Supplementary Information

Temperature-dependent STIM1 activation induces Ca^{2+} influx and modulates gene expression

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Supplementary Methods

Clones and reagents. Human GFP-STIM1 or dsRED-Orai1 cloned in pHAGE-CMV-MCS-IRES-ZsGreen-W vector was kindly provided by Dr. Alberta Parker (GNF). In brief, GFP was inserted after the N-terminal signal peptide of STIM1, while dsRED was fused to the N-terminus of Orai1. Constructs containing either STIM1 alone or Orai1 alone were generated by deleting the segment encoding either GFP or dsRED using the restriction enzyme XhoI or NotI/XhoI, respectively. Using the QuickChange XL-site-directed mutagenesis kit (Stratagene), the GFP-STIM1-deltaK mutant was engineered by introducing a stop codon at amino acid position 670 into GFP-STIM1 construct, while the GFP-STIM1-deltaCAD mutant was engineered by deleting the amino acids 342-448 from GFP-STIM1 construct. The STIM1-ct (from amino acid 236 to 685) and CAD (from amino acid 342 to 448) were amplified by polymerase chain reaction (PCR) with primers franked with KpnI and XbaI restriction enzyme sites. The PCR products were cloned into the pEGFP-C1 vector using the KpnI and XbaI sites. The Cameleon D1ER construct was kindly provided by Dr. Roger Y. Tsien (UCSD). siRNAs against human STIM1 (Product ID: J-011785-05; J-011785-06; J-011785-07; J-011785-08) and non-targeting control siRNA (Product ID: D-001810-01-05) were purchased from Dharmacon, while siRNA against human Orai1 (Product ID: SI00395192; SI03196207; SI04215316; SI04311790) was purchased from Qiagen. N-[5-(2-Chloro-5-trifluoro-methyl-phenyl)-pyrazin-2-yl]-2,6-difluoro-benzamice (Synta1) was patented by Synta Pharmaceutical Corp (International Publication number: WO 2006/081391) and custom-synthesized. Thapsigargin (Tg), Cyclopiazonic acid (CPA), ionomycin and other chemicals were purchased from Sigma. OKT3 antibody (LEAF^{TM} purified anti-human CD3) was purchased from Biolegend.

Cell culture and transient transfection. Cells were plated onto 12-mm round glass coverslips placed in 24-well plates and transfected with 0.2 – 0.5 μg cDNA using lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Transfected cells were grown for 18 - 24 hours prior to Ca^{2+} imaging. For siRNA knock down experiment, HeLa cells were co-transfected with 500 ng of GFP-STIM1 and 10 nM of pooled siRNA (4 different siRNAs against a specific gene) against either Orai1 or STIM1 (also transfected with 200 ng YFP to identify YFP positive

cells) or non-targeting control siRNA. Transfected cells were grown for 48 hours prior to Ca^{2+} imaging.

Fluorometric imaging plate reader (FLIPR). FLIPR experiments with adherent cells were performed essentially as described previously[1]. In brief, HEK293T cells were plated in 384-well plate (8000 cells/well) and transfected with cDNAs (60 ng per well). cDNA-transfected cells were grown for 2 days before functional assay. The cells were washed with 1 x Hanks Balanced Salt Solution (HBSS, 1.3 mM Ca^{2+}) with 20 mM HEPES and 2.5 mM probenecid by an Embla plate-washer (Molecular Devices) at room temperature (RT), loaded with the Ca^{2+} sensitive fluorophore Fluo-3 (Molecular Devices) for 1.5 h at 37^{o}C incubator, washed again at RT. The cell plates were then transferred to a FLIPR (Molecular Devices) to monitor Fluo-3 fluorescence in response to temperature changes or chemical stimulation. The temperature change of the medium inside the well was controlled by a custom-designed temperature-control device as described[1]. For measuring temperature response in the presence of Synta1, 25 µl of the assay buffer with or without Synta1 was first added to the cell plate prior to perform temperature change experiment. For measuring SOCE using FLIPR, we first depleted ER Ca^{2+} stores in the absence of extracellular Ca^{2+} by adding 25 µl of buffer with 40 µM CPA and 6 mM EGTA to each well, and then measured SOCE by adding 25 µl of buffer containing 6.4 mM Ca^{2+} to each well to increase extracellular Ca^{2+} to ~0.4 mM (Supplementary Fig. 2a).

For non-adherent Jurkat cells, we alternatively employed the no-wash FLIPR Calcium 4 Assay Kit (Molecular Device) to avoid steps of washing and spinning. More specifically, one vial of the component A which contains the Ca^{2+} sensitive fluorescent dye from the kit was dissolved using 10 ml of a cell maintaining medium [RPMI-1640 medium supplemented with 1% FBS (0.42 mM extracellular Ca^{2+})]. Cells were spun down and resuspended with the dye solution at a concentration of 1.6 X 10^{6} cells /ml. 25 µl of the cell suspension was then aliquoted to each well of the 384-well-plates (40,000 cells/well). The cell plates were kept at 37^{o}C incubator for 1.5 h for loading the dye and allowing cells to settle down at the bottom of the plates. The cell plates were then transferred for FLIPR assay as described above. For Supplementary Fig. 2d, cells were loaded with the Calcium-4 dye at either 41^{o}C or 37^{o}C for 2 h and then assayed on FLIPR at 37^{o}C (by setting the stage temperature of the FLIPR machine to 37^{o}C) by adding different concentrations of ionomycin with or

without 3 mM EGTA (the compound plates were pre-warmed to 37^{o}C). Responses of EGTA-treated samples were subtracted from the corresponding samples without EGTA to obtain ionomycin-induced SOCE as shown in Supplementary Fig. 2d.

Fura-2 ratio calibration. A separate set of experiments was performed in order to convert the heat off-response of Fura-2 ratio at 25^{o}C to Ca^{2+} concentration. Based on the calibration equation $[Ca^{2+}]_i = Kd \times Q \times (R - R_{min})/(R_{max} - R)$ according to Brandman et al.[2], where Kd = 260 nM at 25^{o}C[3], the R_{max} and R_{min} were determined after measuring the heat off-response of Fura-2 ratio (R = 1.894). R_{max} was determined by measuring the maximal attainable Fura-2 ratio in HeLa cells treated with 5 μM ionomycin in the presence of 10 mM Ca^{2+} (R_{max} = 5.879), R_{min} was determined by measuring the minimal Fura-2 ratio in HeLa cells treated with 5 μM ionomycin in the presence of 0 mM Ca^{2+} and 3 mM EGTA (R_{min} = 0.284). The R_{min} and Q values depend on autofluorescence, unspecific Fura-2 binding to cellular components and Fura-2 sequestration inside cells. The Q value was adjusted for HeLa cells so that the average basal Ca^{2+} level was 50 nM in the calibration experiment according to Brandman et al.[2]. The measured basal Fura-2 ratio for untransfected HeLa cells is 0.431. Accordingly, the calculated Q value is 7.13. Based on the calculation, the $[Ca^{2+}]_i$ for the heat off-response at 25^{o}C is 750 nM. Since both the Kd and the Fura-2 ratio can be affected by temperature[3,4], a calibration of the Fura-2 ratio during temperature ramp is challenging and also could be misleading. Therefore, such analysis was not performed.

Analysis of the decay of the heat off-response. The decay of the heat off-response of Ca^{2+} signal was fitted with a mono-exponential equation (Fig. 1d and Supplementary Fig. 1d). Exponential equations are commonly used to describe biological processes. It is very likely that the decay kinetics shown in Fig. 1d and Supplementary Fig. 1d reflect the involvement of several independent processes, such as the closure of CRAC channels and the calcium pump activity. However, the decay was well fitted with a mono-exponential equation, and we used the tau value of this fit to illustrate the overall kinetics of the calcium signal decay of the heat off-response.

NFAT-luciferase assay and AP-1-luciferase assay. To assay the effect of temperature shifts on a Jurkat cell line stably expressing the NFAT-luciferase reporter gene (kindly provided by Dr. Shin-Shay Tian, the Genomics Institute of the

Novartis Research Foundation), 20,000 cells resuspended in 1 x RPMI1690 medium with 1% FBS were plated to each well of 384-well-plates. The cell plates were first kept at either 37^{o}C or 41^{o}C for 10 min (Supplementary Fig. 2f) or 2 h (Fig. 2), and then treated with 1 μM PMA or 1 μM PMA/ionomycin at 37^{o}C for 5 h (Fig. 2b). Synta1 was added from the beginning of the treatments for blocking experiments. Different temperature pretreatments did not result in different viable cell numbers at the end of the experiment. The expression of luciferase was assayed with Bright-Glow Luciferase Assay System (Promega) using a CLIPR luminometer. For each condition, the luciferase activity was measured from 6-8 wells of 384-well-plates.

For AP-1-responsive or NFAT-responsive luciferase assays in HEK293T cells, cells grown in 384-well-plates were either transfected with the AP-1-luciferase reporter gene or co-transfected with the indicated constructs and the NFAT-luciferase reporter gene [containing three tandem copies of the NTAT consensus sequence and AP1 consensus sequence upstream of the firefly luciferase reporter gene (Clontech)] (kindly provided by Dr. Peter G. Shultz, the Scripps Research Institute). 48 h after transfection, cells were replaced with DMEM containing 1% FBS and treated with the same protocol as described above for Jurkat-NFAT-Luc cells.

Electrophysiology. HEK293T cells were transfected in 24-well plates using lipofectamine 2000 (Invitrogen). HEK293T cells were reseeded on poly-D-lysine coated 12-mm round glass coverslips (BD BioCoat) 24 h after transfection. Patch-clamp experiments were performed the following day in standard whole-cell recordings using an EPC-9 patch clamp amplifier (HEKA Instruments).

Voltage ramps of 100 ms duration spanning a range of -100 to 100 mV after a brief (5 ms) step to -100 mV were delivered from a holding potential of 0 mV at a rate of 0.5 Hz. Currents were sampled at 10 kHz and filtered at 2 kHz. Extracting the current amplitude at -80 mV from individual ramp current records assessed the time course of currents. Standard external solution contained (mM): 140 NaCl or NaGlu, 5 KCl, 10 HEPES and 2.5 $CaCl_2$, pH 7.3 with NaOH. Divalent free solution (DVF) contained (mM): 130 NaCl, 5 KCl, 10 HEPES and 10 EDTA, pH 7.3 with NaOH. Fire-polished pipettes fabricated from borosilicate glass capillaries (Sutter Instruments) with 3-5 MΩ resistance were filled with (mM): 120 Cs Methanesulfonate, 10 HEPES, 10 $MgCl_2$, 4 EGTA and 2 $CaCl_2$, pH 7.3 with CsOH. The final Ca^{2+} concentration was ~175 nM free Ca^{2+} as determined using Maxchelator software (WEBMAXC); this

prevents passive store depletion of the internal Ca^{2+} stores. However, we observed very slow spontaneous CRAC current activation during long lasting recordings (see Fig. 5a), but this current activation was different from current activated by passive store depletion under Ca^{2+} free pipette solution condition. The current started to develop at least 4 minutes after achieving whole cell configuration, a time at which the passive store depletion-induced current is usually close to be fully developed[5]. Moreover, development of this current is very slow since only less than 20% of the maximal current was developed after 8 minutes of recording (see Fig. 5a,b). For experiments with DVF solutions, the perfusion was switched from standard external solution to DVF solution after achieving whole-cell configuration. Voltages were corrected for a liquid junction potential of 10 mV. Leak currents were subtracted by using a ramp recorded before activation of current.

The temperature of the bath solution was controlled using a CL-100 temperature controller (Warner Instruments) and a SC-20 Solution In-Line Heater/Cooler (Harvard Apparatus). Temperature was recorded with a thermistor placed < 5 mm from the cell.

Current-temperature relationships were fitted with a Boltzmann equation of the form : $I(T) = [1 + \exp(-(T - T_{1/2})/s)]^{-1}$, where I is the Orai1 current at -80 mV, T is the bath temperature (in ºC), $T_{1/2}$ is the temperature value that cause 50% of the blockade and s is the current sensitivity to temperature.

Fluorescence resonance energy transfer (FRET) measurement of ER [Ca^{2+}]. HeLa cells grown on coverslips were transfected with an ER- targeted probe Cameleon D1ER and histamine receptor 1 (HR1). ER [Ca^{2+}] was measured using Ca^{2+} sensitive FRET-based Cameleon D1ER protein as described[6]. In brief, cells were excited at 430 nm and the dual emission ratio (535/485: YFP/CFP) was collected using MetaFluor software on an inverted Zeiss Axiovert 200M microscope with a 20x Fluar objective (N.A.: 0.75). Application of 10 μM CPA and 100 μM histamine caused a rapid drop of the FRET ratio, indicating a release of ER Ca^{2+} from the store (Supplementary Fig. 3b). Thus this measurement was used for assaying the releasable ER Ca^{2+} content. It should be noted that heat induced a dramatic increase in the FRET ratio even under ER Ca^{2+} store depleted condition (Supplementary Fig. 3b), suggesting that this FRET measurement of ER Ca^{2+} content is highly temperature sensitive. Therefore we measured the releasable ER Ca^{2+} content of

HeLa cells bathed at either 25^{o}C or heated to 50^{o}C by applying CPA/histamine. We found that CPA/histamine induced an even bigger drop of FRET ratio measured at 50^{o}C than at 25^{o}C, suggesting at least that ER Ca^{2+} content is not depleted by heating cells from 25^{o}C to 50^{o}C.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) HeLa cells were co-transfected with 500 ng of GFP-STIM1 and 10 nM of pooled siRNA (4 different siRNAs against a specific gene) against Orai1, STIM1, or non-targeting control siRNA. Transfected cells were grown for 48 hours prior to total RNA extraction using Trizol treatment. 500 ng of total RNA was used to generate first strand cDNA using the Quantitect reverse transcription kit (Qiagen). Gene specific real time PCR primers were designed to obtain PCR products ranging between 50-100 bps using Primer Express software (Applied Biosystems). The primer sequences for STIM1, Orai1 and β-actin are as follows: hSTIM1-F (GCAGCAGAGTTTTGCCGAAT); hSTIM1-R (TTACGGACTGCCTCGAAGCT); hOrai1-F (TGTCCTGGCGCAAGCTCTA); hOrai1-R (GTCCGGCTGGAGGCTTTAA); hActin-F (CCAGCTCACCATGGATGATG); hActin-R (ATGCCGGAGCCGTTGTC).

SYBR green PCR master mix (1 μl cDNA in 20 μl reaction) (Applied Biosystems) was used to run the reaction in the ABI 7900HT fast real time system according to the manufacturer's instructions. The samples were run in triplicate. Melting curves were generated to verify the product specificity and showed formation of only one PCR product per primer pair used. Calibrations and normalizations were done using the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta C_T = (C_{T,\ target} - C_{T,\ reference})$ siRNA treated - $(C_{T,\ target} - C_{T,\ reference})$ control treated. β-actin was used as the reference gene.

Supplementary Results

Supplementary Fig. 1 Temperature-induced STIM1/Orai1-mediated Ca^{2+} responses. (a) Temperature responses of HEK293T cells transfected with various DNA constructs and assayed on FLIPR (Fluorescent Imaging Plate Reader). The initial drop of Fluo-3 signal during heating was always observed regardless of cell types and whether cells were transfected, which could be due to temperature quench of fluorescence, heat inhibition of Ca^{2+} influx and/or heat facilitation of Ca^{2+} removal from the cytosol. A modest heat-induced but STIM1/Orai1-independent Ca^{2+} influx during heating was observed, which is beyond the focus of this study. (b) STIM1/Orai1-mediated heat off-response was abolished in the presence of 5 mM EGTA for chelating extracellular Ca^{2+}. (c) HEK293T cells transfected with STIM1 and Orai1 showed repeated off-responses to multiple heating pulses as assayed by FLIPR. The abrupt decrease of Fluo-3 signal following the heat off-response at elevated temperatures is due to heat-induced block of the heat off-response as described in Fig. 5. (d) HeLa cells transfected with GFP-STIM1 showed long lasting heat off-response as assayed by single cell Ca^{2+} imaging of Fura-2 (average of 14 cells). The red dashed line represents fit with a mono-exponential function. (e) Heat off-responses of representative HeLa cells transfected with STIM1/Orai1. The heat off-response of most responding cells started to occur at the temperature of ~37 ^{o}C (Fig. 1b, green trace), while a small portion of cells showed much delayed responses even after cooling back to 25 ^{o}C. (f) The heat off-response in HeLa cells transfected with STIM1 was blocked by 2-APB. (g-i) Characterization of a selective CRAC channel blocker Synta1**.** (g) Synta1 concentration-dependent inhibition of SOCE in HEK293T cells transfected with STIM1/Orai1. A hill equation was used to fit the curve. (h) Synta1 did not block any heat-activated TRPV ion channels (capsaicin-activated TRPV1 channels or 2-APB-activated TRPV2, TRPV3 and TRPV4) expressed in HEK293T cells. (i) Synta1 concentration-dependent inhibition of heat off-responses in STIM1/Orai1-transfected HEK293T cells. (j) Representative traces showing inhibition of heat off-responses by 5 μM Synta1 in STIM1/Orai1-transfected HEK293T cells. Synta1 also inhibited the basal Ca^{2+} level. (k) GFP-STIM1/siSTIM1-transfected HeLa cells showed knocked-down expression of GFP-STIM1. Images of HeLa cells co-transfected with GFP-STIM1 and either control non-targeting siRNA, siRNA directed against STIM1 or siRNA directed against Orai1. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) showed 85% knockdown of STIM1

with siSTIM1 and 93% knockdown of Orai1 with siOrai1. (l) HeLa cells co-transfected with GFP-hSTIM1 and non-targeting control siRNA (blue trace, average of 100 cells) displayed strong SOCE [comparable to HeLa cells co-transfected with STIM1 and Orai1 (Fig. 4a), suggesting high expression of endogenous Orai1 in HeLa cells]. SOCE was abolished in cells co-transfected with GFP-hSTIM1 and siRNA against either STIM1 (44 cells) or Orai1 (45 cells).

Supplementary Fig. 2 Temperature effects on Ca^{2+} influx and NFAT-responsive gene expression in Jurkat T cells. (a) Average fluorescent signal changes of Jurkat cells in response to a 41^{o}C heating pulse (Left panel) or 20 μM CPA-induced store depletion and SOCE (Middle panel) with or without 20 μM synta1 as assayed by Calcium 4 Assay Kit using FLIPR. Right panel shows the maximal amplitudes of the heat off-response and SOCE with or without Synta1. The heat off-response was 44% of the SOCE, and both were inhibited by Synta1. Synta1 also inhibited the basal Ca^{2+} level to about 70% of that without Synta1, suggesting basal Ca^{2+} influx through CRAC channels, which may mediate a portion of the basal level of NFAT-dependent luciferase expression at 37^{o}C observed in Fig. 2c, d. (b) Average fluorescent signal changes of Jurkat cells in response to a 41^{o}C heating pulse with or without 20 μM ruthenium red (RR). (c) Average fluorescent signal changes of Jurkat cells in response to a heating pulse from 37^{o}C to 41^{o}C. Acute heating from 37^{o}C to 41^{o}C resulted in a heat off-response at 37^{o}C. (d) Concentration-dependent responses of ionomycin-induced Ca^{2+} influx in Jurkat cells pre-incubated at 41^{o}C or 37^{o}C for 2 h and assayed on FLIPR at 37^{o}C. Responses of EGTA-treated samples were subtracted from the corresponding samples without EGTA to obtain ionomycin-induced SOCE as shown in the panel. The curves were fitted with Hill equations. (e) Fold changes of NFAT-luciferase activity of 41^{o}C treated cells compared to 37^{o}C treated cells with different ionomycin concentrations (the same set of data shown in Fig. 2e was analyzed). (f) NFAT-luciferase activity of Jurkat-NFAT-Luc cells were pretreated at either 41^{o}C or 37^{o}C for 10 min and then kept at 37^{o}C for 5 h. (g) NFAT-luciferase activity of Jurkat-NFAT-Luc cells were pretreated at either 39^{o}C or 37^{o}C for 2 h and then kept at 37^{o}C for 5 h. (h) 1μg/ml of OKT3-induced NFAT-luciferase activity under the indicated conditions using the protocol shown in Fig. 2b. (i) Protocol used for assaying temperature effects on NFAT-responsive luciferase expression. The Jurkat-NFAT-Luc cells were kept at a 33^{o}C incubator for ~18 h prior to the temperature treatment protocol. (j) Concentration-dependent responses of

ionomycin-induced NFAT-luciferase activity of Jurkat-NFAT-Luc cells under the indicated treatment conditions fitted with Hill equations.

Supplementary Fig. 3 Heat does not deplete ER Ca^{2+} stores. (a) HeLa cells transfected with histamine receptor 1 (HR1) showed a similar increase in Fura-2 signal in response to heat before and after ER Ca^{2+} store depletion in the absence of extracellular Ca^{2+}, indicating that the heat-induced Fura-2 signal bump (Fig. 1b, c and Fig. 3b, c) is not due to ER Ca^{2+} release and most likely due to the temperature sensitivity of the dye itself[3,4]. 10 μM CPA and 100 μM histamine were used to rapidly deplete ER Ca^{2+} store. Right panel summarizes the responses of 99 cells to the 1st and 2nd heat pulse shown in the left panel. (b) HeLa cells co-transfected with Cameleon D1ER and HR1 showed a decrease in ER Ca^{2+} level in response to CPA and histamine as assayed by FRET (YFP/CFP) of Cameleon D1ER (average of 35 cells) as expected. Under store depleted conditions, heat induced a dramatic increase in the FRET signal, indicating Cameleon D1ER is also temperature sensitive. (c, d) show the FRET signal drop (between dashed lines) of HeLa cells co-transfected with Cameleon D1ER and HR1 in response to CPA/Histamine-induced ER Ca^{2+} store depletion by CPA/Histamine at either 25^{o}C (c) or 50^{o}C (d). (e) shows average ER Ca^{2+} content as reflected by the FRET signal drop in response to store depletion at either 25^{o}C (33 cell) or 50^{o}C (65 cells). Although it is not clear if a direct comparison of FRET drop can be made at two different temperatures, it is obvious that at 50^{o}C the store was not depleted, as a robust signal was detected.

Supplementary Fig. 4 Temperature-induced NFAT-controlled gene expression depends on STIM1 temperature sensitivity. (a) NFAT-controlled luciferase activity of HEK293T cells transfected with the NFAT-Luciferase reporter, Orai1 and the indicated wild type or STIM1-mutants in response to the indicated conditions using the protocol shown in Fig. 2b (average of 4 separate experiments, *** $p < 0.001$, unpaired t test). (b) Basal Fura-2 ratio at 37^{o}C measured from HEK293T cells transfected with the indicated constructs (3 separate experiments for each individual transfected conditions. ** $p<0.01$, one way ANOVA). A significantly elevated basal Ca^{2+} level was specifically observed in STIM1/Orai1-transfected cells, but not in either STIM1-deltaK/Orai1 (heat insensitive STIM1 mutant) or other constructs-transfected cells. A Synta1-sensitive basal Ca^{2+} influx was also observed in Jurkat cells (Supplementary Fig. 2a). These data suggest that STIM1/Orai1 temperature

sensitivity may drive a tonic Ca^{2+} influx at 37°C. (c) Confocal analysis of HeLa cells co-transfected with Orai1 and GFP-STIM1-deltaCAD at 25°C (upper panels) or after heating to 48°C (lower panels). GFP intensity plots represent localization of each construct across the cell as indicated by the white line. (d) 2-APB responses of HeLa cells co-transfected with the indicated constructs: GFP-STIM1/Orai1 GFP+ (average of 54 cells), GFP-STIM1/Orai1 GFP- (average of 46 cells), eGFP-STIM1-ct/Orai1 GFP+ (average of 100 cells), eGFP-STIM1-ct/Orai1 GFP- (average of 40 cells). 2-APB elicited a small but significant increase in intracellular Ca^{2+} in eGFP-STIM1-ct/Orai1-transfected cells, while inhibited the basal Ca^{2+} level in GFP-STIM1/Orai1-transfected cells. (e) Temperature responses of HeLa cells co-transfected with either STIM1/Orai1 (average of 98 cells) or STIM1-ct/Orai1 (average of 119 cells). Unlike STIM1/Orai1-transfected cells, STIM1-ct/Orai1-transfected cells failed to show heat off-response.

Supplementary Fig. 5 Heat Block of I_{CRAC} by disrupting STIM1-Orai1 functional coupling. (a,b) Block of I_{CRAC} by heat in the presence of 2.5 mM extracellular Ca^{2+}. (a) Representative cell over-expressing STIM1 and Orai1. Left panel: Current recorded at -80mV showing heat off- response followed by full activation with 10 μM CPA (i). Temperature stimulation on already activated current induces current block (ii), which is reversible (iii). Right panel: I-V curves at 10 seconds interval from recording during the heating phase (upper panel) and cooling phase (lower panel) of the temperature stimulus (red box in left panel). (b) Current-temperature relationships of Orai1 current at -80 mV during heating (left, n=6) or cooling (right, n=5) fitted with a Boltzmann equation. The black dashed line is the average fit. The cells were dialyzed with a pipette solution containing ~175 nM free Ca^{2+} and external solution containing Ca^{2+} (2.5 mM). (c) Block of CPA-induced SOCE in STIM1/Orai1-transfected cells by heating (average of 41 cells). (d) Heat blocked the constitutive Ca^{2+} influx in cells transfected with E87A-STIM1/Orai1 (average of 45 cells). (e) Block of CPA-induced SOCE in STIM1-deltaK/Orai1-transfected cells by heating (average of 44 cells). (f) Heat blocked the constitutive Ca^{2+} influx in cells transfected with eGFP-CAD/Orai1 (average of 30 eGFP+ cells and 37 eGFP- cells).

Supplementary Movie 1 Temperature-dependent changes of GFP-STIM1 puncta and Fura2 ratio in HeLa cells transfected with GFP-STIM1. GFP-STIM1-transfected HeLa cells were loaded with Fura-2 and simultaneously subjected to GFP

and Fura-2 imaging. The movie shows GFP-STIM1 puncta and Fura-2 ratio before, during and after heating.

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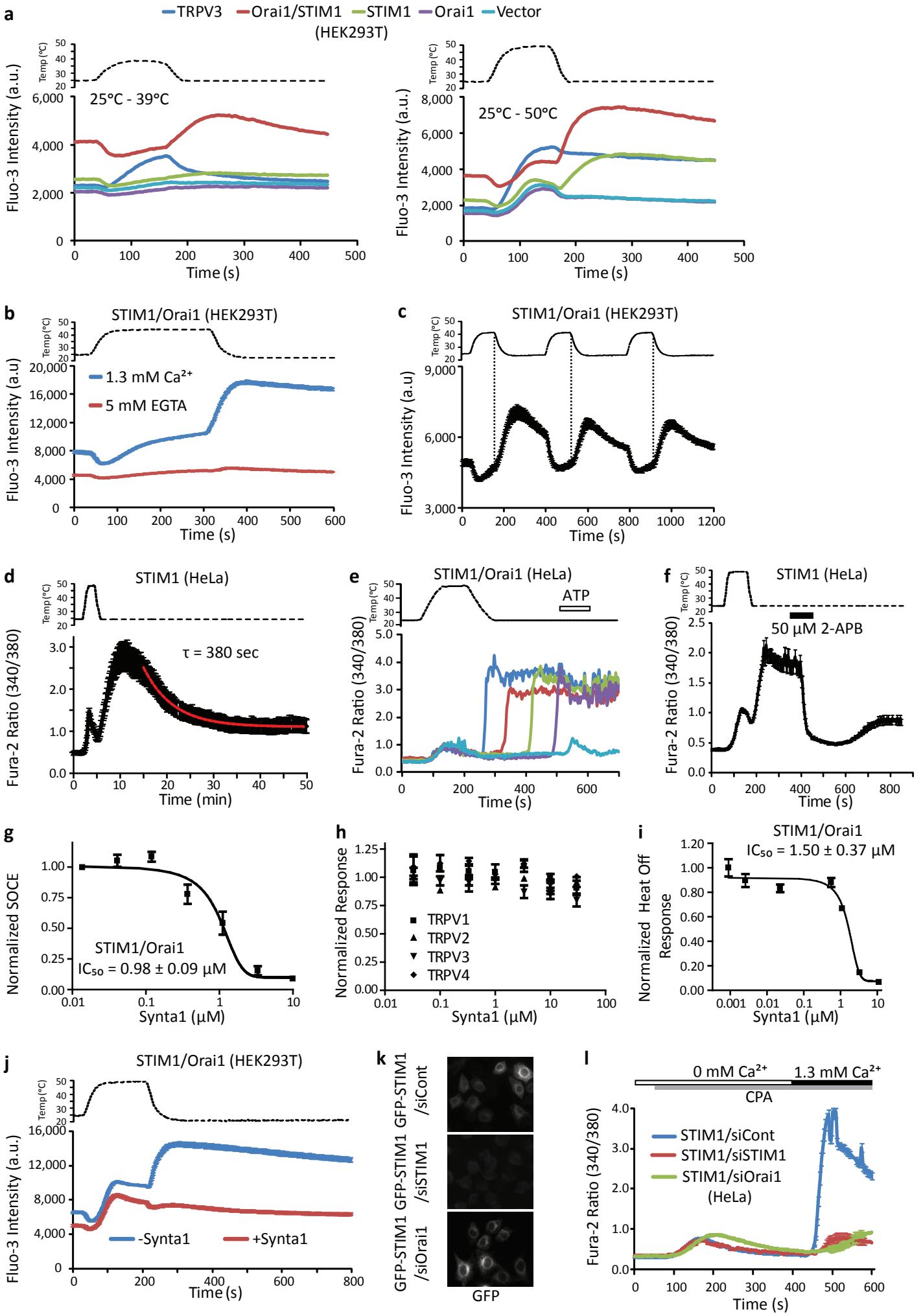
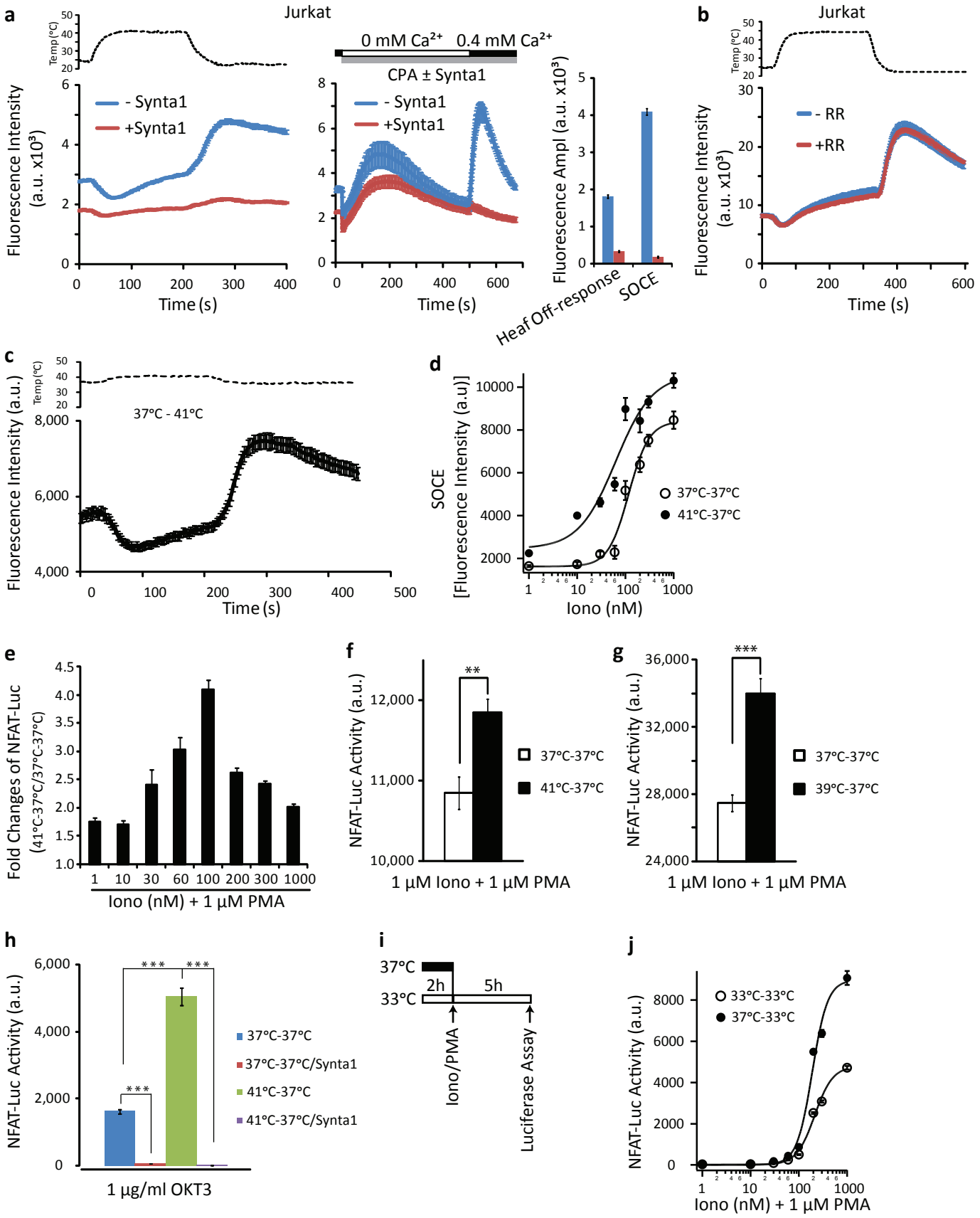
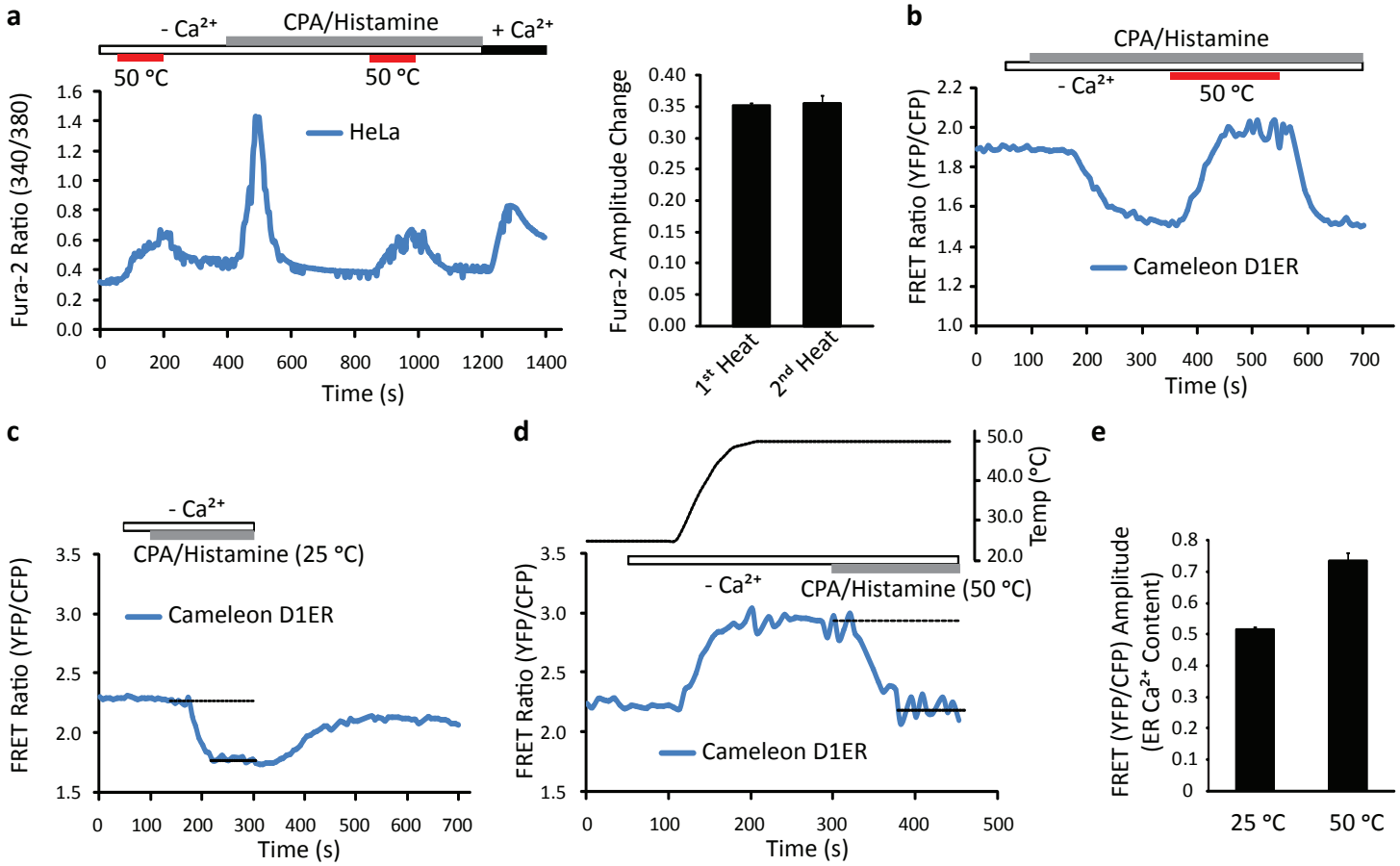
Supplementary Fig. 1



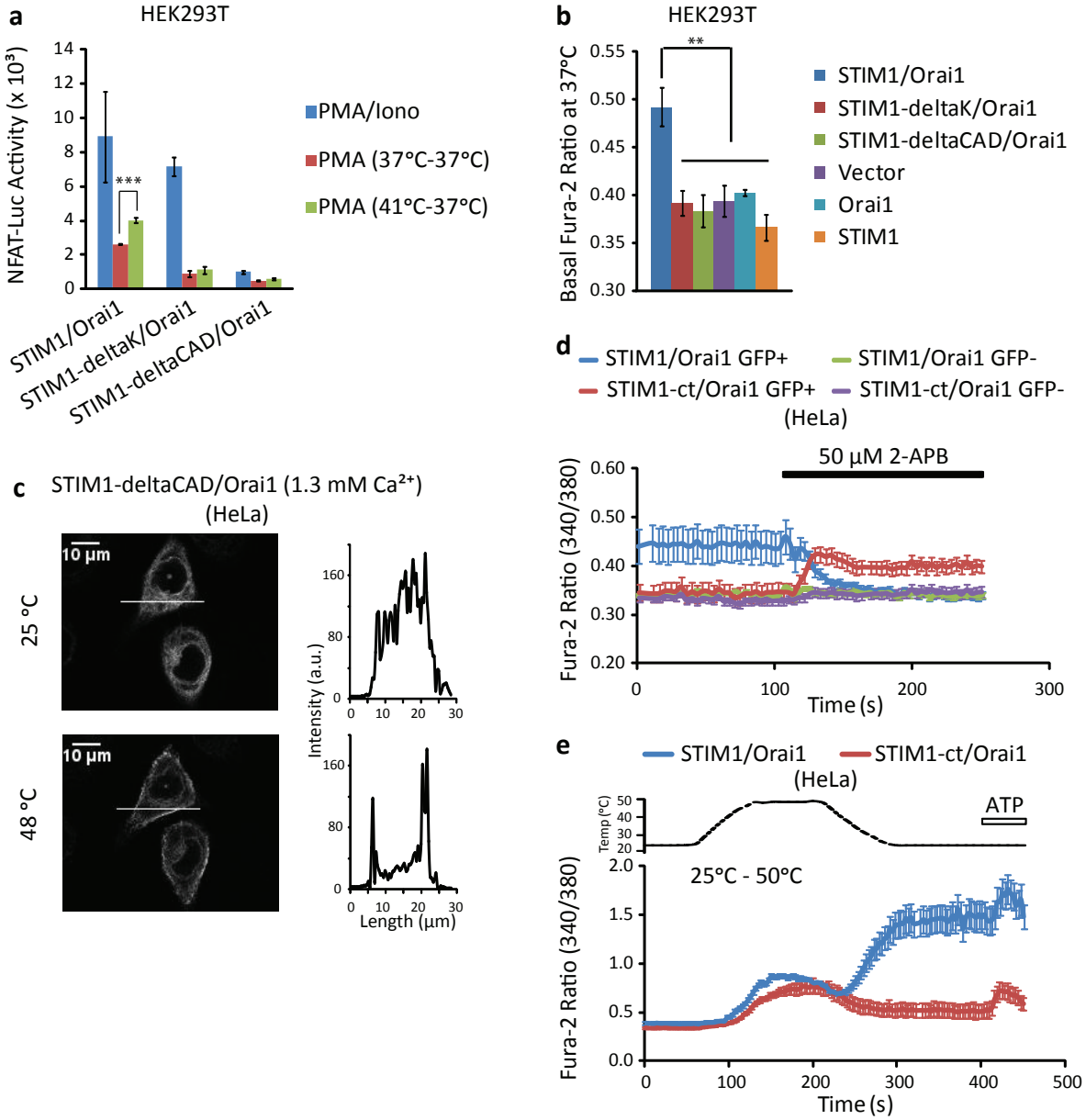
Supplementary Fig. 2



Supplementary Fig. 3



Supplementary Fig. 4



Supplementary Fig. 5