Supporting Information for

A Suicidal Mechanism for the Exquisite Temperature Sensitivity of TRPV1

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SI Materials and Methods:

Expression and purification

Rat TRPV1 clone was provided by David Julius (UCSF). The BacMam expression vector was a gift of Erhu Cao (University of Utah). Protein expression and purification followed published protocols with modifications [1]. Briefly, full-length channels were cloned into a BacMam expression vector [2] containing an N-terminal maltose binding protein (MBP) followed by a TEV protease site. Recombinant baculovirus encoding target genes were generated in SF9 cells using the Bac-to-Bac system (Invitrogen). The amplified P2 virus was used for infecting HEK293 GnTI$^-$ cells, which were grown and maintained in Freestyle293 media (Gibco) containing 2.0 % FBS (Gibco) at 37 ^oC with constant shaking on an orbital shaker in a humidified incubator gassed with 8% CO_2. Baculoviral transduction of cells took place at a cell density of ~3.0 x 10^6/ml. Infected cells were continuously incubated at 37 ^oC for ~24 hrs and then shifted to 30 ^oC after addition of 10 mM sodium butyrate to boost protein expression. After ~48 h of infection, cultures were harvested by centrifugation at 500 g for 15 min, and cell pellets were resuspended in a hypertonic buffer (36.5 mM sucrose, 50 mM Tris, 2.0 mM TCEP, pH8.0) supplemented with protease inhibitors (1.0 mM phenylmethanesulphonylfluoride [PMSF], 3.0 μg/ml aprotinin, 3.0 μg/ml leupeptin, 1.0 μg/ml pepstatin (all from Roche)). The cell suspension was incubated on ice for 45 min, then followed by nitrogen cavitation (Parr bomb) at 500 psi for 30-45 min on ice. Cell lysates were clarified by brief low speed centrifugation to remove intact cells and nuclei. The membrane fraction was harvested by ultracentrifugation at 100,000 x g for 1 hr. The membrane pellet was resuspended in wash buffer (WB: 200 mM NaCl, 2.0 mM TCEP, 10% glycerol, 20 mM HEPES, pH 8.0) supplemented with protease inhibitors and solubilized by 1% DDM (Anatrace). The mixture was incubated at 4 ^oC for 1 hr with gentle shaking *prior to* centrifugation at 25,000 g for 30 min to remove insoluble fractions. The supernatants (membrane extracts) were collected and mixed with amylose resin (New England Biolabs). After 2 hrs of incubation at 4 ^oC, the resins were collected and transferred to a column which was washed with WB containing either 0.5mM DDM as well as 10 μg/ml soybean lipids (Avanti), followed by elution with 20 mM maltose in the same buffer. The purified sample was assessed for protein homogeneity by size-exclusion chromatography (SEC) in a Superose 6 column equilibrated with WB containing appropriate detergents and lipids and were used immediately for reconstitution experiments. Denaturing SDS-PAGE was performed on 8.0 - 10% polyacrylamide gels according to the Laemmli procedure using the Mini-PROTEAN Tetra System by Bio-Rad in a Tris/glycine/SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) and an applied voltage of 200 V. Native PAGE was performed on NativePAGE Bis-Tris precast gels (Invitrogen, Thermo Fisher Scientific) at 4^oC following the manufacture's protocol.

Protein reconstitution

Reconstitution of purified proteins into preformed liposomes was carried out using the detergent-mediated reconstitution approach [1,3]. Lipids dissolved in chloroform were dried

under a gentle nitrogen stream and then under a strong vacuum for 4-5 hrs before rehydration overnight in the reconstitution buffer at a concentration of 5.0 mg/ml (RB: 200 mM KCl, 20 mM MOPS, 2.0 mM TCEP pH 7.0). Liposomes were formed by 10 cycles of freeze-thawing and then 1-2 minutes of bath sonication. *Prior to* reconstitution, liposomes were destabilized by adding DDM to a final concentration of 4.0 mM, and the suspension was incubated at room temperature for 30 min with gentle shaking. Proteins were added to the detergent-lipid mixture at the desired lipid/protein ratio, typically 8:1 (weight/weight), and the mixture was incubated at room temperature for 1 hr under constant agitation. After equilibration, detergents were removed at room temperature using a fast detergent removal procedure through additions of four batches of prewashed polystyrene beads (Bio-Beads SM2, Bio-Rad) respectively at 30 mg, 30 mg, 50 mg and 100 mg per milligram of detergents. The first three treatments were incubated at room temperature at 1 hr intervals and the last one was at 4 ^{o}C overnight, all under gentle agitation. After complete detergent removal, the supernatant was aspired from BioBeads by a gel loading tip and then subject to ultracentrifugation at 100,000 g for 1 hr. The collected proteoliposomes were resuspended in the reconstitution buffer and flash frozen in liquid nitrogen for storage at -80 ^{o}C. For experiments requiring removal of the MBP tag from channel proteins, TEV was added to samples at a weight ratio of 20 : 1 (protein : Tev) before the last step of BioBeads addition.

Electrophysiology

Patch-clamp recording was carried out with an Axopatch 200B amplifier (Axon Instruments). Currents were low-pass filtered at 5–10 kHz through the built-in eight-pole Bessel filter and sampled at 10–20 kHz with a multifunctional data acquisition card (National Instruments). Data acquisition was controlled using custom software capable for synchronous I/O and simultaneous control of laser and patch-clamp amplifier. Patch pipettes were fabricated from borosilicate glass capillary (Sutter Instrument) with a resistance of ~5 MΩ when filled with 150 mM NaCl solution. Pipette series resistance and capacitance were compensated using the built-in circuitry of the amplifier, and the liquid junction potential between the pipette and bath solutions was zeroed prior to seal formation. Currents were evoked from a holding potential at -60 mV.

Giant proteoliposomes suitable for patch-clamp recording were prepared using the dehydration-rehydration method as previously described [1]. Briefly, frozen proteoliposomes (2-3 μl) were thawed and supplemented with 20 mM sucrose (final concentration). The mixture was dehydrated on a glass coverslip in a vacuum desiccator at room temperature (~30 min), and then rehydrated with addition of ~10 μl of reconstitution buffer (200 mM KCl, 5 mM MOPS, 2mM TCEP, pH 7) before overnight incubation at 4 ^{o}C in a sealed, humidified chamber. For patch-clamp experiments, the coverslip was transferred to a petri dish containing 150 mM NaCl, 2 mM $MgCl_2$ and 10 mM HEPES (pH 7.4). Recordings were usually made on large unilamellar blisters at the peripheral of liposome clumps. The pipette and bath solutions were symmetrical. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Temperature jumps were produced by laser irradiation using a single emitter high power infrared laser diode as previously described [4]. In brief, the diode was mounted on a cooling block and operates at room temperature. Laser emission from the laser diode was launched into a multimode fiber with a 100-µm core diameter and 0.22 NA. The other end of the fiber was positioned close to patch pipettes as the perfusion pipette normally was. The laser diode was driven by a pulsed quasi-CW current power supply (Lumina Power). Constant temperature steps were generated by irradiating the tip of an open pipette and using the current of the electrode as the readout for feedback control. The laser was first powered on for a brief duration (<1 ms) to reach the target temperature and was subsequently modulated to maintain a constant pipette current. Between consecutive temperature pulses, laser power was adjusted manually and the adjustment generally took less than half a minute to a minute. Temperature was calibrated offline from the pipette current based on temperature dependence of electrolyte conductivity.

Sucrose density gradient centrifugation

Three T-25 flasks of adherent HEK293 cells were grown to ~80% confluency in DMEM plus 10% FBS and transiently transfected by calcium phosphate precipitation. ~24 hrs post transfection, the monolayer was rinsed with PBS and scrapped from the flask and then pelleted at ~1000 g for 10-20 min at 4 °C. The pellet was resuspended in WB plus protease inhibitors. The suspension was divided into two fractions in an equal amount; one was heated in a PCR thermocycler to 48 °C for ~60 s, while the other was used as the no heat treatment control. The cells were lysed for < 1hr on ice in WB containing 1% DDM (Anatrace). Detergent-insoluble fractions were removed after centrifugation at 18,000 g for 35 min. The supernatant was collected and layered onto a 5-step sucrose density gradient (w/v: 10%, 20%, 30%, 40% and 50%) prepared in a Beckman ultra-clear centrifuge tubes (14 x 89 mm). All sucrose solutions were made up in WB with 2 mM TCEP and 4 mM DDM. The gradients were centrifuged at 35,000 rpm for 22 hrs at 4 °C. Fractions were collected by bottom puncture, and their EGFP fluorescence was quantified in a 1 cm quartz cuvette using a Hitachi F-7000 fluorometer. The EGFP emission spectra were measured, and the intensity at the peak around 510 nm was reported.

Calorimetry

DSC measurements were performed in a Microcal capillary ultrasensitive differential scanning microcalorimeter (Malvern Panalytical). Before each experiment, exhaustive cleaning of the cells was undertaken, and the sample and reference solutions were thoroughly degassed for at least 10-15 min in a temperature-controlled vacuum chamber (ThermalVac). About 250 ul degassed solution was loaded to each calorimetric cell, which was subsequently pressurized under an extra pressure of ~2 atm to prevent solutions from degassing during heating. The measurements always started by filling both the sample and reference cells with the buffer used for the protein sample. The buffer-buffer scan was repeated until the instrument became thermally equilibrated, which typically took 4-5 repetitions. The sample was then loaded to the

sample cell, normally during the cooling from the previous scan and before the start of thermal equilibration for the next scan. The sample-buffer scan was also repeated for 2-3 runs to determine the calorimetric reversibility of the thermally induced transition occurring during the first sample-buffer scan. Before the start of the first scan and between repeated scans, the solutions in the cells were allowed to equilibrate for 2-5 min at appropriate temperatures (4 or 15 ^{o}C for detergent-solubilized samples and 20 ˚C for reconstituted proteins).

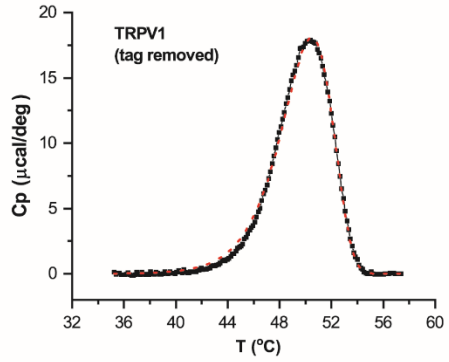
For all measurements the scan rate was 1 K/min, and protein concentrations were in the range of 0.4-0.8 mg/ml. The buffer used was either the reconstitution buffer for liposome measurements or the elution buffer used during protein purification as described above. For solubilized proteins in detergents, the concentrations were determined from the absorbance at 280 nm using a Nanodrop spectrophotometer (ThermoFisher). For liposome samples, the concentrations were estimated based on the DSC transitions of the MBP tag fused to the channel proteins in reference to the pre-determined ΔH of MBP in detergents (~220 kcal/mol, which is also similar to the published values of free MBP in aqueous solutions [5]). For a rough estimation of protein concentration in liposomes (necessary for DSC sample loading), TRP fluorescence of the sample was measured in a fluorimeter (Hitachi F-7000), and the protein concentration was quantitated using BSA as a reference after subtraction of lipid background. The lipid recovery in our reconstitution assay was about 90%, as determined from fluorescence recovery by mixing 1% fluorescent lipids (ß-BODIPY FL C12-HPC, ThermoFisher) into reconstitution lipids. We also attempted more conventional quantitation methods such as BCA but with less accuracy.

Data analysis

The excess heat capacity thermographs were extracted from the original calorimetric profiles by first subtracting the repeated scan containing no transitions, followed by correction for possible baseline drifts as determined with suitable pre- and post-transition baselines. The corrected profiles were normalized by protein concentrations to yield molar heat capacity. The evaluation of theoretical excess heat capacity of appropriate models [6] (see ***S7***) and their fitting to DSC transitions were carried out in a MATLAB program employing the built-in non-linear least-square solver implementing the Levenberg-Marquardt and trust-region-reflective algorithms.

Supplementary Figures

a



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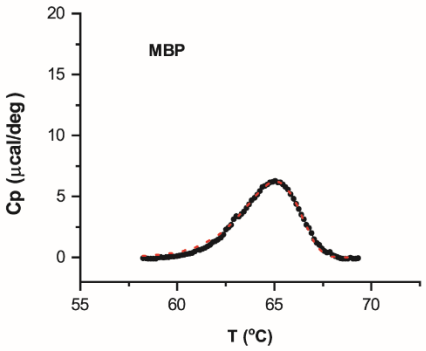

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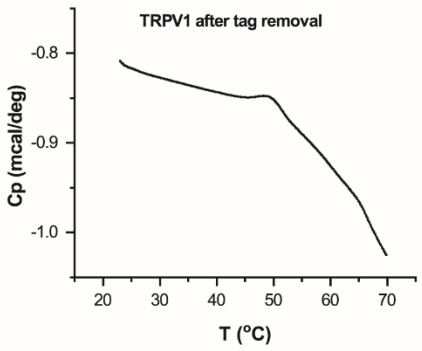

Figure S1. DSC of TRPV1 in liposomes after removal of MBP tag. **A.** Plot of excess heat capacity of TRPV1 showing that robust DSC transition of TRPV1 remained detected between 40 °C and 55 °C, with a similar profile to that of TRPV1-MBP. **B.** Residual DSC transition peak of MBP between 60 °C and 70 °C after Tev treatment. **C.** Representative DSC scans of reconstituted TRPV1 channels after removal of MBP by Tev enzymes.

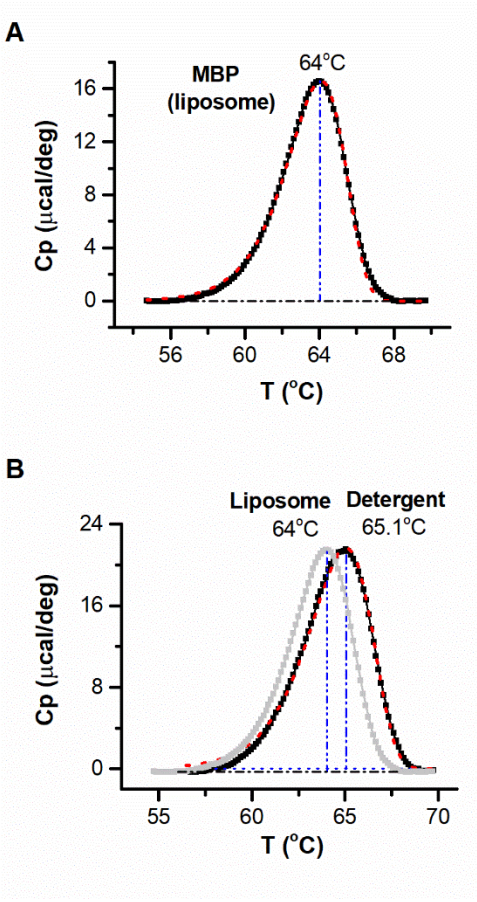

Figure S2. DSC transitions of MBP proteins. **A.** Plot of excess heat capacity of MBP protein fused to TRPV1 in liposomes. The red line represents the fit by a two-state model. **B.** Excess heat capacity of MBP fused to TRPV1 in solutions containing 0.5 mM DDM (black), in comparison with liposome measurement (grey). The dotted red line corresponds to the fit by a two-state model. The thermograph profiles of MBP under the two conditions were comparable, with a minor shift in the peak temperature, which is in contrast to the large shift of channel proteins under the same conditions.

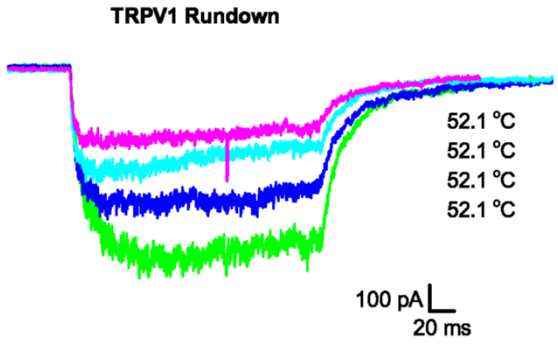

Figure S3. Functional rundown of TRPV1 in liposomes. Continued heating at high temperature following maximum activation (~52 °C) caused progressive reduction of channel activity (the bottom trace corresponds to the first run). Pulse duration was 200 ms. Holding potential -60 mV. Time interval between successive pulses ranged half a minute to a minute.

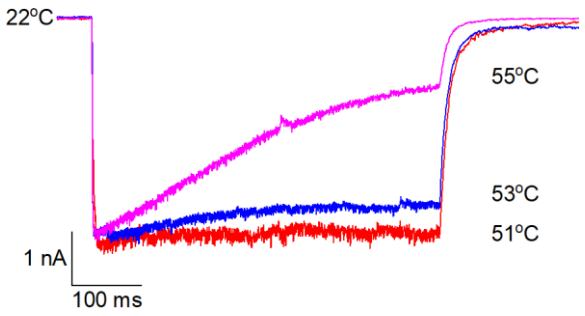

Figure S4. An exemplar recording showing temperature dependence of rundown of TRPV1. Currents were elicited by a temperature pulse of 500 ms held constant at specified temperatures. Recordings from HEK293 cells transiently expressing TRPV1. Holding potential -60 mV. Currents from three different patches were normalized to a similar maximum amplitude for comparison.

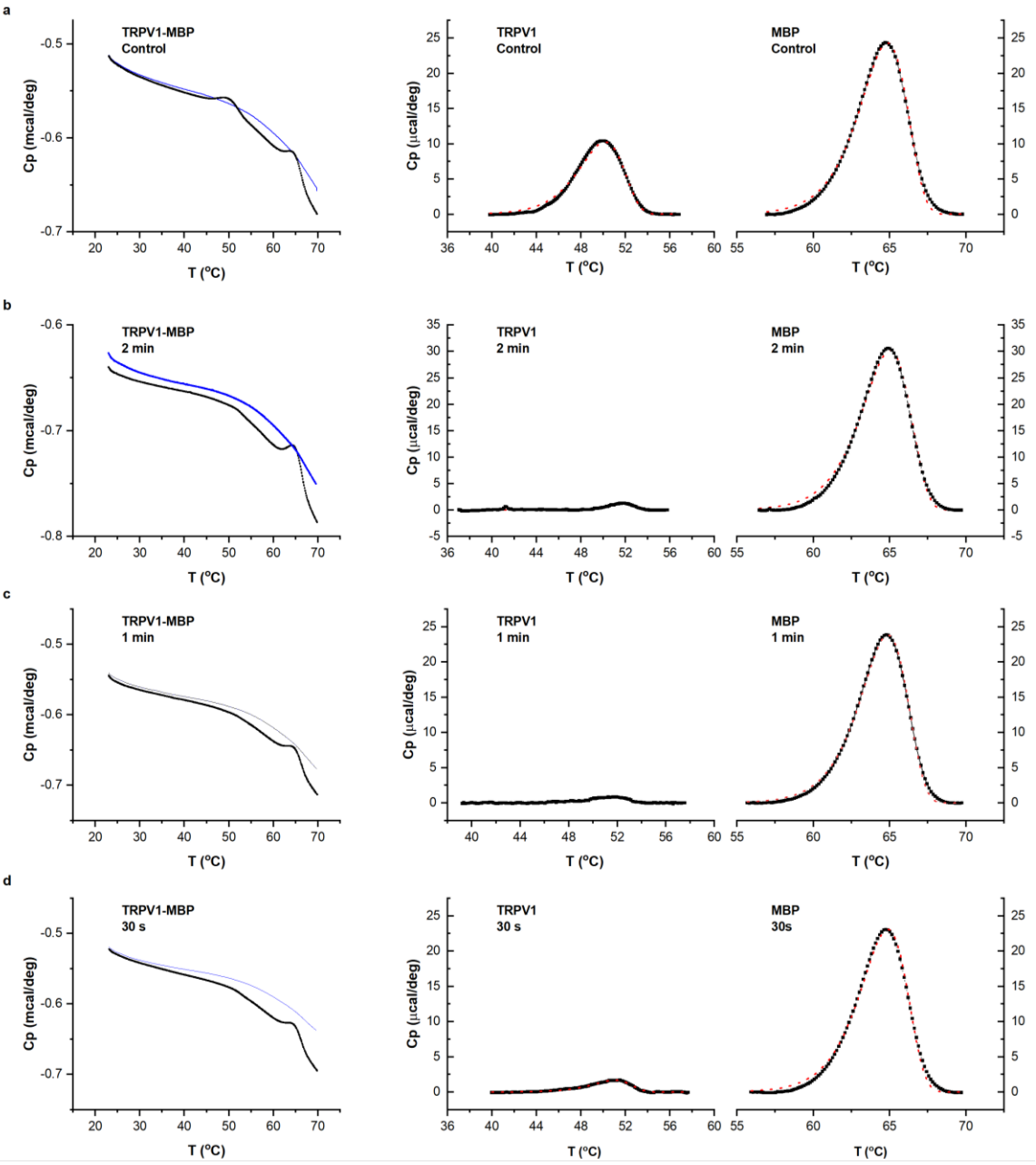

Figure S5. Intrinsicality of the DSC transition of TRPV1. To evaluate possible effects of slow DSC heating rates, TRPV1 proteoliposomes were pre-heated to 51 °C in a PCR thermocycler for variable durations that were considerably shorter than in DSC. The top panel (*a*) shows DSC scans of control samples without *prior* heat treatment. The lower panels (*c*-*d*) correspond to pre-heated samples (*b* – 2 min; *c* – 1 min; *d* – 30s). Irrespective of the preheating duration, the TRPV1 transition peak became diminished after preheat treatment, as compared to the robust peak in the control sample (*a*). The MBP peak remained unchanged in all cases. For the 2-min heating duration, four repetitions of 30 s heating followed by 30 s cooling (20 °C) were applied. Data were representative of 2-3 experiments at each condition.

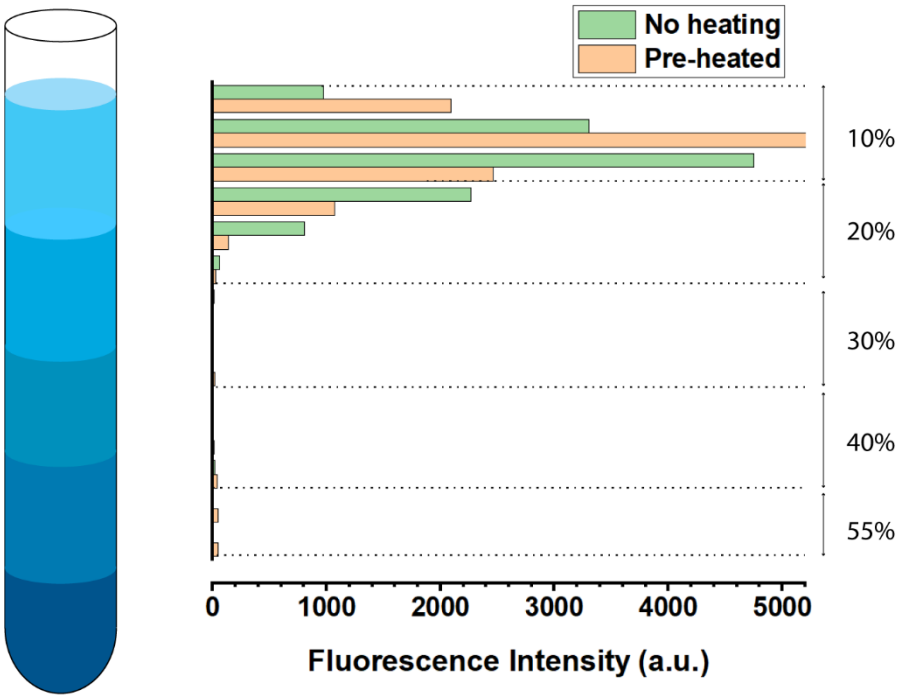

Figure S6. Fluorescence intensity profiles of sucrose density gradient fractions of cell lysates from HEK 293 cells co-transfected with TRPV1 and EGFP (green color for cells receiving no prior heat treatment; orange color for cells pre-heated to 48 °C for 60 s prior to lysis). Fluorescence intensity distribution was normalized to the same total fluorescence intensities.

S7. Formalisms for the excess heat capacity of models used for data fitting.

1) Two-state model:

$$A \xrightarrow{k} B$$

where *k* is the rate constant which is determined by the activation energy (E) according to:

$$k = e^{-\frac{E}{R}\left(\frac{1}{T}-\frac{1}{T^*}\right)}.$$

The excess heat capacity of the model follows:

$$C_{p,exc} = \frac{\Delta H}{v} \cdot e^{-\frac{E}{R}\left(\frac{1}{T}-\frac{1}{T^*}\right)} \cdot e^{-\frac{1}{v}\int k dT}$$

where v is the DSC scan rate.

2) Three state model:

$$A \xrightarrow{\alpha} B \xrightarrow{\beta} C$$

where α and β are rate constants and are related to their activation energies, E_1 and E_2 by

$$\alpha = e^{-\frac{E_1}{R}\left(\frac{1}{T}-\frac{1}{T_1}\right)}$$

$$\beta = e^{-\frac{E_2}{R}\left(\frac{1}{T}-\frac{1}{T_2}\right)}.$$

The excess heat capacity in this case can be derived as

$$C_{p,exc} = \Delta H_1 \cdot \frac{dX_B}{dT} + (\Delta H_1 + \Delta H_2) \cdot \frac{dX_C}{dT}$$

where ΔH_1 and ΔH_2 are enthalpy changes from A to B and B to C respectively, and X represents state occupancy and follows:

$$\frac{dX_B}{dT} = \frac{\alpha}{v} X_A - \frac{\beta}{v} X_B$$

$$\frac{dX_C}{dT} = \frac{\beta}{v} X_B$$

where v is the DSC scan rate. The excess heat capacity is solved by solving the above differential equations using Runge-Kutta formula with a trapezoidal rule (the ODE23TB solver in Matlab).

SI References:

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