluminal pressure (n = 6 vessels from 3 mice for each group, *P < 0.05, 2-way ANOVA). **(I)** Representative traces showing changes in luminal diameter in response to a range of concentrations

1384 (0.01 to 100 μ M) of the vasoconstrictor agonist phenylephrine (PE) in 3rd-order
1385 mesenteric arteries isolated from control (black trace) and *Stim1*-smKO (red trace)
1386 mice. **(J)** Summary data for vasoconstriction as a function of PE concentration,
1387 presented as means $\pm$ SEM (n = 6 vessels from 3 mice for each group; *P < 0.05, two-
1388 way ANOVA).

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Figure 8 – figure supplement 1.

(A and B) Representative traces **(A)** and summary data **(B)** showing the effects of the BK channel blocker paxilline (1 μ M) on the myogenic tone (60 mmHg) of cerebral pial arteries isolated from control (black trace) and *Stim1-smKO* (red trace) mice with (n = 6 vessels from 3 mice, *P<0.05 between untreated and treated, #P<0.05 between the control and *Stim1-smKO* groups, 2-way ANOVA). **(C and D)** Representative traces **(C)** and summary data **(D)** showing the effects of the TRPM4 channel blocker 9-phenanthrol (9-phen; 30 μ M) on the myogenic tone of cerebral pial arteries isolated from control (black trace) and *Stim1-smKO* (red trace) mice (n = 6 vessels from 3 mice, *P<0.05 between untreated and treated, #P<0.05 between the control and *Stim1-smKO* groups, 2-way ANOVA). ns – not significant. **(E)** Summary data showing vasoconstriction of cerebral pial arteries isolated from control mice in response to 60 mM KCl with or without (baseline) the selective Orai1 blocker Synta66 (10 μ M). (n = 6 vessels from 3 mice per group, paired t-test). ns – not significant. **(F)** Representative traces showing changes in luminal diameter over a range of intraluminal pressures (5 to 140 mmHg) in isolated cerebral pial arteries before (black) and after (blue) treatment with Synta66 (10 μ M). Gray trace represents passive response to changes in intraluminal pressure. **(G)** Summary data showing that Synta66 had no effect on myogenic tone (n = 12 vessels from 6 mice for both groups, 2-way ANOVA). ns – not significant.

Figure 9. *Stim1*-smKO mice are hypotensive.

(A) Systolic BP (mmHg) over 48 hours in conscious, radio telemeter-implanted *Myh11^{CreERT2}; Stim1^{fl/fl}* mice before (control) and after (*Stim1*-smKO) tamoxifen injection. Shaded regions depict night cycles (n = 5 for both groups; *P < 0.05 vs. control day, #P < 0.05 vs. control night, paired t-test). **(B)** Diastolic BP measurements for control and *Stim1*-smKO mice (n = 5 for both groups, paired t-test). ns, not significant). **(C)** MAP for control and *Stim1*-smKO mice (n = 5 for both groups, #P < 0.05 vs. control night, paired t-test - ns, not significant). **(D)** Pulse pressure for control and *Stim1*-smKO mice (n = 5 for both groups; *P < 0.05 vs. control day, #P < 0.05 vs. control night, paired t-test). **(E)** HR for control and *Stim1*-smKO mice (n = 5 for both groups, paired t-test - ns, not significant). **(F)** Locomotor activity (arbitrary units [AU]) for control and *Stim1*-smKO mice (n = 5 for both groups, paired t-test). ns, not significant. Forty-eight-hour recordings are shown as means; bar graphs are shown as means ± SEM.

Figure 9 – figure supplement 1.

(A) Systolic BP (mmHg) over 48 hours in conscious, radio telemeter-implanted *Myh11^{CreERT2}; Stim1^{fl/fl}* mice before (control) and after vehicle injection. Shaded regions depict night cycles (n = 5 for both groups, paired t-test). **(B)** Diastolic BP measurements for *Myh11^{CreERT2}; Stim1^{fl/fl}* mice before and after vehicle injection (n = 5 for both groups, paired t-test). **(C)** MAP for *Myh11^{CreERT2}; Stim1^{fl/fl}* mice before and after vehicle injection (n = 5 for both groups, paired t-test). **(D)** Pulse pressure for *Myh11^{CreERT2}; Stim1^{fl/fl}* mice before and after vehicle injection (n = 5 for both groups, paired t-test). **(E)** HR for *Myh11^{CreERT2}; Stim1^{fl/fl}* mice before and after vehicle injection (n = 5 for both groups, paired t-test). **(F)** Locomotor activity (arbitrary units [AU]) for *Myh11^{CreERT2}; Stim1^{fl/fl}* mice before and after vehicle injection (n = 5 for both groups, paired t-test). ns – not significant. Forty-eight-hour recordings are shown as means; bar graphs are shown as means $\pm$ SEM.

1459 **Source Data Files**

1460 **Figure 1-source data.**

1461 Individual data points and analysis summaries for datasets shown in Figure 1.

1462 **Figure 1-figure supplement 1-source data**

1463 Individual data points and analysis summaries for datasets shown in Figure 1-figure
1464 supplement 1.

1465 **Figure 1-figure supplement 2-source data**

1466 Individual data points and analysis summaries for datasets shown in Figure 1-figure
1467 supplement 2.

1468 **Figure 1-figure supplement 3-source data**

1469 Individual data points and analysis summaries for datasets shown in Figure 1-figure
1470 supplement 3.

1471 **Figure 2-source data**

1472 Individual data points and analysis summaries for datasets shown in Figure 2.

1473 **Figure 3-source data**

1474 Individual data points and analysis summaries for datasets shown in Figure 3.

1475 **Figure 4-source data**

1476 Individual data points and analysis summaries for datasets shown in Figure 4.

1477 **Figure 5-source data**

1478 Individual data points and analysis summaries for datasets shown in Figure 5.

1479 **Figure 6-source data**

1480 Individual data points and analysis summaries for datasets shown in Figure 6.

1481 **Figure 7-source data**

1482 Individual data points and analysis summaries for datasets shown in Figure 7.

1483 **Figure 7-figure supplement 1-source data**

1484 Individual data points and analysis summaries for datasets shown in Figure 7-figure

1485 supplement 1.

1486 **Figure 7-figure supplement 2-source data**

1487 Individual data points and analysis summaries for datasets shown in Figure 7-figure

1488 supplement 2.

1489 **Figure 8-source data**

1490 Individual data points and analysis summaries for datasets shown in Figure 8.

1491 **Figure 8-figure supplement 1-source data**

1492 Individual data points and analysis summaries for datasets shown in Figure 8-figure

1493 supplement 1.

1494 **Figure 9-source data**

1495 Individual data points and analysis summaries for datasets shown in Figure 9.

1496 **Figure 9-figure supplement 1-source data**

1497 Individual data points and analysis summaries for datasets shown in Figure 9-figure

1498 supplement 1.

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Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (M. musculus)	C57BL/6 J; Wild type	Jackson Laboratory	Strain #: 000664, RRID:IMSR_JAX:000664	
Genetic reagent (M. musculus)	Stim1 ^{fl/fl}	Jackson Laboratory	Strain #: 023350, RRID:IMSR_JAX:023350	
Genetic reagent (M. musculus)	Myh11 ^{Cre} ; Myh11 ^{Cre} /ERT2	Jackson Laboratory	Strain #: 019079, RRID:IMSR_JAX:019079	
Antibody	Anti-Stim1 (rabbit polyclonal)	Sigma-Aldrich	Cat. #: S6072, RRID:AB_1079008	(1:1000)
Antibody	Anti-Stim1 (rabbit polyclonal)	Cell Signaling Technologies	Cat. #: 4916, RRID:AB_2271287	(1:100)
Antibody	Anti-Stim1 (mouse monoclonal)	BD BioSciences	Cat. #: 610954, RRID:AB_398267	(1:50)
Antibody	Anti-BKα1 (rabbit polyclonal)	Alomone Labs	Cat. #: APC-021, RRID:AB_2313725	(1:100)

Antibody	Anti-RyR2 (mouse monoclonal)	Thermo Fisher Scientific	Cat. #: MA3-916, RRID:AB_2183054	(1:50)
Antibody	Anti-TRPM4 (goat polyclonal)	Antibodies-online.com	Cat. #: ABIN572220, RRID:AB_10787216	(1:400)
Antibody	Anti-IP ₃ R (rabbit polyclonal)	Abcam	Cat. #: ab5804, RRID:AB_305124	(1:200)
Antibody	Alexa-Fluor 647-conjugated anti-mouse IgG (goat polyclonal)	Thermo Fisher Scientific	Cat. #: A-21236, RRID:AB_2535805	(1:1000)
Antibody	Alexa-Fluor 532-conjugated anti-rabbit IgG (goat polyclonal)	Thermo Fisher Scientific	Cat. #: A-11009, RRID:AB_2534076	(1:1000)
Antibody	Alexa-Fluor 647-conjugated anti-goat IgG (donkey polyclonal)	Abcam	Cat. #: ab150131, RRID:AB_2732857	(1:1000)
Antibody	Alexa-Fluor 568-conjugated anti-	Thermo Fisher Scientific	Cat. #: A10042, RRID:AB_2534017	(1:1000)

	rabbit IgG (donkey polyclonal)			
Other	ER-Tracker Green	Thermo Fisher Scientific	Cat. #: E34251	(1:1000)
Other	Cell-Mask Deep Red	Thermo Fisher Scientific	Cat. #: C10046	(1:1000)
Other	Fluo-4 AM	Thermo Fisher Scientific	Cat. #: F14201	1 μ M
Other	Fura-2 AM	Thermo Fisher Scientific	Cat. #: F1221	4 μ M
Chemical compound, drug	Tamoxifen	Sigma-Aldrich	Cat. #: T5648	
Chemical compound, drug	Triton X-100	Sigma-Aldrich	CAS #: 9036-19-5	
Chemical compound, drug	SEABLOCK	Thermo Fisher Scientific	Cat. #: 37527	
Chemical compound, drug	Cysteamine hydrochloride	Sigma-Aldrich	CAS #: 156-57-0	
Chemical compound, drug	Twinsil	Picodent	Cat. #: 1300 5000	
Chemical	TRIzol	Thermo Fisher	Cat. #: 15596026	

compo und, drug		Scientific		
Chemi cal compo und, drug	Synta66	Sigma- Aldrich	Cat. #: SML1949	
Chemi cal compo und, drug	Paxilline	Sigma- Aldrich	Cat. #: P2928	
Chemi cal compo und, drug	9- Phenant hrol	Sigma- Aldrich	Cat. #: 211281	
Peptid e, recomb inant protein	Papain	Worthing ton Biochem ical Corporat ion	Cat. #: LS003119	
Peptid e, recomb inant protein	Collagen ase Type 2	Worthing ton Biochem ical Corporat ion	Cat. #: LS004202	
Peptid e, recomb inant protein	Glucose Oxidase	Sigma- Aldrich	Cat. #: G2133	
Peptid e, recomb inant protein	Catalase	Sigma- Aldrich	Cat. #: C40	
Comm ercial assay or kit	5X RIPA buffer with Protease Inhibitor Cocktail	Cell Biolabs	Cat. #: AKR-190	

Commercial assay or kit	BCA Protein Assay Kit	Thermo Fisher Scientific	Cat. #: 23225	
Commercial assay or kit	12-230 kDa Separation module	Proteinsimple	Cat. #: SM-W004	
Commercial assay or kit	Anti-Rabbit Detection Module	Proteinsimple	Cat. #: DM-001	
Commercial assay or kit	Total Protein Detection Module	Proteinsimple	Cat. #: DM-TP01	
Commercial assay or kit	Direct-zol RNA microprep kit	Zymo Research	Cat. #: R2060	
Commercial assay or kit	qScript cDNA Supermix	Quanta Biosciences	Cat. #: 95047	
Commercial assay or kit	QX200 ddPCR EvaGreen Supermix	Bio-Rad	Cat. #: 186-4033	
Software, algorithm	Compass for SW software	Proteinsimple	https://www.proteinsimple.com/compass/downloads	
Software, algorithm	Imaris software	Bitplane/Oxford Instruments	https://imaris.oxinst.com/packages RRID:SCR_007370	
Software, algorithm	Leica Application Suite X software	Leica	https://www.leica-microsystems.com/products/microscope-software/details/product/leica-las-x-ls/ RRID:SCR_013673	

Software, algorithm	ImageJ software	National Institutes of Health	https://imagej.net/ RRID:SCR_003070	
Software, algorithm	pClamp software	Molecular Devices, LLC	http://www.moleculardevices.com/products/software/pclamp.html RRID:SCR_011323	
Software, algorithm	SparkAn custom software	Dr. Adrian Bonev and Dr. Mark Nelson; PMID: 22095728	N/A	Software used to analyze Ca^{2+} spark events kindly provided by Dr. Adrian Bonev and Dr. Mark Nelson from the University of Vermont
Software, algorithm	IonWizard software	IonOptix, LLC	https://www.ionoptix.com/products/software/ionwizard-core-and-analysis/	
Software, algorithm	Ponemah software	Data Science International	https://www.datasci.com/products/software/ponemah RRID:SCR_017107	
Software, algorithm	GraphPad Prism Software	GraphPad Software, Inc.	https://www.graphpad.com/ RRID:SCR_002798	