ONLINE INFORMATION

Store-Independent Orai1/3 Channels Activated by Intracrine LeukotrineC_4: Role in Neointimal Hyperplasia

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ONLINE METHODS

MATERIALS

Recombinant rat PDGF-BB from R&D Biosystems; Thapsigargin was purchased from Calbiochem; Arachidonic acid from Krackeler Scientific; Leukotriene C_4, Leukotriene B_4 and Leukotriene C_4 ELISA kit from Cayman Chemical; heparin and thrombin from Sigma; 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA, Cs^+ salt) was from Invitrogen. All siRNA sequences were obtained from Dharmacon; shRNA against LTC_4S (shLTC_4S), Orai3 (shOrai3) and a control non-targeting shRNA (shNT) all cloned in the pGipz vector were purchased from Open Biosystems. ShRNA against the fly luciferase (shLuc), STIM1 (shSTIM1) and Orai1 (shOrai1) described earlier(1) were cloned in our laboratory in the pFUGW vector (see sequences in **Online Table III**). Specific primers for rat STIM, Orai, and TRPC are listed in **Online Table III**. Anti-STIM1 antibody was purchased from BD Biosciences, anti-β-actin NH_2-terminal domain from Sigma, and anti-Orai1 (extracellular; catalog no. ACC-060) from Alomone, anti-Orai3 (CT) from Prosci Inc. All other chemical products were obtained from Fisher Scientific unless specified otherwise.

METHODS

VSMC dispersion and culture

The use of rats for these experiments has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Albany Medical College Animal Resource Facility, which is licensed by the US Department of Agriculture and the Division of Laboratories and Research of the New York State Department of Public Health and is accredited by the American Association for the Accreditation of Laboratory Animal Care. Male adult rats (150 g) were euthanized by suffocation in a CO_2 chamber. Aortas or carotids were dissected out into ice-cold physiological saline solution. Fat tissues and endothelium were removed. The artery was cut into small pieces and digested with a papain solution for 20 min at 37°C and then with a mixture of collagenase II and collagenase type H for 15 min at 37°C. The digestion solution was removed, and the cells were washed and gently liberated with a fire-polished glass pipette and transferred to culture plates. Isolated cells are routinely tested for expression of VSMC markers (SM22α) using immunofluorescence. Isolated VSMCs undergo phenotypic modulation in culture that is complete within 30 hours. These synthetic proliferative VSMCs that are reminiscent of vascular disease conditions are maintained in culture (45% DMEM-45% Ham's F-12–10% FBS supplemented with L-glutamine) at 37°C, 5% CO_2, and 100% humidity, passaged (synthetic), and used in all experiments up to passage 8. For freshly isolated VSMCs, dispersed cells are used seeded immediately after isolation for patch clamp experiments or lysed for Western blots.

Cell transfections

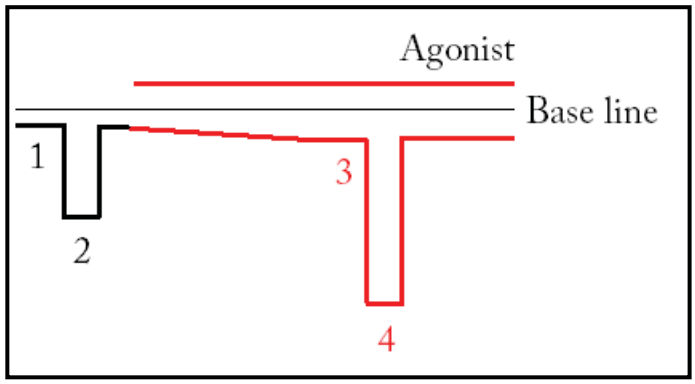
Sets of four different siRNAs per target gene were initially assessed for their ability to reduce mRNA levels using quantitative RT-PCR (qPCR) as described below; the primers used for each target mRNA are listed in **Online Table III**. SiRNA sequences that induced significant decreases in their target mRNA (over 80%) without cross-effects on other mRNAs were used in Western blotting to confirm protein knockdown as described below. All transfections in VSMCs were done using the Nucleofector device II (Amaxa Biosystems, Gaithersburg, MD) using program # D33 according to the manufacturer's instructions. 0.5 μg of green fluorescent protein (GFP) was co-transfected with siRNA for identification of successfully transfected cells. The control siRNA is a scrambled siRNA sequence.

Ca^{2+} measurements

Ca^{2+} was measured as described previously(1-4). Briefly, coverslips with attached cells were mounted in a Teflon chamber and incubated at 37°C for 1 hour in culture medium (DMEM with 10% FBS) containing 4μM Fura 2-AM (Molecular Probes, Eugene, OR). Cells were then washed and bathed in HEPES-buffered saline solution (in mM: 140 NaCl, 1.13 $MgCl_2$, 4.7 KCl, 2 $CaCl_2$, 10 D-glucose, and 10 HEPES, with pH adjusted to 7.4 with NaOH) for ≥10 min before Ca^{2+} was measured. For Ca^{2+} measurements, fluorescence images of several cells were recorded and analyzed with a digital fluorescence imaging system (InCyt Im2, Intracellular Imaging; Cincinnati, OH). Fura 2 fluorescence at an emission wavelength of 510 nm was induced by exciting alternately at 340 and 380 nm. The ratio of fluorescence at 340 nm to that at 380 nm was obtained on a pixel-by-pixel basis. All experiments were conducted at room temperature.

Whole-cell patch clamp recordings

Conventional whole-cell patch-clamp recordings were carried out using an Axopatch 200B and Digidata 1440A (Axon Instruments, NY) as previously published with few important modifications (2, 4, 5). To reduce the noise to a minimum, we added in series a humbug® noise eliminator that eliminates electrical interference such as simple 50/60Hz sine waves, mixtures of 50/60Hz harmonics, noise spikes from dimmers and complex noise from fluorescent lamps. All experiments were performed at room temperature (20-25°C). Pipettes were pulled from borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota, FL) with a P-97 flaming/brown micropipette puller (Sutter Instrument Company, Novatao, CA) and polished with DMF1000 (World Precision Instruments). Resistances of filled glass pipettes were 2–3 MΩ. Series resistances were in the range of 2–10 MΩ. The liquid-junction potential offset was around 4.6 mV and was corrected. Only cells with tight seals (>16 GΩ) were selected for break in. Immediately after establishing the whole-cell patch-clamp configuration, we start the recording by running a 250 ms voltage ramp (from +100 mV to –140 mV) every 2 s and performing a first DVF pulse before current development. These first I/V curves obtained in Ca^{2+}-containing bath solutions (position 1 in scheme) and divalent free (DVF) bath solutions (position 2) represent background currents that are subtracted from agonist-activated Ca^{2+} currents (position 3) and Na^+ currents (obtained in DVF bath solutions; position 4), respectively. After the currents are fully activated by different stimuli, I/V curves are obtained for Ca^{2+} currents (in Ca^{2+}-containing bath solutions) and Na^+ currents (in



DVF bath solutions). Using Origin Lab 7.5 software (OriginLab, Northampton, MA, USA), I/V curves corresponding to background currents obtained in Ca^{2+} and Na^{+} are subtracted from the I/V curves obtained in Ca^{2+} and Na^{+} after stimulus/agonist addition and maximal current activation. Namely, for Ca^{2+} currents (curve 3 – curve 1) and Na^{+} currents (curve 4 – curve 2), respectively. The subtracted I/V curves are represented as independent I/V curves in all figures. Cells were maintained at a 0 mV holding potential during experiments. Reverse ramps were designed to inhibit Na^{+} channels potentially expressed in VSMCs. High $MgCl_2$ (8mM) was included in the patch pipette to inhibit TRPM7 currents, and 3μM nimodipine was added to the bath solution to generally stabilize membrane patches and reach better seals.

Solutions employed for whole cell patch clamp electrophysiology

Thrombin- and AA-activated currents

Bath Solution: 135mM Na-methanesulfonate, 10mM CsCl, 1.2mM $MgSO_4$, 10mM HEPES, 20mM $CaCl_2$, and 10mM glucose (pH was adjusted to 7.4 with NaOH). 100nM Thrombin or 8μM AA was added to the bath where indicated in figures.
Pipette Solution: 145mM Cs-methanesulfonate, 10mM Cs-1,2-*bis*-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (Cs-BAPTA), 5mM $CaCl_2$, 8mM $MgCl_2$, and 10mM HEPES (pH adjusted to 7.2 with CsOH). Calculated free Ca^{2+} is 150nM as estimated using Maxchelator software (http://maxchelator.stanford.edu/).

Store depletion-activated currents

Bath Solution: 135mM Na-methanesulfonate, 10mM CsCl, 1.2mM $MgSO_4$, 10mM HEPES, 20mM $CaCl_2$, and 10mM glucose (pH was adjusted to 7.4 with NaOH).
Pipette Solution: 145mM Cs-methanesulfonate, 20mM Cs-1,2-*bis*-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (Cs-BAPTA), 8mM $MgCl_2$, and 10mM HEPES (pH adjusted to 7.2 with CsOH).

LeukotrieneC_4-activated currents

Bath Solution: 135mM Na-methanesulfonate, 10mM CsCl, 1.2mM $MgSO_4$, 10mM HEPES, 20mM $CaCl_2$, and 10mM glucose (pH was adjusted to 7.4 with NaOH).
Pipette Solution: 145mM Cs-methanesulfonate, 10mM Cs-1,2-*bis*-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (Cs-BAPTA), 5mM $CaCl_2$, 8mM $MgCl_2$, and 10mM HEPES (pH adjusted to 7.2 with CsOH). Calculated free Ca^{2+} was 150nM using Maxchelator software (http://maxchelator.stanford.edu/). 100nM LeukotrieneC_4 (LTC_4) was added to the pipette solution when indicated.
Divalent-free (DVF) bath solution: 155mM Na-methanesulfonate, 10mM *HEDTA*, 1mM EDTA, and 10mM HEPES (pH 7.4, adjusted with NaOH).

Western blotting

Cells were lysed using RIPA lysis buffer (50mM Tris-HCl (pH 8.0), 150mM NaCl, 1% Triton X-100, 0.2mM EDTA, 0.1% SDS, 0.5% Sodium deoxycholate, 2mM phenylmethylsulfonyl fluoride (PMSF), 10% protease inhibitor cocktail (Roche), 10% phosphatase inhibitor cocktail (Roche). Protein concentrations were determined and proteins (20–100μg) in denaturing conditions were subjected to SDS-PAGE (8%-14%) and then electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad). After the blots were blocked with 5% nonfat dry milk (NFDM) dissolved in Tris-buffered saline containing 0.1% Tween 20 (TTBS) either overnight or 2 h at room temperature, they were washed three times with TTBS for 5 min each and incubated overnight at 4°C with specific primary antibodies [anti-STIM1 (BD Biosciences, 1:250 dilution), anti-Orai1 (Alomone; 1:1,000 dilution), anti-Orai3 (ProSci CT, 1: 125 dilution), anti-GFP (Abcam, 1:1000 dilution), anti-LTC_4S (sc-20108, Santa Cruz Biotechnology, Inc.; 1:500) and β-actin NH_2-terminal

(NT) domain (Sigma; 1:35000 dilution)] in TTBS containing either 1% BSA or 2% NFDM. On the next day, membranes were washed (3 times for 5 min each) with TTBS and incubated for 1h at room temperature with horseradish peroxidase-conjugated secondary antibodies [anti-mouse antibody (1:10,000 dilution; Jackson) or anti-rabbit IgG (1:20,000 dilution; Jackson) in TTBS containing 2% NFDM. Protein bands were visualized by enhanced chemiluminescence using Super Signal West Pico or Femto reagents (Pierce). Signal intensity was measured with a Fuji LAS4000 Imaging Station. Membranes were then stripped and reprobed with β-actin antibody to verify equal loading and densitometric analysis was performed using Image J software.

FRET experiments

VSMCs were transfected by electroporation with the Cameleon D1ER sensor (3μg plasmid DNA per 10^6 cells) and seeded on glass-bottom petri dishes (MatTek) for live confocal imaging using a Zeiss LSM 510 META confocal microscope as described above. For these experiments the Cameleon D1ER sensor (kindly provided by Dr. Roger Y. Tsien, University of California San Diego) was excited with the 458 nm line of the argon laser and the emitted fluorescence was filtered with band filters for the CFP channel (458-490) and the YFP channel (511-554), respectively. ImageJ was employed to subdivide the cell into regions of interest (ROIs) for data quantification. The FRET/CFP ratio is reported as net FRET value. All experiments were conducted at room temperature.

LTC_4 measurement using ELISA

Leukotriene C_4 (LTC_4) concentrations were measured from VSMC culture supernatants before (t=0) and after stimulation (5min or 15 min) with maximal concentrations of thrombin. The assay was performed using the competitive LTC_4 Enzyme Immunoassay Kit obtained from Cayman Chemicals (#520211).

In brief, VSMCs were seeded on 6 well plates (50 x 10^3 cells/well) to 50% confluence, and allowed to recover for 48 hours. Before the assay, cells were washed 3 times with 500μL HBSS and stabilized in the incubator (37°C and 5% CO_2) for 5 minutes. HBSS was exchanged with a HBSS solution containing 500nM thrombin and incubated for 5 and 15 minutes, respectively. The supernatant from 3 wells representing un-stimulated cells and cells stimulated for different time points (5 or 15 min) were collected and kept on ice. Three wells for each condition were assayed in triplicates essentially according to the manufacturer's protocol. Absorbance reading on plates was performed in a microplate reader at a wavelength of 405 nm. Raw absorbance readings of known standards (provided in kit) and unknown experimental data points were analyzed using a 4-parameter logistic fit. This analysis was achieved by employing a computer spreadsheet for data analysis provided by the manufacturer and available online at *www.caymanchem.com/analysis/eia*. LTC_4 concentrations were calculated in pg/mL and statistical analyses were performed as described below. Data shown are representative of three independent experiments performed on different days.

Production of lentiviral particles

Lentiviral particles encoding rat specific shRNA against STIM1, Orai1 and Luciferase control (cloned in the viral vector pFUGW-GFP3) and shRNA against Orai3, LTC_4S, and a non-targeting control shRNA (cloned in the lentiviral vector pGipz-GFP) were produced using standard protocols. The shRNA sequences targeting STIM1, Orai1 and Luciferase were previously described (1). Sequences or origin of shRNAs are included in **Online Table III**. Viral particles were generated using standard protocols. Briefly, PolyJet was used as a transfection reagent (SignaGen) to transfect HEK293FT cells (Invitrogen). The lentiviral constructs pCMV-VSVG, pCMV-dR8.2 and either pFUGW or pGipz encoding-shRNA were co-transfected into a flask of 95% confluent HEK293FT cells. Cell culture media with viral particles were collected at 48h and 72h after transfection and were concentrated using Amicon Ultra-15 filter by

centrifugation. These viral particles were then employed to induce efficient knockdown of protein of interest either in vivo or in vitro. For in vitro infections, cells were studied 7 days post-infection; we determined that at this time point, protein knockdown was optimal.

Balloon injury of rat carotid arteries

The use of rats for these experiments has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Albany Medical College Animal Resource Facility, which is licensed by the US Department of Agriculture and the Division of Laboratories and Research of the New York State Department of Public Health and is accredited by the American Association for the Accreditation of Laboratory Animal Care. Male Sprague-Dawley rats (350–400g body weight; Taconic Farms, Germantown, NY) were anesthetized with xylazine (5mg/kg) and ketamine (70mg/kg) via intraperitoneal injection, and balloon angioplasty was carried out essentially as previously described (1). Briefly, a 2-F Fogarty balloon was inserted through a small arteriotomy in the external carotid artery and passed into the common carotid artery. After balloon inflation to a pressure of 1.5atm, the catheter was partially withdrawn till the bifurcation of carotid artery and the balloon was deflated. Then the balloon catheter was reinserted and the withdrawal of inflated balloon was repeated two more times. After recovery from operation and anesthesia, animals received a postoperative dose of the analgesic buprenorphine (Buprenex; 0.02mg/kg s.c). Sham-operated animals were subjected to a similar surgical procedure with the exception of catheter and balloon insertion.

Sections, Hematoxylin/Eosin (H&E) staining

Rats were euthanized at different time points by asphyxiation in a CO_2 chamber and pieces of carotid arteries were isolated and placed in a cryoprotective embedding medium OCT and then snap frozen in liquid nitrogen. The specimens were stored at -80˚C or sectioned in a Leica CM3050 cryostat. Sections were treated by pre-cooled acetone for 10 minutes at 4˚C and air dried. Hematoxylin/Eosin (H&E) staining was performed following standard protocols (1), with the exception that incubation time of Hematoxylin was increased to 3 minutes and the time with Eosin-Y was decreased to 15 seconds.

RT-PCR and real-time PCR

Total RNA was extracted from cells using a Qiagen RNeasy Mini Kit following the manufacturer's protocol. cDNA was made from 0.5µg of RNA reverse transcribed using oligo(dT) primers (Invitrogen, Carlsbad, CA, USA) and SuperScript III reverse transcriptase (Invitrogen). PCR reactions were completed using Illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The sense and antisense primers targeting rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TRPC, STIM, and Orai isoforms are described in **Online Table III**. All primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). The PCR amplification was done using a MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Amplification started with initial denaturation at 94°C for 5 min and then 40 cycles of denaturation at 94°C for 30 s, annealing at 54.3°C for 1 min, and extension at 72°C for 2 min. Gel electrophoresis was used to identify the PCR products in a 1% agarose gel using ethidium bromide staining. Real-time PCR analysis was performed using a Bio-Rad iCycler and iCycler iQ Optical System Software (Bio-Rad Laboratories). PCR reactions were performed using Bio-Rad iQ SYBR Green Supermix. The PCR protocol started with 5 min at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s at 54.3°C, and 45 s at 72°C. Quantification was measured as sample fluorescence crossed a predetermined threshold value that was just above the background. Expressions of STIM and Orai isoforms were compared to those of the housekeeping gene GAPDH and were measured using comparative threshold cycle values.

Statistical analysis

For patch clamp and animal studies where the n number is less than 10, data are expressed as mean ± range (instead of SEM), and statistical analyses comparing two experimental groups were performed using two-tail t-test with Origin 7.5 software (OriginLab, Northampton, MA).Throughout the figures *, ** and *** indicates p values < 0.05, 0.01 and 0.001 respectively. Differences were considered significant when P < 0.05. Mean ± range and n values are reported in **Table 1** for patch clamp data; for animal studies the n number (n=5 for each animal group) is mentioned in the legend to **Figure 7**. For studies with bigger sample size, including Ca^{2+} imaging, data are represented as mean ± SEM. The n number in the representative Ca^{2+} imaging traces represent the number of cells analyzed simultaneously in the same coverslip and averaged. The two numbers between parentheses (x,y) next to each data point in bar graphs represent: x= number of independent experiments and y= total number of cells from independent experiments/transfections. For comparison between control and one experimental condition two-tail t-test was used. For multiple comparisons, one way ANOVA was performed. The exact p values for all comparisons are included in **Table I** for all patch clamp experiments and also listed for all the remaining experiments in **Online Table II**.

Online Table I. Reversal potentials for Ca^{2+} and Na^{+} currents activated by thrombin, LTC_4 and store depletion (20 mM BAPTA) are represented as mean± range.

Reversal Potential

	I[Ca^{2+}]	I[Na^{+}]	n
Thrombin	63.19 ± 12.66	61.96 ± 12.91	4
LTC_4	60.74 ± 15.96	59.90 ± 15.20	5
Store depletion (BAPTA)	63.24 ± 18.11	61.89 ± 17.62	7

Online Table II. p values for statistical analysis performed on data represented in figures.

Figure	P-Value	Figure	P-Value
2-d	0.00806 (shOrai1 *vs* shLuc)	**3-g**	0.0035
	0.00029 (shOrai3 *vs* shNT)	**5-f**	0.18276
	0.00849 (shSTIM1 *vs* shLuc)	**5-h**	0.00493
	0.00442 (Orai1-E106Q *vs* Control)	**6-g**	0.00143
	0.00378 (Orai3-E81Q *vs* Control)	**6-h**	0.48185
	0.00192 (siOrai3 *vs* siControl)	**7-j**	0.00002
	0.00160 (siSTIM1 *vs* siControl)	**S1-d**	0.00435
3-d	0 (5')	**S2-a**	0
	0 (15')	**S2-d**	0.02681

Online Table III. List of primers used for qPCR/PCR; siRNA and shRNA sequences used to target different genes used in the study are also shown.

PCR Primers

	Forward (5'-3')	Reverse (3'-5')	Size (bp)
rOrai1	ACGTCCACAACCTCAACTCC	ACTGTCGGTCCGTCTTATGG	362
rOrai2	CACCTATTTGCCCTGCTCAT	AGCTTGTGCAGTTCCTCGAT	386
rOrai3	CTGTCCACCAGTCACCACAC	CCACCAAGGATCGGTAGAAA	422
rSTIM1	GGCCAGAGTCTCAGCCATAG	CATAGGTCCTCCACGCTGAT	305

siRNA Sequences

rOrai1#1	CAACAGCAAUCCGGAGCUU
rOrai1#2	GCCAUAAGACGGACCGACA
rOrai2#1	GCCACAACCGUGAGAUCGA
rOrai2#2	GCAUGCACCCGUACAUCGA
rOrai3#1	GGGUCAAGUUUGUGCCCAU
rOrai3#2	GUUUAUGGCCUUUGCCCUA
rSTIM1#1	UAAGGGAAGACCUCAAUU
rSTIM1#2	CAUCAGAAGUGUAUAACUG
rLTC_4S#1	GAGUUCUGCUGCAAGCCUA
rLTC_4S#2	Dharmacon ON-TARGETplus Set of 4 Clones
rTRPC1#1	CTGCTCATCGTAACAACTA
rTRPC1#2	GAGAAATGCTGTTACCATA
rTRPC4#1	GGCTCAGTTCTATTACAAA
rTRPC4#2	CCACGAGGTCCGCTGTAAC
rTRPC6#1	GGACCAGCATACATGTTTA

rTRPC6#2	GTGTGGATTACATGGGCCA

shRNA Sequences

shNT	Open Biosystems sequence 1
shLuciferase	CGTACGCGGAATACTTCGA
shOrai1	GACCGACAGTTCCAGGAGCTCAACGAGCT
shOrai3	CCTCCCTTAGTTTAGCTTCTAA
shSTIM1	GATGATGCCAATGGTGATGTGGATGTGGA
shLTC$_4$S	Open Biosystems sequence C-6

ONLINE FIGURE LEGENDS

Online Figure I. Thrombin stimulation does not cause sustained Ca^{2+} store depletion.
Ca^{2+} imaging experiments showing that maximal concentrations of PDGF (500 ng/mL) in nominally Ca^{2+} free media induce Ca^{2+} release. PDGF was kept throughout and Ca^{2+} was restored to the extracellular space for 6min to prevent excessive Ca^{2+} leak from ER. Subsequent stimulation with maximal concentrations of thrombin (500 nM) in nominally Ca^{2+} free media failed to cause Ca^{2+} release from internal stores suggesting that IP_3-sensitive stores have been completely depleted by PDGF (**a**). When the order of agonist addition was reversed (i.e. thrombin first), the peak of Ca^{2+} release induced by PDGF added after thrombin was similar to the peak generated when PDGF was added first (**c**), suggesting that thrombin only reversibly mobilizes internal Ca^{2+} stores without causing sustained depletion. Please also note that the Ca^{2+} release peak induced by thrombin is rather "skinny" when compared to that of PDGF (duration 1min versus 6min for PDGF). Statistical analysis on PDGF-induced intracellular Ca^{2+} release; whether PDGF was used as 1st or 2nd agonist, a similar extent of Ca^{2+} release was observed in both cases (**b**). However, PDGF-induced Ca^{2+} release essentially eliminated the ability of subsequently added thrombin to cause intracellular Ca^{2+} release (**d**). (x,y) next to each data bar: x=number of independent runs, y= total number of cells. **e**; ER-Ca^{2+} levels were measured by the ER-targeted FRET sensor Cameleon-D1ER, upon stimulation with maximal concentrations of thapsigargin (TG; 4μM), PDGF (500ng/mL) and thrombin (Th; 500nM).

Online Figure II. Orai3 knockdown abrogates thrombin-activated Ca^{2+} entry; thrombin and AA-activated currents are not additive.
a; Because of lack of specific antibody against rat Orai2 protein, we performed quantitative RT-PCR to document specific Orai2 knockdown upon transfection of VSMCs with specific siRNA against rat Orai2 (no effect was seen on Orai1 or Orai3 expression; statistical analysis on data are from three independent transfections, each performed in triplicates). **b**; Western blot analysis validated the specificity of a second siRNA sequence targeting Orai3; Orai3 protein knockdown was readily detected with no effects on Orai1 or STIM1 proteins. Representative Ca^{2+} imaging traces from VSMCs transfected with the same siRNA Orai3 sequence confirmed

the requirement for Orai3 in thrombin-activated Ca^{2+} entry (**c**). Statistical summary of Ca^{2+} imaging data is shown (**d**). **e-g;** Whole-cell patch clamp electrophysiological recordings showing no additivity between thrombin (100nM) and arachidonic acid (AA; 8μM). Na^+ I/V curves and statistical analysis are shown in **f** and **g,** respectively.

Online Figure III. LTC_4 Synthase (LTC_4S) is required for thrombin-activated Ca^{2+} entry.
Whole-cell patch clamp electrophysiological recordings testing for additivity between LTB_4 and thrombin (**a-c**). **d, e**; addition of LTC_4 to the bath solution failed to activate Ca^{2+} or Na^+ currents; Na^+ I/V relationship is shown in **e**. **f**; Representative Ca^{2+} imaging traces (and statistics; **g**) assessing SOCE activated by thapsigargin in VSMCs transfected with control siRNA or siRNA against LTC_4S. Note that knockdown of LTC_4S had no effect on thapsigargin-activated SOCE (**f, g**). Representative whole-cell patch clamp traces showing thrombin-activated Ca^{2+} and Na^+ currents in VSMCs transfected with either siRNA control or siRNA targeting LTC_4S (**h-k**). I/V relationships show significant inhibition of thrombin-activated Na^+ (**j**) currents. I/V curves for Na^+ currents are taken from current traces where indicated by color-coded asterisks. Statistical analyses on normalized Na^+ currents are shown in **k**. Whole-cell patch clamp electrophysiology in VSMCs infected with lentivirus carrying either control non targeting shRNA (shNT) or shRNA targeting LTC_4S ($shLTC_4S$) confirmed the requirement of LTC_4S for thrombin-activated currents (**l-o**). The Na^+ I/V relationships as well as the statistical summary are included in **n** and **o**, respectively.

Online Figure IV. STIM1 knockdown abrogates CRAC currents activated by store depletion in VSMCs.
Whole-cell patch clamp recordings of CRAC currents (**a, b**) activated by passive store depletion with 20mM BAPTA in VSMCs. VSMCs were infected with lentiviruses encoding either control luciferase shRNA (shLuc; **a**) or shRNA targeting STIM1 (shSTIM1; **b**). Inclusion of BAPTA in the pipette activates CRAC currents measured in Ca^{2+}-containing and DVF solutions that were completely abrogated in cells depleted of STIM1. Na^+ I/V relationships are shown in **c** and statistics on current densities are shown in **d**.

Online Figure V. Orai1 knockdown abrogates CRAC currents activated by store depletion in VSMCs while Orai3 does not.
Whole-cell patch clamp electrophysiology of CRAC currents activated by passive store depletion with 20mM BAPTA in VSMCs. VSMCs were infected with lentivirus carrying either control shRNA against luciferase (shLuc), or shRNA targeting Orai1 (shOrai1). Orai1 knockdown completely abrogated Na^+/Ca^{2+} CRAC currents (**b**) as compared to control (**a**). Na^+ I/V relationships for CRAC are shown in (**c**) and statistical analysis is shown in (**d**). Whole-cell patch clamp electrophysiology of CRAC currents activated by passive store depletion with 20mM BAPTA in VSMCs. VSMCs were infected with lentivirus-encoding either non-targeting control shRNA (shNT; **e**) or shRNA targeting Orai3 (shOrai3; **f**). CRAC currents activated by passive store depletion with 20mM BAPTA (**e**) were not affected by Orai3 knockdown (**f**). Na^+ I/V relationships show indistinguishable Na^+ CRAC I/V relationships in both shNT and shOrai3 conditions (**g**). Statistical analysis is shown in (**h**).

Online Figure VI. Orai3 is upregulated in synthetic VSMCs and Orai3 shRNA lentiviruses efficiently infect VSMC in vitro.
a; Western blot analysis showing upregulation of Orai3 protein levels in cultured synthetic VSMCs (reminiscent of disease states) as compared to quiescent VSMCs freshly dispersed from rat aorta (representing the healthy situation). **b**; Cultured synthetic VSMCs were infected with lentiviruses-encoding either shRNA control (shNT) or shRNA against Orai3 (shOrai3).

Infected cells are visualized by GFP fluorescence. Phase pictures are shown to gauge the infection efficiency, which is essentially 100%.

Online Figure VII. Store depletion activates CRAC currents in freshly isolated medial and neointimal VSMCs from injured carotid arteries but not in medial VSMCs from control carotids.

Whole-cell patch clamp electrophysiological recordings on VSMCs freshly isolated from either control non-injured media (**a**, n=4) or from media (**b**, n=5) and neointima (**c**, n=5) of injured carotid arteries. Only cells from injured carotids showed development of CRAC currents. Upon dialysis of medial and neointimal VSMCs from injured carotids (14 day post injury) with 20mM BAPTA to cause store depletion, CRAC currents were observed. However, non-injured control medial VSMC showed no CRAC currents upon store depletion with BAPTA. Na^+ I/V relationships are taken from traces where indicated by the color-coded signs and are shown in (**d**). Statistical summary is also shown in (**e**).

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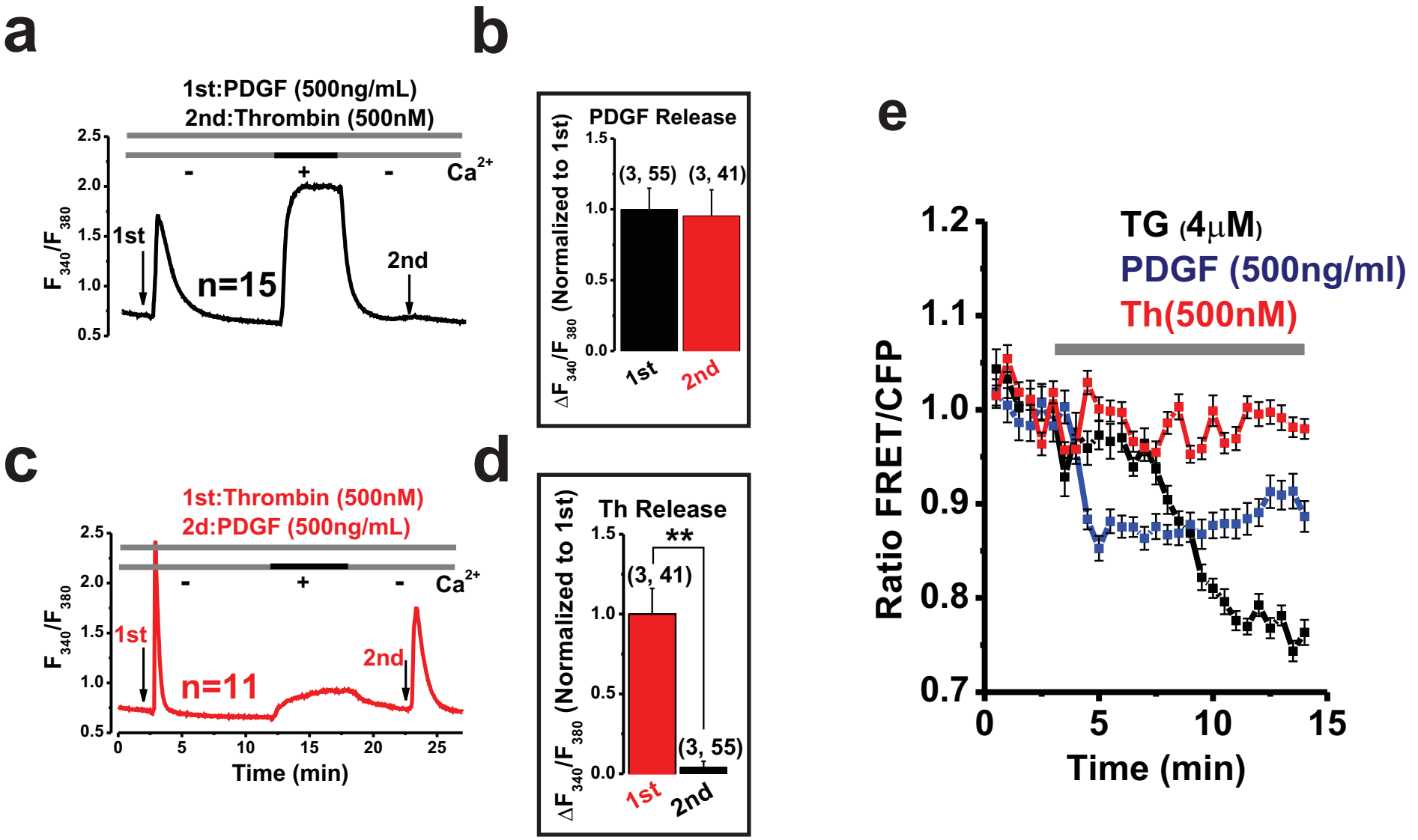
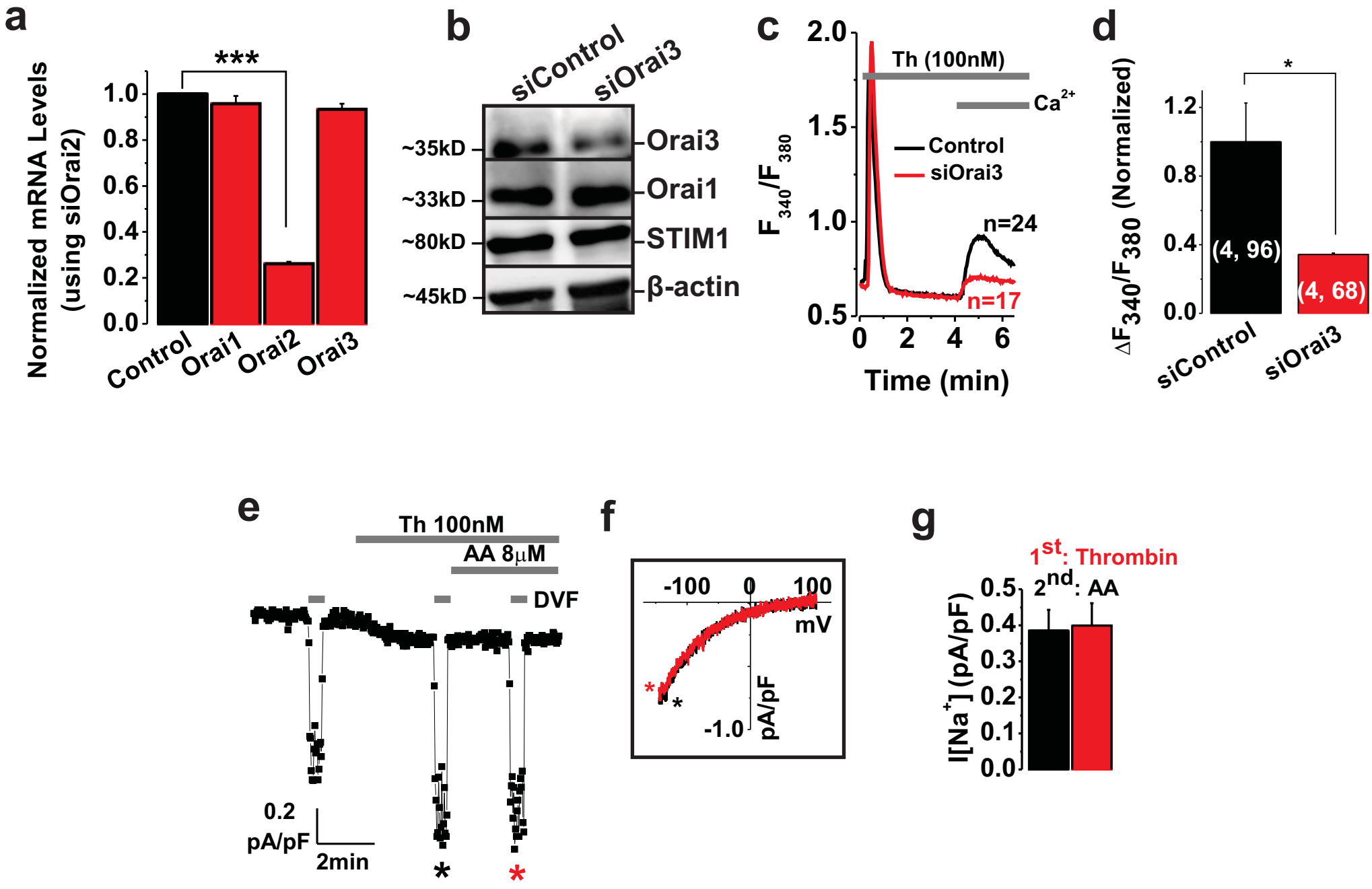
Online Figure I



Online Figure II



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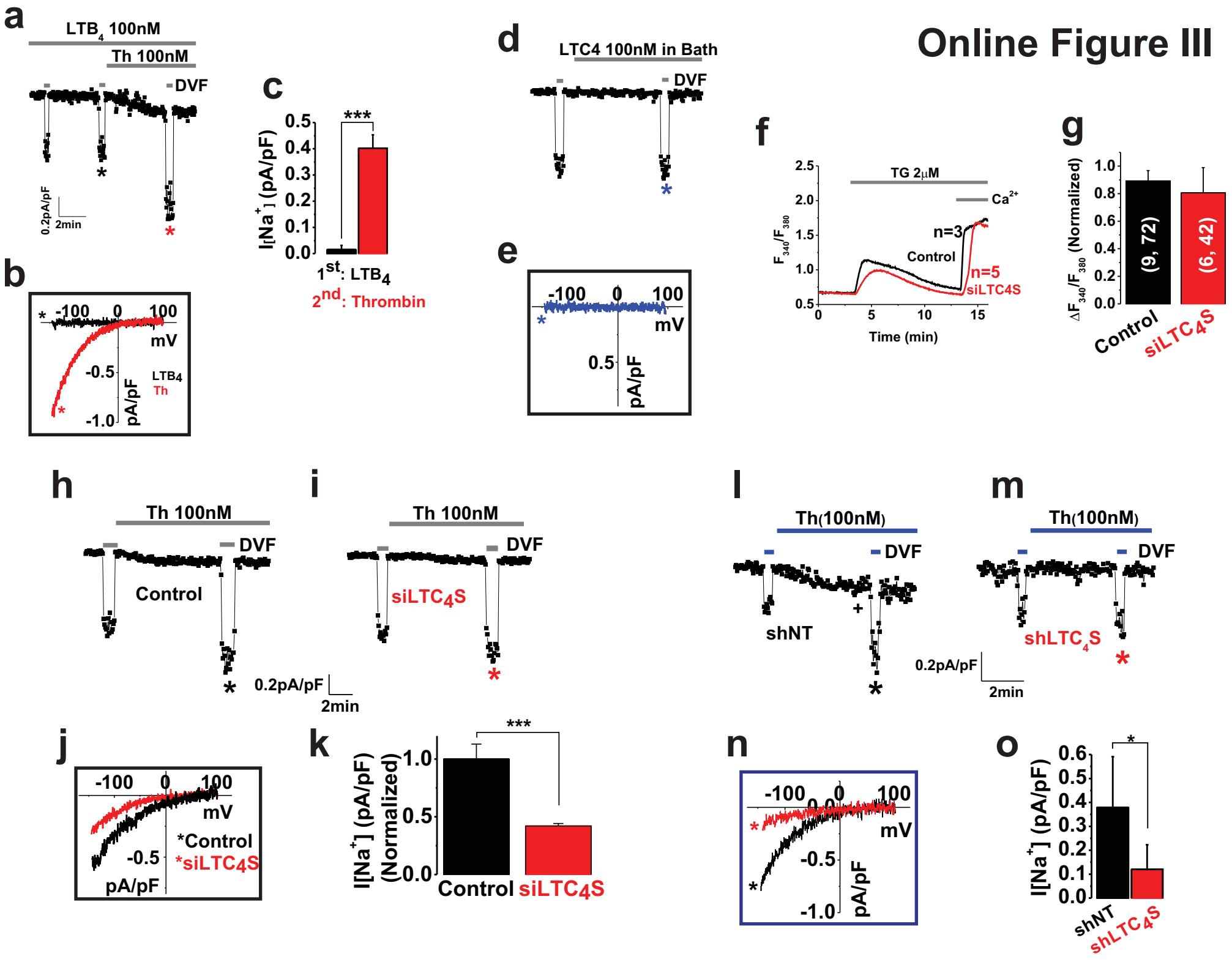
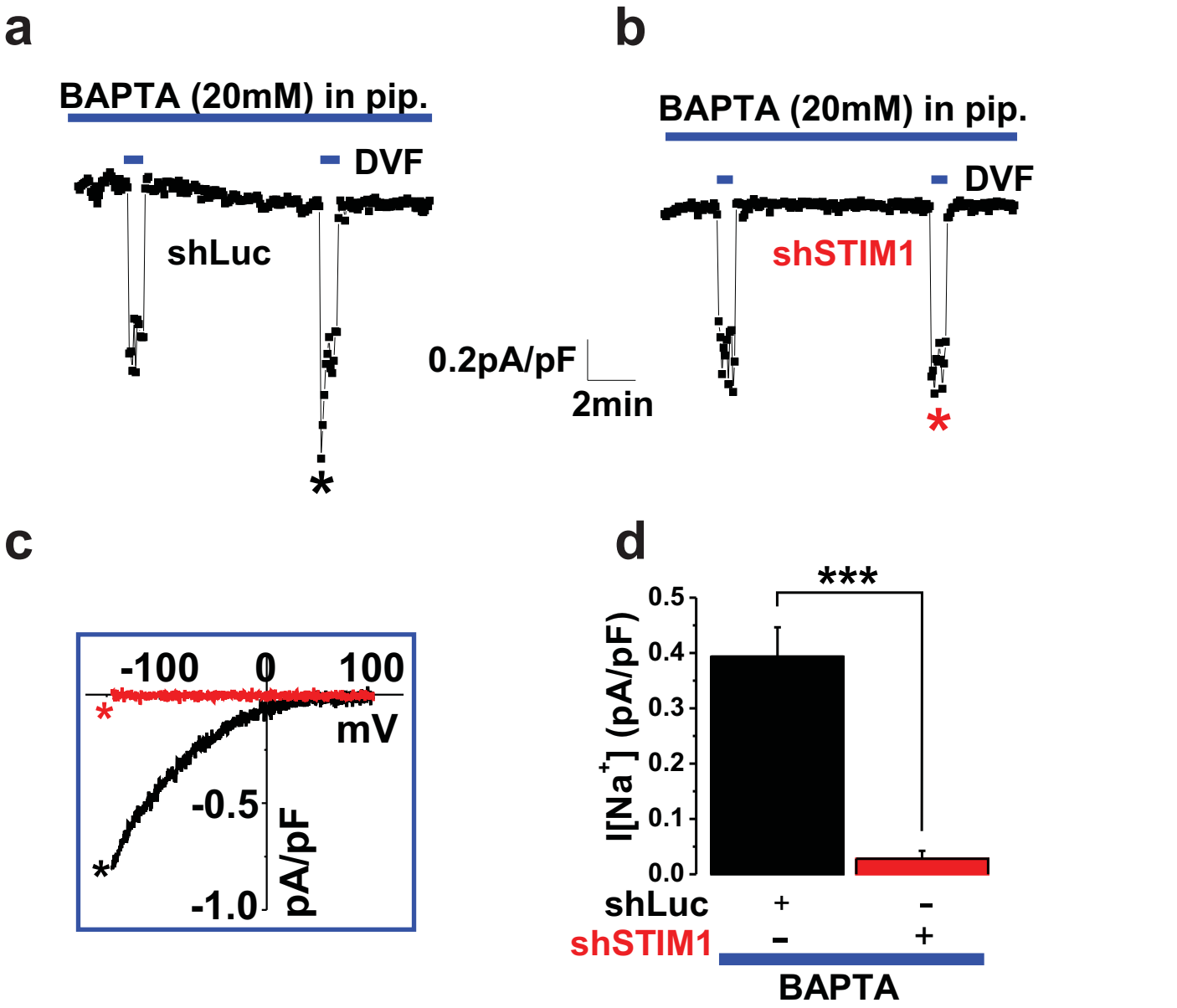
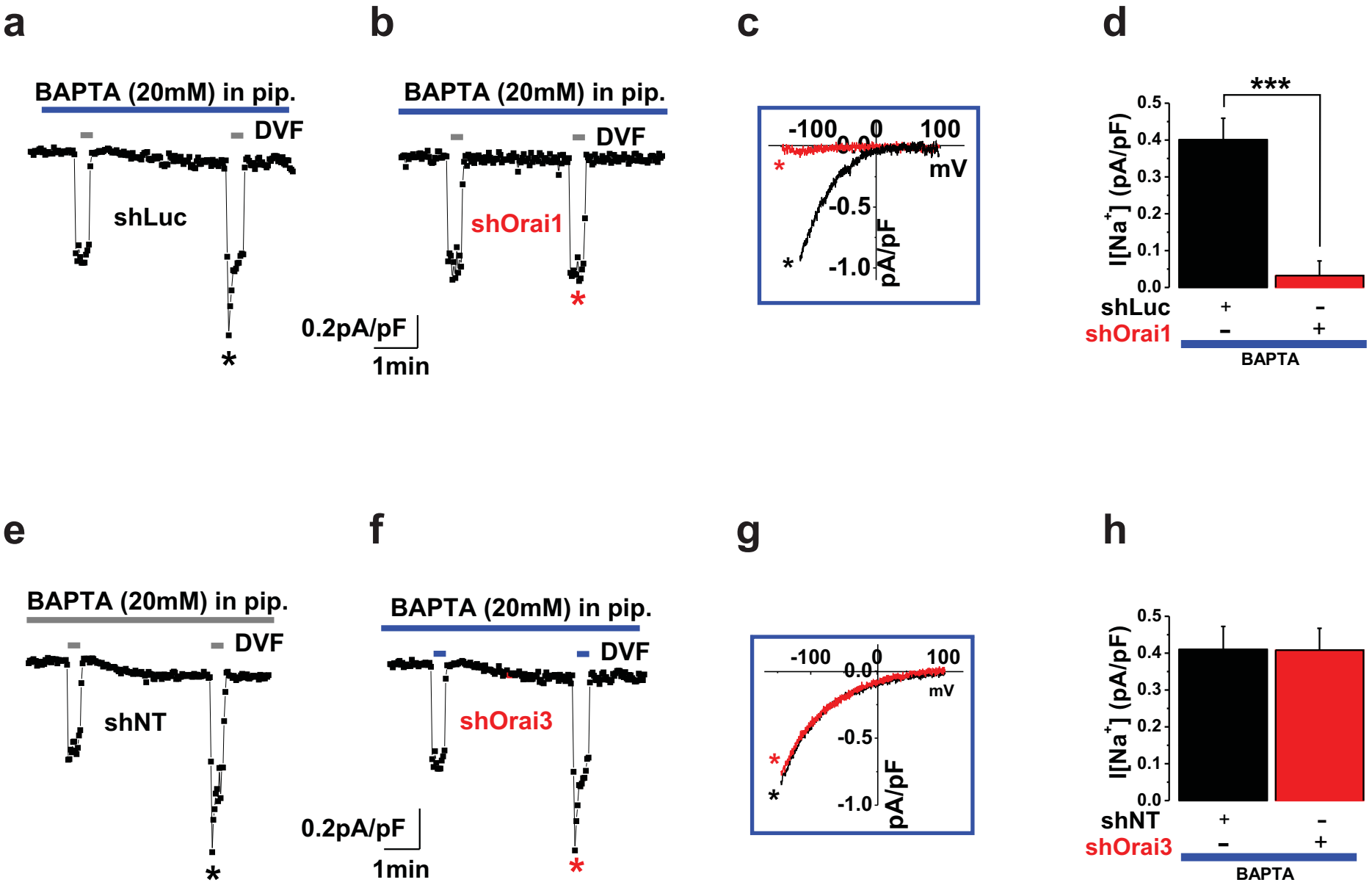


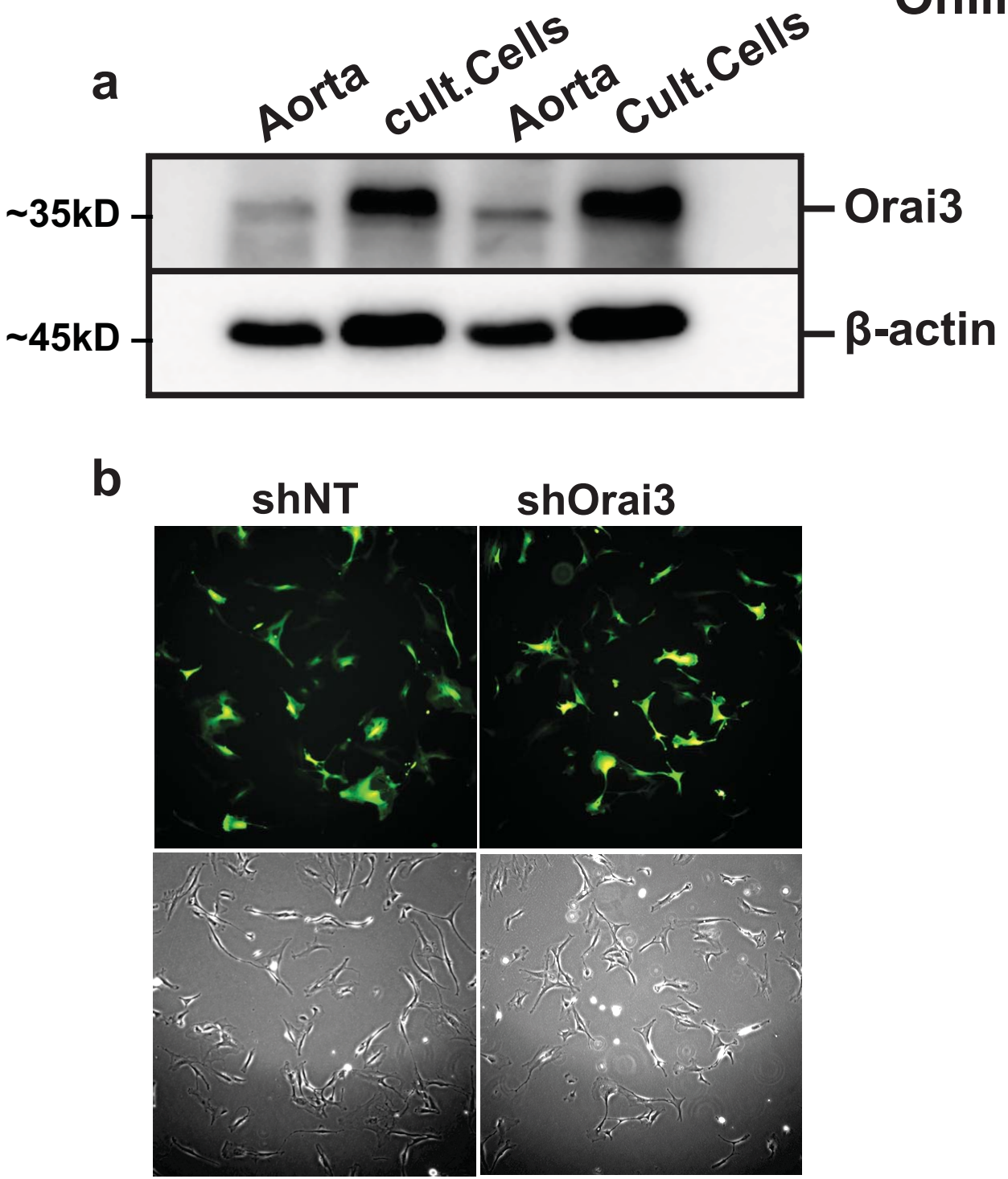
Online Figure IV



Online Figure V



Online Figure VI



Online Figure VII